BỘ Y TẾ

HỌC VIỆN Y DƯỢC HỌC CỔ TRUYỀN VIỆT NAM

MINH CHỨNG BÀI BÁO

HÀ NỘI – 2024

Mẫu 5: Các công trình khoa học công bố của giảng viên, nhà khoa học cơ hữu liên quan đến ngành đào tạo dự kiến mở của cơ sở đào tạo trong thời gian 5 năm tính đến thời điểm nộp hồ sơ mở ngành đào tạo (kèm theo bản liệt kê có bản sao trang bìa tạp chí, trang phụ lục, trang đầu và trang cuối của công trình công bố)

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3.	**Loi Vu Duc** *, Tung Bui Thanh, Huong Le Thi Thu and Bach Nguyen Van (2018), Chemical Constituents and Tyrosinase Inhibitory Activity of Aqueous Fraction of the Leaves of *Morus alba* L. from Vietnam, *International Journal of Pharmacognosy, Vol. 5(7):* 399-403.	
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9.	**Loi Vu Duc***, Tung Bui Thanh, and Tung Nguyen Huu (2018), Terpenoids from *Dicliptera chinensis* (L.) Nees Grown in Vietnam and their Anti-Inflammatory Activities, International Journal of Pharmaceutical Sciences and nanotechnology, 11(2), 4052-5058.	
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29.	Phạm Thị Vân Anh, Đặng Thị Thu Hiền, Mai Phương Thanh, Lê Thị Kim Loan, **Nguyễn Duy Thuần** (2020), *Nghiên cứu ảnh hưởng của viên nang cứng Anti-200 lên chức năng gan thận trên thực nghiệm,* Tạp chí Y Dược cổ truyền Việt Nam, Số 03(28), Tr. 19-10.	
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40.	Pham Quoc Binh, **Nguyen Duy Thuan**, Vu Viet Hang, Pham Thuy Phuong, Pham Quoc Su, Duong Van Phu, Phuong Thien Thuong, Dinh Thi Thu Hang, and Pham Thi Van Anh, *Antidiabetic Activity of Gomphogyne bonii Gagnep. Extract against High-Fat Diet and Alloxan-Induced Type 2 Diabetes in Mice,* Hindawi Evidence-Based Complementary and Alternative Medicine Volume 2021, Article ID 8648209, https://doi.org/10.1155/2021/8648209	
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CHEMICAL CONSTITUENTS AND TYROSINASE INHIBITORY ACTIVITY OF AQUEOUS FRACTION OF THE LEAVES OF *MORUS ALBA* LINN. FROM VIETNAM

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Morus alba L., tyrosinase inhibitors, Kaempferol-3,7-di-O-α-L-rhamno pyranoside, 7,4'-dihydroxy-5.3`-dimethoxyflavone,(S)-5,5`,7-trihydroxy-2',4'-dimethoxy-6-methylflavanone

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ABSTRACT: The mulberry has been widely cultivated to feed silkworms. The leaves of mulberry have been used in traditional medicine as an analgesic, antitussive, cathartic, diuretic. In this study we have isolated three compounds (LC1-3) by chromatographic methods from the mulberry leaves (*Morus alba* L.) collected in Thai Nguyen province, Vietnam. These compounds were identified as: Kaempferol-3,7-di-O-α-L-rhamnopyranoside (LC1), 7, 4'- dihydroxy-5, 3'- dimethoxyflavone (LC2), (S)-5,5',7-trihydroxy-2',4'-dimethoxy-6-methylflavanone (LC3). Their structures were elucidated by spectroscopic methods, including MS and NMR. Compound LC3 was isolated from mulberry leaves for the first time. These compounds were evaluated the tyrosinase inhibitory activity *in-vitro*. Our data showed that compound LC3 has potential tyrosinase inhibitory effects with IC_{50} values of 15.48 ± 2.96 μg/mL.

INTRODUCTION: In East Asia, whitening skin and protection against skin darkening are considered desirable by some for cosmetic purposes. Because tyrosinase plays a critical regulatory role in melanin biosynthesis, many tyrosinase inhibitors that suppress melanogenesis have been actively studied with the aim of developing preparations for skin whitening by many cosmetic companies. Plant extracts having an inhibitory effect on enzyme tyrosinase may be a good choice for this purpose because of their relatively lower side effects. In cosmetic preparations, many plant extracts such as Mulberry (*Morus alba* Linn.) leaves have been used as whitening agent [1].

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Mulberry (*Morus alba* L., family of Moraceae) is a native plant in Vietnam. It leaves have long been used in traditional medicine for treatment of several diseases such as fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine and lower blood pressure [2]. Leaves of mulberry species have been widely consumed as antihyperglycemic nutraceutical foods for patients with diabetes mellitus [3].

Previous studies reported that *Morus alba* L. had many pharmacological activities, including anti-hyperglycemic [4], anti-oxidant and antiglycation activities [5]. Phytochemical studies of this plant mainly showed the presence of flavonoids, anthocyanins, anthroquinones, triterpenes, tannins, phytosterols, sitosterols, benzofuran derivatives, morusimic acid, oleanolic acid, alkoloids, steroids, saponins and phenolic compounds [6, 7]. However, in Vietnam, there have been very few studies on this species. Therefore, this paper reports on the phytochemical investigation of the leaves of *Morus*

Reviewer's recommendations:

1. Check for spelling, grammar and punctuation error(s).
2. Mention reference number 18 into the text.

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Chemical Constituents and Tyrosinase Inhibitory Activity of Ethyl Acetate Extract of the Leaves of *Morus alba* L. from Vietnam

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Abstract

*Four compounds (**DB1–4**) were isolated from the Mulberry leaves (Morus alba L.) collected in Thai Nguyen province by chromatographic methods. These compounds were identified as: Kaempferol-3-O-β-D-glucopyranoside **(DB1)**, quercetin 3-O-α-L-rhamnopyranoside **(DB2)**, isofraxidin-7-O-β -D-glucopyranoside **(DB3)**, and (±) -3, 5, 6, 7, 8, 4'-hexahydroxyflavane **(DB4)**. Their structures were elucidated by spectroscopic methods, including mass spectrometry (MS) and nuclear magnetic resonance (NMR). Compound **DB4** was isolated from Mulberry leaves for the first time. Compound **DB4** also showed potential tyrosinase inhibitory effects with IC_{50} values of 12.02 ± 2.63 μg/ml.*

Keywords: *Morus alba L., tyrosinase inhibitors, kaempferol-3-O-β-D-glucopyranosid, quercetin 3-O-α-L-rhamnopyranoside, isofraxidin-7-O-β-D-glucopyranoside*

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INTRODUCTION

In East Asia, whitening skin and protection against skin darkening are considered desirable by some for cosmetic purposes. Because tyrosinase plays a critical regulatory role in melanin biosynthesis, many tyrosinase inhibitors that suppress melanogenesis have been actively studied with the aim of developing preparations for skin whitening by many cosmetic companies. Plant extracts having an inhibitory effect on enzyme tyrosinase may be a good choice for this purpose because of their relatively lower side effects. In cosmetic preparations, many plant extracts such as Mulberry (*Morus alba* L.) leaves have been used as whitening agents [1].

Mulberry (*M. alba* L., family of Moraceae) is a native plant in Vietnam. Its leaves have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine and lower blood pressure [2]. Previous studies reported that *M. alba* L. had many pronounced bioactivities, including antihyperglycemic, antioxidant and antiglycation activities [3]. Phytochemical studies of this plant mainly showed the presence of flavonoids as well as phenolic compounds [4]. However, in Vietnam, there have been very few studies on this species. Therefore, this paper reports on the phytochemical investigation of the leaves of *M. alba* L. and on the evaluation of the tyrosinase inhibitory activity of isolated compounds.

MATERIALS AND METHODS

Plant Material

The leaves of *M. alba* L. were collected in Thai Nguyen province during June 2016 and authenticated by the School of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam (SMP-VNU). A voucher specimen has been deposited in the SMP10.

General Experimental Procedures

Melting points were measured on Mikroskopheiztisch PHMK-50 (VEB WaegetechnikRapido, Germany). The FT-IR spectra were recorded on an IMPACT-410FT-IR spectrometer (CARL ZEISS JENA). The NMR [^{1}H (500 MHz), ^{13}C (125 MHz), and DEPT-90 and 135 MHz)] spectra were recorded on an AVANCE spectrometer AV 500 (Brucker, Germany) in the Institute of Chemistry, Vietnam Academy of Science and Technology (VAST), Vietnam. Chemical shifts were reported in ppm downfield from

The IC_{50} value of acid kojic was close to that from previous studies. Among four isolated compounds, two compounds showed potential tyrosinase inhibitory effects, including kaempferol 3-*O*-*β*-D-glucopyranoside (IC_{50}= 19.49 ±2.83 μg/ml), and (±) -3,5,6,7,8,4'-hexahydroxyflavane (IC_{50}= 12.02 ± 2.63 μg/ml) [12]. Therefore, kaempferol 3-O-β-D-glucopyranoside and (±) -3,5,6,7,8,4'-hexahydroxyflavane are worthy of further studies as potential tyrosinase inhibitors (Figure 2 and Table 2).

CONCLUSION

From the Mulberry leaves (*M. alba* L.) collected in Thai Nguyen province, four compounds (DB1-4) were isolated by chromatographic methods. On the basis of spectroscopic analyses and by spectral comparison with published literature, the isolated compounds were identified as Kaempferol-3-*O*-*β*-D-glucopyranoside **(DB1)**, quercetin 3-*O*-*α*-L-rhamnopyranoside **(DB2)**, isofraxidin-7-*O*-*β* -D-glucopyranoside **(DB3)**, (±) -3, 5, 6, 7, 8, 4'- hexahydroxyflavane **(DB4)**. This is the first report on the isolation of **DB4** fromMulberry leaves. Among four isolated compounds, DB4 also showed significant tyrosinase inhibitory effects, with IC_{50} values of 12.02 ± 2.63 μg/ml.

ACKNOWLEDGEMENT

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The authors have declared no conflict of interest.

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Flavonoids from *Dicliptera chinensis* (L.) Nees Grown in Vietnam and their Anti-Inflammatory Activities

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Abstract

From the leaves of Dicliptera chinensis (L.) Nees (Acanthaceae) collected in Nam Dinh province, five flavonoids (1-5) were isolated by chromatographic methods. These compounds were identified as: Kaempferol-3-O-β-D-glucopyranoside (1), Nicotiflorin (2), Kaempferol-3-O-α-L-rhamnopyranoside-7-β-D-glucopyranoside (3), Catechin (4), Quercetin (5). Their structures were elucidated by spectroscopic methods, including MS and NMR. Compound 1 and 3 were isolated from Dicliptera genus for the first time. Compound 3 and 4 also showed potential COX-2 inhibitory effects with IC50 values of 22.38 ± 1.72, 22.95 ± 1.87 µg/mL, respectively.

Keywords: Kaempferol-3-O-β-D-glucopyranoside; Nicotiflorin; Kaempferol-3-O-α-L-rhamnopyranoside-7-β-D-glucopyranoside; Catechin; Quercetin

Accepted on January 23 2018

Introduction

The leaves of *Dicliptera chinensis* (L.) Nees (Acanthaceae) is an ethnomedicine, which has been commonly used for treatment of inflammatory in folk [1]. Phytochemical studies of this plant demonstrated the presence of glycosides, flavonoids, monoterpenoids: dicliparisides A, dicliparisides B, dicliparisides C, acid vanillic, β–sitosterol, 2,5–Dimethoxy–bezoquinone, daucosterol, lugrandoside and poliumonside [2,3]. Gao Yu-tao has isolated thirteen compounds from petroleum ester extract fraction of Dicliptera chinensis, these were hexatriacontanol, stearic acid, lupenone, lupeol, 4-sitost-4-en-3-one, stigmast-5-en-7-oxo-3β-yl palmitate, β-sitosterol, oleanolic acid, 3β,6β-stigmast-4-en-3,6-diol, 6β-hydroxy-stigmast-4-en-3-one, 3β-hydroxy-stigmast-5-en-7-one, dehydrovomifoliol, and vomifoliol [4]. Previous studies reported that *D. chinensis* had pronounced bioactivities, including antioxidant, anti-inflammatory activities. Gao Ya has showed *D. chinensis* polysaccharide was effective for liver injury induced by antituberculosis drug, and the mechanism may be associated with its anti-inflammatory action [5]. Other study demonstrated that functional components including flavonoids, polysaccharides and polyphenols from *D. chinensis* had strong free radical scavenging capacity [6]. Although *D. chinensis* is used for clinical treatment, there have been very few studies on this plant. Therefore, this paper reports on the phytochemical investigation of *D. chinensis* and on the evaluation of the anti-inflammatory activities of isolated compounds.

Materials and Methods

Plant material

The whole plants of *D. chinensis* (L.) Nees were collected from Nam Dinh province, Vietnam during June 2016 and taxonomically identified by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The samples were stored at the School of Medicine and Pharmacy-VNU.

General experimental procedures

Column chromatography was performed on silica gel (0.040-0.063 mm, Nicalai Tesque Inc., Japan), YMC ODS-A (50 µm, YMC Co. Ltd., Japan).Organic solvents were of analytical grade.Analytical TLC was performed on Kieselgel 60 F254 (Merck) plates (silica gel, 0.25 mm layer thickness) and RP-18 F254 (Merck) plates (0.25 mm layer thickness). Spots were visualized using ultraviolet radiation (at 254 and 365 nm) and by spraying with 10% H2SO4, followed by heating with a heat gun.The NMR [^{1}H (500 MHz), ^{13}C (125 MHz), and DEPT-90 and 135 MHz)] spectra were recorded on an AVANCE spectrometer AV 500 (Brucker, Germany) in the Institute of Chemistry, Vietnam Academy of Science and Technology (VAST).

Spectra (ESI-MS) were recorded on an AGILENT 1260 Series LC-MS ion Trap (Agilent Technologies, USA).Melting points were measured on SMP10 BioCote in the School of Medicine and Pharmacy-VNU.Optical rotation was measured on PLR-4, MRC scientific instruments in the School of Medicine and Pharmacy -VNU.

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Research Paper

Terpenoids from *Dicliptera chinensis* (L.) Nees Grown in Vietnam and their Anti-inflammatory Activities

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ABSTRACT

The main goal of this study was to isolate and screen the therapeutic potential of some terpenoids from *Dicliptera chinensis* (L.) Nees grown in Vietnam. From the leaves of *Dicliptera chinensis* (L.) Nees (Acanthaceae) collected in Nam Dinh province, one new terpenoid (1) together with five known (2-6) were isolated by chromatographic methods. These compounds were identified as 6-hydroxy-1,12-oleanadien-3-on (1), 1,4-dihydroxyeudesm-11-ene (2), 3-hydroxylup-20(29)-en (3), stigmast-5,22-dien-3--ol (4), rutaevine (5), mangiferin (6). All of them were isolated from this plant for the first time. Compound 4 showed signficant potential for COX-2 inhibitory effects with IC_{50} values of 7.32± 1.82 µg/mL. These pilot results suggest the potential of *Dicliptera terpenoids* for treating inflamatory conditions.

KEYWORDS: *Dicliptera chinensis*, 6-Hydroxy-1,12-oleanadien-3-on, 1β,4β-dihydroxyeudesm-11-ene, 3β-hydroxylup-20 (29)-20(29)-en, Stigmast-5,22-dien-3-β-ol, rutaevine, mangiferin

Introduction

The leaves of *Dicliptera chinensis* (L.) Nees (Acanthaceae) is an ethnomedicine, which has been commonly used for treatment of inflammatory in folk (Lin et al., 1993; Zhang et al. 2010). Phytochemical studies of this plant demonstrated the presence of glycosides, flavonoids, monoterpenoids: dicliparisides A, dicliparisides B, dicliparisides C, acid vanillic, β – sitosterol, 2,5 – dimethoxy – bezoquinone, daucosterol, lugrandoside and poliumonside (Gao et al. 2007). Previous studies reported that *D. chinensis* had pronounced bioactivities, including antioxidant, anti-inflammatory activities. Although *D. chinensis* is used for clinical treatment, there have been very few studies on this plant. Therefore, this paper reports on the phytochemical investigation of *D. chinensis* and on the evaluation of the anti-inflammatory activities of isolated compounds.

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- Analytical TLC was performed on Kieselgel 60 F254 (Merck) plates (silica gel, 0.25 mm layer thickness) and RP-18 F254 (Merck) plates (0.25 mm layer thickness). Spots were visualized using ultraviolet radiation (at 254 and 365 nm) and by spraying with 10% H_2SO_4, followed by heating with a heat gun.
- The NMR [^1H (500 MHz), ^{13}C (125 MHz), and DEPT-90 and 135 MHz)] spectra were recorded on an AVANCE spectrometer AV 500 (Brucker, Germany) in the Institute of Chemistry, Vietnam Academy of Science and Technology (VAST).
- Spectra (ESI-MS) were recorded on an AGILENT 1260 Series LC-MS ion Trap (Agilent Technologies, USA)
- Melting points were measured on SMP10 BioCote in the School of Medicine and Pharmacy -VNU.
- Optical rotation was measured on PLR-4, MRC scientific instruments in the School of Medicine and Pharmacy -VNU.

Extraction and isolation: Leaves of *D. chinensis* was dried, powdered and then extracted with 96% ethanol (8L × 3 hours × 3 times) by supersonic method. The resulting extracts were combined and then evaporated to dryness *in vacuo* to yield crude extract (630.0 g). The ethanol extract (120 g) was dissolved in water (1.2 L) and subjected to liquid-liquid partitioning (3 times) using *n*-hexane, ethyl acetate (EtOAc), yielding 31.0 g and 56.0 g of residue, respectively. The aqueous fraction was concentrated to yield 33,0 g of residue.

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ORIGINAL ARTICLE

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Chemical constituents and anti Helicobacter pylori effect of ethyl acetate fraction from *Sanchezia nobilis* Hook.F

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Sanchezia nobilis Hook.F, 13-O-acetylfawcettimine, ethyl acetate fraction, anti-Helicobacter pylori, MIC

ABSTRACT

The leaves of Sanchezia Nobilis Hook. F grown in Vietnam are extracted with ethanol then distilled for ethanol retrievement under low pressure. The ethanol concentrate was shaken with n-hexane, then stirred with ethyl acetate and concentrated fractions were obtained. From the high ethyl acetate fraction, four compounds were isolated by normal phase and reversed-phase column chromatography. Their structures were determined by spectral analysis, including 1D and 2D NMR techniques (1H, 13C, DEPT, COZY, HSQC, HMBC and NOESY), High Resolution - Electrochewmical Ionization - Mass Spectroscopy (HR-ESI-MS), and identified by comparing with the recorded data. Among the isolated compounds, compound 2 (13-O-acetylfawcettimine) was first time isolated from nature; and mixture 1 (Fawcettidin), 3 (Apigenin) and 4 (Kaempferol) were first time isolated from the Sanchezia genus. The high ethyl acetate fraction of Sanchezia Nobilis Hook. F leaves were then evaluated for the anti-Helicobacter pylori (anti-HP) effect by determining the minimum inhibitory concentration method (MIC), which showed a good inhibitory, dose–dependent effect on Helicobacter pylori. The ethyl acetate fraction at 1/8 dilution showed a complete inhibitory effect on Helicobacter pylori for as long as 24 hours. At 1/128 dilution, the fraction only showed good results on inhibition after 24 hours.

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INTRODUCTION

Sanchezia is a small genus with more than 50 species, mainly distributed in the subtropical and tropical area such as the USA, Africa, the Mediterranean, Oceania, India, and some Southeast Asian countries. Most species are located in tropical rainforests in Central and South America (Ecuador) (Leonard and Smith, 1964). In previous publications, the genus was studied for effects such as antibacterial, antifungal and insecticidal by the agar diffusion method with 15 strains of Gram (+) and Gram (-). The effect on six strains of fungi and *Tribolium castaneum* was not as good as the comparison antibiotics (Rafshanjani *et al.*, 2014). Evaluation of antioxidant effect by the ORAC method showed a result similar to quercetin. The anticancer effect on a methanolic fraction from *Sanchezia speciosa* leaves extract on MCF-7, SK-MEL-5, HUVEC cell lines had the best results on CMF-7 cell lines (Paydar *et al.*, 2013). The anticancer effect by the MTT method on Hela cells from *S. Nobilis* roots had positive results (Shaheen

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Research Article

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...mical and anti-inflammatory effect from the leaf of ...*chezia speciosa* Leonard growing in Vietnam

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...ol of Medicine and Pharmacy, Viet nam National University, Hanoi, Vietnam
[2]Hanoi University of Pharmacy, Hanoi, Vietnam

ABSTR...

*Sanchez... ...sa is an evergreen shrub in the dogbane family Apocynaceae and planted throughout the tropical and sub... ...l region. It has been showed that Sanchezia speciosa has high amount of cardiac glycoside, flavonoi... ...ound... and also the antioxidant, anti-inflammatory and anticancer activities. In this study, we aim to study th... ...chemical component of ethanolic extract of Sanchezia speciosa leaf and investigate the anti-inflamm... ...f Sanchezia speciosa leaf extract. Leaf of Sanchezia Speciosa Leonard was extracted with 80% eth... ...nds were isolated using on silica gel normal and reverse phase and preparative glass-backed ... The compound's structure were characterized on the basis of spectroscopic data, including IR, MS ... ...d by comparing their physicochemical and spectral data with those published in literatures. Paw ed... ...ced in the mice using 0.05 ml of 1% Carrageenan sodium salt to investigate the anti-inflamm... ...ject ... Sanchezia speciosa leaf extract. From ethanolic extract of Sanchezia speciosa leaf we have isolated ...mpo...ds. Their structures were identified as (1) Quercetin 3-O-α-L-rhamnopyranosid (quercitrin), (2) Que... ...-O-...)-galactopyranosid (hyperosid), (3) sitosterol-3-O-β-D-glucopyranosid (daucosterol), (4) 3-Methyl-... ...le-4,9-dione. Our data showed that S.specsiosa leaves extract (dose 1.5 g/kg b.w) reduced signific... ...a induced by Carrageenan. This is first time compound (1), (2), (4) were isolated from leaves of San... ... S.specsiosa leaf extract have been showed strong effect in inhibit carrageenan-induced paw edema...

Keywo... ... *speciosa*, quercitrin, hyperosid, daucosterol, anti-inflammatory.

INTRODUCTION

Vietna... ...al climate with diversity of plant species. It is estimated that Vietnam has more than 12000 plant s... ... nearly 4000 species can be used in traditional medicine. *Sanchezia speciosa* is a member of Acanth... ...mily. In Vietnam, *Sanchezia speciosa* grown mostly in North Vietnam and have been commo... ...t gastritis disease. Literature survey reveals that *Sanchezia speciosa* had antioxidant and antican... ...terial, antifungal and insecticidal effects [2]. Currently, there is scarcely research on the chemic... ...of *S.speciosa* and biological effects of them for gastritis treatment. Therefore, it is necessary to stud... ...ytochemical and pharmacology activity of this plant. In this study, we have isolated and identifi... ...nds from ethanolic extract of *Sanchezia speciosa* leaf grown in Vietnam, including quercitrin, hypero... ... 3-methyl-1H-benz[f]indole-4,9-dione. We also investigated the anti-inflammatory effect of *Sanch*... ...f extract on carrageenan-induced paw edema in mice.

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d C-9 linked to ring A at position C-4a and C-8a. The pyrole C ring also is determined by correlates with C-3, C-3a and C-9a; CH_3-10 correlates with C-2 and C-3a. The methyl group le C ring is also confirmed by NOE correlation between H-2 and CH_3-10. Based on the above rature data [9], compound **4** was identified as 3-methyl-1H-benz[f]indole-4,9-dione. This ne isolated from *Sanchezia Speciosa* Leonard.

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esponse of the immunological defense system to microbial infections, burns, allergens and ogenesis of many diseases, including diabetes, cardiovascular, neurodegenerative, cancer and iseases involve the inflammation. Inflammation is a complex series of cascade reactions, ation, release of chemical mediators, effusion of fluids, cell migration, and tissue damage and enan-induced mouse paw edema is a practicable model for evaluating the anti-inflammatory ts. The acute and local inflammatory response is induced by carrageenan injection into the has been well established as a valid model to study pro-inflammatory mediators and cytokine e in inflammatory conditions [11]. The present study showed that *Sanchezia Speciosa* leaves istration, reduced paw volumes in the carrageenan-induced paw edema test. It can explain the n of *Sanchezia Speciosa* leaves exttract is related to the effects on mediators and cytokines.

CONCLUSION

From
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hezia Speciosa Leonard. we have isolated four compounds: quercitrin (**1**), hyperoside (**2**), yl-1H-benz[f]indole-4,9-dione (**4**). These compounds (1), (2), and (4) were isolated for ves of *S.specsiosa* leaves. *S.specsiosa* leaves at the dose 3 and 1.5 g extract/kg b.w reduced <0,05, respectively) at 24 h in Carrageenan induced paw edema.

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Original Article

Chemi al C tituents and Anti-Ulcer Activity of Ethylacetate Extrac of eaves of *Sanchezia nobilis* Hook.F.

Loi Vu Duc[1,*], Xu i[1], Ngoc Tran Minh[2]

ABSTRACT

Study have two objectives. First objective is about the identification of the five components. The second objective is about the antiulcer activity of the ethylacetate extract. Five compounds (1–5) were isolated from the leaves of *Sanchezia nobilis* Hook.F. collected in Nam Dinh province by chromatographic methods. These compounds were identified as: Quercetin (**1**), Scopoletin (**2**), Kaempferol-3-*O*- α -L-rhamnopyranosyl-(1→6)- β -D-glucopyranoside (**3**), Quercetin-3-O-α-L-rhamnosyl-(1→6)-β-*D*-glucopyranosyl-(1→3)-β-*D*-glucopyranoside (**4**),3′–O–methyl–3, 4–methylenedioxy ellagic acid (**5**).Their structures were elucidated by spectroscopic methods, including mass spectrometry (MS) and nuclear magnetic resonance (NMR). Compounds **3**, **4**, **5** were isolated from the leaves of *Sanchezia nobilis* Hook.F. for the first time. Evaluation the effect of gastric and duodenal anti-ulcer on cyteamine induced gastric ulcer models indicates at this ethylacetate extract has the effect to improve the extent of ulcer lesions treatment (54,17 %); obviously reduced the average of number pocket of ulcers (1,85 ± 0,80) and ulcer index (5,61 ± 2,69), however, it does not change the area of the ulcer.

Key words: *Sanchezia nobilis*, Quercetin, Scopoletin, Anti-ulcer activity

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INTRODUCTION

Sanchezia nobilis Hook.F. is being paid attention and studied in a lot of different ways. The research of Abu Shuaib Rafshanjani and partners about the evaluation of the safety of Brine shrimp lethality bioassay method on n-hexane and ethylacetate segments, the results showed that both segments were safer than vincristine sulphate.[1] With antimicrobial, antifungal and insecticidal testing by diffusion on agar plates with 15 strains of Gram (+) and Gram (-) bacteria; 6 strains of fungus and insects *Tribolium castaleum* was not as good as comparison antibiotics.[2] Mohammad javad Paydar and co-worker have tried the antioxidant effect by Oxygen Radical Antioxidant Capacity (ORAC) method, the results were nearly equal to Quercetin and anti-cancer on methanolic segment from *Sanchezia speciosa* extract on MCF-7, SK-MEL-5, HUVEC giving the best results on CMF-7 cell lines, the anti-cancer effect on Hela cells from the roots of *Sanchezia nobilis* produced good results by MTT of Nusrat Shaheen and co-worker.[3,4] Research on the antioxidant effect of DPPH and anti-inflammatory by inhibition of albumine denaturation assay method by Bui Thanh Tung and partners gave positive results.[5] Some new claims showing that *Sanchezia nobilis* Hook.F. contains a number of substance groups such as flavonoids, glycosides, carbohydrates, alkaloids, steroids, phenolic, saponins and tannins. Some specific substances were isolated such as 5 matsutake alcohol compounds, in which the four compounds were first isolated from the Acathanceae family and one was the first isolated from nature, 3-O-arab-arabinopyranosyl-(1-6) -β-glucopyranosyl-(1-6)-β-glucopyranosyl-1-octen-3-ol and 6 other compounds from the leaf and root methnol extract, in which 1 substance was first isolated from naturally, 9-O-β-xylopyranosyl-(1→6)-O-β-glucopyranosyl-(1→6)-O-β-glucopyranosyltrans-cinnamyl alcohol, the 4 substances were first isolated from the Acanthaceae family and 3 substances were reported for the first time from *Sanchezia* by Ahmed and co-worker Bui Thanh Tung and partners also isolated 4 compounds in which 3 compounds were first isolated from *Sanchezia speciosa*.[6-8] In Vietnam, *Sanchezia* are found in many localities such as Tuyen Quang, Quang Nam, Da Nang, Nam Dinh, Vinh Phuc, Phu Tho and Thai Nguyen.[9] In Vietnam, many people used *Sanchezia nobilis* Hook.F. as a traditional precious medicine to cure gastritis. We need more research on *Sanchezia nobilis* Hook.F. to use them more effective.

MATERIALS AND METHODS

Plant material

The leaves of *Sanchezia nobilis* Hook.F. were collected in Co Le hamlet, Truc Ninh district, Nam Dinh province, Viet Nam, during January, 2018 and authenticated by the School of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam (No: 190DV18 SMP-VNU). A voucher specimen has been deposited in the SMP-VNU.

General experimental procedures

Melting points were measured on Mikroskopheiztisch PHMK-50 (VEB WaegetechnikRapido, Germany). The FT-IR spectra were recorded on an IMPACT-410FT-IR spectrometer (CARL ZEISS JENA). The NMR [^{1}H (500 MHz), ^{13}C (125 MHz), and DEPT-90 and 135 MHz)] spectra were recorded on an

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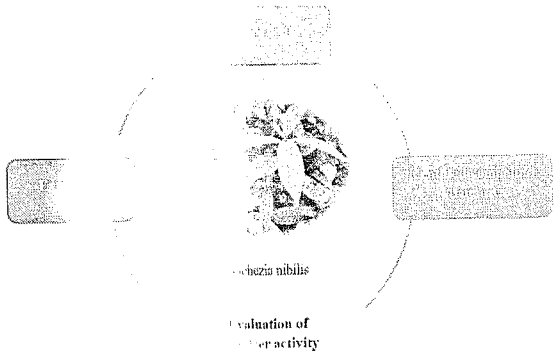
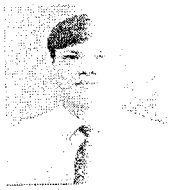
GRAPHIC... AB...



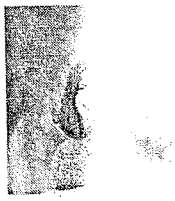
SUMMARY

All the demonstrated data revealed that five compounds were extracted, isolated from the leaves of *Sanchezia nobilis*. Furthermore, evaluation the effect of gastric and duodenal anti-ulcer on cyteamine induced gastric ulcer models indicates at this fractional ethylacetate has the effect to improve the extent of ulcer lesions treatment.

ABOU...



...uc Loi is currently the Head of Department of Pharmacognosy and Traditional Pharmacy, School of ...e and Pharmacy, Viet nam National University, Hanoi, Vietnam. He has over 12 years of experience ...ning Phytochemistry, Pharmacognosy and Medicinal Plant. He is specialized in chemistry of natural ...ts with a special interest in terpenoid, flavonoids and alkaloids. He has published more than 90 sci... ...icles.



...i Thi Xuan is a lecture from School of Medicine and Pharmacy, Vietnam National University. She has ...aching, researching for more than 10 years, published many scientific papers and Phytochemistry-... scientific research topics. She is studying for a PhD in medicine plant. The study focused on finding ...stances in plants and testing the biological effects of different extracts.

...h Ngoc Ph.D, Vice – Director of National Institute of Medicinal Material, Hanoi, Vietnam. He has ...ce in modern spectroscopy and structural elucidation of natural products using 1D and 2D NMR ...HMBC, COSY, ROESY and NOESY) technique, as well as MS (HRMS, GC-MS, LC-ESI-MS) analysis ...rience in a protocol for traditional & modern medicine, and marker compounds in pharmaceutical ...medicine plant. He has published more than 30 scientific articles.

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AN JOURNAL OF CHEMISTRY

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nical Constituents and Antiulcer Activity of *n*-Hexane ract of *Sanchezia nobilis* Hook F. Leaves from Vietnam

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y, Viet nam National University, No.144 Xuan Thuy street, Cau Giay, Hanoi, Vietnam
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ted from the leaves of *Sanchezia nobilis* collected in Nam Dinh province by chromatographic methods. l as: mangiferin (**1**), β-sitosterol (**2**), margaric acid (**3**), ursolic acid (**4**), oleanolic acid (**5**). Their structures methods, including mass spectrometry and nuclear magnetic resonance. These compounds were isolated *bilis* for the first time. Screening of gastric and duodenal antiulcer effects on indomethacin induced *n*-hexane fraction produced the highest antiulcer activity. Percentage inhibition of gastric ulceration of hat of *n*-hexane fraction was 28.57 % ($p < 0.05$). Evaluation of gastric and duodenal antiulcer effects on r models showed that this fraction was effective against gastric and duodenal ulcer (83.3 %), improved ntly reduced the number of mean ulcer and ulcer index (2.00 ± 1.28) but it did not change the area of the

Mangiferin, β-Sitosterol, Margaric acid, Ursolic acid, Oleanolic acid.

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s from *Sanchezia nobilis* roots
y of Shaheen *et al.* [5]. The antibacterial, antifungal and insecticidal activities of the extracts of *Sanchezia speciosa* Hook.F. gave a very positive result [6]. Similarly, the study of antioxidant effect by DPPH and anti-inflammatory by the inhibition of albumine denaturation assay was reported by Thanh *et al.* [7].

Some specific substances were isolated as five matsutake alcohol compounds, four compounds were isolated for the first time from the family Acathanceae and one another compound was isolated from a nature sources [3,8,9]. Ellah *et al.* [10] isolated six compounds from the methanol extracts of leaves and roots of *Sanchezia nobilis*. In Vietnam, *S. nobilis* has been known as a valuable herbal medicine to treat gastritis for a long time. However, there is scarcely any research on the chemical composition of *S. nobilis* and its biological effects. Therefore, it is necessary to study the phytochemical and pharmacological activities of this plant. In this study, the antigastric ulcer effect of *n*-hexane fraction from the extracts of *Sanchezia nobilis* Hook.F. leaves and its chemical composition of this fraction were studied.

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F INTEREST

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Constituents and Antacid Activity of Aqueous the Leaves of *Sanchezia nobilis* Hook.f. from Vietnam

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Abstract

ueous extract of the leaves of Sanchezia nobilis Hook.f. collected in Nam Dinh romatographic methods. Their chemical structures were elucidated by the of melting temperature, rotation angle, mass spectrometry (MS), nuclear ance (NMR) and compared with previously published data. These were 4',5,7-trihydroxy-3',5'-dimethoxyflavone (NX1), kaempferol-3-O-α-L- le (NX2), kaempferol-3-O-β-D-glucopyranoside (NX3). These all compounds rom the leaves of Sanchezia nobilis for the first time. Evaluation the antacid n-hexane, ethylacetate and water fractions from the extract of this plant showed d effect of water fraction was better than the other two fractions, but the remained weaker than that of antacid Antigas.

hezia nobilis Hook.f, 4',5,7-trihydroxy-3',5'-dimethoxyflavone, kaempferol-3- furanoside, kaempferol-3-O-β-D-glucopyranoside.

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INTRODUCTION

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he genus *Sanchezia* s more than 50 species, opical and subtropical Mediterranean, India, A and some Southeast species have existed for tropical rainforests of erica (Ecuador) [1]. In was found in many n Quang, Quang Nam, er provinces Nam Dinh, d Thai Nguyen [2].

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ctivity chemical and nt have been previously . *Sanchezia nobilis* has ntioxidant and anti- in vitro, antibacterial ome of the chemical nt have been identified, noids, glycosides, ds, steroids, phenolic, saponins and tannins [6, 7, 8]. In Vietnam, *S. nobilis* has been known as a very valuable herbal medicine to treat gastritis for a long time. However, there is scarcely any research on the chemical composition of *S. nobilis* and its biological effects in the world and Vietnam both. Therefore, it is necessary to study the phytochemical and pharmacological activities of this plant. In this study, we provide information the chemical composition and the antacid activity of aqueous extract from the leaves of *Sanchezia nobilis* Hook.f.

MATERIALS AND METHODS

Plant Material

The leaves of *Sanchezia nobilis* Hook.f. were collected in Nam Dinh province during January, 2018 and authenticated by the School of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam (No.: 190DV18 SMP-VNU). A voucher specimen has been deposited in the SMP-VNU.

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Cite this Article

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MEDICINAL PLANTS

HYPOGLYCEMIC ACTIVITY OF ISOLATED COMPOUNDS FROM *Gomphrena celosioides* MART

Loi Vu Duc,[1,*] Duong Le Hong,[1] and Giang Dinh Hoang[1]

Original article submitted February 9, 2020.

Five compounds (**1 — 5**) were isolated from the leaves of *Gomphrena celosioides* Mart species harvested in Nam Dinh province and studied by chromatographic method. These compounds were identified as cleomiscosin A (**1**), dictyoceratin- C (**2**), ilimaquinone (**3**), bruceolline F (**4**), and neodactyloquinone (**5**). Their structures were elucidated by spectroscopic techniques including mass spectrometry (MS) and nuclear magnetic resonance (NMR). These compounds were isolated from the leaves of *G. celosioides* for the first time. All compounds were evaluated for their hypoglycemic activity in PTP1B inhibition and glucose uptake into 3T3-L1 adipocytes enhancement models. Results showed that compound 5 produced maximum effect with PTP1B inhibitory activity of 80.39 ± 6.88%, IC50 = 42.78 ± 2.86 μM, as well as the strongest ability to enhance glucose uptake in 3T3-L1 adipocytes.

Keywords: *Gomphrena celosioides* Mart.; *G. celosioides*; cleomiscosin A; dictyoceratin-C; ilimaquinone; bruceolline F; neodactyloquinone.

1. INTRODUCTION

The *Gomphrena* genus (Amaranthaceae family) contains more than 130 species, including native species in Central Malesia to Oceania, tropical and subtropical Americas, and introduced species in Africa, Central Asia and East Asia countries. In Vietnam, plants of the *Gomphrena* genus grow wild in many regions with hot dry climate, seashore, roadsides, railways, especially in the Southwest and Middle regions. *Gomphrena celosioides* Mart. is a member of the Amaranthaceae family. Bamba, et al. [1] proved that there is no risk of intoxication with ethanol extract of *G. celosioides* (EEGC < 5000 mg/kg mice weight), but at doses ≥ 5000 mg/kg it causes hepatocellular hypotension and liver dysfunction [1]. Olalele, et al. [2] reported that EEGC (200 mg/kg) had an anti-inflammatory effect equivalent to sodium diclofenac (10 mg/kg) on carrageenan-induced inflammation in rats [2]. Meite, et al. [3] evaluated the antioxidant effect of EEGC using serum thiobarbituric acid reduction activity. In [3, 4] *G. celosioides* was shown to contain many important compounds, including alkaloids (aurantiamide), flavonoids (gomphrenol), betalains (gomphrenin), saponins, steroids, tanins, amino acids, terpenoids, butacyanins, etc. According to some studies, aurantiamide has important properties, in particular antioxidant [5], anti-inflammatory and antiviral [6], antibacterial and immunomodulating [7]. These findings supported the use of this plant in folk medicine to treat gastrointestinal, respiratory or infectious diseases. In Vietnam, people advice to use *G. celosioides* (bachelor's button) as a precious medicine to cure diabetes. However, studies on chemical composition and biological effects of this plant in Vietnam and over the world are still limited.

Therefore, in order to provide more data on bachelor's button, as well as to help guide the use of this medicinal herbs more effectively, this study provides information on the hypoglycemic effects and the chemical composition of n-hexane fraction from *G. celosioides* leaves extract.

2. MATERIALS AND METHODS

2.1. Plant Material

The leaves of *G. celosioides* Mart. were collected in Nam Dinh province in January, 2018 and verified by School of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam (No: 18 SMP-VNU). A voucher sample has been deposited in the SMP-VNU.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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ANTIOXIDANT ACTIVITY, INHIBITION OF NO PRODUCTION AND CYTOTOXICITY OF CHEMICAL COMPOUNDS ISOLATED FROM *Oxalis corniculata* L.

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Five flavonoid compounds were isolated from ethyl acetate extract of the aerial part of *Oxalis corniculata L.* and identified as: 4′,5,7-trihydroxy-6-methoxyisoflavon (tectorigenin **1**), 3′,5,7-trihydroxy-4′,5′,6-trimethoxyisoflavon (irigenin **2**), 4′,5,7-trihydroxy-3′,6-dimethoxyisoflavon (iristectorigenin A **3**), kaempferol-3,7-di-O-α-L-rhamnopyranosid (kaempferitrin **4**), and kaempferol-3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranosid (arapetaloside B **5**). All the isolated compounds showed potential antioxidant effects in DPPH radical scavenging activity test with IC_{50} values in the range from 28.62 to 48.53 μg/mL, varied in following order: compound **1** > **3** > **2** > **5** > **4**. The five phytochemicals produced inhibition of nitric oxide (NO) production, with IC_{50} values ranging from 18.6 to 39.5 μM. All isolated compounds exhibited cytotoxicity against HepG2, LU-1, MCF-7 and SK-Mel-2 cells using Sulforhodamine B assay, with IC_{50} values in the range ftom 12.32 to 39.88 μM.

Keywords: *Oxalis corniculata* L.; flavonoids; tectorigenin; irigenin; iristectorigenin A; kaempferitrin; arapetaloside B; antioxidant; inhibition of NO production; cytotoxic activity.

1. INTRODUCTION

The genus *Oxalis* (family *Oxalidaceae*) includes more than 900 species worldwide, distributed in the tropics and subtropics [1]. Currently, in Vietnam, there are four species of the genus *Oxalis*, including: *O. acetosella* L., *O. corymbosa* DC., *O. deppei* Sw., and *O. corniculata* L., of which three are used as medicines and *O. corniculata* is the most common one [2]. It has been shown that *O. corniculata* contains some groups of compounds such as flavonoids, tannins, oxalate salts, and fatty acids. A large amount of oxalate is the main cause of the plant's characteristic acidity [3 – 6]. In 2012, Srikanth, et al. [7] conducted a phytochemical and pharmacodynamic study and isolated three glycosylflavones from the leaves of *O. corniculata*, namely 6-C-glucosyl luteolin (isoorientin), 6-C-glucosyl apigenin (isovitexin) and isovitexin 7-methylether (sertisin). In addition, in 2013, Ibrahim, et al. [1] successfully isolated corniculatin A, a new flavonoid glucoside, along with luteolin, luteolin-7-O-β-D-glucoside, and β-sitosterol-3-O-β-D-glucoside from ethyl aceatate extract of *O. corniculata*. Since *O. corniculata* contains a large amount of compounds such as flavonoids, vitamin C, carotene, that have extremely antioxidant effects, shown in their stable and better peroxyde inhibitory activity compared to standard butylated hydroxyanisole [8, 9]. Methanol extract of *O. corniculata* inhibits the release of neutrophil lysosome components at the site of inflammation, exhibits significant antiproteinase activity, inhibits on albumin denaturation due to heat. The resulting anti-inflammatory activity is possible due to the presence of related flavonoids and polyphenols [10]. In addition, β-sitosterol isolated from the petroleum ether extract of the leaves of *O. corniculata* has analgesic and anti-inflammatory effects by central mechanism through opoid receptors [11]. The cytotoxicity of ethanol and methanol extracts of *O. corniculata* was assessed by MTT assay against Hep2 [12]. Kathiriya, et al. [13] showed that the ethanol extract of *O. corniculata* gave anticancer activities on ascites carcinoma (EAC) in Swiss albino rats. Other researches evaluating the anticancer actilitv of ethanol extract of *O. corniculata* on ascites carcinoma (EAC) in rats also showed similar results [13, 14].

With the aim of providing more evidential information of antioxidant activity, inhibition of NO formation, and cytotoxicity of *O. corniculata*, we proceeded a research and

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3.3. Evaluation of NO Production Inhibition

Five compounds were evaluated for their NO production inhibition activity in LPS stimulated BV2 cells. Each compound was screened at a concentration of 100 μM, showing potential inhibitory activity with inhibition percentages from 70.2 ± 3.6 to 83.5 ± 4.9 μM. Next, those compounds were studied dose-dependent response to find their IC_{50} values. The results indicated that compounds **1 – 5** exhibited as promising NO inhibitors with IC_{50} values of 18.6 ± 0.8, 23.5 ± 1.3, 22.4 ± 1.1, 39.5 ± 2.1 and 36.8 ± 1.5 μM, respectively (Fig. 3/Table 2). Butein, the well-known inhibitor on NO production in LPS stimulated BV2 cells, was used as a positive control throughout experiments.

In summary, the obtained results suggest that compounds **1 – 5** have promising inhibition the formation of NO. We suggest to evaluate the isolated compounds on other inflammatory mechanisms, to as to have an adequate evaluation of their potential anti-inflammatory activity.

3.4. Cytotoxic Activity Evaluation

Cytotoxic effects toward HepG2, LU-1, MCF-7 and SK-Mel-2 human cancer cell lines of the isolated five compounds were evaluated using the SRB assay. As shown in Fig. 4/Table 3, compounds 1=-5 displayed cytotoxicity toward all tested cancer cells, with IC_{50} values ranging from 12.32 ± 1.19 to 39.88 ± 1.20 μM. These results indicate that compounds **1 – 5** isolated from *O. corniculata* could serve as promising candidates for anticancer drugs. Further studies are required to confirm and clarify the mechanism of cytotoxic activity of these compounds, especially of compound **5** which exhibited the most promising IC_{50} value.

4. CONCLUSION

O. corniculata is a valuable medicinal plant that has been used to cure diseases according to folk experience. After the experimental research, five flavonoid compounds were isolated and identified as: 4′,5,7-trihydroxy-6-methoxyisoflavon (tectorigenin **1**), 3′,5,7-trihydroxy-4′,5′,6-trimethoxyisoflavon (irigenin **2**), 4′,5,7-trihydroxy-3′,6-dimethoxyisoflavon (iristectorigenin A **3**), kaempferol-3,7-di-O-α-L-rhamnopyranosid (kaempferitrin 4), and kaempferol-3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranosid (arapetaloside B **5**). Our research data showed that these compounds have very potent antioxidant, NO production inhibition, and cytotoxic activities. Therefore, this research contributes scientific addition of *O. corniculata* to its database, future research and application of the plant.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding publication of this paper.

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Original Research Article

Anti-Ulcer Effect on Indomethacin-Induced Ulcerated Mice of *Chromolaena odorata* Leaf from Vietnam and its Secondary Metabolites

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ABSTRACT

Chromolaena odorata (*C. odorata*) (L.) R. King & H. Robinson is a perennial flowering shrub with diverse habitats, including crops, grasslands, and roadsides. This plant has been widely used in Vietnamese folk medicine for gastric ulcer treatment. Hence, the present study aimed to evaluate the acute toxicity and the anti-ulcer effect of the ethanol crude extract of *C. odorata* leaves and its fractions against the indomethacin-induced gastric ulcer model in mice, and investigate the chemical constituents of the most active fraction. According to *in vivo* results, the ethyl acetate residue with the highest anti-ulcer activity significantly reduced gastric lesions in the experimental mice model with an ulcer index of 0.73 ± 0.39 and a percentage inhibition of 26.92%. Thus, this fraction was chosen for further chemical investigation. Four pure compounds (1-4) were extracted and isolated by using chromatographic methods. Based on the nuclear magnetic resonance spectroscopy, melting temperature, mass spectrometry analysis, and compared with the published literature, their structures were elucidated as 1-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol (**1**), kaempferol-7-*O*-*α*-L-rhamnopyranoside (**2**), naringenin-5,7-di-*O*-*β*-D-glucopyranoside (3), and rubrosterone (4). To our best knowledge, all of these compounds were isolated for the first time from *C. odorata* leaves. These findings contribute to providing scientific evidence for the traditional use and phytochemicals of *C. odorata* leaves.

Keywords: *Chromolaena odorata*, anti-ulcer, indomethacin, phytochemicals.

Introduction

Peptic ulcer disease, including gastric and duodenal ulcers, is responsible for the increase in morbidity and mortality worldwide.[1] The main causes of almost peptic ulcer disease cases are associated with the infection of *Helicobacter pylori* and the frequent administration of nonsteroidal anti-inflammatory drugs (NSAIDs). In general, the medication therapies for peptic ulcers embraces antacids, anticholinergics, histamine-2-receptor antagonists, antibiotics, proton pump inhibitors, sucralfate, and bismuth.[2] However, these anti-ulcer agents exhibit many serious side effects such as impotence, hypersensitivity, skin rash, constipation, headache, arrhythmia, urinary retention, atrophic gastritis, blurred vision, hematopoietic alterations, gynecomastia, xerostomia.[3] Therefore, the demand for finding and developing herbal drugs with fewer adverse effects is increasing significantly.

Chromolaena odorata (*C. odorata*) (L.) R. King & H. Robinson is a medicinal plant of the *Chromolaena* genus, which was identified by King and Robinson in 1970. It also has other scientific names such as *Eupatorium conyzoides* M., *Eupatorium odoratum* L., and *Osmia odorata* L.[4] *C. odorata* is a spreading, clustering shrub that is mainly found in crops and grasslands in South Asia and West Africa.[5] This species is native to Asia, North and South America, West and South Africa, and Australia.[6,7]

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According to modern pharmacological investigations, *C. odorata* has potents of antioxidant, antidiabetic, anti-inflammatory, anti-fungal, antimicrobial, anti-dyslipidemia, anticancer, and cytoprotective activities.[8] It is proven that the chemical composition of *C. odorata* includes flavonoids (chalcone, flavone, flavonol, and aurone), alkaloids, tannins, phytates, saponins, steroids, diterpenes, anthraquinones, phenolic acids, and cyanogenic glycosides, which take responsibility for these activities.[9,10] Besides, a previous study revealed that the dried leaves of *C. odorata* contain flavonoid aglycones, terpenes, triterpenoids, saponins, tannins, and phenolic acids.[11] In addition, the leaves of *C. odorata* have traditionally been commonly used for the treatment of diarrhea, wounds, headache, skin diseases, inflammatory diseases, and stomach ulcers.[8,12] In Vietnamese folk medicine, a remedy including *Chromolaena odorata* 30 g, *Ardisia silvestris* 30 g, *Herba Hedyotidis capitellatae* 20 g, and *Stahlianthus thorelii Gagnep* 5 g is utilized to improve the stomach ulcer condition.[13] Due to the capability of good anti-ulcer activity, fast-growing, high adaptability, and wide distribution, the potential for developing raw materials and creating anti-ulcer products from the leaves of *C. odorata* is enormous. To contribute to providing the evidence for the usage and the premise for the developing anti-ulcerogenic agents derived from *C. odorata* leaves, this study aimed to evaluate the anti-ulcer effects of ethanol crude extract and its fractions of *C. odorata* leaves on the indomethacin-induced gastric ulcer mice model and investigate the phytochemical compounds of the most active fraction.

Materials and Methods

General experimental procedures

Indomethacin was purchased from Kwality Pharmaceutical, India. Misoprostol was obtained from Unimed Pharm, Korea. All other chemicals used in this research were of analytical grade. Silica gel (Merck, 0.040 – 0.063 mm) and Sephadex LH-20 (Sigma-Aldrich) were used for column chromatography. Thin layer chromatography was

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SHORT COMMUNICATION

Anti-diabetic Effect of Major Compounds from *Commelina diffusa*

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Abstract
Although *Commelina diffusa* Burm.f., Commelinaceae, has been proven to exhibit many pharmacological activities, the scientific evidence for its antihyperglycemic activities and active substances is still limited. This present study aims to evaluate the *in vitro* antidiabetic ability of *C. diffusa* using α-glucosidase and α-amylase inhibitory assays and isolate its pure compounds with enzymatic inhibitory effects. The ethyl acetate fraction, with the most potent hypoglycemic activity, was chosen to separate and purify six known compounds: 4-hydroxybenzoic acid, methyl gallate, lyratol F, *N-trans*-feruloyltyramine, *N-trans-p*-coumaroyl-3′,4′-dihydroxyphenylethylamine, and 1,2-dihydro-6,8-dimethoxy-7-hydroxy-1-(3,5-dimethoxy-4-hydroxyphenyl)-N^1,N^2-bis-[2-(4-hydroxyphenyl)ethyl]-2,3-naphthalene dicarboxamide (**6**). Furthermore, the antihyperglycemic effect of the isolated compounds was also evaluated *in vitro* on α-glucosidase and α-amylase enzymes. Compound **6** exhibited the most potent inhibitory activity against both tested enzymes with values of 61.37 ± 0.83 μM in the α-glucosidase assay and 38.23 ± 1.04 μM in the α-amylase assay. These values were much higher than those of positive control (acarbose IC_{50} 210.43 ± 2.78 and 129.19 ± 3.13 μM, respectively). This is the first report on the antidiabetic capacity of *C. diffusa* and its hypoglycemic pure compounds. Our findings suggested that *C. diffusa* might be a promising source of antidiabetic agents.

Keywords Chemical constituents · Spectral analysis · Phenolics · Hypoglycemia · α-Glucosidase inhibition · α-Amylase inhibition

Introduction

Diabetes mellitus is one of the most common non-communicable diseases globally. It causes many dangerous complications and is the leading cause of cardiovascular disease, blindness, kidney failure, and lower-limb amputation (Mandal et al. 2021). Type 2 diabetes, which was previously known as non-insulin-dependent diabetes or adult-onset diabetes, takes responsibility for over 90% of all diabetes cases (Patel et al. 2012). Diabetes mellitus cannot be completely cured. The main goal of hyperglycemia management is maintaining the glycemic levels as close to the non-diabetic range as possible, which can be achieved by proper diet, exercise, and pharmacological approaches. In general, medication therapies are mainly used to lower blood glucose or provide temporary insulin replacement for diabetic patients. However, these existing agents are expensive and expose side effects such as jaundice, diarrhea, headache, liver toxicity, or itchy skin (Nathan et al. 2006; Scheen 2007; Chiabchalard and Nooron 2015). Thus, it is very essential to research and develops antihyperglycemic drugs derived from natural sources, especially medicinal plants that have been widely used in tradition.

Commelina diffusa Burm.f., Commelinaceae, is widely distributed in both tropical and subtropical regions of Africa, America, Southeast Asia, and the Pacific Islands (Boyette et al. 2015). In Vietnam, *C. diffusa* can be commonly found in airy and moist areas including fields, gardens, roadsides, ditches, wastelands, and riverbanks. It is proven that the chemical constituents of *C. diffusa* contain

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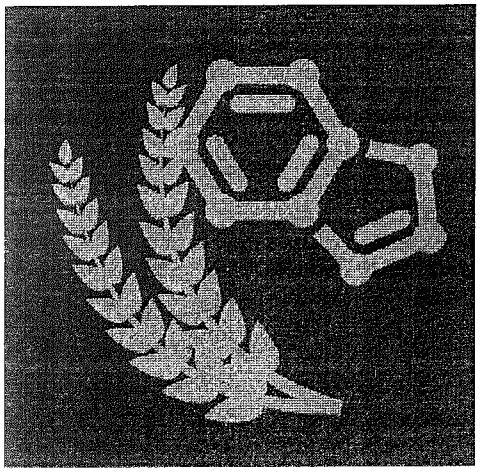

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Article

Cinnamomum cassia and *Rosa laevigata* Mixture Improves Benign Prostatic Hyperplasia in Rats by Regulating Androgen Receptor Signaling and Apoptosis

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Abstract: Benign prostatic hyperplasia (BPH) is the most common condition in elderly men that is characterized by an increase in the size of the prostate gland. *Cinnamomum cassia* and *Rosa laevigata* have been reported to treat the symptoms associated with BPH. The aim of this study was to evaluate the effects of HT080, an herbal extract of *C. cassia* and *R. laevigata*, on a testosterone propionate (TP)-induced BPH rat model. The rats received a daily subcutaneous injection of TP (3 mg/kg) for 4 weeks to induce BPH. Rats were divided into four groups: group 1 (sham), group 2 (BPH, TP alone), group 3 (Fina, TP + finasteride 1 mg/kg/day), and group 4 (HT080, TP + HT080 200 mg/kg/day). At the end of the experiment, all rats were sacrificed, and their prostate glands were removed, weighed, and subjected to histopathological examination and western blot analyses. Serum testosterone and dihydrotestosterone (DHT) levels were determined. In addition, serum alanine and aspartate aminotransferase levels were measured to evaluate the toxicity in the liver. The Hershberger bioassay was also conducted to investigate the effects of HT080 on androgenic and antiandrogenic activities. In the BPH model, the prostate weight, prostate index, prostate epithelial thickness, and serum testosterone and DHT levels in the HT080 group were significantly reduced compared to the BPH group. Histological studies showed that HT080 reduced prostatic hyperplasia. The protein expression of androgen receptor from the HT080 group was significantly reduced in comparison with the BPH group ($p < 0.05$). HT080 also induced apoptosis by regulating Bcl-2 and Bax expression. In addition, HT080 showed no toxicity in the liver and did not exhibit androgenic and antiandrogenic activities. Our finding revealed that HT080 can be a potential candidate for the treatment of BPH by regulating androgen receptor signaling and apoptosis.

Keywords: *Cinnamomum cassia*; *Rosa laevigata*; benign prostate hyperplasia; apoptosis; androgen receptor

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1. Introduction

Benign prostate hyperplasia (BPH) or prostate gland enlargement is the most common condition in middle-aged and elderly men regardless of their culture or ethnic origins [1,2]. It is characterized by an increase in the size of the prostate gland and not cancer-causing lower urinary tract symptoms (LUTSs) such as nocturia, weak urinary stream, urgency, and hesitancy [3,4]. It has been reported that the risk of BPH increases with age from 8% at age 31 to 40 years to 40–50% at age 51 to 60 years and to over 80% at age 80 years [5,6]. Much research revealed that risk factors for BPH and LUTS include hormonal alterations, obesity, diet-induced hyperinsulinemia, inflammation, lack of exercise, or glucose homeostasis as hyperglycemia [7–12]. Among them, hormonal alterations are considered the main causes of BPH, which leads to the imbalance of growth and apoptosis of prostate cells. Testosterone and dihydrotestosterone (DHT) are two main androgens that play a crucial

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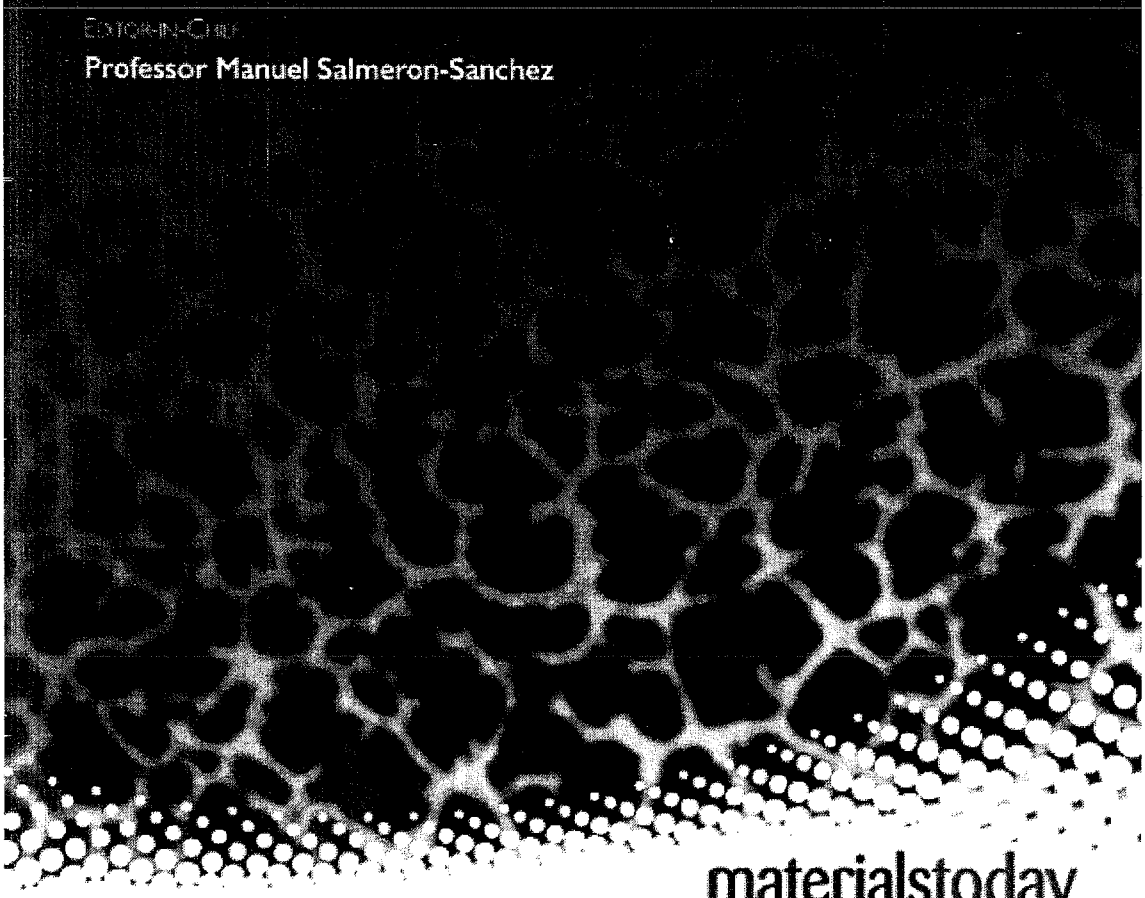

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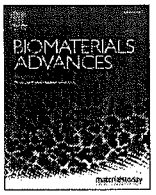

Sialic acid-decorated liposomes enhance the anti-cancer efficacy of docetaxel in tumor-associated macrophages

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ABSTRACT

Tumor-associated macrophages (TAMs) in the tumor microenvironment potentially enhance tumor growth and invasion through various mechanisms and are thus an essential factor in tumor immunity. The highly expressed siglec-1 receptors on the surfaces of TAMs are potential targets for cancer drug delivery systems. Sialic acid (SA) is a specific ligand for siglec-1. In this study, the sialic acid-polyethylene glycol conjugate (DSPE-PEG_{2000}-SA) was synthesized to modify the surface of liposomes and target TAMs by interacting with the siglec-1 receptor. Three docetaxel (DTX)-loaded liposomes, conventional (DTX-CL), DSPE-PEG_{2000}-coated (DTX-PL), and DSPE-PEG_{2000}-SA-coated (DTX-SAPL) liposomes, were prepared, with a particle size of <100 nm, uniform polydispersity index (PDI) values, negative zeta potential, and % encapsulation efficiency (EE) exceeding 95 %. Liposomes showed high stability after 3 months of storage at 4 °C without significant changes in particle size, PDI, zeta potential, or % EE. DTX was released from liposomes according to the Weibull model, and DTX-SAPL exhibited more rapid drug release than other liposomes. *In vitro* studies demonstrated that DTX-SAPL liposome exhibited a higher uptake and cytotoxicity on RAW 264.7 cells (TAM model) and lower toxicity on NIH3T3 cells (normal cell model) than other formulations. The high cell uptake ability was demonstrated by the role of the SA-SA receptor. Biodistribution studies indicated a high tumor accumulation of surface-modified liposomal formulations, particularly SA-modified liposomes, showing high signal accumulation at the tumor periphery, where TAMs were highly concentrated. *Ex vivo* imaging showed a significantly higher accumulation of SA-modified liposomes in the tumor, kidney, and heart than conventional liposomes. In the anti-cancer efficacy study, DTX-SAPL liposomes showed effective inhibition of tumor growth and relatively low systemic toxicity, as evidenced by the tumor volume, tumor weight, body weight values, and histopathological analysis. Therefore, DSPE-PEG_{2000}-SA-coated liposomes could be promising carriers for DTX delivery targeting TAMs in cancer therapy.

1. Introduction

Targeted drug delivery systems have recently garnered a lot of attention in cancer therapy. The underlying mechanism of these systems is selective drug delivery to the tumor site, which improves drug effectiveness and reduces toxicity [1]. In addition, enhanced permeation and retention (EPR) aids drug delivery to tumors [1,2]. However, the efficacy of cancer therapy based on EPR is controversial because its permeability is limited at the tumor site. The extracellular matrix stiffness and selective pressures from the tumor microenvironment (TME) complicate the delivery of nanoparticles to the blood vessels and cancer cells [3].

The TME comprises numerous immune cell types, such as mast cells, neutrophils, and tumor-associated macrophages (TAMs), which account for approximately 50 % of the total tumor volume [4,5]. Unlike normal macrophages, TAMs are nontumor-immunoreactive and incapable of inhibiting tumor growth. Instead, they secrete factors that promote tumor immunosuppression and facilitate tumor proliferation and invasion [6]. Owing to the aforementioned characteristics, targeting TAMs could be an effective immunotherapy approach in the treatment of tumors.

A common approach toward enhancing the permeation and targeting

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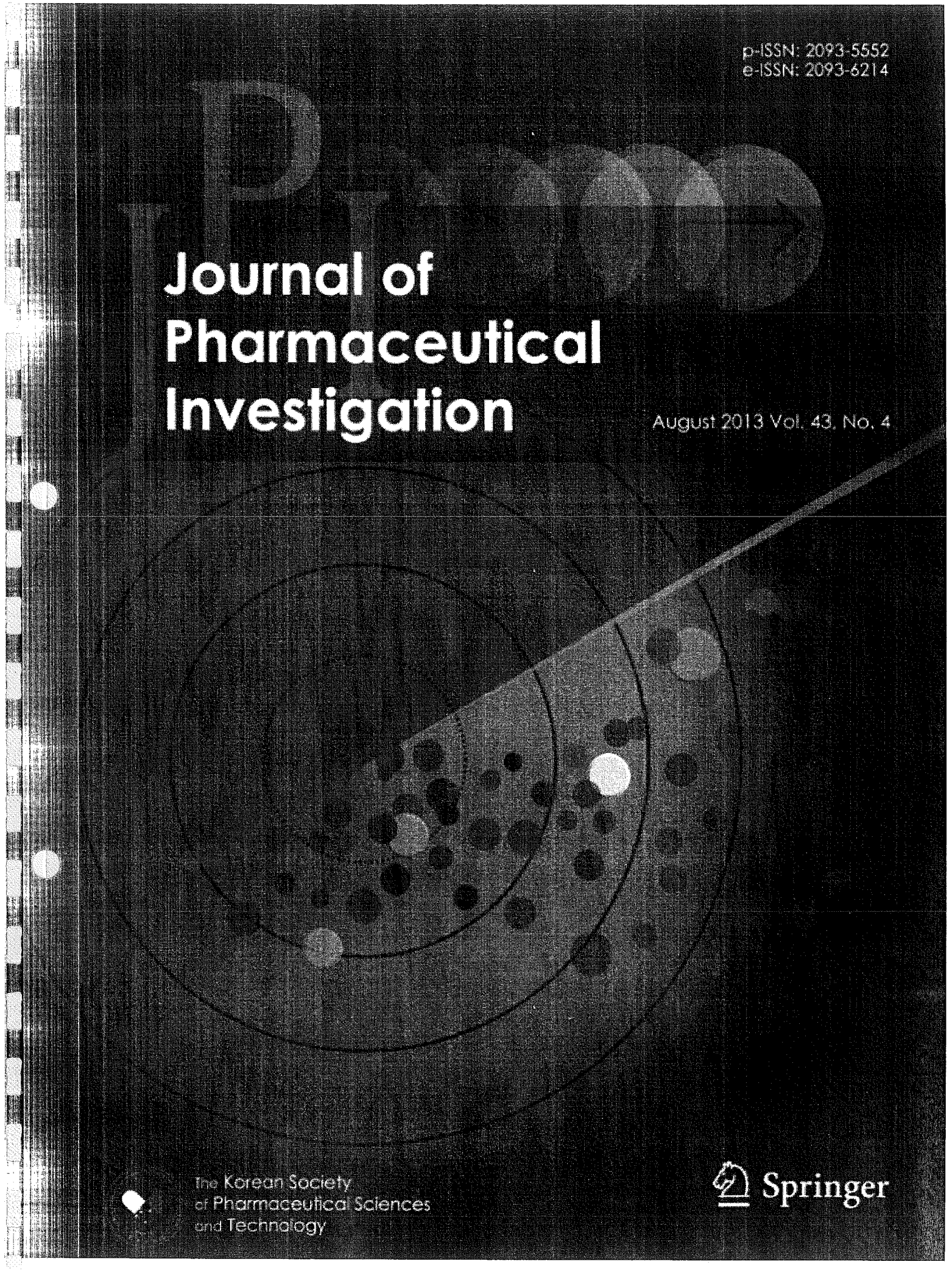

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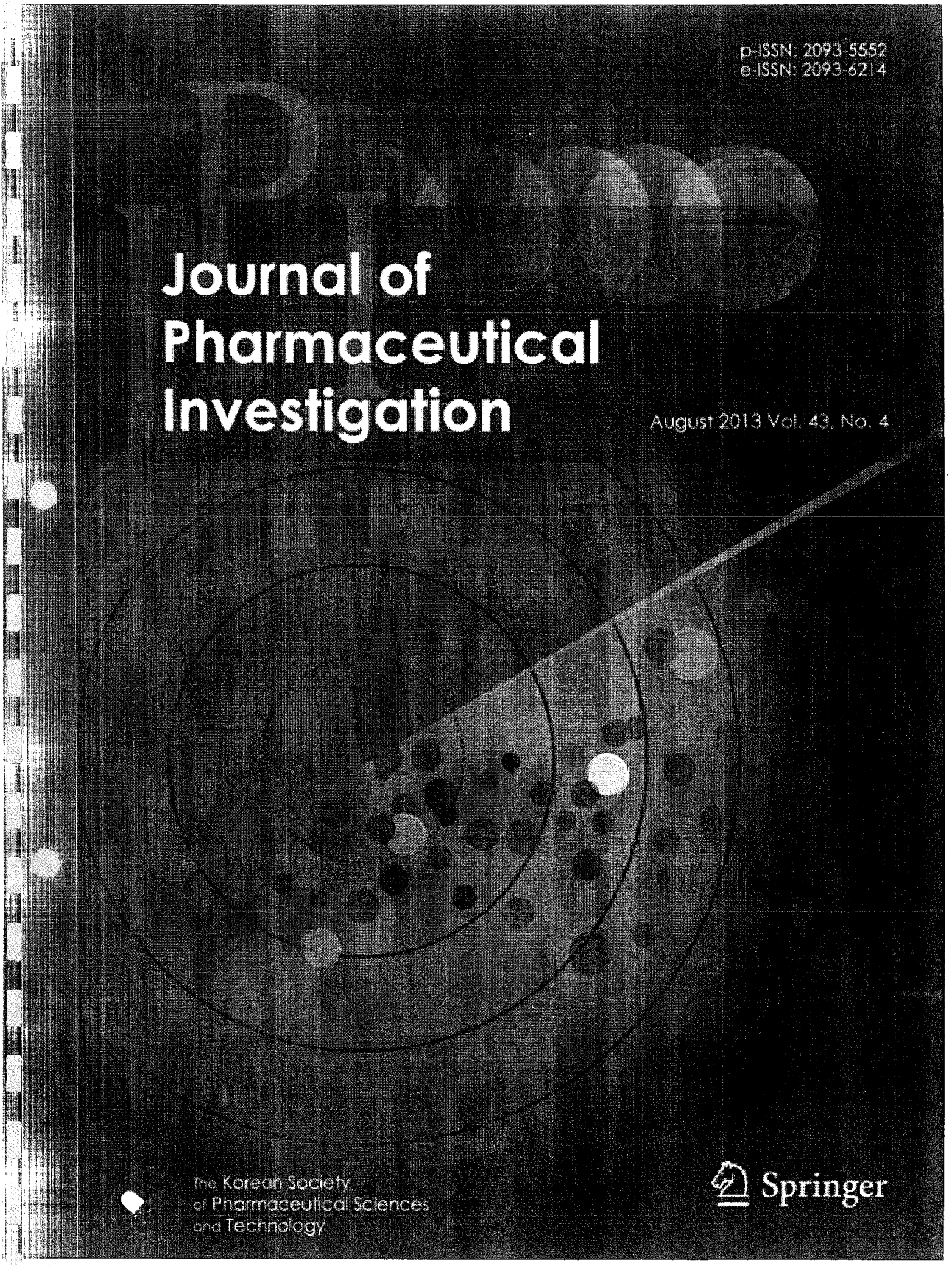

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ORIGINAL ARTICLE

Alginate-coated chitosan nanoparticles protect protein drugs from acid degradation in gastric media

Phuong Tran[1] · Jeong-Sook Park[1]

Received: 8 December 2021 / Accepted: 26 April 2022

Abstract

Purpose The aim of this study was to design and evaluate chitosan nanoparticles (CS NPs) coated with alginate which protect protein drugs from acid degradation.K The model protein drug used was bovine serum albumin (BSA).

Methods BSA-loaded CS NPs (BSA-CS NPs) were prepared using the ionic gelation method with sodium tripolyphosphate and the surface of the BSA-CS NPs were coated with sodium alginate (Alg). The optimized alginate-coated BSA-CS NPs (Alg-BSA-CS NPs) were evaluated for BSA degradation in an acidic medium.

Results The encapsulation efficiency (EE), particle size, polydispersity index, and zeta potential of the prepared Alg-BSA-CS NPs were 95.2%, 476.4 nm, 0.24, and − 53.8 mV, respectively. An in vitro release study showed that the initial burst release of BSA from the BSA-CS NPs was higher than that from the Alg-BSA-CS NPs. Cytotoxicity analysis revealed that the Alg-BSA-CS NPs were non-toxic to Caco-2 cells. The in vitro cellular uptake of the Alg-BSA-CS NPs in Caco-2 cells was significantly higher than that of the BSA-CS NPs and free BSA. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that the Alg-BSA-CS NPs protected BSA from degradation in an acidic environment.

Conclusion Alg-BSA-CS NPs are suitable for the oral delivery of protein drugs by preventing protein degradation in acidic environments.

Keywords Protein protection · Acid degradation · Nanoparticles · Chitosan · Alginate · Oral delivery

Introduction

With advances in biotechnology in recent years, protein drugs have received increasing attention for the treatment of various diseases owing to their high potency and low toxicity (Frokjaer and Otzen 2005). Oral administration is the preferred route of drug delivery. However, the absorption of protein drugs from the gastrointestinal (GI) tract is usually hampered by instability and degradation caused by stomach acids, resulting in decreased drug efficacy and poor bioavailability. Therefore, the development of novel protein drug formulations to overcome these problems is an important research subject in the pharmaceutical industry (Torchilin and Lukyanov 2003; Al-Tahami and Singh 2007). Various strategies have been developed to overcome these problems, such as the use of microparticles and nanoparticles (NPs) (Su et al. 2012; Jain et al. 2012; Muheem et al. 2016), liposomes (Okada et al. 1997; Kurz and Ciulla 2002; Mohanraj et al. 2010), micro/nanoemulsions (Mueller et al. 1994; Park et al. 2011; Patil et al. 2019), and solid core particles (Müller et al. 2000; Sarmento et al. 2007). Among these, nanoparticles (NPs) have recently received considerable attention. NPs prepared for protein drugs typically use carriers, such as gelatin, starch, or chitosan (CS), for the controlled release of protein drugs (Ghormade et al. 2011).

In this study, CS, a polysaccharide derived from chitin, was used to prepare NPs. CS is a potent natural polymer consisting of β-(1–4)-linked d-glucosamine (deacetylated units) and N-acetyl-d-glucosamine (acetylated units) (Sinha et al. 2004; Wang et al. 2005; Amidi et al. 2010; Kumari et al. 2010). It is insoluble in water but soluble in acidic solutions. Additionally, it provides many advantages as a carrier for NPs, such as biodegradability, biocompatibility, mucoadhesiveness, and low toxicity (Shu and Zhu 2002; Sinha et al. 2004; Amidi et al. 2010). However, the CS formulation can be deprotonated in a physiological environment, causing it to lose its mucoadhesive properties and permeation-enhancing

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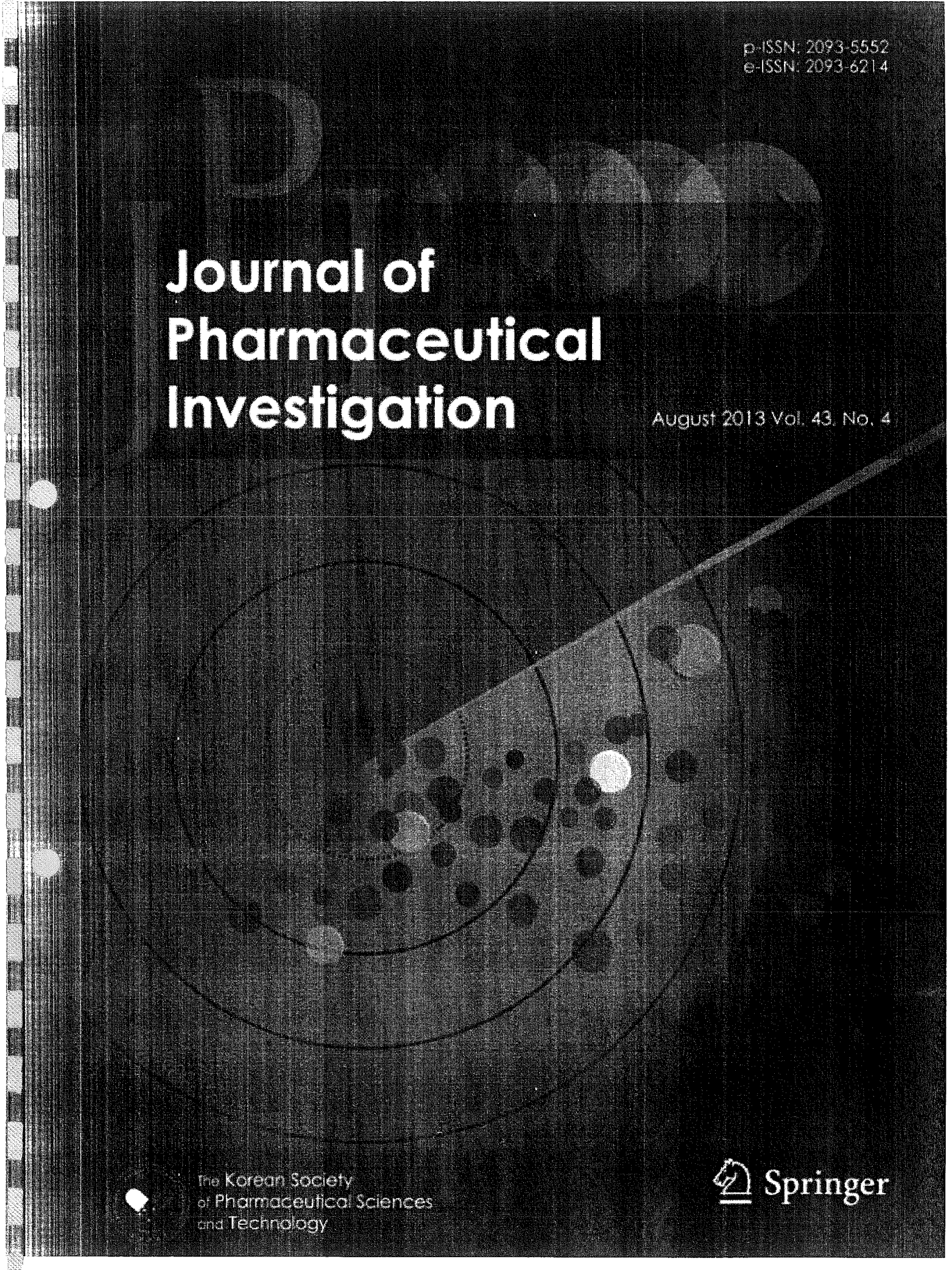

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REVIEW

Recent advances of nanotechnology for the delivery of anticancer drugs for breast cancer treatment

Phuong Tran[1] · Sang-Eun Lee[1] · Dong-Hyun Kim[1] · Yong-Chul Pyo[1] · Jeong-Sook Park[1]

Received: 9 June 2019 / Accepted: 20 August 2019

Abstract

Background Breast cancer is one of the most common causes of death for women worldwide. While chemotherapy is the treatment option for most cancers, surgery, chemotherapy, and radiotherapy are the three main therapeutic strategies for the treatment of breast cancer. In recent years, nanotechnology applications for cancer treatments have attracted a lot of attention.
Area covered This review focuses on the various nanoparticle types, such as liposomes, micelles, polymeric nanoparticles, solid lipid nanoparticles, and gold nanoparticles, and their applications for the treatment of breast cancer.
Expert opinion In recent decades, nanotechnology has developed and been applied to cancer treatments. Currently, nanotechnology plays an important role in the targeted delivery of drugs for cancer treatments, including breast cancer. Nanoparticles can target tumors and control the release of drugs to precise sites, thereby improving the therapeutic efficiency of drugs and decreasing the toxicity to normal tissues or organs. In addition, nanoparticles are also able to activate immune cells against tumors. Therefore, nanoparticles are a promising tool for future cancer research and treatment.

Keywords Nanoparticles · Breast cancer · Drug delivery · Anticancer drugs

Introduction

Cancer is one of the leading causes of death worldwide and is defined as a disease that begins when cells grow uncontrollably and crowd out normal cells. Cancer can develop anywhere in the body, such as in the lungs, breasts, or liver. The World Health Organization predicted that the burden of cancer will increase to 23.6 million new cases annually by 2030 (World Health Organization 2014). Thus, cancer treatment has become a prominent issue over the past several decades. For women, breast cancer is one of the most commonly diagnosed cancers globally. In 2018, approximately 266,120 new cases of invasive breast cancer were estimated in women constituting 30% of all cancer cases (878,980 total cases); in addition, 40,920 of these breast cancer cases were estimated to be fatal (American Cancer Society 2018). Breast cancer is usually classified on the basis of the type of receptor overexpression present on the cancer cell membrane (Fig. 1), including progesterone (PR) and estrogen (ER) hormone receptors and HER2 receptors, with HER2 being a member of the human epidermal growth factor receptor family. Breast cancers that present the overexpression of these receptors are called either PR−, ER−, or HER2-positive, depending on the type of receptor overexpression. Patients that show PR−, ER−, HER2-positive breast cancer cells are said to have triple-positive breast cancer. In addition, triple-negative breast cancer group exists that is composed of breast cancers that are neither PR/ER-positive nor HER2-positive. It has been reported that the primary cause of deaths due to breast cancer is the result of its potential metastasis to distant organs such as the liver, lungs, lymph nodes, bones, and brain (Carty et al. 1995; Grobmyer et al. 2012).

Currently, surgery (in which whole breast is removed, called a mastectomy, or in which only the tumor and surrounding tissues are removed, called a breast-conserving lumpectomy), chemotherapy (in which drugs are used to kill cancer cells), and radiotherapy (in which high-energy waves are used to kill cancer cells) are the three main cancer treatment strategies (Shewach and Kuchta 2009). Among them, chemotherapy is more popularly used for treating most types of cancer. Chemotherapy can kill many cancer cells throughout the body, eradicate microscopic disease at the

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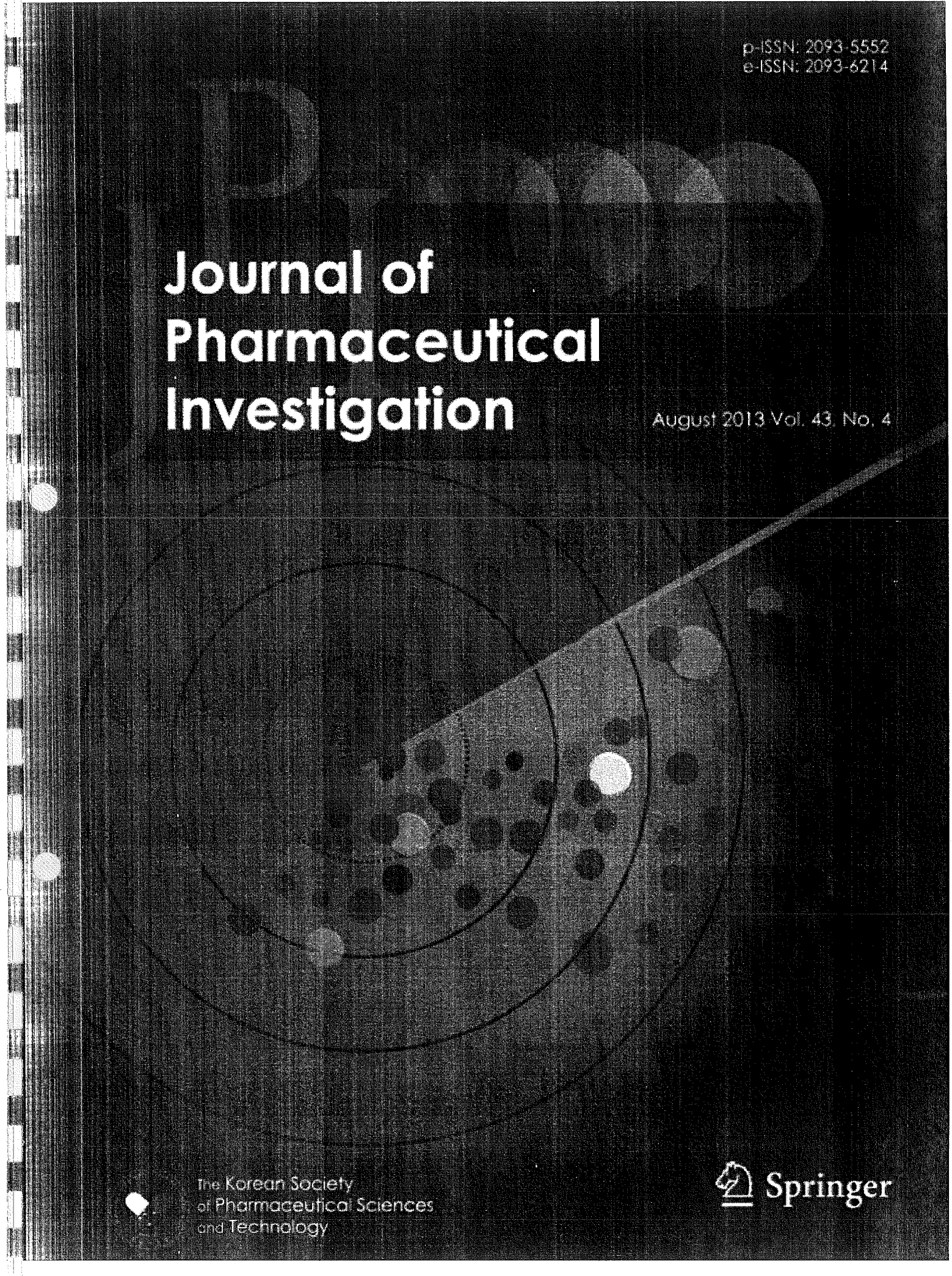

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REVIEW

Recent trends of self-emulsifying drug delivery system for enhancing the oral bioavailability of poorly water-soluble drugs

Phuong Tran[1] · Jeong-Sook Park[1]

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Abstract

Background The oral route is the most popular route for the clinical administration of drugs to treat various diseases. Before a drug is absorbed into the blood circulation, it must undergo dissolution and permeation. However, most drugs exhibit poor aqueous solubility, and their limited absorption leads to low oral bioavailability. The solubility of hydrophobic drugs can be improved by various ways, such as solid dispersion, salt formation, pH modification, and self-emulsifying drug delivery system (SEDDS) use. Among them, the SEDDS has garnered attention during recent years as it improves oral bioavailability, reduces drug dose, and increases drug protection from unsuitable environment in the gastrointestinal tract.

Area covered SEDDS comprises lipid-based formulations. It can solve the problems related to the dissolution and bioavailability of the Biopharmaceutics Classification System Class II and IV drugs. Depending on the preparation procedure, drug-loaded SEDDS can be divided into micro- (SMEDDS) and nano- (SNEDDS) formulations. In this review, we summarize the classification system of lipid formulations, the mechanism underlying improved oral drug absorption by SEDDS, and recent advances in the SEDDS.

Expert opinion The SEDDS is a potential formulation for drug delivery. Owing to its small particle size, large surface area, high encapsulation efficiency, and high drug loading, the SEDDS can improve the rate and extent of oral absorption by maximizing drug solubility in the intestinal absorption site. Moreover, because of the lipid-based formulation of SEDDS, it can stimulate and enhance lymphatic transport of drugs to avoid hepatic first-pass metabolism, and thus improve their bioavailability.

Keywords SEDDS · Oil · Surfactant · Co-surfactant/co-solvent · Bioavailability

Introduction

Oral and intravenous (IV) routes are the two most commonly used routes for drug administration. Practically, IV injection is the best administration route for most drugs because 100% of the drug is directly introduced into the blood circulation system. However, IV injections have several limitations including the discomfort from hospitalization, need for sterile needles, difficulty in needle insertion, and need for trained medical staff for IV injection. Therefore, oral administration is currently considered as an attractive route and thus has garnered attention.

Oral administration is safe, easy, and painless. Compared with IV injection, oral administration can be conveniently employed at home by patients without any discomfort. With oral administration, the drug concentration in the blood can be maintained for longer than that with IV injection. Moreover, drugs can be administered orally as liquids, capsules, or solid or chewable tablets. For oral administration, drugs must dissolve in the gastrointestinal (GI) fluid before absorption into the blood circulation (Tran et al. 2019). However, more than 40% of new chemical entities (NCEs) are insoluble in water (Takagi et al. 2006; Kawabata et al. 2011; Rodriguez-Aller et al. 2015), resulting in poor absorption and low bioavailability (BA). In general, drug absorption is mainly influenced by two factors, namely, solubility and permeability. Amidon et al. first introduced the Biopharmaceutics Classification System (BCS) based on these two factors (Amidon et al. 1995). Drugs are divided into four groups as shown in Fig. 1 (FDA 2017; Nikolakakis and Partheniadis

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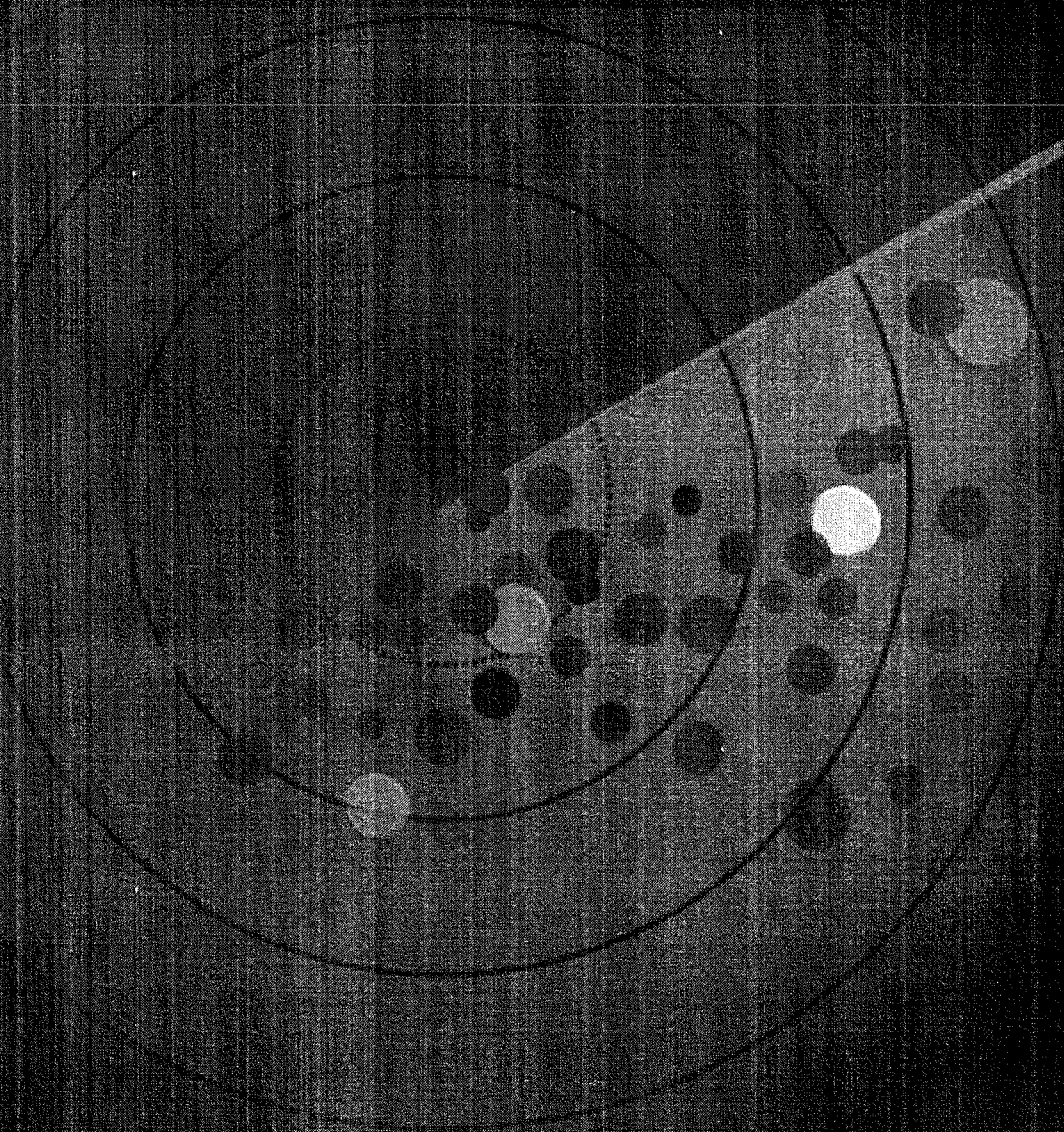

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ORIGINAL ARTICLE

Solid dispersion of mebendazole via surfactant carrier to improve oral bioavailability and in vitro anticancer efficacy

Thu Nhan Nguyen[1] · Phuong Tran[1] · Yeong-Eun Choi[1] · Jeong-Sook Park[1]

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Abstract

Purpose This study aimed to prepare a solid dispersion (SD) formulation of MBZ to improve dissolution and oral bioavailability.

Methods A SD formulation of mebendazole (MBZ) was prepared using sodium dodecyl sulfate (SDS) as a carrier via lyophilization method. Powder X-ray diffraction (PXRD), differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM) were used to confirm the structural properties and morphology of the MBZ-SD formulation. Dissolution study was conducted in an acidic medium (0.1 M HCl), and pharmacokinetic study was conducted in rats. In addition, the in vitro anticancer effects of MBZ-SD were also investigated in various cancer cell lines.

Results From the results of PXRD, DSC, FTIR, and SEM assessments, there was an interaction between MBZ and SDS in the MBZ-SD. MBZ-SD significantly improved the aqueous solubility of MBZ (approximately 15,982-fold) and the dissolution of MBZ at 5 min (1.5-fold) as compared to that of pure MBZ. The area under the curve (AUC_{0-24}) and the maximum concentration (C_{max}) of the MBZ-SD formulation showed a 3.56- and 3.30-fold increased values compared to pure MBZ. The anticancer effects of MBZ with IC_{50} value were in the order of A549 > MDA-MB-231 > HepG2 > MCF-7 > NCI-H1299 > HeLa. At safe concentrations in normal cells, the MBZ-SD formulation exhibited the superior anticancer efficacy in HeLa cells.

Conclusion The obtained results in the present study suggests that SD is a good candidate for improving the bioavailability and anticancer effects of MBZ.

Keywords Mebendazole · Solid dispersion · Dissolution · Oral bioavailability · Anticancer effect

Introduction

Mebendazole (MBZ) is a broad-spectrum anthelmintic drug, of the benzimidazole class that is used to treat ascariasis (roundworm infection), enterobiasis (pinworm infection), and hookworm infection caused by Ancylostoma duodenale, Necator americanus, and trichuriasis (whipworm infection) (Jongsuksuntigul et al. 1993; Flohr et al. 2007; Soukhathammavong et al. 2012). Recently, MBZ was reported to have anticancer properties that can inhibit the growth of different types of cancer cells such as colon, stomach, adrenal, breast, and lung cancers (Mukhopadhyay et al. 2002; Sasaki et al. 2002; Martarelli et al. 2008; Doudican et al. 2008; Nygren et al. 2013; Pinto et al. 2015; Williamson et al. 2016; Shashaani et al. 2016; Zhang et al. 2019). It was reported that MBZ exhibits dose- and time-dependent apoptotic effects in human lung cancer cells (Mukhopadhyay et al. 2002). In another study by Pinto et al., high cytotoxicity of MBZ in gastric cancer cells was demonstrated with an IC_{50} of 0.39 μM for ACP-2 (gastric adenocarcinoma cell line, diffuse type) and 1.25 μM for ACP-03 (intestinal type) (Pinto et al. 2015). The effect of MBZ on human adrenocortical carcinoma cancer cells (H295R and SW-13) was evaluated by Martarelli et al. and the obtained results showed that MBZ significantly inhibited the in vitro growth of human adrenocortical carcinoma cells, with an IC_{50} of 0.23 and 0.27 μM for H295R and SW-13, respectively (Martarelli et al. 2008). In addition, MBZ significantly inhibited tumor

Thu Nhan Nguyen and Phuong Tran have contributed equally to this work.

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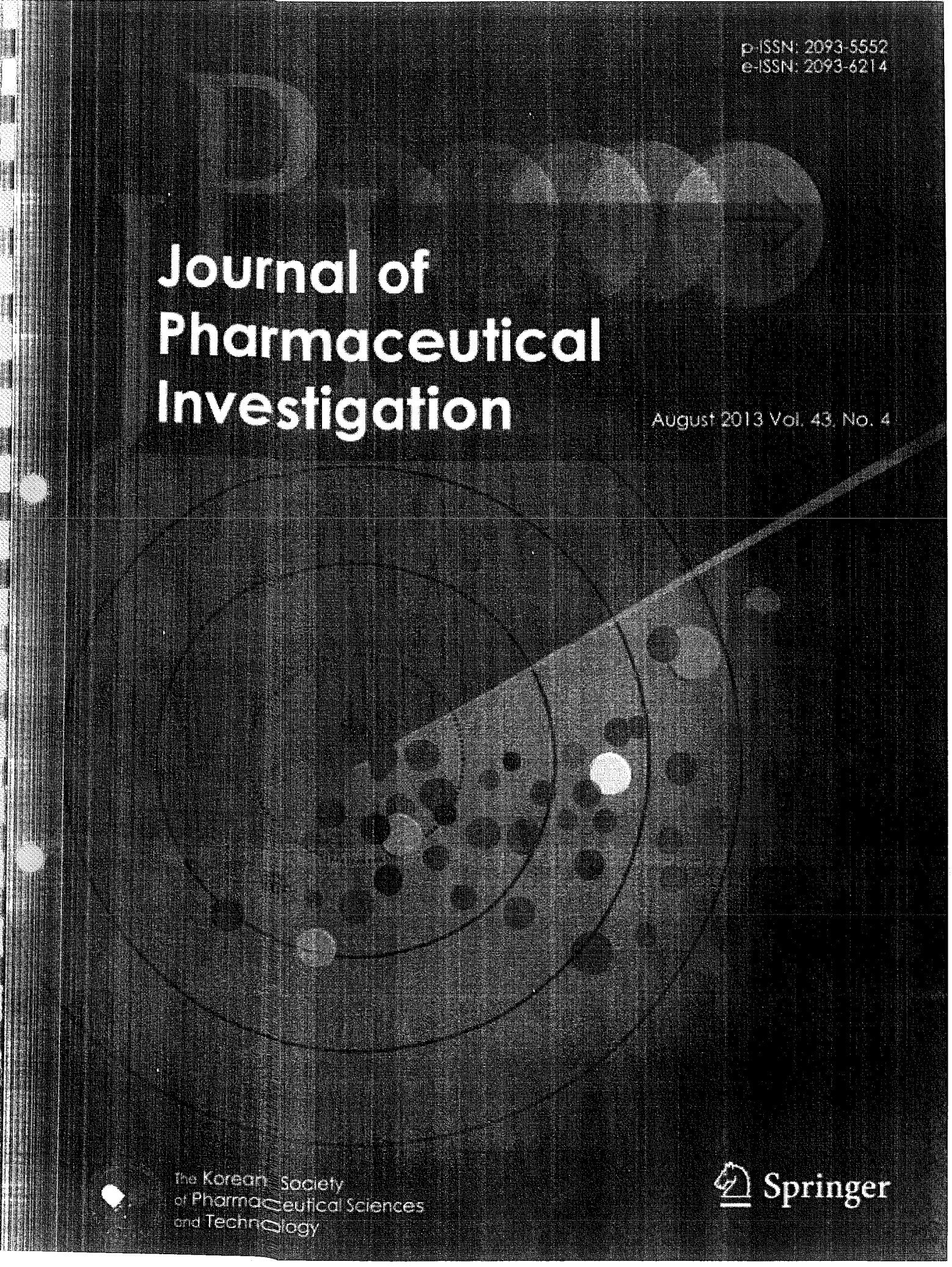

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REVIEW

Solubility enhancement and application of cyclodextrins in local drug delivery

Dong-Hyun Kim[1] · Sang-Eun Lee[1] · Yong-Chul Pyo[1] · Phuong Tran[1] · Jeong-Sook Park[1]

Received: 14 January 2019 / Accepted: 16 March 2019

Abstract
Cyclodextrins (CDs) have been used in many pharmaceutical formulations as their constitution and inherent shape present advantages for drugs with poor aqueous solubility and low bioavailability. Because CDs can act as drug carriers by forming inclusion complexes to conjugate with many drugs, they have been applied in many drug delivery systems and can be used to develop new strategies. The objectives of this review are to describe the role of CDs in local administration, the ways in which CDs are used, and relevant studies currently underway. The basic structure and characteristics of CDs, as well as the mechanisms used to formulate inclusion complexes to solubilize drugs are also described. Several studies have been conducted to investigate the use of CDs and most have shown improvements in drug solubility and bioavailability. CDs show potential not only in the pharmaceutical industry but also in a variety of applications, and further research into the use of other drug carriers is therefore necessary.

Keywords Cyclodextrin · Inclusion complex · Local administration · Drug delivery · Drug carrier

Introduction

Since the discovery of cyclodextrins (CDs) approximately 100 years ago, they have been used in several pharmaceutical industries (Vyas et al. 2008). Over 30 different pharmaceutical products using CDs are currently on the market (Table 1). The use of CDs improves the low solubility and bioavailability of drugs, facilitates absorption through the mucosa or skin, and enhances oral absorption (Baek et al. 2015; Kang et al. 2015). In addition, CDs have the advantage of preventing the rapid loss of drug, eliminating bitter taste, and allowing for a variety of formulations (Arima et al. 2001; Duchêne 1991; Loftsson et al. 2005).

CDs are cyclic oligosaccharides comprising (α-1,4)-linked D-glucopyranose units and have a toroidal shape. Their outer surface is lipophilic and the inside is hydrophilic (Davis and Brewster 2004; Tiwari et al. 2010). The most common natural CDs used in the pharmaceutical industry are αCD, βCD, and γCD, consisting of 6, 7, and 8 glucopyranose units, respectively (Shelley and Babu 2018). CDs react with various reagents such as trimethylamine and form derivatives that are more soluble in water than natural CDs. CD derivatives include hydroxypropylated CD (HP-βCD and HP-γCD), carboxymethylated CD (CM-βCD), and sulfobutylether CD (SBE-βCD and SBE-γCD) (Loftsson and Duchêne 2007; Pinho et al. 2014). The features of natural CDs and CD derivatives are listed in Tables 2 and 3, respectively (Aqil et al. 2013; Davis and Brewster 2004; Jansook et al. 2018), and the three-dimensional structure is shown in Fig. 1.

CDs are used in many local drug delivery systems, including ophthalmic, nasal, pulmonary, buccal, vaginal, and rectal delivery (Choi et al. 2014; Kim et al. 2010). The advantages of local drug delivery include the reduction of first-pass and side effects, and increased effectiveness at relatively low doses (Baek et al. 2015). Easy administration also increases convenience for patients.

This review addressed the interactions and factors needed to form inclusion complexes when using CDs for drug solubilization. We also introduced the Higuchi and Connors method to analyze inclusion complex formed through solubilization and the pharmaceutical benefits that can be gained using CDs. In addition, the practical applications of CDs in

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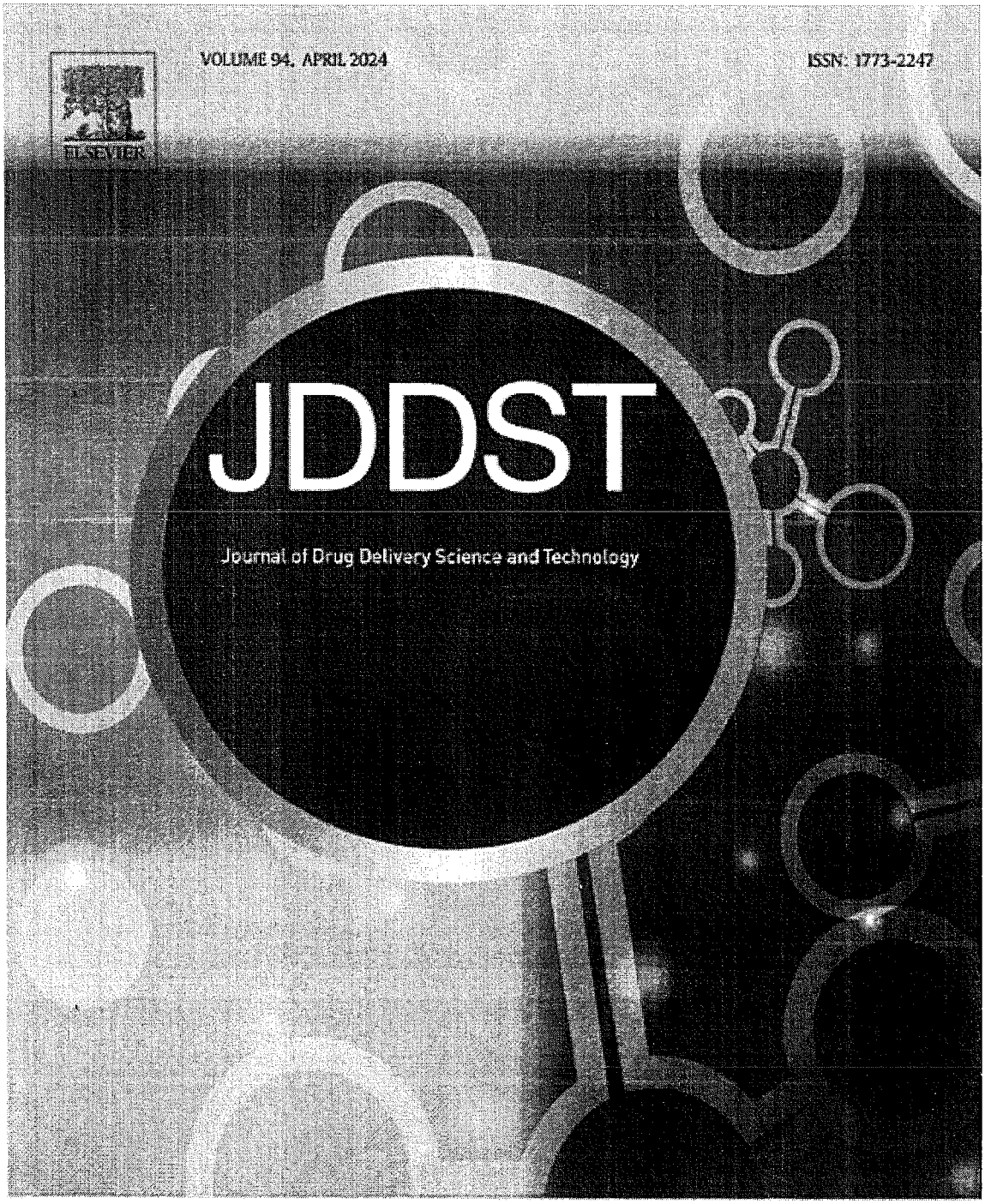
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Effect of calcium chloride on the protein encapsulation and stability of proliposomal granules

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ABSTRACT

The purpose of this study was to develop oral proliposomal granules incorporating $CaCl_2$ in order to enhance the encapsulation efficiency (EE) of protein model drug after reconstitution. Proliposomal granules were prepared by a granulation process with a solid carrier, protein model drug, and lipid solution. The proliposomal granules were characterized in terms of particle size, EE, and loading capacity. Protein structure analysis and cellular viability were also examined. $CaCl_2$, ranged 0.125–6.0% w/w, was successfully incorporated into the dried granules during the wet binding process of granulation. Reconstituted proliposomes with 1% w/w $CaCl_2$ showed the highest EE among those examined. Different reconstituted diluents did not alter the EE, but had an impact on particle size and charge. The α-helical content (%), calculated from the mean molar ellipticity, was similar between protein drug alone and that with addition of $CaCl_2$, indicating the preservation of structural integrity, as also confirmed by electrophoresis. $CaCl_2$–incorporated proliposomes were non-toxic to cells at the dose used. In conclusion, inclusion of $CaCl_2$ into proliposomal granules enhanced the EE of protein drug showing optimal effects at 1% w/w with good preservation of protein integrity and cellular viability.

1. Introduction

Biotherapeutic agents (biodrugs), such as peptides and proteins, have attracted considerable interest as many of these agents are effective with few side effects [1,2]. However, their lack of stability is still considered as a major concern, especially for the oral administration route [3]. Proliposomes provide advantages for oral administration due to their stability in dried free-flowing powder form, and suitability for formulation as stable oral dosage forms, such as tablets or capsules [4,5]. Tantisripreecha and coworker [6] prepared protein proliposomes by granulation and optimized the preparation conditions. The liposomes reconstituted from proliposomes prepared by this method showed good physical properties, and were well-suited to compressing into tablets. However, the encapsulation efficiency (EE) of reconstituted liposomes was relatively low (10–14%). Other groups also obtained good results with liposomes reconstituted from proliposomes. The EE of salmon calcitonin (sCT) in the reconstituted liposomes was 54.9% and 19.9% for the taurodeoxycholate (TDC) proliposomes and sCT proliposomes, respectively, increasing oral bioavailability of sCT from the TDC proliposomes [7]. The EE of protamine sulfate-recombinant human (Pt-rh) insulin proliposomes was measured to be 17.6 ± 2.4% with no significant difference from 18.7 ± 4.1% of rh insulin proliposomes, but cellular uptake of Pt-rh insulin proliposomes in Caco-2 cells was superior to that of rh insulin proliposomes [8]. The mannosylated buserelin acetate (MANS-BA) reconstituted from proliposome powders exhibited the 21.12–33.80% of EE, and the permeability of reconstituted MANS–BA liposomes [9]. However, they did not show high EE of peptide/protein drugs [7–9].

Divalent cationic salts have been studied extensively with regard to their effects on binding with biological membranes, i.e., phospholipid bilayers, in biological systems. Interactions occur between cationic salts and the charged phospholipid membrane generally via Coulombic forces [10], with different cationic salts affecting lipid bilayer differently, with respect for example to dissimilar ability to induce membrane aggregation or alter the surface potential of the lipid [11,12]. One common effect of divalent cationic salts is the ability to stabilize the gel state in the gel-to-liquid crystalline phase transition of the phospholipid bilayer. Divalent cationic salts, both calcium and magnesium ions strongly influence the immobilization of phosphodiester groups of phospholipids [10]. These two ions, especially calcium, have

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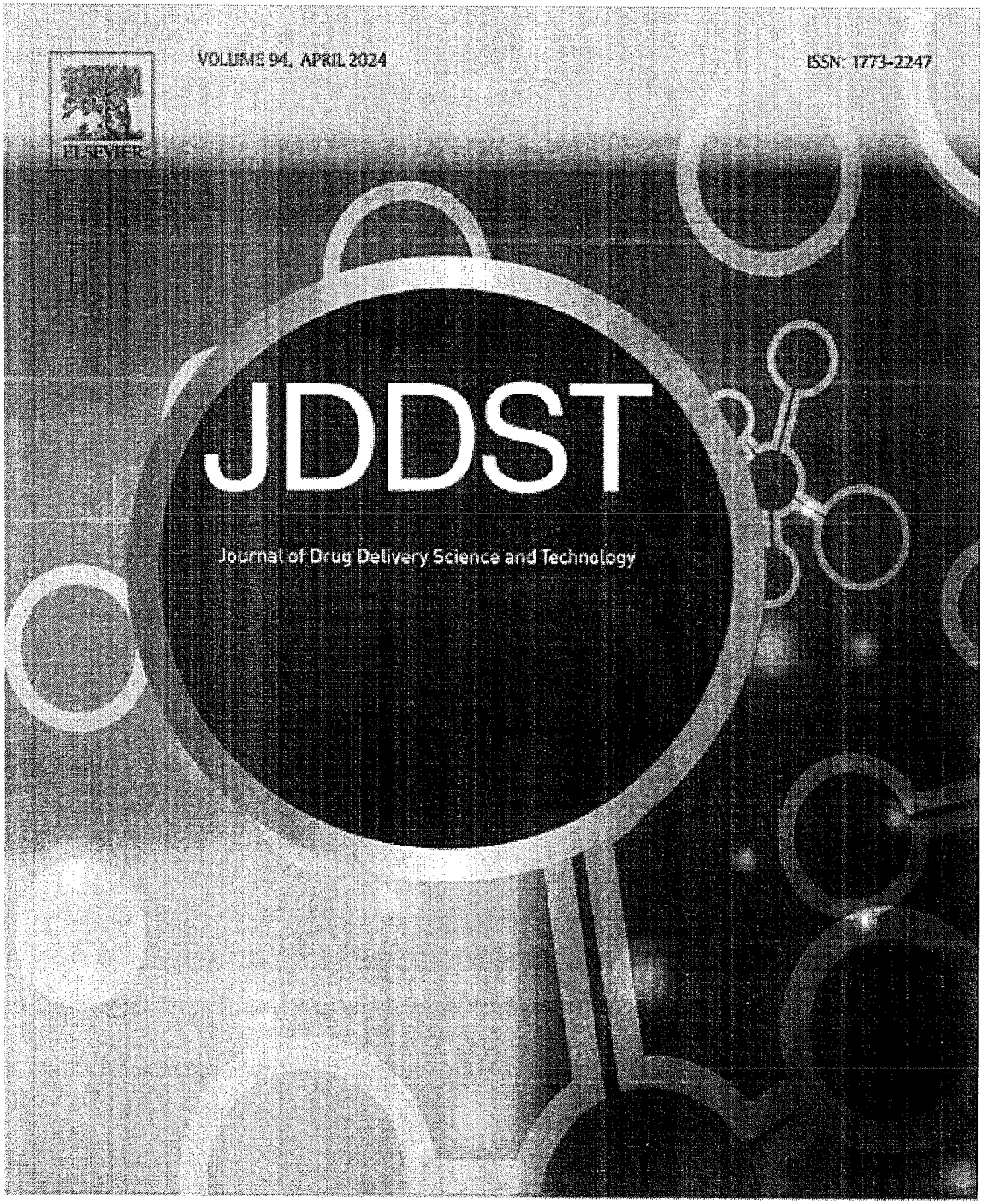

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Co-carrier-based solid dispersion of celecoxib improves dissolution rate and oral bioavailability in rats

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ABSTRACT

This study aimed to prepare a co-carrier-based solid dispersion (SD) of celecoxib (CXB) to improve its dissolution and oral bioavailability. The CXB-loaded SD formulation was prepared using CXB, pol407, Aerosil 200, and Eudragit L100 at a weight ratio of 1:3:1.5:1. PXRD, DSC, and FTIR analyses were conducted to evaluate the structural behavior and interactions between the drug and carrier. The dissolution profile was studied to demonstrate superior CXB dissolution capacity of CXB-SD than that of the physical mixture and raw CXB, and the results showed that the dissolution efficiency (%) of optimized CXB-SD significantly ($P < 0.05$) increased compared to that of raw CXB. The mean dissolution times of CXB-SD at pH 1.2 and 6.8 were reduced by 2.4-fold and 2.5-fold, respectively, compared to that of raw CXB. The dissolution of CXB-SD fitted well with the zero-order model. The preparation of the CXB-SD formulation improved the bioavailability of CXB, as demonstrated by the increased AUC_{last} (1.88-fold) and C_{max} (2.24-fold) of CXB-SD compared to that of raw CXB. In conclusion, these results indicate that SDs can enhance the dissolution and oral bioavailability of poorly water-soluble CXB.

1. Introduction

Celecoxib (CXB; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl]-benzenesulfonamide), a nonsteroidal anti-inflammatory drug belonging to the class of selective cyclooxygenase-2 inhibitors, is used in the treatment of osteoarthritis, rheumatoid arthritis, and acute pain [1, 2]. However, due to poor aqueous solubility (1–3 μg/mL) [3], CXB is categorized as a biopharmaceutics classification system (BCS) class II drug. Drug solubility is the major factor affecting the oral absorption of BCS class II drugs from the gastrointestinal tract [4,5]. In particular, the oral bioavailability of CXB is low, varying from 22% to 40% [6]. Therefore, improving the solubility of CXB may enhance its dissolution rate and bioavailability [7].

Solid dispersion (SD) is a simple and effective technique in the pharmaceutical industry to improve the solubility of poorly water-soluble drugs [4,8,9]. SDs are systems in which hydrophobic drugs are dispersed molecularly in one or more hydrophilic carriers to reduce particle size, increase surface area, improve wettability, and transform the crystalline state of a drug into an amorphous state [10,11]. This was first introduced by Sekiguchi and Obi, using urea as a carrier for sulfathiazole-SD formulation [12]. With the development of science and technology in the pharmaceutical industry, various carriers used in the preparation of SDs, including acids, sugars, soluble polymers, insoluble polymers, surfactants, inert carriers, and miscellaneous, have been reported in the literatures [13]. Owing to the increased solubility of CXB, CXB-SDs with various carriers (Soluplus, Poloxamer 188, Poloxamer 407, polyethylene glycol (PEG), polyvinyl alcohol (PVA), hydropropyl methylcellulose (HPMC), polyvinylpyrrolidone (PVP), and Eudragit®) have been formulated in several studies with many interesting results. A binary SD system of CXB and a carrier was successfully prepared with poloxamer 407, PVP, HPMC, and PEG, which increased the solubility of CXB 65, 31, 38, and 23.5 times that of raw CXB, respectively [14]. Jeon et al. prepared a ternary system of CXB-SD with PVP-K30 or Eudragit EPO as aqueous carriers and poloxamer 407 as a surfactant using the spray drying method [15]. The obtained data showed that the CXB-SD formulations increased the dissolution of CXB. When PVP-K30 was used as the carrier, the dissolution rate increased with increasing PVP concentration, and CXB was released gradually in CXB-PVPK30-SDs. The use of Eudragit EPO as a carrier showed fast and complete release of CXB and no difference in the dissolution profiles of CXB-Eudragit

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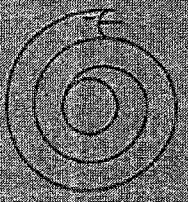

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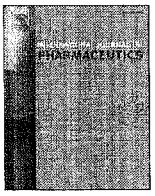

Application of supercritical fluid technology for solid dispersion to enhance solubility and bioavailability of poorly water-soluble drugs

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ABSTRACT

Many new chemical entities (NCEs) have been discovered with the development of the pharmaceutical industry. However, the main disadvantage of these drugs is their low aqueous solubility, which results in poor bioavailability, posing a challenge for pharmaceutical scientists in the field of drug development. Solid dispersion (SD) technology is one of the most successful techniques used to resolve these problems. SD has been widely used to improve the solubility and bioavailability of poorly water-soluble drugs using several methods such as melting, supercritical fluid (SCF), solvent evaporation, spray drying, hot-melt extrusion, and freeze-drying. Among them, SCF with carbon dioxide (CO_2) has recently attracted great attention owing to its enhanced dissolution and bioavailability with non-toxic, economical, non-polluting, and high-efficiency properties. Compared with the conventional methods using organic solvents in the preparation of the formulation (solvent evaporation method), SCF used CO_2 to replace the organic solvent with high pressure to avoid the limitation of solvent residues. The solubility of a substance in CO_2 plays an important role in the success of the formulation. In the present review, the various processes involved in SCF technology, application of SCF to prepare SD, and future perspectives of SCF are described.

1. Introduction

Improvements in solubility and oral bioavailability (BA) of drugs with poor aqueous solubility drugs play an important role in drug development (Ha et al., 2020; Kim et al., 2020, 2021; Tran and Park, 2021). The oral route of administration is preferred over other routes (intravenous, intramuscular, and subcutaneous), owing to several advantages such as safety, pain avoidance, and good patient compliance. After ingestion via the oral route, the drugs must dissolve in the gastrointestinal (GI) fluid to enable their penetration into the bloodstream through the GI tract membrane. Absorption is affected by many factors, such as blood perfusion, differences in luminal pH along the GI tract, the presence of bile and mucus, surface area per luminal volume, and the nature of epithelial membranes. Drugs belonging to the biopharmaceutical classification system (BCS) class II exhibit poor aqueous solubility, indicating dissolution rate-limited absorption, resulting in poor BA.

Solid dispersion (SD) is a promising technique for improving the aqueous solubility and BA of BCS class II drugs by enhancing wettability, reducing particle size, high porosity, and the amorphous state (Byeon et al., 2019; Kim et al., 2021; Luu et al., 2019; Tran et al., 2019). It is simpler and easier to prepare than other methods such as lipid-based systems (Chen et al. 2018a, 2018b; Kim et al., 2017), micronization (Aguiar et al., 2018; Karashima et al., 2017; Seo et al., 2016), co-crystals (Reggane et al., 2018), and nanonization (Chen et al. 2018a, 2018b; Park et al., 2018; Wong et al., 2018). SD is defined as the dispersion of a hydrophobic drug in hydrophilic carriers to improve its surface area or change the state of the drug (crystalline to amorphous). With the recent development of the pharmaceutical industry, products with minimal environmental impact and less toxicity have become more attractive. Therefore, technological development is the top priority. In this regard, high-pressure technology is currently receiving great attention, and supercritical fluids (SCFs) are still popular in this area. Unlike other methods [fusion method (Karolewicz et al., 2016), solvent evaporation method (Mustapha et al., 2017), lyophilization technique (Kaur et al., 2017), or spray drying method (Pradhan et al., 2015)], SCF can be used to prepare amorphous SD with many advantages such as controllable processing conditions, good reproducibility, and environmental friendliness (Han et al., 2019). SCF was introduced in the late 1980s and the early 1990s. In 1879, Hannay and Hogarth (1879) reported an SCF for

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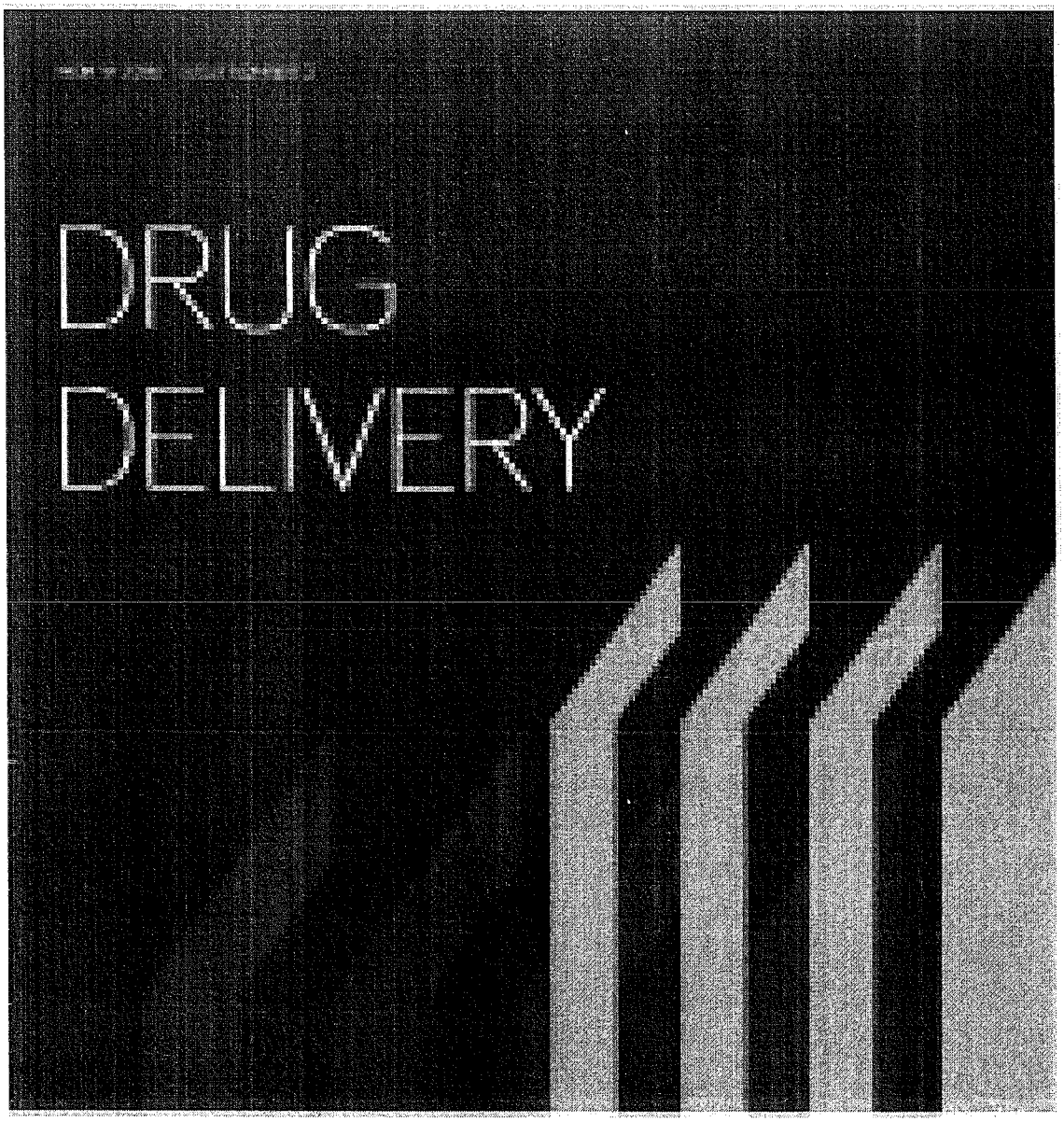
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RESEARCH ARTICLE

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Local drug delivery using poly(lactic-co-glycolic acid) nanoparticles in thermosensitive gels for inner ear disease treatment

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ABSTRACT

Intratympanic (IT) therapies have been explored to address several side effects that could be caused by systemic administration of steroids to treat inner ear diseases. For effective drug delivery to the inner ear, an IT delivery system was developed using poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) and thermosensitive gels to maintain sustained release. Dexamethasone (DEX) was used as a model drug. The size and zeta potential of PLGA NPs and the gelation time of the thermosensitive gel were measured. *In vitro* drug release was studied using a Franz diffusion cell. Cytotoxicity of the formulations was investigated using SK-MEL-31 cells. Inflammatory responses were evaluated by histological observation of spiral ganglion cells and stria vascularis in the mouse cochlea 24 h after IT administration. In addition, the biodistribution of the formulations in mouse ears was observed by fluorescence imaging using coumarin-6. DEX-NPs showed a particle size of 150.0 ± 3.2 nm in diameter and a zeta potential of −18.7 ± 0.6. The DEX-NP-gel showed a gelation time of approximately 64 s at 37 °C and presented a similar release profile and cytotoxicity as that for DEX-NP. Furthermore, no significant inflammatory response was observed after IT administration. Fluorescence imaging results suggested that DEX-NP-gel sustained release compared to the other formulations. In conclusion, the PLGA NP-loaded thermosensitive gel may be a potential drug delivery system for the inner ear.

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1. Introduction

Hearing loss caused by inner ear disease is increasing because of prolonged exposure to noise, increased life expectancy, and the use of medicines such as anticancer drugs (Kim, 2017). Therefore, there is a growing interest in drug delivery to the inner ear. Glucocorticoid drugs, such as dexamethasone (DEX), are considered potential otoprotective drugs with anti-inflammatory effects (Van De Water et al., 2010). However, systemic drug administration usually results in only a small amount of drug that reaches the inner ear and a risk of side effects. The benefits of inner ear drug delivery are that they do not cross the blood–labyrinth barrier, avoid first-pass metabolism from systemic administration, maintain high drug concentrations in the inner ear, and reduce the total amount of administered drug (Juhn et al., 1982; Mäder et al., 2018).

Intratympanic (IT) administration, a process in which drugs are injected into the middle ear through the eardrum to deliver drugs into the inner ear, has been studied for many years (Ersner et al., 1951; Schuknecht, 1956). Since the mid-1990s, local drug delivery to the ear has been used as a clinical treatment. If the drug is administered to the middle ear, it must pass through the round window membrane (RWM) to reach the inner ear (Nedzelski et al., 1993; Toth & Parnes, 1995). Therefore, the administered drug must remain in the middle ear for sufficient time and be in contact with the RWM. However, the drug administered to the middle ear is rapidly removed into the Eustachian tube through the flow of the mucosa (Salt & Plontke, 2018).

Over the past several years, biodegradable polymer nanoparticles (NPs) have attracted interest for drug delivery to the inner ear. As a carrier for local drug administration, NPs composed of poly(lactic-co-glycolic acid) (PLGA), approved by the Food and Drug Administration and the European Medicine Agency, have been considered an ideal carrier (Blasi, 2019; Elmowafy et al., 2019; Schoubben et al., 2019). Previous studies have demonstrated the application of

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Dong-Hyun Kim, Thu Nhan Nguyen, Phuong Tran: performed experiments, conceptualization, investigation, writing – original draft. Thu Nhan Nguyen: performed data analysis, revision, and discussion. Dong-Hyun Kim, Phuong Tran, Jinhyung Rho: performed investigation on animal study. Young-Min Han: performed experiments. Jae-Young Lee, Hwa-Young Son, Jeong-Sook Park: performed data analysis, review, and discussion. Jeong-Sook Park: performed writing and editing, supervision, revision.

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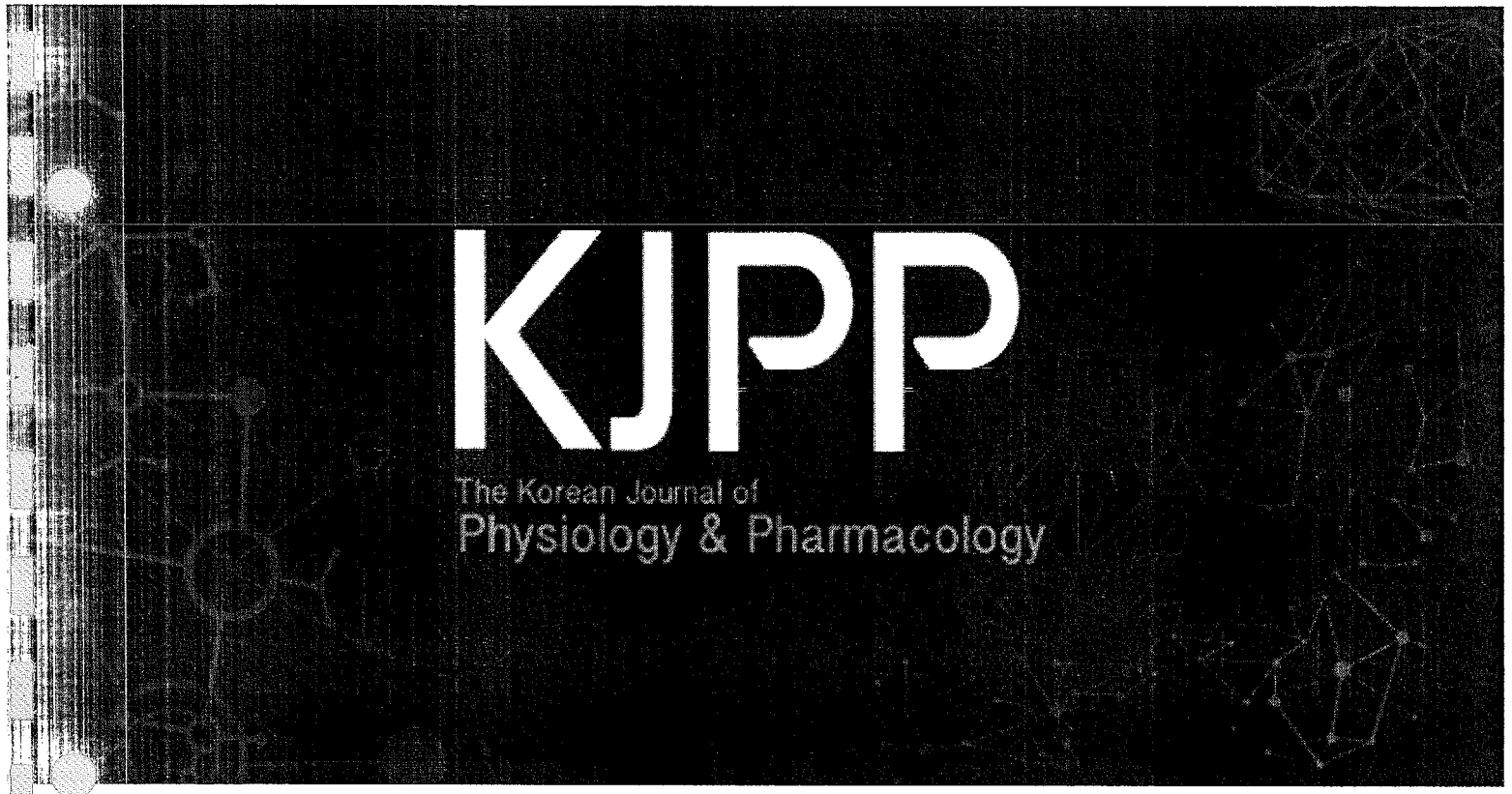
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Original Article

Docetaxel-loaded PLGA nanoparticles to increase pharmacological sensitivity in MDA-MB-231 and MCF-7 breast cancer cells

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ABSTRACT This study aimed to develop docetaxel (DTX) loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (DTX-NPs) and to evaluate the different pharmacological sensitivity of NPs to MCF-7 and MDA-MB-231 breast cancer cells. NPs containing DTX or coumarin-6 were prepared by the nanoprecipitation method using PLGA as a polymer and d-α-tocopherol polyethylene glycol 1000 succinate (TPGS) as a surfactant. The physicochemical properties of NPs were characterized. *In vitro* anticancer effect and cellular uptake were evaluated in breast cancer cells. The particle size and zeta potential of the DTX-NPs were 160.5 ± 3.0 nm and −26.7 ± 0.46 mV, respectively. The encapsulation efficiency and drug loading were 81.3 ± 1.85% and 10.6 ± 0.24%, respectively. The *in vitro* release of DTX from the DTX-NPs was sustained at pH 7.4 containing 0.5% Tween 80. The viability of MDA-MB-231 and MCF-7 cells with DTX-NPs was 37.5 ± 0.5% and 30.3 ± 1.13%, respectively. The IC_{50} values of DTX-NPs were 3.92- and 6.75-fold lower than that of DTX for MDA-MB-231 cells and MCF-7 cells, respectively. The cellular uptake of coumarin-6-loaded PLGA-NPs in MCF-7 cells was significantly higher than that in MDA-MB-231 cells. The pharmacological sensitivity in breast cancer cells was higher on MCF-7 cells than on MDA-MB-231 cells. In conclusion, we successfully developed DTX-NPs that showed a great potential for the controlled release of DTX. DTX-NPs are an effective formulation for improving anticancer effect in breast cancer cells.

INTRODUCTION

Breast cancer occurs when some breast cells begin to grow abnormally and divide more rapidly than healthy cells. These cells accumulate and form a lump or mass. Breast cancer cells can spread to the lymph nodes and other parts of the body. Therefore, the development of a drug with high efficacy in the treatment of breast cancer would play an important role in reducing the death rate of woman with this disease. Many drugs are currently used to treat breast cancer such as doxorubicin [1], gemcitabine [2], paclitaxel [3], docetaxel (DTX) [4], and leuprolide [5]. Among them, DTX is a second-generation taxane approved by the US Food and Drug Administration (FDA) to treat multiple types of cancers such as breast, non-small cell lung, hormone-refractory prostate cancers, and gastric adenocarcinoma and squamous cell carcinoma of the head and neck [6]. DTX is usually selected as a model drug for the treatment of breast cancer and it acts as a microtubule-stabilizing agent that blocks the cell cycle at the G2/M phase, which inhibits microtubule disassembly during cell-cycle progression, causing cell death. It is designated as biopharmaceutical classification system class IV agent that has poor aqueous solubility and permeability, which reduce its bioavailability, thereby limiting the treatment efficacy. Therefore, the commercial DTX product Taxotere (Sanofi Aventis, Bridgewater, NJ , USA)

Author contributions: P.T. designed the study and wrote the manuscript. T.N.N. performed the HPLC analysis. Y.L. and P.N.T. performed the cell-based assay experiments. J.S.P. supervised and coordinated the study.

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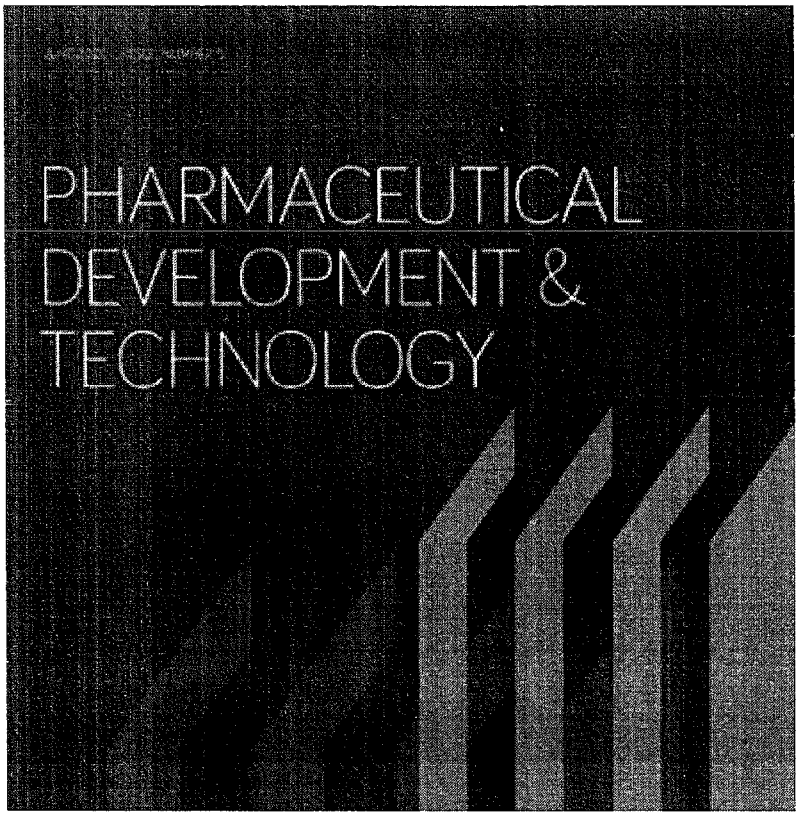
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RESEARCH ARTICLE

Formulation of solid dispersion to improve dissolution and oral bioavailability of poorly soluble dexibuprofen

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ABSTRACT

Dexibuprofen (DEXI) belongs to BCS class II drug with poor aqueous solubility resulting in poor bioavailability. To enhance solubility and bioavailability of DEXI, DEXI-loaded solid dispersion (SD) was formulated. DEXI-SDs were prepared by melting method and solvent evaporation method. Amphipathic polymer poloxamer 407 (pol 407) was selected based on solubility and dissolution tests. The ratio of DEXI:pol 407 was optimized as 1:2. The physicochemical properties, dissolution, and oral bioavailability of SD3 and SD6 were evaluated to compare preparation methods. The dissolution rate of DEXI from SD formulations was higher at pH 6.8 and pH 7.2 than at pH 1.2. Following oral administration in rats, the C_{max} and AUC_{last} of SD3 and SD6 formulations were significantly higher compared with raw DEXI. In addition, the SD6 formulation showed increased C_{max} and AUC_{last} by 1.34- and 1.33-fold, compared with those of SD3 formulation, respectively. These results demonstrated that SD formulation has excellent potential as a formulation for poorly soluble drug DEXI.

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Dexibuprofen; solid dispersion; dissolution; bioavailability

1. Introduction

Currently, oral administration is the preferred route of treatment, owing to several advantages such as convenience, avoidance of pain, and safety. The prerequisite for oral administration is complete and predictable absorption in the gastrointestinal (GI) tract. To achieve this, drugs should dissolve in water to be absorbed in the GI tract and be effectively taken up in the circulatory system. However, approximately 40% of new chemical entities (NCEs) are reportedly as poor water-soluble drugs (hydrophobic drugs) resulting in incomplete absorption, poor bioavailability (BA), and large inter- and intra-individual variability in drug concentrations *in vivo*. Thus, enhancing the dissolution and BA of poorly water-soluble drugs are great challenges in the pharmaceutical industry (Singh et al. 2018).

Dexibuprofen (DEXI) (*S*-2-(4-isobutyl phenyl)-propionic acid), a nonsteroidal anti-inflammatory drug, is typically used in the treatment of osteoarthritis, acute and chronic pain, rheumatoid arthritis, and related conditions (Bondan et al. 2017; Gordo et al. 2017; Ho et al. 2018). DEXI is an *S*+ enantiomer of ibuprofen and belongs to Biopharmaceutics Classification System class II with low solubility (around 11 μg/mL) and high permeability (Kaehler et al. 2003; Rinaki et al. 2004; Potthast et al. 2005; Tsume et al. 2012; Stoyanova et al. 2016). The high permeability of ibuprofen and its enantiomers have been observed in Caco-2 cell cultures. In a radiolabeled Caco-2 cell culture study, the apparent permeability coefficient (P_{app}) of ibuprofen was 30.1×10^{-6} cm/s (Berben et al. 2018). Due to its low solubility, the dissolution rate of DEXI is limited in the GI tract, thereby decreasing the BA of the drug. Thus, improving the solubility and dissolution rate of DEXI can enhance the BA of the drug. Therefore, several techniques have been developed to improve the drug solubility and BA (Karashima et al. 2017; Ahsan and Verma 2018; Choi et al. 2019a).

Solid dispersion (SD) is well-studied technique used to enhance solubility and BA of poorly water-soluble drugs (Mehenni et al. 2018; Ding et al. 2019; Kwon et al. 2019). SD is defined as a group of solid products consisting of a hydrophobic drug dispersed in at least one hydrophilic carrier, resulting in an enhanced surface area, and leading to higher drug solubility and dissolution rate. Enhanced drug BA is achieved by improving wettability and dispersibility, and reducing the aggregation and agglomeration of drug particles (Tran et al. 2019). Besides the traditional methods to produce SD as melting method, solvent evaporation method, spray-dried amorphous SD has recently used (Henriques et al. 2021). The selection of suitable carrier is a prerequisite for success in formulation. Soliman et al. prepared diacerein solid dispersion loaded tablets to enhance solubility, dissolution, and BA of diacerein using Pluronic® F68 (poloxamer 188) as the carrier (Soliman et al. 2021). As the result, the solubility of diacerein from SD (187.61 μg/mL) was 8.3-fold higher compared to drug powder (22.5 μg/mL). While the dissolution rate of SD was 6.6-fold higher than powder drug, the dissolution rate of SD tablet was 12.5-fold higher than the marketed product. In addition, the oral BA of diacerein from SD tablet was impreoved 2.66-fold in comparison with the marketed product.

In this study, DEXI-SD formulations were prepared by melting method and solvent evaporation method. (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), hydroxypropyl cellulose (HPC), polyvinylpyrrolidone (PVP), urea, poloxamer 188 (pol188), poloxamer 407 (pol407), and PEG 6000 were screened to select the suitable carrier. The physicochemical properties of SD formulations were evaluated using a field emission scanning electron microscope (FE-SEM), powder X-ray diffraction (PXRD), differential scanning calorimetry (DSC), and Fourier-transform infrared spectroscopy (FTIR). *In vitro* dissolution, *in vitro* cytotoxicity, and a pharmacokinetic study of DEXI were also evaluated.

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COLLOIDS AND SURFACES B

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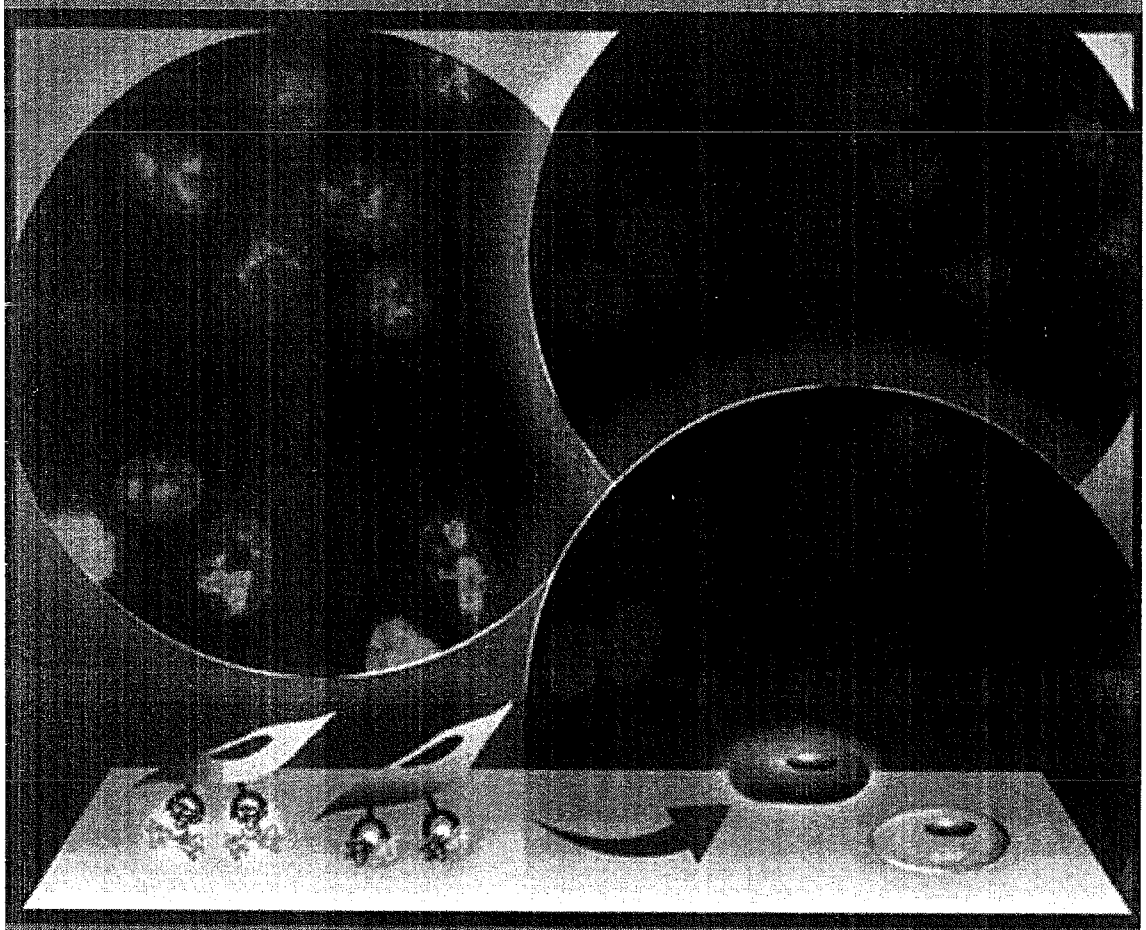

Selective inhibition of partial EMT-induced tumour cell growth by cerium valence states of engineered cerium nanoparticles for anticancer treatment

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Chitosan-coated nanostructured lipid carriers of fenofibrate with enhanced oral bioavailability and efficacy

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ABSTRACT

Fenofibrate is frequently used to lower cholesterol levels in cardiovascular disease. Owing to its poor solubility and high gastrointestinal permeability, it is classified as a Biopharmaceutics Classification System class II compound. The aim of this study was to improve the solubility and bioavailability of fenofibrate by formulating it as fenofibrate-loaded nanostructured lipid carriers (FFB-NLCs) and coating it with a biodegradable polymer to allow controlled drug release. Chitosan-coated nanostructured lipid carriers (CF-NLCs) were prepared via an ultrasonication method using chitosan as the biodegradable polymer, stearic acid as the solid lipid, oleic acid as the liquid lipid, and Tween 80 as the surfactant. To study encapsulation efficiency and solubility conditions, stearic acid/oleic acid ratios were varied as 80/20, 70/30, 60/40, and 50/50 (mg/mg), by adjusting chitosan ratio. Chitosan is an adhesive polymer, coating the surface of the NLC to improve its bioavailability. All NLC formulations demonstrated a particle size of approximately 200 nm and a polydispersity index below 0.3. The encapsulation efficiencies of the NLC formulations were above 85%. For CF-NLCs, the solubility and encapsulation efficiency of fenofibrate were increased when compared with those of a commercial fenofibrate formulation. The pharmacokinetic and pharmacodynamic parameters of fenofibrate in the form of CF-NLCs were improved after oral administration. CF-NLCs can be used for allowing controlled release and improving the bioavailability and stability of fenofibrate.

1. Introduction

Recently discovered novel drug candidates are insoluble in the aqueous phase [1]. Despite their excellent efficacy, some candidates are limited in therapeutic use owing to their poor aqueous solubility [2]. Novel concepts are crucial to address this shortcoming. One strategy is to develop a carrier that can be encapsulated, protected, and released under specific and desired conditions [3]. As guest molecules electrostatically enter the hydrophobic matrix, lipid materials are suitable candidates for the formulation of active hydrophobic delivery systems. Furthermore, various techniques, additives, and formulations are required to enhance the solubility of poorly soluble drugs. Self-emulsifying drug delivery systems (SEDDS) [4], self-microemulsifying drug delivery systems (SMEDDS) [5–7], amorphous solid dispersions [8], nanosuspensions [9], and liquid crystals [10,11] have been tested. Several lipid-based carriers have been established including emulsions, liposomes, solid lipid nanoparticles (SLNs), as well as the more recently developed, nanostructured lipid carriers (NLCs).

Fenofibrate, a biopharmaceutical classification system class II drug, is used to treat hypercholesterolemia and hypertriglyceridemia [8]. Fenofibrate is marketed globally and has been well-described pharmacologically [12]. The drug is poorly water-soluble, partly owing to its high hydrophobicity (log P = 5.24, **Fig. S1**). Therefore, it exhibits poor oral bioavailability. For poorly water-soluble drugs, the rate of absorption depends on the rate of dissolution, which in turn determines the bioavailability [13]. Lipidil® Supra, a brand name for fenofibrate, has been used commercially for the treatment of hyperlipidemia (Green Cross Co., Ltd., Seoul, Korea), and is consumed orally once a day.

It is challenging to administer lipid-based drug carriers as enzymatic hydrolysis by lipase causes rapid elimination of particles, resulting in the loss of a certain amount of bioactive molecules. Hence, these particles should be protected from gastrointestinal environment. Polymer coating and encapsulation of nanoparticles in polymeric microparticles (synthetic and natural polymers) have been used to protect the formulation from enzymatic attack. A polymer coating system with natural polymers (e.g., hyaluronic acid and chitosan) improves the oral bioavailability of bioactive molecules [14]. This strategy was applied to develop NLCs

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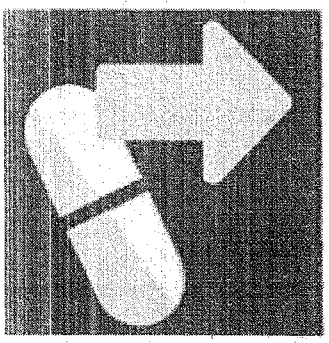

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Review

Overview of the Manufacturing Methods of Solid Dispersion Technology for Improving the Solubility of Poorly Water-Soluble Drugs and Application to Anticancer Drugs

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Abstract: Approximately 40% of new chemical entities (NCEs), including anticancer drugs, have been reported as poorly water-soluble compounds. Anticancer drugs are classified into biologic drugs (monoclonal antibodies) and small molecule drugs (nonbiologic anticancer drugs) based on effectiveness and safety profile. Biologic drugs are administered by intravenous (IV) injection due to their large molecular weight, while small molecule drugs are preferentially administered by gastrointestinal route. Even though IV injection is the fastest route of administration and ensures complete bioavailability, this route of administration causes patient inconvenience to visit a hospital for anticancer treatments. In addition, IV administration can cause several side effects such as severe hypersensitivity, myelosuppression, neutropenia, and neurotoxicity. Oral administration is the preferred route for drug delivery due to several advantages such as low cost, pain avoidance, and safety. The main problem of NCEs is a limited aqueous solubility, resulting in poor absorption and low bioavailability. Therefore, improving oral bioavailability of poorly water-soluble drugs is a great challenge in the development of pharmaceutical dosage forms. Several methods such as solid dispersion, complexation, lipid-based systems, micronization, nanonization, and co-crystals were developed to improve the solubility of hydrophobic drugs. Recently, solid dispersion is one of the most widely used and successful techniques in formulation development. This review mainly discusses classification, methods for preparation of solid dispersions, and use of solid dispersion for improving solubility of poorly soluble anticancer drugs.

Keywords: solid dispersion; classification; manufacturing methods; bioavailability; anticancer drugs

1. Introduction

Cancer is one of the leading causes of death worldwide, and treatment remains a great challenge. Currently, there are three major cancer treatment strategies of surgery (performed by a surgical oncologist), chemotherapy (use of anticancer drugs), and radiotherapy (delivered by a radiooncologist) [1]. The objective of any treatment is to kill as many cancer cells as possible and minimize death of normal cells. Patients can receive monotherapy or combination therapy. For example, Hwang et al. [2] reported a combination of photodynamic therapy (PDT) and anti-tumor immunity

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Integration of lornoxicam nanocrystals into hydroxypropyl methylcellulose-based sustained release matrix to form a novel biphasic release system

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ABSTRACT

The study aims to (a) enhance the solubility of a poorly soluble drug by optimization of nanocrystal formulation using the top-down approach and (b) modify the release profile of this drug, which exhibits a short elimination half-life, by the integration of a fast-release phase containing the optimized nanocrystals and a sustained-release phase in a compression-coated tablet. Nanocrystals of the model drug (lornoxicam; LNX) was prepared by simultaneous application of jet-milling and ball-milling techniques. Investigation of the precipitation inhibition capacity, thermal property, and interaction of different polymers with the drug revealed polyvinyl pyrrolidone K30 (PVP) as the most effective stabilizer for nanocrystals. The immediate-release layer containing the optimized nanocrystals (size of 279.5 ± 11.25 nm and polydispersity index of 0.204 ± 0.01) was then compressed on a zero-order sustained-release matrix core using different derivatives of hydroxypropyl methylcellulose (HPMC). Application of the Design of Experiment approach (DoE) was applied to optimize the formulation of tablet. Analysis of drug concentration in dog plasma by liquid chromatography-tandem mass spectrometry demonstrated an improvement in the release behavior of LNX from the optimal compression-coated tablet integrating a HPMC-based sustained release matrix core and a PVP-stabilized lornoxicam nanocrystals coating layer compared to the reference product.

1. Introduction

In recent years, there has been a tendency to employ various techniques in the preparation of one drug dosage form to improve the therapeutic efficiency of drugs. For example, Gómez-Gaete et al. [1] developed a hybrid vector named Trojan particles, which combined the therapeutic potential of nanoparticles with the ease of manipulation of microparticles, to enhance the intravitreal administration of dexamethasone. In another study, Frederic Tewes et al. [2] improved therapeutic efficiency and minimized unwanted side effects of superparamagnetic iron oxide by porous microparticles loaded with superparamagnetic iron oxide nanoparticles (SPIONs). Some other researchers, including Lo et al. and Tung et al. [3,4], applied microencapsulation and pH modulating to overcome the very bitter taste and low stability of azithromycin. The development of multiple-unit pellet system (MUPS) tablets containing drugs also based on the combination of soft tableting and micropellet coating technique to improve drug usage and stability in a gastric medium or control the drug release, respectively [5,6].

In this study, lornoxicam (LNX) indicated for inflammatory diseases of the joints, osteoarthritis, surgery, and other inflammations [7,8] was chosen as the model drug. Owing to its poorly aqueous solubility [9–11] and short elimination half-life (usually only 3 to 5 h) [12], the use of LNX is limited by the lag time to the onset of action and the

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inconvenience due to daily administration of the drug, i.e., two to three times per day.

Among strategies to enhance the poor aqueous solubility of an active pharmaceutical ingredient [13], drug nanocrystals have been considered for their effectiveness in drug solubility enhancement, maintenance of drug physical drug stability, and avoidance of toxic organic solvents, as well as ease of scaling up [14–16]. However, of the two preparation techniques for nanocrystals, the bottom-up approach would be complicated, and it would produce organic solvent residues, thus making it harder to scale up. Meanwhile, the other technique, i.e., top-down, has been proved to be more practical and useful [14,17,18]. Nevertheless, the literature contains too little or no information regarding the application of the top-down approach for the preparation of LNX nanocrystals. Therefore, the successful development of LNX nanocrystals using the top-down method represents the novelty of this study.

To overcome the short elimination half-life of active pharmaceutical ingredients (API), a sustained-release (SR) matrix containing these drugs was successfully applied and commercialized [19–21]. The presence of polymeric carriers in the matrix would slow down the release rate of the drug, thereby prolonging the half-life of the drug. Therefore, an SR matrix can be combined with a fast-release layer containing LNX nanocrystals to produce biphasic release tablets, which can serve as a novel dosage form of this drug. Biphasic release tablets have also been proved effective in modifying the drug release of non-steroidal anti-inflammatory drugs (NSAIDs) and antihypertensive, antihistaminic, and anti-allergic agents at two different rates [22–25].

Popular techniques to prepare such dosage form include the preparation of bilayer tablets, compression-coated tablets, or multiarticulate drug delivery systems [26,27]. These have enabled the commercialization of products such as zolpidem tartrate (Stilnoct®), diclofenac (Diclofenac-Ratiopharm Uno® or Diclofenac Duo®), and iron (Ferro-Gradumet™). However, until now, few studies have been conducted on the application of bilayer or compression coated tablets to modify the release rate of lornoxicam [28,29]. In these studies, solid dispersion and cyclodextrin-drug complex were employed to create the quick-release phase and hydrophilic matrix (xanthan gum) or hydrophobic matrix (Compritol® ATO 888) for a slow release phase. Besides, existing literature [30–34] showed the simultaneous use of the two release models in a tablet might result in complicated and unpredictable release behavior of LNX. Therefore, to facilitate the optimization of this dosage form, quality by design approach has been advocated by FDA and proved advantageous [30–33].

The study, therefore, aimed to optimize the LNX nanocrystals prepared using the top-down technique. Additionally, a biphasic release tablet containing an SR core and an immediate-release (IR) layer was developed using a Design of Experiment approach (DoE); its pharmacokinetic profile was compared with that of a reference tablet.

2. Materials and methods

2.1. Materials

LNX was obtained from Hetero Drugs Limited (India). Piroxicam was purchased from Sigma-Aldrich Corporation (U.S.A.). Polyvinyl pyrrolidone K30 (PVP K30) and crospovidone (Kollidon® CL) were purchased from BASF (Germany). HPLC-grade methanol and acetonitrile were provided by J.T. Baker (U.S.A.), respectively. Sodium starch glycolate (SSG), sodium croscarmellose (Disolcel), and microcrystalline cellulose PH 101 (Avicel PH 101) were purchased from Mingtai Chemical Co. Ltd. (Taiwan). Polyvinyl alcohol 205 (PVA 205) was purchased from Kuraray Poval (Germany). Lactose was supplied by Meggle (Germany) and starch was supported by Roquette (France). Derivatives of hydroxypropyl methylcellulose were kindly supported by Coloron Limited (U.S.A.) including Methocel K4M, Methocel K100M, Methocel K15 premium LV, Methocel K100 LV, and by Shin-Etsu Chemical Co., Ltd. (Japan) including Metolose 90SH 4000, Metolose 90SH 15,000, Metolose 90SH 100,000, Pharmacoat 606 (HPMC 606) and Pharmacoat 615. Water was purified using reverse osmosis and was filtered in-house. All other reagents were analytical grade commercial products.

2.2. Development of lornoxicam nanocrystals

2.2.1. Preparation method of lornoxicam nanocrystals

LNX nanocrystals were prepared using the top-down technique. First, a micronized powder of LNX was fabricated using a jet-milling machine (Hosokawa, ALPINE PX5-001, Germany). The apparatus was set at the powder feeding speed of 45 g/min, input air pressure of 3 bar, and grinding air pressure of 2.5 bar. Second, nanosized LNX was prepared by wet-milling of micronized LNX under the stabilizing of a polymeric stabilizer.

The polymeric stabilizers for the LNX nanocrystals were screened on the basis of the drug precipitation inhibition capacity of the polymer using the solvent-shift method, which was modified from a previous study [35]. Briefly, approximately 5 mL of dimethyl sulfoxide solution containing 50 mg of LNX was gradually poured into the received medium containing 500 mL of hydrochloric acid pH 1.2 (with or without polymer), which was maintained at 37 °C ± 0.5 °C and agitated by paddle at a speed of 50 rpm. After predetermined time intervals of 5, 15, 30, 60, and 120 min, aliquots of approximately 3 mL were withdrawn and centrifuged (17,586 ×*g* for 3 min). The obtained supernatant containing LNX was filtered through a 0.45 μm membrane (Sartorius, Germany, Model Minisart RC 25) and diluted with methanol before analysis using a validated HPLC method. The most effective polymeric stabilizer for the preparation of LNX nanocrystals was considered to be the one that could create the highest supersaturation degree of LNX.

The nanosizing process was conducted on a ball-milling machine (Retsch, Germany, MM301 High-Speed Mixer Mill) using two 50 mL Teflon chambers and zirconia beads (Glen Mills Inc., U.S.A., 650 μm and 800 μm beads). Approximately 200 mg of micronized LNX and 10 mL of ethanol solution 70% containing the polymeric stabilizer was milled in the ball-milling chamber at different durations and frequencies. To avoid overheating in the milling chamber, the ball-milling process was paused for 5 min every 15 min. After every hour, the LNX suspension was collected and analyzed for the size distribution.

2.2.2. Particle size measurement

The particle size of the micronized powder of LNX was determined using Malvern Mastersizer (Malvern Instruments Limited, U.K., Mastersizer 3000). The micronized powders were directly dispersed into approximately 500 mL of distilled water for 30 s before sample examination. The laser obscuration was set from 0.1 to 20%. The particle size was expressed as the mean volume diameter in micrometers.

Additionally, the particle size of nanosized LNX was investigated using Malvern Zetasizer (Malvern Instruments, U.K., Model Zetasizer Nano ZS90). Samples were diluted with ultra-purified water before addition to a cuvette for the measurement of the nanosized particles.

2.2.3. Differential scanning calorimeter (DSC) analysis

The effect of polymeric stabilizers on the thermal properties of LNX was determined using a differential scanning calorimeter (Mettler Toledo, Germany, DSC 1) and an empty aluminum pan as a reference. A mass of approximately 10 mg samples including LNX nanocrystals, LNX raw material, and polymers was placed into a sealable aluminum pan before performing thermal scanning in the temperature range of 30 to 250 °C at a heating rate of 10 °C/min and a continuous flow of nitrogen at 50 mL/min. The powder of LNX nanocrystals prepared for this test was collected through ultracentrifugation of LNX nanosuspension at the maximum speed (16,060 rcf) for 5 min and sebsequent drying in a vacuum oven at 50 °C for 4 h. The obtained results were finally analyzed using the STARe – Software version 10.00 (Mettler Toledo, Germany, DSC 1).

2.2.4. Fourier transform infrared spectroscopy (FTIR)

The mechanism underlying the interaction of LNX and polymeric stabilizers was determined using Fourier transforms infrared spectroscopy (Bruker Optics, Germany, Model IFS-66/S) using the potassium bromide (KBr) disk method. One to two milligrams of the samples including powders of LNX nanocrystals, LNX raw material, and polymers were mixed with 150 mg of spectra-grade KBr and pressed into a disk that was 12 mm in diameter using a Carver hydraulic press (Carver, U.S. A., Model 3912). The samples were analyzed over 600–4000 cm^{-1} at an instrument resolution of 0.1 cm^{-1}.

2.2.5. Solubility studies

The solubility of LNX in different mediums was investigated by the addition of an excess amount of the samples into an experimental tube containing 5 mL of medium, followed by shaking for 48 h. The tubes were centrifuged at a relative centrifugal force of 3634 ×*g* for 10 min to obtain the supernatant. The supernatant containing solubilized LNX was filtered through membranes with 0.2 μm pore size (Sartorius, Germany, Model Minisart RC 25), diluted with phosphate buffer saline pH 6.8, and analyzed using a UV spectrophotometer (Hitachi, Japan, Model U-1800) at 375 nm.

2.2.6. Scanning electron microscope (SEM)

The morphology of nanosized, micronized LNX and the raw material wder was examined using scanning electron microscopy. The powder of LNX nanocrystals prepared for this test was collected through ultracentrifugation of LNX nanosuspension at the maximum speed (16,060 rcf) for 5 min and sebsequent drying in a vacuum oven at 50 °C for 4 h before analyzing by SEM. The dry powders were fixed on a plate using adhesive tape, coated with gold for 240 s, and analyzed using a scanning electron microscope (Hitachi, Japan, FESEM S4800).

2.3. Development of biphasic release tablet containing lornoxicam

2.3.1. Preparation method of biphasic release tablet containing lornoxicam

The SR core containing 8 mg of LNX was prepared using the wet granulation method. Briefly, raw LNX and hydroxypropyl methylcellulose derivatives (HPMCs) were separately ground, passed through a 0.125 mm sieve, and thoroughly mixed with Avicel PH101. Subsequently, the binder solution (5% PVP K30 in ethanol 70%) was added to the sifted powders to form a wet mass. The wet mass was passed through a 1 mm sieve, dried at 50 °C–60 °C, and sized through a 1 mm sieve. The obtained granules were mixed with the sifted lubricants (Aerosil and magnesium stearate) before being compressed into a tablet of 200 mg weight, 7 to 9 kp hardness, and 8.0 mm diameter.

The composition of the IR layer containing the LNX nanocrystals was analyzed by investigating the effectiveness of the critical excipients on the disintegration time of a single IR tablet and dissolution rate of LNX after 5 min in hydrochloric acid solution at pH 1.2 as the medium. The nanosuspension containing LNX was used as a binder solution to wet the homogenous powder mixture of diluent, superdisintegrant, and channel-forming agents. The wet mass was subsequently sieved through a 1 mm sieve, dried at 50 °C–60 °C, and sized through a 1 mm sieve to yield granules containing LNX nanocrystals. The granules were finally mixed with Aerosil, magnesium stearate, and sodium croscarmellose before being compressed to a tablet of 800 mg weight, 7 to 9 kp hardness, and 13 mm diameter. These optimal excipients and the procedure to form IR granules containing LNX nanocrystals were then applied for preparing the IR coating layer of the biphasic release tablets as follows.

The biphasic release tablets were prepared using the compression coating technique through the following steps. First, half of the IR granules were fed and preliminary compressed in a die of 13 mm. Second, the SR core with a diameter of 8 mm was placed into the die before the addition of the remaining half of the IR granules and compression into tablets of 800 mg weight, 7 to 9 kp hardness and 13 mm diameter (Shanghai Tianhe Pharmaceutical Machinery Co., Ltd., China, ZPW26 Core – Covered Tablet Press).

2.3.2. Dissolution study

The dissolution study was designed to observe the effect of excipients on the release behavior of LNX from both the SR core and biphasic release tablets. Consequently, most dissolution conditions were the same, except for the slight difference in the dissolution medium and sampling times. Specifically, the dissolution rate of LNX was tested in the dissolution apparatus type 2 (Erweka, Germany, Model DT 600). The study was performed at 37 °C ± 0.5 °C and under a stirring speed of 100 rpm. The withdrawn samples were subsequently filtered through membranes of pore size 0.20 μm (Sartorius, Germany, Model Minisart RC 25) and analyzed using a UV spectrophotometer (Hitachi, Japan, Model U-1800) at 372 nm with the samples in 0.1 N hydrochloric acid and at 375 nm with the samples in phosphate buffer saline, pH 6.8.

The dissolution medium for evaluating the SR core was 900 mL of phosphate buffer saline pH 6.8. Three milliliters of the aliquot was withdrawn after each hour for 8 h. Meanwhile, the dissolution medium for evaluating the biphasic release tablet was the two continuous mediums (900 mL of 0.1 N hydrochloric acid for the first 2 h and 900 mL of phosphate buffer saline, pH 6.8 for the last 8 h). Three milliliters of the aliquot was withdrawn after each hour for 10 h, and three milliliters of fresh medium was immediately supplemented into the dissolution vessels.

2.3.3. Design of experiment

To evaluate the major effect and interaction effects of critical factors in the SR core and the IR coating layer on the dissolution rate of LNX from the biphasic release tablets, the central composite design was applied. The three independent variables were $CaCO_3$ (X_1), Methocel K4M (X_2), and Pharmacoat 615 (X_3). The low (−1), medium (0), and high levels (+1) of X_1, X_2, and X_3 are presented in Table 3. The dissolution rates of LNX after 2 h (Y_1), 4 h (Y_2), 8 h (Y_3), and 10 h (Y_4) were chosen as dependent variables. The MODDE 12.0 software (Umetrics, Sweden, Version 12.0) was used for constructing the experimental design, analyzing the ANOVA table.

2.3.4. Pharmacokinetic study

The animal study was approved by the Local Scientific and Ethics Committee (No 45/PCT-HDDD). Male dogs, each weighing 16–18 kg, were used for the pharmacokinetic study. Animals had ad libitum access to tap water and food and were kept at under a temperature of 25 ± 1 °C and regular 12/12 h light/dark cycle. The dogs, which were divided into 2 groups of 4–5, were kept in fasting condition one night before the day of the experiment. The samples were biphasic release tablets (12 mg LNX) and reference tablets (12 mg LNX). The reference tablets were prepared by the wet granulation technique. Briefly, a powder mixture of LNX and Avicel PH101 was wetted by a starch solution before being passed through a sieve of 1 mm, dried, and sized to yield the dried granules. The dried granules were subsequently mixed with magnesium stearate, Aerosil, before being compressed to a tablet of 800 mg weight, 13 mm diameter, and 7–9 kp hardness. The reference tablets were selected because these were conventional tablets having the same dosage of LNX (12 mg) and the same physical properties as the biphasic release tablets.

Approximately 3–4 mL of blood was collected from a femoral vein after 5, 15, 30 min and 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48, and 72 h and placed in an experimental tube containing heparin 50 UI. The plasma was collected through centrifugation of the above samples at 2684 rcf within 10 min and preserved in a deep-freezer at −40 °C until the day of analysis.

2.3.5. LC-MS/MS analysis of lornoxicam in dog plasma

LNX concentrations in dog plasma were analyzed using liquid chromatography-tandem mass spectrometry. A system of LC-MS/MS SCIEX ExionLC™ AD Qtrap 6500$^+$ (AB Sciex, U.S.A.) coupled with LC-

20AD high-pressure pumps, column compartment, and autosampler (Shimadzu, Japan) was used. LC separation was obtained using a Symmetry C18 column (150 × 2.1 mm; 3.5 μm particle size) and a pre-column (Waters, U.S.A.) with a mobile phase consisting of a solution of formic acid in distilled water (0.1%, w/v) and acetonitrile. The gradient program was initially set at 10% acetonitrile for 1.5 min and then increased linearly to 80% acetonitrile over 3 min. Subsequently, the eluent composition was maintained at 80% acetonitrile for 3 min, then back to 10% acetonitrile in 1.6 min, and re-equilibrated over 1.9 min. The flow rate was kept constant at 0.5 mL/min. The total run time was 10 min.

The mass spectrometer was operated in negative ESI mode with the capillary voltage and temperature set at −4500 V and 400 °C, respectively. A Peak Scientific AB-3G gas generator (U.K.) was used to generate N_2, which was used as the curtain gas, and the air was used as the source gas. The MS experiments were conducted in multiple reaction monitoring modes with two transitions for each compound. Higher intensities of the precursor-to-product ion transition were used for quantification.

A 50 μL aliquot of the plasma sample was diluted by 425 μL of distilled water in a 2 mL centrifuge tube. Subsequently, 25 μL of the internal standard solution at 25 μg/mL (piroxicam in methanol) was added to the tube, followed by the addition of acetonitrile containing 0.5% formic acid (500 μL). These elements were mixed using a vortex mixer for 30 s. After the addition of 0.3 g of ammonium acetate into the tube, the mixture was mixed for approximately 1 min using the vortex mixer and centrifuged at the maximum speed (16,060 rcf) for 5 min. The supernatant was filtered through a 0.45 μm membrane, and 10 μL of the filtrate was injected into the LC-MS/MS system for analysis.

2.4. Data analysis

The data was calculated using Excel (Microsoft, U.S.A.), MODDE 12.0 (Umetrics, Sweden), and Phoenix 8.0 (Certara® Inc., U.S.A.) program. Data were expressed as the mean ± standard deviation (S.D.) and analyzed for statistical significance using the one-way ANOVA test. Values of $p < 0.05$ were considered statistically significant. The dissolution profile of the observed and predicted data was compared using a similar factor (f_2), which is described by the following equation:

$$f_2 = 50^{*}log\left\{\left[1 + (1/n)\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} * 100\right\} \quad (1)$$

where n represents the number of sampling points. T_i and R_i represent the percentage of drug dissolution rate of the observed and predicted samples at each sampling point (i).

3. Results and discussion

3.1. Optimization of lornoxicam nanocrystals

The purpose of this step was to optimize the preparation process and formulation of LNX nanocrystals, which was easily used to fabricate the IR coating layer of biphasic release tablets.

LNX nanocrystals were prepared using a top-down approach consisting of two primary continuous steps: dry-milling and wet-milling. The dry-milling step using the jet-mill technique exerted a significant effect on the size reduction of LNX (1.26 μm) against the size of the raw material (19.5 μm). The SEM results indicated that the LNX raw material existed in a rod shape and LNX microparticles exhibited a cube shape with a size of approximately 1 μm (Fig. 1). To further decrease the size of the microparticles, the effect of the wet-milling technique on the nanocrystal size was investigated.

3.1.1. Effect of polymer on the formation of LNX nanocrystals

This step aimed to determine the impact of three polymers (PVP K30, HPMC 606, and PVA 205) on the formation of LNX nanocrystals. First, the solvent-shift method was applied to gain insight into the effectiveness of polymers on the recrystallization tendency of LNX (Fig. 2). The tendency of LNX toward fast precipitation, as indicated by immediate precipitation following the solvent-shift test, proved that precipitation inhibitors are essential in the anti-solvent medium. The results presented in Fig. 2 showed that the addition of polymers on the solvent-shift process was useful for slowing the aggregation of LNX nuclei, thereby prolonging the supersaturation state of LNX in the anti-solvent. Additionally, this result was consistent with the data for particle size and polydispersity index (PDI), as presented in Table 1, where samples with supplementation of PVP K30, HPMCP 606, and PVA 205 in the ball-

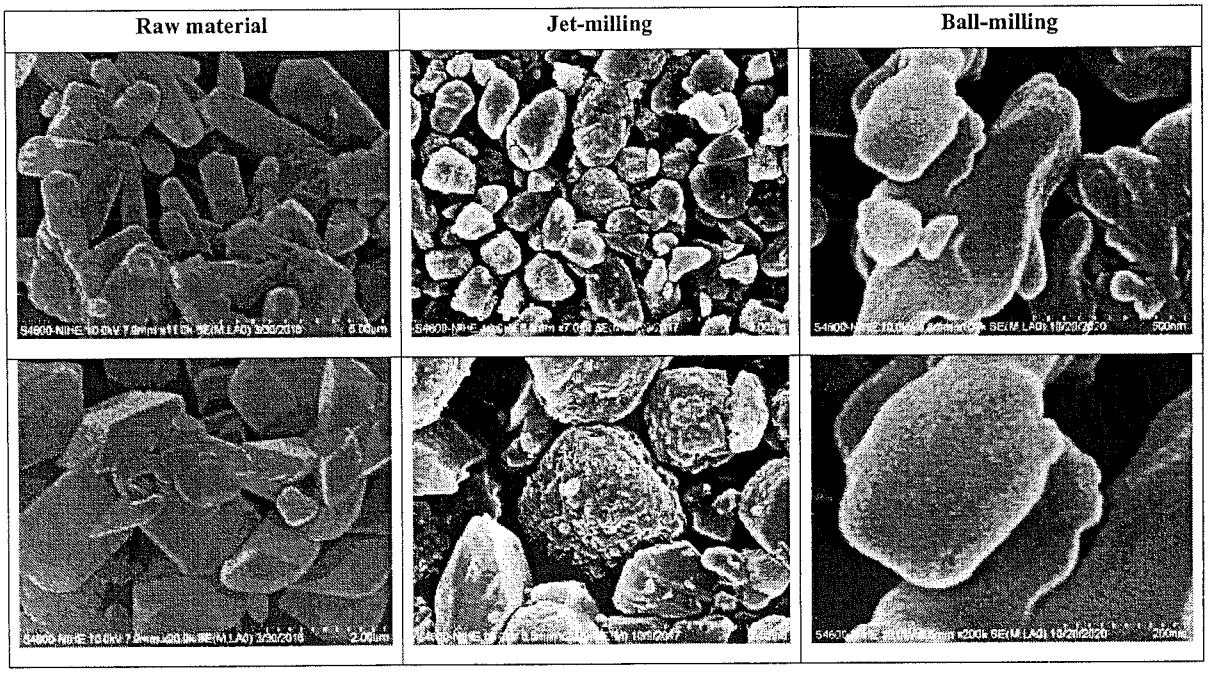
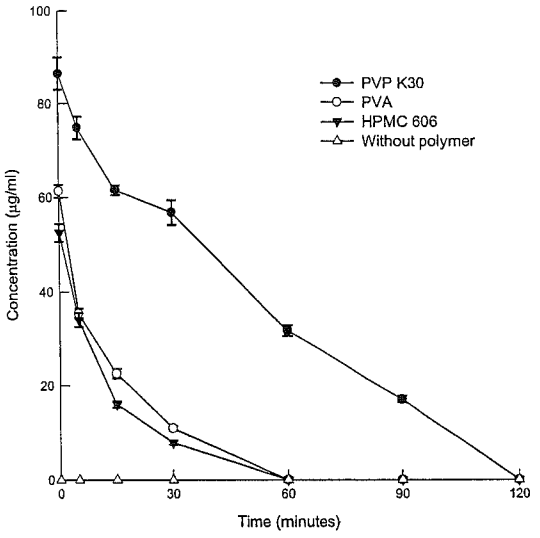


Fig. 1. SEM images of raw material, after jet-milling and ball-milling of lornoxicam.



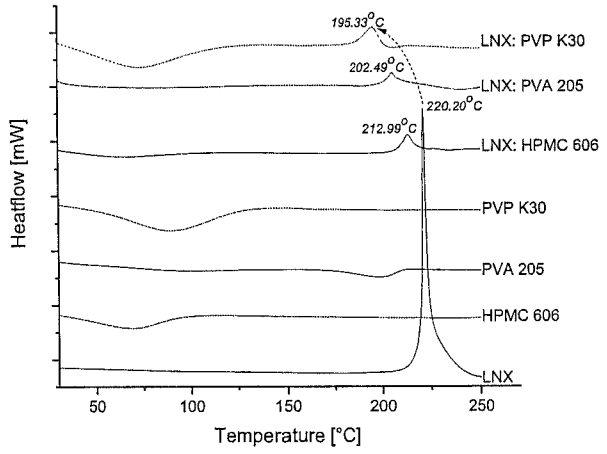


Fig. 2. Effect of polymers on a) recrystallization of lornoxicam in solvent-shift method and b) depression of melting point of LNX in DSC.

milling process showed size reduction to 279.5 ± 11.25, 775.6 ± 31.29, and 686.4 ± 25.61 nm, respectively, compared the sample without polymer (1266 ± 32.71 nm). Moreover, the solvent-shift method partly explained why PVP K30 was considered the most effective stabilizer for LNX nanocrystals.

This kind of experimental design was developed by Taylor et al. [35–40] to effectively investigate the impact of different polymers on the precipitation behavior of highly supersaturated drug solutions. The authors studied the nucleation, growth, and induction times for crystallization of supersaturated solutions containing different hydrophobic drugs such as acetaminophen, danazol, six structural analogues belonging to the dihydropyridine classes (felodipine, nifedipine, nimodipine...) in the presence and absence of polymers additives. The polymers investigated in these studies were hydroxypropylmethyl cellulose (HPMC) Pharmacoat grade 606 and hydroxypropylmethyl cellulose acetate succinate (HPMCAS), poly(acrylic acid) (PAA), polyvinylpyrrolidone K-29/32 (PVP), Kollidon VA 64 (PVPVA), carboxymethyl cellulose acetate butyrate (CMCAB), and cellulose propionate adipate (CAPAdp). The study results indicated that the precipitation behavior of drugs was strongly dependent on the specific interaction of drug and polymer, and there was no single polymer showing the highest effectiveness to the recrystallization of all drugs.

Table 1
Effect of different variables on the particle size and polydispersity index of LNX (n = 3, Mean ± STDEV).

Kind of screening study	Variables	Size (nm)	PDI
Kinds of polymer	Without polymer	1266 ± 32.71	0.4 ± 0.01
	PVP K30 (12.5%, w/v)[a]	279.5 ± 11.25	0.204 ± 0.01
	HPMC 606 (12.5%, w/v)	775.6 ± 31.29	0.225 ± 0.01
	PVA 205 (12.5%, w/v)	686.4 ± 25.61	0.241 ± 0.01
Ball-milling duration	1 h	955.4 ± 10.57	0.234 ± 0.01
	2 h	775.4 ± 30.00	0.229 ± 0.01
	3 h[a]	279.5 ± 11.25	0.204 ± 0.01
	4 h	278.6 ± 12.2	0.201 ± 0.01
Ball-milling frequency	15 Hz	546.4 ± 24.74	0.221 ± 0.01
	30 Hz[a]	279.5 ± 11.25	0.204 ± 0.01
Amount of zirconia beads	10 g	946.3 ± 29.72	0.25 ± 0.01
	15 g	473.5 ± 15.53	0.231 ± 0.01
	20 g[a]	279.5 ± 11.25	0.204 ± 0.01
Size of zirconia beads	0.8 mm	280.5 ± 12.12	0.205 ± 0.01
	0.65 mm[a]	279.5 ± 11.25	0.204 ± 0.01

[a] Fixed parameters for each kind of screening study.

For example, PVPVA was the most effective PPI with the dihydropyridine classes [39] while the systems containing HPMC-AS had the longest induction times of danazol recrystallization [37], and PVP were found to be the most effective at nucleation inhibition in supersaturated solutions of acetaminophen [40].

In the present study, the supersaturation state of LNX induced by PVP K30 was superior to that induced by HPMCP 606 and PVA 205, which showed the potential of PVP K30 in significantly slowing the two critical steps (the nucleation and growth rate) of drug crystallization. Because the supersaturation state of LNX could appear on the surface of the newly formed nanocrystals during the wet-milling process, PVP K30 was selected as the optimal PPI in the solvent-shift method as a quick and convenient procedure for preparing small and uniform nanocrystals.

The effectiveness of PVP K30 on the size reduction of LNX nanocrystals was explained by DSC and FTIR. Theoretically, it is well known that the Gibbs–Thomson equation can be used to describe the melting point depression seen in nanocrystals [41,42]. The complete Gibbs–Thomson equation for melt systems is as follows:

$$T_0 - T(r) = \frac{2\sigma_s \nu T_0}{\rho(\Delta H_f) r} \quad (2)$$

where T_0 is the melting point of a bulk crystal of infinite size, T(r) is the melting point of a crystal of size r (nanocrystals), ρ is crystal density, and ΔH f is the latent heat of fusion for the crystallizing species.

Based on the Gibbs–Thomson equation, it is estimated that the melting point difference between crystals of different sizes (i.e. bulk crystals vs. nanocrystals) is inversely proportional to the size of nanocrystals. Several published articles [42–44] also pointed out the relationship between the size of nanocrystals and melting point depression.

In our present study, the melting point depression of LNX was also observed in nanocrystals and this result agreed with the Gibbs-Thomson equation. Depending on the size of nanocrystals prepared from different kinds of polymer, the melting point depression would range from 220.20

(bulk material) to 195.33 °C (nanocrystals using PVP K30 as a stabilizer) (Fig. 2b). Specifically, the exothermic peaks of samples consisting of HPMC 606, PVA 205, and PVP K30 were 212.99 °C, 202.49 °C, and 195.33 °C, respectively. Burgess et al. [44] explained that the alteration in both the thermal property of the drug and the size of nanocrystals was caused by the surface interaction of drug and polymer. This observation suggested that DSC was a possible approach to gain insights into the role of polymer on nanocrystal formation. Additionally, the highest melting point depression of LNX in nanocrystals with PVP K30 indicated that the interaction of PVP K30 with LNX was stronger than that of other polymers with LNX. To an extent, the high affinity of PVP K30 to the surface layers of LNX nanocrystals might be the reason for the change in the thermal property of this drug.

The interaction of LNX and PVP K30 was determined by observing the movement of functional groups of LNX in FTIR spectra. The results presented in Fig. 3 indicated that the carbonyl group (—C═O) of the LNX raw material, physical mixture, and nanocrystals of LNX: PVP K30 (1:1) was located at the wavenumber of 1646.91 cm^{-1}. Other peaks of lornoxicam observed in FTIR were C—Cl (765.61 and 790.67 cm^{-1}); O═S═O (1037.52 cm^{-1}), CH—Ar (3067.23 cm^{-1}), —NH— bending (1595.81 cm^{-1}), and —OH (3444.24 cm^{-1}). Additionally, PVP K30 possessed the carbonyl group on the pyrrole ring located at 1670.05 cm^{-1}. However, this carbonyl group in nanocrystals of LNX:PVP K30 at different ratios (1:2.5; 1:3; 1:5, w/w) moved to 1668.12 cm^{-1}, 1663.3 cm^{-1}, and 1667.16 cm^{-1}, respectively. The positions of most peaks of LNX (C—Cl, O═S═O, CH—Ar, and —NH—) were almost unchanged except the —OH peak of LNX, which moved from 3444.24 cm^{-1} to 3443.28 cm^{-1}, 3441.35 cm^{-1} and 3435.56 cm^{-1} in FTIR spectra of nanocrystals with various ratios of LNX: PVP K30 (1:2.5; 1:3; 1:5, w/w). This result indicated that the carbonyl group of both LNX and PVP K30 participated in a new interaction at the surface of the LNX nanocrystals. On the basis of the structure of LNX, it can be assumed that the carbonyl group (—C═O) of one LNX molecule connected to the hydroxyl group (—OH) of another molecule through a hydrogen bond (—C═O…O—H) to form intermolecular hydrogen bonds of LNX that caused the low solubility of this drug [45]. The appearance of PVP-30 with a carbonyl group (—C═O) on the pyrrole ring might have interfered with these intermolecular hydrogen bonds (—C═O…O—H) of LNX. A new hydrogen bond between the —C═O group of PVP K30 and —OH of LNX might have formed, which changed the carbonyl group wavenumber of both LNX and PVP K30 (Fig. 4). This observation was consolidated by Sharma et al. [46] whose study showed high mechanical energy input during wet milling may induce surface interaction between indomethacin and PVP, thereby leading to disruption of the indomethacin dimers.

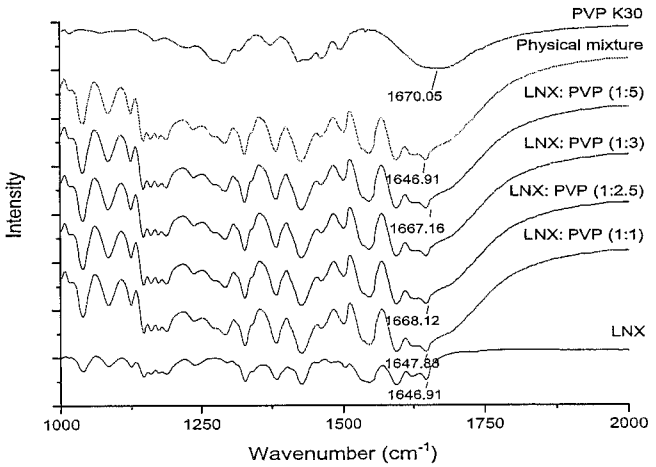


Fig. 3. FTIR diagram showing the interaction of LNX and PVP K30.

3.1.2. Effect of the preparation process on particle size

We evaluated the effect of critical variables of the wet-milling process including duration and frequency of milling, as well as the size and weight of the milling-ball on the particle size and PDI of LNX nanocrystals. The results in Table 1 illustrated the effect of the duration of the ball-milling process and frequency of the milling motor on the size and PDI of the nanocrystals. Specifically, the LNX nanocrystals exhibited the smallest size of 279.5 ± 11.25 nm when the duration of the wet-milling process and frequency of the milling motor were optimized at 3 h and 30 Hz, respectively. The shorter milling durations (1 and 2 h) and lower milling frequency (15 Hz) were also evaluated; however, the particle size of LNX did not decrease below 500 nm. Additionally, the longer milling duration (4 h) could not cause a significant difference in the size of the LNX nanocrystals compared to the optimized milling duration (3 h). This observation implied that an energy threshold was reached after 3 h of the milling process at a frequency of 30 Hz that was sufficiently high to break the intermolecular hydrogen bonds of the LNX molecules and form smaller LNX nanocrystals.

Other factors possibly influencing the size of the LNX nanocrystals were the properties of the milling-ball. First, the amount of zirconia beads was evaluated at three values (10, 15, and 20 g). The amount of milling-ball was inversely proportional to the nanocrystal size. The maximum amount of zirconia beads (20 g) possibly loaded in the zirconia ball mill jar exhibited the highest surface area, thereby easily colliding with the LNX particles and forming the smallest LNX nanocrystals with the lowest PDI (279.5 ± 11.25 nm and 0.204 ± 0.01). Meanwhile, the other amounts of zirconia beads (10 and 15 g) only created bigger LNX nanocrystals (946.3 ± 29.72 and 473.5 ± 15.53 nm, respectively) with higher PDIs (0.25 ± 0.01 and 0.231 ± 0.01, respectively). Second, the effect of two sizes of zirconia beads including 0.65 and 0.8 mm on the particle size of the nanocrystals was investigated. As shown in Table 1, the size of the milling-ball did not cause a significant difference in the size reduction of the nanocrystals. The study used zirconia beads of 0.65 mm for further study because the size and PDI of the two nanocrystals using these two types of zirconia beads (0.65 and 0.8 mm) were almost the same at approximately 280 nm and 0.2, respectively.

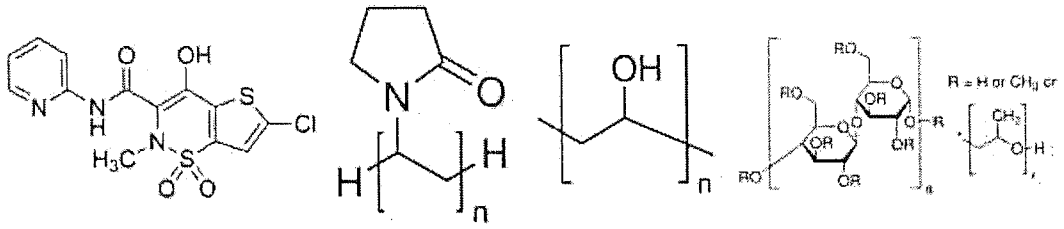
Generally, the optimal size and PDI of LNX nanocrystals are 279.5 ± 11.25 nm and 0.204 ± 0.01, respectively. The SEM images indicated almost cube-shaped nanocrystals with a size of approximately 200 nm. The small size of the nanocrystals showed an advantage in the improvement of drug solubility. Owing to the high surface area, the solubility of the LNX nanocrystals in distilled water (42.03 ± 0.43 μg/mL) and the pH 1.2 medium (18.02 ± 0.71 μg/mL) was approximately 3 and 3.35 times higher than that of the LNX raw material in the same mediums (13.41 ± 0.47 and 5.38 ± 0.22 μg/mL), respectively.

The significant enhancement of the solubility of the free unionized drug in the gastric medium (pH 1.2) using nanocrystals indicated the potential to accelerate drug absorption in the upper GI tract and shorten the time to onset of action of this drug. Besides, the successful preparation of LNX nanocrystals using a top-down approach, a convenient preparation process, indicated high prospects for easy application in the preparation of final pharmaceutical dosage forms. Similarly, the potential of a top-down approach in the manufacturing of nanocrystals and application for final dosage forms were indicated in other studies [14,47–50]. Specifically, Scholz et al. [51] successfully developed more cost-effective, faster, and easily scalable technology (ARTcrystal®) for the production of drug nanocrystals based on a top-down approach.

3.2. Preparation of biphasic release tablet containing lornoxicam

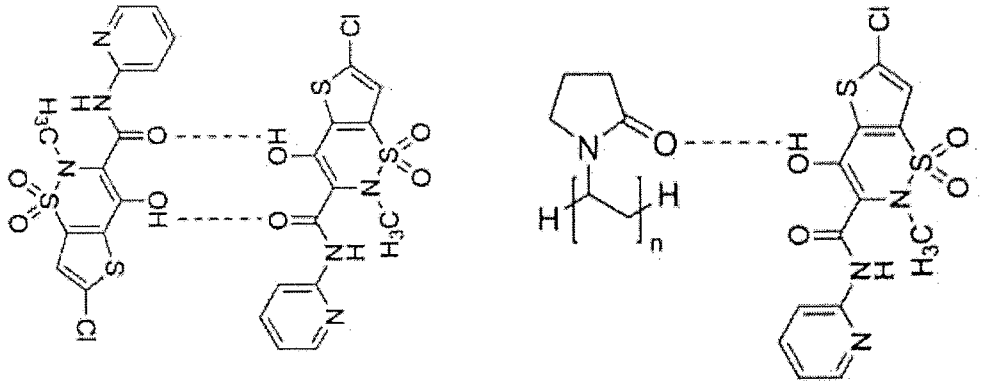
3.2.1. Screening compositions for immediate-release coating layer and sustained-release core

The criteria to select the optimal compositions of the IR coating layer were the shortest disintegration time of the coating layer and the highest percentage of LNX release after 5 min in pH 1.2 medium. The core



Lornoxicam PVP K30 PVA 205 HPMC 606

a)



b) Dimer LNX – LNX c) Hydrogen bonding of PVP - LNX

Fig. 4. a) The structure of drug and polymers; b) Intermolecular interaction of LNX – LNX; c) Hydrogen bonding of PVP – LNX.

compositions of the IR coating layer consisting of LNX nanocrystals, diluent, superdisintegrant, channel-forming agent, magnesium stearate, and Aerosil were investigated step-by-step. First, Avicel PH 101 was chosen as the main diluent since this excipient could produce the shortest disintegration time (254 s) and the highest release rate of LNX after 5 min (32.93%) compared to other diluents (Table 2). Furthermore, due to these two reasons, Disocel was selected as the most suitable superdisintegrant for this layer compared with the two remaining agents (sodium starch glycolate and Kollidon CL). To further improve the disintegration time and percentage of drug release in the gastric medium, a channel-forming agent, $CaCO_3$, which is a lubricant and pH modulator as well, was added into the IR layer. As shown in Table 2, the appearance of $CaCO_3$ accelerated the uptake of the gastric medium into the core of the IR layer, thereby guaranteeing the disintegration of this layer and the release rate of LNX. Because the release and disintegration behavior of this layer depended utmost on the ratio of $CaCO_3$, the excipient was selected as a representative variable of the IR coating layer in the DoE of the biphasic release tablet.

The SR core of the biphasic release tablets was optimized to exhibit zero-order release, in which the predicted release rate of LNX in pH 6.8 medium at 2, 4, 8 h were approximately 20, 40, and 80%, respectively. The release profile of LNX in PBS at pH 6.8 using various HPMC derivatives was compared to the target value by a similar factor (f_2). As shown in Table 2, the application of only one type of HPMC as a controlling agent in the SR core could not yield the predicted dissolution profile. The tablets using low viscosity HPMCs were quickly eroded during the first phase of the dissolution process thereby the burst release of LNX was observed in these tablets. The higher viscosity polymers were effective in sustaining drug release for 8 h; however, the limited water uptake into the tablets resulted in LNX dissolution rates lower than the predicted values. To improve water uptake into the SR core, the synergistic effect of a polymeric mixture including a low viscosity polymer (Pharmacoat 615) and a high viscosity polymer Methocel K4M on the release rate of LNX was evaluated. A combination of 10% Pharmacoat 615 and 10% Methocel K4M resulted in the desired dissolution profile of LNX with an f_2 of 86. The release rate of LNX was fitted into 6 different release models, and the criteria to select the appropriate dissolution model was the Akaike information criterion (AIC). The zero-order model was the most suitable because of its AIC value (0.754) was the lowest compared to that of other models including the first-order (27.040), Higuchi (37.189), Weibull (13.792), Hixson-Crowell (20.300), and Hopfenberg (34.157) models. Because the release profile of LNX from the SR core was strongly dependent on the ratio of both Methocel K4M and Pharmacoat 615, these two excipients were selected as representative variables of the SR core in designing the biphasic release tablet.

3.2.2. *DoE-based formulation of biphasic release tablets*

The effects of the three critical factors including $CaCO_3$ (X_1), Methocel K4M (X_2), and Pharmacoat 615 (X_3) on the dissolution rate of LNX were determined through 17 formulations (Table 3).

In the present study, based on the separated dissolution rate of LNX from the IR layer and SR core, the predicted ranges (targeted values) of the LNX dissolution rate from the biphasic release tablet after 2, 4, 8, 10 h were 20–40 (33%), 45–65 (60%), 75–95 (90%), and 90–105 (100%), respectively. The dissolution rate of LNX at 2 h in the pH 1.2 medium was responsible for the fast-release phase, while that within 2–10 h in the pH 6.8 medium caused the SR phase.

Results in Table 4 showed the fast-release phase of LNX from the dry coating layer at 2 h was not heavily dependent on either the main effect or the interaction effect of critical factors ($p > 0.05$). The LNX dissolution rate after 2 h from most formulations was higher than the targeted value (33%), which partly resulted from the complete release of LNX from the fast-release layer and partial release from the SR core. Because of the insignificant impact of $CaCO_3$ on the dissolution rate of LNX at 2 h ($p > 0.05$), it might be extrapolated that the absolute release of LNX

Table 2
Effect of the composition of the immediate-release layer on the disintegration time and dissolution rate of LNX after 5 min in pH 1.2 medium and effect of the types of HPMCs in the sustained-release core on the value of the similarity factor (f_2).

Screening compositions of the immediate-release layer		Disintegration time (seconds)	% LNX release after 5 min
Effect of diluents	Lactose: starch (2:2, w/w)	351 ± 31	22.83 ± 2.00
	Lactose: starch (1:3, w/w)	332 ± 2	20.63 ± 1.93
	Avicel PH 101	254 ± 31	32.93 ± 0.92
Effect of superdisintegrants	Sodium starch glycolate	162 ± 2	33.20 ± 2.81
	Kollidon CL	126 ± 5	32.93 ± 4.13
	Disocel	102 ± 8	45.57 ± 2.07
Effect of channel-forming agents	LNX: $CaCO_3$ (1:1, w/w)	40 ± 3	48.15 ± 3.56
	LNX: $CaCO_3$ (1:2, w/w)	30 ± 2	58.01 ± 1.04
	LNX: $CaCO_3$ (1:3, w/w)	30 ± 1	50.61 ± 1.07

Screening compositions of the sustained-release core		f_2
Effect of low viscosity HPMC	Methocel K15LV	31
	Methocel K100LV	42
	Pharmacoat 606	33
	Pharmacoat 615	22
Effect of medium viscosity HPMC	Methocel K4M	39
	Metolose 90 SH-4000	23
	Metolose 90 SH-15000	26
Effect of high viscosity HPMC	Methocel K100M	23
	Metolose 90 SH-100000	22
Effect of a couple of HPMC	Methocel K4M: Pharmacoat 615 (10%:5%, w/w)	22
	Methocel K4M: Pharmacoat 615 (10%:10%, w/w)	86
	Methocel K4M: Pharmacoat 615 (10%:15%, w/w)	39

from the IR layer was primarily caused by the nanosized LNX. The high surface area of LNX nanocrystals increased both the solubility and dissolution rate of LNX in the fast-release phase. The remaining amount of LNX released from the SR core at 2 h was antagonistically affected ($p > 0.05$) by Methocel K4M and Pharmacoat 615 because the high viscosity of these two controlled release polymers inhibited the drug release.

Second, the SR phase of LNX was described by the dissolution rate of LNX after 4, 8, and 10 h. The ANOVA results indicated the significance of the interaction effect on the dissolution rate of LNX at these points ($p < 0.05$); meanwhile, the main effect of the variables was not remarkable. Specifically, the interaction of Methocel K4M * Pharmacoat 615 significantly impacted the dissolution rate of LNX at all points in this phase. Meanwhile, the interaction of $CaCO_3$ * Methocel K4M and $CaCO_3$ * Pharmacoat 615 only exerted a significant impact on the dissolution rate of LNX at the final time points of the dissolution profile. The combination of a high viscosity polymer (Methocel K4M) as the primary polymer for controlled release and a low-viscosity polymer (Pharmacoat 615) as a water-uptake channel was useful for creating a dissolution profile of LNX that was similar to the predicted range. The appearance of $CaCO_3$ at the low level did not play a crucial role in altering the dissolution profile of LNX because this excipient, present in the outer layer of the tablets, was disintegrated and dispersed into the dissolution medium. However, the release rate of LNX after 8 and 10 h from the SR core remarkably increased with the appearance of a high amount of this alkaline agent ($CaCO_3$ * $CaCO_3$) ($p < 0.05$). For producing the optimal formulation, a predicted ratio of these excipients was simulated by MODDE 12.0. Accordingly, the amount of $CaCO_3$, Methocel K4M, and Pharmacoat 615 in the optimal tablet were 10.88, 13.26, and 14.90 mg, respectively. The fact that f_2 was 84 indicated that the observed dissolution profile of this optimal tablet was similar to the results predicted by the software (Fig. 5a), and this optimal tablet with the drug content uniformity of around 12.0 mg/tablet would be used for the pharmacokinetics study. Before conducting the in vivo study, the dissolution profiles of the biphasic release tablet and the reference tablet in the pH-shift medium was also compared. Even though the two tablets had the same dosage of LNX (12 mg) and physical properties (800 mg weight, 13 mm diameter,

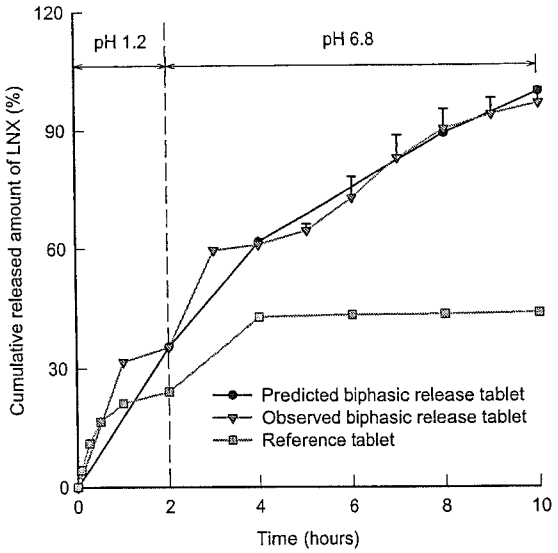
Table 4
Regression results indicating the impact of input variables on the release rate of LNX from biphasic release tablets after 2, 4, 8, and 10 h.

	Coeff_Y_2	Coeff_Y_4	Coeff_Y_8	Coeff_Y_{10}
Constant	10.12*	4.07*	5.20*	6.14*
$CaCO_3$	−0.16	−0.08	−0.02	−0.06
Methocel K4M	−0.07	0.05	−0.07	−0.13
Pharmacoat 615	−0.55	0.01	0.03	0.01
$CaCO_3$*$CaCO_3$	0.50	0.73	1.04*	1.32*
Methocel K4M*Methocel K4M	0.30	0.41	0.29	0.08
Pharmacoat 615*Pharmacoat 615	0.00	0.18	−0.22	−0.89*
$CaCO_3$*Methocel K4M	0.59	−0.23	−0.61*	−0.59*
$CaCO_3$*Pharmacoat 615	0.24	−0.16	−0.57*	−0.56*
Methocel K4M*Pharmacoat 615	−0.11	−0.97*	−0.74*	−0.63*

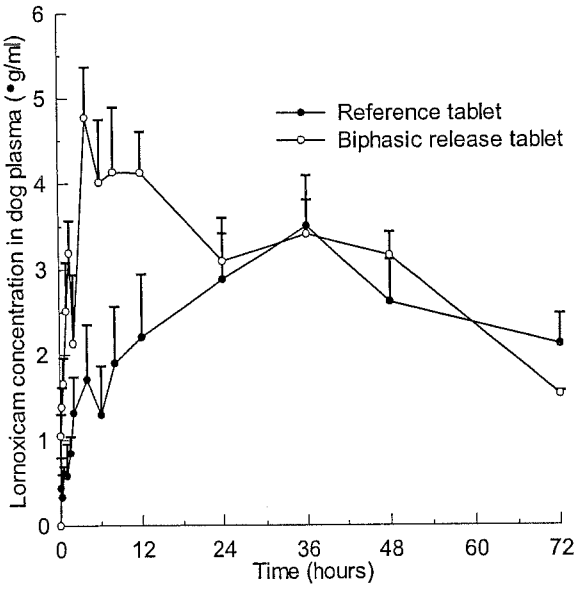
* $p < 0.05$.

Table 3
Design of biphasic release tablets and release rate of LNX after 2, 4, 8, and 10 h.

Formulation	X_1 ($CaCO_3$) (−1, 4 mg) to (+1, 12 mg)	X_2 (Methocel K4M) (−1, 10 mg) to (+1, 30 mg)	X_3 (Pharmacoat 615) (−1, 10 mg) to (+1, 30 mg)	Y_2 (%)	Y_4 (%)	Y_8 (%)	Y_{10} (%)
N1	−1	−1	−1	45.46	60.33	66.8	73.38
N2	+1	−1	−1	32.94	58.73	97.5	101.22
N3	−1	+1	−1	37.19	91.32	104.78	106.36
N4	+1	+1	−1	43.89	93.74	105.49	106.94
N5	−1	−1	+1	34.3	82.61	101.45	103.03
N6	+1	−1	+1	36.09	89.32	104.37	105.37
N7	−1	+1	+1	35.57	76.7	101.93	106.12
N8	+1	+1	+1	34.42	53.23	63.01	67.95
N9	−1	0	0	36.48	61.44	89.8	106.25
N10	+1	0	0	36.09	66.66	91.01	105.01
N11	0	−1	0	38.00	68.36	86.66	98.94
N12	0	+1	0	33.16	50.97	72	76.48
N13	0	0	−1	34.43	54.64	67.63	70.31
N14	0	0	+1	34.71	58.14	76.12	77.24
N15	0	0	0	35.9	62.37	79.64	92.32
N16	0	0	0	34.61	60.2	74.61	85.58
N17	0	0	0	34.18	58.54	80.58	95.37



a)



b)

Fig. 5. Illustration of a) dissolution rate of LNX and b) pharmacokinetics profiles of LNX in dog plasma for different dosage forms.

and 7–9 kp hardness), the drug release from the biphasic release tablet was still higher than that from the reference product. It could be explained by the following reasons. Firstly, the driving force of drug release from the two tablets was different. The sustained release core of biphasic release tablet controlled the drug release by gradual erosion process of HPMC. Therefore, the drug would release completely when the HPMCs were all eroded. Meanwhile, the reference product managed the drug release by the water uptake inside the tablet for accelerating the tablet disintegration. However, its hardness, which is to the biphasic release tablet, resulted in a concrete structure of the reference product, thus inhibiting the water uptake completely into the core of tablet. Secondly, compositions of the biphasic release tablet such as pH modulator ($CaCO_3$) or highly soluble polymer (PVP) might partly accelerate the drug release from this tablet.

3.2.3. Pharmacokinetics study

This study aimed to gain a deeper understanding of the release of LNX from the optimal biphasic release tablet in an in vivo model.

In general, the PK results for the biphasic release tablet, as presented in Fig. 5b, showed the two stages: the fast-absorption phase and slow-elimination phase of LNX, resulting from the IR layer and SR matrix core, respectively. Meanwhile, the PK profile of LNX for the reference tablet did not exhibit a fast-absorption phase but a gradual absorption pattern of LNX.

The pharmacokinetics parameters of LNX for the two tablets are shown in Table 5. Accordingly, the maximum LNX concentration in the dog plasma (C_{max}) for the reference tablets (3.65 ± 0.62 μg/mL) was 1.4 times lower than that for the biphasic release tablets (5.12 ± 0.69 μg/mL). In addition, the time taken for LNX to reach C_{max} (T_{max}) in the dog plasma for reference tablets (27.00 ± 5.74 h) was significantly longer compared to that for the biphasic release tablets (5.50 ± 0.96 h). These results indicated that the slow initial absorption of LNX from reference tablet was caused by the slow dissolution rate, which agreed with the in-vitro release study (Fig. 5a).

Because of such phenomenon, the reference tablet had the extended-release profile on plasma concentration – time curve. Meanwhile, the extended-release phase was also observed in the pharmacokinetics profile of the biphasic release tablet due to the gradual erosion of the HPMC matrix core. Consequently, both the biphasic release tablet (half-life of 54.22 ± 16.98 h) and the reference one (57.30 ± 3.05 h) exhibited comparable extended plasma concentration profiles and prolonged action in pain relief. However, the high solubility of nanosized LNX and the quick disintegration time (30 s) of the IR layer of the biphasic release tablets result in high initial plasma concentration and potentially shorten the time to onset of action of LNX, which might rapidly reduce the symptoms of acute pain and inflammation in osteoarthritis and rheumatoid arthritis. This is an edge compared to the reference tablet and opens up a high potential in the treatment of other acute diseases which requires the fast absorption rate of drug.

Regarding the extended-release phase of the two dosage forms, the advantage of the biphasic release tablet over the reference tablet might not be obviously seen within the scope of the present study because of reasons extrapolating from the basic pharmacokinetics equations:

Firstly, the half-life of drug is proportional to the volume distribution through the Eq. (3)

$$\text{Half} - \text{life} = 0.693 \times \text{Volume of distribution/Clearance.} \quad (3)$$

where, the volume of distribution is the volume of plasma that would be necessary to account for the total amount of drug in the patient's body, if that drug was present throughout the body at the same concentration as found in the plasma. And the volume distribution is used to determine the loading dose necessary for the plasma concentration of a drug in single compartment model through the simplified Eq. (4):

$$\text{Volume of distribution} = \text{Dose}/C_0 \quad (4)$$

Table 5
Pharmacokinetics parameters of lornoxicam after oral administration of reference tablet and biphasic release tablet in dogs (Mean ± SE).

Formulation	Reference tablet	Biphasic release tablet
Mean C_{max} (μg/ml)	3.65 ± 0.62	5.12 ± 0.69
CI_90_Lower (%)		75.88[a]
CI_90_Upper (%)		266.20[a]
$AUC_{0\text{-}72h}$ (μg.h/ml)	181.31 ± 34.61	198.44 ± 13.96
CI_90_Lower (%)		75.89[a]
CI_90_Upper (%)		173.77[a]
T_{max} (h)	27.00 ± 5.74	5.50 ± 0.96
$T_{1/2}$ (h)	57.30 ± 3.05	54.22 ± 16.98

[a] 90% Confidence Intervals of the ratio ($C_{max\_biphasic\ release\ tablet}/C_{max\_reference\ tablet}$ or $AUC_{biphasic\ release\ tablet}/AUC_{reference\ tablet}$) were transformed by the logarithm.

where the C_0 is extrapolated concentration at time = 0 on a plasma concentration vs. time curve.

Based on these two equations, the pharmacokinetics results of LNX might be discussed. Firstly, the high dose of LNX (12 mg) in the pharmacokinetics study vs. the regular dose (4 mg) of LNX, was one of the reasons for the long half-file of LNX from both the biphasic release tablet and the reference one. Secondly, such kind of LNX dose might be used in human, in which the volume of distribution was around 0.1–0.2 L/kg [12]. However, there was not information about the volume of distribution of LNX in dog, the animal model in the current study. Therefore, the correlation between the volume of distribution of LNX in human and the experimental animal (dog) was considered using volumes of plasma in human and dog, which were assumed around 3 and 0.5 L, respectively. Consequently, based on Eq. (4), the plasma concentration of LNX and half-life of drug in dog might be six times higher than that in human. This also meant the drug concentration in plasma almost obtained the state-state. Therefore, results in Fig. 5b indicated that the plasma concentration of LNX in both the reference and biphasic release tablet were almost similar after 24 h. The design of a PK study in a more relevant model such as humans might be more effective for comparing the SR behavior of LNX from the biphasic release tablet with the uncontrolled release behavior of LNX from the reference tablet.

Along with indicating the advantage of the LNX absorption rate from the biphasic release tablets, the pharmacokinetics study indicated that these tablets exhibited a higher absorption of LNX than the reference tablet. The 90% confidence intervals of the ratio $AUC_{biphasic\ release\ tablet}$/$AUC_{reference\ tablet}$ transformed by the logarithm were not in the range of 80–125% CI_90, which indicated that the two dosage forms were not bioequivalence. Moreover, the mean relative bioavailability of the biphasic release tablet/reference tablet was approximately 109.45%, which showed that the biphasic release tablet exhibited a higher bioavailability than the conventional dosage form. The application of LNX nanocrystals for increasing the drug solubility in the upper GI tract might be the major reason for the enhancement of drug bioavailability.

The IVIV correlation between the in vitro dissolved fraction and the in vivo absorbed fraction of LNX was estimated from four linear functions in the Phoenix 8.0 IVIVC Toolkit (U.S.A, Pharsight, CA) software with four main steps: 1) fitting the dissolution results into the mathematical models; 2) generation of the unit impulse response (UIR); 3) deconvolution to explore the time course of in vivo absorption; and 4) construction of IVIV correlation.

First, to determine the most suitable dissolution models, the in vitro results were fitted into one of the four mathematical models: Hill, Weibull, Double Weibull, and Makoid-Banakar. The Akaike information criterion (AIC) values of biphasic release tablet corresponding to each mathematical model were calculated, and the model with the lowest AIC value was selected as the most suitable. Accordingly, the Makoid-Banakar model was selected as the optimal dissolution model.

The second step was to generate the unit impulse response (UIR) presenting in Eq. (5):

$$UIR = R/D \tag{5}$$

where R is the response, and D is the dose of LNX.

The unit impulse response of many points can be expressed by the following exponential Eq. (6):

$$UIR(t) = \sum_{j=1}^{n} Ae^{-\alpha jt} = -0.3611e^{0.1108*t} + 0.3611e^{0.0077*t} \tag{6}$$

In addition, the concentration of drug in plasma can be described by the convolution of function UIR(t) and speed of drug input into the body (Eq. (7)):

$$c(t) = UIR(t)I(t) \tag{7}$$

Consequently, deconvolution of Eq. (3) revealed the speed of drug input into the body, I(t), which was then used to calculate the absorbed fraction using Eq. (8):

$$A(t) = \int_0^t I(u)du \tag{8}$$

The correlation between the absorbed fraction and the dissolved fraction was finally built using one of the four linear equations available in the software. The selection of the optimal equation was based on the AIC value. The result in Table 6 indicates that Equation Fabs = AbsScale*Diss(Tscale*Tvivo) had the lowest AIC (109.53), which showed the largest correlation between the absorbed fraction and dissolved fraction:

$$Fabs = 1.5047*Diss(1.3888*Tvivo) \tag{9}$$

The results showed that the in vitro dissolution test consisting of the two continuous mediums (900 mL of 0.1 N hydrochloric acid for the first 2 h and 900 mL of phosphate buffer saline, pH 6.8 for the last 8 h) was correlated to the in vivo release in the animal model, and this in vitro dissolution test was suitable to develop the biphasic release tablet containing lornoxicam.

4. Conclusion

In general, the study was successful in developing a biphasic release tablet containing LNX, which exhibited a fast-release phase and a sustained-release phase. The release rate of the drug from the IR layer was enhanced using LNX nanocrystals. The SR matrix core was prepared using a wet-granulation technique with different derivatives of HPMC. The biphasic release tablet optimized using the DoE approach demonstrated an improvement in the rate and extent of absorption in dogs in comparison to the reference tablet. Besides the positive results, however, there still exist several points to be addressed in future studies such as the effect of the preparation process on the critical factors of tablets, the design of a PK study in a more relevant model such as humans and preclinical studies. We believe that this strategy can potentially increase the therapeutic efficiency of drugs that require an early onset of action and low administration frequency such as indomethacin [52] and nifedipine [53].

CRediT authorship contribution statement

Conception and design of study: Nguyen-Thach Tung.

Acquisition of data: Thi-Hoang-Yen Dong, Cao-Son Tran, Thi-Kim-Thuy Nguyen, Sang-Cheol Chi, Danh-Son Dao, Dang-Hoa Nguyen.

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Table 6
Akaike information criterion (AIC) values of the biphasic release tablets obtained from different dissolution models and IVIVC correlation equations.

Models		Biphasic release tablet
Dissolution profiles	Hill	−40.10
	Weibull	−40.57
	Double Weibull	Fail
	Makoid-Banakar	−40.74
IVIV Correlation equation	Fabs = Diss(Tvivo)	341.31
	Fabs = AbsScale*Diss(Tscale*Tvivo)	109.53
	Fabs = AbsScale*Diss (Tscale*Tvivo–Tshift)	111.53
	Fabs = AbsScale*(Diss (Tscale*Tvivo–Tshift)–AbsBase)	113.57

Data availability

Data will be made available on request.

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Research paper

Formulation and biopharmaceutical evaluation of bitter taste masking microparticles containing azithromycin loaded in dispersible tablets

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ABSTRACT

The objective of this study was to prepare and evaluate some physiochemical and biopharmaceutical properties of bitter taste masking microparticles containing azithromycin loaded in dispersible tablets. In the first stage of the study, the bitter taste masking microparticles were prepared by solvent evaporation and spray drying method. When compared to the bitter threshold (32.43 μg/ml) of azithromycin (AZI), the microparticles using AZI:Eudragit L100 = 1:4 and having a size distribution of 45–212 μm did significantly mask the bitter taste of AZI. Fourier transform infrared spectroscopy (FTIR), and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) proved that the taste masking of microparticles resulted from the intermolecular interaction of the amine group in AZI and the carbonyl group in Eudragit L100. Differential scanning calorimeter (DSC) analysis was used to display the amorphous state of AZI in microparticles. Images obtaining from optical microscopy and scanning electron microscopy (SEM) indicated the existence of microparticles in regular cube shape with many layers. In the second stage, dispersible tablets containing microparticles (DTs-MP) were prepared by direct compression technique. Stability study was conducted to screen pH modulators for DTs-MP, and a combination of alkali agents ($CaCO_3$:NaH_2PO_4, 2:1) was added into DTs-MP to create microenvironment pH of 5.0–6.0 for the tablets. The disintegration time of optimum DTs-MP was 53 ± 5.29 s and strongly depended on the kinds of lubricant and diluent. The pharmacokinetic study in the rabbit model using liquid chromatography tandem mass spectrometry showed that the mean relative bioavailability (AUC) and mean maximum concentration (C_{max}) of DTs-MP were improved by 2.19 and 2.02 times, respectively, compared to the reference product (Zithromax®, Pfizer).

1. Introduction

Azithromycin (AZI) is a broad-spectrum antibiotic with strong antibiotic activity. The drug is commonly indicated to treat or prevent many different kinds of infections (e.g. respiratory infections, skin infections, ear infections, and sexually transmitted diseases). AZI is a subgroup of macrolide antibiotics and has a similar antibacterial mechanism of action to that of erythromycin, an older macrolide antibiotic. However, AZI has a 15-membered ring and a methyl-substituted nitrogen replacing the 9A carbonyl group [1]. For this reason, AZI has several advantages including fewer gastrointestinal (GI) side effects and a longer half-life ($T_{1/2}$ = 68 h) as compared to erythromycin [1,2]. The long half-life of azithromycin is very beneficial to patients, especially those who are children and older adults because of the minimum frequency of administration required and the improved compliance of patients to treatment [1].

Azithromycin is marketed in several oral dosage forms including film-coated tablet, capsule, extended-release oral suspension, and granules for suspension in sachet [3,4]. To successfully develop these oral dosage forms, the manufacturers need to overcome the general limitations of macrolide antibiotics including extreme bitter taste and instability in the gastric medium [5]. The film-coated tablet is commonly used to mask the taste of AZI. However, this dosage form is not quite suitable for the pediatric patients or infants since it is not easy for these subjects to swallow tablets. Consequently, a palatable oral suspension containing AZI has been

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commercialized for pediatric medications. However, this dosage form faces up to some disadvantages such as unpleasant taste (especially to pediatric patients), physical instability, imprecise dosage and high transportation cost.

The addition of sweeteners and flavors to suspension has been used as the most popular approach to obscure the unpleasant taste of suspension. Choi et al. [6] investigated the taste masking effect of several sweeteners such as neohesperidin dihydrochalcone, sucrose, sucralose, and aspartame. Acetaminophen, ibuprofen, tramadol hydrochloride and sildenafil citrate were model drugs (all at 20 mM). The bitterness value of free drugs and combinations of drug and sweeteners was investigated by a multichannel taste sensor system (an electronic tongue). The authors concluded that the taste masking of the drugs varied with the concentration and type of sweeteners. However, molar amounts and ionic structure of sweeteners played a more important role in taste masking than their sweetening potency. Even though being effective, this method does not work with active pharmaceuticals having an extremely bitter taste like azithromycin [7,8], since the compounds still can dissolve in the saliva and have an impact on the taste receptors. Furthermore, as azithromycin dissolves in upper parts of the gastrointestinal tract, it will interact with the bitter receptors or motilin receptors located in the upper GI tract and elicit responses such as an intense lingering aftertaste or GI side effects (nausea, vomiting, and diarrhea). Consequently, the aim of taste masking microparticles containing AZI is to prevent interactions of the dissolved drug with the receptors in the mouth, throat, or stomach.

Several approaches applying to inhibit the contact of dissolved AZI with the saliva include the application of physical barrier on the drug/the dosage form or modification of AZI solubility [7,9,10]. The physical barrier of dosage forms can be carried out using polymeric coating processes in fluid bed systems or conventional coaters [11–13]. By using new fluid-bed coating approach, Stange formulated the bitter taste masking granules containing naproxen sodium. Eudragit E was effective in inhibiting the drug release to a level that is under the threshold bitter value, and an appropriate taste masking for more than 5 min was guaranteed [12]. However, the taste masking coating of drug or dosage forms may break during compression to prepare dispersible tablets (DTs). Thus, modification of AZI solubility is more suitable to prepare tablets [7,9,10]. Bora et al. [14] used the spray-drying technique to develop the taste-masking microspheres of an intensely bitter drug, ondansetron hydrochloride (OSH). The bitter taste threshold value of OSH was determined. Chitosan, hydroxypropyl methylcellulose (methocel E15 LV), and poly(meth)acrylates (Eudragit E100) were used as polymeric carriers for preparation of microspheres. The results indicated that methocel could not mask the taste of OSH at any drug–polymer ratios. Meanwhile, the Eudragit microspheres showed their taste masking effect at 1:2 drug–polymer ratio and chitosan microspheres took effect at the ratio of 1:1. Similarly, this approach has been proved effective with azithromycin using ethylcellulose or glyceryl behenate as hydrophobic carriers for taste masking microspheres. However, while being effective, microspheres using hydrophobic carriers also shows limitations concerning drug dissolution processes in GI tract. The pharmacokinetic profile and bioavailability of drug using these microspheres may change or reduce when the dissolution rate of AZI decreases. Accordingly, application of polymers with pH-dependent solubility as carriers for microspheres may be other choices for taste masking of compounds [15–17]. Since such polymers keep drugs insoluble in the saliva, they neither dramatically alter the solubility of compounds in the absorption window in GI tract nor reduce the bioavailability of drugs. Until now, there is short of study about taste masking of AZI using polymers with pH-dependent solubility as carriers for microparticles. Thus a study about the impact of polymers with pH-dependent solubility on the taste masking and relative bioavailability of AZI is essential.

Besides the unpleasant taste of compounds, application of oral suspension to azithromycin does come with other challenges such as physical instability, imprecise dosage, and high transportation cost. Development of dispersiable tablets (DTs) is a potential approach to overcome these shortcomings of oral suspension [18–23]. Currently, there are not any commercial DTs containing azithromycin or the drug's taste masking microparticles. Thus, a replacement of conventional oral suspension by DTs containing the taste masking microparticles will expand the choices for pediatric medications.

Dispersible tablets (DTs) are defined as those disintegrating into a homogenous dispersion within 3 min in water before administration [24]. After coming in contact with water, the fast disintegration accelerates the rapid loss of tablet structure and releases smaller drug carriers (microspheres, granules, powder particles, etc.) [25–27], which makes it easier to swallow [1]. The disintegration of DTs before swallowing can bring about some outstanding advantages such as elimination of the potential risk of aspiration and choking in children and older adults when using big monolithic dosage forms, convenient administration, and ease of use for the pediatric and geriatric population. As a result, DTs now attract increasing attention from scientists and pharmaceutical companies [25]. Several commercial products have been approved by the Food and Drug Administration (FDA) such as Zofran® Zydis® (Ondansetron, GlaxoSmithKline), Risperdal® M-Tab (Risperidone, Janssen), Ascotop® melting tablet (Zolmitriptan, AstraZeneca), Zomig®-ZMT (Zolmitriptan, AstraZeneca) [26].

Different techniques have been employed to prepare dispersiable tablets namely direct compression, sublimation, molding, mass extrusion, melt granulation and so on [27]. In this research, direct compression was chosen to prepare dispersiable tablets containing microparticles (DTs-MP) because of its relatively easy handling, widespread knowledge, and cheap manufacturing process [18]. The rapid disintegration of DTs-MP upon contact with the saliva requires incorporation of superdisintegrants in the compressed masses [18,28]. Besides, because a macrolide antibiotic like AZI is unstable in gastric medium [1,5], pH modulators were also screened and incorporated into the DTs for stability improvement of the drug. The aim of this study was to formulate bitter taste masking microparticles containing azithromycin and incorporate these microparticles into dispersible tablets. The obtained DTs-MP was used to compare the relative bioavailability in the rabbit model with the reference product, Zithromax® (Pfizer, U.S.A).

2. Materials and methods

2.1. Materials

Azithromycin (AZI) was obtained from Hebei Dongfeng Pharmaceutical Co., Ltd (China). Poly(meth)acrylates (Eudragit EPO, E100, L100, S100), and hydrophilic fumed silica (Aerosil® 200) were supported by Evonik Co., Ltd (Germany). Quinine hydrochloride, roxithromycin were purchased from Sigma-Aldrich (U.S.A). HPLC-grade methanol was purchased from J.T. Baker (U.S.A). Water was purified by reverse osmosis. Sodium croscarmellose (Disocel), microcrystalline cellulose (Avicel PH 101) were purchased from Mingtai Chemical Co., Ltd (Taiwan). Sodium lauryl sulfate, polyvinylpyrrolidone K30 (PVP K30) were purchased from BASF Chemical Co., Ltd (Germany). All other reagents were of analytical grade commercial products and purchased from Beijing Chemical Reagent Factory (China).

2.2. Animals

New Zealand white rabbits, each weighed between 2 and 2.5 kg, were obtained from the National Institute of Drug Quality Control (Vietnam) for the pharmacokinetic (PK) test. They were kept in a clean room at a temperature of 25 ± 2 °C with a 12-h light/dark cycle. The relative humidity was 55 ± 15% with air ventilation frequency of 15–20 times/h. All rabbits were fed with water and commercial diet. The protocol of the animal study was approved by the Animal Care and Use Committee of the Hanoi University of Pharmacy, Vietnam.

2.3. Preparation of azithromycin microparticles

Microparticles were prepared by the two methods of spray drying and solvent evaporation. Regarding spray drying method, AZI and polymer were dissolved in a mixture of ethanol: dichloromethane (1:1, v/v) and filtered through a 125 μm sieve. To reduce the viscosity of this solution, polymer concentration was fixed around 50 mg/ml. The filtrate was sprayed at the speed of 10 ml/min in a spray drier (Buchi, Switzerland, Mini Spray Dryer B-290) in which the nozzle diameter was 0.75 mm, and inlet temperature was 80 °C.

To prepare microparticles by a solvent evaporation method, AZI and polymer were also dissolved in the mixture of ethanol: dichloromethane (1:1, v/v) and the polymer concentration was 300 mg/ml. The organic solvents were evaporated at 60 °C in a heated bath for 6 h under stirring and in a static oven for the next 24 h. The obtained product was then ground by a ball milling machine (Retsch, Germany, Mixer Mill MM 200) and sieved through suitable sieves to harvest microparticles. The microparticles were finally put in plastic bags and kept in a desiccator before analysis.

2.4. Characterization of azithromycin microparticles

2.4.1. Bitter taste masking of microparticles

The bitterness value of AZI was determined based on the appendix of the British Pharmacopoeia 2013 [4]. Simply speaking, the bitterness value of a drug, i.e. the reciprocal of the dilution of an extract or a liquid that still had a bitter taste, was evaluated by comparing with that of quinine hydrochloride (the standard), which was set at 200,000. The experiment was conducted with six volunteers (3 males and 3 females). To make up for individual differences in evaluating bitterness, a correction factor (k) for each volunteer was determined.

2.4.1.1. Determination of the correction factor. Prepare a series of dilutions of quinine hydrochloride in water. Determine the dilution with the lowest concentration that still had a bitter taste. Take 10.0 mL of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution was not found to be bitter, spit it out and waited for 1 min. Rinse the mouth with water. After 10 min, use the next dilution in order of increasing concentration. Calculate the correction factor k for individual volunteer using the expression:

$$K = \frac{n}{5}$$

where n = the number of milliliters of the stock solution in the dilution of lowest concentration that was judged to be bitter.

2.4.1.2. Determination of the bitterness value of AZI. Dissolve 0.1 g AZI in 1000 mL distilled water to have a stock solution of AZI. Prepare a series of dilutions of AZI in water from the stock solution of AZI in 10 mL distilled water. Determine the dilution with the lowest concentration that still had a bitter taste. Take 10.0 mL of the tested solution in order of increasing concentration.

$$\textit{The bitterness value of AZI} = \frac{100,000 \cdot K}{X}$$

where X was the lowest volume of tested solution that still had a bitter taste and K was the correction factor.

$$\textit{The bitterness threshold } (\mu g/ml) = \frac{1,000,000}{\textit{The bitterness value}}$$

The bitter taste masking of microparticles was determined by the bitter taste threshold of AZI. The experiment to determine the taste masking effect of microparticles was modified from the method developed by Kharb et al. [29]. Accordingly, microparticles equivalent to 600 mg AZI were put into glass tubes where 10 mL of distilled water was then added to each. The volume of distilled water (10 mL) was used to mimic the real condition when the patient used the reference product (Zithromax oral suspension). The tubes were mixed for 30 s using a vortex mixer and filtered through membranes 0.45 μm (Sartorius, Germany, Model Minisart RC 25). The drug concentration in the filtrate was analyzed by high-performance liquid chromatography (HPLC) and compared to the bitter taste threshold. If the drug concentration in the filtrate was smaller than the bitter taste threshold, the microparticles were considered to have completely masked the bitter taste of AZI.

The HPLC system consisted of an isocratic pump (Agilent, U.S.A., Model G1311C), a manual injector (Agilent, U.S.A., Model G1328C), a column thermostat (Agilent, U.S.A., Model G1316A), and a multi-wavelength detector (Agilent, U.S.A., Model G1315D). Detector output was integrated and digitalized using the Agilent ChemStation software (Agilent, U.S.A., Model 1200 Series HPLC system). The column used was a C18 (Zorbax SB, 4.6 × 250 mm, 5 μm particle size, Agilent, U.S.A.). The detector was set at 215 nm. The mobile phase containing methanol: distilled water: concentrated ammonia solution at a ratio of 80:19.9:0.1 was delivered at 1 mL/min at room temperature. The injection volume was 100 μL and the total run time for a sample was about 10 min.

2.4.2. Particle size and particle size distribution

The particle size was determined using Malvern Mastersizer (Malvern Instruments Limited, U.K, Mastersizer 3000). The microparticles were directly dispersed into about 500 mL of distilled water for 30 s before sample examination. The laser obscuration was set from 0.1 to 20%. The particle size was expressed as the mean volume diameter in μm.

2.4.3. Differential scanning calorimeter (DSC) analysis

The thermal properties of samples were determined by differential scanning calorimeter (Mettler Toledo, Germany, DSC 1). An empty aluminum pan was used as a reference. The analysis of samples was conducted with sealable aluminum pans using approximately 10 mg sample. A continuous flow of nitrogen at 50 ml/min was used for the measurement. The samples were heated from 30 to 250 °C at a rate of 10 °C/min. Analysis of the results was conducted using the STAR – Software version 10.00 (Mettler Toledo, Germany, DSC 1).

2.4.4. Fourier transform infrared spectroscopy (FTIR)

FT-IR spectra were obtained on a Fourier transform infrared spectroscopy (Bruker Optics, Germany, Model IFS-66/S) using the potassium bromide (KBr) disk method. One to two milligrams of the sample were mixed with 150 mg of spectra-grade KBr and pressed into a disk that was 12 mm in diameter using a Carver hydraulic press (Carver, U.S.A., Model 3912). Samples were analyzed from 600 to 4000 cm^{-1} with an instrument resolution of 0.1 cm^{-1}.

2.4.5. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

^{1}H NMR experiments were conducted to investigate the possibility of the intermolecular interaction between drug and polymer. The ^{1}H NMR spectra of the samples were taken at 25 °C on a Bruker Avance 500 MHz spectrometer equipped with 5 mm TCI HCN Z gradient cryoprobe. Spectra were processed by Bruker Topspin 2.1 software and analyzed by CARA 1.8.4 software. A sample containing drug and polymer was dissolved at an optimum ratio in CD_3OD at a concentration of 20 mg/ml. This solution was added to a suitable glass tube which was then placed into to Bruker NMR Spectrometer.

2.4.6. Morphology

The morphology of the microparticles was examined by both scanning electron microscopy and optical microscopy. The former, which required the microparticles be fixed on a plate using adhesive tape and coated with gold for 240 s, was carried out using a scanning electron microscope (Hitachi, Japan, FESEM S4800). For the latter, the microparticles were placed directly on lamella and immediately observed by optical microscopy (Olympus, U.S.A, Olympus CX 31). Images of microparticles were taken by a camera (ToupTek Photonics Co., Ltd, China, Camera ToupCam 3.1MP) connected to the optical microscopy.

2.5. Preparation of dispersible tablets containing azithromycin microparticles

2.5.1. Stability kinetics and screening of pH modulators

The kinetic stability of AZI in different pHs (solution of 0.1 N hydrochloric acid pH 1.0, phosphate buffer saline pH 2.0, 3.0, 4.0 and 5.0) was determined under the two conditions. First, a series of AZI solution (2 mg/ml) was prepared by completely dissolving AZI in different pHs for 30 s that was referred to as initial time (t_0). After predetermined interval times (t), 5 ml of the medium was withdrawn and immediately analyzed by a validated HPLC method. This was called stability test without neutralization step.

Second, a similar stability test of AZI in these pHs was also conducted; however, the withdrawn samples were immediately neutralized by 5 mL of 0.2 M Na_2HPO_4 solution. The neutralized products were also analyzed by HPLC method. The degradation rate constant (K) and the time required for the drug to decrease its initial amount by 10% ($t_{90\%}$) followed pseudo-first-order kinetic behavior and were determined by the equation:

$$\frac{1}{t} Ln \frac{C_0}{C} \ (\text{min}^{-1}) \quad T_{90\%} = \frac{Ln\left(\frac{10}{9}\right)}{K} \ (\text{min})$$

where C_0 was the initial drug concentration and C was the drug concentration in time t.

Based on the stability test, a suitable pH range was chosen. The addition of pH modulators to adjust the medium pH was necessary, and the kinds of pH modulator were screened by modification of titration method developed by Lo et al. [30]. Briefly, the pH modulator was dissolved in 10 mL distilled water and mixed with 40 mL gastric medium (0.1 N hydrochloric acid). The pH value was determined after 5 min. After that, the previous solutions were added with a further 10 mL of 0.1 N hydrochloride acid. These solutions were allowed to equilibrate for 5 min before reading of pH values. Finally, diagrams displaying the relationship between pH values and volume of additional gastric medium were drawn.

The effect of pH modulators on the dissolution rate of AZI from microparticles was studied using the dissolution apparatus type 2 (Vankel Varian, U.S.A, Model 7010). A quantity of 500 ml dissolution medium of 0.1 N hydrochloric acid was used for the first 2 h. After 2 h, the dissolution medium was changed to pH 6.8 phosphate buffer saline by the addition of 10 mL solutions of NaOH (5 M) and 10 mL solutions of pH 6.8 phosphate buffer saline (1 M). The temperature was maintained at 37 ± 0.5 °C. The rotation speed was 100 rounds per minute (rpm). Ten milliliters of aliquot were withdrawn at predetermined time intervals of 5, 30, 60, 120, 125, 150, 180 and 240 min and filtered through membranes 0.45 μm (Sartorius, Germany, Model Minisart RC 25). Five mL of filtrate was immediately neutralized by 5 mL solutions of 0.2 M Na_2HPO_4 and analyzed by HPLC method. The dissolution profile of the reference product (Zithromax) was also investigated under the same dissolution apparatus and dissolution conditions. However, the dissolution medium was changed to pH 6.8 phosphate buffer saline right after 30 min in a dissolution medium of 0.1 N hydrochloric acid. The sampling times for the dissolution test of Zithromax were 5, 15, 30, 35, 60, 90, and 120 min.

2.5.2. Preparation of dispersible tablets

Dispersible tablets containing microparticles were prepared by direct compression method. The main compositions of the tablet were the microparticles, lubricants, superdisintegrant, a suspension stabilizer, pH modulators and diluents. The microparticles and additional excipients were sieved through a 250-μm sieve and equally mixed in a plastic bag and then pressed by a single punch tableting machine (Korsch, Germany, Model VFD007S21A) to form dispersible tablets. The total weight and diameter of these tablets were 650 mg and 13 mm, respectively. The microparticles consisting of AZI and Eud L100 (1:4) had the size distribution of 45–212 μm. Regarding the amount of lubricant, the screening ranges of Aerosil, Talc, and magnesium stearate were 0–0.5%, 0–1% and 0.5–1%, respectively. Sodium croscarmellose (Disocel) was fixed as the only superdisintegrant in the tablets with the screening range of 1–5%. The investigated levels of pH modulators ($CaCO_3$: NaH_2PO_4 = 2:1) were 0, 50, 75 and 100%. The diluent including Avicel, lactose SD or mannitol, was added to fulfill the quantity of the tablets. To stabilize the suspension which was formed after the dispersion of DT-MP in water, 1% sodium lauryl sulfate was fixed in the compositions of the tablet. The tablets were prepared with the two levels of crushing strength including 30–40 N and 90–95 N. The crushing strength of the tablets was measured by a crushing strength tester (Pharmatest, Germany, Type PTB 511E). The disintegration time of tablets and dissolution rate of AZI were used as the output factors for the screening of excipients. Besides, the effect of breaking forces on the disintegration time and friability of DT-MP was also determined.

2.6. Characterization of dispersible tablets containing azithromycin microparticles

2.6.1. Disintegration time studies

A method for evaluating the disintegration time of DT-MP was modified from one described by Gohel et al. [31]. Specifically, a volume of 10 mL distilled water was added to a petri dish (10-cm diameter) with a tablet in the center. This experiment was conducted in a static condition. Disintegration time was determined at the point at which the tablet disintegrated completely into fine particles and was recorded by stopwatch.

2.6.2. Friability test of dispersible tablets

The friability of the tablets was determined by a friability tester (Pharmatest, Germany, Type PTF20E) based on the guidance in British Pharmacopoeia. Briefly, twenty DTs-MP were carefully dedusted by sieving these tablets through a sieve of 710 μm. These tablets were then accurately weighed (M_1), placed in the drum of the machine, and rotated at 25 rpm for 100 times. Finally, the rotated tablets were again dedusted and accurately weighted (M_2).

$$Friability\ (\%) = \frac{M_1 - M_2}{M_1} \times 100$$

2.6.3. In vitro release studies

The dissolution rate of AZI from samples into the medium was modified from the method developed by Hu et al. [32]. The dissolution apparatus type 2 (Vankel Varian, U.S.A, Model 7010) was used in this study. A quantity of 500 ml dissolution medium of pH 6.8 phosphate buffer saline was used for 2 h. The temperature was maintained at 37 ± 0.5 °C. The rotation speed was 100 rounds per minute (rpm). Ten milliliters of aliquot were withdrawn at predetermined time intervals of 0.25, 0.5, 1, 1.5, 2 h and filtered through membranes 0.45 μm (Sartorius, Germany, Model Minisart RC 25). The medium was replenished with 10 ml of fresh medium each time. Withdrawn samples were analyzed using HPLC method.

2.7. Pharmacokinetics studies in rabbits

The animal study was approved by the Local Animal Use Committee. Six male rabbits, each weighed 2–2.5 kg, were used for the pharmacokinetics study. The rabbits, which were divided into 2 groups of three, were kept in fasting condition one night before the day of the experiment. The two samples were optimal DT-MP and azithromycin 200 mg/5 ml powder for oral suspension (Zithromax®, Pfizer). The optimal DT-MP containing 100 mg AZI was completely dispersed in 25 ml distilled water, and the reference product (Zithromax) containing 600 mg AZI was also completely dispersed in 100 ml distilled water to obtain a homogenous dispersion for 10 min before administering to the rabbits. A volume of the liquid suspension equivalent to 10 mg/kg was carefully withdrawn and fed to the rabbit by a syringe. The drug content uniformity among rabbits was assured by gently shaking the flask bottle containing liquid suspensions upside down and up in a vertical direction by hand 10 times before each feed. Blood samples (2 ml/sample) were withdrawn from the ear artery after 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24 h (hrs.) and supplemented with equal amounts of saline containing heparin 50UI. Plasma was collected by centrifugation of the above samples at 3500 rpm within 10 min and stored in a deep freezer at −40 °C until the day of analysis.

2.8. LC-MS/MS analysis of azithromycin in rabbit plasma

An AB Sciex 5500 QQQ mass spectrometer (AB Sciex, USA) coupled with LC-20AD high-pressure pumps, column compartment and autosampler (Shimadzu, Japan) was used for the determination of azithromycin in plasma. LC separation was obtained by using an Eclipse plus C18 column (10 cm × 2.1 mm; 5 μm particle size) and a pre-column (Agilent, USA) with a mobile phase composition of 10 mM ammonium acetate and acetonitrile. The gradient program was initially set at 5% acetonitrile for 0.5 min then increased linearly from 5% to 90% acetonitrile over 3 min. After that, the eluent composition was maintained at 90% acetonitrile from 3 to 4 min then returned to 5% acetonitrile in 1 min, and re-equilibrated until the end of the analysis process. The flow rate was kept constant at 0.5 mL min^{-1}. The injection volume was 5 μL. Total run time was 6 min.

The mass spectrometer was operated in positive ESI mode with the capillary voltage and temperature set at 5000 V and 400 °C, respectively. MS experiments were carried out in multiple reaction monitoring modes with the transition *m/z* 749.5–591 for quantification and *m/z* 749.5–116 for confirmation. The transition for internal standard roxithromycin was *m/z* 837.5–158.

One milliliters aliquot of the plasma sample was transferred into a 15 mL centrifuge tube. Twenty microliters of an internal standard solution of 1 μg mL^{-1} (roxithromycin in methanol) and 0.5 ml solution of 0.10 M ammonium hydroxide were added to the tube. Diethyl ether (2 mL) was then added, and the tube was mixed by a vortex mixer for 5 min. The supernatant (0.8 mL) was transferred to 10 mL glass tube and evaporated under the flow of nitrogen gas and 40 °C. The residue was reconstituted with 0.8 mL methanol, mixed by a vortex mixer for 60 s and filtered through 0.45 μm. Finally, the filtrate (5 μL) was injected into the LC-MS/MS system.

The method was fully validated according to guidelines for bioanalytical method validation from the U.S. Food and Drug Administration (FDA).

2.9. Data analysis

The experiment was run triplicates. The data were expressed as a mean ± standard deviation and analyzed for statistical significance by one-way ANOVA and Student's *t*-test using Excel (Microsoft, U.S.A.). Pharmacokinetics parameters were determined by the non-compartment model using Phoenix® software (A Certara™ Company, U.S.A, WinNonlin® 6.5).

3. Results and discussion

3.1. Preparation of bitter taste masking microparticles containing azithromycin

Based on guidelines from the British Pharmacopoeia 2013, the bitter taste threshold of AZI was determined by an experiment with six volunteers. Data in Table 1 showed that the bitter taste threshold of azithromycin was 32.43 ± 6.81 μg/ml which was around 6 times higher than that of the standard, i.e. quinine hydrochloride (roughly 5 μg/ml). When compared to USP solubility criteria, this threshold (32.43 μg/ml) lied in the practically insoluble range and the bitter taste of AZI might result from the trace of AZI in solution. This indicated that the taste of AZI was extremely bitter and development of taste masking microparticles was essential.

Previous studies have attempted to mask the bitter taste of AZI by preparing microparticles containing AZI using different carriers such as ethylcellulose or glyceryl behenate. Most notably, Hu et al. [32] developed microspheres of azithromycin with ethyl cellulose by the modified solvent diffusion method. The obtained results demonstrated that the bitter taste of azithromycin was significantly masked by microparticles and the relative bioavailability in human of microspheres was 102.7% compared to a commercial product. Similarly, Lo et al. [30] used a melt-congealing process to prepare matrix microparticles containing AZI. The microparticles had small size (~200 μm) with a narrow particle size distribution and good tolerability. The present study employed a roughly similar solution to that by Hu et al. [32] to mask the bitter taste of AZI that was to maintain the released amount of drug in water medium under the bitter threshold (32.43 μg/ml). However, a novel bitter taste masking microparticles containing AZI and Eudragit L100, whose taste masking mechanism was different from that of ethyl cellulose and glyceryl behenate, was developed by

Table 1
The bitter threshold of azithromycin and effect of polymers on the bitter taste masking of microparticles (n = 6, Mean ± SD).

Microparticles	Released amount of AZI in water (μg/ml)	Bitter threshold (μg/ml) of azithromycin
AZI:Eudragit L100 (1:4)	63.47 ± 3.24	
AZI:Eudragit S100 (1:4)	76.16 ± 2.53	
AZI:Eudragit E100 (1:4)	190.41 ± 3.46	32.43 ± 6.81 (μg/ml)
AZI:Eudragit EPO (1:4)	229.77 ± 4.12	
Spray dried AZI	262.63 ± 0.85	

spray drying. After spray drying of free drug, the dissolved amount of spray dried AZI (262.63 ± 0.85 μg/ml) was 8.1 times higher than the bitter threshold. It was explained by the formation of porosity on the surface of spray dried AZI, which accelerated the dissolution rate of the drug. To inhibit the bitter taste of AZI by modification of AZI solubility, several polymers with pH-dependent solubility (Eudragit E, L, S100) were used to prepare the microparticles. The experiment to evaluate the bitter taste of microparticles was conducted with 10 ml which mimicked the usage guideline of the reference product. To make it easier for the bitter taste evaluation of AZI, the polymers were considered as intact excipients which would not have any specific tastes. Plus, these polymers were mainly used to reduce the drug solubility in the saliva, thereby masking the bitter taste of AZI. The impact of these polymers on the drug solubility was also seen in Table 1. Specifically, the released amount of AZI from microparticles using Eudragit E100, Eudragit EPO, Eudragit S100 and Eudragit L100 were 229.77 ± 4.12, 190.41 ± 3.46, 76.16 ± 2.53 and 63.47 ± 3.24 μg/ml, respectively. This meant anionic polymers (Eudragit L100 and Eudragit S100) were more effective than cationic polymers (Eudragit E100 and Eudragit EPO) regarding bitter taste masking ability.

To find out the molecular mechanism for the taste masking ability of microparticles, FTIR and NMR spectroscopies were used to study the interactions between AZI and Eudragit L100. As shown in FTIR spectroscopy (Fig. 1), AZI, Eudragit L100 and physical mixture of AZI and Eudragit L100 had a carbonyl peak in wavenumber of 1722 cm^{-1}. Because of hydrogen bonds of intermolecular interactions between carbonyl groups (—C=O) and hydroxyl groups (—OH) in Eudragit L100, the carbonyl peaks (—C=O) shifted to the lower wavenumber (1705 cm^{-1}) and formed a shoulder-type peak in FTIR spectra of Eudragit L100 and physical mixture. This shoulder-type peak, however, disappeared in FTIR spectra of microparticles (AZI: Eud L100 = 1:4) and the carbonyl peak (—C=O) in microparticles moved from 1722 to 1724 cm^{-1}. These changes in FTIR spectra of microparticles were caused by the interaction between the carboxyl group (=COOH) in Eudragit L100 and an amine group in azithromycin. Besides, the fact that there was an appearance of a new peak at 2300 cm^{-1} in microsphere spectra also proved a formation of a new interaction. This new peak was formed from N^+=H after interaction of the acid group (Eudragit L100) and the amine group (AZI). Besides, the ^1H NMR spectra (Fig. 2a) showed the shift of $=CH_x=N=$ to the upfield in the range of 2–3 ppm, especially the $CH_3=N=$ group. This movement of $CH_3=N=$ group proved that the interaction of the amine group (AZI) and the acid group (Eudragit L100) turned the nitrogen into a positively charged center which would attract electron and moved to upfield in NMR spectra. Based on these results, it proved that acid-base interaction of drug and polymer was the first main reason for the taste masking effect of microparticles. The molecular interaction between functional groups of Eudragit L100 and AZI was described in Fig. 2b. Besides, Eudragit L100, known as a polymer soluble in intestinal fluid from pH 6.0, partly inhibited the drug release in gastric fluid which may even have an impact on bitter receptors in the stomach and elicit responses such as an intense lingering aftertaste. The characterization of a large family of putative mammalian taste receptors (T2Rs) was first reported by a research group of Zuker et al. [33,34]. Later, Depoortere et al. [35] proved that these bitter taste receptors (T2Rs) presented in smooth muscle tissue along the mouse gut and human gastric smooth muscle cells (hGSMC). In the present study, the advantage of Eudragit L100 in the bitter taste masking might be seen in the lower release of AZI in upper GI tract, thereby preventing the contact of dissolved drug from T2Rs in extra-oral tissues, including gut endocrine cells. Consequently, the endings of the vagus nerve that lie underneath the lining of the GI contain bitter receptors was inactivated to signal the bitter information to the brain [36]. The obtained results also proved that water soluble polymer (hypromellose) or gastric-soluble polymer (Eudragit E100) might not be effective in masking the bitter taste of AZI because they cannot inhibit the contact of the dissolved drug with bitter receptors in saliva and stomach. The kind of interaction between drug and a polymer soluble in intestinal fluid from pH 6.0 was also used to mask the bitter taste of other drugs. For example, Gao et al. [37] prepared taste-masking microspheres containing roxithromycin by the emulsion solvent diffusion method. The effect of different polymers on the characteristics of the microspheres was investigated by DSC, X-ray diffraction, and IR. The authors concluded that Eudragit S100 was the best effective polymer among the six kinds of polymers for masking the unpleasant taste of roxithromycin. Similar results obtained from Lu et al. [38] and Yi et al. [39]. Lu et al. [38] also proved that the ionic bonding of the amine group in clarithromycin to the high molecular weight polyacrylic acid (carbomer) was the mechanism to remove the free drug from the solution phase, thus effectively masking the bitter taste of this drug.

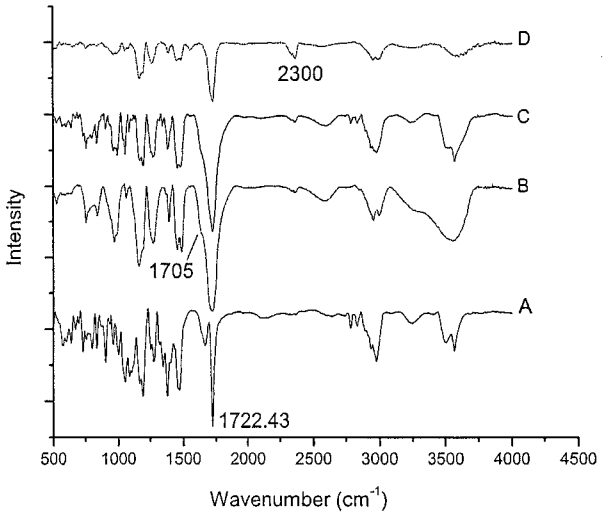


Fig. 1. FTIR diagram of (A) Azithromycin, (B) Eudragit L100, (C) physical mixture, (D) microparticles (AZI: Eud L100 = 1:4).

Even though Eudragit L100 was effective in inhibiting the bitter taste of AZI, the release amount of AZI (63.47 μg/ml) still doubled the threshold (32.43 μg/ml), causing the bitter taste of microparticles. Table 2 demonstrated the effect of different preparation methods, particle sizes and ratios of drug to the polymer on the amount of AZI released in water. Microparticles of similar size (≤45 μm) were prepared by the two methods of spray drying and solvent evaporation. As shown in Table 2, the released amount of AZI from these two kinds of microsphere was almost the same (63.47 ± 3.24 vs. 67.51 ± 1.41 μg/ml), which indicated that different preparation methods did not change the bitter taste of AZI.

Because of the easy preparation process, solvent evaporation method was chosen for further study. The four-size distribution range (≤45, 45–75, 75–125 and 125–212 μm) was selected to investigate the effect of microsphere size on the bitter taste of AZI. Due to the smaller surface area, the bigger microparticles had a lower release rate of AZI and less bitter taste compared to the smaller ones (Fig. 3a). For microspheres sized over 45 μm, the released amount of AZI in water was under the bitter threshold (32.43 μg/ml), resulting in almost non-bitter microparticles (Table 2). Consequently, the size of microparticles was identified as the second main reason for their taste masking effect. Guo et al. [40] also studied the effect of structure, morphology, and size of the microparticles on the taste-masking performance of

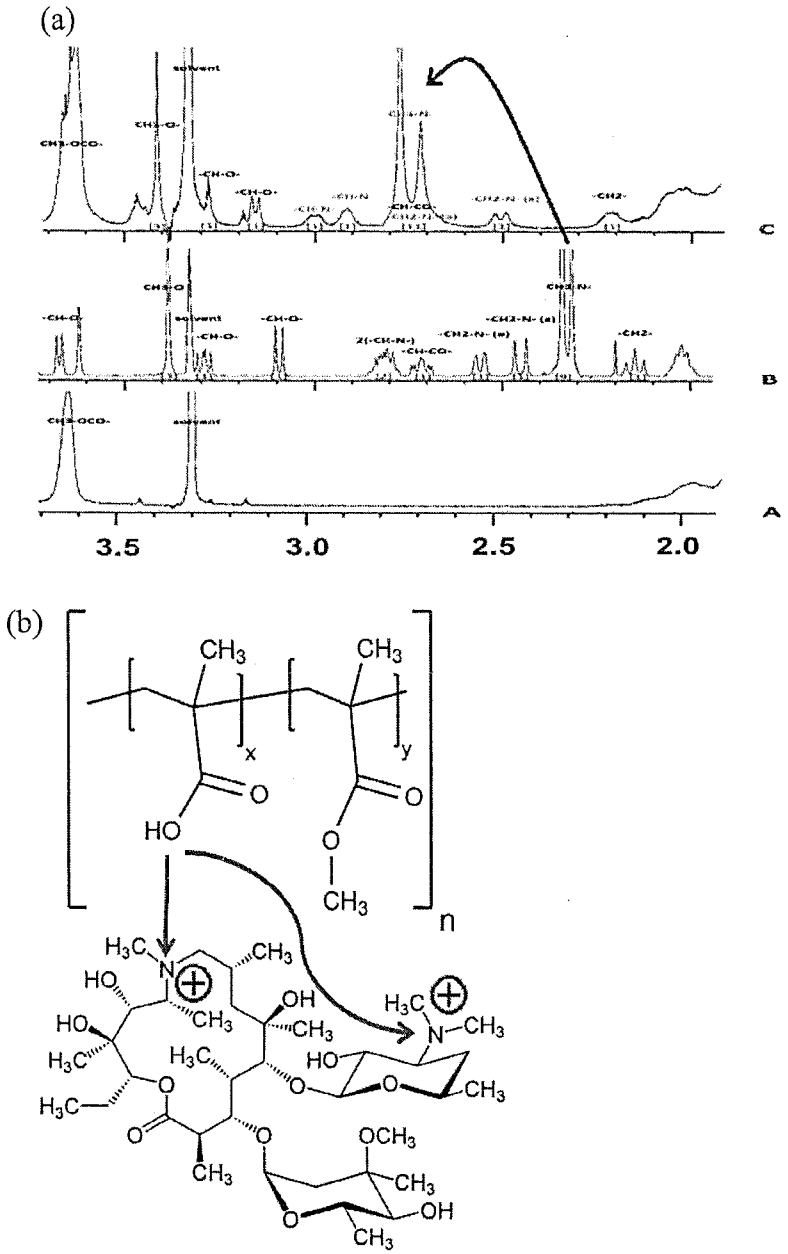


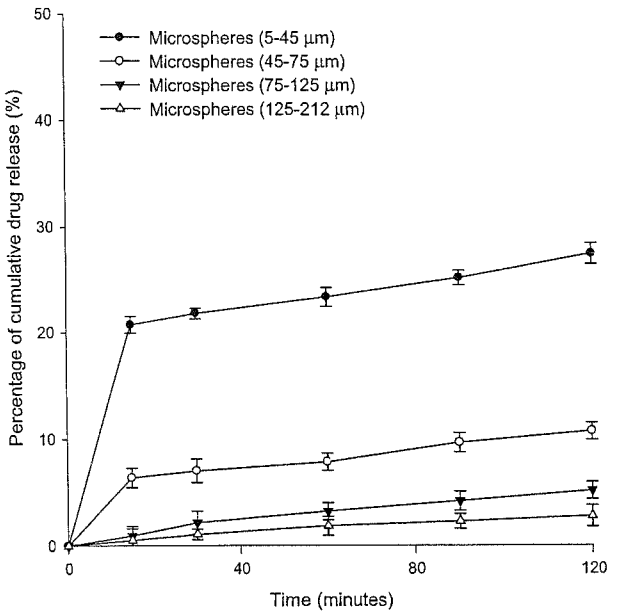
Fig. 2. (a) NMR diagram of (A) Eudragit L100, (B) Azithromycin, (C) microparticles; (b) structures of azithromycin and Eudragit L100.

Table 2
Effect of preparation method, the ratio of AZI: Eud L100 and particle size on bitter taste masking of microparticles.

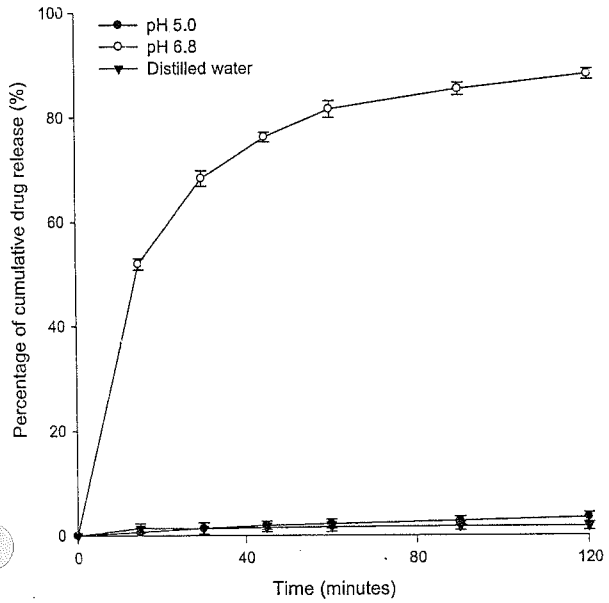
Method	Ratio of AZI: Eud L100	Size distribution (μm)	Mean of particle size (μm)	Released amount of AZI in water (μg/ml)	Bitter threshold (μg/ml)
Spray drying	AZI:Eud L100 = 1:4	≤45	17.4	63.47 ± 3.24	32.43
Solvent evaporation	AZI:Eud L100 = 1:4	≤45	17.6	67.51 ± 1.41	
		45–75	58.1	15.28 ± 0.89	
		75–125	117	4.79 ± 1.22	
		125–212	205	3.22 ± 0.68	
	AZI:Eud L100 = 1:2	75–125	n/a	47.53 ± 2.31	
	AZI:Eud L100 = 1:1	75–125	n/a	196.78 ± 1.42	

microparticles. Based on results obtaining from synchrotron radiation X-ray computed microtomography (SR-mCT), the authors managed to figure out the changes of fine structure for particle formulations during the dissolution test. Accordingly, particles with regular shape and a smooth surface having a smaller surface area as well as slower dissolution rate were likely to be less bitter than the rough and smaller ones.

Lastly, the impact of different ratios of AZI to Eudragit L100 (1:1, 1: 2 and 1:4) on the bitter taste of microparticles was determined. The size distribution of these microparticles was fixed from 75 to



(a)



(b)

Fig. 3. (a) Effect of microparticles size to drug release from microparticles prepared by a solvent evaporation method in pH 5.0; (b) Effect of different mediums to drug release from microparticles (45–212 μm) prepared by a solvent evaporation method (n = 3, Mean ± S.D).

125 μm. The results in Table 2 proved that the amount of Eudragit L100 was inversely proportional to the concentration of AZI in water. The higher amount of Eudragit L100 the lower the drug release and bitter taste of AZI were inhibited. Consequently, the microparticles in which the ratio of AZI to Eudragit L100 was 1:4 and whose size distribution was 45–212 μm were considered optimal because the released amount of AZI in water was under the threshold. These optimal microparticles were used to determine some physiochemical properties before incorporating into tablets.

Regarding the dissolution patterns of AZI from microparticles (Fig. 3), those from microparticles having sizes ≤75 μm displayed an initial burst followed by a very slow and incomplete release (Fig. 3a). The burst release phenomenon was caused by the high dissolution rate of microparticles which were in the lower limit of size distribution range. The existence of these microparticles was shown in SEM and optimal microscopy image (Fig. 5). According to images displaying in Fig. 5, there were microparticles or clusters of microparticles having sizes of around 2 μm on the surface of bigger microparticles. Following the fast dissolution of AZI from these small-sized microparticles, AZI from the bigger-sized microparticles which mainly existed in a matrix form with regular cube shape and many layers was gradually released. The dissolution of AZI from this matrix was mainly managed by the solubility of Eudragit L100 in the medium. Since Eudragit L100 was insoluble in pH 5.0 and water, the drug was kept mainly inside the matrix then released slowly and incompletely out of the microparticles. Meanwhile, as shown in Fig. 3b, the dissolution rate of AZI in phosphate buffer medium pH 6.8 was significantly higher than those in phosphate buffer medium pH 5.0 and distilled water. The reason was the phosphate buffer medium pH 6.8 facilitated the solubility of microparticles using anionic polymer (Eudragit L100). Furthermore, the high dissolution rate of AZI in pH 6.8 also resulted from the amorphous state of AZI in microparticles. The amorphous state of AZI was confirmed by DSC analysis (Fig. 4). Accordingly, DSC spectra of AZI and the physical mixture had one endotherm peak at 126 °C. However, the fact that this endotherm peak (126 °C) disappeared in DSC spectra of microparticles proved the existence of AZI in an amorphous state in microparticles.

3.2. Preparation of dispersible tablets containing bitter taste masking microparticles

As an antibiotic, AZI has a high potential to be decomposed in acidic medium. Stability kinetics of AZI in several pH media (pH 1.0, 2.0, 3.0, 4.0 and 5.0) was studied to screen stabilizers for the dispersible tablets. As shown in Fig. 6a, the concentration of AZI strongly depended on pH of media and the reduction of AZI concentration followed the first-order degradation kinetics. When pH increased from 1.0 to 2.0, the degradation constant reduced about 10 times (from $2.07 \cdot 10^{-2}$ down to $1.87 \cdot 10^{-3}$). However, AZI was only quickly decomposed in pH ≤ 2.0. When pH increased from 3.0 to 5.0, the concentration of AZI did not change after 4 h. This was explained by the fact that the chemical connection

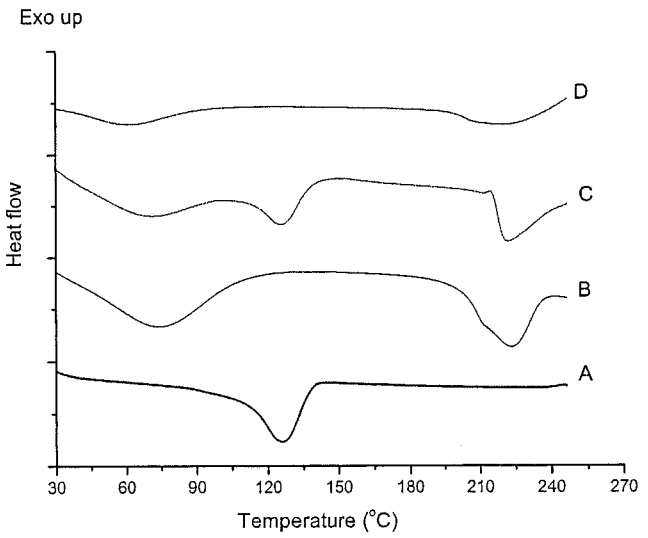


Fig. 4. DSC diagram of (A) Azithromycin, (B) Eudragit L100, (C) physical mixture, (D) microparticles.

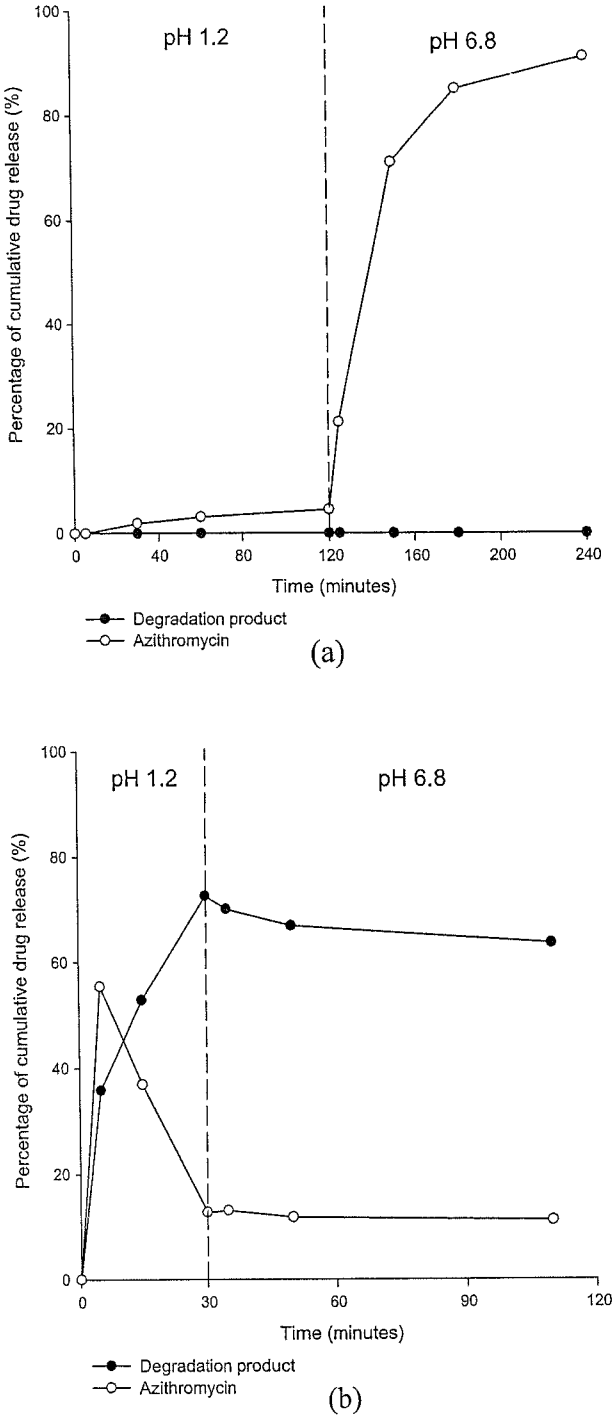


Fig. 8. Dissolution profiles of AZI in pH 1.2 and 6.8 from: (a) Bitter taste masking microparticles with pH modulators ($CaCO_3$: NaH_2PO_4 = 2:1); (b) Zithromax.

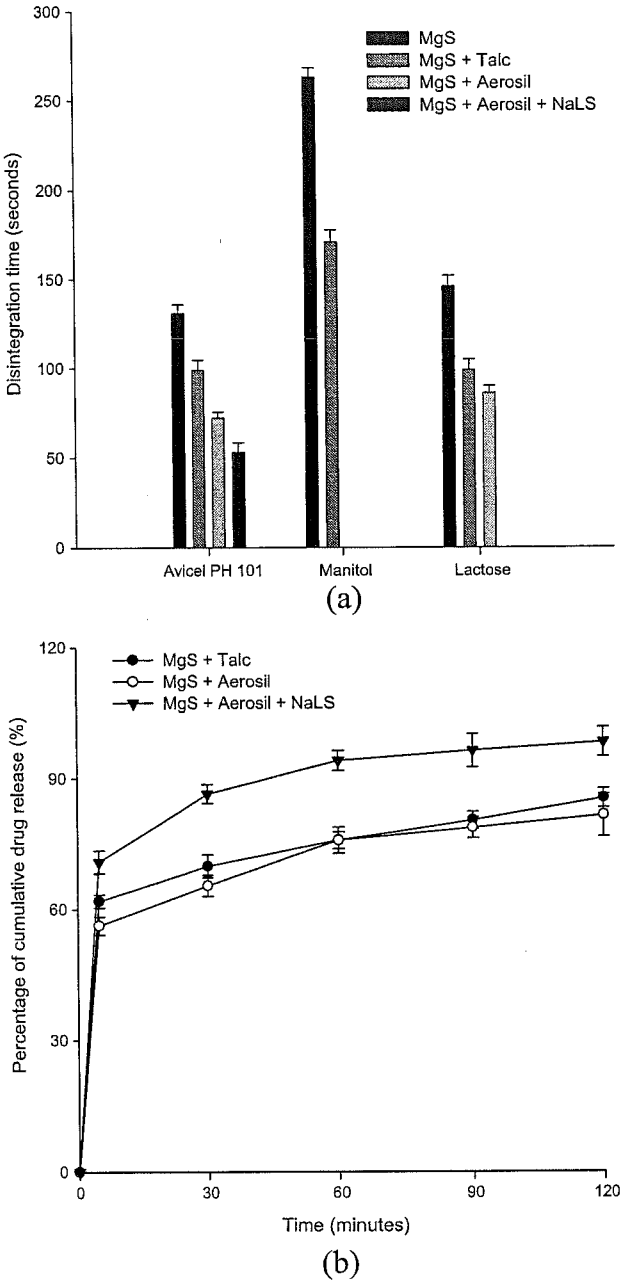


Fig. 9. Effect of lubricants to (a) disintegration time of tablets; (b) dissolution profiles of azithromycin in pH 6.8 (n = 3, Mean ± S.D).

NaH_2PO_4 = 2:1) were chosen as components of the DT-MP. The effect of some main factors including diluents and lubricants on disintegration time of DT-MP and dissolution rate of AZI was shown in Fig. 9. Regarding the effect of kinds of diluent, the disintegration time of DT-MP decreased following the order: mannitol > lactose SD ~ Avicel PH101. Due to the crystallized state of mannitol, the dissolution rate of this excipient was longer than that of lactose and Avicel. Consequently, the formation of porosity in tablets using mannitol was slower than that using lactose and Avicel. Meanwhile, both lactose and Avicel accelerated the disintegration process of DT by microcapillaries mechanism after the dissolution of lactose and swelling mechanism of Avicel. Despite using different kinds of lubricants, these two diluents (lactose SD and Avicel PH 101) always had the smallest disintegration time compared to mannitol.

Regarding the impact of lubricants, DT-MP using a combination of different lubricants had the shorter disintegration time than that using single lubricant (magnesium stearate). The increasing order of disintegration time of DT-MP using different lubricants was magnesium stearate + aerosil + sodium lauryl sulfate < magnesium stearate + aerosil < magnesium stearate. Consequently, tablets only using magnesium stearate had the longest disintegration time. Microparticles using Eudragit L100 as a polymeric carrier were highly sticky. Meanwhile, Aerosil, for its very high surface area, when adhered to the surface of microparticles would inhibit the sticky tendency of microparticles and reduce the disintegration time of DT-MP. On the other hand, the introduction of a small

amount of sodium lauryl sulfate (NaLS), a surfactant, would promote the wettability of dissolution medium in tablets, thus activating the disintegration process of DT-MP (Fig. 9b). Due to the fast disintegration of tablets to liberate the small microparticles in pH 6.8, the initial burst phenomenon was once again observed in the dissolution profiles of DT-MP. However, the addition of hydrophobic lubricants including Talc, Aerosil, and magnesium stearate inhibited the drug release in the later phase of the dissolution process. One more reason for the slow release was the formation of bigger granules containing the bigger microparticles after compression process in a tableting machine. The adsorption of hydrophobic lubricants on the surface of these bigger granules inhibited the water uptake inside the granules thus delaying the drug release in the later phase of the dissolution process. Accordingly, the ratio of main components in the DT-MP included 78% of optimal microparticles (equivalent to 100 mg AZI), 4% Avicel, 1% Disocel, 0.5% Aerosil, 0.5% magnesium stearate, 1% sodium lauryl sulfate and 15% pH modulators ($CaCO_3$: NaH_2PO_4 = 2:1). These tablets were used to investigate the impact of crushing strength on physical properties of tablets. When the crushing strength increased from 30–40 N to 90–95 N, the disintegration time of the tablets increased from 53 to 89 s while the friability of these tablets decreased from 1.03% to 0.79%, respectively. It was explained by the fact that the higher compression force and crushing strength, the lower porosity of the tablets and the harder it was for water uptake inside the tablets. The tablets undergoing 30–40 N of breaking force were used for further study because of their lower disintegration time compared to those experiencing 90–95 N and their friability which almost met the general requirement for tablets in pharmacopoeia (1%). Consequently, these tablets were used to compare the relative bioavailability with the reference product (Zithromax®, Pfizer) in the rabbit model.

3.3. Pharmacokinetics study in rabbits

The pharmacokinetics study of AZI in both the optimal DT-MP and reference product was conducted in the rabbit model. The drug concentration in rabbit plasma was analyzed by LC-MS/MS. The pharmacokinetics profiles and pharmacokinetics parameters of AZI were shown in Fig. 10 and Table 3, where similar patterns could be observed in both the optimal DT-MP and reference product. Specifically, AZI was quickly absorbed after oral administration, and the drug concentration peaked after 0.5 h. Later, AZI had a very fast elimination phase from 0.5 to 4 h. Finally, due to the long half-life of AZI, the drug concentration gradually reduced in the last phase. The pharmacokinetics profile looked like a plateau in the last phase. Besides similar PK patterns, as it was shown in Fig. 10, both DT-MP and Zithromax had the same t_{max} (0.5 h). The relationship between this *in vivo* value (t_{max}) and the *in vitro* dissolution profile was explained by the following assumption. Regarding the t_{max} of the reference product (Zithromax), this value was almost in agreement with the dissolution profile in Fig. 8b. For its quick solubility in the gastric medium, t_{max} of Zithromax was observed after 0.5 h in PK profile. According to Merchant et al. [42], the pH of rabbit stomach medium was around 1.6, which led to Zithromax being quickly soluble and decomposed. Therefore, the C_{max} of Zithromax was lower than that of DT-MP. One more reason for the quick absorption of AZI in both formulations (Zithromax and DT-MP) was the rapid gastric emptying phenomenon. Pilot [43] reported that gastric emptying was accelerated by macrolide antibiotics such as erythromycin and azithromycin because they were also motilin receptor agonists [44]. Moreover, Larson et al. [45] proved that the mean gastric emptying half life ($t_{1/2}$) in human for azithromycin (10.4 ± 7.2 min) and erythromycin (11.9 ± 8.4 min) were almost similar. Due to the rapid gastric emptying phenomenon, AZI rapidly passed to the intestinal medium where the reported pH was higher than 6.4 [42]. Because of the fast dissolution rate of DT-MP and Zithromax in the intestinal medium, the t_{max} of the two formulations (DT-MP, Zithromax) were around 0.5 h.

Even though the drug concentration in plasma of DT-MP was higher than that of the reference product, Fig. 10 displayed the overlapping error bars of the two formulations for the initial phase of pharmacokinetics profiles. One of the main reason for these unwanted results was the small number of experimental animals (n = 3) which increased the standard deviations of the means. Furthermore, azithromycin and its microparticles were pH-dependent soluble drug and dosage form, thus the pharmacokinetics response would be more likely to be influenced by inter-subject and intra-subject variability. In this case, as the pharmacokinetics test was of parallel type, the risk of inter-subject variability would be one considered reason for the overlapping error bars. The difference in pH and motility of stomach and intestine among experimental rabbits strongly influenced the dissolution and absorption process of AZI in GI tract. One of the feasible solutions was to enhance the number of experimental animals. The recommended number for bioequivalence study was from 12 to 18 volunteers, and the type of experimental design was crossover study.

The results in Table 3 proved that the optimal DT-MP offered higher bioavailability than the reference product. The mean area under the curve (AUC) of DT-MP was 2.19 times higher than that of the reference product. And the 90% confidence interval (CI_90%) of the ratio ($AUC_{DT\text{-}MP}/AUC_{reference}$) transformed by the logarithm was from 130.83 to 345.92%. The obtained results of

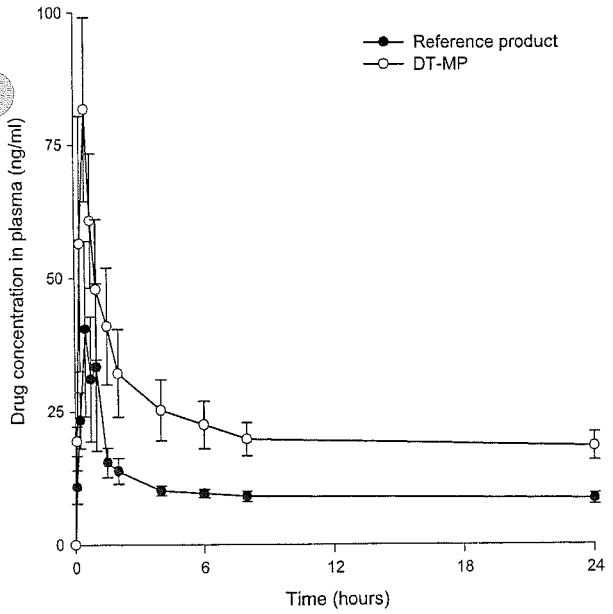


Fig. 10. Pharmacokinetics profile of azithromycin in rabbit model from reference product and a dispersible tablet containing bitter taste masking microparticles (n = 3, Mean ± S.E).

Table 3
Pharmacokinetics parameters of azithromycin from reference product and DT containing bitter taste masking microparticles (n = 3, Mean ± S.E).

Parameters	Reference product	DT-MP	90% CI_(Lower; Upper)[a]
AUC (ng h/ml)	250.83 ± 25.18	548.29 ± 98.17	(130.83; 345.92)
Cmax (ng/ml)	40.50 ± 16.47	81.77 ± 17.35	(87.61; 577.05)
Tmax (h)	0.50	0.50	

[a] 90% Confidence intervals of the ratio ($AUC_{DT\text{-}MP}/AUC_{reference}$ or $C_{max\_DT\text{-}MP}/C_{max\_reference}$) were transformed by the logarithm.

CI_90%$_{ln(AUC)}$ proved that the bioavailability of DT-MP was significantly higher than that of the reference product. Besides, the mean C_{max} of DT-MP (81.77 ng/ml) also doubled that of the reference product (40.50 ng/ml). However, the fact that the CI_90%_lower$_{ln(Cmax)}$ (87.61%) was lower than the upper limit of bioequivalence (125%) indicated the non-significant difference between C_{max} of DT-MP and C_{max} of the reference product. This result was explained by the inter-subject variability in the initial phase of pharmacokinetics profile which was discussed earlier. Assumingly, the higher bioavailability of DT-MP was caused by some of the following factors.

First, since AZI is a weakly basic drug, it is very likely to precipitate in the intestinal medium right after leaving the gastric medium, causing a reduction in drug absorption. Zithromax was easily dissolved in the stomach and also decomposed in this medium. The remaining part of dissolved AZI passed to the intestinal medium where the drug solubility declined. Consequently, this part of AZI would have a high potential of precipitation. In the dissolution profile of AZI, a slight reduction of Zithromax concentration (from 13.07% to around 11.15%) was observed after the remaining drug passed to the intestine. This reduction might be caused by the precipitation phenomenon of AZI. However, the remaining concentration of AZI was very small, which suggested that it might be under or around the saturated solubility of AZI. As a result, it was hard to see a marked reduction of Zithromax concentration in the intestinal medium. In contrast, the bitter taste masking microparticles behaved differently from Zithromax regarding dissolution profile in the two mediums (pH 1.2 and 6.8). After passing to the intestine, the dissolution rate of AZI from microparticles increased dramatically to more than 80% after 180 min. Because of the amorphous state of AZI in microparticles, its solubility in the intestinal medium was improved, and its precipitation was limited. AZI might easily re-dissolve after supersaturation state in intestinal medium, and its absorption accelerated.

In recent years, several research groups [46–48] have looked into the mechanism of enhancing oral bioavailability of weakly basic drugs using amorphous solid dispersion. One approach was based on the supersaturation, precipitation, and re-dissolution process of weakly basic drugs in the intestinal tract. Tanaka et al. [48] used a hydrochloric acid solution containing fluorescein isothiocyanate dextran (FD-4), a non-absorbable marker, to investigate how supersaturation, precipitation, and re-dissolution processes influenced the intestinal absorption of cinnarizine (CNZ), a lipophilic weak base. From the luminal concentration-time profiles of FD-4 and CNZ, the team concluded that the key process for the absorption of CNZ was re-dissolution, not supersaturation. Similarly, Rubbens et al. [47] employed a weak base, indinavir, as a model drug to investigate its intraluminal dissolution, supersaturation and precipitation behavior in GI tract. The experiment was conducted among five healthy volunteers in a cross-over study in fasted state, fed state and fasted state with concomitant proton pump inhibitor (PPI) use. Their conclusion was the presence of duodenal supersaturation in all three testing conditions.

In another approach, Indulkar [46] used liquid-liquid phase separation (LLPS) theory to explain the oral bioavailability enhancement of the three weakly basic drugs (clotrimazole, nicardipine, and atazanavir). Right after exiting the acidic stomach environment, these model drugs could form liquid-liquid phase separation prior to crystallization. Accordingly, if liquid-liquid phase separation happened, a turbid solution consisting of a continuous solution phase and a colloidal phase would be formed. The high drug concentration in the continuous solution phase corresponding to the amorphous solubility of the drug was a driving force for the enhancement of drug absorption. Besides, the colloidal phase composing of a disordered drug-rich phase was a drug reservoir to maintain the supersaturation at a constant value during absorption.

Second, the combination of two stabilizers ($CaCO_3$ and NaH_2PO_4 = 2:1) protected AZI from degradation in gastric medium and maintained the high level of parent drug in the upper part of gastrointestinal tract. The result in Fig. 10 also indicated that the drug concentration of AZI from DT-MP within the first hour after oral administration was always higher than that from reference product. In this case, both $CaCO_3$ and NaH_2PO_4 were alkalizes for modulation of microenvironmental pH in microparticles. The potential of these agents in the enhancement of drug solubility and stability was also proved in other studies [49–51]. Tung et al. [51] used l-lysine as a pH modulator to increase the solubility of rebamipide, an anti-ulcer drug. The solid dispersion using rebamipide, l-lysine, PVP-VA 64 and poloxamer 407 increased the relative oral bioavailability of the drug about 1.74-fold compared with the reference product in a rat model. Similarly, Park et al. [50] used various alkalizes (MgO, Na_2CO3, Na_2HPO_4, and $NaHCO_3$) to modulate the microenvironmental pH of clarithromycin in a crystalline solid dispersion system. The authors concluded that alkalizes in crystalline-solid dispersion maintained the microenvironmental pH of the tablet above pH 5 under acidic conditions thus providing an useful method to improve the dissolution rate and stability of clarithromycin without changing drug crystallinity. Even though the pharmacokinetics study was conducted with a small number of experimental animals, the improved bioavailability of AZI primarily proved the high potential of the newer dosage form, DTs-MP. When the relative bioavailability of the DTs-MP doubled that of the commercial product, other therapeutic advantages of the DTs-MP can be considered such as reduction of dose, total weight of tablets, and minimization of GI side effects.

4. Conclusion

The study was successful in developing bitter taste masking microparticles containing azithromycin loaded in dispersible tablets. The kind of polymeric carrier and microsphere size played an important role in masking the bitter taste of AZI. An incorporation of soluble and insoluble alkalizes into DTs-MP for the maintenance of the microenvironmental pH of tablets around 5.0 was useful for the improvement of the drug stability and minimization of side effects of AZI on the GI. The two times enhancement of the relative bioavailability of DTs-MP (2.19 times) compared to that of the commercial powder for oral suspension (Zithromax, Pfizer) resulted from the high solubility of the amorphous drug in the intestinal medium and the improved stability of AZI in the gastric medium.

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Table 1
Specifications of the purpose-made CE prototypes used for food analyses.

Specification	CE prototype	
	Manual version	Semi-automated version
Maximum voltage	20 kV	25 kV
Injection mode	Siphoning	Siphoning
Capillary flushing	Manually, using an external syringe	Automatically, using an integrated valve system and gas pressurization
Polarity	Single (either positive or negative)	Single (either positive or negative)
Power supply	Main power or 12 V battery (8 h before recharging is needed)	Main power or 12 V battery (7 h before recharging is needed)
Thermostatic chamber	Not included	Not included
Casing material (for high voltage isolation)	Plexiglas or mica	Plexiglas

miniaturized high voltage (HV) - C^4D built in-house (Pham et al., 2014). The resulting signals were recorded with a 12 VDC-powered E-corder 201 data acquisition system (eDAQ, Denistone East, NSW, Australia) connected to the USB-port of a personal computer. The systems can be operated with a 12 V (for CE) and split ±12 V (for C^4D) supplies, using either batteries or 220 VAC-to-12 VDC inverters.

2.3. Sampling and sample treatment

All food samples were transported to the lab and stored at 4 °C for subsequent in-lab analysis and cross-checked with the confirmation methods. Canned and bottled beers from different trademarks were purchased from local stores. Bottles of orange juice, wine, alcohol, beverages, vinegar as well as some coffee sachets and Lipton tea bags were purchased from supermarkets in Hanoi. A tea drink and a black bean sweet soup were obtained from a sidewalk café. The beverage, tea drink, vinegar and black bean sweet soup samples were prepared by filtering with 0.45 μm PTFE membrane filters (Chromafil O-20/15 MS, Macherey-Nagel, Oensingen, Switzerland), then diluting with deionized water. Dilution ratios of 5–20 were applied for all these samples, except for vinegar where a much higher dilution ratio of 500 was used due to an extremely high concentration of acetic acid in this sample. Beer samples were ultrasonicated for 30 min to completely remove the dissolved gas, then filtered with 0.45 μm PTFE membrane filters prior to dilution with deionized water. For pretreatment of a tea bag sample, the tea from a tea bag was precisely weighed (about 1.6 g), immerged in boiling deionized water for 3 min, cooled down to room temperate, filtered through a 0.45 μm PTFE membrane filter, and 20-time diluted prior to analysis with CE-C^4D. The coffee samples from coffee sachets were precisely weighed (1 g), dispersed in 10 mL of warm deionzed water, vortexed for 5 min and ulstrasonicated for 20 min. The obtained solutions were filtered through a 0.45 μm filter and then diluted (5 times) with deionized water.

Noodle sachets from different trademarks, *i.e.* Hao Hao, Ba Mien were purchased from local stores in Hanoi. The noodle samples were grinded and well mixed in a ceramic mortar. Then 4 mL of deionized water was added in to a precisely weighed amount (about 0.5 g) of a grinded noodle sample. The sample was then ultrasonicated for 30 min, centrifuged at 8000 rpm for 30 min, filtered through a 0.45 μm PTFE membrane filter and finally diluted with deionized water.

Two fruity jelly packages, each containing about 40 jelly cubes, from Vietfoods LLC were purchased from a local store. 5 random jelly cubes in each package were grinded with a blender, mixed with 100 mL deionized water, ultrasonicated for 30 min, and filtered through 0.45 μm PTFE membrane filters. The obtained solutions were either further diluted with deionized water (1/10 ratio) if needed or spiked with aspartame (20 ppm) and acesulfame K (20 ppm) (for the standard addition method) before injection into the CE system.

2.4. Analytical methods

All CE-C^4D operations for determination of food additives in food samples were carried out immediately upon conclusion of the sampling to avoid/minimize sample nature modification. Unless otherwise stated, a BGE composed of 50 mM His/30 mM MES and 25 μM CTAB (pH 6.0) was employed for the determination of oxalate, citrate and tartrate. Electrophoretic separations of these compounds were carried out by application of a −15 kV voltage over a capillary whose total length (L_t), effective length (L_{eff}, the distance between the injection end of the capillary and the detection position) and internal diameter (I.D.) are 60 cm, 50 cm and 75 μm respectively. The buffer used for organic acid (C1-C4) determination was composed of 30 mM His/40 mM MES (pH 5.8). A capillary having L_t of 50 cm, L_{eff} of 43 cm and I.D. of 50 μm and a voltage of −18 kV were used for the CE operations in this case. Artificial sweeteners were determined with a BGE containing 100 mM Tris/10 mM His (pH 9.2), using a voltage of +15 KV applied over a capillary of 75 μM I.D. (L_t of 60 cm and L_{eff} of 53 cm). The capillaries were equilibrated with the appropriate BGE for some hours prior to CE-C^4D analyses in order to minimize the shift of migration times and obtain stable baselines (Nguyen et al., 2015). Quantification was implemented with the standard addition method. All cross check operations were carried out at NIFC using an HPLC-PDA equipment (Model: 20A, Shimadzu, Japan) according to the protocols adapted from (Chen & Wang, 2001; Ergoenuel & Nergiz, 2010; Zeppa, Conterno, & Gerbi, 2001).

3. Results and discussion

3.1. Considerations when using purpose-made CE-C^4D

Different purpose-made portable CE-C^4D instruments were developed by our group and transferred to users in various institutes in Vietnam for testing their performances (Duong et al., 2015; Le et al., 2016; Thanh Duc; Mai et al., 2016; Nguyen et al., 2016; Nguyen et al., 2014; Nguyen et al., 2015). Feedbacks from the users were then collected to provide information on the positive features as well as drawbacks of purpose-made CE-C^4D setups so that further instrumental improvement and/or methodology adaptation can be realized for more robust operation and better performance. In low-cost and portable CE-C^4D systems (see Fig. 1 for demonstration of a low-cost purpose-made instrument), only the essential components required to create electrophoretic separations and C^4D detection are equipped. These components include

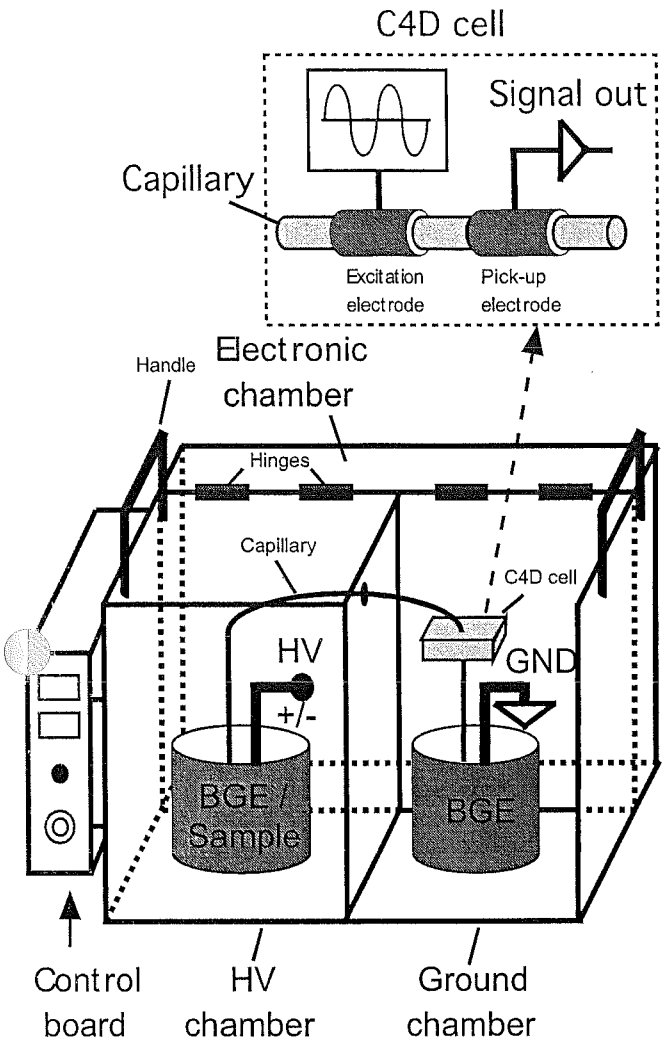

Fig. 1. Demonstration of a low-cost purpose-made CE-C^4D instrument. BGE: background electrolyte; HV: high voltage electrode; GND: ground electrode; C^4D: capacitively coupled contactless conductivity detector.

a high voltage module (up to 30 kV of a predefined polarity), two platinum electrodes, two vials containing sample or background electrolyte (BGE) solutions, a capillary, a casing with high voltage and ground chambers and a C^4D. Some miniature isolation valves may be additionally integrated in the ground (GND) side for semi-automated operations (Nguyen et al., 2014). All other frills often found in bench-top commercial CE instruments, *i.e.* a sample tray and thermo-stated chamber were excluded in order to minimize the power consumption and the construction cost. As a result, some steps of capillary pre-conditioning, notably flushing with sodium hydroxide (0.1–1 M), hydrochloric acid (0.1–1 M), deionized water and/or pre-coating solution(s) were excluded in the protocol. To simplify the procedure, thus rendering the CE-C^4D method more adapted to non-expert users, there is only one capillary rinsing step with the working BGE between two analyses. From our experience, good baseline stability and reproducibility of migration times are obtained when allowing equilibration of the capillary with the BGE for an extended period of time (hours) before analyses. As C^4D is a bulk detector, it can non-selectively visualize all (or most of) species in samples, including the unwanted ones. The performance of the CE-C^4D technique therefore is much dependent on the sample matrices to be injected into the capillary. This is particularly true for home-made CE-C^4D instruments as they are more vulnerable to problems (e.g. signal drifting, significant background noise) if the sample matrix is not optimized properly. The presence of wall-adsorbing species in the sample matrix, for example proteins, can result in signal disturbance and significant fluctuation of migration times and peak areas. A too conductive sample matrix should also be avoided as this can cause reduction of signal sensitivity. When working with CE-C^4D using the hydrodynamic injection mode, the sample is preferably prepared in (deionized) water or diluted BGE whose conductivity is significantly lower than that of the BGE in order to assure the sample stacking effect. Non-wall-adsorbing inorganic and small organic species are the favorite analytes for CE-C^4D. In some cases, addition of an organic solvent, e.g. methanol, acetonitrile or ethanol, into the sample matrix is needed to facilitate the dissolution of the (organic) analytes. This does not affect much the performance of CE-C^4D as long as the organic phase percentage is kept minimal (Mai, Bomastyk, Duong, Pham, & Hauser, 2012). In order to obtain a 'clean' matrix that is suitable for CE-C^4D operation, recourse to a sample pre-treatment step is very often needed for i) removal of the interfering compounds in the matrix and ii) sample enrichment for much improved detection sensibility. To avoid the adverse effect of ambient temperature variations and high humidity on the CE-C^4D performance, for example drifts of baseline and migration time, the CE system should be operated in an air-conditioned room. In the absence of this condition, application of the standard addition method may be needed for peak identification and quantification.

3.2. CE-C^4D determination of artificial sweeteners in beverage, jelly, black bean sweet soup samples

Artificial sweeteners are widely used as a low calorie alternative to natural sugar in food, beverages and pharmaceutical products.

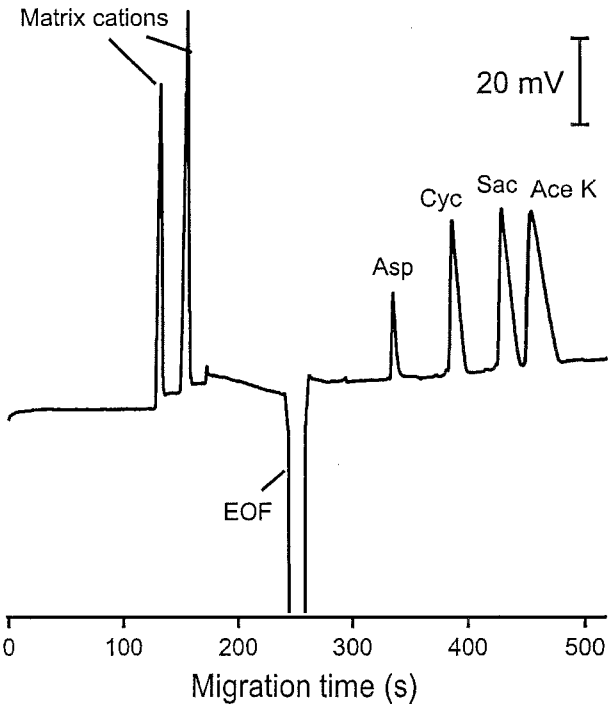

Fig. 2. Electropherogram for a standard solution containing aspartame (100 ppm), cyclamate (50 ppm), saccharine (50 ppm) and acesulfame K (50 ppm). CE conditions: Electrolyte solution: 100 mM Tris/10 mM His (pH 9.2). Voltage: −15 kV. Sample injection: hydrodynamic (HD) for 10s with the height of 15 cm. Capillary: fused-silica, 75 μm id, Lt = 60 cm (Leff = 53 cm). EOF: Electro-osmotic flow.

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Formulation and biopharmaceutical evaluation of supersaturatable self-nanoemulsifying drug delivery systems containing silymarin

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ABSTRACT

The first objective of this study was to optimize a supersaturatable self-nanoemulsifying drug delivery system (S-SNEDDS) containing silymarin through the investigation of the single and synergistic effect of either SNEDDS or a precipitation inhibitor on dissolution efficiency (DE) of silymarin. The bioavailability and hepatoprotective activity of S-SNEDDS were then compared to those of a branded product (Legalon®, Meda). SNEDDS containing silymarin was developed by titration technique, and Poloxamer 407 was selected as the optimal precipitation inhibitor by using casting film and solvent-shift method. The interaction of silybin (the major active constituent of silymarin) and the polymer was then determined by differential scanning calorimetry, powder X-ray diffractometry (PXRD), Fourier transforms infrared spectroscopy and ^{1}H NMR analysis. The combination of two techniques including SNEDDS and addition of 10% of Poloxamer 407 remarkably increased DE_{4h} (88.28%) compared to the reference product (6.41%). The relative bioavailability of S-SNEDDS versus Legalon® was about 760%. The hepatoprotective activity of S-SNEDDS in CCl_4-induced mice was also superior to the commercial product in declining both the levels of serum transaminases (ALT, AST) and lipid peroxidation as well as glutathione and superoxide dismutase (SOD) activities under tested doses calculated as silybin (10, 25 and 50 mg/kg). These biopharmaceutical and pharmacological advantages of S-SNEDDS indicated prospects in the development of a novel product that offers lower strength of silymarin while enhancing therapeutic outcomes.

1. Introduction

Silymarin (SLM) is a mixture of flavonoids extracted from the fruits of the milk thistle (*Silybum marianum*) consisting of silybin (A, B), isosilybin (A, B), silydine and silychristin, among which silybin is the main constituent offering the therapeutic effect. Silymarin has been clinically proven in the treatment of numerous liver disorders such as steatosis, alcohol-related liver disease, viral hepatitis, drug-induced liver injury, chronic hepatitis, and cirrhosis. Recently, silymarin has also been proved of antiproliferative/cytotoxic activity against certain cancers and several potential protective effects on cardiovascular system (Deep et al., 2007; DT et al., 2017; Taleb et al., 2018). As a result, there have been several attempts to modify the original formulation, aiming at further enhancing its therapeutic advantages (Arcari et al., 1992; Wu et al., 2006; Woo et al., 2007; Sun et al., 2008; Javed et al., 2011). However, the improvement of bioavailability and pharmacological effect of silymarin faces a challenge regarding the very low oral bioavailability (0.73%) of silymarin (Woo et al., 2007). The primary reasons include low aqueous solubility, low permeability across intestinal epithelial cells, extensive phase II metabolism, rapid excretion in bile and urine (Javed et al., 2011) as well as strong binding of silybin to human serum albumin (Yamasaki et al., 2017).

Improving the bioavailability of drugs has always been one of the major concerns in recent research. A number of studies have come up with techniques to improve the oral bioavailability of drugs in solid dosage forms such as salt forms, a complex of cyclodextrin with the drug, reduction of particle size, lipid-based carriers or solid dispersion (Rabinow, 2004; Williams et al., 2013). Most of these techniques have been applied in synthetic drugs, and some of them are commercialized such as itraconazole (Sporanox®, Janssen), cyclosporine A (Neoral®,

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Novartis), paclitaxel (Taxol®, Bristol-Myers Squibb). Nevertheless, proper attention has not been paid to the improvement of oral bioavailability and pharmacological effects of herbal drugs in general and silymarin in particular. Several studies suggested that modern pharmaceutical techniques such as liposomes (Mohsen et al., 2017), nanoparticles (Yang et al., 2013; El-Nahas et al., 2017) or solid dispersion (Sun et al., 2008) could be used to enhance the solubility of silymarin. Solid dispersion was used by a number of researchers to enhance silymarin solubility. Back in 1992, Arcari et al developed a complex of SIL and β-cyclodextrin that increased the drug solubility by about 90% after 5 min (Arcari et al., 1992). More recently, Sun et al employed solvent evaporation to prepare a solid dispersion containing SIL and PVP, which was then incorporated into a fluid bed apparatus (Sun et al., 2008). Other modern pharmaceutical techniques were also proved to minimize the limitations of silybin, thereby increasing the oral bioavailability of silymarin compared to suspension containing raw material (Wu et al., 2006; Woo et al., 2007; Javed et al., 2011; Hwang du et al., 2014).

Still, very few studies directly compared the new formulation to the branded product (Legalon®) in both terms of biopharmaceutical and pharmacological studies. Woo et al (2007) concluded that self-microemulsifying drug delivery systems (SMEDDS) could enhance the bioavailability of silymarin by approximately 3.6 times in the rat model compared to the commercial product. However, the authors did not conduct a subsequent pharmacological study to enable a conclusion on the therapeutic effect of SMEDDS. In another study, Yang et al (2013) proved that silymarin-loaded lipid nanoemulsion significantly reduced carbon tetrachloride-induced hepatotoxicity and improved the bioactivity compared with the raw material and the respective commercial product. Nevertheless, the improved pharmacological results of nanoemulsion did not well correlate with its relative bioavailability results, which was only 1.3 times higher than that of the commercial product. Following the FDA guideline, the AUC of the two formulations (nanoemulsion *vs.* Legalon®) might not be significantly different because the 90% confidence intervals of the $AUC_{nanoemulsion}/AUC_{Legalon®}$ would be out of the acceptable range (85–125%). Furthermore, the experimental design of these pharmacological studies (Yang et al., 2013; El-Nahas et al., 2017) was only conducted with a unique dose of silymarin (50 mg/kg), which only partially demonstrated the relationship between bioavailability enhancement of nanoemulsion and its hepatoprotective activity. If the bioavailability results supported the pharmacology study at the different doses of silymarin, the improvement in bioavailability would be more convinced. It is useful to note that the ultimate purpose of developing a generic product is to find out a new formulation with a lower dose of silymarin but similar bioavailability, hepatoprotective activity, and lower side effects compared to the branded product.

Among many new drug delivery systems to overcome biopharmaceutical limitations of the original product (Wu et al., 2006; Woo et al., 2007; Yang et al., 2013; Mohsen et al., 2017), self-nanoemulsifying or self-microemulsifying drug delivery system (SNEDDS/SMEDDS) was among the most effective approaches to address drugs with poor bioavailability and extracts containing the low bioavailability active constituents such as powdered milk thistle extract *(Silybum marianum L.)*. Nevertheless, the limitation of SNEDDS/SMEDDS was the risk of drug precipitation after dilution in the gastric medium. To avoid this phenomenon, the general approach was to increase the amount of surfactant. However, a high ratio of surfactant in formulation might raise a concern about stomach irritation and toxicity for patients. Therefore, another solution to consider was to prepare supersaturatable self-nanoemulsifying/self-microemulsifying drug delivery system, S-SNEDDS/SMEDDS. The advantage of S-SNEDDS/SMEDDS over traditional SNEDDS/SMEDDS was the incorporation of a hydrophilic polymer (HPMC, PVP) as a precipitation inhibitor into the traditional SNEDDS/SMEDDS. The presence of this polymer will maintain the supersaturation condition of the drug in the gastrointestinal tract by inhibiting the precipitation process of the drug after dilution of SNEDDS/SMEDDS. Several studies on S-SNEDDS/SMEDDS containing various drugs (paclitaxel, PNU-9135, AMG 517) have agreed on the advantage of S-SNEDDS/SMEDDS in bioavailability improvement of drugs (Gao et al., 2003; Gao and Morozowich, 2005; Wei et al., 2012; Chavan et al., 2015; Jaisamut et al., 2017a; Yeom et al., 2017).

Despite the initial positive results of S-SNEDDS/SMEDDS, a thorough understanding of the system has not been achieved, especially a basis for selecting certain polymeric precipitation inhibitors. Most research groups, when preparing S-SNEDDS/SMEDDS, opted for traditional polymers such as HPMC or PVP for their proven benefits rather than experimenting with newer ones (Gao et al., 2003; Wei et al., 2012; Jaisamut et al., 2017a). Consequently, further screening of different polymers and demonstration of the recrystallization inhibition mechanism after self-nanoemulsifying/self-microemulsifying play an important role in the successful development of S-SNEDDS/SMEDDS.

In this context, the first objective of this study was to optimize a supersaturatable self-nanoemulsifying drug delivery system (S-SNEDDS) containing silymarin through investigating the single and synergistic effect of either SNEDDS or a precipitation inhibitor on dissolution efficiency (DE) of silymarin. The bioavailability and hepatoprotective activity of S-SNEDDS were then compared to those of a branded product (Legalon®).

2. Materials and methods

2.1. Materials

The powdered milk thistle extract *(Silybum marianum L.)*, of which silymarin comprises 55.8%, was obtained from Pujing chemical Co., Ltd. (China). Quercetin and silybin were purchased from Sigma-Aldrich Corporation (U.S.A). Propylene glycol caprylate (Capryol 90), oleoyl macrogol-6 glycerides (Labrafil M 1944 CS), caprylocaproyl polyoxyl-8 glycerides (Labrasol), and diethylene glycol monoethyl ether (Transcutol P) were supplied by Gattefossé (France). PEG-40 hydrogenated castor oil (Kolliphor® RH 40), polyoxyl 35 hydrogenated castor oil (Kolliphor® EL) and poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Poloxamer 407) were purchased from BASF (Germany). Polysorbate 80 (Tween 80) and polyethylene glycol 400 were purchased from Croda (U.K). HPLC-grade methanol was purchased from J.T. Baker (U.S.A.). Hydroxypropyl methylcellulose phthalate (HPMCP) was purchased from Shin-Etsu Chemical Co., Ltd. (Japan). Methacrylic acid-methyl acrylate copolymer (Eudragit L100) was purchased from Evonik Corporation (Germany). Hydroxypropyl beta cyclodextrin (HPβCD, Kleptose®) was purchased from Roquette Co., Ltd (France). Water was purified by reverse osmosis and was filtered in-house. All other reagents were analytical grade commercial products. The commercial product was the sugar-coated tablet (Legalon® 70 mg, Meda Pharmaceutical Company) with the Lot no of B1701839 and expired date at 03/2020.

2.2. Development of SNEDDS

2.2.1. Solubility studies

The solubility of SLM in different oils, surfactants, and co-solvents was investigated. An excess amount of powdered milk thistle extract and 5 mL of a screened solvent were put into a capped vial which was then shaken in an isothermal shaker (Daihan, Korea, Model WCB 30) at 25 °C. After 48 h of shaking, the tube was centrifuged at the relative centrifugal force of 3634*g* for 10 min to obtain the supernatant. The supernatant containing solublized SLM was filtered through membranes 0.45 μm (Sartorius, Germany, Model Minisart RC 25) and diluted by a mixture of methanol: dichloromethane (1:1, v/v) prior to analyzing by a UV spectrophotometer (Hitachi, Japan, Model U-1800) at 288 nm.

2.2.2. Construction of ternary phase diagrams

The water titration method was applied to construct the pseudo-ternary phase diagrams. Based on preliminary experiments, Labrafil was chosen as the oil phase. The surfactant and cosurfactant were Kolliphor® RH 40 and Transcutol HP, respectively. Referring to previous studies (Wu et al., 2006; Woo et al., 2007; Tung et al., 2018), the effect of different ratios of surfactant to cosurfactant (S_{mix}, 1:1, 2:1, 3:1 and 4:1) on the formation of self-emulsification region was investigated in order to select the suitable S_{mix}. For each phase diagram, the various ratios of oil and a specific S_{mix} (O/S_{mix}, 2:8, 3:7, 4:6, 5:5, 6:4 and 7:3) were mixed thoroughly in glass vials before gradual addition of the distilled water. Visual observation was applied to measure the phase boundary of the self-emulsification region in which the sample appearance changed from turbid to transparent or vice versa.

2.2.3. Droplet and particle size measurement

The droplet size of nanoemulsion and particle size of precipitate in the solvent-shift study were determined by using Malvern Zetasizer (Malvern Instruments, UK, Model Zetasizer Nano ZS90). Samples were diluted with ultra-purified water prior to addition to a cuvette for measurement of droplet size.

2.2.4. Dissolution study

The dissolution efficiency of silymarin was tested in non-sink conditions using the dissolution apparatus type 2 (Erweka, Germany, Model DT 600). The study was carried out at 37 °C ± 0.5 °C and the stirring speed of 100 rpm. The dissolution medium was 500 mL phosphate buffer saline pH 6.8. Samples were SNEDDS containing around 700 mg of milk thistle powder extract. Three milliliters of the aliquot was withdrawn at predetermined time intervals of 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4 h. After 3 min of ultracentrifugation at the relative centrifugal force of 3634*g*, the obtained supernatant containing SLM was filtered through membranes 0.20 μm (Sartorius, Germany, Model Minisart RC 25) and diluted by a mixture of methanol: dichloromethane (1:1, v/v) prior to analyzing by a UV spectrophotometer (Hitachi, Japan, Model U-1800) at 288 nm. Dissolution efficiency (D.E.) of each formulation was calculated by the following Eq. (1):

$$E = \frac{\int_{t_1}^{t_2} y \cdot dt}{y_{100} \cdot (t_2 - t_1)} \times 100 \quad (1)$$

where y is the percentage of the dissolved product; D.E. is the area under the dissolution curve between time points t_1 and t_2 and is expressed by the percentage of the curve at maximum dissolution, y_{100}, over the same period.

2.3. Development of supersaturatable-SNEDDS

2.3.1. Casting film study

With a reference to Parikh et al (2015), a modified casting film method was developed to quickly screen the most effective precipitation inhibitor for silymarin. Briefly, dissolve completely 0.2 g of milk thistle powder extract and polymer at different ratios in 2 mL of a solvent mixture (methanol: dichloromethane, 1: 1).

The 3 groups of polymers including non-ionized polymer groups (HPβCD), anionic polymer groups (HPMCP, Eudragit L100), and copolymer (Poloxamer 407) were chosen for this comparative study, and the ratios of milk thistle powder extract to polymer were 4: 6; 5: 5; 6: 4; 7: 3. Films having the thickness of 200 μm was formed by casting the above solutions on microscope slides. The organic solvents in the casting film were evaporated in the vacuum oven at 25 °C for 24 h. These films were then stored in the climate chamber at 40 °C and 75% relative humidity. The appearance of the casted films and the existence of crystals on the dried films were monitored by observing with an optical microscope (Nikon Eclipse Ci-L, Japan) at different time frames at the magnification rate of 10×.

2.3.2. Supersaturation study of SLM by the solvent-shift method

Besides the casting film method, the precipitation inhibition capacity of a polymer to silymarin in the supersaturated solution was also evaluated by the solvent-shift method, which was reported in an earlier study by Konno et al. (2008). The experimental conditions of the dissolution tester were 37 °C ± 0.5 °C and under 100 rpm stirring speed of paddle. The solution containing silymarin was prepared by dissolving completely 1.0 g of powdered milk thistle extract in 5 mL of solvent mixture DMF and methanol (3: 7, v/v). This solution was gradually poured into the dissolution medium containing 500 mL phosphate buffer saline pH 6.8 (with or without polymer). After predetermined time intervals of 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4 h, about three milliliters of aliquot were withdrawn and centrifuged at the relative centrifugal force of 17,586*g* for 3 min. The obtained supernatant containing SLM was filtered through membranes 0.20 μm (Sartorius, Germany, Model Minisart RC 25) and diluted by methanol prior to analyzing by a UV spectrophotometer (Hitachi, Japan, Model U-1800) at 288 nm. The 3 groups of polymers including non-ionized polymer groups (hydroxypropyl beta-cyclodextrin, HPβCD), anionic polymer groups (HPMCP, Eudragit L100) and copolymer (Poloxamer 407) were also chosen for this comparative study. The degree of supersaturation (DS) of SLM in buffer pH 6.8 containing different kinds of polymers was calculated by the following Eq. (2):

$$DS = \frac{Concentration\ of\ SLM}{Equilibrium\ solubility} \quad (2)$$

in which DS > 1.0 showed supersaturation state of SLM; DS = 1.0 showed saturation state, and DS < 1.0 showed undersaturation state of SLM.

2.3.3. Studies of physical properties

The physical properties of precipitate in the solvent-shift method were determined by differential scanning calorimetry and powder X-ray diffractometry (PXRD) study. Differential scanning calorimetry (DSC) was performed using Perkin Elmer Pyris DSC thermal analyzer system in aluminum pans. DSC was performed against a reference pan at temperatures between 30 °C and 250 °C at 10 °C/min. The crystallinity of silymarin, silybin (the major active constituent of silymarin), and the precipitate of silybin in the solvent-shift method were evaluated with an X-ray diffractometer (Siemens, Germany, Model D500) with Cu-Kal radiation and Ni filter. About 5 to 10 mg of the sample was placed onto the copper plate of the X-ray equipment. X-ray diffraction data were collected at room temperature in a range of $3° < 2\theta° < 60°$ and at the scanning speed of 0.02°/min.

The interaction mechanism of silybin and precipitation inhibitor was determined by Fourier transforms infrared spectroscopy and ^{1}H NMR analysis. FT-IR spectra were obtained on a Fourier transform infrared spectroscopy (Model IFS-66/S, Bruker Optics, Germany) using the potassium bromide (KBr) disk method. One to two mg of sample was mixed with 150 mg of spectra-grade KBr and pressed into a disk of 12 mm diameter using Carver hydraulic press (Model No. 3912, Carver Inc., and U.S.A). Samples were analyzed from 600 to 4000 cm^{-1} with an instrument resolution of 0.1 cm^{-1}.

^{1}H NMR experiments were conducted to investigate the possibility of the intermolecular interaction between silybin and polymer. The ^{1}H NMR spectra of the samples were taken at 25 °C on a Bruker Avance 500 MHz spectrometer equipped with 5 mm TCI HCN Z gradient cryoprobe. Spectra were processed using Bruker Topspin 2.1 software and analyzed using CARA 1.8.4 software. Sample containing silybin and polymer at an optimum ratio was dissolved in CD_3OD at a concentration of 20 mg/ml. This solution was poured into a suitable glass tube which was then put into Bruker NMR Spectrometer.

2.3.4. Preparation of supersaturatable-SNEDDS

The supersaturatable-SNEDDS was prepared by the two following steps. The first step was to make SNEDDS containing milk thistle

powder extract. Accordingly, oil (Labrafil) and S_{mix} were mixed thoroughly in a beaker and then heated up to 80 °C in a thermostatic tank prior to addition of the powder extract. The extract was completely dissolved in the mixture of oil and S_{mix} under the agitation of magnetic stirring for 30 min until a transparent, uniform system was formed. The second step was to incorporate a silymarin precipitation inhibitor (Poloxamer 407, HPβCD, HPMCP and Eudragit L100) into the above SNEDDS to obtain the supersaturatable-SNEDDS (S-SNEDDS).

When Poloxamer 407 was used as the precipitation inhibitor, this polymer was added directly into the previously heated SNEDDS (80 °C). The mixture was then continuously mixed by magnetic stirring for 30 min at the temperature of about 80 °C to have a final homogenous mixture (S-SNEDDS).

When HPβCD, HPMCP and Eudragit L100 were used as the precipitation inhibitors, each of them was dispersed into the cooled SNEDDS by the wet milling method. Briefly, the polymer was ground by using pestle and mortar milling before sieving through a mesh with 180 μm openings. The fine polymer was then thoroughly milled with SNEDDS to obtain a final dispersion of the polymer in SNEDDS, which was also called S-SNEDDS.

2.3.5. Dissolution study

The dissolution rate of SLM from S-SNEDDS was also studied using the dissolution apparatus type 2 (Erweka, Germany, Model DT 600). This method was similar to the dissolution study applied to SNEDDS in Section 2.2.4.

2.4. Pharmacokinetic study

The animal study was approved by the Local Scientific and Ethics Committee (No 113/QD-DHN). Male rabbits, each weighed approximately 2 kg, were purchased from the Centre of Experimental Animals, National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). Animals had ad libitum access to tap water and food and were kept at room temperature of 25 ± 1 °C and regular 12/12 h light/dark cycle. These rabbits were divided into different groups of four to five and were fasted for 24 h before the experiment. Samples were the suspension of fine powder Legalon® in distilled water and S-SNEDDS with the standardized silybin dosage of approximate 28 mg/kg. It should be noted that Legalon® (sugar-coated tablets) was ground by pestle and mortar before sieving through 180 μm sieve for fine powder.

The concentration of silybin in samples was analyzed using a validated high-performance liquid chromatography (HPLC) method (Agilent, U.S.A, Model 1200 series) (Wei et al., 2012). Briefly, each of the samples was dissolved with the mobile phase consisting of methanol: phosphate buffer saline KH_2PO_4 0.05 M (adjust to pH 4.0 by the solution of H_3PO_4 10%) (50:50, V/V) then injected into the column for analysis with the injection volume of 50 μl. The flow rate was 1.0 mL/min, and the detector wavelength was 288 nm. The HPLC system consisted of an isocratic pump (Agilent, U.S.A., Model G1311C), a manual injector (Agilent, U.S.A., Model G1328C), a column thermostat (Agilent, U.S.A., Model G1316A), and a multi-wavelength detector (Agilent, U.S.A., Model G1315D). Detector output was integrated and digitalized using the Agilent ChemStation software (Agilent, U.S.A., Model 1200 Series HPLC system). The column used was a C18 (Zorbax SB, 4.6 × 250 mm, 5 μm particle size, Agilent, U.S.A.), and the total run time for a sample was about 30 min. All operations were carried out at 40 °C. Accordingly, the content of silybin, which was the major therapeutic constituent in the milk thistle powder extract (*Silybum marianum L.*), was 31.27 ± 0.72 (%, w/w).

After oral administration of samples, about 3 mL of blood was withdrawn from the ear artery at predetermined intervals of 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h. An equal amount of saline containing heparin 50UI was immediately supplemented after each sampling point. The sample was then centrifuged at 2684*g* for 10 min to collect plasma then stored in deep-freezer at −40 °C until the day of analysis.

3. LC-MS/MS analysis of silybin in rabbit plasma

The withdrawn samples were analyzed by liquid chromatography-tandem mass spectrometry. A LC-MS/MS system included a 6460 Triple Quad mass spectrometry coupled with a 1290 Quat Pump and 1290 Sampler (Agilent Technologies, U.S.A) was used to quantify the analyte. LC separation was obtained by using an Eclipse Plus C18 column (150 × 2.1 mm; 3.5 μm i.d. particle size) and a precolumn (Agilent Technologies, U.S.A.) with a mobile phase composition of 0.1% formic acid in water and acetonitrile. The gradient program was initially set at 5% acetonitrile for 0.5 min then increased linearly to 50% acetonitrile over 7 min. After that, the eluent composition was maintained at 50% acetonitrile for 0.5 min then returned to 50% acetonitrile in 0.5 min and re-equilibrated for over 2 min. The flow rate was kept constant at 0.5 mL min^{-1}. The total run time was 10 min. The mass spectrometer was operated in positive ESI mode with the capillary voltage and the gas temperature set at 4000 V and 300 °C, respectively. A Peak NM32-LA gas generator (UK) was employed to generate highly pure N2 (99.9995%) to be used as a source gas (collision gas). MS/MS experiments were carried out in multiple reaction monitoring modes with two transitions for each compound. The transitions monitored for silybin were *m/z* 481 → 125 (CE 30 V) and *m/z* 481 → 152 (CE 42 V) while those for quercetin were *m/z* 301 → *m/z* 151 (CE 22 V) and *m/z* 301 → *m/z* 183 (CE 38 V). The first transition of each compound was used for quantification, and the other was used for confirmation.

The sample preparation was slightly modified from Brinda et al. (2012). A 500 μl aliquot of the plasma sample was transferred into a 2 mL centrifuge tube. 25 μl of an internal standard solution of 1 μg mL^{-1} (quercetin in methanol) was added to the tube, followed by the addition of ethyl acetate (0.5 mL). After being blended by a vortex mixer for 5 min, the tube was centrifuged at the speed of 16,060*g* for 10 min. The above layer (0.25 mL extract) was pipetted into a 2 mL vial. After being vaporized to dryness under a gentle stream of nitrogen gas at 45 °C, the residue was dissolved by exactly 500 μl of methanol, and a volume of 10 μl was extracted then injected into the LC-MS/MS system for analysis.

3.1. Evaluation of the in vivo hepatoprotective effect of S-SNEDDS

3.1.1. Experimental animals

Adult Swiss mice (20–22 g each) were purchased from the Centre of Experimental Animals, National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). The animals had ad libitum access to tap water and food and were kept at room temperature of 25 ± 1 °C and regular 12/12 h light/dark cycle. A seven-day period of acclimation was given to mice prior to any experiment. All experimental procedures and protocols used in the present study were approved by the Scientific and Ethics Committee, Hanoi University of Pharmacy (No 113/QD-DHN).

3.1.2. Animal grouping and treatment

Mice were randomly divided into 8 groups of 9–10. Groups I-II (controls): was administered normal saline orally every day for 7 days; Groups III-V: received daily oral administration of S-SNEDDS with doses of 10, 25, and 50 mg/kg b.w for 7 days; Groups VI-VIII: received daily oral administration of the reference product with equivalent doses in the same period (the doses of test and reference product were equivalent in silybin component in each dosage form). On the 7th day, one hour after the oral administration, experimental hepatotoxicity was induced for all animals of groups II-VIII by intraperitoneal injection of a single dose of 0.5 mL/kg carbon tetrachloride diluted with olive oil (1:10 v/v). Meanwhile, animals from group I (control group) was administered only solvent (olive oil).

3.1.3. Sampling procedure

Twenty-four hours following injection of carbon tetrachloride, blood was withdrawn from orbital sinus and allowed to clot for

approximately 60 min at room temperature. Subsequently, serum was separated by centrifuging the clotted blood at 3000 rpm for 10 min and stored at −20 °C until further use. Liver samples were also collected and kept at −80 °C for preparation of liver post-mitochondrion supernatant (PMS).

3.1.4. Determination of serum aminotransferases

Enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the spectrophotometric method according to instructions of the supplier (Biosystems S.A – Barcelona, Spain), using semi-auto biochemistry analyzer TC-3300 Plus (Teco Diagnostics, USA).

3.1.5. Determination of lipid peroxidation and reduced glutathione (GSH)

The liver samples (10% w/v) were homogenized in ice-cold phosphate buffer (100 mM, pH 7,4) then centrifuged at 10,000 rpm for 30 min at 0 °C (5702R, Eppendorf, Germany). The supernatant (PMS) was subsequently collected and used for measurement of biochemistry parameters.

Hepatic lipid peroxidation level was assayed by the thiobarbituric acid reactive substance (TBARS) method as described by Wasowicz with some minor modifications Wasowicz et al. (1993). Specifically, 0.15 mL PMS was mixed with 2 mL thiobarbituric acid (0.25% in acetic acid, pH 2.4–2.6) in presence of saturated butylated hydroxytoluene (BHT). The reaction mixture was placed in a boiling water bath for 60 min. The formed colored adduct was extracted by n-butanol and then measured spectrophotometrically at 532 nm. The result was expressed as malondialdehyde (MDA) equivalent (nmol MDA/g tissue), using freshly prepared tetramethoxypropane as standard.

GSH level in liver tissue was estimated by a spectrophotometric method using Ellman's reagent (Koh et al., 2012). PMS was precipitated by the addition of one volume of 4% sulfosalicylic acid. The supernatant obtained after centrifugation (20 µl) was mixed with 10 mM 5,5′-Dithiobis (2-nitrobenzoic acid) (20 µl) and phosphate buffer (160 µl). The absorbance of the product was immediately recorded at 412 nm. GSH content was expressed as µmol reduced GSH/g tissue.

3.1.6. Superoxide dismutase (SOD) activity

SOD activity was assayed by xanthine method according to the protocol described previously (Takada et al., 1982). The reaction mixture contained diluted PMS (replaced by water in control wells), 50 mM, pH 10.2 carbonate buffer, 0.1 mM EDTA, 100 mM xanthine, 0.025 mM nitrotetrazolium blue chloride (NBT) and 0.01 U/mL xanthine oxidase. The rate of reduction of NBT was measured at 560 nm for 5 min at 25 °C. The result was calculated from a standard curve prepared with different concentrations of SOD and expressed as units of SOD per gram tissue (kU/g). One unit of SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

3.2. Data analysis

The data was calculated using Excel (Microsoft, USA), SPSS 22.0 and Phoenix 8.0 (Certara® Inc., USA) program. Data were expressed as the mean ± standard deviation (S.D) and analyzed for statistical significance by one-way ANOVA test followed by LSD or Dunnett's T3 post-hoc test. The values of $p < 0.05$ were considered to be statistically significant.

4. Results and discussion

4.1. Development of SNEDDS

4.1.1. Solubility and construction of phase diagram study

In order to develop SNEDDS containing silymarin, the solubility of silymarin in different solvents was determined. It was clear from the results in Table 1 that there were three optimal excipients: Labrafil M1944CS, Kolliphor® RH40, and Transcutol P. Labrafil M 1944CS was selected as the oil phase of SNEDDS for the higher solubility of SLM (113.0 mg/ml) compared to Capryol 90 (103.9 mg/ml). Kolliphor® RH 40 also emerged as the appropriate excipient as it showed the dual advantages in enhancing the solubility of SLM and emulsifying Labrafil in water. Finally, Transcutol P was chosen as co-solvent because it offered the highest solubility of SLM (around 350.1 mg/ml). This co-solvent not only increased the SLM solubility but also made it easier for the oil phase to disperse into the water phase due to its miscibility with both oil and water phase (Tung et al., 2018). Given the advantages of these excipients, SNEDDS would be more stable when emulsifying into the water medium, and the risk of SLM precipitation during the dilution of SMEDDS might be limited. This finding was in line with previous studies (Zhang et al., 2011; Zhang et al., 2015; Tung et al., 2018), in which Kolliphor® RH40 and Transcutol P made a useful couple of surfactant/cosolvent in the preparation of SNEDDS. However, the optimal ratio of surfactant/cosolvent (S_{mix}) varied in these studies for its dependence on both kinds of oil and the properties of active pharmaceutical ingredients (Bala et al., 2016; Bi et al., 2016; Hong et al., 2016; Jaisamut et al., 2017b).

Table 1
The solubility of silymarin in different mediums (n = 3, Mean ± STDEV).

Excipients		Solubility (n = 3, Mean ± STDEV)
Oils	Labrafil M 1944	113.0 ± 5.7 mg/ml
	Capryol 90	103.9 ± 4.3 mg/ml
Surfactants	Kolliphor® RH40	145.0 ± 5.5 mg/g
	Kolliphor® EL	59.1 ± 2.3 mg/g
	Labrasol	57.7 ± 1.5 mg/g
	Tween 80	32.8 ± 2.7 mg/g
Cosolvents	Transcutol P	350.1 ± 6.1 mg/ml
	Ethanol	225.2 ± 4.2 mg/ml
	PEG 400	63.0 ± 4.5 mg/ml

Therefore, a phase diagram consisting of Labrafil, Kolliphor® RH 40 and Transcutol P was constructed to find out the suitable ratio of S_{mix} (Fig. 1). The software ImageJ 1.51j8 (U.S.A) was employed to determine the self-emulsification region of each phase diagram. When the ratio of Kolliphor® RH 40: Transcutol P changed from 1: 1 to 2: 1, the self-emulsification region dramatically increased from 0.068 to 0.188. It was because the increased amount of surfactant led to a reduction in surface tension between the oil and water phase. However, when the proportion of surfactant climbed from S: CoS = 2: 1 to S: CoS = 4: 1, the self-emulsification region only rose slightly from 0.188 to 0.203. These results indicated that S_{mix} of 2:1 might be the change threshold of self-emulsification region happening to these self-dispersing systems. Based on the two criteria for selection of suitable S_{mix} including the largest self-emulsification region and the smallest amount of surfactant, the S_{mix} = 2: 1 was chosen for further experiments.

4.1.2. Formulation of SNEDDS containing silymarin

The optimal SLM-loaded SNEDDS using for developing the supersaturatable-SNEDDS was prepared by the trial-and-error approach. Two critical factors of SLM loaded SNEDDS included the amount of silymarin and the ratio of Labrafil: S_{mix}. Output variables were the dissolution efficiency of silymarin after 4 h, the droplet size and PDI of nanoemulsion (Table 2). Because the commercial milk thistle tablets were prepared from milk thistle powder extract (*Silybum marianum L.*) consisting of 70 mg silymarin, the dissolution efficiency of silymarin acted an indicator for the release process of samples in the screening step.

As the amount of milk thistle powder extract in the formula increased from 15.6 to 20.8%, the DE_{4h} of SLM remarkably decreased while the droplet size of nanoemulsion slightly increased. The highest amount of the powder extract (20.8%) in the formulation might

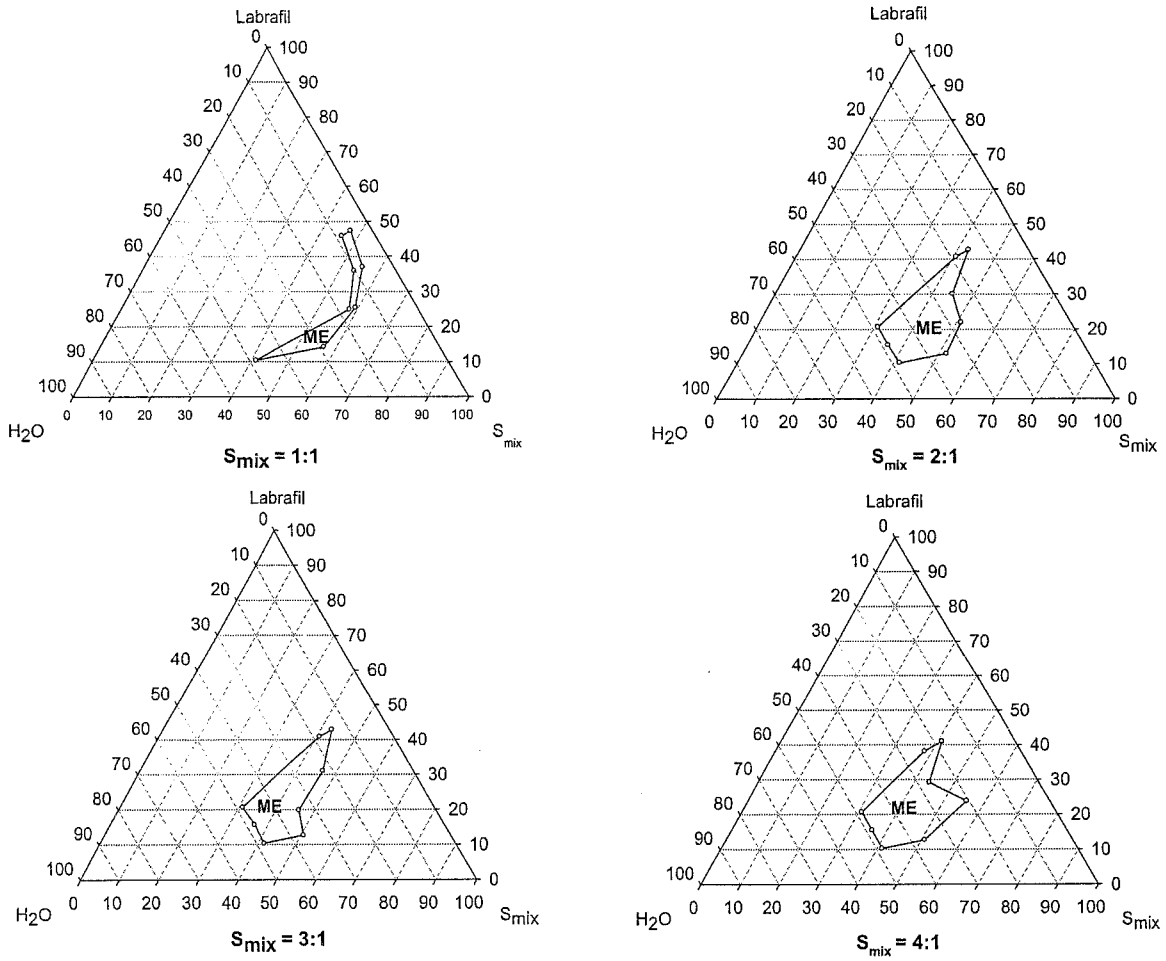


Fig. 1. Pseudoternary phase diagrams consisting of Labrafil, Kolliphor® RH 40 (surfactant), Transcutol HP (cosolvent) and water with various ratios of S_{mix} (S: CoS).

Table 2
Effects of compositions of SNEDDS, S-SNEDDS on dissolution efficiency of silymarin at 4 h, droplet size, and PDI.

Formulation		Ratio in formulation	DE_{4h} (%)	Droplet size (nm)	PDI
SNEDDS	Amount of milk thistle powder extract (%)	15.6%	53.66 ± 0.23	160.50	0.41
		20.8%	47.47 ± 0.09	176.80	0.43
	Ratio of Oil: S_{mix}*	5:5	47.65 ± 0.07	192.70	0.45
		4:6	53.66 ± 0.23	160.50	0.41
		3:7	63.41 ± 0.09	132.50	0.33
		2:8	69.19 ± 0.10	99.67	0.26
S-SNEDDS*	Poloxamer 407	2.2%	68.99 ± 0.41	368.20	0.58
		5.3%	76.48 ± 0.06	200.90	0.38
		10.0%	88.28 ± 0.06	221.40	0.51
		14.3%	93.48 ± 0.23	218.30	0.42
		18.2%	95.47 ± 0.05	307.10	0.52
	HPβCD	10.0%	71.32 ± 0.31	133.40	0.30
	Eudragit L100	10.0%	75.25 ± 0.19	155.40	0.44
Legalon®			6.41 ± 0.07	n/a	n/a

* The samples contain about 15.6% milk thistle powder extract.

accelerate the precipitation of SLM in dissolution medium due to the easy formation of the supersaturation state of SLM in this medium. Right after dilution of SNEDDS in medium, part of the SLM moved from the droplets into the dissolution medium, participated in the nucleation process, and grew up to form bigger crystals. These crystallized particles were the main reason for the reduction of drug dissolution and polydispersity of colloidal particles in the dissolution medium. Due to the higher DE_{4h} of SNEDDS containing 15.6% milk thistle powder extract, this amount of extract was fixed for further formulation.

Regarding the impact of oil: S_{mix}, it was found that when the amount of oil decreased and the amount of S_{mix} increased, the DE_{4h} of SLM steadily increased from 47.65 to 69.19%. This demonstrated that S_{mix} enhanced SLM dissolution in the medium and partly inhibited SLM precipitation. However, if the amount of oil was minimum, and the amount of S_{mix} was maximized for enhancing SLM dissolution, the state of the system would change from an emulsion system to a solution system. The toxicity potential of the system would also be more severe due to the appearance of the high amount of surfactant and cosolvent in the formula. This finding indicated that if the loaded amount of milk thistle powder extract was fixed at 15.6%, the designed SNEDDS showed a limitation in terms of solubility enhancement of SLM in phosphate buffer pH 6.8. Specifically, the current SNEDDS could only increase DE_{4h} up to 69% and would hardly bring the SLM dissolution up to absolute level. There was a potential of SLM precipitation in the dissolution medium after dilution of SNEDDS if the loaded amount of SLM increased and the amount of S_{mix} decreased. To overcome this phenomenon, a supersaturatable self-emulsifying drug delivery system was investigated in a follow-up study.

4.2. Development of supersaturatable-SNEDDS

The most important factor in deciding the success of the supersaturatable-SNEDDS was the polymeric inhibitors of SLM precipitation in dissolution medium or gastrointestinal tract after the self-emulsifying process of this system in the release medium. However, very few studies

comprehensively investigated the compatibility of precipitation inhibitors with both silymarin and SNEDDS (Gao and Morozowich, 2005; Yeom et al., 2017). In the present study, a suitable polymer which was used to effectively inhibit SLM precipitation in the absorption window of the GI tract (pH 6.8) was screened by the two techniques of casting film and solvent-shift method.

4.2.1. Screening of SLM precipitation inhibitors by casting film method

The casting film method for quick observation of SLM recrystallization was reported and accepted in several previous studies (Konno et al., 2008; Parikh et al., 2015). Based on this method, three groups of polymeric inhibitors including a non-ionic polymer (hydroxypropyl beta-cyclodextrin), anionic polymers (hydroxypropyl methylcellulose phthalate and Eudragit L100) and copolymer (Poloxamer 407) were investigated. All three polymeric groups were soluble in phosphate buffer pH 6.8 and might have a high potential to inhibit silymarin precipitation. When the precipitation of SLM in this medium was prolonged, the absorption rate and extent of SLM might be enhanced. Both enteric polymers of HPMCP and Eudragit L100 are negatively charged in phosphate buffer pH 6.8, but their compositions are different. HPMCP is a polymer of cellulose derivatives and comprises the phthalate functional group while Eudragit L100 belongs to the group of methacrylic acid-methyl acrylate copolymers and involves the methacrylic acid functional group in its structure. Meanwhile, hydroxypropyl beta-cyclodextrin has a cage-like supramolecular structure and does not carry any charge. Poloxamer 407 is an amphiphilic triblock-copolyether and is also not charged in the dissolution medium.

The obtained films were observed under an optical microscope after 1, 7 and 90 days (Fig. 2). It was assumed that SLM recrystallized on the casting film because dark spots and clusters formed on the film after a period of storage. This assumption could be better confirmed with a polarized light microscope (Parikh et al., 2015; Nie et al., 2017; Li and Taylor, 2018). However, given the present study objective that was to quickly discover the potential polymers for inhibiting SLM recrystallization, the use of an optical microscope was sufficient and more convenient.

The ability of different polymers to hinder the recrystallization of SLM was significantly different. The copolymer (Poloxamer 407) was the most effective, indicated by the delayed appearance of tiny dark spots on the film applied with a small amount of Poloxamer 407 (30–40%) after at least 90 days. The enteric polymers (HPMCP and Eudragit L100) could also slow down SLM recrystallization but not as much as Poloxamer 407. Dark spots or clusters were visible on the film using HPMCP and Eudragit L100 after 7–90 days of storage. Meanwhile, the non-ionic polymer (hydroxypropyl beta-cyclodextrin) at the SLM extract: HBβCD ratios of 6: 4 and 7:3 was the least effective because big dark clusters of SLM crystals were observed after only 1 day of storage. To quantitatively clarify the potential of Poloxamer 407 among other polymers, the supersaturation concentration of silymarin in phosphate buffer saline pH 6.8 containing above polymers was determined by the solvent-shift model.

4.2.2. Screening of SLM precipitation inhibitors by the solvent-shift method

The inhibiting effect of different polymers on SLM precipitation in solvent-shift method was indicated by the degree of supersaturation (DS). The fact that DS of all polymers (Fig. 3a) were over 1.0 indicated that all polymers could create the supersaturation state for silymarin in intestinal medium (pH 6.8). In other words, the solubility of SLM could be enhanced by adding these polymers into the dissolution medium as precipitation inhibitors. However, their different degrees of supersaturation proved that these polymers could inhibit drug precipitation at varied levels. The obtained result by solvent-shift method was basically in line with that using the casting-film method. Poloxamer 407 still ranked the first among the precipitation inhibitors because it could create the highest degree of supersaturation (DS = 4.7 after 5 min) as compared to that of the complex polymer (HPβCD), enteric polymers (HPMCP and Eudragit L100), which was only 3.3, 3.6 and 3.0, respectively.

Such differences in terms of precipitation inhibition were explained by the interference of these polymers in silymarin precipitation process in the dissolution medium of phosphate buffer saline pH 6.8, which included two continuous phases of nucleation and crystal growth. Accordingly, the size of precipitate would be one of the factors indicating the role of polymer in the precipitation process of SLM. When there was no precipitation inhibitor in the dissolution medium, SLM quickly precipitated to form big crystals of approximately 1,531 nm after 30 min, which gradually increased to 1,700 nm after 60 min. Meanwhile, the precipitate size reduced to 778, 621.4 and 433.9 nm

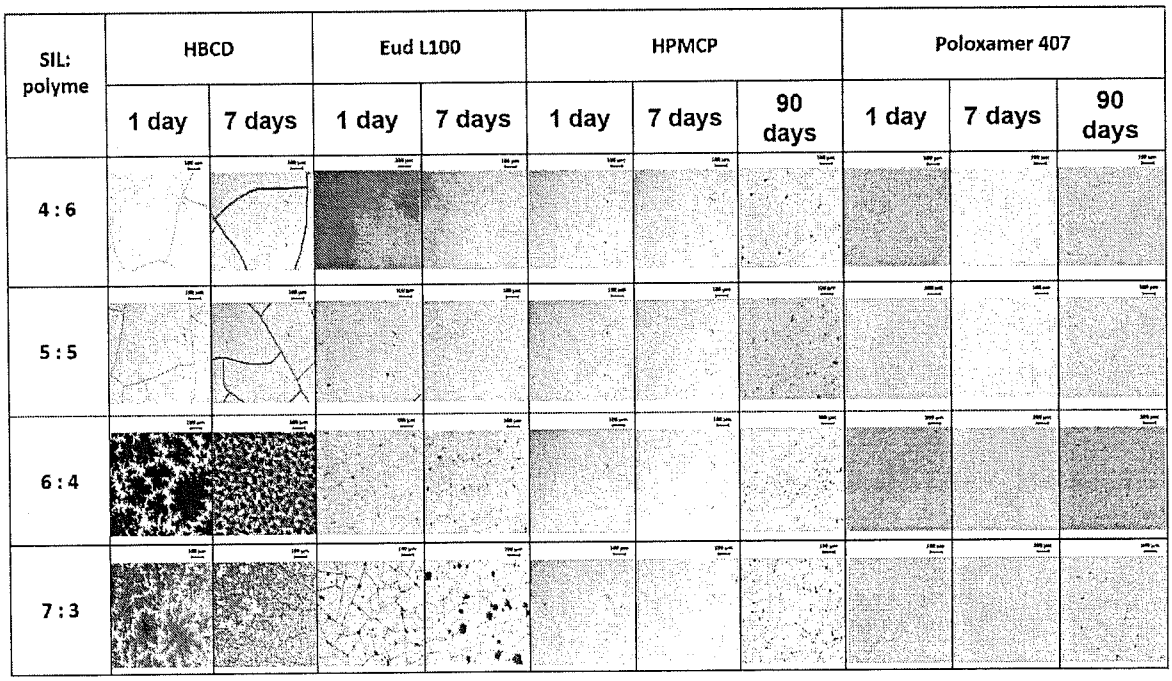
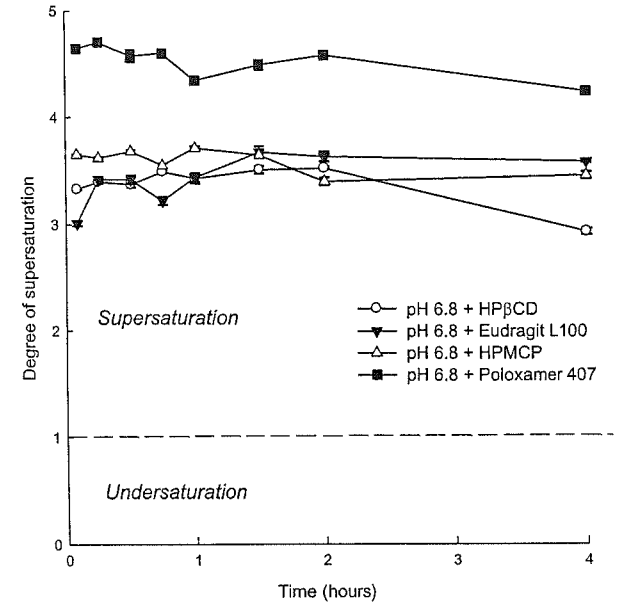
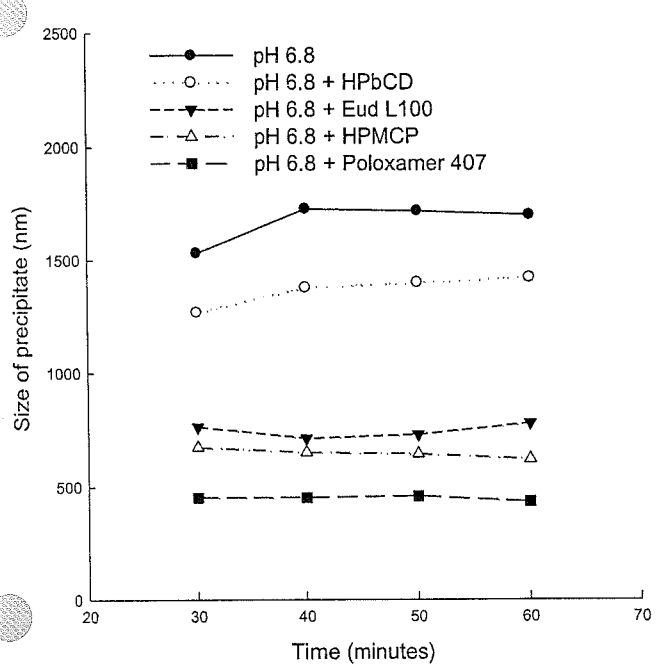


Fig. 2. Casting film of silymarin and different polymers under different storage periods.



A)



B)

Fig. 3. Effect of polymers on A) degree of supersaturation and B) size of precipitate in the solvent-shift model.

when Eudragit L100, HPMCP and Poloxamer 407 were added into the medium, respectively. This suggested the participation of enteric polymers and copolymer in the nucleation step of SLM. Nevertheless, the fact that the size of SLM precipitates in a medium using the remaining polymer, HPβCD was around 1400 nm indicated the intervention of HPβCD in the crystal growth step of SLM (Fig. 3b).

4.2.3. Study of physical properties

Given the varied capacities of polymers in DS enhancement of SLM, different scenarios regarding the interaction mechanism between silymarin and polymer could be assumed. First, copolymer (Poloxamer 407) and the enteric polymers (HPMCP and Eudragit L100) increased the DS through their adsorption onto the surface of SLM (Price and Ditzinger, 2018), thereby slowing down the aggregation and growth of silymarin crystals, which was evident in the casting film and the solvent-shift studies. Meanwhile, the presence of HPβCD in pH 6.8 improved the equilibrium crystalline solubility of SLM by the formation of a complex between drug and HPβCD (Price and Ditzinger, 2018). This polymer did not strongly interfere with the precipitation process of SLM and did not remarkably change the solute activity of SLM.

Due to the highest DS of silymarin resulting from the presence of Poloxamer 407 in the dissolution medium, the interaction of this polymer to silybin, the major active constituent of silymarin, in milk thistle powder extract at both molecular and crystal state was considered. Thermal analysis, powder X-ray diffraction as well as vibration spectroscopies were applied to observe some physiochemical properties of silymarin, silybin, and a precipitate of silybin in dissolution medium (pH 6.8) containing Poloxamer 407.

First, the interaction of Poloxamer 407 and silybin molecular was determined by Fourier transform infrared spectroscopy and ^{1}H NMR analysis. The infrared spectrum (Fig. 4a) showed that the position of the proton donor group (–OH) in silybin structure moved from 3456.44 in silybin spectra to 3458.37 cm^{-1} in the mixture spectra. ^{1}H NMR spectroscopy experiments were continued to investigate the possible interaction between silybin and Poloxamer 407. Also, the results in Fig. 4b indicated a corresponding shift of hydroxy groups on the spectrum of silybin at 1.600, 3.747 and 7.025 ppm to 1.843, 3.917 and 7.23 ppm on the spectrum of the molecular mixture of silybin and Poloxamer 407. It was preliminary predicted that the hydrogen bond between silybin and Poloxamer 407 might be one of the main interactions of silybin and Poloxamer 407, leading to the enhanced degree of supersaturation. The molecular interaction of silybin and Poloxamer 407 described in Fig. 4c might be one reason for the effective adsorption of Poloxamer 407 on silybin, thus limiting the silybin precipitation and increasing the silybin solubility.

Second, the interaction between Poloxamer 407 and crystal precipitate of silymarin was determined by X-ray diffraction spectroscopy and differential scanning calorimetry. The X-ray diffraction results (Fig. 5a) illustrated that silymarin existed in crystal state with the crystal peaks at 6.072, 5.506, 4.515, 3.971, 3.630 and 3.315°, in which the specific crystal peaks of silybin were located at 6.072, 4.515, 3.971, 3.630° (Fig. 5b). The X-ray diffraction spectrum of precipitate obtaining from the solvent-shift study also showed similar specific crystal peaks for silybin (Fig. 5d). This meant the precipitation process and the presence of Poloxamer 407 in the dissolution medium did not change the crystal structure of silybin.

The property of precipitate was further investigated by thermal analysis. In the DSC diagram (Fig. 6), there were two endothermal peaks illustrating silybin A and silybin B at the temperatures of 172 and 181 °C, respectively. Similarly, in the solvent-shift process, the DSC spectrum of silybin A and B precipitate also included the two above endothermal peaks. However, compared to the original silybin, the position of silybin A precipitate moved from 172 to 160 °C, and the intensity of these peaks was remarkably reduced. This phenomenon might result from the hydration state and the semi-crystalline of silybin precipitate. In short, the adsorption of Poloxamer 407 on the surface of crystal nuclei of silybin slowed down the aggregation of these crystals but did not change the crystal structure of silybin. Based on the compatibility study between silymarin and Poloxamer 407 using solvent-shift, casting film, thermal analysis as well as vibration spectroscopy, Poloxamer 407 was chosen as the main precipitation inhibitor for the preparation of S-SNEDDS.

4.2.4. Effect of precipitation inhibitor on S-SNEDDS

To make it easier for the investigation of a ternary drug delivery system containing silymarin-Poloxamer 407-SNEDDS, the interaction of Poloxamer 407 with the two remaining factors (silymarin and SNEDDS) was conducted by a trial-and-error approach. Accordingly, the rates of silymarin, Labrafil M1944CS, Transcutol P and Kolliphor® RH40 were

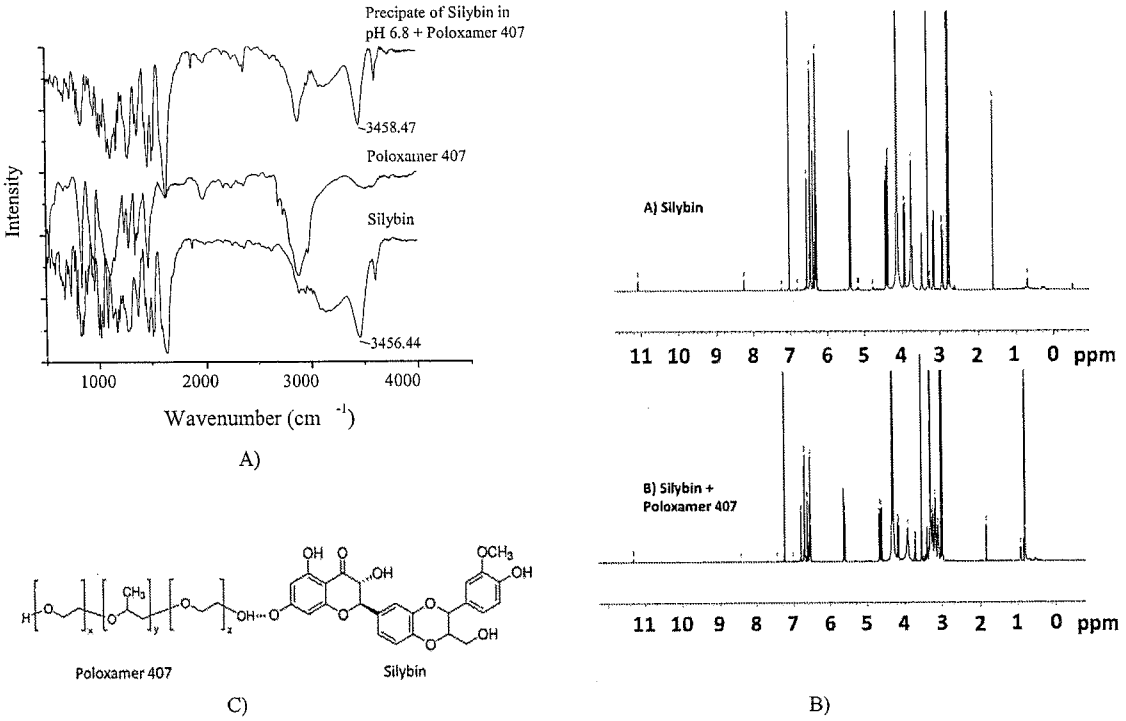


Fig. 4. A) FT-IR diagram, B) ^{1}H NMR diagram of silybin and precipitate of silybin in the buffer pH 6.8 containing Poloxamer 407; C) Molecular structure of interaction between silybin and Poloxamer 407.

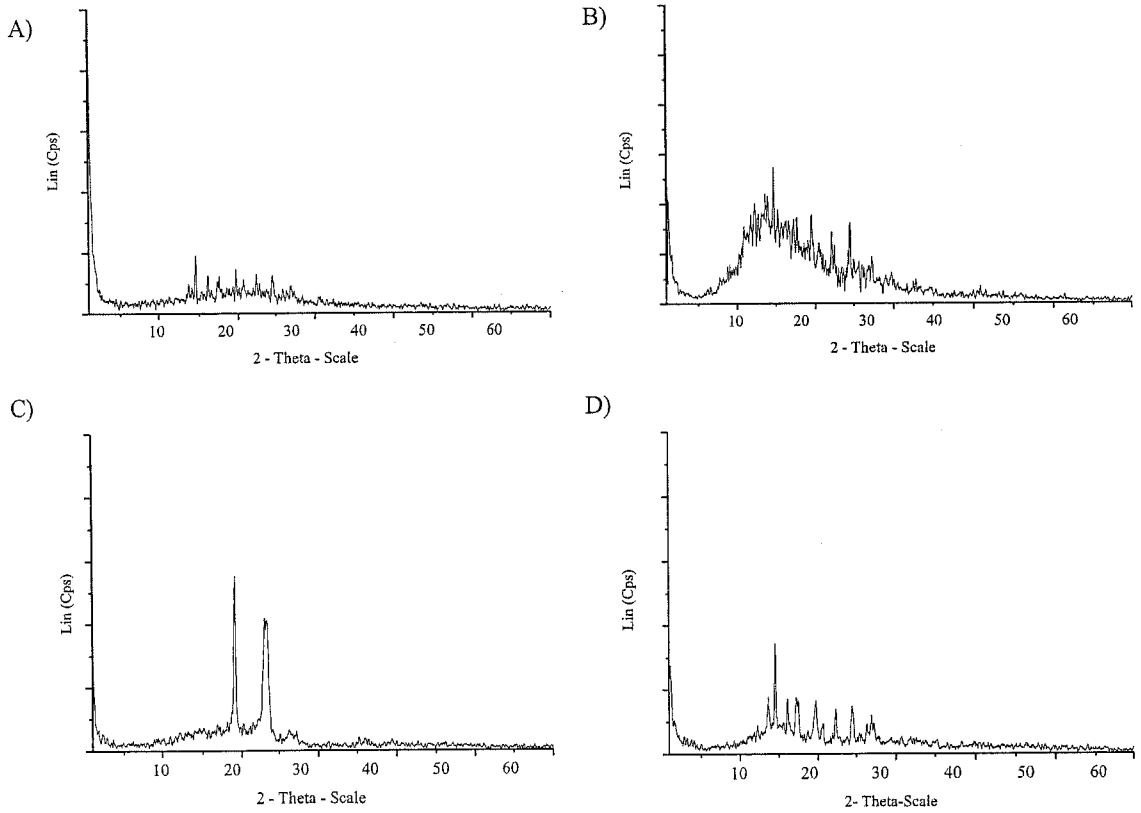


Fig. 5. X-ray diffraction patterns of A) silymarin powder extract, B) silybin, C) Poloxamer 407 and D) precipitate of silybin in the buffer pH 6.8 containing Poloxamer 407.

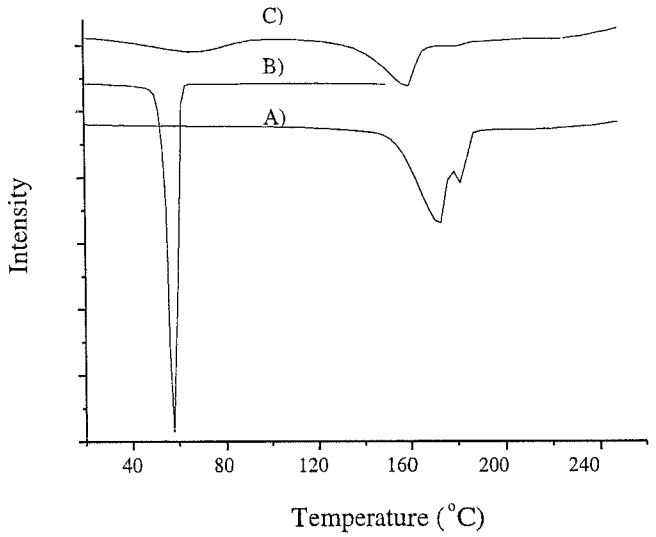


Fig 6. DSC diagram of A) silybin, B) Poloxamer 407 and C) precipitate of silybin in the buffer pH 6.8 containing Poloxamer 407.

fixed at 15.6%, 33.8%, 16.9%, and 33.8%, respectively. The effect of different percentages of Poloxamer 407 on the DE_{4h} of silymarin from S-SNEDDS was evaluated. As shown in Table 2, the higher the amount of Poloxamer 407 incorporated in SNEDDS, the higher the dissolution efficiency of SLM gained. Specifically, DE_{4h} of SLM from S-SNEDDS increased by more than 15% as compared to SNEDDS when a small amount of Poloxamer 407 (2.2%) was added into SNEDDS. Moreover, the DE_{4h} of SLM was almost one hundred percent when 18.2% of Poloxamer 407 was used as the SLM precipitation inhibitor in S-SNEDDS. This result pointed to the potential of Poloxamer 407 in minimizing SLM recrystallization in dissolution medium as well as enhancement of SLM dissolution rate. The combination of two techniques including preparation of self-nanoemulsifying drug delivery systems and the addition of 10% Poloxamer 407 as the SLM precipitation inhibitor significantly increased the DE_{4h} (88.28%) compared to the reference product (6.41%).

The explanation for the superior advantage of S-SNEDDS versus commercial product in enhancing DE_{4h} was assumed as follows. The emulsifying process of SNEDDS in dissolution medium accelerated the formation of nanoemulsion containing a concentrated amount of silymarin which started the formation of the drug-rich phase in oil droplets. The free unionized drug might be pushed out of these droplets and moved to the poorly soluble medium prior to precipitation into the crystal particles or clusters. The DE_{4h} of SLM in mediums containing around 10% of HPβCD (71.32%), Eudragit L100 (75.25%) or Poloxamer 407 (88.28%) was higher than that in the conventional SNEDDS (53.66%). The successful incorporation of one of the above polymers as the drug precipitation inhibitor into SNEDDS, therefore, proved the assumption.

As discussed in the previous section, the effects of these polymers on the DE_{4h} varied due to the different interaction mechanisms between silybin and polymer. The copolymer (Poloxamer 407) was the most useful polymer to inhibit the SLM precipitation, which promoted the formation of a central compartment containing a huge amount of unionized free drug in the dissolution medium (Taylor and Zhang, 2016). Eudragit L100 and HPβCD were also effective inhibitors of drug precipitation, but their immiscibility in SNEDDS might raise concerns about phase separation during storage of S-SNEDDS. This phenomenon might also have happened in previous studies (Gao et al., 2003; Gao and Morozowich, 2005; Jaisamut et al., 2017a; Yeom et al., 2017) relating to S-SNEDDS using hydrophilic polymers like HPMC or PVP. These immiscible polymers in SNEDDS could be considered strange particles or nuclei for attachment of silybin in the oil solution (SNEDDS)

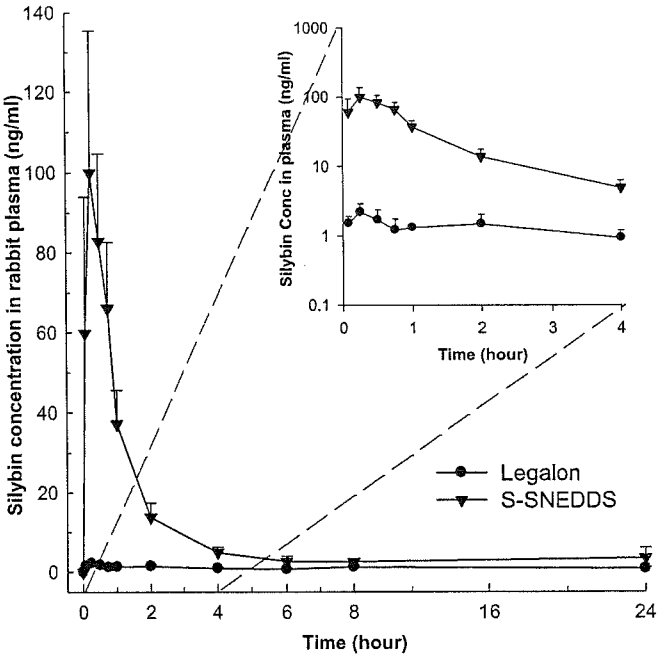


Fig. 7. Pharmacokinetic profiles of silybin after oral administration of Legalon® and S-SNEDDS in a rabbit model (Mean ± SE).

and could lead to the silybin precipitation in SNEDDS during the storage period. Nevertheless, due to the amphiphilic property of Poloxamer 407, this copolymer was miscible with SNEDDS and could maintain the supersaturable state of silymarin in SNEDDS and supersaturated state of silymarin after the emulsifying of SNEDDS in the dissolution medium. The supersaturation of silymarin might be the thermodynamic driving force for pushing the drug into the central compartment.

The appearance of the central compartment containing unionized free drug might be understood as an acceleration of concentration gradient between the two sides of a GI membrane. The drug might have a higher chance to increase the passive absorption from the GI medium to the GI membrane. This experimental design proved the potential of S-SNEDDS in terms of the drug release. However, the advantage of S-SNEDDS in enhancing drug permeability had not yet been pointed out, which was worth discovering in the pharmacokinetic study.

4.3. Pharmacokinetic study

The objective of this part was to compare the relative bioavailability of S-SNEDDS to those of reference product in rabbits. The pharmacokinetics and pharmacological effect of silymarin were determined through silybin, the major therapeutic constituent in the extract. The concentration of silybin in rabbit plasma was calculated by the non-compartment model using Phoenix 8.0 (Certara USA, Inc). The results in Fig. 7 indicated that after oral administration of S-SNEDDS, silybin was quickly absorbed into the blood circulation and obtained the maximum concentration within the first two hours before coming to the elimination phase. Meanwhile, the pharmacokinetics profile of Legalon was slightly different, which was illustrated by pharmacokinetic parameters of silybin in Table 3. Accordingly, all parameters of S-SNEDDS were remarkably higher than those of reference product. Specifically, the mean area under the concentration-time curve after 24 h of S-SNEDDS and Legalon® was 161.87, and 21.30 ng h/ml, respectively. Consequently, the 90% confidence interval (CI_90%) of the ratio ($AUC_{S\text{-}SNEDDS}/AUC_{Legalon}$) transformed by the logarithm was from 398.83 to 2430.63%. The $CI\_90\%_{ln(AUC)\_Lower}$ and $CI\_90\%_{ln(AUC)\_Upper}$ were much higher than the bioequivalent requirement of FDA for CI

Table 3
Pharmacokinetics parameters of silybin after oral administration of Legalon® and S-SNEDDS in rabbit (Mean ± SE).

Formulation		Legalon® (n = 4)	S-SNEDDS (n = 5)
T_{max} (h)		0.40 ± 0.21	0.35 ± 0.06
Mean C_{max} (ng/ml)		2.23 ± 0.47	115.56 ± 32.53
$AUC_{0\text{-}24h}$ (ng.h/ml)	Mean $AUC_{0\text{-}24h}$ (ng.h/ml)	21.30 ± 8.57	161.87 ± 24.83
	CI_90_Lower (%)		398.83[a]
	CI_90_Upper (%)		2430.63[a]
Mean relative Bioavailability (%)		760 (%)	

[a] 90% Confidence Intervals of the ratio ($AUC_{S\text{-}SNEDDS}/AUC_{Legalon®}$ or $C_{max\_S\text{-}SNEDDS}/C_{max\_Legalon®}$) were transformed by the logarithm.

$(90\%)_{Lower\_Upper}$ (85–125%). Based on this calculation, it could be concluded that S-SNEDDS significantly improved the relative bioavailability of silybin compared to the branded product. Moreover, the combination of SNEDDS and a precipitation inhibitor in S-SNEDDS also markedly increased the maximum concentration of silybin compared to the reference product.

The remarkable enhancement of silybin bioavailability could be explained as follows. First, the emulsifying process of SNEDD in GI medium accelerated the formation of nanoemulsion, which was directly absorbed into the intestinal enterocyte before going to the blood or lymphatic circulation. Second, due to the supersaturated concentration of silymarin in these droplets, a part of silymarin was pushed out of the nanoemulsion droplets after the dilution step of SNEDDS and existed in the form of free silymarin. This free drug might be micellized by bile acid or directly absorbed into the blood circulation. Nevertheless, if the concentration of free drug in the GI tract were as high as supersaturation concentration, silymarin would precipitate into the metastable or stable crystal. The formulation design of conventional SNEDDS could not completely inhibit this precipitation. However, the appearance of a copolymer (Poloxamer 407) in S-SNEDDS played a dual role in the fate of free silymarin in GI medium. First, the formulation containing Poloxamer 407 rapidly supersaturated after dilution in the GI tract and underwent liquid-liquid phase separation (LLPS) to lead the formation of a reservoir containing the free drug-rich phase. The reservoir now acted as a central compartment with "spring and plateau" behavior which would have the ideal thermodynamic activity for the drug absorption into the system circulation (Indulkar et al., 2015; Park et al., 2015; Rubbens et al., 2016; Taylor and Zhang, 2016; Nie et al., 2017; Li and Taylor, 2018). The second role of Poloxamer 407 was known as an inhibitor of P-glycoprotein (P-gp), which has been identified as a drug efflux transporter on the intestinal epithelium, resulting in the very poor oral bioavailability of silymarin (Javed et al., 2011). Consequently, the employment of Poloxamer 407 might increase the permeation rate and therapeutic efficacy of the free drug by avoiding drug efflux transporters.

In short, the driving force for the high absorption process of silybin from S-SNEDDS was the integration of nanoemulsion, micelles containing SLM, a reservoir containing a huge amount of free unionized drug and the inhibition of drug efflux transporters. Meanwhile, the fact that the reference product, a sugar-coated tablet, was a conventional dosage form consisting of starch, calcium carbonate, calcium hydrogen phosphate, magnesium stearate might possess only one absorption mechanism: passive transport of a limited free unionized drug. Therefore, the relative bioavailability of S-SNEDDS was significantly higher than that of Legalon®. This partly indicated the advantage of S-SNEDDS over reference product in terms of the pharmacological effect of silybin.

4.4. Pharmacological study

The pharmacological study was conducted in CCl_4-induced mice to prove the therapeutic effect of a novel drug delivery system, S-SNEDDS, versus CCl_4-control and the commercial product. The relationship between the different doses (10, 25 and 50 mg/kg silybin) and the therapeutic effect as well as the relationship between dissolution test, pharmacokinetics, and pharmacological study were also withdrawn from the obtained results.

CCl_4, a potent hepatotoxin, was commonly used as hepatotoxicity model for the screening protective effects of natural products against hepatic dysfunctions (Bhakuni et al., 2016). This compound was metabolized via CYP2E1 in the liver to a highly reactive trichloromethyl free radical which could initiate a chain reaction of radical species production resulting in lipid peroxidation, hepatocellular oxidative stress, and necrosis as a consequence (Boll et al., 2001). Therefore, to investigate the hepatoprotective activity of S-SNEDDS, the present

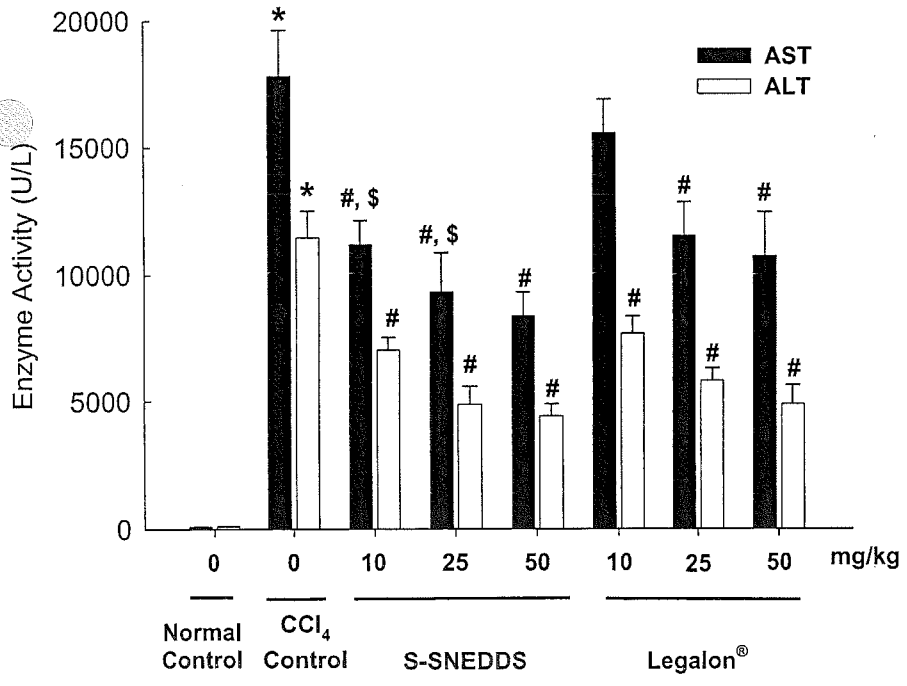


Fig. 8. Effect of S-SNEDDS and Legalon® on CCl_4-induced alterations in serum activity of aspartate aminotransferase. *Data were presented as mean ± SEM (n = 9–10 per group); $^{*}p < 0.05$ vs. normal control; $^{\#}p < 0.05$ vs. CCl_4 control; $^{\$}p < 0.05$ vs. group treated with reference product at respective dose level; one-way ANOVA followed by LSD test.*

study evaluated effects of this formulation on levels of serum transaminase, and MDA, GSH, and SOD level in liver tissue of CCl_4-treated mice in comparison with the commercial product.

Given that any hepatocellular damage would lead to increased leakage of AST and ALT into the bloodstream, serum levels of these enzymes has been considered the most sensitive markers of acute liver injury (Molander et al., 1957). As shown in Fig. 8, administration of CCl_4 resulted in an extremely significant rise in serum AST and ALT activity compared to normal control. However, a seven-day course of S-SNEDDS showed effectiveness in reducing the influences of CCl_4 on the enzyme levels in a dose-dependent manner in comparison to CCl_4-control. Specifically, enzyme levels fell by 52.9, 47,6 and 37,1% ($p < 0.001$) in AST and 61.4, 57.5 and 38.7% ($p < 0.001$) in ALT in groups treated by S-SNEDDS with doses of 50, 25 and 10 silybin mg/kg, respectively. These results suggested that S-SNEDDS could protect the structural integrity of hepatocytic cell membrane against the attack of the hepatoxic even with a low dose of 10 mg/kg silybin.

In addition to an elevation in serum aminotransferase levels, the CCl_4-induced liver injury was also characterized by lipid peroxidation and oxidative stress. MDA was one of the final products and commonly used as an indicator of polyunsaturated fatty acids peroxidation of hepatocyte membranes (Khan, 2006). On the contrary, reduced GSH and SOD in liver tissues played an essential role in the clearance of free radicals. GSH was considered one of the main scavengers of reactive oxygen species (ROS) mediated by its transformation to the disulphide derivative and reactions with electrophiles to generate deactivated conjugates. Meanwhile, superoxide dismutase (SOD) was a ubiquitous antioxidant enzyme that converts superoxide radical to H_2O_2, which in turn was eliminated by catalase (Mates et al., 1999; Tsukamoto et al., 2002). Therefore, overproduction of ROS and depletion in pools of these endogenous radical scavengers could contribute to oxidative stress and hepatocellular damage (Khan, 2006). In line with these theories as well as previous publications (Yang et al., 2013; Ahmed et al., 2014; El-Nahas et al., 2017), there were increased formation of TBARS (expressed as MDA equivalents) accompanied by decreased reserve of GSH and SOD in liver tissue following a single administration of CCl_4 (Fig. 9). It has also been demonstrated that S-SNEDDS inhibited generation of TBARS at all the three tested doses, as evidenced by a significant reduction of 66.3–77.5% in MDA levels in the liver homogenate compared with the CCl_4 control. Additionally, pretreatment with S-SNEDDS significantly increased antioxidant parameters including SOD at any dose between 10 and 50 mg/kg and GSH at doses of 25 and 50 mg/kg.

In short, the study provided a comprehensive comparison between the group treated by S-SNEDDS and the group of CCl_4-control in terms of hepatoprotective activity, which was not addressed in previous studies (Yang et al., 2013; Hwang du et al., 2014). A novel silymarin delivery system consisting of SNEDDS and a precipitation inhibitor was developed to effectively protect the structural integrity of hepatocytic cell membrane as well as increase antioxidant activity.

In an attempt to develop a novel product, the pharmacological effect of S-SNEDDS was parallel to that of the commercial product with three different doses (10, 25 and 50 mg/kg silybin). In this experimental design, the lowest dose of silybin was just 10 mg/kg, which was 5 times smaller than the normal dose of silybin in previous studies (50 mg/kg) (Yang et al., 2013; El-Nahas et al., 2017). If the lowest dose of silybin had been around 50 mg/kg, the hepatoprotective effect of both samples (S-SNEDDS and reference product) would always be positive because the absorption concentration of silybin into blood circulation would be higher than the minimum effective concentration (MEC). The advantages of S-SNEDDS over Legalon® might therefore not easily be realized.

Based on the above experimental design, several pharmacological results were obtained and presented in Figs. 8 and 9. In a head-to-head comparison between Legalon® and S-SNEDDS, the latter was superior in most parameters. The hepatoprotective effect of S-SNEDDS containing 10 mg/kg or 25 mg/kg of silybin was almost similar to that of the reference product containing 50 mg/kg of silybin. Specifically, AST (11190.88 ± 940.62 U/L), ALT (7028.30 ± 488.56 U/L), MDA (58.75 ± 18.63 nmoL/g tissue), SOD (237.13 ± 17.25 kU/g tissue) of S-SNEDDS containing 10 mg/kg silybin were mostly equal to those of Legalon® containing 50 mg/kg of silybin (AST = 10734.01 ± 1714.64 U/L, ALT = 5814.12 ± 765.57 U/L, MDA = 58.02 ± 14.19 nmoL/g tissue, SOD = 274.50 ± 7.13 kU/g tissue), respectively. Although the commercial product was also found to be effective in attenuating liver damage induced by CCl_4, its hepatoprotective effects were mainly observed in higher doses (50 mg/kg). With the exception of serum ALT, Legalon® with a dose equivalent to 10 mg/kg silybin did not effectively reverse biochemical changes in serum and liver homogenate. Taking into consideration the consistent dose-dependent effects of silymarin in both formulations, these findings suggested that S-SNEDDS formula improved the performance of silymarin, which could help reduce the therapeutically effective dose of this agent by at least two times compared to the commercial product. Overall, the benefits from S-SNEDDS included not only the greater improvement in liver function parameters but also the reduced minimum therapeutically effective dose (MTED) of silymarin.

In summary, an effective drug delivery system containing silymarin namely S-SNEDDS has been developed and has its advantages demonstrated by *in-vitro* and *in-vivo* tests. The relationship between the dissolution results and pharmacokinetics as well as pharmacological results were also discussed. Regarding the *in-vivo* studies, results from a hepatoprotective evaluation of S-SNEDDS indicated a certain correlation between the improvement of oral bioavailability and

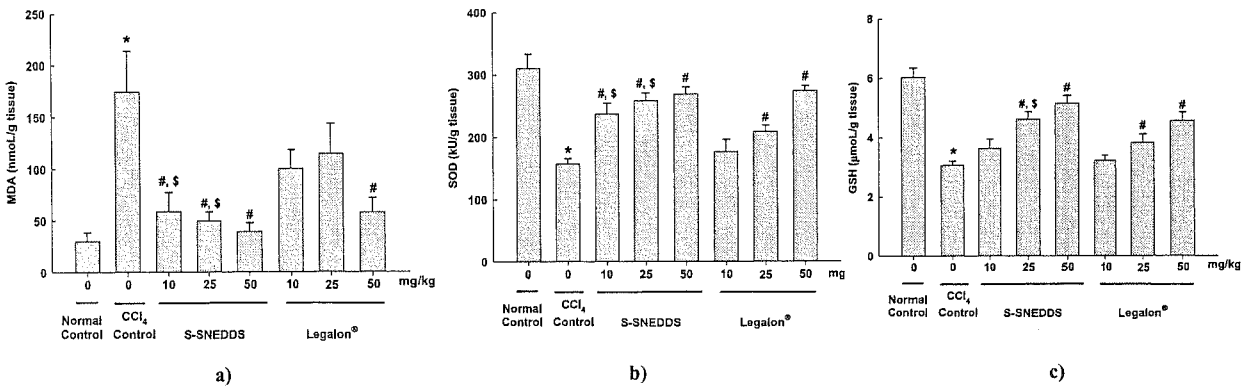


Fig. 9. Effect of S-SNEDDS and Legalon® on CCl_4-induced alterations in liver tissue homogenates (a–c). *Data were presented as mean ± SEM (n = 9–10 per group); $^{*}p < 0.05$ vs. normal control; $^{\#}p < 0.05$ vs. CCl_4 control; $^{\$}p < 0.05$ vs. group treated with reference product at the respective dose level; one-way ANOVA followed by LSD test.*

pharmacological efficacy. However, this correlation appears to be incomplete, considering the fact that the relative bioavailability of S-SNEDDS was 7.6 folds higher than that of Legalon®. This could be attributed to the non-linear relationship between doses of silymarin and its hepatoprotective responses as well as the substantial difference in composition of silymarin from various material sources mentioned earlier (Chambers et al., 2017). The novelty of this research was the comparative study with different doses of each formulation instead of just one dose level in previous studies. The study design could help resolve the problem related to non-linear response to silymarin and allow for estimates of equivalent doses of S-SNEDDS to the marketed product.

Besides, the relationship between pharmacokinetics and dissolution results was considered through the relative bioavailability and dissolution efficiency of silymarin from S-SNEDDS and Legalon®. The application of non-sink dissolution conditions proved the improved dissolution efficiency of S-SNEDDS compared to the reference product. The fact that DE_{4h} of S-SNEDDS using 10% Poloxamer 407 as a precipitation inhibitor was around 13 times higher than that of Legalon® illustrated positivity in oral absorption enhancement (Table 2). Under the non-sink dissolution conditions, silymarin might undergo the supersaturated state which was the driving force for the augmentation of passive absorption from the central aqueous compartment to the GI intestinal membranes (Taylor and Zhang, 2016; Price and Ditzinger, 2018). This scenario was confirmed by the heightening of oral bioavailability of silybin after administration of S-SNEDDS (7.6 folds) compared to Legalon®. The remarkable increase in DE_{4h} and oral bioavailability of silymarin resulted from the synergistic effect of both SNEDDS and precipitation inhibitor. The *in-vitro* tests to develop SNEDDS as well as to screen precipitation inhibitors were effective and helped to achieve a good correlation between dissolution results and pharmacokinetic results. This improvement of DE_{4h} and oral bioavailability of S-SNEDDS was once again demonstrated by hepatoprotective parameters in the pharmacological study. It is, therefore, possible to consider the proposed formulation a potential candidate for its capacity in decreasing the required dose of silymarin, which in turn could improve tolerance and minimize the potentially adverse effects.

5. Conclusion

An effective drug delivery system consisting of SNEDDS, polymeric precipitation inhibitor, and silymarin was developed. The impact of both SNEDDS and precipitation inhibitor on dissolution efficiency was evaluated to optimize the formulation of S-SNEDDS. The combination of SNEDDS and Poloxamer 407 in S-SNEDDS significantly increased the relative bioavailability of silybin (760%) and hepatoprotective parameters (ALT, AST, MDA, GSH, and SOD level in liver tissue) in CCl_4-induced mice in comparison with the commercial product.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research Article

LC-MS/MS Method for Rapid Quantification of Progesterone in Rabbit Plasma and Its Application in a Pharmacokinetic Study of the Transdermal Formulation

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A rapid and effective method using QuEChERS-based sample preparation procedure and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been developed and validated to determine progesterone in rabbit plasma. The analyte was extracted from plasma by acetonitrile with phase partitioning by a mixture of magnesium sulfate and sodium chloride. The supernatant was then directly injected into LC-MS/MS in a positive electrospray ionization mode and quantified using progesterone-d9 as the internal standard. The method linearity was in the range from 1 ng/mL (LOQ) to 200 ng/mL. Method recovery was from 86.0% to 103%, and repeatability was lower than 5.5%. The plasma sample was stable for 12 weeks stored at 18 ± 2°C. This method was applied to quantify progesterone in rabbit plasma in a pharmacokinetic study of two transdermal formulations: a reference drug and a eutectic-hydrogel system. The data indicate that the eutectic-hydrogel system's bioavailability was 1.5 times better than that of the reference drug, and the transdermal system is a potential drug delivery system for progesterone.

1. Introduction

Progesterone (Figure 1) is an endogenous steroid hormone secreted from the ovaries, testes, adrenal cortex, and placenta. It is the most critical hormone of progestins since this chemical has a crucial impact on the development of the uterus, fallopian tube, and breast [1]. Progesterone promotes cells to proliferate, enlarge, and become a secretary in nature [2]. The average serum progesterone level in adult women ranges from 0.15 to 25 ng/mL, but it can reach 150 ng/mL during pregnancy [3, 4]. This level in men and postmenopausal women was 0.38 ± 0.13 ng/ml and 0.38 ± 0.37 ng/mL, derived from a study of Winkelmann et al. [5]. Progesterone has been indicated to contraception, implantation, breast cancer, autoimmune, or progesterone deficiency diseases [6–9]. The transdermal formulations of progesterone have been developed for contraception, breast cancer, and artificial insemination support [7, 10, 11].

Some pharmacokinetic studies have evaluated the effectiveness of various formulations of progesterone [12–14], including the transdermal application [11]. Fraser et al., in an initial pharmacokinetic trial, evaluated the progesterone level in volunteers' blood after applying Nestorone®, a spray formulation of progesterone. The study indicated that progesterone's serum level to block ovulation was achievable, and it can provide effective contraception [11]. However, the evidence which proves the transdermal application of progesterone is inadequate [10]. There is a need to study the progesterone's pharmacokinetics in the transdermal gel to evaluate its efficacy and safety.

Many methods have been used for analyzing progesterone in plasma. A conventional method for progesterone analysis is radioimmunoassay (RIA). Abraham et al. developed an RIA method to determine progesterone in plasma: the sensitivity varied from 10 to 25 pg, and the recovery was 84.2 ± 4.8% [15]. Two enzyme immunoassay

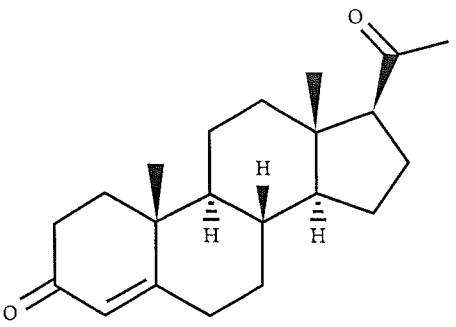

FIGURE 1: Chemical structure of progesterone.

(EIA) methods were developed using two different enzymes, horseradish peroxidase (HP) and alkaline phosphatase (AP), and were compared with RIA. The EIA-HP method's precision was comparable with the RIA method, and the detection limit was 10 times better than RIA. The EIA-AP method was not suitable to determine progesterone in the plasma because the value of this assay was three times higher than those measured by the other methods. Although RIA and EIA methods have their pros for high specificity and sensitivity, its cons are present in complexity in its cost and instruments [16].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has recently become a widely used method to determine progesterone in biological matrices. Tai et al. developed a liquid-liquid extraction followed by LC-MS/MS analysis to quantify progesterone in human serum. Progesterone and the internal standard (progesterone-^{13}C2) were duplicate extracted into *n*-hexane. The extract was then dried under nitrogen and reconstituted in methanol containing acetic acid before directly injected into LC-MS/MS. The method was successfully validated in the range from 0.151 to 24.42 ng/g [17]. Fernandes et al. applied a similar procedure in cattle plasma using medroxyprogesterone acetate as the internal standard and compared it to the RIA method. The LC-MS/MS method gave a higher progesterone concentration than the RIA method, explaining that the RIA method is affected by interferences in the matrix [18]. Zhang et al. used solid-phase extraction with Oasis HLB cartridge prior to LC-MS/MS analysis to determine 17α-hydroxyprogesterone caproate, 17α-hydroxyprogesterone, and progesterone in human plasma with medroxyprogesterone acetate as the internal standard. The linearity of progesterone was from 1 to 200 ng/mL [14]. Sasaki et al. employed salting-out assisted liquid-liquid extraction with LC-MS/MS for progesterone analysis to obtain easier and quicker sample preparation steps. With ammonium acetate as the salting-out agent, this method was applied to analyze progesterone in rat plasma from 0.05 to 20 ng/mL. The heavy matrix effect was controlled using the calibration curve on the surrogate matrix (water) [13].

Plasma analysis requires a method that is high throughput and appropriate for a limited amount of plasma. QuEChERS methodology, invented by Anastassiades and Lehotay for multiresidue analysis of pesticides [19], has been applied in extracting various pharmaceutical compounds in plasma matrices [20–23]. Intending to develop a method that is quick and uses less solvent as well as to evaluate the pharmacokinetic study of progesterone from the transdermal route, this paper presents a rapid QuEChERS-based method coupled with LC-MS/MS for the quantification of progesterone in rabbit plasma.

2. Materials and Methods

2.1. Standards, Reagents, and Materials. Progesterone analytical standard and isotope internal standard (progesterone-d9) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethanol, formic acid, ammonium chloride, anhydrous magnesium sulfate, and sodium chloride were obtained from Merck Vietnam (Hanoi, Vietnam). Ultrapure water was produced by a water filtration system (Milli-Q® Integral, Merck, Germany).

The progesterone and progesterone-d9 stock solutions, both of 100 μg/mL, were separately prepared in ethanol. The stock solutions were then diluted with acetonitrile to the concentrations of 1 μg/mL. The matrix-matched calibration curve was prepared in the blank extract with the progesterone concentration in the range of 1 to 200 ng/mL and progesterone-d9 concentration of 50 ng/mL.

The eutectic hydrogel of progesterone (EHP) was prepared in the Department of Pharmaceutics, Hanoi University of Pharmacy (obtained from another study). The reference drug formulation (RDF) was 1% progesterone gel (Besins Manufacturing, Belgium), which was purchased in the market.

2.2. Plasma Preparation. Rabbit plasma was brought to room temperature, and 500 μL was pipetted into 2 mL centrifuged tube followed by the addition of 25 μL internal standard solution of 1 μg/mL and 475 μL of acetonitrile. After being vigorously shaken by a vortex mixer, the tube received a mixture of salts and sorbents (described below), and it was mixed thoroughly for 1 min. The tube was then centrifuged at 13,000 rpm in 5 mins, and the supernatant was passed through a 0.2 μm PTFE filter and analyzed by LC-MS/MS.

The composition of salting-out and cleaning agents will be accessed by comparing the efficiency of different mixtures of salts and sorbents: (1) 200 mg of $MgSO_4$, (2) 150 mg of $MgSO_4$ and 50 mg of NaCl, (3) 150 mg of $MgSO_4$ and 50 mg of PSA, (4) 150 mg of $MgSO_4$ and 50 mg of C18, (5) 200 mg of CH_3COONH_4, and (6) 200 mg of NH_4Cl.

The blank sample was extracted via the abovementioned procedure without adding IS in the first step. The final extract was used to prepare working standard solutions at the concentration from 1 to 200 μg/mL (for progesterone) and 50 μg/mL (for progesterone-d9).

2.3. Instrumentation. An LC 1290 coupled with a 6460 QQQ mass spectrometer (Agilent, USA) was used to determine progesterone and progesterone-d9. The analytes were separated in Eclipse plus Agilent XD8 C18 column (150 × 2.1 mm, 3.5 μm particle size) with the mobile phase of

deionized water (A) and acetonitrile (B); both contained 0.1% of formic acid, at the flow rate of 0.5 mL/minute. The gradient program was initially set at 10% B in 2 minutes. After that, the eluent composition gradually increases to 90% B in 2.5 minutes and maintains 4 minutes before returning to 10% in 1 minute. The system was finally reequilibrated in 2 minutes before the next injection.

The electrospray ionization source was operated in the positive mode to select the precursor ion. The collision energies were optimized to obtain the most abundant product ions. Two transitions of each compound were observed, of which the product mass with a higher intensity was chosen for the quantitative purpose, and the other mass was used for the confirmation purpose.

2.4. Method Validation. The method was validated according to the guideline for the bioanalytical method validation of the US FDA [24]. The specificity was assessed by comparing the chromatograms of the blank sample, the standard solution of 50 μg/mL, and the blank sample spiked with progesterone and progesterone-d9 at the concentration of 50 μg/mL. The noise ratio (S/N) methodology was applied to estimate the limit of detection (LOD) and limit of quantification (LOQ). The progesterone levels in blank spiked samples having S/N ratios of 3 and 10 were LOD and LOQ, respectively. Repeatability and recovery were evaluated by analyzing 6 replicates of spiked samples of three concentration levels: low-quality control (LQC) at 1 μg/mL, medium-quality control (MQC) at 50 μg/mL, and high-quality control at 200 μg/mL.

Because of the low photochemical stability of progesterone, the long-term stability of the plasma sample was evaluated following the recommendation of the US FDA [24]. The blank rabbit plasma samples were spiked with progesterone at 2 concentrations of LQC and HQC. The first lot was analyzed in 6 replicates to determine the initial concentration. The remaining lots were stored at $-18^{\circ}C \pm 2^{\circ}C$ and were tested after 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks. The bias of the average concentration of each lot to the initial concentration should be within 15%.

2.5. Application in Pharmacokinetic Study. The animal study was approved by the Scientific and Ethics Committee, Hanoi University of Pharmacy. Male rabbits of about 2 kg each, purchased from the Centre of Experimental Animals, National Institute of Hygiene and Epidemiology (Hanoi, Vietnam), were selected for pharmacokinetic study. Male rabbits were chosen for pharmacokinetic experiments to avoid high fluctuating progesterone levels in female individuals. They were divided into two groups of three: one group for the reference drug and the other for the eutectic hydrogel system containing progesterone. Each gel (2.5 g) was applied to skin-free fur (5 cm × 10 cm) on the back of the rabbits. The blood samples (2 mL) were collected before applying the drug and at 4 hours, 6 hours, 7 hours, 9 hours, and 10 hours after the administration into an EDTA-coated tube. The tubes were centrifuged at 6000 rpm in 10 minutes, and the plasmas were collected and stored in 2 mL tubes at −10C before being injected into LC-MS/MS.

The Phoenix 8 software was used to calculate the pharmacokinetic parameters, including the maximum plasma concentration (C_{max}), the time until C_{max} is reached (T_{max}), and the area under the curve from time zero to ten hours (AUC0-10 h).

3. Results and Discussion

3.1. Optimization of Mass Spectrometry Condition. The ion transitions of progesterone and progesterone-d9 were obtained by directly infusing the standard solution of 1 μg/mL into the mass spectrometer. The precursor ions were obtained when the molecular ion combined with a proton. The optimal collision energies were selected for two product ions (Table 1). Other mass spectrometer parameters were selected to gain the highest intensities of the analytes. Under these conditions, progesterone and progesterone-d9 peaks, both with a retention time of around 4.9 min, were symmetric and sufficient to the analysis (Supplement Figure S1).

3.2. Selection of Salting-Out Agents. The average intensities of peak area in spiked samples ($n = 2$) and working standard solutions of the same concentration (50 μg/mL) were compared to select the most effective partitioning and cleaning mixtures. The results are introduced in Figure 2.

The use of CH_3COONH_4 and NH_4Cl gave the highest recovery of progesterone and progesterone-d9. However, the values of higher than 100% recovery indicate that the two layers are not completely separated: the amount of acetonitrile layer was less than the aqueous layer. The recovery when using $MgSO_4$ was low because some water might still be in the acetonitrile layer. Although the salting-out assisted liquid/liquid extraction (SALLE), described by Sasaki et al. [13], used CH_3COONH_4 as a salting-out agent, this study showed that using CH_3COONH_4 will result in incomplete separation between aqueous and organic phase.

The QuEChERS extraction consists of two steps: first, the compound is usually extracted into acetonitrile from the water phase with the help of salting-out agents, and second, the extract is cleaned up by dispersive solid-phase extraction. Since the amount of plasma is limited, the second step was omitted, and the sorbents were added into the first step to investigate the cleaning efficiency. However, neither PSA nor C18 sorbent helped increase the recovery of progesterone. The combination of $MgSO_4$ and NaCl gave the best extraction recovery of 105% and 91% for progesterone and progesterone-d9, respectively, and was chosen to be the salting-out agents in this extraction procedure.

3.3. Method Validation. The method specificity was accessed by comparing chromatograms of the blank sample, spiked sample at 50 ng/mL, and standard solution of 50 ng/mL (data shown in Supplements). There is no interference in the blank sample compared to the progesterone peak in the spiked sample and standard solution. Furthermore, the specificity was also supported by accessing the ion ratios of

Table 1: Multiple reaction monitoring conditions of progesterone and progesterone-d9.

Analytes	Precursor ion (m/z)	Product ion (m/z)	Collision energy, eV
Progesterone	315.2	109[a]	30
		97	26
Progesterone-d9	324.3	100[a]	30
		113	22

[a]Quantitative ion.

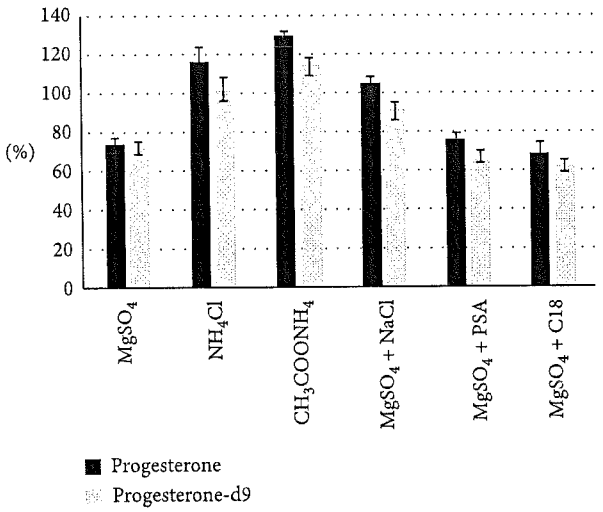

Figure 2: Progesterone and progesterone-d9 extraction recovery of different salting-out agents.

progesterone and progesterone-d9 (both about 100%) of the samples to those of the standards (Supplement Figure S1).

LOD and LOQ were determined by calculating the S/N ratio in low-concentration spiked sample analysis (Supplement Figure S2). The LOD and LOQ were at 0.3 and 1 ng/mL, respectively, which were low enough for determining progesterone concentration in rabbit plasma. The LOD of the method was not as low as that of Sasaki's study [13], but it is fit for the purpose of analyzing the level of progesterone in plasma, which is usually higher than 1 ng/mL. The method was linear from 1 to 200 ng/mL with the coefficient of determination (R^2) being higher than 0.99 (Figure 3).

The repeatability and recovery of progesterone at three concentrations ($n = 6$) are presented in Table 2. The method is precise and accurate, with the relative standard deviation lower than 5.5% and the recovery from 86.0% to 103%. This method was proven to meet the US FDA's requirements, had very high throughput (10 minutes to complete a set of 6 samples), and was environmental friendly (less than 0.5 mL of solvent for one extraction). These results indicate that the method can be a useful tool for pharmacokinetics studies of progesterone.

The stability of the plasma sample stored at 18°C ± 2°C within 12 weeks is introduced in Figure 4. Through 12 weeks, the difference in concentration of the analyte in the plasma and the original concentration sample had not exceeded 15% for both HQC and LQC levels. The RSD value between the quantitative concentrations of each QC batch at the time of analysis was less than 15%. These results demonstrate that the plasma sample is stable for at least 12 weeks with the proper storage condition.

3.4. Pharmacokinetic Study. The T_{max} of the reference drug and the eutectic-hydrogel system (Table 3) was similar (6.33 hours and 6.67 hours) following the drug's pharmacokinetics through the skin: it takes several hours to reach the peak in plasma. This is because the drug has to undergo dissolution and absorption through the skin followed by the distribution, metabolism, and elimination process. The C_{max} of the eutectic-hydrogel system was higher than that of the reference drug, but it was not statistically different ($p = 0.13$). Relative bioavailability data showed that the area under the curve from time zero to ten hours ($AUC_{0-10\,h}$) of the eutectic-hydrogel system was about 1.5 times higher than that of the reference drug (Table 3 and Figure 5).

The pharmacokinetics of progesterone on the rabbit has not yet been reported before. Compared with the study on volunteers of Fraser [11], the T_{max} of this study (6.33 to 6.67 hours) was lower than that of the single-dose application (20 hours) but higher than that of the multiple-dose treatment (4 hours) of a spray formulation. Because of the pharmacokinetic differences between species, the C_{max} value was incomparable.

This pharmacokinetic study may be affected by the limited number of rabbits used in this study. The standard

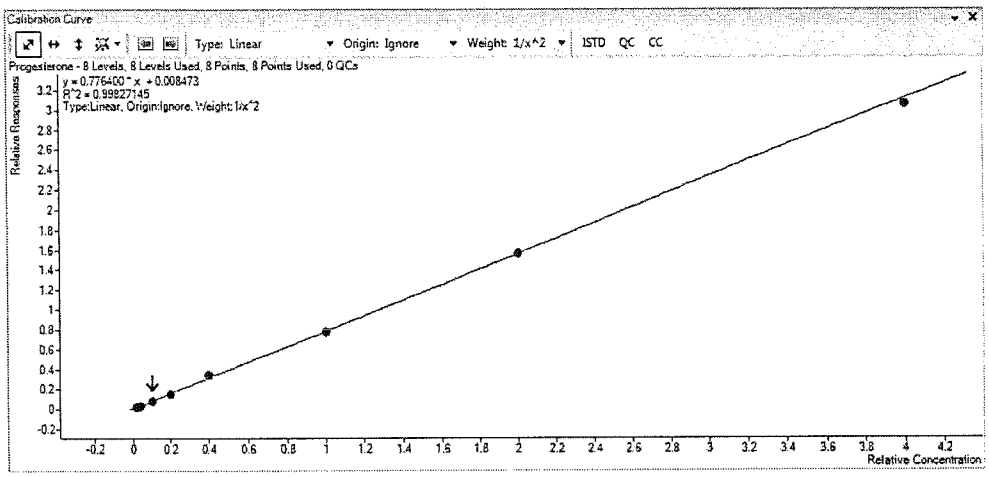

FIGURE 3: The calibration curve of progesterone on blank matrix.

TABLE 2: Repeatability and recovery of progesterone at different levels.

Spiked level (ng/mL)	RSD (%)	R (%)
1	5.5	86.0–100
50	1.7	98.6–103
200	2.8	97.3–102

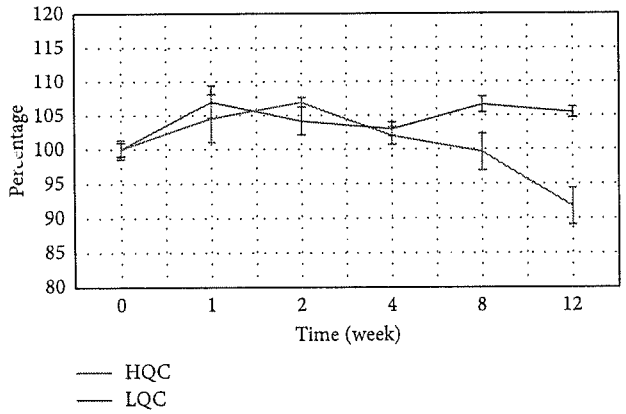

FIGURE 4: The stability of plasma sample spiked at two levels (LQC and HQC) within 12 weeks stored at −18°C ± 2°C.

TABLE 3: Pharmacokinetic parameters of progesterone on rabbit models.

Parameter	Eutectic-hydrogel system (±SD)	Reference drug formulation (±SD)
C_{max} (ng/mL)	11.1 ± 0.66	8.49 ± 3.32
T_{max} (h)	6.67 ± 0.58	6.33 ± 0.58
$AUC_{0-10\,h}$ (ng.h/mL)	69.0 ± 6.49	46.5 ± 21.9

deviations of some points in the pharmacokinetic curve of three cases were high (Figure 5), and it may change the actual values of pharmacokinetic parameters. Future studies may be needed with a larger number of objects and a longer time of sample collection. However, the trend of the curves is unaffectable, these results substantiate the transdermal gel of progesterone, and this formulation can be a potential route for future progesterone application.

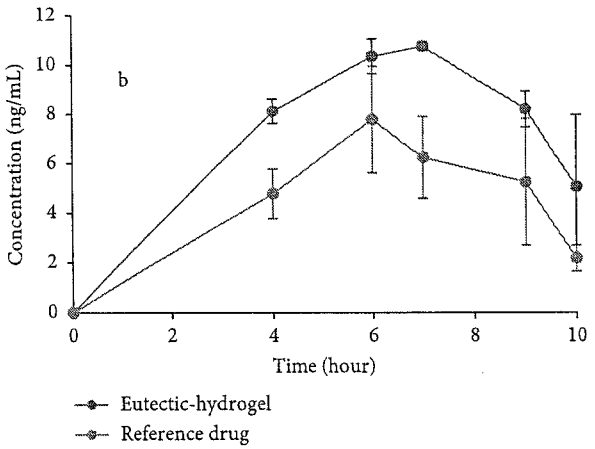

FIGURE 5: Progesterone content in rabbit plasma after applying the transdermal gels.

4. Conclusions

We have validated a rapid and effective QuEChERS-based method to determine progesterone in rabbit plasma using liquid chromatography-tandem mass spectrometry. The method uses less organic solvent than conventional liquid-liquid extraction or solid-phase extraction methods and has suitable sensitivity and accuracy to quantify the progesterone concentration in plasma. The progesterone level in plasma was stable within 12 weeks of evaluation. The pharmacokinetics study showed a similar pattern of the pharmacokinetics of two transdermal formulations, and the eutectic-hydrogel system is proven to be a potential application of progesterone.

Data Availability

The main part of the research data is included in the article. The chromatograms are included in the supplement file. Other data can be made available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary Materials

Figure S1: the chromatograms of standard solution of progesterone and progesterone-d9 (both of 50 ng/mL) with two ion transitions for each compound. Figure S2: chromatograms of progesterone in (a) working standard solution of 50 ng/mL, (b) blank sample spiked at 50 ng/mL, (c) blank sample, and (d) blank sample spiked at 1 ng/mL (LOQ). (*Supplementary Materials*)

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Short communication

Isolation and characterization of *N*-hydroxyethyl dithio-desethyl carbodenafil from a health supplement

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ABSTRACT

A new phosphodiesterase type-5 inhibitor (PDE-5i) with thiocarbonyl and thiolactam skeleton has been identified. The unknown compound has very similar properties like dithio-desmethyl carbodenafil, which was detected alongside during the screening process. It has been isolated by a semi-preparative high performance liquid chromatography tandem ultra-violet detector (HPLC-UV). The purified compound has been characterized using Fourier-transform infrared spectroscopy (FTIR), high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance spectroscopy (NMR). It is named as *N*-hydroxyethyl dithio-desethyl carbodenafil due to attachment of a hydroxyethyl group to the heterocyclic nitrogen of dithio-desethyl carbodenafil.

1. Introduction

New emerging PDE-5i found in health supplements have become a safety concern in public health with the first reported fatal case associated with intoxication of desmethyl carbodenafil [1]. Carbodenafil-bonded compounds have characteristic carbonyl or thiocarbonyl group in between alkoxyphenyl and piperazine ring [2–8]. These include dithio-desmethyl carbodenafil [2], desethyl carbodenafil [3], dithio-desethylcarbodenafil [4], dimethyl dithiodenafil [5], dimethyl thiocarbodenafil [5], dithiopropylcarbodenafil [6], 3,5-dimethylpiperazinyl dithio-desmethyl carbodenafil [7] and hydroxycarbodenafil [8]. Chemical modifications are commonly done on the piperazine ring, alongside the combination of carbonyl-lactam [3,8], thiocarbonyl-thiolactam [2,4–7] and thiocarbonyl-lactam [5], see Chart 1 .

Herein, we have identified a new dithio carbodenafil analogue alongside desmethyl dithio-carbodenafil from a health supplement. The compound has been revealed as *N*-hydroxyethyl dithio-desethyl carbodenafil due to a hydroxyethyl group bonded to the piperazine ring.

2. Experimental

2.1. Materials and chemicals

A health supplement was collected from a pharmaceutical store in Hanoi. The laboratory of National Institute for Food Control (NIFC), Vietnam screened for PDE-5i using an Orbitrap liquid chromatography high-resolution mass spectrometry (LC-HRMS) and detected dithio-desmethyl carbodenafil and an unknown analogue with molecular weight of 500. The sample was sent to Health Sciences Authority, Singapore for further investigation. It was presented in capsule form, containing brown powder.

Dithio-desemethyl carbodenafil was purchased from TLC Pharmachem (Aurora, ON, Canada). Formic acid, ammonia, sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), ethyl acetate (AR grade) were supplied by Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was supplied by Merck (Mumbai, India). Methanol (HPLC grade) was purchased from J. T. Baker (Gyeonggi, South Korea). Chloroform-d, $CDCl_3$ (D, 99.9 %) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). The PTFE membrane filters 0.45 μm were supplied by Fischer Scientific. The ultrapure water 18.2 mΩ was obtained from Milli-Q Integral 5 water purification system from EMD Millipore, Merck KGaA (Darmstadt, Germany).

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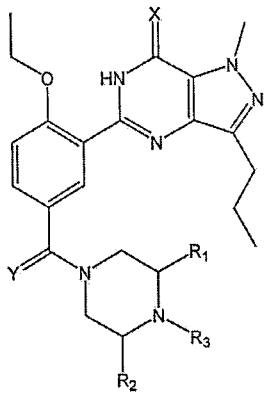

X = O, Y = O, R_1 = H, R_2 = H, R_3 = H; Desethyl carbodenafil
X = O, Y = O, R_1 = H, R_2 = H, R_3 = CH_2CH_2OH; Hydroxycarbodenafil
X = O, Y = S, R_1 = CH_3, R_2 = CH_3, R_3 = H; Dimethylthiocarbodenafil
X = S, Y = S, R_1 = H, R_2 = H, R_3 = H; Dithio-desethylcarbodenafil
X = S, Y = S, R_1 = H, R_2 = H, R_3 = CH_3; Dithio-desmethyl carbodenafil
X = S, Y = S, R_1 = H, R_2 = H, R_3 = $CH_2CH_2CH_3$; Dithiopropyl carbodenafil
X = S, Y = S, R_1 = CH_3, R_2 = CH_3, R_3 = H; Dimethyl dithiodenafil
X = S, Y = S, R_1 = CH_3, R_2 = CH_3, R_3 = CH_3; 3,5-dimethylpiperazinyl dithio-desmethyl carbodenafil

Chart 1. Carbodenafil-like analogues found in illegal health supplements.

2.2. Sample extraction for HPLC screening and compound isolation

For screening purpose, 0.5 g sample was dissolved in 5 mL of methanol and sonicated for 30 min. Sample solution was filtered using a 0.45 μm PTFE membrane filter. The screening analysis was performed using an Agilent 1260 series HPLC chromatograph with diode-array detector (Waldbronn, Germany). The column was a Thermo Hypersil BDS C18 (200 mm × 4.6 mm i.d., 5 μm). The mobile phases were 25 mM $NaH_2PO_4.H_2O$, pH 3.2 (A) and acetonitrile (B). The gradient profile was as follows: 0–30 min, 10–70 % B; 30–35 min, 70 % B; 35–40 min, 70–10 % B. The flow rate of the mobile phase was 1.0 mL/min. The injection volume was 15 μL. The UV spectra from 200 to 400 nm were recorded on-line during the chromatographic run. The chromatograms were recorded at a wavelength of 254 nm.

For compound isolation and purification, the content of 2 capsules was placed in a 50 mL polypropylene centrifuge tube and mixed with 6 mL of methanol and sonicated for 30 min. The sample solution was filtered and injected to an Agilent 1260 Infinity system with binary pump and an automatic fraction collector 1260 FC-AS (Waldbronn, Germany). The column used was an Agilent HPLC ZORBAX SB-C18 semi-preparative column (250 mm × 9.4 mm i.d., 5 μm). The mobile phases were (A) 0.1 % formic acid in ultrapure Milli-Q water and (B) 0.1 % formic acid in acetonitrile. The gradient elution profile was as follows: 0–7 min, 20–80 % B; 7–9 min, 80–90 % B; 9–12 min, 90 % B; 12–12.5 min, 20 % B; 12.1–15 min, 20 % B. The flow rate of the mobile phase was 4 mL/min and injection volume was 25 μL. The UV spectra from 200 to 400 nm were recorded online during the chromatographic run. The fraction of interest was collected based on the UV detection wavelength of 254 nm. These fractionated volumes were combined and evaporated using a rotary evaporator at bath temperature 40 °C. Then, the pH of the aqueous solution was adjusted to basic condition using 5 M ammonia. After that, 10 mL of ethyl acetate was added. The mixture was vortexed vigorously for 15 s. This was followed by centrifugation at 4000 rpm for 3 min. The organic layer was transferred into a glass tube. The extraction, centrifugation and organic layer transfer were repeated twice. The combined organic solvent was purged to dryness under nitrogen gas. After an overnight of freeze-drying, approximately 4 mg of yellow powder was yielded.

2.3. FTIR-ATR

The isolated compound was analyzed by a Thermo Nicolet 6700 FTIR spectrometer and a single bounce Smart DuraScope Attenuated Total Reflection (Madison, WI, USA). The data acquisition (4000–650 cm^{-1}) and processing were managed by the OMNIC 8 software.

2.4. Accurate mass analysis

Prior to analysis, the Thermo Fischer Scientific LTQ Orbitrap XL™ hybrid FTMS System (Bremen, Germany) controlled by the Xcalibur Version 2.0.7. System was calibrated using Pierce™ LTQ ESI positive ion calibration solution (Rockford, IL, USA) prior to use.

The purified compound and dithio-desmethyl carbodenafil was each dissolved in methanol and diluted with 0.1 % (v/v) formic acid in water: acetonitrile (1:4) for direct infusion analysis. The ESI ionization source was operated in the positive ion mode with spray voltage set at 3 kV, sheath gas flow rate at 6 arb, auxiliary gas flow rate at 0 arb, capillary voltage 40 V, capillary temperature at 275 °C and tube lens at 100 V. The mass resolution was fixed at 30000. The mass range was set from 100 to 500 Da for dithio-desmethyl carbodenafil, and 100–550 Da for the unknown compound. The high-resolution MS^2 spectra were acquired by direct infusion technique with a flow rate of 5 μL/min and under High energy Collision Dissolution (HCD) mode (normalize collision energy 30 for dithio-desmethyl carbodenafil and 31 for the unknown compound).

2.5. NMR analysis

The purified compound was dissolved in $CDCl_3$. All NMR spectra were recorded on a Bruker Ascend™ 500 spectrometer (Fällanden, Switzerland) at 298 K. Data were processed using TopSpin Software 4.0.7. All chemical shifts were reported in parts per million (ppm). The chemical shifts were reported in parts per million (ppm). The coupling constants (J) were given in hertz (Hz).

3. Results and discussion

3.1. HPLC-UV

The methanolic extracted sample solution showed two intense peaks at 22.1 and 22.9 min, respectively (Supplementary data Fig. S1). Both showed very similar UV profile with dithio-desmethyl carbodenafil from in-house database. The latter has been confirmed as dithio-desmethyl carbodenafil by HRMS analysis using the reference standard of dithio-desmethyl carbodenafil. Thus, this suggests that the peak at 22.1 min can be a structurally similar compound with higher hydrophilicity.

3.2. FTIR analysis

Like many dithio carbodenafil compounds [2,4–7], the strong C=S stretching have been observed at 1246, 1195 and 1080 cm^{-1} (Fig. 1). The presence of a secondary amine group has been indicated by a stretching absorption of at 3234 cm^{-1}. Additionally, a mild O–H stretch observed at 3378 cm^{-1}, suggesting the presence of a hydroxyl functional group [8]. The stretching for aromatic C=C bonds also observed for the 1,2,4-trisubstituted benzene at 1570, 1542 and 1495 cm^{-1}, which presented alongside the strong bending of C–H at 819 cm^{-1}. The peaks around 2924–2853 cm^{-1} represent the aliphatic C–H stretches.

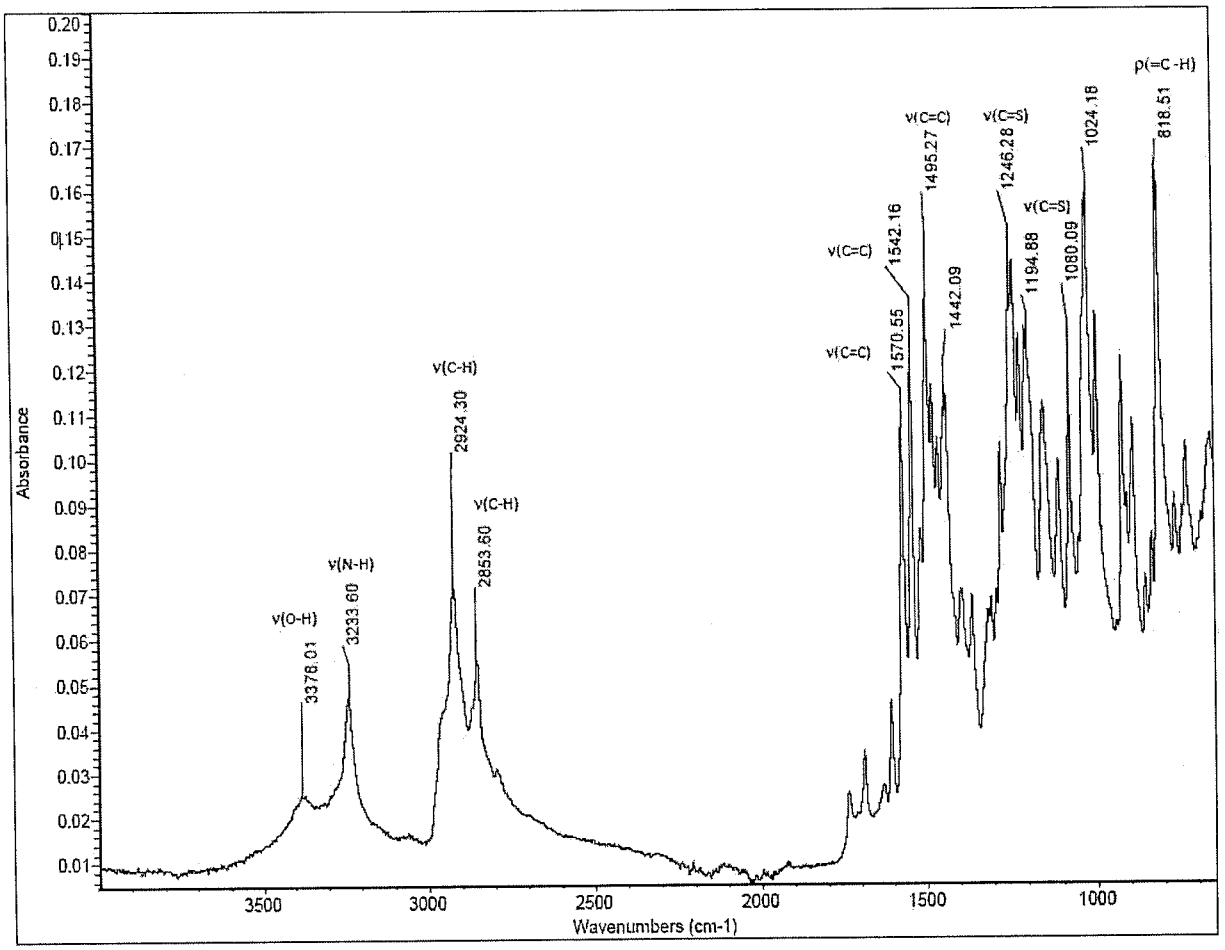

Fig. 1. FTIR spectrum of the unknown compound collected by ATR.

3.3. High-resolution MS^2 analysis

The MS^2 spectra of dithio-desmethyl carbodenafil and the unknown compound are displayed (Supplementary data Fig. S2). Both share four common fragment ions at *m/z* 440, 414, 371 and 343. The first twos can be associated with the partial cleavage of piperazine ring. Meanwhile, the fragment ions at *m/z* 371 and 343 can be due to the cleavage of piperazine ring and subsequently with a loss of an ethene from the ethoxyphenyl moiety, a common fragmentation pattern shown by many dithio carbodenafil compounds [2,5–7]. The proposed fragmentation pathways of the unknown compound are illustrated in Fig. 2.

Ironically, the unknown compound produced two fragments ion at *m/z* 483 and 467, which were not observed for dithio-desmethyl carbodenafil. The fragment ion at *m/z* 483 can be produced by a neutral loss of H_2O, indicating the presence of a hydroxyl group like hydroxycarbodenafil [8]. On the other hand, the fragment ion at *m/z* 467 is a good evidence of the direct intramolecular alkyl transfer, whereby alkyl migration occurs before the piperazine ring is lost [9]. As shown in Fig. 2, the electron-rich sulfur of thiolactam can form a bond with the hydroxyethyl group. It subsequently rearranges to form a five membered-ring ion before losing the thiol group. These fragments match well with theoretical values with mass errors within ±1 ppm, see Table 1.

Table 1
High-resolution MS^2 data of the unknown compound.

Formula molecule ion	Experimental	Theoretical	Mass error (ppm)
$C_{24}H_{33}O_2N_6S_2^+$	501.2101	501.2099	−0.332
$C_{24}H_{31}ON_6S_2^+$	483.1995	483.1993	−0.413
$C_{24}H_{31}O_2N_6S^+$	467.2224	467.2222	−0.436
$C_{22}H_{26}ON_5S_2^+$	440.1573	440.1573	−0.023
$C_{20}H_{24}ON_5S_2^+$	414.1417	414.1417	0.036
$C_{18}H_{19}ON_4S_2^+$	371.0995	371.0996	0.350
$C_{16}H_{15}ON_4S_2^+$	343.0682	343.0683	0.437

3.4. NMR analysis

The NMR data of the unknown compound and the reported dithiopropylcarbodenafil [6], an analogue with an *N*-propyl group bonded to piperazine ring are compiled in Table 2. The unknown compound shows very similar proton and carbon signals with dithiopropylcarbodenafil (Fig. 3), except for positions 29 and 30. More deshielded proton signals H-29 (2.69 ppm) and H-30 (3.71 ppm) and carbon signal C-30 (57.8 ppm) were observed as a result of the replacement of a methyl group at position 31 of dithiopropylcarbodenafil by a more electronegative hydroxyl group. This is also in a good agreement with the HRMS data. However, the proton signal derived from the hydroxyl group was not observed due to H/D exchange in solution.

In the COSY experiment, H-29 and H-30 showed the strong correlation amongst themselves. This has been supported by the correlation between H-29 and C-30 in the HMBC experiment (Fig. 4). In addition, the close proximity of H-29 and piperazinyl carbons C-25 and C-27 supports the bonding of an *N*-hydroxyethyl group to the piperazine ring [8]. Therefore, the unknown compound can be revealed as 5-[2-ethoxy-5-(4-hydroxyethylpiperazine-1-carbonothioyl)

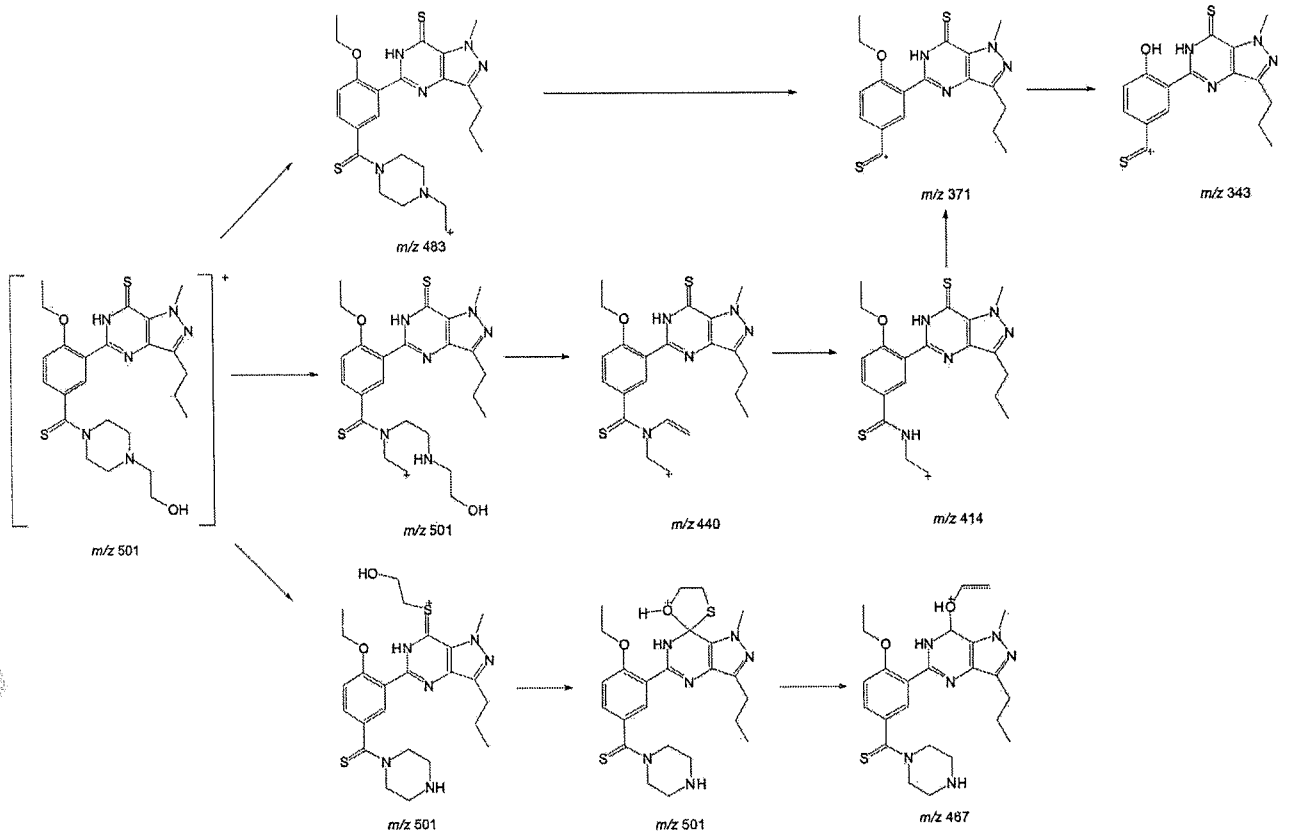

Fig. 2. Proposed fragmentation pathways of the unknown compound.

Table 2
NMR data of dithiopropylcarbodenafil and the unknown compound in $CDCl_3$ (δ in ppm, *J* in Hz).

	Dithiopropylcarbodenafil		*N*-hydroxyethyl dithio-desethyl carbodenafil			
Pos	δ_H	δ_C	δ_H	δ_C	COSY	HMBC
1	–	–	–	–	–	–
2	–	–	–	–	–	–
3	–	146.2	–	146.2	–	H-11/H-12
4	–	–	–	–	–	–
5	–	147.0	–	147.0	–	H-15/H-18
6	12.61 (1H, brs)	–	12.58 (1H, brs)	–	–	–
7	–	171.1	–	171.8	–	–
8	–	132.3	–	132.3	–	H-11
9	–	134.1	–	134.1	–	H-10
10	4.53 (3H, s)	39.4	4.53 (3H, s)	39.4	–	–
11	2.93 (2H, t, 7.5)	27.6	2.93 (2H, t, 7.45)	27.6	H-12	–
12	1.86 (2H, sextet, 7.5)	22.3	1.86 (2H, sextet, 7.45)	22.3	H-11/H-13	H-11/H-13
13	1.01 (3H, t, 7.4)	14.1	1.00 (3H, t, 7.35)	14.1	H-12	H-11/H-12
14	–	136.3	–	136.2	–	H-13/H-18
15	8.42 (1H, d, 2.3)	128.1	8.41 (1H, d, 2.3)	128.3	–	H-17
16	–	118.4	–	118.6	–	H-18
17	7.57 (1H, dd, 2.3, 8.6)	131.7	7.55 (1H, dd, 2.3, 8.6)	131.7	H-18	–
18	7.07 (1H, d, 8.6)	113.0	7.06 (1H, d, 8.6)	113.1	H-17	H-15
19	–	156.9	–	157.0	–	H-15/H-17/H-18/H-20
20	4.35 (2H, q, 7.0)	66.0	4.34 (2H, q, 7.0)	66.0	H-21	H-21
21	1.70 (3H, t, 7.0)	14.8	1.69 (3H, t, 7.0)	14.8	H-20	H-20
22	–	198.8	–	199.6	–	H-15/H-17
23	–	–	–	–	–	–
24	3.71 (2H, brs)	52.3	3.76 (2H, brs)	52.0	H-25	–
25	2.52 (2H, t, 4.7)	53.5	2.65 (2H, brs)	53.3	H-24	H-29
26	–	–	–	–	–	–
27	2.69 (2H, brs)	52.6	2.83 (2H, brs)	52.3	H-28	H-29
28	4.49 (2H, brs)	49.8	4.50 (2H, brs)	49.4	H-27	–
29	2.38 (2H, t, 7.5)	60.1	2.69 (2H, t, 5.05)	59.2	H-30	–
30	1.54 (2H, sextex, 7.5)	20.0	3.71 (2H, t, 5.2)	57.8	H-29	H-29
30'	–	–	[a]ND	–	–	–
31	0.93 (3H, t, 7.4)	11.8	–	–	–	–

[a] The absence of proton signal from —OH could be attributed to H/D exchange.

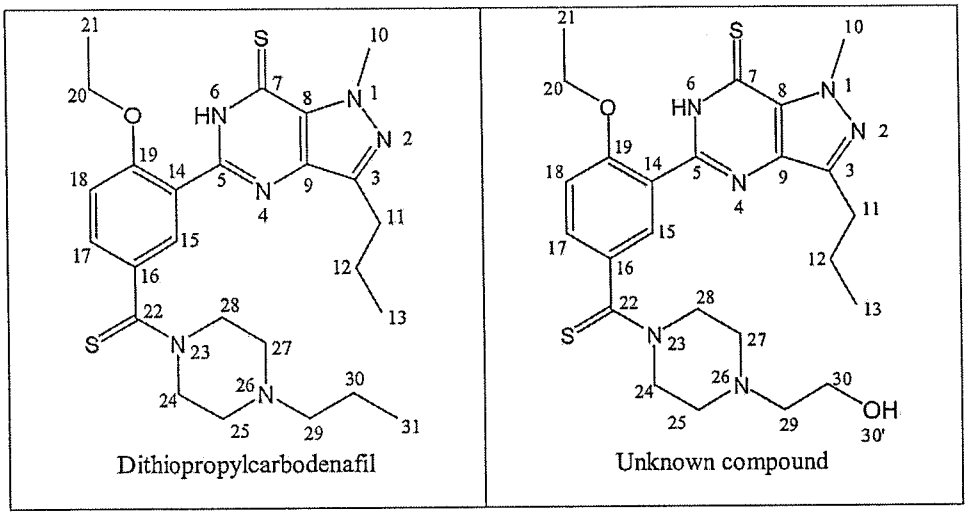

Fig. 3. Structures of dithiopropylcarbodenafil and the unknown compound.

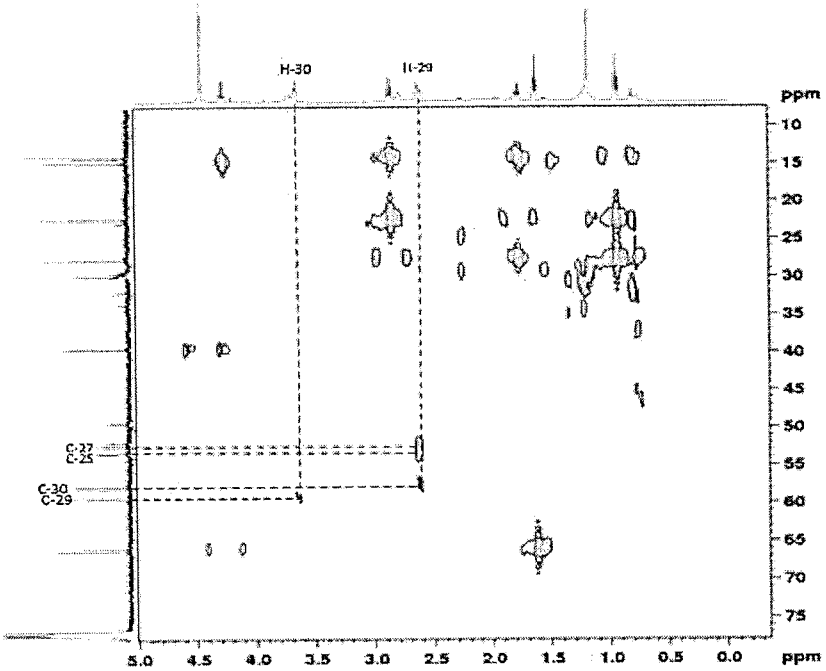

Fig. 4. HMBC of the unknown compound. Some correlations have been highlighted.

phenyl]-1-methyl-3-propyl-1,6-dihydro-7H-pyrazolo[4,3-*d*]pyrimidine-7-thione, or simplified as *N*-hydroxyethyl dithio-desethyl carbodenafil.

4. Conclusion

A new PDE-5i, *N*-hydroxyethyl dithio-desethyl carbodenafil has been identified from an illegal health supplement. The FTIR, HRMS and NMR results can be useful reference materials by laboratories and regulatory agencies in tackling the new emerging carbodenafil-like analogues.

Author statement

All authors have been acknowledged and agreed with the revision made.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113431.

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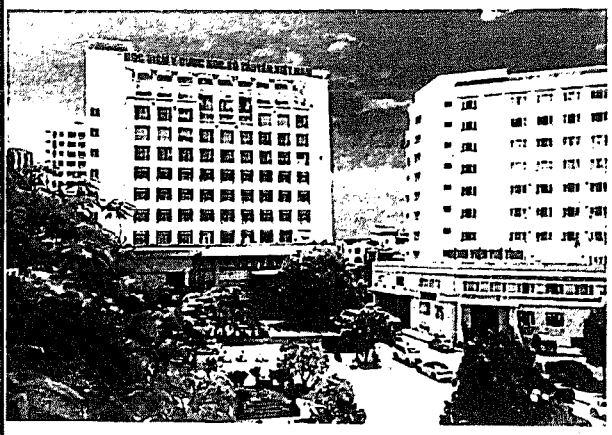

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GIẤY PHÉP XUẤT BẢN

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BÀI NGHIÊN CỨU

Nghiên cứu tác dụng kích ứng da và ảnh hưởng của tinh dầu húng quế (ocimum basilicum l.) Đến một số chức năng sống của thỏ thực nghiệm

RESEARCH ON EFFECTS OF BASIL OIL (*OCIMUM BASILICUM* L.) ON SKIN AND SOME VITAL FUNCTIONS OF THE EXPERIMENTAL RABBITS

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TÓM TẮT

Nghiên cứu được tiến hành vào tháng 5 năm 2021, tại Học viện Y Dược học cổ truyền Việt Nam.

***Mục tiêu:** Đánh giá tác dụng kích ứng da và ảnh hưởng của tinh dầu Húng quế (Ocimum basilicum* L. Lamiaceae*) đến một số chức năng sống của thỏ thí nghiệm.*

***Phương pháp:** Tiến hành theo hướng dẫn của Bộ Y tế và OECD.*

***Kết quả:** Tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu - ethanol 70° (7:3) không gây kích ứng da thỏ sau 24 giờ theo dõi liên tục (không gây mẩn đỏ, không phù nề, không gây viêm da), không làm thay đổi có ý nghĩa thống kê các chỉ số thân nhiệt, nhịp thở và nhịp tim của thỏ tại các thời điểm 1, 4, 6 và 24 giờ sau khi dùng chất thử so với trước khi dùng (các giá trị p > 0,05).*

***Từ khóa:** Húng quế, Ocimum basilicum, tinh dầu, thỏ, kích ứng da, thân nhiệt, nhịp tim, nhịp thở.*

ABSTRACT

This study was conducted in May 2021 at Vietnam University of Traditional Medicine and Pharmacy.

***Objective:** To test whether basil oil (Ocimum basilicum) causes skin irritation or affects some vital functions of experimental rabbits.*

***Methods:** Follow the guidelines of the Ministry of Health and OECD.*

***Results:** Pure basil oil and a mixture of basil oil and ethanol 70° (7:3) did not irritate rabbits' skin after 24 hours of continuous follow-up with no appearance of erythema, oedema, and dermatitis. Also, these chemical substances did not significantly change the rabbits' body temperature, breathing rates and heart rates at 1, 4, 6 and 24 hours after having the reagent administration compared to those before using (p values > 0.05).*

***Key words:** Basil, Ocimum basilicum, oil, rabbits, body temperature, heartbeats, breathing rates.*

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ĐẶT VẤN ĐỀ

Húng quế được coi là cây thuốc quý trong Y học cổ truyền của nhiều nước như Ấn Độ, Banglades, Kenya, ... Toàn bộ phần trên mặt đất của cây húng quế đều có thể dùng với các mục đích chữa bệnh khác nhau. Tinh dầu húng quế có tác dụng giảm bớt mệt mỏi về tinh thần, trị chứng cảm lạnh, co cơ, viêm mũi dị ứng, kháng khuẩn, kháng nấm, diệt côn trùng và được ưu tiên dùng để trị côn trùng đốt hoặc rắn cắn. [4], [7], [8].

Một vài nghiên cứu về tác dụng xua và diệt côn trùng của húng quế đã được tiến hành trên thế giới và trong nước. Nghiên cứu thực địa tại Kenya cho thấy, cây húng quế trồng trong chậu có thể xua 40% muỗi *Anopheles*; tinh dầu húng quế có tác dụng bảo vệ 100% khỏi muỗi *Aedes* trong vòng 6 giờ [8]. Kiplang'at K.P. và cộng sự (2013) đã công bố tinh dầu húng quế pha trong dầu khoáng tự nhiên với tỷ lệ 3% có tác dụng bảo vệ da thỏ khỏi 100% muỗi *Aedes aegypti* [5]. Nguyễn Thị Minh Thu và cộng sự (2021) đã công bố về tác dụng xua muỗi của dịch chiết và tinh dầu húng quế cho thấy tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu - ethanol 70^0 (7:3) có tác dụng xua trung bình lần lượt là 99,99% và 99,98% muỗi *Aedes aegypti*, 99,33% và 99,35% muỗi *Anopheles minimus*, 99,96% và 99,90% muỗi *Culex tritaeniorhynchus* [2], [3].

Do đó, để phát triển thuốc từ nguồn gốc húng quế sẵn có, dễ trồng tại Việt Nam và tạo ra các dạng chế phẩm từ tinh dầu có tác dụng phòng chống muỗi, ngăn ngừa bệnh do muỗi truyền, nghiên cứu này được tiến hành với mục tiêu đánh giá khả năng gây kích ứng da và ảnh hưởng của tinh dầu húng quế với một số chức năng sống ở động vật thí nghiệm.

VẬT LIỆU VÀ PHƯƠNG PHÁP

Thời gian và địa điểm nghiên cứu

Nghiên cứu được tiến hành vào tháng 5 năm 2021, tại Học viện Y Dược học cổ truyền Việt Nam.

Đối tượng và vật liệu nghiên cứu

Mẫu nghiên cứu

Phần trên mặt đất của cây húng quế còn tươi được thu hái tại Yên Xá, Tân Triều, Thanh Trì, Hà Nội vào tháng 5/2021.

Dược liệu được rửa sạch và cho vào bình chiết xuất bằng thép không gỉ, có chứa hơi nước ở bên trong. Thông qua một cửa vào, hơi nước được bơm qua dược liệu, cuốn theo các phân tử chất thơm và biến chúng thành hơi. Hơi này gặp lạnh ở phần bình chứa nước lạnh sẽ ngưng tụ và chảy vào bình ngưng. Khi đó, tinh dầu sẽ nổi trên mặt nước. Tinh chế tinh dầu húng quế bằng cách cất kéo hơi nước. Quá trình chưng sẽ kết thúc khi giọt chất lỏng cất ra không còn đục mà trở nên trong suốt.

Tiến hành tách tinh dầu thu được ra khỏi nước bằng cách làm nguội dung dịch, cho muối NaCl sạch vào hỗn hợp nước - tinh dầu đến bão hòa. Sau đó chuyển hỗn hợp vào phễu chiết, để yên cho tách lớp và chiết tinh dầu ra khỏi hỗn hợp. Cho tinh dầu vào bình tam giác 50 ml sạch có sinh hàn không khí, cho Na_2SO_4 khan vào bình và đun nóng nhẹ trên bếp cách thủy ở 60-70^0 C cho đến khi dịch trở nên trong suốt. Rót tinh dầu đã được làm khan qua phễu có giấy lọc. Tinh dầu này được dùng để thử nghiệm.

Động vật dùng trong nghiên cứu

Thỏ trưởng thành (*Oryctolagus cunniculus* L.), tổng số 12 con, trọng lượng trung bình 2,0 ± 0,2 kg, 02 tháng tuổi, khỏe mạnh, không phân biệt đực cái, do Trung tâm nghiên cứu dê và thỏ Sơn Tây cung cấp. Động vật cái không mang thai, không nuôi con bú và chưa sinh sản lần nào. Thỏ được nuôi ổn định trong điều kiện thí nghiệm 7 ngày trước khi tiến hành nghiên cứu.

Hóa chất, dụng cụ dùng trong nghiên cứu

- Nước cất 2 lần, cồn ethylic 70^0 dược dụng.

- Bình cất kéo tinh dầu, tông đơ điện, kéo phẫu thuật, cốc thủy tinh chia vạch, băng gạc, nhiệt kế thủy ngân, đồng hồ bấm giờ, ống nghe y tế Deluxe CK-SS601PF (Đài Loan).

Phương pháp tiến hành

Thử nghiệm tác dụng kích ứng da: Tiến hành theo hướng dẫn của Bộ Y tế và OECD [1], [6].

* Chuẩn bị động vật:

Trước thí nghiệm, làm sạch lông thỏ ở vùng hai bên sườn đều về hai bên cột sống một khoảng đủ rộng để đặt các mẫu thử và đối chứng (khoảng 10 × 15 cm). Chỉ những thỏ có da khoẻ mạnh, đồng đều và lành lặn mới được dùng vào thí nghiệm.

* Đặt mẫu thử:

Chuẩn bị 2 mẫu thử gồm: tinh dầu nguyên chất và tinh dầu pha trong ethanol 70° theo tỷ lệ 7:3. Mỗi mẫu được thử trên 06 thỏ. Mỗi thỏ đều có vùng hai bên sườn mỗi bên đặt 1 miếng gạc tẩm thuốc và 1 miếng gạc tẩm dung môi (tổng số 2 miếng gạc tẩm thuốc và 2 miếng gạc tẩm dung môi).

Lô 1 (n = 6): Đặt lên vùng bên sườn trái mỗi thỏ 1 miếng gạc tẩm tinh dầu nguyên chất và 1 miếng gạc tẩm nước cất, cách nhau 3 cm. Làm tương tự như vậy với vùng bên sườn phải. Như vậy, có 12 miếng gạc tẩm tinh dầu và 12 miếng gạc tẩm nước cất.

Lô 2 (n = 6): Đặt lên vùng bên sườn trái mỗi thỏ 1 miếng gạc tẩm tinh dầu pha với ethanol và 1 miếng gạc tẩm ethanol 70°, cách nhau 3 cm. Làm tương tự như vậy với vùng bên sườn phải. Như vậy, có 12 miếng gạc tẩm tinh dầu pha với ethanol và 12 miếng gạc tẩm ethanol 70°.

Liều chất thử hoặc dung môi trên mỗi miếng gạc là 0,5 ml. Tẩm mẫu thử hoặc dung môi lên miếng gạc không gây kích ứng 2,5 cm x 2,5 cm có độ dày thích hợp rồi đắp lên da. Cố định miếng gạc bằng băng dính không gây kích ứng trong 24 giờ. Sau đó bỏ gạc và băng dính, làm sạch tinh dầu còn lại trên da thỏ bằng cách rửa với nước ấm, rồi thấm khô bằng gạc.

* Quan sát và ghi điểm:

Quan sát và ghi điểm phản ứng trên chỗ da đặt chất thử so với da không đặt chất thử ở các thời điểm 1 giờ, 4 giờ và 6 giờ sau khi làm sạch mẫu thử. Đánh giá phản ứng trên da ở các mức độ gây ban đỏ, phù nề theo qui định ở bảng 1.

Bảng 1. Mức độ phản ứng trên da thỏ

Phản ứng	Điểm đánh giá
Sự tạo vảy và ban đỏ	
- Không ban đỏ	0
- Ban đỏ rất nhẹ (vừa đủ nhận thấy)	1
- Ban đỏ nhận thấy rõ	2
- Ban đỏ vừa phải đến nặng.	3
- Ban đỏ nghiêm trọng (đỏ tấy) đến tạo thành vảy để ngăn ngừa sự tiến triển của ban đỏ.	4
Gây phù nề	
- Không phù nề	0
- Phù nề rất nhẹ (vừa đủ nhận thấy)	1
- Phù nề nhận thấy rõ (viền phù nề phồng lên rõ)	2
- Phù nề vừa phải (da phồng lên khoảng 1mm)	3
- Phù nề nghiêm trọng (da phồng lên trên 1mm và có lan rộng ra vùng xung quanh)	4
Tổng số điểm kích ứng tối đa có thể.	8

Những thay đổi khác trên da sẽ được theo dõi và ghi chép đầy đủ.

* Đánh giá kết quả:

Trên mỗi thỏ, điểm phản ứng được tính bằng tổng số điểm ở hai mức độ ban đỏ và phù nề chia cho số lần quan sát. Điểm kích ứng của mẫu thử được lấy trung bình điểm phản ứng của các thỏ đã thử. Trong trường hợp có dùng mẫu đối chứng, điểm phản ứng của mẫu thử được trừ đi số điểm của mẫu đối chứng. Chỉ sử dụng các điểm tại thời gian quan sát ở 6 giờ để tính kết quả. Đối chiếu điểm kích ứng với các mức độ qui định ở bảng 2 để xác định khả năng gây kích ứng da thỏ của mẫu thử.

Bảng 2. Phân loại các phản ứng trên da thỏ

Loại phản ứng	Điểm trung bình
Kích ứng không đáng kể	0 - 0,5
Kích ứng nhẹ	> 0,5 - 2,0
Kích ứng vừa phải	> 2,0 - 5,0
Kích ứng nghiêm trọng	> 5,0 - 8,0

Theo dõi ảnh hưởng của tinh dầu húng quế đến nhịp thở, nhịp tim, nhiệt độ của thỏ khi thử nghiệm tác dụng kích ứng da

Đo thân nhiệt thỏ: Bắt lần lượt từng thỏ, một người giữ thỏ ở tư thế nằm sấp trên bàn, hai tay nắm da vùng gáy và mông (hoặc chân sau), người đo nhiệt độ một tay cầm đuôi, một tay cầm nhiệt kế loại nhỏ thấm ướt đầu thủy ngân rồi đặt vào lỗ hậu môn xoay nhẹ vào trực tràng sâu 2cm và đọc nhiệt độ sau một phút (hình 1)

Hình 1. Đo nhiệt độ ở hậu môn thỏ

Đếm nhịp thở: Để thỏ yên tĩnh, tư thế tự nhiên ở trong lồng chuồng, quan sát và đếm nhịp dao động thành bụng trong 1 phút.

Đếm nhịp tim: Bắt lần lượt từng thỏ, để thỏ nằm yên tĩnh trên bàn, dùng ống nghe đặt tại điểm 1/3 từ dưới lên của xương sườn thứ 2 - 4 từ bên trái. Đếm nhịp tim trong 1 phút.

Xử lý số liệu

Số liệu được phân tích bằng chương trình Excel 2016 theo phương pháp thống kê y học với cỡ mẫu nhỏ.

KẾT QUẢ NGHIÊN CỨU

Ở cả hai lô thỏ thử nghiệm, trong và sau thời gian theo dõi, vùng da đặt thuốc thử vẫn bình thường, không có dấu hiệu ban đỏ, không bị kích ứng và không phù nề. Vùng da đặt chất thử và vùng da chứng tương tự nhau (hình 2).

Hình 2. Da thỏ ở lô 1 sau đặt thuốc 24 giờ
1. Vùng da đặt tinh dầu nguyên chất; 2. Vùng da đặt nước cất.

Ảnh hưởng của tinh dầu húng quế đến một số chức năng sống của thỏ (gồm thân nhiệt, nhịp thở và nhịp tim) được đánh giá tại thời điểm trước thử nghiệm (TN) và tại 1, 4, 6 và 24 giờ sau khi đặt chất thử, thể hiện ở các bảng từ 3 - 5.

Bảng 3. Ảnh hưởng của tinh dầu húng quế đến nhiệt độ cơ thể thỏ

Lô	Thân nhiệt thỏ tại các thời điểm theo dõi (i) (°C, TB ± SD)*					p (i-trước TN)
	Trước TN	1 giờ	4 giờ	6 giờ	24 giờ	
(1), n = 6: dùng tinh dầu nguyên chất	39,25 ± 0,451	39,32 ± 0,194	39,28 ± 0,306	38,87± 0,301	39,30 ± 0,469	> 0,05
(2), n = 6: hỗn hợp tinh dầu-ethanol (7:3)	39,23 ± 0,356	39,08 ± 0,147	39,17 ± 0,455	39,27 ± 0,516	38,87 ± 0,26	> 0,05
p (1-2)	> 0,05	> 0,05	> 0,05	> 0,05	> 0,05	

* TB: Trung bình; SD: độ lệch chuẩn.

Nhiệt độ cơ thể của thỏ tại các thời điểm nghiên cứu thay đổi không có ý nghĩa thống kê so với trước khi dùng thuốc và không có sự khác biệt thống kê giữa lô dùng tinh dầu nguyên chất và lô dùng hỗn hợp tinh dầu - ethanol (7:3), các giá trị $p > 0,05$.

Bảng 4. Ảnh hưởng của tinh dầu húng quế đến nhịp thở của thỏ

Lô	Nhịp thở của thỏ tại các thời điểm theo dõi (lần/ phút, TB ± SD)*					p (i-trước TN)
	Trước TN	1 giờ	4 giờ	6 giờ	24 giờ	
(1), n = 6: dùng tinh dầu nguyên chất	87,7 ± 2,79	88,2 ± 2,64	88,0 ± 3,41	88,7 ± 1,97	88,5 ± 2,88	> 0,05
(2), n = 6: hỗn hợp tinh dầu-ethanol (7:3)	88,2 ± 4,07	86,0 ± 4,29	87,2 ± 4,88	86,8 ± 3,76	85,3 ± 3,98	> 0,05
p (1-2)	> 0,05	> 0,05	> 0,05	> 0,05	> 0,05	

Bảng 4 cho thấy, nhịp thở của thỏ tại các thời điểm nghiên cứu tăng, giảm không có ý nghĩa thống kê so với trước khi dùng thuốc ($p > 0,05$). Nhịp thở của thỏ ở nhóm 1 và nhóm 2 cũng khác nhau không có ý nghĩa thống kê tại cùng thời điểm nghiên cứu (các giá trị $p > 0,05$).

Bảng 5. Ảnh hưởng của tinh dầu húng quế đến nhịp tim của thỏ

Lô	Nhịp tim của thỏ tại các thời điểm theo dõi (lần/ phút, TB ± SD)*					p (i-trước TN)
	Trước TN	1 giờ	4 giờ	6 giờ	24 giờ	
(1), n = 6: dùng tinh dầu nguyên chất	140,5 ± 8,02	142,5 ± 7,48	138,5 ± 6,16	140,8 ± 3,43	142,0 ± 7,72	> 0,05
(2), n = 6: hỗn hợp tinh dầu-ethanol (7:3)	139,5 ± 9,71	143,2 ± 8,13	141,0 ± 10,37	142,7 ± 10,82	141,8 ± 6,46	> 0,05
p (1-2)	> 0,05	> 0,05	> 0,05	> 0,05	> 0,05	

Kết quả ở bảng 5 cho thấy, thỏ ở các lô có nhịp tim thay đổi không có ý nghĩa thống kê so với trước khi dùng thuốc và khác nhau không có ý nghĩa thống kê giữa hai nhóm dùng thuốc tại cùng thời điểm (các giá trị $p > 0,05$).

BÀN LUẬN

Thử tác dụng kích ứng da và đánh giá ảnh hưởng của chất thử đến nhịp thở, nhịp tim và thân nhiệt của động vật thực nghiệm là các phép thử nhằm đánh giá tác dụng không mong muốn của chất thử đó và góp phần chứng minh tính an toàn của thuốc. Phép thử đánh giá độc tính trên da thường được dùng để thử nghiệm các chất thử dự định dùng ngoài như dạng bôi ngoài da hay dạng xịt. Trong nghiên cứu này, do tinh dầu húng quế được thử nghiệm nhằm sử dụng với mục đích dùng ngoài để xua muỗi, nên các thí nghiệm đánh giá tính an toàn như trên là hợp lý.

Trong thử nghiệm đánh giá độc tính trên da thỏ, các thỏ được chọn đều khỏe mạnh, đồng lứa, vùng da được chọn không có tổn thương để đảm bảo hạn chế sai số trong nghiên cứu. Kết quả cho thấy, cả tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu - ethanol 70° (7:3) đều không gây kích ứng da thỏ sau khi cho da thỏ tiếp xúc với mẫu thử (0,5 ml/mẫu) tại các thời điểm 1, 4, 6 và 24 giờ. Không có hiện tượng da bị ban đỏ, phù nề hay viêm. Các vùng da đặt mẫu thử tương tự vùng da đặt mẫu chứng (hình 2). Điều đó chứng tỏ, tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu - ethanol 70° (7:3) đều không gây kích ứng da thỏ và mẫu thử có thể sử dụng để bôi hay xịt ngoài da.

Kết quả nghiên cứu trên cũng phù hợp với nghiên cứu của Kiplang'at K.P. và cộng sự (2013) khi thử tác dụng xua muỗi *Aedes aegypti* của tinh dầu húng quế pha trong dầu khoáng tự nhiên (Vaseline Pure Petroleum Jelly) với tỷ lệ 3% cũng không gây tổn thương da thỏ [5]. Sritabutra D. và cộng sự (2013) [9] khi nghiên cứu tác dụng xua *Aedes aegypti* (Linn.) và *Culex quinquefasciatus* (Say.) của hỗn hợp tinh dầu húng quế - dầu oliu (1:9) và tinh dầu húng quế - dầu dừa (1:9) trên lâm sàng cũng nhận thấy các mẫu thử trên không gây kích ứng da ở người thử nghiệm [9].

Song song với thử tác dụng kích ứng da thỏ, tại các thời điểm nghiên cứu, các thông số như nhiệt độ cơ thể, nhịp tim và nhịp thở của thỏ cũng được đánh giá để bổ sung vào hồ sơ về tính an toàn của chất thử khi dùng ngoài. Kết quả nghiên cứu cho thấy, nhiệt độ cơ thể thỏ dao động trong khoảng từ 38,87 ± 0,26 °C đến 39,32 ± 0,194 °C, thay đổi không có ý nghĩa thống kê giữa các thời điểm 1, 4, 6, và 24 giờ sau khi tiếp xúc với mẫu thử so với trước khi dùng, không có sự khác biệt giữa lô 1 và lô 2 tại cùng thời điểm tương ứng (các giá trị $p > 0,05$). Điều này chứng tỏ, tinh dầu húng quế không ảnh hưởng đến thân nhiệt thỏ. Tương tự, nhịp thở của thỏ (dao động trong khoảng 85,3 ± 3,98 và 87,7 ± 2,79 lần/phút) và nhịp tim thỏ (dao động trong khoảng 138,5 ± 6,16 và 142,0 ± 7,72 lần/phút) cũng thay đổi không có ý nghĩa thống kê giữa các thời điểm nghiên cứu so với trước khi tiếp xúc với mẫu thử và giữa các lô thử nghiệm ở cùng thời điểm (các giá trị $p > 0,05$). Kết quả này chứng tỏ, tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu-ethanol 70°C (7:3) không ảnh hưởng đến chức năng sống của thỏ, mẫu thử an toàn với động vật thử nghiệm khi tiếp xúc bằng cách dùng ngoài. Kết quả trên cũng phù hợp với thực tế sử dụng trong dân gian khi dùng tinh dầu để xua đuổi côn trùng và dùng để ăn (làm gia vị) hoặc để chữa bệnh. [4], [8]

Rất ít nghiên cứu trên thế giới tiến hành đánh giá tính an toàn của tinh dầu húng quế trên thực nghiệm và lâm sàng. Đây là nghiên cứu đầu tiên ở Việt Nam đánh giá độc tính trên da và ảnh hưởng của tinh dầu húng quế đến một số chức năng sống của động vật thí nghiệm.

KẾT LUẬN

Đã nghiên cứu độc tính cấp trên da và ảnh hưởng của tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu - ethanol 70° (7:3) trên thỏ thực nghiệm. Kết quả cho thấy: Hai mẫu thử trên không gây kích ứng da thỏ sau 24 giờ theo dõi liên tục, không làm thay đổi có ý nghĩa thống kê các chỉ số thân nhiệt, nhịp thở và nhịp tim của thỏ tại các thời điểm dùng thuốc so với trước khi dùng.

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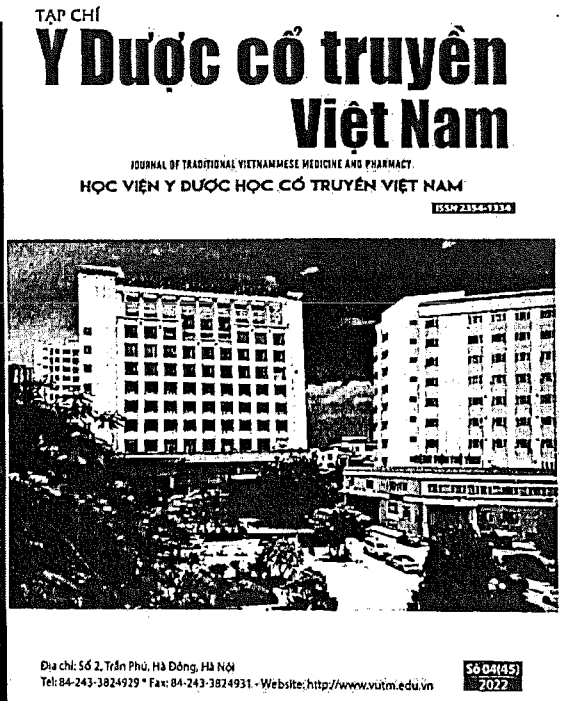

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BÀI NGHIÊN CỨU

Antimalarial drugs quality monitoring in some provinces of VietNam

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ABSTRACT

This study was conducted between January and August, 2016 in some malarial prevalence areas of Vietnam including Dak Nong and Gia Lai provinces. Total of 114 anti-malarial medicine samples (of which 55 available antimalarials and 59 selected antibiotics for malaria treatment) were collected from public and private health facilities.

All of the collected samples were screen analized by GPHF-minilab kits at National Institute of Malariology, Parasitology and Entomology (NIMPE) according to GPHF monographs for specifications of visual and physical inspections, disintergration tests and thin layer chromatography assays. None of them was suspicious as substandard quality drugs.

Fifteen randomly chosen samples (13.16% of total samples) were sent to National Institute of Drug Quality Control (NIDQC) for comfirmatory tests according to United States Pharmacopoeia or Vietnamese Pharmacopoeia criteria. The results showed that: three of them (03/114, 2.63%) were found as substandard drugs. All three artesunate 50 mg tablet samples (had the same lot 012013, expiry date in March, 2016, registration number VD-13186-10, collected from private pharmacies in Dak Nong and Gia Lai) did not pass purity tests.

All three substandard drug samples were antimalarials with proportion of 2.63% of total samples and 5.45% of altimalarials.

***Keywords:** Antimalarial medicines, drug monitoring, screen analizing, confirmatory tests, substandard drugs.*

1. BACKGROUND AND RATIONALE

In recent years, rate of substandard and fake drugs including antimalarials increased rapidly from year to year. Although many efforts of authority agencies were carried out to prevent poor quality medicines from their circulations, counterfeit and substandard drugs are still increasing. In Southeast Asia, an estimated of 10-35% of medicines are improperly made and illegally produced and sold. [9]

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According to the Promoting the quality of Medicines program (PQM), in 2004, proportion of fake artesuate with no active ingredient was found as 44% in countries of Mekong sub-region. In 2008, this rate of poor quality artesunate reduced and reached 11.2% [9]. Some studies on anti-infectious medicines quality in Vietnam in last few years found that rate of substandard and counterfeit antimalarials was between 1.3% to 2.4% and this rate increased annually. [6], [7], [8]

The goal of this study was to monitor and obtain evidence-based data on antimalarial drug quality in some malaria prevalence provinces in Vietnam including Dak Nong and Gia Lai.

2. METHODS

2.1. Sampling locations:

Two provincial sites of Vietnam were involved in this study including Dak Nong and Gia Lai. These sites have high malaria burden and border with *Plasmodium falciparum*'s artemisinin derivatives resistance area.

Samples were collected from various drug stores and pharmacies in urban, suburban, rural and remote areas of 2 sentinel sites belonging public and private sectors of supply and distribution systems.

2.2. Samples size:

In this survey, three criteria were taken into account in the determination of the sample size: The level of precision or sampling error of ± 4 percent, the confidence level of 95% of sample values and the degree of variability in the quality of antimalarial products distributed across the study sites in each country.

Adapted Yamane simplified formula was used:

Minimum sample size = $[Z^2 \times (p) \times (1-p)] / d^2$

Where: Z = critical value (e.g., 1.96 for 95% confidence level);

p = prevalence, expressed as decimal (failure rate) = between 4-5% per year;

d = confidence interval, expressed as decimal (e.g., 0.04).

A minimum of between 92 and 114 samples of antimalarials and selected antibiotics was suitable and enough for testing.

2.3. Radomization sampling:

Sample collection was conducted between January and February, 2016 by NIMPE staff. A simple randomization technique ensured against bias in selection of outlets for sample collection. From each sentinel site, two districts were chosen in which 2-3 communes or district hospitals and private drug stores provided randomly selected samples. All available antimalarials and selected antibiotics for malaria treatment (including clindamycin and doxycycline preparations) were picked randomly, labeled, kept (with sample collection forms), transported and stored as recommended by manufacturer to prevent deterioration, contamination and adulteration.

A minimum of 40 units per sample was collected for solid dosage forms (tablets, capsules) and 30 vials for injectables. If this minimum number of units is unavailable, no sample was collected from the selected site, and the next closest site was chosen to collect samples. Information was recorded in the sample collection form that the sample was collected from the next closest/available site as opposed to the initial site selected. No expired products was collected either, nor those with less than two (2) months shelf-life remaining until expiration date. These quantities of medicines ensured being enough for at least two assays according to GPHF monographs and one re-confirmatory test.

At the same sampling site, if there is more than one brand or manufacturer and lot or batch number of an anti-infective preparation, samples from different brands and batches were also taken.

2.4. Handling, packaging, labeling and transporting of samples

All operations related to sampling were performed with care. Samples were collected in the original container or box, if possible. The container used to store a sample should protect physical damage to the samples that may affect the physical/ visual inspection. Following quality assurance measures to ensure that samples were transported in plastic container/box provided and protected in a paper box or wrap to ensure their integrity.

2.5. Sample storage

Samples collected were packed, transported, and stored in such a way to prevent any deterioration, contamination, or adulteration and physical damages. The samples collected were stored in accordance with storage instructions of manufacturers. If not specified, then they were stored in sample cabinet with a temperature not exceeding 25°C. Where air-conditioning environment was not applicable, to achieve this temperature condition, wrapped the samples in aluminum foil (especially, artesunate/DHA products), placed them in plastic container, and put them in 2-3 paper boxes. Dated and initialed the plastic bag and plastic container when opening a sample container for analysis.

2.6. Quality drug testing:

Each sample was subject to testing at two levels. Testing level 1 was done by basic tests using Minilab techniques and procedures at the laboratory of NIMPE. All products included in this study have their Minilab testing methods. Testing Level 2 known as confirmatory analysis was conducted at National Institute of Drug Quality Control (NIDQC). Each sample was analysed using its respective pharmacopeial method and procedures. These include United States Pharmacopoeia, Vietnamese Pharmacopoeia, unless otherwise specified.

2.6.1. Corrected labeling and packaging according to the following requirements. At minimum the following information should be available on the label:

- Product name (brand or trade name, and INN or generic name)
- Dosage form and strength
- Number of tablet or capsules (quantity) per dispensing unit
- Manufacture date and expiry date
- Lot or batch number
- Name and address of manufacturer and/or distributor
- Registration number
- Storage condition instructions
- Administration instruction and package insert, if applicable.

2.6.2. Organoleptic (physical/visual) examination for contaminant, uniformity of shape, and other physical characteristics (color, mark, score line, etc.).

2.6.3. Identification of active pharmaceutical ingredients (APIs) [2], [3], [4], [5]; if passed, continue with Assay test.

2.6.4. Assay for content of APIs; if passed, continue with dissolution test.

2.6.5. Dissolution test for tablet and capsule forms.

For injectables, the following tests should be performed and same cut-off measures applied: proper packaging and labeling, organoleptic (physical/visual inspection) test, identification test and assay for content of APIs.

2.7. Data analysis, reporting and feeding back:

Collected data were handled by NIMPE staff and reports were fed back to 2 provincial sites and also were submitted to the Drug Administration of Vietnam.

3. RESULTS AND DISCUSSION

Total of 114 samples including available antimalarials and selected antibiotics for malaria treatment were collected from both public and private sectors in Dak Nong and Gia Lai provinces of Vietnam between January and February, 2016 (see tables 3.1 and 3.2).

Table 3.1: Number of collected samples from 2 sites in Vietnam in 2016

Serial No.	Provincial sites	Number of collected samples		Sub-total
		Antimalarials (at Public -Private sectors)	*Antibiotics (at Public - Private sectors)*	
1	Dak Nong	27 (18-09)	31 (10-21)	58
2	Gia Lai	28 (21-07)	28 (08-20)	56
	Total	**55 (39-16)**	**59 (18-41)**	**114**

Between 2 provincial sites, Dak Nong had the higher number of collected samples (58 samples, 50.88%) with no significance. The number of collected available antimalarial drugs (55 samples, 48.25%) was lower than that of selected antibiotics (59 samples, 51.75%), $P > 0.05$. These samples were collected from provincial malaria control centres, district medical centers, drug stores and private pharmacies as well. In which, 39 antimalarial samples were collected from public facilities (39/55, rated 70.91% of antimalarials - 34.21% of all samples), higher significantly than that collected from private pharmacies (16/51, rated 29.09% of antimalarials - 14.04% of all samples), $P < 0.05$. In contrast, antibiotics for malaria treatment were mainly purchased from private facilities (41 samples, 69.49% of antibiotic samples - 35.96% of all samples) higher significantly than that collected from public sectors (18/59 samples, 30.51% of of antibiotic samples - 15.79% of all samples), $P < 0.05$.

Especially, Artesunate 50 mg tablets were only purchased from private pharmacies and could not find at public health facilities because Artesunate 50mg tablet has not been used as monotherapy for malaria in Vietnam since at the end of 2009.

Table 3.2: Some information of collected samples

Serial No.	Trade names, generic names, concentrations, preparations	Number of collected samples at health facilities		Sub total
		Public sectors	*Private sectors*	
1	Chloroquine phosphate 250 mg, tablet	12	04	16
2	CV artecan (DHA-PIP 40-320 mg), film coated tablet	11	0	11
3	Primaquine diphosphate 13,2 mg, film coated tablet	08	0	08
4	Artesunate 60 mg, vial of powder for injection	02	0	02
5	Artesunate 50 mg, tablet	0	05	05
6	Quinine sulfate 250 mg, tablet	06	05	11
7	Agino Quinin (Quinine sulfate 50mg- paracetamol 200 mg), tablet	0	02	02
8	Doxycycline 100 mg, capsule	10	35	45
9	Clindamycin 150 mg or its trade names, capsule	0	04	04
10	Clindamycin 300 mg or its trade names, capsule	08	02	10
	Total	**57**	**57**	**114**

The antimalarial drug and antibiotic with the highest numbers of collected samples was chloroquine phosphate 250 mg tablets and doxycycline 500 mg capsules (16/114 samples, 14.04% and 45/114 samples, 39.47%, respectively).

All of collected antimalarial drugs were produced by internal pharmaceutical companies while antibiotic preparations (doxycycline or clindamycin) were manufactured by both internal and external pharmaceutical factories/companies with Vietnamese registration numbers.

Samples were collected from 12 public facilities (12/53, 22.64%) and 41 private sectors (41/53, 77.36%).

All of the collected samples were screen analyzed at the laboratory of NIMPE by GPHF minilab-kits. None of them were been suspicious as substandard quality drugs.

Fifteen randomly chosen samples passing screening tests (15/114, 13.16% of total samples) were sent to NIDQC for confirmatory tests according to the 34th United Sates Pharmacopoeia and the 4th Vietnamese Pharmacopoeia criteria.

The results showed that 03 (3/114, 2.63%) of them did not passed confirmatory tests and were considered as substandard samples. All three substandard samples were artesunate 50 mg tablets (had the same lot 012013, expiry date in March, 2016, registration number VD-13186-10, collected from private pharmacies in Dak Nong and Gia Lai) and did not pass purity tests. In contrast, all antibiotics for malaria passed GPHF-minilab and confirmatory tests.

In this study, the test result of Artesunate 50 mg tablet quality was similar to that of Artesunate tablet collected from a private pharmacy in Ha Tinh province in 2013. Rate of substandard samples in 2016 (3/114, 2.63%) was higher than that in 2011 (2/589, 0.34%), 2013 (10/421, 2.38%) and in period of 2003-2009 (47/3117, 1.51%) but lower than that in 2014 (4/107, 3.74). In which, rate of substandard antimalarial samples (3/55, 5.45%) was significantly higher than that in 2013 (6/273, 2.20%), 2011 (2/307, 0.65%) and in period of 2003 - 2009 (2/804, 0.25%), P values < 0.05 [6], [7], [8]. This is due to total numbers of samples collected in 2013, 2011 and period of 2003-2009 five to fifteen fold higher than that in 2016.

4. CONCLUSIONS

4.1. Total of 114 samples including 55 antimalarials (48.25%) and 59 antibiotics for malaria treatment (51.75%) were collected from Dak Nong and Gia Lai provinces and quality analysis in 2016.

4.2. Three antimalarial samples (3/114, 2.63%) were considered as substandard drugs and none antibiotics samples did not meet quality specifications.

4.3. Identified availability and sources of antimalarials in Dak Nong and Gia Lai in 2016.

Recommendations

1. Expending the study of monitoring antimalarial drugs quality in other provinces of Vietnam exept the old ones.

2. Strengthening comunication to community in order to reduce the circle of substandard and forbidden medicines in the markets.

3. More source investment for identifying counterfeit and substandard drugs in the fields.

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BÀI NGHIÊN CỨU

Nghiên cứu ảnh hưởng của dịch chiết húng quế *Ocimum basilicum* L. Lamiaceae đến chức năng thận thỏ

EFFECTS OF BASIL (*OCIMUM BASILICUM* L. LAMIACEAE) EXTRACT ON EXPERIMENTAL RABBITS' RENAL FUNCTIONS

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TÓM TẮT

Nghiên cứu được tiến hành từ tháng 5 - 7 năm 2021, tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và Trường Đại học Y Hà Nội.

Mục tiêu: *Thử ảnh hưởng của dịch chiết nước Húng quế (Ocimum basilicum* L. Lamiaceae*) đến chức năng thận của thỏ.*

Phương pháp: *Tiến hành theo hướng dẫn của Bộ Y tế và OECD về thử độc tính bán trường diễn. Dịch chiết nước húng quế được dùng bằng đường uống trên thỏ ở 2 mức liều 0,6 và 1,8 g/kg/ngày x 28 ngày liên tiếp. Thử nghiệm tiến hành song song với nhóm chứng. Thử nghiệm tiến hành song song với nhóm chứng. Lấy máu tĩnh mạch tai thỏ để xét nghiệm sinh hóa vào các ngày N0, N14 và N29. Mổ 50% số thỏ ở mỗi lô vào ngày N29 và mổ nốt thỏ ở ngày N43 để quan sát đại thể thận và lấy các mô thận, làm tiêu bản đánh giá ảnh hưởng của thuốc đến hình thái vi thể thận thỏ. Các chỉ tiêu đánh giá gồm: hàm lượng creatinin huyết thanh, những biến đổi bất thường của hình thái đại thể và vi thể thận thỏ (nếu có).*

Kết quả: *Hàm lượng creatinin ở các lô uống dịch chiết húng quế liều 0,6 và 1,8 g/kg/ngày × 28 ngày tại N14 và N29 thay đổi không có ý nghĩa thống kê so với N0 và so với lô chứng tại các thời điểm tương ứng với N0 (các giá trị p > 0,05). Hình thái đại thể thận thỏ của tất cả các lô thí nghiệm tại N29 và N43 đều bình thường, nhu mô mềm, mịn, không sung huyết. Cấu trúc vi thể: Ở các lô chứng và lô dùng thuốc, ống thận bình thường, tỷ lệ thỏ bị sung huyết nhẹ cầu thận tương ứng là 2/5, 2/5 và 3/5 ở ngày N29 và 3/6, 2/6 và 3/6 ở N43.*

Kết luận: *Dịch chiết nước húng quế không ảnh hưởng đến chức năng thận thỏ thí nghiệm.*

Từ khóa: *Húng quế, Ocimum basilicum L. Lamiaceae, dịch chiết nước, chức năng thận, creatinin, mô thận, cấu trúc vi thể, hình thái đại thể, sung huyết.*

Ngày nhận bài: 10/3/2022

Ngày phản biện: 14/3/2022

Ngày chấp nhận đăng: 29/3/2022

ABSTRACT

This study was carried out between May and July 2021 at National Institute of Malariology, Parasitology and Entomology, and Hanoi University of Medicine, Vietnam.

Objective: *To assess the effects of basil extract (Ocimum basilicum* L. Lamiaceae*) on experimental rabbits' renal functions.*

Methods: *The Vietnam Ministry of Health's and OECD's guidelines for sub-chronic toxicity testing were applied. The aqua basil extract was treated orally in two different groups with the dose regimens of 0.6 and 1.8 g/kg/day x 28 consecutive days, respectively. A control group treated orally with distilled water was also tested simultaneously. Two milliliters of blood were pulled out from each rabbit's ear vein on day 0 (before testing), day 14 (the middle of testing) and day 29 (after just stopping taking basil extract) in order to test for creatinine parameter. Less than half of rabbits from each group were operated on day 29 and the others were finished operations on day 43 (after 15 days taking no basil extract) for general kidney observations and microbody structures of renal cells.*

Results: *The concentrations of creatinine in rabbits' serum samples of the treated groups were insignificantly different from that of the control group on the same testing days; furthermore, there were unremarkable changes of this parameter between days 14 or 29 and day 0 (the p values > 0.05). Moreover, macroscopic kidney parenchyma of all rabbits on days 29 and 43 were in normal, soft, smooth, homogeneous, reddish-brown, and non-congestive textures.*

Microscopically, the rabbits of the control and treated groups had normal renal tubules. Nevertheless, some of them had mild glomerular congestions with the ratios of 2/5 (40.0%), 2/5 (40.0%) and 3/5 (60.0%) on day 29 and 3/6 (50.0%), 2/6 (33.33%) and 3/6 (50.0%) on day 43, respectively. In general, the aqua basil extract did not affect experimental rabbits' renal functions.

Key words: *Basil, Ocimum basilicum L. Lamiaceae, aqua basil extract, creainine, renal function, renal parenchyma, macroscopic, microscopic, congestion.*

ĐẶT VẤN ĐỀ

Mặc dù từ lâu đã nổi tiếng là cây thuốc quý trong y học cổ truyền của nhiều nước, nhưng các nghiên cứu về húng quế (*Ocimum basilicum* L. Lamiaceae) còn chưa nhiều. Ở một số nước, người dân dùng húng quế làm thuốc bổ và trị giun. Trà húng quế (uống lúc nóng) có tác dụng trị buồn nôn, đầy hơi, chứng kiết lỵ. Tinh dầu húng quế rất có hiệu quả làm giảm mệt mỏi về tinh thần, chữa cảm lạnh, viêm mũi, co thắt, và là thuốc ưu tiên dùng để trị ong đốt và rắn cắn [5]. Húng quế rất hiệu quả trong điều trị bệnh tim, bệnh về máu, bệnh bạch bì... Nước ép húng quế làm giảm chứng đau. Dịch chiết húng quế dùng để trị chứng đau đầu, đau do bệnh gout, cải thiện chức năng đường tiêu hóa, nhuận tràng nhẹ và làm nước súc miệng để chữa hơi thở hôi. Ngoài ra còn có tác dụng giảm đau khi sinh đẻ [5], [10]. Ở Việt Nam, húng quế được dùng chủ yếu làm gia vị cho các món ăn (lá và ngọn) hoặc để uống cho mát (hạt é) [1]. Một số công ty hiện sản xuất tinh dầu húng quế để chữa bệnh.

Một số nghiên cứu về húng quế trên thế giới cho thấy, nói chung, húng quế được dung nạp tốt, hầu như rất ít tác dụng không mong muốn khi sử dụng liên tục tới 13 tuần. Sử dụng quá liều húng quế hoặc lạm dụng trong thời gian dài có thể gây khó thở, thở gấp, ho hoặc tiểu ra máu; hạ đường huyết, làm loãng máu hoặc chậm quá trình đông máu; ung thư niêm mạc gan; có thể làm co thắt cổ tử cung với phụ nữ mang thai, gây biến chứng trong

khi sinh [5] [10]. Tuy nhiên, hiện nay, chưa có công trình nghiên cứu nào công bố về ảnh hưởng của dịch chiết húng quế đến mô bệnh học của thận. Đặc biệt, các nghiên cứu về tính an toàn của húng quế ở Việt Nam hầu như chưa có.

Vì vậy, để phát triển thuốc có nguồn gốc dược liệu, nghiên cứu này được tiến hành nhằm đánh giá ảnh hưởng của dịch chiết húng quế tới chức năng thận thỏ thí nghiệm với liều bán trường diễn 28 ngày.

ĐỐI TƯỢNG, PHƯƠNG TIỆN VÀ PHƯƠNG PHÁP NGHIÊN CỨU

Thời gian và địa điểm nghiên cứu

Nghiên cứu được tiến hành từ tháng 5 - 7 năm 2021, tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và Trường Đại học Y Hà Nội.

Đối tượng và phương tiện nghiên cứu

Mẫu nghiên cứu

Phần trên mặt đất của cây húng quế còn tươi được thu hái từ tháng 5/2021 tại Yên Xá, Tân Triều, Thanh Trì, Hà Nội. Mẫu sau thu hái được rửa sạch, thái nhỏ và được chiết nóng với nước như sau. Lấy 3 kg húng quế, thêm 5 lít nước, đun nhỏ lửa trong 30 phút. Sau đó lọc lấy dịch chiết. Thêm tiếp 2 lít nước vào và đun nhỏ lửa tiếp trong 30 phút nữa. Sau đó lọc lấy dịch chiết lần 2. Gộp dịch chiết lần 1 và lần 2 lại, cô cách thủy cho tới khi được dịch chiết tỷ lệ 3: 1 (300 g/100 ml). Từ dịch chiết gốc này, pha loãng để được các dịch chiết với các nồng độ khác nhau dùng trong thử nghiệm.

Động vật dùng trong nghiên cứu

Thỏ trưởng thành (*Oryctolagus cuniculus* L.), 2,1± 0,2 kg, 2 tháng tuổi, khỏe mạnh, không phân biệt đực - cái, do Trung tâm nghiên cứu Dê và Thỏ Sơn Tây cung cấp. Thỏ cái không mang thai, không nuôi con bú và chưa sinh sản lần nào. Động vật được nuôi 7 ngày trong điều kiện trong điều kiện thí nghiệm trước khi tiến hành nghiên cứu.

Hóa chất dùng trong nghiên cứu

Nước cất hai lần, các dung dịch xét nghiệm sinh hóa máu dành cho máy bán tự động của Boehringer (Đức), ethanol 950, ethanol tuyệt đối, xylen, formol, dung dịch hematoxylin, eosin 1%, thuốc thử Schiff (acid periodic 1%), baume Canada, paraffin.

Thiết bị và dụng cụ dùng trong nghiên cứu

- Máy cất nước hai lần Aquatron (hãng Bibby sterilin, Anh).
- Cân Sauter, độ chính xác d = 0,1mg.
- Máy Photometer 5010 của hãng Boehringer-Mannheim (Đức) để xét nghiệm sinh hóa.
- Máy chuyển tự động STP 120 (hãng Microm, Đức), máy đúc khối nến AP 280-1 (Microm – Đức), máy cắt tiêu bản vi thể (microtome – Đức), bàn hơ tiêu bản Prolabo (hot plate - Pháp), lưỡi dao cắt vi thể 1 lần S35 (Nhật), lam kính, lamen, kính hiển vi Nihon (Nhật), bộ bể nhuộm (Nhật): để nghiên cứu hình thái vi thể thận thỏ.
- Kim đầu tù cho động vật uống.
- Cốc thủy tinh có chia vạch, bơm kim tiêm 5 mL.
- Kéo phẫu thuật, pank, dao mổ.

Phương pháp tiến hành

Tiến hành theo hướng dẫn của Bộ Y tế và OECD [2], [8]. Sau khi nuôi ổn định 1 tuần, các thỏ được đánh dấu, cân trọng lượng và chia ngẫu nhiên vào 3 lô:

- Lô 1 (n = 11): chứng, uống nước cất 2 lần, thể tích tương đương liều điều trị.
- Lô 2 (n = 11): uống dịch chiết nước húng quế liều 0,6 g/kg/ngày × 28 ngày (tương đương liều dự kiến trên người).
- Lô 3 (n = 12): dịch chiết nước húng quế liều 1,8 g/kg/ngày × 28 ngày (gấp 3 lần liều dự kiến trên người).

Trước uống thuốc, thỏ được được lấy máu tĩnh mạch tai để làm xét nghiệm creatinin huyết thanh

vào ngày N0. Cho thỏ uống thuốc mỗi ngày một lần vào buổi sáng bằng kim đầu tù, liên tục trong 28 ngày. Các ngày lấy máu tĩnh mạch tai để làm xét nghiệm creatinin huyết thanh tiếp theo là N14 (sau khi uống thuốc 2 giờ) và N29.

Sau 28 ngày cho uống thuốc liên tục, vào ngày N29, mổ 50% số thỏ ở lô chứng và các lô uống thuốc, lấy các mô thận để làm tiêu bản đánh giá ảnh hưởng của thuốc. Số thỏ còn lại được nuôi bình thường, sau 15 ngày ngừng thuốc, vào ngày N43, mổ hết để đánh giá sự hồi phục của tổ chức thận. Các mẫu tiêu bản thận ở hai đợt được xử lý như nhau [8].

Phương pháp xác định các chỉ số sinh hóa máu: Hàm lượng creatinin trong huyết thanh thỏ được xác định theo kỹ thuật thường quy.

Phương pháp nghiên cứu mô bệnh học: Sau khi giết động vật thí nghiệm, quan sát tổng thể các mẫu thận một cách sơ bộ, rồi lấy đại diện 5 mẫu thận ở mỗi thỏ tại các vị trí khác nhau. Các mẫu bệnh phẩm được cắt mỏng từ 3-5 mm, cố định trong dung dịch formol 10% trong 48 giờ. Sau đó, khử nước, đúc parafin (khối nến), và cắt mảng 3µm. Nhuộm hematoxylin eosin (HE) [6].

Các chỉ số nghiên cứu

Chỉ số sinh hóa: Hàm lượng creatinine huyết thanh (µmol/L) tại ngày N0, N14 và N29.

Chỉ số mô bệnh học thận: Những biến đổi bất thường của hình thái vi thể thận thỏ (nếu có) của lô uống thuốc so với lô chứng tại N29 và sự hồi phục sau khi ngừng thuốc tại N43.

Xử lý số liệu

- Số liệu được biểu thị bằng trị số trung bình ± độ lệch chuẩn (M±SD).

- Các số liệu nghiên cứu được xử lý bằng chương trình Excel 2016 theo phương pháp thống kê y học với cỡ mẫu nhỏ (< 30), sử dụng t-test Student và Fisher's exact test để so sánh các số liệu trước, trong và sau thử nghiệm và so sánh giữa lô dùng thuốc và lô chứng.

- Trong so sánh, nếu p > 0,05 là khác biệt không có ý nghĩa thống kê, p < 0,05 là khác biệt có ý nghĩa thống kê và p càng nhỏ thì khác biệt có ý nghĩa thống kê càng cao.

KẾT QUẢ NGHIÊN CỨU

Đánh giá ảnh hưởng của dịch chiết húng quế đến chức năng thận thông qua định lượng hàm lượng creatinin huyết thanh và nghiên cứu sự biến đổi hình thái vi thể mô thận trước và sau khi dùng thuốc. Kết quả hàm lượng creatinin được thể hiện ở bảng 1.

Bảng 1. Hàm lượng creatinin (µmol/L) của thỏ ở các lô nghiên cứu

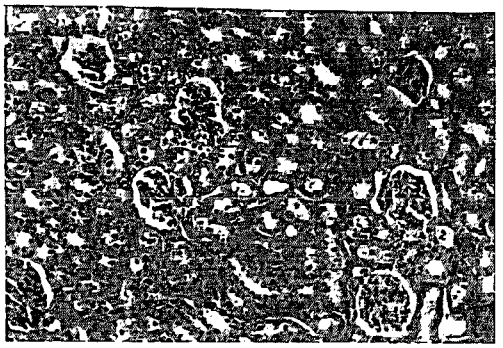
Lô	N0	N14	N29	p (N0-N14)	p (N0-N29)
Lô 1: chứng (n = 11)	83,71 ± 10,91	88,41 ± 8,07	89,60 ± 9,11	> 0,05	> 0,05
Lô 2: uống dịch chiết húng quế, 0,6 g/kg/ngày × 28 ngày (n = 11)	88,30 ± 9,97	96,22 ± 13,45	94,76 ± 13,54	> 0,05	> 0,05
Lô 3: uống dịch chiết húng quế, 1,8 g/kg/ngày × 28 ngày (n = 11)	92,73 ± 10,25	95,41 ± 11,50	95,45 ± 9,94	> 0,05	> 0,05
p (1-2), p (1-3), p (2-3)	> 0,05	> 0,05	> 0,05		

Hàm lượng creatinin ở các lô uống dịch chiết húng quế liều 0,6 và 1,8 g/kg/ngày × 28 ngày tại N14 và N29 thay đổi không có ý nghĩa thống kê so với N0 và so với lô chứng tại các thời điểm tương ứng (các giá trị p > 0,05).

Mô bệnh học thận thỏ

Quan sát đại thể

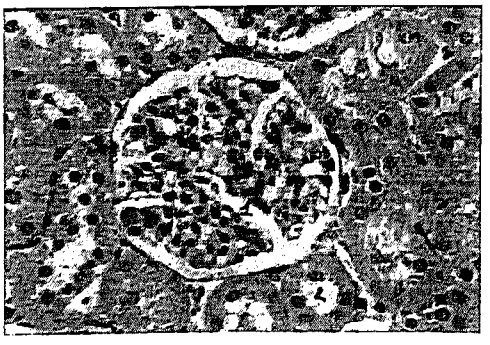
Thỏ ở cả ba lô đều có nhu mô thận mềm, mịn, soi bằng kính lúp thấy những hạt nhỏ li ti đều đặn (cầu thận), không sung huyết, không bất thường.



Hình 1. Thận thỏ lô chứng (HE × 100) ngày N29
1. Cầu thận: sung huyết nhẹ; 2. Ống thận: bình thường

Cấu trúc vi thể thận thỏ lô uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày ở N29:

3/5 thỏ sung huyết nhẹ cầu thận (tĩnh mạch trung tâm và xoang mạch sung huyết nhẹ), ống thận không có tổn thương (hình 3).



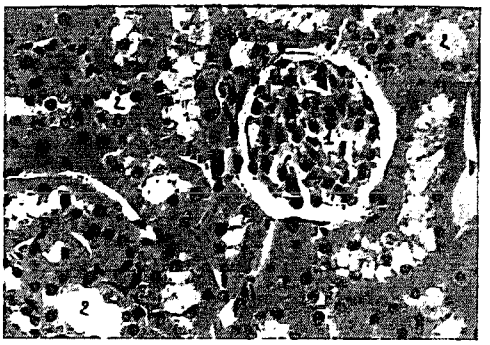
Hình 3. Thận thỏ uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày, ở N29 (HE × 250)
1. Cầu thận: sung huyết nhẹ; 2. Ống thận: bình thường.

Cấu trúc vi thể thận thỏ lô chứng:

Lô chứng có 2/5 thỏ có sung huyết nhẹ ở cầu thận; ống thận không có tổn thương, không thoái hoá lòng tế bào, trong lòng không có trụ albumin; 1/5 thỏ có tăng sinh tế bào mao mạch (hình 1).

Cấu trúc vi thể thận thỏ uống dịch chiết húng quế liều 0,6 g/kg/ngày × 28 ngày, ở N29:

Cầu thận sung huyết nhẹ (2/5 thỏ), ống thận và mô kẽ không có tổn thương, một số ống thận vùng tuỷ trong lòng có trụ albumin (hình 2).



Hình 2. Thận thỏ uống dịch chiết húng quế liều 0,6 g/kg/ngày × 28 ngày, ở N29 (HE × 250)
1. Cầu thận; 2. Ống thận

Ở ngày 43, có 3/6 thỏ ở lô chứng, 2/6 thỏ ở lô (2) và 3/6 thỏ ở lô (3) có sung huyết nhẹ cầu thận, ống thận bình thường (hình 4).

Hình 4. Thận thỏ uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày, ở N43 (HE × 250)
1. Cầu thận: sung huyết; 2. Ống thận: bình thường

Như vậy, cả lô chứng và lô dùng thuốc tại N29 và N43 đều có tỷ lệ nhất định thỏ có sung huyết nhẹ cầu thận, ống thận bình thường.

BÀN LUẬN

Thận giữ vai trò và ý nghĩa vô cùng quan trọng trong việc duy trì sự sống của nhiều loài động vật. Chức năng chính của thận là lọc máu, loại bỏ cặn bã ra bên ngoài cơ thể thông qua đường tiểu. Bên cạnh đó, thận cũng có chức năng quan trọng trong việc bài tiết nước tiểu, chức năng nội tiết và điều hòa thể tích máu. Tuy nhiên, nhiều thuốc rất nhạy cảm với nhu mô thận, có khả năng gây độc cho thận. Do đó việc đánh giá ảnh hưởng của thuốc nghiên cứu trên chức năng thận là hoàn toàn cần thiết.

Để đánh giá mức độ ảnh hưởng của dịch chiết nước húng quế đến chức năng thận thỏ, chúng tôi tiến hành định lượng hàm lượng creatinin trong huyết thanh và nghiên cứu sự biến đổi hình thái vi thể mô thận thỏ ở ngày N29 và sau khi ngừng dùng thuốc 15 ngày (N43).

Bảng 1 cho thấy, hàm lượng creatinin ở lô chứng và các lô uống dịch chiết húng quế liều 0,6 và 1,8 g/kg/ngày × 28 ngày tại thời điểm trước khi dùng thuốc (ngày N0) khác nhau không có ý nghĩa thống kê ($p > 0,05$). Điều này chứng tỏ, các thỏ có chức năng thận tương đồng nhau trước khi thử nghiệm và hàm lượng creatinin đều nằm trong giới hạn bình thường.

Hàm lượng creatinin của thỏ ở các lô dùng thuốc tại N14 và N29 thay đổi không có ý nghĩa thống kê so với N0 và so với lô chứng tại các thời điểm tương ứng (các giá trị $p > 0,05$). Kết quả này cho thấy, dịch chiết nước húng quế ở các liều đã thử nghiệm không ảnh hưởng đến hàm lượng creatinin trong máu thỏ, và các giá trị này vẫn trong giới hạn bình thường sau thời gian dùng thuốc 28 ngày liên tục.

Kết quả mổ thỏ ở các ngày N29 và N43 cho thấy, hình thái đại thể thận thỏ ở cả ba lô đều bình thường, với nhu mô thận mềm, mịn, không sung huyết và có màu nâu đỏ. Khi soi bằng kính lúp thấy rõ cầu thận là những hạt nhỏ li ti đều đặn.

Về cấu trúc vi thể thận thỏ tại N29: Cả lô chứng và lô dùng thuốc đều có tỷ lệ nhất định thỏ bị sung huyết nhẹ cầu thận và tăng sinh tế bào mao mạch, còn ống thận bình thường. Tỷ lệ sung huyết tương ứng ở lô chứng và các lô dùng thuốc là 2/5 (40,0%), 2/5 (40,0%) và 3/5 (60,0%) ở ngày N29 và 3/6 (50,0%), 2/6 (33,33%) và 3/6 (50,0%) ở ngày N43.

Sung huyết ở thận thỏ có thể do nhiều nguyên nhân khác nhau, có thể gặp ở cả thỏ khỏe mạnh, ăn uống sinh hoạt bình thường. Cả 3 lô nghiên cứu đều có thỏ gặp phải tình trạng sung huyết ở cầu thận, tuy nhiên tỷ lệ thỏ sung huyết ở lô dùng liều cao 1,8 g/kg/ngày × 28 ngày tại ngày N29 có xu hướng cao hơn so với hai lô còn lại. Ở ngày N43, tỷ lệ xung huyết của lô dùng liều cao không khác biệt so với lô chứng, đều 50%.

Chưa thấy nghiên cứu nào đánh giá ảnh hưởng của dịch chiết húng quế đến chức năng thận. Theo nghiên cứu của Balin A. và cộng sự (2012), dịch chiết húng quế có tác dụng để điều trị viêm bàng quang, viêm thận[5]. Tuy nhiên, khi sử dụng quá liều húng quế hoặc lạm dụng trong thời gian dài có thể gây ung thư niêm mạc gan [5] [10].

Như vậy, những kết quả ban đầu trên cho thấy, dịch chiết húng quế ở hai liều thử nghiệm không ảnh hưởng đến chức năng thận thỏ. Tuy nhiên, để có cơ sở chắc chắn, cần nhiều nghiên cứu trên các loài động vật khác nhau và với những mức liều cao hơn.

KẾT KUẬN

Đã nghiên cứu ảnh hưởng của dịch chiết nước húng quế đến chức năng thận của thỏ thí nghiệm với liều đường uống 0,6 và 1,8 g/kg/ngày × 28 ngày liên tiếp. Kết quả cho thấy:

Hàm lượng cretinin trong huyết thanh thỏ ở lô chứng và lô dùng thuốc khác nhau không có ý nghĩa thống kê tại cùng thời điểm nghiên cứu N0, N14 và N29, đồng thời, không có sự khác biệt thống kê tại các thời điểm sau uống thuốc ở ngày N14 và N29 so với N0 (trước uống thuốc), các giá trị $p > 0,05$.

Quan sát đại thể thận thỏ bình thường, nhu mô mềm, mịn, đồng nhất. Quan sát vi thể cho thấy ống thận thỏ ở các lô bình thường. Một số thỏ có sung huyết nhẹ cầu thận, ở cả lô chứng và lô dùng thuốc với tỷ lệ tương ứng ở 3 lô là 2/5, 2/5 và 3/5 ở ngày N29 và 3/6, 2/6 và 3/6 ở ngày N43.

Dịch chiết nước húng quế liều 0.6 và 1.8 g/kg/ngày không ảnh hưởng đến chức năng thận thỏ thí nghiệm.

LỜI CẢM ƠN

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BÀI NGHIÊN CỨU

Nghiên cứu ảnh hưởng của 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) đến chức năng tạo máu của thỏ thực nghiệm

EFFECTS OF PROPYL 10β - [(2'β-HYDROXY-3'-IMIDAZOL)] DEOXOARTEMISININ (32) ON RABBITS' HEMATOPOIETIC FUNCTIONS

Nguyễn Thị Thúy[1], Nguyễn Thị Minh Thu[2], Trần Thanh Dương[3], Nguyễn Thị Thu Hằng[2]

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TÓM TẮT

Nghiên cứu được tiến hành tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương từ tháng 02 - 05 năm 2021 nhằm đánh giá ảnh hưởng của hợp chất 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) đến chức năng tạo máu của thỏ thực nghiệm.

***Phương pháp:** Tiến hành theo hướng dẫn của Bộ Y tế và OECD về thử độc tính bán trường diễn. Hợp chất (32) được dùng bằng đường uống trên thỏ ở 2 mức liều 72 và 216 mg/kg/ngày x 28 ngày liên tiếp. Thử nghiệm tiến hành song song với nhóm chứng. Lấy máu tĩnh mạch tai thỏ để xét nghiệm vào các ngày N0, N14 và N29. Các chỉ tiêu đánh giá gồm: hàm lượng hemoglobin, số lượng hồng cầu, số lượng bạch cầu, công thức bạch cầu, hematocrit, số lượng tiểu cầu và thể tích trung bình hồng cầu.*

***Kết quả:** Hầu hết các chỉ số huyết học ở 2 lô uống (32) đều không khác biệt có ý nghĩa thống kê so với lô chứng, đồng thời không có sự khác biệt có ý nghĩa giữa các thời điểm N14 và N29 so với N0, các giá trị $p > 0,05$. Ngoại trừ, ở N14, số lượng hồng cầu ở lô uống (32) liều 72 mg/kg/ngày giảm có ý nghĩa thống kê so với N0 ($p < 0,05$) nhưng ở N29 lại tăng lên và không khác biệt có ý nghĩa so với N0. Tương tự, lượng hematocrit của lô uống 216 mg/kg/ngày tăng lên có ý nghĩa thống kê ở N14 nhưng ở N29 lại khác biệt không có ý nghĩa so với N0 ($p > 0,05$).*

***Kết luận:** Hợp chất (32) với liều 72 và 216 mg/kg/ngày x 28 ngày liên tiếp bằng đường uống không ảnh hưởng tới chức phận tạo máu của thỏ thí nghiệm.*

***Từ khóa:** 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32), chức năng tạo máu, hồng cầu, bạch cầu, hematocrit, hemoglobin, tiểu cầu, thể tích trung bình hồng cầu.*

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ABSTRACT

This study was conducted at the National Institute of Malariology, Parasitology and Entomology between February and May, 2021. The compound 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) was treated orally in experimental rabbits in order to examine whether it affected rabbits' hematopoietic functions or not.

Methods: *The Vietnam Ministry of Health's and OECD's guidelines for sub-chronic toxicity testing were applied. The compound (32) was treated orally in two different groups with the dose regimens of 72 and 216 mg/kg/day x 28 consecutive days, respectively. A control group treated orally with solven was also tested simultaneously. Two milliliters of blood were pulled out from each rabbit's ear vein on day 0 (before testing), day 14 (the middle of testing) and day 29 (after stopping taking 32). All of those blood samples were tested for hematological parameters including red blood cell, white blood cell, white blood cell formula, hematocrit, hemoglobin, platelet and mean corpuscular volume.*

Results: Generally, most of the hematological parameters of the two (32)-treated groups were not significantly different from the control group. Besides, those indices on days 14 and 29 did not change significantly compared to before taking the (32) on day 0 (the p values > 0.05). Nevertheless, with the dose of 72 mg/kg/day, rabbits' erythrocyte counts decreased significantly on day 14 comparing to that on day 0 ($p < 0.05$) and became in normal limits on day 29 ($p > 0.05$). In addition, in the group treated with 216 mg/kg/day, rabbits' hematocrit indices increased significantly on day 14 comparing to that on day 0 ($p < 0.05$) and did not change significantly compared to that on day 29 ($p > 0.05$).

Conclusion: *The compound (32) at the dose regimens of 72 and 216 mg/kg/day x 28 consecutive days did not affect rabbits' hematopoietic functions.*

Keywords: *10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32), rabbit, hematopoietic function, hematological parameters, red blood cell, white blood cell, white blood cell formula, hematocrit, hemoglobin, platelet and mean corpuscular volume.*

ĐẶT VẤN ĐỀ

Sốt rét là bệnh truyền nhiễm nguy hiểm có thể bùng phát thành dịch, căn nguyên do kí sinh trùng Plasmodium gây nên. Tổ chức Y tế Thế giới (WHO) và các nước đã có nhiều biện pháp để ứng phó, nhưng sốt rét vẫn là một trong những nguyên nhân chính gây ra bệnh tật, tử vong và trở thành gánh nặng sức khỏe toàn cầu.

Thách thức lớn nhất trong việc kiểm soát sốt rét là tình trạng kháng thuốc ngày càng lan rộng ở nhiều quốc gia và vùng lãnh thổ, đặc biệt tại các quốc gia trong khu vực Tiểu vùng Sông Mê-kông (GMS), trong đó có Việt Nam. Điều đáng lo ngại hiện nay là ký sinh trùng đã kháng artemisinin (ART) và các dẫn xuất - nhóm thuốc có hiệu lực điều trị KST nhanh và hiệu quả trong những thập kỷ trước [6]. Do vậy, WHO khuyến cáo các nước sử dụng phối hợp thuốc trong điều trị hoặc tăng cường nghiên cứu phát triển thuốc mới có khả năng chống kháng thuốc [2],[5].

Hợp chất 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (dẫn xuất 32) đã được Viện Hóa học các Hợp chất thiên nhiên tổng hợp và tinh chế, được Viện Sốt rét – KST – CT Trung ương chứng minh hiệu lực in vitro tương đương ART [3], có tác dụng tốt in vivo với chủng P. bergei kháng cloroquin trên chuột nhắt trắng [4] và không có độc tính cấp đường uống [3],[4]. Hiện chưa có

nghiên cứu nào theo dõi độc tính dài ngày của (32) trên các cơ quan của động vật thực nghiệm. Để tiếp tục phát triển hợp chất (32), nghiên cứu này được tiến hành nhằm đánh giá ảnh hưởng của (32) đến chức năng tạo máu của thỏ khi cho uống 28 ngày liên tiếp.

ĐỐI TƯỢNG, PHƯƠNG TIỆN VÀ PHƯƠNG PHÁP NGHIÊN CỨU

Đối tượng nghiên cứu

Thuốc nghiên cứu

Dẫn xuất 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) do Viện Hóa học các Hợp chất thiên nhiên cung cấp, độ tinh khiết 99,98%.

Động vật dùng trong nghiên cứu

Thỏ trưởng thành (*Oryctolagus cuniculus* L.), tổng số 36 con, cân nặng trung bình 2,1± 0,2 kg, 2 tháng tuổi, khỏe mạnh, không phân biệt đực - cái, do Trung tâm nghiên cứu Dê và Thỏ Sơn Tây cung cấp. Thỏ cái đều không mang thai, không nuôi con bú và chưa sinh sản lần nào. Động vật được nuôi ổn định 7 ngày trong điều kiện thí nghiệm trước khi tiến hành nghiên cứu.

Phương tiện nghiên cứu

Hóa chất dùng trong nghiên cứu

Nước cất hai lần; Các dung dịch xét nghiệm huyết học Sysmex KX-21; Gôm arabic dược dụng (Thái Lan sản xuất), lô HG-107: Dùng để pha thuốc.

Dụng cụ và thiết bị dùng trong nghiên cứu

- Máy cất nước hai lần Aquatron (hãng Bibby sterilin, Anh).
- Cân Sauter, độ chính xác d = 0,1mg.
- Máy xét nghiệm huyết học Sysmex KX-21 (Sysmex corporation - Nhật).
- Cân Sauter, độ chính xác d = 0,1mg.
- Kim đầu tù cho động vật uống.
- Cốc thủy tinh có chia vạch, bơm kim tiêm 5 mL.

Phương pháp nghiên cứu

Địa điểm nghiên cứu

Nghiên cứu được tiến hành tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương từ tháng 02/2021 đến tháng 05/2021.

Phương pháp nghiên cứu

Tiến hành theo hướng dẫn của Bộ Y tế [1] và OECD số 407 [7]. Ảnh hưởng của hợp chất (32) đến chức năng tạo máu của thỏ được đánh giá thông qua việc xác định các chỉ số huyết học trước, trong và sau 28 ngày liên tiếp dùng thuốc.

Thỏ sau khi nuôi ổn định 7 ngày, được chia ngẫu nhiên thành 3 lô, mỗi lô 12 con:

- Lô 1: chứng (n = 12): Uống dung môi gồm arabic 1%, thể tích tương đương liều điều trị thuốc × 28 ngày liên tiếp.
- Lô 2 (n = 12): Uống hỗn dịch (32) liều 72mg/kg/ngày, (tương đương liều dùng dự kiến trên người) × 28 ngày liên tiếp.
- Lô 3 (n = 12): Uống hỗn dịch (32) liều 216mg/kg/ngày, (tương đương gấp 3 lần liều dùng dự kiến trên người) × 28 ngày liên tiếp.

Trước uống thuốc, thỏ được được lấy máu tĩnh mạch tai để làm xét nghiệm huyết học vào ngày N0. Cho thỏ uống thuốc mỗi ngày một lần vào buổi sáng bằng kim đầu tù, liên tục trong 28 ngày. Các ngày lấy máu tĩnh mạch tai để làm xét nghiệm huyết học tiếp theo là N14 (sau khi uống thuốc 2 giờ) và N29.

Các chỉ số nghiên cứu

Hàm lượng hemoglobin (g/L), số lượng hồng cầu (T/L), số lượng bạch cầu (G/L), công thức bạch cầu (%), số lượng tiểu cầu (G/L), hematocrit (%) và thể tích trung bình hồng cầu (fL) tại các ngày N0, N14 và N29.

Xử lý số liệu

- Số liệu được biểu thị bằng trị số trung bình ± độ lệch chuẩn (M±SD).

- Các số liệu nghiên cứu được xử lý bằng chương trình Excel 2016 theo phương pháp thống kê y học với cỡ mẫu nhỏ (< 30), sử dụng t-test Student và Fisher's exact test để so sánh các số liệu trước, trong và sau thử nghiệm và so sánh giữa lô dùng thuốc và lô chứng.

- Trong so sánh, nếu p > 0,05 là khác biệt không có ý nghĩa thống kê, p < 0,05 là khác biệt có ý nghĩa thống kê và p càng nhỏ thì khác biệt có ý nghĩa thống kê càng cao.

KẾT QUẢ NGHIÊN CỨU

Ảnh hưởng của hợp chất (32) đến chức năng tạo máu của thỏ được đánh giá thông qua việc xác định các chỉ số huyết học trước, trong và sau khi dùng thuốc.

Bảng 1. Hàm lượng hemoglobin (g/L) của thỏ ở các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p* (N0-N29)
Lô 1: chứng (n = 12)	105,67 ± 16,76	108,4 ± 12,13	110,33 ± 11,04	> 0,05	> 0,05
Lô 2: uống (32), 72 mg/kg/ ngày × 28 ngày (n = 12)	109,25 ± 11,94	113,5 ± 9,45	108,33 ± 10,33	> 0,05	> 0,05
Lô 3: uống (32), 216 mg/kg/ ngày × 28 ngày (n = 12)	108,92 ± 13,18	111,6 ± 12,16	111,5 ± 12,53	> 0,05	> 0,05
p (1-2), *p* (1-3), *p* (2-3)	> 0,05	> 0,05	> 0,05		

Bảng 1 cho thấy, hàm lượng hemoglobin của các lô uống (32) ở N14 và N29 thay đổi không có ý nghĩa thống kê so với N0 (trước khi uống thuốc) ($p > 0,05$). Hàm lượng hemoglobin giữa các lô dùng thuốc với lô chứng và giữa các lô dùng thuốc với nhau tại cùng thời điểm tương ứng khác biệt không có ý nghĩa thống kê ($p > 0,05$).

Bảng 2. Số lượng hồng cầu (T/L) trong máu thỏ ở các lô nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p* (N0-N29)
Lô 1: chứng (n = 12)	4,575 ± 0,218	4,433 ± 0,314	4,471 ± 0,212	> 0,05	> 0,05
Lô 2: uống (32), 72 mg/kg/ ngày × 28 ngày (n = 12)	4,73 ± 0,276	4,445 ± 0,225	4,625 ± 0,176	***p = 0,01104 (< 0,05)***	> 0,05
Lô 3: uống (32), 216 mg/kg/ ngày × 28 ngày (n = 12)	4,461 ± 0,357	4,564 ± 0,243	4,621 ± 0,328	> 0,05	> 0,05
p (1-2), *p* (1-3), *p* (2-3)	> 0,05	> 0,05	> 0,05		

Ở lô 1 và lô 3, số lượng hồng cầu thỏ tại ngày N14 và N29 thay đổi không có ý nghĩa thống kê so với ở N0 ($p > 0,05$). Riêng ở lô uống (32) liều 72 mg/kg/ngày ×28 ngày, số lượng hồng cầu thỏ ở ngày N14 giảm đi có ý nghĩa thống kê so với N0 ($p < 0,05$) nhưng ở N29, hồng cầu lại tăng lên và khác biệt không có ý nghĩa so với N0. Giữa các lô với nhau, hồng cầu thỏ khác biệt không có ý nghĩa thống kê tại cùng thời điểm tương ứng ($p > 0,05$).

Bảng 3. Ảnh hưởng của hợp chất (32) đến số lượng bạch cầu (G/L) trong máu thỏ tại các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p* (N0-N29)
Lô 1: chứng (n = 12)	10,354 ± 2,846	9,313 ± 2,081	9,408 ± 1,625	>0,05	>0,05
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	8,771 ± 1,891	8,825 ± 1,392	8,779 ± 1,522	>0,05	>0,05
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	10,479 ± 3,083	9,138 ± 1,371	9,963 ± 1,855	>0,05	>0,05
p (1-2), *p* (1-3), *p* (2-3)	> 0,05	> 0,05	> 0,05		

Số lượng bạch cầu trong máu thỏ ở các lô uống hợp chất (32) tại ngày N14 và N29 khác biệt không có ý nghĩa thống kê so với trước khi uống thuốc (N0), $p > 0,05$. Số lượng bạch cầu của hai lô uống (32) liều 72 và 216 mg/kg/ngày × 28 ngày cũng thay đổi không có ý nghĩa thống kê so với lô chứng tại cùng thời điểm nghiên cứu (các giá trị $p > 0,05$).

Bảng 4. Tỷ lệ (%) các loại bạch cầu (BC) thỏ ở các lô nghiên cứu

Chỉ số	Lô	N0	N14	N29	p (N0-N14)	p (N0-N29)
BC trung tính (%)	Lô 1: chứng (n = 12)	38,67 ± 10,69	34,08 ± 7,09	34,67 ± 6,87	> 0,05	> 0,05
	Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	34,5 ± 14,5	33,92 ± 8,73	35 ± 11,5	> 0,05	> 0,05
	Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	36,42 ± 10,44	35,17 ± 11,35	34,83 ± 9,84	> 0,05	> 0,05
	p (1-2), *p* (1-3), *p* (2-3)	> 0,05	> 0,05	> 0,05		
BC lympho (%)	Lô 1: chứng (n = 12)	57,92 ± 10,65	63,08 ± 7,91	62,08 ± 7,08	> 0,05	> 0,05
	Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	62,50 ± 14,50	63,75 ± 8,95	61,92 ± 11,43	> 0,05	> 0,05
	Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	60,50 ± 10,75	62,17 ± 11,85	61,58 ± 9,70	> 0,05	> 0,05
	p (1-2), *p* (1-3), *p* (2-3)	> 0,05	> 0,05	> 0,05		

BC mono (%)	Lô 1: chứng (n = 12)	3,50 ± 1,12	2,75 ± 1,36	3,25 ± 0,97	> 0,05	> 0,05
	Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	3,00 ± 1,04	2,33 ± 0,89	3,08 ± 1,51	> 0,05	> 0,05
	Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	3,08 ± 1,16	2,83 ± 1,47	2,75 ± 1,60	> 0,05	> 0,05
	***p* (1-2), *p* (2-3), *p* (1-3)**	> 0,05	> 0,05	> 0,05		

Tỷ lệ % bạch cầu trung tính, lympho và mono của các lô uống hợp chất (32) thay đổi không có ý nghĩa thống kê so với lô chứng tại cùng thời điểm, cũng như tại các ngày N14 và N29 so với trước khi uống thuốc (các giá trị $p > 0,05$).

Bảng 5. Lượng hematocrit (%) trong máu thỏ ở các lô nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p* (N0-N29)
Lô 1: chứng (n = 12)	35,97 ± 4,52	36,93 ± 3,59	37,34 ± 3,61	> 0,05	> 0,05
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	37,14 ± 4,60	37,70 ± 2,61	38,26 ± 2,06	> 0,05	> 0,05
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	39,53 ± 4,59	39,74 ± 2,81	38,71 ± 3,39	> 0,05	> 0,05
***p* (1-2), *p* (2-3), *p* (1-3)**	> 0,05	> 0,05 **< 0,05**	> 0,05		

Lượng hematocrit trong máu thỏ ở lô uống (32) liều 72 mg/kg/ngày x 28 ngày khác biệt không có ý nghĩa thống kê so với lô chứng tại các thời điểm nghiên cứu, cũng như tại các ngày N14 và N29 so với trước khi uống thuốc (các giá trị $p > 0,05$). Riêng ở lô 3, ngày N14, lượng hematocrit của thỏ cao hơn có ý nghĩa thống kê so với lô chứng ($p < 0,05$) nhưng ở ngày N29, sự khác biệt này lại không có ý nghĩa thống kê ($p > 0,05$).

Bảng 6. Số lượng tiểu cầu (G/L) trong máu thỏ ở các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p* (N0-N29)
Lô 1: chứng (n = 12)	373,71 ± 29,80	393,97 ± 22,62	382,83 ± 20,66	> 0,05	> 0,05
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	371,37 ± 30,85	364,01 ± 34,24	379,98 ± 25,53	> 0,05	> 0,05
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	385,34 ± 28,18	393,41 ± 22,06	389,72 ± 24,7	> 0,05	> 0,05
***p* (1-2), *p* (1-3), *p* (2-3)**	> 0,05	> 0,05	> 0,05		

Số lượng tiểu cầu của các lô uống thuốc tại N14 và N29 thay đổi không có ý nghĩa thống kê so với N0, và khác biệt không có ý nghĩa thống kê so với lô chứng tại các thời điểm tương ứng (các giá trị $p > 0,05$).

Bảng 7. Ảnh hưởng của (32) đến thể tích trung bình hồng cầu (fL) thỏ

Lô	N0	N14	N29	*p*(N0-N14)	*p*(N0-N29)
Lô 1: chứng (n = 12)	64,39 ± 1,51	65,43 ± 1,95	63,48 ± 2,07	> 0,05	> 0,05
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	64,26 ± 2,17	64,83 ± 2,15	65,25 ± 2,15	> 0,05	> 0,05
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	64,66 ± 1,83	65,42 ± 1,58	64,75 ± 2,17	> 0,05	> 0,05
***p* (1-2), *p* (1-3), *p* (2-3)**	> 0,05	> 0,05	> 0,05		

Thể tích trung bình hồng cầu thỏ ở các lô uống thuốc hợp chất (32) thay đổi không có ý nghĩa thống kê ở N14 và N29 so với N0 và so với lô chứng tại các thời điểm tương ứng (các giá trị $p > 0,05$).

BÀN LUẬN

Hệ thống tạo máu đóng một vai trò hết sức quan trọng trong cơ thể vì máu là cơ quan trao đổi vật chất, vận chuyển khí, bảo vệ cơ thể, duy trì sự sống,... Hiện nay, việc xác định giá trị các thành phần cơ bản có trong máu giữ vai trò quan trọng trong chẩn đoán và điều trị bệnh. Thông qua các chỉ số về máu có thể gián tiếp đánh giá được chức năng hoạt động của cơ quan tạo máu. Bất kỳ một nguyên nhân nào ảnh hưởng tới cơ quan tạo máu đều làm thay đổi các chỉ số trong máu.

Ảnh hưởng của hợp chất (32) đến chức năng tạo máu của thỏ được đánh giá thông qua các chỉ số huyết học: Hàm lượng hemoglobin, số lượng hồng cầu, số lượng bạch cầu, công thức bạch cầu, hematocrit, số lượng tiểu cầu và thể tích trung bình hồng cầu của thỏ tại 3 thời điểm trước, trong và sau khi dùng thuốc.

Kết quả nghiên cứu cho thấy hầu hết các chỉ số huyết học ở 2 lô thử nghiệm thuốc đều không có gì khác biệt so với lô chứng, đồng thời kết quả N14, N29 không có sự khác biệt so với N0 (các giá trị $p > 0,05$), ngoại trừ:

Số lượng hồng cầu ở lô uống thuốc (32) liều 72 mg/kg/ngày (lô 2) tại N14 giảm đi có ý nghĩa thống kê so với N0 ($p < 0,05$), tuy nhiên ở N29, chỉ số này lại tăng lên và khác biệt không có ý nghĩa thống kê so với N0 ($p > 0,05$).

Lượng hematocrit trong máu thỏ ở lô uống thuốc liều 216 mg/kg/ngày (lô 3) vào N14 cao hơn có ý nghĩa thống kê so với lô chứng ($p < 0,05$), tuy nhiên chỉ số này đã giảm đi ở N29 và sự khác biệt so với N0 không có ý nghĩa thống kê ($p > 0,05$).

Ghi nhận sự khác biệt mang ý nghĩa thống kê về số lượng hồng cầu ở lô 2 tại N14 so với N0 và lượng hematocrit ở lô 3 so với lô chứng tại N14, tuy nhiên các chỉ số này đều trở về mức bình thường và khác biệt không có ý nghĩa thống kê so với N0 và lô chứng tại N29. Sự khác biệt này chỉ diễn ra trong thời gian ngắn, sau đó lại phục hồi về chỉ số bình thường, chứng tỏ cơ quan tạo máu vẫn hoạt động hiệu quả, dự đoán chỉ là các bất thường trong sinh lý cơ thể thỏ, không phải do ảnh hưởng của thuốc, cần tiến hành nghiên cứu thêm ở quy mô lớn hơn để có thể đưa ra kết luận chính xác hơn.

Từ các kết quả trên có thể kết luận rằng hợp chất (32) tại liều 72 mg/kg/ngày x 28 ngày và 216 mg/kg/ngày x 28 ngày gần như không làm ảnh hưởng tới chức phận tạo máu của thỏ.

KẾT LUẬN

Đã nghiên cứu ảnh hưởng của 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) đến chức năng tạo máu của thỏ thực nghiệm với hai liều đường uống 72 và 216 mg/kg/ngày × 28 ngày liên tiếp. Kết quả cho thấy:

Hàm lượng hemoglobin, số lượng hồng cầu, bạch cầu, công thức bạch cầu, lượng hematocrit, số lượng tiểu cầu và thể tích trung bình hồng cầu ở các lô thử nghiệm nhìn chung khác biệt không có ý nghĩa thống kê so với lô chứng và giá trị tại N14, N29 không khác biệt so với N0 ($p > 0,05$).

Số lượng hồng cầu thỏ ở lô uống 72 mg/kg/ngày giảm có ý nghĩa thống kê ở ngày N14, nhưng không khác biệt thống kê các lô khác và ở N29 lại trở lại giới hạn bình thường. Hàm lượng hematocrit ở lô uống 216 mg/kg/ngày tăng có ý nghĩa thống kê ở N14 nhưng ở ngày N29 khác biệt không có ý nghĩa thống kê với N0 và so với các lô khác ($p > 0,05$).

Hợp chất (32) tại 2 liều thử không ảnh hưởng tới chức năng tạo máu của thỏ.

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TẠP CHÍ

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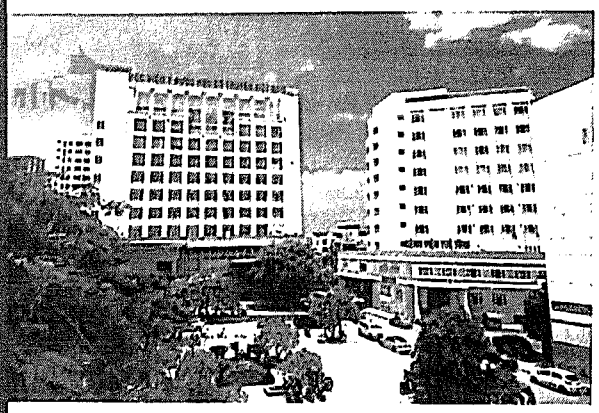

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MỤC LỤC SỐ ĐẶC BIỆT

BÀI NGHIÊN CỨU

Nghiên cứu tác dụng xua muỗi của tinh dầu húng quế Ocimum Basilicum L. Lamiaceae trên thực nghiệm

STUDY ON MOSQUITOES REPELLENT EFFECT OF BASIL OIL (OCIMUM BASILICUM L. LAMIACEAE) IN EXPERIMENTAL ANIMALS

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[2] Viện Sốt rét – Ký sinh trùng – Côn trùng Trung ương

TÓM TẮT

Nghiên cứu được tiến hành từ tháng 6 - 8 năm 2021, tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương.

Mục tiêu: *Đánh giá tác dụng xua muỗi Aedes aegypti, Anopheles minimus và Culex tritaeniorhynchus của tinh dầu Húng quế (Ocimum basilicum L. Lamiaceae) trên động vật thí nghiệm.*

Phương pháp: *Tiến hành theo phương pháp của Tổ chức Y tế thế giới (WHO) với mồi là chuột lang. Mỗi thử nghiệm được tiến hành 3 lần. Chín trăm (900) muỗi mỗi loài được chia thành 9 lô, mỗi lô 100 con; trong đó có 3 lô chứng (muỗi được nhốt trong ống tunnel với màn không tẩm mẫu thử), 3 lô thử với tinh dầu húng quế nguyên chất và 3 lô thử với hỗn hợp tinh dầu húng quế - ethanol 70⁰ (7:3). Đánh giá tỷ lệ muỗi chết và tỷ lệ muỗi bị ức chế hút máu ở các lô chứng và lô thử nghiệm sau 15 giờ tiếp xúc với màn tẩm mẫu thử.*

Kết quả: *Tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu-ethanol 70⁰ có tác dụng xua muỗi tốt với cả 3 loài muỗi với tác dụng xua trung bình lần lượt là: 99,99% và 99,98% muỗi Aedes aegypti, 99,33% và 99,35% muỗi Anopheles minimus, 99,96% và 99,90% muỗi Culex tritaeniorhynchus.*

Từ khóa: *Húng quế, Ocimum basilicum L. Lamiaceae, Aedes aegypti, Anopheles minimus, Culex tritaeniorhynchus, tác dụng xua, tỷ lệ chết, tỷ lệ ức chế hút máu.*

Ngày nhận bài: 6/9/2021
Ngày phản biện: 9/9/2021
Ngày chấp nhận đăng: 20/9/2021

ABSTRACT

This study was conducted between June and August, 2021 at Institute of Natural Products Chemistry and National Institute of Malariology, Parasitology and Entomology, Hanoi, Vietnam.

Objective: *To test repellent effect of basil oil (Ocimum basilicum L. Lamiaceae) on Aedes aegypti, Anopheles minimus and Culex tritaeniorhynchus mosquitoes in experimental animals..*

Methods: *Repellent effect of basil oil on three different mosquitoe species were assessed according to WHO method in Guinea pigs. Total of 900 mosquitoes of each species were selected and divided in 9 groups, in which each group had 100 mosquitoes. Three groups were considered as the control ones that were kept respectively in a tunnel with blank nets. Three others were caged in turn at the same tunnel with the net which had been soaked in the pure basil oil while the rest were confined in the tunnel in succession with the net dipped into the combination of basil oil – ethanol 70⁰ (7:3). Then, the rates of dead mosquitoes and sucking blood inhibition were evaluated after exposing 15 hours to basil oil-sample nets.*

Results: *The pure basil oil and the combination of basil oil - ethanol 70⁰ (7:3) revealed high repellent effects on three tested mosquitoe species with the average repellent rates of 99.99% and 99,98% on Aedes aegypti, 99.33% and 99.35% on Anopheles minimus, 99.96% and 99.90% on Culex tritaeniorhynchus, respectively.*

Key words: *Basil, Ocimum basilicum L. Lamiaceae, Aedes aegypti, Anopheles minimus, Culex tritaeniorhynchus, mosquito, water extract, repellent effect, rate of dead mosquitoes, rate of sucking blood inhibtion.*

ĐẶT VẤN ĐỀ

Húng quế từ lâu đã nổi tiếng là một loài cây có giá trị trong Y học cổ truyền của nhiều nước. Mỗi bộ phận của húng quế đều có tác dụng chữa bệnh khác nhau. Lá húng quế dùng làm thuốc bổ và thuốc trị giun. Trà húng quế uống nóng có tác dụng tốt trong trị nôn, đầy chướng, và lỵ. Hoa của cây cũng có tác dụng kích thích tiêu hóa, chống đầy hơi, lợi tiểu và giảm viêm. Tinh dầu húng quế có tác dụng giảm bớt mệt mỏi về tinh thần, trị chứng cảm lạnh, co cơ, viêm mũi dị ứng, kháng khuẩn, kháng nấm, diệt côn trùng và được ưu tiên dùng để trị côn trùng đốt hoặc rắn cắn. [5]

Hướng nghiên cứu về tác dụng xua và diệt côn trùng của húng quế đã được một số tác giả trong và ngoài nước quan tâm. Một số nghiên cứu thực địa tại Kenya cho thấy, cây húng quế trồng trong chậu có thể xua 40% muỗi *Anopheles*; tinh dầu húng quế có tác dụng bảo vệ 100% khỏi muỗi *Aedes* trong vòng 6 giờ [5]. Kiplang'at K.P. và cộng sự (2013) đã công bố tinh dầu húng quế pha trong dầu khoáng tự nhiên với tỷ lệ 3% có tác dụng bảo vệ da thỏ khỏi 100% muỗi *Aedes aegypti* [2]. Nguyễn Thị Minh Thu và cộng sự (2020) đã công bố về tác dụng xua muỗi của dịch chiết húng quế [1]. Rất ít nghiên cứu trong nước tiến hành thử tác dụng xua muỗi của tinh dầu húng quế; thậm chí các nghiên cứu về tác dụng xua muỗi của tinh dầu húng quế trên thế giới cũng chỉ giới hạn với một số loài muỗi lưu hành phổ biến tại địa phương và với một số dung môi nhất định.

Do đó, để phát triển thuốc từ nguồn gốc húng quế sẵn có, dễ trồng tại Việt Nam và tạo ra các dạng chế phẩm có tác dụng phòng chống muỗi,

ngăn ngừa bệnh do muỗi truyền, nghiên cứu này được tiến hành với mục tiêu đánh giá tác dụng xua muỗi *Aedes aegypti, Anopheles minimus* và *Culex tritaeniorhynchus* của tinh dầu húng quế (*Ocimum basilicum* L. *Lamiaceae*) trên thực nghiệm.

VẬT LIỆU VÀ PHƯƠNG PHÁP

Thời gian và địa điểm nghiên cứu

Nghiên cứu được tiến hành từ tháng 6-8 năm 2021, tại Viện Hóa học các hợp chất thiên nhiên và Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương.

Đối tượng và vật liệu nghiên cứu

Mẫu nghiên cứu

Phần trên mặt đất của cây húng quế còn tươi được thu hái tại Yên Xá, Tân Triều, Thanh Trì, Hà Nội vào tháng 6/2021.

Dược liệu được rửa sạch, thái nhỏ, và cho vào bình chiết xuất bằng thép không gỉ, có chứa hơi nước ở bên trong. Thông qua một cửa vào, hơi nước được bơm qua dược liệu, cuốn theo các phân tử chất thơm và biến chúng thành hơi. Hơi này gặp lạnh ở phần bình chứa nước lạnh sẽ ngưng tụ và chảy vào bình ngưng. Khi đó, tinh dầu sẽ nổi trên mặt nước. Tinh chế tinh dầu húng quế bằng cách cất kéo hơi nước. Quá trình chưng sẽ kết thúc khi giọt chất lỏng cất ra không còn đục mà trở nên trong suốt.

Tiến hành tách tinh dầu thu được ra khỏi nước bằng cách làm nguội dung dịch, cho muối NaCl sạch vào hỗn hợp nước – tinh dầu đến bão hòa. Sau đó chuyển hỗn hợp vào phễu chiết, để yên cho tách lớp và chiết tinh dầu ra khỏi hỗn hợp. Cho tinh dầu vào bình tam giác 50 ml sạch có sinh hàn không khí, cho Na_2SO_4 khan vào bình và đun nóng nhẹ trên bếp cách thủy ở 60 - 70 ^{0}C cho đến khi dịch trở nên trong suốt. Rót tinh dầu đã được làm khan qua phễu có giấy lọc. Tinh dầu này được dùng để thử nghiệm.

Muỗi dùng trong nghiên cứu

Muỗi cái *Aedes aegypti* và *Anopheles minimus và Culex tritaeniorhynchus*: 900 con mỗi loại, 5 - 8 ngày tuổi, khỏe mạnh, đủ 6 chân, đủ 2 cánh, có tư thế đậu bình thường, chưa hút máu.

Muỗi được nuôi tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương trong điều kiện thí nghiệm, được nuôi bằng dung dịch glucose 10%. Chọn những con được nuôi ăn vừa phải, không quá no hoặc quá đói.

Động vật mồi dùng trong nghiên cứu

Chuột hamster, 10 con, 110,0 ± 10 g, trưởng thành, khỏe mạnh, không phân biệt giống, do Học viện Nông nghiệp Việt Nam cung cấp, được nuôi trong điều kiện thí nghiệm từ 5-7 ngày trước khi tiến hành nghiên cứu.

Nguyên liệu, dụng cụ dùng trong nghiên cứu

- Ống tunnel hình hộp chữ nhật bằng thủy tinh dài 60 cm, chiều rộng 25 cm, chiều cao 25cm: 04 ống.

- Khung nhựa, kích thước trong 20cm x 20cm để giữ màn thử nghiệm đặt ở vị trí 1/3 chiều dài của ống tunnel.

- Mảnh vải thử nghiệm và mảnh vải đối chứng kích thước 21cm x 21cm. Mỗi mảnh cắt 9 lỗ đường kính 1cm, một lỗ ở tâm mảnh màn, 8 lỗ còn lại cách viền màn 6cm và cách đều nhau 5cm theo hàng ngang và hàng dọc.

- Kẹp để kẹp chuột.

- Lồng màn kích thước 30cm x 30cm.

- Ống hút bằng thủy tinh, đường kính ngoài 1 - 1,2 cm, một đầu ống uốn cong giúp cho việc bắt chuyển muỗi từ phễu nhựa vào cốc nghỉ một cách dễ dàng. Ống hút này được nối với một ống cao su hoặc một ống nhựa mềm có chiều dài 60 cm có gắn đầu ngậm hút.

- Tuýp bắt muỗi bằng thủy tinh thủng hai đầu, đường kính 1,2 cm; dài 18 cm - 20cm.

Phòng thử nghiệm

Hai phòng riêng biệt, có diện tích tối thiểu 12m²/ phòng, duy trì điều kiện nhiệt độ 27 °C ± 2 °C và độ ẩm 75% ± 10%, đủ tối khi tiến hành thử nghiệm.

Phương pháp tiến hành

Chuẩn bị muỗi thử

Chuẩn bị mỗi lô thử nghiệm và lô chứng 100 muỗi cái 5-8 ngày tuổi, chưa hút máu, chia thành các lô như sau:

- Lô 1: 100 muỗi cái *Aedes aegypti* được nhốt trong ống tunnel, màn không tẩm tinh dầu;

- Lô 2: 100 muỗi cái *Aedes aegypti* được nhốt trong ống tunnel với màn tẩm tinh dầu nguyên chất;

- Lô 3: 100 muỗi cái *Aedes aegypti* được nhốt trong ống tunnel với màn tẩm tinh dầu pha với ethanol 70⁰ (7:3);

- Lô 4: 100 muỗi cái *Anopheles minimus* được nhốt trong ống tunnel, màn không tẩm tinh dầu;

- Lô 5: 100 muỗi cái *Anopheles minimus* được nhốt trong ống tunnel với màn tinh dầu nguyên chất;

- Lô 6: 100 muỗi cái *Anopheles minimus* được nhốt trong ống tunnel với màn tẩm tinh dầu pha với ethanol 70⁰ (7:3);

- Lô 7: 100 muỗi cái *Culex tritaeniorhynchus* được nhốt trong ống tunnel với màn không tẩm tinh dầu;

- Lô 8: 100 muỗi cái *Culex tritaeniorhynchus* được nhốt trong ống tunnel với màn tẩm tinh dầu nguyên chất;

- Lô 9: 100 muỗi cái *Culex tritaeniorhynchus* được nhốt trong ống tunnel với màn tẩm tinh dầu pha với ethanol 70⁰ (7:3).

Dùng tuýp bắt muỗi, mỗi tuýp bắt 5 muỗi / lần. Mỗi thí nghiệm trên mỗi loài muỗi được tiến hành 3 lần, mỗi lần chia thành 9 lô như trên.

Tiến hành (theo phương pháp của WHO [7])

- Dùng băng dính cố định miếng vải vào khung nhựa, đặt vào khe ở vị trí 1/3 chiều dài của ống tunnel (hình 2). Với các lô chứng: Miếng vải không được tẩm dịch chiết húng quế. Với các lô thử nghiệm còn lại, miếng vải sẽ được tẩm dịch chiết với các nồng độ khác nhau ở mỗi lần.

- Cố định 1 con chuột vào tunnel bằng kẹp chuột, sau đó đưa vào phần ngắn hơn của tunnel, buộc đầu màn ở phần ngắn hơn của ống tunnel lại.

- Mỗi lần, thả 100 muỗi cái đã chuẩn bị ở trên vào phần dài hơn của ống tunnel, buộc đầu màn ở phần dài hơn của ống tunnel lại.

- Sau thời gian tiếp xúc 15 giờ, muỗi được hút từ mỗi phần của tunnel vào các lồng muỗi khác nhau bằng ống hút muỗi, ghi lại tỷ lệ muỗi chết (nếu có) và tỷ lệ muỗi bị ức chế hút máu, tỷ lệ muỗi no máu.

Những con muỗi được đánh giá là chết khi chúng không bay lên được.

Chỉ tiêu đánh giá

Đánh giá tỷ lệ muỗi chết và muỗi bị ức chế hút máu theo các tình huống sau (bảng 1).

Bảng 1. Một số tình huống nghiên cứu và xử trí

STT	Tình huống	Xử lý
1	- Nếu tỷ lệ muỗi chết trong lô chứng > 10%.	- Hủy bỏ kết quả và làm lại thí nghiệm (TN).
2	- Nếu tỷ lệ muỗi đối chứng chết trong khoảng 0 - 10%	- Tỷ lệ muỗi chết được điều chỉnh theo công thức Abbott.
3	- Nếu tỷ lệ muỗi chết trong lô đối chứng 0%	- Giữ nguyên tỷ lệ chết quan sát mà không cần điều chỉnh.

Công thức Abbott:

$$\text{Tỷ lệ \% muỗi chết} = \frac{\text{(Số muỗi chết ở lô thí nghiệm - số muỗi chết ở lô chứng) x 100}}{\text{100 – số muỗi chết ở lô đối chứng}}$$

$$\text{\% muỗi bị ức chế hút máu} = \frac{\text{(Số muỗi bị ức chế ở lô TN - số muỗi bị ức chế ở lô chứng) x 100}}{\text{100 – số muỗi bị ức chế ở lô chứng}}$$

Xử lý số liệu

Số liệu được xử lý theo phương pháp thống kê y học. Tỷ lệ muỗi chết và bị ức chế hút máu được tính trung bình của 3 lần thí nghiệm.

KẾT QUẢ NGHIÊN CỨU

Tinh dầu húng quế nguyên chất và tinh dầu pha loãng với ethanol 70⁰ (7:3) đã được thử nghiệm với ba loài muỗi *Aedes aegypti, Anopheles minimus* và *Culex tritaeniorhynchus* (900 con mỗi loại). Thử nghiệm được tiến hành 3 lần để lấy giá trị trung bình. Kết quả được thể hiện ở bảng 2-4.

Bảng 2. Ảnh hưởng của tinh dầu húng quế với Aedes aegypti (trung bình của 3 lần thử, n = 300 muỗi/lô)

Lô	Tỷ lệ trung bình muỗi chết (%) ((Abbott	Tỷ lệ trung bình muỗi ngã/bị ức (%) chế hút máu	Tỷ lệ trung bình muỗi (%) no máu	Tỷ lệ trung bình muỗi chết + ức chế hút máu (%)
(1)-chứng (TB)	0,33	0	99,67%	**0,33**
(2)- Tinh dầu nguyên chất (TB)	94,32	5,67	-	**99,99**
(3)- Hỗn hợp tinh dầu-ethanol 70⁰ tỷ lệ 7:3 (TB)	90,31	9,67	-	**99,98**
p (2-1), *p* (3-1)	< 0,001	< 0,01	-	< 0,001

Bảng 2 cho thấy, tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu-ethanol 70⁰ (7:3) có tác dụng xua *Aedes aegypti* lần lượt là 99,99% và 99,98%, cao hơn có ý nghĩa thống kê so với lô chứng ($p < 0,001$).

Bảng 3.8. Ảnh hưởng của tinh dầu húng quế với *Anopheles minimus*
(trung bình của 3 lần thử nghiệm, n = 300 muỗi/lô)

Lô	Tỷ lệ trung bình muỗi chết ((%) (Abbott	Tỷ lệ trung bình muỗi bị ức chế hút máu (%) ((Abbott	Tỷ lệ trung bình muỗi (%) no máu	Tỷ lệ trung bình muỗi chết + ức chế hút (%) máu
(4)-chứng (TB)	0,33	0,67	99,0	**1,0**
(5)- Tinh dầu nguyên chất (TB)	96,99	2,34	-	**99,33**
(6)- Hỗn hợp tinh dầu-ethanol 70⁰ tỷ lệ 7:3 (TB)	93,31	6,04	-	**99,35**
p (5-4), p (6-4)	< 0,001	< 0,05		< 0,001

Kết quả ở bảng 3.8 cho thấy, lô chứng (4) có tỷ lệ muỗi chết trung bình là 0,33%, tỷ lệ muỗi bị ức chế hút máu/ngã trung bình là 0,67%. Do đó tỷ lệ muỗi chết + ức chế hút máu trung bình là 1% . Lô 5 và 6 có tỷ lệ xua muỗi *Anopheles minimus* tương ứng là 99,33% và 99,35%, cao hơn có ý nghĩa thống kê so với lô chứng (các giá trị $p < 0,001$).

Bảng 3.12. Ảnh hưởng của tinh dầu húng quế với *Culex tritaeniorhynchus* (trung bình của 3 lần thử nghiệm, n = 300 muỗi/lô)

Lô	Tỷ lệ trung bình muỗi chết (%) (Abbott)	Tỷ lệ trung bình muỗi bị ức chế hút máu (%)	Tỷ lệ trung bình muỗi no máu (%)	Tỷ lệ trung bình muỗi chết + ức chế hút máu (%)
(7)-chứng (TB)	1	0	99,0	**1**
(8)- Tinh dầu nguyên chất (TB)	96,29	3,67	-	**99,96**
(9)- Hỗn hợp tinh dầu-ethanol 70^0 tỷ lệ 7:3 (TB)	91,57	8,33		**99,90**
p (8-7), p (9-7)	< 0,001	< 0,05		< 0,001

Ở lần 1 và lần 3, số muỗi ở lô chứng chết tự nhiên là 1 và 2 con. Tỷ lệ muỗi chết + bị ức chế hút máu trung bình của lô chứng và lô 8, 9 lần lượt là 1%, 99,96% và 99,90%. Sự khác biệt giữa lô thí nghiệm với lô chứng có ý nghĩa thống kê với $p < 0,001$.

BÀN LUẬN

Aedes, Anopheles và *Culex* là 3 loài muỗi chính lan truyền bệnh ở người trong số hơn 2700 loài muỗi. Trong đó, *Aedes aegypti* là vector truyền rất nhiều bệnh nguy hiểm sang người như: sốt xuất huyết, sốt vàng da, Chikungunya và virus Zika,...[4]. Việt Nam là nước nhiệt đới, có khí hậu nóng ẩm gió mùa, rất thích hợp cho các loài muỗi trên phát triển và lan truyền bệnh. Vì thế, bên cạnh việc phát triển các thuốc chữa bệnh do muỗi truyền, nghiên cứu các thuốc có tác dụng xua hay diệt muỗi cũng rất cần thiết và cấp bách.

Tác dụng của tinh dầu húng quế với loài *Aedes aegypti*

Trong nghiên cứu này, tinh dầu húng quế và hỗn hợp tinh dầu – ethanol 70^0 (7:3) đã được thử nghiệm 3 lần trên 900 muỗi *Aedes aegypti*. Kết quả cho thấy, các mẫu thử trên có tác dụng xua muỗi *Aedes aegypti* trung bình lần lượt là 99,99% và 99,98%, cao hơn có ý nghĩa thống kê so với lô chứng ($p < 0,001$). Ở lần thí nghiệm 1 và 3, lô chứng không có muỗi nào chết hoặc ngã, tất cả muỗi ở lô chứng (100%) đều no máu. Nhưng ở lần thử nghiệm thứ hai, lô chứng có 01 muỗi chết, chưa hút máu, tỷ lệ 1%; còn lại 99/100 muỗi (99%) no máu.

Ở cả ba lần thử, muỗi được chọn vào nghiên cứu đều đạt tiêu chuẩn thí nghiệm. Việc có 01 muỗi ở lô chứng chết là hoàn toàn ngẫu nhiên, mang tính sinh học và không ảnh hưởng đến kết quả nghiên cứu. Tinh dầu nguyên chất và tinh dầu 70% (pha trong ethanol 70^0) có tác dụng diệt muỗi gần tương đương nhau, mặc dù tinh dầu nguyên chất có xu hướng tác dụng tốt hơn, nhưng sự khác biệt giữa hai lô 2 và 3 không có ý nghĩa thống kê ($p > 0,05$). Như vậy, theo quy định của WHO [7], cả hai mẫu thử đều có tác dụng xua muỗi tốt.

Kết quả nghiên cứu này cũng phù hợp với một số nghiên cứu của các tác giả khác, tuy nhiên tinh dầu húng quế được pha trong các dung môi khác nhau. Một nghiên cứu ở Kenya cho thấy, tinh dầu húng quế có tác dụng bảo vệ 100% khỏi muỗi *Aedes aegypti* [5] cho thấy tinh dầu có tác dụng xua muỗi rất tốt. Năm 2013, Kiplang'at K.P. và cộng sự đã công bố tinh dầu húng quế pha trong dầu khoáng tự nhiên (Vaseline Pure Petroleum Jelly) với tỷ lệ 3% có tác dụng bảo vệ da thỏ khỏi 100% muỗi *Aedes aegypti* [2].

Sritabutra D. và cộng sự (2013) [6] đã nghiên cứu tác dụng xua *Aedes aegypti* (Linn.) của hỗn hợp tinh dầu húng quế - dầu oliu (1:9) và tinh dầu húng quế - dầu dừa (1:9) trên lâm sàng. Kết quả cho thấy hỗn hợp tinh dầu húng quế - dầu oliu (1:9) có tác dụng bảo vệ khỏi *Aedes aegypti* trong 41.25 ± 23.67 phút với tỷ lệ xua là 98,6%. Hỗn hợp tinh dầu húng quế - dầu dừa (1:9) có tác dụng bảo vệkhỏi *Aedes aegypti* trong 73.50±12.37 phút và tỷ lệ xua là 98,87%.

Tác dụng của tinh dầu húng quế với muỗi *Anopheles minimus*

Ở lần thử nghiệm thứ nhất, lô chứng không có muỗi ngã hoặc bị ức chế hút máu, số muỗi no máu là 100%. Trong lần thử nghiệm thứ hai, lô chứng có 1 (1%) muỗi chết và số muỗi no máu là 99%. Lần thử thứ ba, lô chứng có 1 muỗi chết và 1 (1%) muỗi và 1 (1%) muỗi bị ngã nên không sang được buồng nhốt động vật và không hút được máu, tỷ lệ muỗi no máu là 98%. Các trường hợp muỗi chết hoặc ngã này là tự nhiên, trong giới hạn cho phép và số liệu của các lô còn lại được hiệu chỉnh theo Abbott. Kết quả trung bình của 3 lần thử nghiệm cho thấy, tinh dầu húng quế và hỗn hợp tinh dầu – ethanol 70^0 (7:3) có tác dụng xua muỗi tốt, với tỷ lệ xua trung bình lần lượt là 99,33 và 99,35%, cao hơn có ý nghĩa thống kê so với lô chứng (1,0%, $p < 0,001$).

Hỗn hợp tinh dầu pha trong ethanol 70^0 (tỷ lệ 7:3) dường như có tác dụng xua cao hơn tinh dầu nguyên chất, nhưng sự khác biệt không có ý nghĩa thống kê

Kết quả này cũng phù hợp với nghiên cứu của Arthi và cộng sự khi đánh giá tác dụng của tinh dầu húng quế và spinosad (hỗn hợp thuốc diệt côn trùng spinosad A – spinosad D tỷ lệ 5:1) cho thấy có tác dụng diệt 85% muỗi *Anopheles stephensi* [5]. Điều này cho thấy có thể loài *Anopheles stephensi* không nhạy cảm với tinh dầu húng quế so với loài *Anopheles minimus*.

Tác dụng của tinh dầu húng quế với loài *Culex tritaeniorhynchus*

Ở lần 1 và lần 3, lô chứng có lần lượt 1 (1%) và 2 (2%) muỗi *Culex tritaeniorhynchus* chết tự nhiên, số muỗi no máu là 99% và 98%, đạt yêu cầu về giới hạn của lô chứng (< 10%). Lô chứng ở lần 2 không có muỗi nào chết, tỷ lệ muỗi no máu là 100%.

Ở cả ba lần thử nghiệm, tỷ lệ muỗi chết ở lô tinh dầu nguyên chất cao hơn so với lô tinh dầu pha trong ethanol 70^0, nhưng sự khác biệt không có ý nghĩa thống kê và tỷ lệ trung bình muỗi chết + ức chế hút máu của hai lô cũng khác nhau không có ý nghĩa thống kê (99,96% so với 99,90%, $p > 0,05$).

Kết quả nghiên cứu này cũng phù hợp với nghiên cứu của Sritabutra D. và cộng sự (2013) [8] khi đánh giá tác dụng xua *Culex quinquefasciatus* (Say.) của hỗn hợp tinh dầu húng quế - dầu oliu (1:9) và tinh dầu húng quế - dầu dừa (1:9) trên lâm sàng. Hỗn hợp tinh dầu húng quế - dầu oliu (1:9) có tác dụng bảo vệ khỏi *Culex quinquefasciatus* trong 97.50 ± 9.00 phút với tỷ lệ xua là 98,6%. Hỗn hợp tinh dầu húng quế - dầu dừa (1:9) có tác dụng bảo vệ khỏi *Culex quinquefasciatus* 84.00±10.39 phút và tỷ lệ xua là 98,97%.

Nguyễn Thị Minh Thu và cộng sự (2020) đã nghiên cứu tác dụng xua muỗi của dịch chiết húng quế trên một số loài muỗi lưu hành ở Việt Nam cho thấy dịch chiết húng quế ở các nồng độ 100 g/100 mL và 200 g/100 mL có tác dụng xua trung bình lần lượt với các loài muỗi là: 62,71% và

76,12% muỗi *Aedes aegypti*, 55,25% và 60,05% muỗi *Anopheles minimus*, 51,85% và 60,49% muỗi *Culex tritaeniorhynchus*. Dịch chiết húng quế có xu hướng xua *Aedes aegypti* tốt hơn so với *Anopheles minimus* và *Culex tritaeniorhynchus*. Tuy nhiên, sự khác biệt chưa có ý nghĩa thống kê ($p > 0,05$). [1]

Rất ít nghiên cứu trên thế giới tiến hành đánh giá tác dụng xua muỗi của húng quế, đặc biệt chưa có nghiên cứu nào công bố tác dụng của tinh dầu húng quế với loài *Anophenles minimus* và *Culex tritaeniorhynchus*.

KẾT KUẬN

Đã nghiên cứu tác dụng xua muỗi của tinh dầu húng quế nguyên chất và hỗn hợp tinh dầu - ethanol 70^0 (7:3). Kết quả cho thấy, hai mẫu thử trên có tác dụng xua trung bình lần lượt là: 99,99% và 99,98% muỗi *Aedes aegypti*, 99,33% và 99,35% muỗi *Anopheles minimus*, 99,96% và 99,90% muỗi *Culex tritaeniorhynchus*. Tinh dầu húng quế có tác dụng xua tốt với cả ba loài *Aedes aegypti, Anopheles minimus* và *Culex tritaeniorhynchus*.

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TẠP CHÍ

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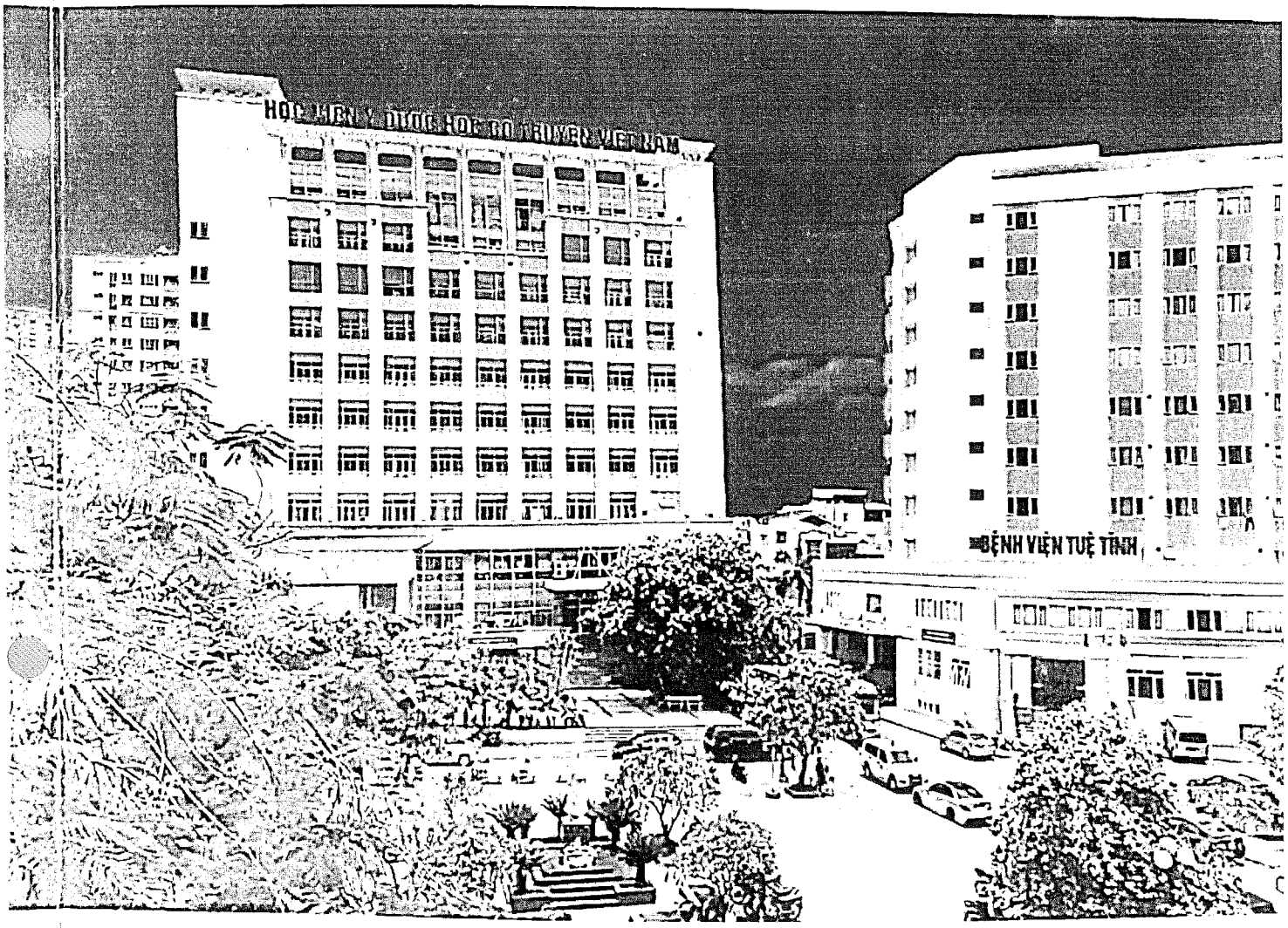

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BÀI NGHIÊN CỨU

Nghiên cứu ảnh hưởng của dịch chiết húng quế *Ocimum basilicum* L. *lamiaceae* đến chức năng gan thỏ

EFFECTS OF BASIL (*OCIMUM BASILICUM* L. *LAMIACEAE*) EXTRACT ON EXPERIMENTAL RABBITS' HEPATIC FUNCTIONS

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TÓM TẮT

Nghiên cứu được tiến hành từ tháng 5 - 7 năm 2021, tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và Trường Đại học Y Hà Nội.

Mục tiêu: *Thử ảnh hưởng của dịch chiết nước Húng quế (Ocimum basilicum L. Lamiaceae) đến chức năng gan thỏ.*

Phương pháp: *Thỏ được chia làm 3 nhóm uống dịch chiết nước húng quế với 2 liều 0,6 và 1,8 g/kg/ngày và nước cất x 28 ngày liên tiếp. Xét nghiệm sinh hoá máu tĩnh mạch tai ngày N0, N14 và N29 đánh giá: AST, ALT, bilirubin toàn phần, protein toàn phần,. Mổ 50% số thỏ ở mỗi lô vào ngày N29 và mổ nốt thỏ ở ngày N43 để đánh giá đại thể và vi thể gan.*

Kết quả: *Chỉ số AST, ALT, bilirubin và protein toàn phần của thỏ ở hai lô dùng thuốc khác biệt không có ý nghĩa thống kê so với lô chứng tại các 3 thời điểm N0, N14 và N29; không có sự khác biệt có ý nghĩa ở các ngày N14 và N29 so với N0 (các giá trị p > 0,05). Ngoại trừ, hàm lượng protein toàn phần ở lô uống dịch chiết liều 0,6 g/kg/ngày tăng lên có ý nghĩa thống kê ở N14 so với N0 (p < 0,05) nhưng ở ngày N29 khác biệt không có ý nghĩa thống kê so với N0 (p > 0,05). Về đại thể, nhu mô gan thỏ ở tất cả các lô tại N29 và N43 đều bình thường, gan mềm, mịn, đồng nhất, đỏ tươi. Cấu trúc vi thể tế bào gan bình thường, không thoái hóa hay hoại tử, bào tương sáng. Có 1/5 thỏ lô chứng và 2/5 thỏ ở mỗi lô dùng thuốc có sung huyết nhẹ ở tĩnh mạch trung tâm và xoang mạch, khác biệt không có ý nghĩa thống kê (p > 0,05).*

Kết luận: *Dịch chiết nước húng quế không ảnh hưởng đến chức năng gan thỏ thí nghiệm.*

Từ khóa: *Húng quế, Ocimum basilicum L. Lamiaceae, dịch chiết nước, chức năng gan.*

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ABSTRACT

This study was carried out between May and July, 2021 at National Institute of Malariology, Parasitology and Entomology, and Hanoi University of Medicine, Vietnam.

Objective: *To assess the effects of basil water extract (Ocimum basilicum L. Lamiaceae) on experimental rabbits' hepatocellular functions.*

Methods: *The Vietnam Ministry of Health's and OECD's guidelines for sub-chronic toxicity testing were applied. The aqua basil extract was treated orally in two different groups with the dose regimens of 0.6 and 1.8 g/kg/day x 28 consecutive days, respectively. A control group treated orally with distilled water was also tested simultaneously. Two milliliters of blood were pulled out from each rabbit's ear vein on day 0 (before testing), day 14 (the middle of testing) and day 29 (after just stopping taking basil extract) in order to test for biochemical parameters including AST, ALT, total bilirubin and total protein. Less than half of rabbits from each group were operated on day 29 and the others were finished operations on day 43 (after 15 days taking no basil extract) for general liver observations and microbody structures of liver cells.*

Results: *The concentrations of AST, ALT, total bilirubin and protein in rabbits' serum samples of the treated groups were insignificantly different from that of the control group on the same testing days; furthermore, there were unremarkable changes of these parameters between days 14 or 29 and day 0 (the p values > 0.05) except for total protein in the second group on day 14. The second group treated with basil extract at the dose regimen of 0,6 g/kg/day had a significantly higher total protein concentration on day 14 than that on day 0 ($p > 0.05$) but an insignificant change on day 29 compared to that on day 0 ($p < 0.05$). Besides, macroscopic liver parenchyma of all rabbits on days 29 and 43 were normal, soft, smooth, homogeneous and brightly red. Microscopically, liver cells had no necrosis and degeneration and liver textures were in normal limits with bright cytoplasms. There were 1/5 (20%) rabbits in the control group and 2/5 rabbits (40%) of each treated groups which had mild congestions in the central veins and sinuses on day 29 ($p > 0.05$) while the others had expected textures. In general, the aqua basil extract did not affect experimental rabbits' hepatic functions.*

Keywords: *Basil, Ocimum basilicum L. Lamiaceae, aqua basil extract, AST, ALT, total protein, total bilirubin, hepatic parenchyma, macroscopic, microscopic, congestion.*

ĐẶT VẤN ĐỀ

Húng quế (*Ocimum basilicum* L. *Lamiaceae*) là cây thuốc nổi tiếng trong y học cổ truyền của nhiều nước. Húng quế được dùng làm thuốc bổ và thuốc trừ giun. Trà húng quế (dùng nóng) điều trị buồn nôn, đầy hơi, chứng kiết lỵ. Tinh dầu húng quế rất có hiệu quả làm giảm mệt mỏi về tinh thần, cảm lạnh, viêm mũi, co thắt, và là thuốc ưu tiên dùng để trị ong đốt và rắn cắn [5]. Húng quế rất hiệu quả trong điều trị bệnh tim, bệnh về máu, bệnh bạch bì... Nước ép húng quế làm giảm chứng đau. Dịch chiết húng quế dùng để trị chứng đau đầu, đau do bệnh gout, cải thiện chức năng đường tiêu hóa, nhuận tràng nhẹ và làm nước súc miệng để chữa hơi thở hôi. Ngoài ra còn có tác dụng giảm đau khi sinh đẻ [5], [10]. Ở Việt Nam, húng quế từ lâu đã được dùng chủ yếu làm gia vị cho các món ăn (lá và ngọn) hoặc để uống cho mát (hạt é) [1].

Trên thế giới, một số nghiên cứu về húng quế cho thấy, húng quế nói chung được dung nạp tốt, hầu như rất ít tác dụng không mong muốn khi sử dụng liên tục tới 13 tuần. Sử dụng quá liều húng quế

hoặc lạm dụng trong thời gian dài có thể gây khó thở, thở gấp, ho hoặc tiểu ra máu; hạ đường huyết, làm loãng máu hoặc chậm quá trình đông máu; ung thư niêm mạc gan; có thể làm co thắt cổ tử cung với phụ nữ mang thai, gây biến chứng trong khi sinh [5] [10]. Chưa có công trình nghiên cứu nào công bố về ảnh hưởng của dịch chiết húng quế đến mô bệnh học của gan. Đặc biệt, các nghiên cứu về tính an toàn của húng quế ở Việt Nam hầu như chưa có.

Vì vậy, để phát triển thuốc có nguồn gốc dược liệu, nghiên cứu này được tiến hành nhằm đánh giá ảnh hưởng của dịch chiết húng quế tới chức năng gan thỏ thí nghiệm với liều bán trường diễn 28 ngày.

VẬT LIỆU VÀ PHƯƠNG PHÁP

Thời gian và địa điểm nghiên cứu

Nghiên cứu được tiến hành từ tháng 5 - 7 năm 2021, tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và Trường Đại học Y Hà Nội.

Đối tượng và vật liệu nghiên cứu

Mẫu nghiên cứu

Phần trên mặt đất của cây húng quế còn tươi được thu hái từ tháng 5-6/2021 tại Yên Xá, Tân Triều, Thanh Trì, Hà Nội. Sau khi chiết với nước lấy dịch với tỷ lệ 1:1 (100 g/100 ml), tiếp tục cô cách thủy tiếp để được các dịch chiết với các nồng độ khác nhau dùng trong thử nghiệm.

Động vật dùng trong nghiên cứu

Thỏ trưởng thành (*Oryctolagus cuniculus* L.), 2,1± 0,2 kg, 2 tháng tuổi, khỏe mạnh, không phân biệt đực - cái, do Trung tâm nghiên cứu Dê và Thỏ Sơn Tây cung cấp. Thỏ cái chưa sinh sản lần nào và đang không mang thai. Động vật được nuôi 7 ngày trong điều kiện chuẩn trước khi nghiên cứu.

Hóa chất dùng trong nghiên cứu

- Nước cất hai lần, các dung dịch xét nghiệm sinh hóa máu dành cho máy bán tự động của Boehringer (Đức), ethanol 950, ethanol tuyệt đối, xylen, formol, dung dịch hematoxylin, eosin 1%, thuốc thử Schiff (acid periodic 1%), baume Canada, paraffin.

Thiết bị và dụng cụ dùng trong nghiên cứu

- Máy cất nước hai lần Aquatron (hãng Bibby sterilin, Anh), cân Sauter, độ chính xác d = 0,1 mg, máy Photometer 5010 của hãng Boehringer-Mannheim (Đức) để xét nghiệm sinh hóa, máy chuyển tự động STP 120 (hãng Microm, Đức), máy đúc khối nến AP 280-1 (Microm - Đức), máy cắt tiêu bản vi thể (microtome - Đức), bàn hơ tiêu bản Prolabo (hot plate - Pháp), lưỡi dao cắt vi thể 1 lần S35 (Nhật), lam kính, lamen, kính hiển vi Nihon (Nhật), bộ bể nhuộm (Nhật).

Phương pháp tiến hành

Tiến hành theo hướng dẫn của Bộ Y tế và OECD [2], [8], thỏ được đánh dấu, cân trọng lượng và chia ngẫu nhiên vào 3 lô tiến hành cho uống nước và dịch chiết trong 28 ngày:

- Lô 1 (n = 11): chứng, nước cất 2 lần, thể tích tương đương liều điều trị.

- Lô 2 (n = 11): dịch chiết nước húng quế liều 0,6 g/kg/ngày, (tương đương liều dự kiến trên người).

- Lô 3 (n = 12): dịch chiết nước húng quế liều 1,8 g/kg/ngày, (gấp 3 lần liều dự kiến trên người).

Thỏ được lấy máu tĩnh mạch tai ngày N0, N14 (sau khi uống thuốc 2 giờ) và N29; uống thuốc mỗi ngày một lần vào buổi sáng bằng sonde dạ dày.

Ngày thứ 29, mổ 50% số thỏ ở cả ba lô, lấy các mô gan để làm tiêu bản đánh giá ảnh hưởng của thuốc. Số thỏ còn lại được nuôi bình thường, ngày thứ 15 sau dừng thuốc (N43), mổ hết để đánh giá sự hồi phục của các tổ chức gan. Các mẫu tiêu bản gan được xử lý như nhau [9].

Phương pháp xác định các chỉ số sinh hóa máu:

Hoạt tính AST (aspartat amino transferase), ALT (alanin amino transferase), hàm lượng

bilirubin, protein toàn phần và creatinin được xác định theo kỹ thuật thường quy.

Phương pháp nghiên cứu mô bệnh học:

Lấy đại diện 5 mẫu gan ở mỗi thỏ tại các vị trí khác nhau. Các mẫu được cắt mỏng từ 3-5 mm, cố định trong dung dịch formol 10% trong 48 giờ. Sau đó, khử nước, đúc parafin (khối nến), và cắt mảng 3µm. Nhuộm hematoxylin eosin (HE). Để xác định khả năng dự trữ glycogen của gan, dùng phương pháp nhuộm periodic acid Schiff (phản ứng PAS) [6], [9].

Chỉ tiêu đánh giá

Chỉ số sinh hóa: AST (U/L), ALT (U/L), creatinin (µmol/L), protein (g/L), bilirubin toàn phần (µmol/L) tại các ngày N0, N14 và N29.

Chỉ số mô bệnh học gan: Những biến đổi của hình thái vi thể gan thỏ giữa các lô ở N29 và N43.

Xử lý số liệu

Số liệu được biểu thị bằng trị số trung bình ± độ lệch chuẩn (M ± SD). Các số liệu được xử lý bằng chương trình Excel 2016 theo phương pháp thống kê y học với cỡ mẫu nhỏ (< 30), sử dụng t-test Student và Fisher's exact test để so sánh.

KẾT QUẢ NGHIÊN CỨU

Tác dụng trên sinh hoá máu

Bảng 1. Hàm lượng AST (U/L) trong huyết thanh thỏ

Lô	N0	N14	N29	p (N0-N14)	p (N0-N29)
Lô 1 (n = 11)	21,55 ± 7,59	20,64 ± 6,5	24,36 ± 5,28	> 0,05	> 0,05
Lô 2 (n = 11)	23,73 ± 11,94	18,36 ± 5,35	21,27 ± 6,54	> 0,05	> 0,05
Lô 3 (n = 12)	20,08 ± 5,87	18,58 ± 5,73	22,83 ± 9,62	> 0,05	> 0,05
p(1-2), *p*(1-3), *p*(2-3)	> 0,05	> 0,05	> 0,05		

Hàm lượng AST không khác nhau tại mỗi thời điểm giữa các nhóm, và tại các thời điểm trong mỗi nhóm ($p > 0,05$).

Bảng 2. Hàm lượng ALT (U/L) trong huyết thanh thỏ

Lô	N0	N14	N29	p (N0-N14)	p (N0-N29)
Lô 1 (n = 11)	43,82 ± 14,20	50,18 ± 11,94	51,27 ± 7,80	> 0,05	> 0,05
Lô 2 (n = 11)	53,91 ± 9,38	57,36 ± 9,89	51,91 ± 9,76	> 0,05	> 0,05
Lô 3 (n = 12)	50,58 ± 12,43	51,25 ± 8,49	46,92 ± 8,78	> 0,05	> 0,05
p(1-2), *p*(1-3), *p*(2-3)	> 0,05	> 0,05	> 0,05		

Hàm lượng ALT không khác nhau tại mỗi thời điểm giữa các nhóm, và tại các thời điểm trong mỗi nhóm ($p > 0,05$).

Bảng 3. Hàm lượng bilirubin toàn phần (µmol/L) trong huyết thanh thỏ

Lô	N0	N14	N29	p (N0-N14)	p (N0-N29)
Lô 1 (n = 11)	5,18 ± 1,25	4,64 ± 1,75	5,55 ± 1,44	> 0,05	> 0,05
Lô 2 (n = 11)	4,27 ± 1,01	4,64 ± 0,92	4,36 ± 1,29	> 0,05	> 0,05
Lô 3 (n = 12)	4,67 ± 1,50	5,25 ± 1,36	5,00 ± 1,28	> 0,05	> 0,05
p(1-2), *p*(1-3), *p*(2-3)	> 0,05	> 0,05	> 0,05		

Hàm lượng bilirubin toàn phần không khác nhau tại mỗi thời điểm giữa các nhóm, và tại các thời điểm trong mỗi nhóm ($p > 0,05$).

Bảng 4. Nồng độ protein toàn phần (g/L) của thỏ

Lô	N0	N14	N29	p (N0-N14)	p (N0-N29)
Lô 1 (n = 11)	52,81 ± 6,72	57,19 ± 5,07	58,43 ± 6,33	> 0,05	> 0,05
Lô 2 (n = 11)	53,17 ± 3,61	57,85 ± 5,49	56,21 ± 4,00	= 0,0287	> 0,05
Lô 3 (n = 12)	52,93 ± 4,25	55,67 ± 4,62	54,60 ± 6,97	> 0,05	> 0,05
p(1-2), *p*(1-3), *p*(2-3)	> 0,05	> 0,05	> 0,05		

Protein toàn phần của thỏ ở lô 3 tại N14 và N29 thay đổi không có ý nghĩa thống kê so với N0 và so với lô chứng tại các thời điểm tương ứng ($p > 0,05$). Ở lô 2, Protein ngày N14 tăng có ý nghĩa thống kê so với ngày N0 ($p = 0,0287$), nhưng sự khác biệt này không có ý nghĩa thống kê ở N29 ($p > 0,05$). So với lô chứng và lô dùng thuốc còn lại, hàm lượng protein của lô 2 ở các thời điểm nghiên cứu đều khác nhau không có ý nghĩa thống kê (các giá trị $p > 0,05$).

Tác dụng trên mô bệnh học

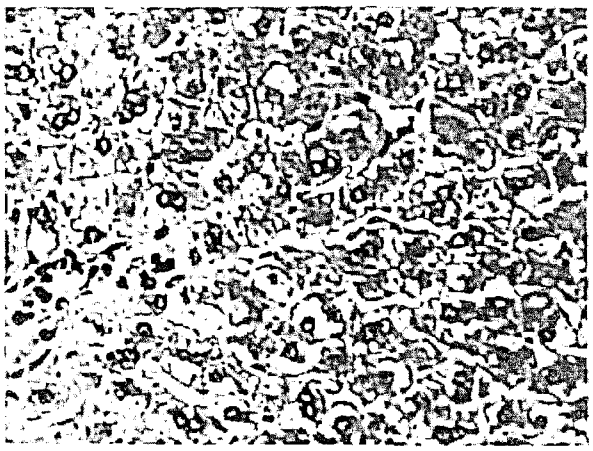
Tại N29 và N43, quan sát không có bất thường về hình thái đại thể của gan.

Hình 1. Mổ thỏ thí nghiệm ở lô uống dịch chiết hùng quế 1,6 g/kg/ngày × 28 ngày

Cấu trúc vi thể gan thỏ như sau:

Cấu trúc vi thể gan thỏ lô chứng:

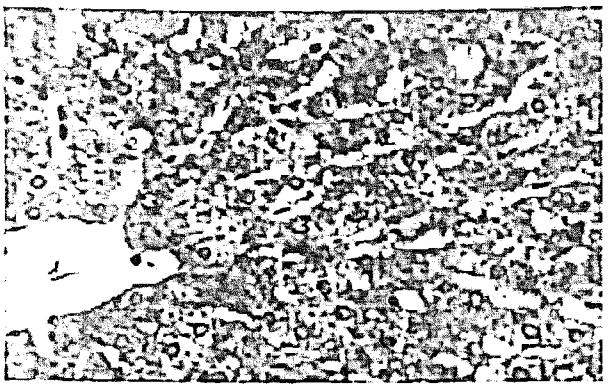
Ở lô chứng, các mẫu xét nghiệm đều bình thường, nhu mô gan không có ổ hoại tử, bào tương sáng có các hạt nhỏ đồng đều. 1/5 thỏ có xoang mạch và tĩnh mạch trung tâm sung huyết nhẹ; PAS dương tính, vừa, đồng đều; khoảng cửa không viêm (hình 2).



Hình 2. Gan thỏ lô chứng (HE × 250) ngày N29
1. Tế bào gan: bình thường; 2. Xoang mạch: bình thường, không sung huyết.

Cấu trúc vi thể gan thỏ uống dịch chiết hùng quế liều 0,6 g/kg/ngày × 28 ngày liên tiếp:

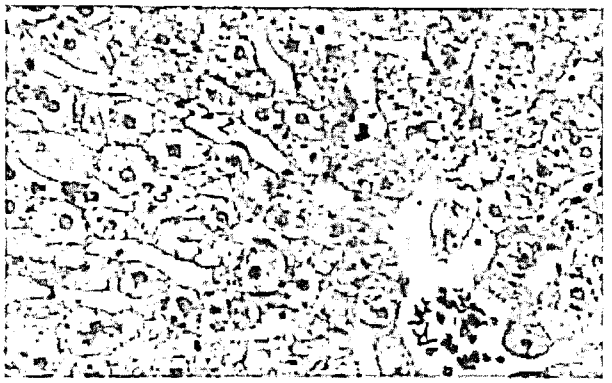
Các mẫu gan ở lô 2 đều bình thường, không thoái hóa hay hoại tử, bào tương sáng, giàu glycogen, PAS dương tính. 2/5 thỏ có sung huyết nhẹ các tĩnh mạch trung tâm và xoang mạch (hình 3), các thỏ khác bình thường (không sung huyết xoang mạch).



Hình 3. Gan thỏ uống dịch chiết húng quế liều 0,6 g/kg/ngày × 28 ngày (HE × 250)
1. Tĩnh mạch trung tâm: có rất ít hồng cầu; 2. Xoang mạch: sung huyết nhẹ; 3. Tế bào gan: bình thường.

Cấu trúc vi thể gan thỏ uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày liên tiếp:

Ở 3/5 thỏ: Tế bào gan sáng, giàu glycogen, PAS (+++), không hoại tử, không sung huyết. Tuy nhiên, 2/5 thỏ có sung huyết nhẹ ở tĩnh mạch trung tâm và xoang mạch, tế bào gan bình thường, không thoái hóa, không hoại tử (hình 4).



Hình 4. Gan thỏ uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày (HE × 250)
1. Tĩnh mạch trung tâm; 2. Xoang mạch: sung huyết; 3. Tế bào gan: bình thường.

Tất cả số thỏ còn lại được mổ ở ngày N43 cho thấy tế bào gan bình thường, không thoái hóa, PAS dương tính, tĩnh mạch trung tâm và xoang mạch không sung huyết (hình 5).

Hình 5. Gan thỏ uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày, sau 15 ngày ngừng thuốc (HE × 250)
1. Tĩnh mạch trung tâm; 2. Tế bào gan; 3. Xoang mạch.

BÀN LUẬN

Gan là một trong những cơ quan đảm nhận nhiều chức năng quan trọng, trong đó có chức năng chuyển hóa và thải trừ các chất ra khỏi cơ thể trong đó có thuốc. Chức năng này tỷ lệ nghịch so với nồng độ các enzym giải phóng từ tế bào gan trong huyết thanh. Quá trình theo dõi cần thực hiện trong suốt quá trình dùng thuốc và nhiều ngày sau đó.

Kết quả nghiên cứu ở các bảng 1 - 4 cho thấy, các chỉ số AST, ALT, protein toàn phần, bilirubin toàn phần ở các lô thử không khác biệt có ý nghĩa thống kê so với lô chứng và kết quả thu được tại N14, N29 không có sự khác biệt so với N0 ($p > 0,05$). Riêng protein toàn phần của lô 2, tại N14 tăng có ý nghĩa thống kê so với ngày N0 ($p = 0,0287$), nhưng không khác biệt so với hai lô còn lại ($p > 0,05$). Ở N29 nồng độ protein của lô 2 khác biệt không có ý nghĩa thống kê so với N0 ($p > 0,05$).

Việc tăng hàm lượng protein toàn phần của lô 2 ở N14 có ý nghĩa thống kê so với N0, trong khi đó, lô 3 uống dịch chiết húng quế liều cao gấp 3 lần so với lô 2 nhưng không có sự khác biệt giữa N14 và N0. Điều này chứng tỏ, đây chỉ là hiện tượng

rối loạn sinh lý thường gặp ở thỏ, tại N29 chỉ số này lại trở về mức bình thường và không khác biệt so với N0, cho thấy dịch chiết húng quế không làm ảnh hưởng tới khả năng sản xuất protein của gan.

Kết quả mổ thỏ ở các ngày N29 và N43 cho thấy, hình thái đại thể gan thỏ ở tất cả các lô thí nghiệm đều bình thường, không quan sát thấy tổn thương, nhu mô gan mịn, đều, màu đỏ tươi.

Cấu trúc vi thể gan thỏ tại N29 cho thấy: Ở lô chứng, tế bào gan bình thường, bào tương sáng, có 1/5 (20%) thỏ có xoang mạch và tĩnh mạch trung tâm sung huyết rất nhẹ. Ở lô 2 và lô 3 (liều 0,6 và 1,8 g/kg/ngày) đều có 2/5 (40%) thỏ sung huyết nhẹ các tĩnh mạch trung tâm và xoang mạch, các tế bào gan bình thường, không thoái hóa hay hoại tử. Các thỏ còn lại có tế bào gan bình thường, không sung huyết ở tĩnh mạch trung tâm hay xoang mạch. Cấu trúc vi thể gan thỏ tại N43 (15 ngày sau khi ngừng thuốc) cho kết quả hoàn toàn bình thường, không thoái hóa, PAS dương tính, tĩnh mạch trung tâm và xoang mạch không sung huyết.

Như vậy, hiện tượng xuất huyết nhẹ tĩnh mạch trung tâm và xoang mạch gặp cả ở cả 3 lô. Mặc dù ở hai lô dùng thuốc, tỷ lệ thỏ xuất huyết cao hơn lô chứng (2/5 và 2/5 so với 1/5) nhưng khác biệt không có ý nghĩa thống kê ($p > 0,05$). Xuất huyết là hiện tượng thường gặp khi thỏ chết và điều này có thể liên quan đến tác động từ bên ngoài (ví dụ thỏ giãy giụa và va đập khi chết). Như vậy, dịch chiết húng quế không ảnh hưởng đến chức năng gan của thỏ thí nghiệm.

Kết quả của nghiên cứu này cũng phù hợp với bài báo của Balin A. và cộng sự (2012), dịch chiết húng quế có tác dụng cải thiện tốt chức năng gan, giúp tiêu hóa tốt và nhuận tràng nhẹ [5]. Tuy nhiên, khi sử dụng quá liều húng quế hoặc lạm dụng trong thời gian dài có thể gây ung thư niêm mạc gan [5] [10].

KẾT KUẬN

Dịch chiết nước húng quế liều 0.6 và 1.8 g/kg/ngày không ảnh hưởng đến chức năng gan thỏ thí nghiệm.

LỜI CẢM ƠN

Nghiên cứu được sự hỗ trợ một phần kinh phí từ Chương trình Phòng chống sốt rét Quốc gia. Chúng tôi xin trân trọng cảm ơn sự phối hợp của cán bộ Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và TS. Trần Văn Minh, Trưởng Khoa Giải phẫu bệnh - Bệnh viện Đại học Y Hà Nội trong công tác xét nghiệm mẫu. Cảm TS. Hoàng Quỳnh Hoa, Trường Đại học Dược Hà Nội trong việc thẩm định mẫu dược liệu.

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MỤC LỤC

SỐ 37 (Chuyên đề Y học)

lệ sử dụng nhà tiêu hợp vệ sinh cao gấp 2,6 lần so với những HGĐ với kinh tế nghèo/ cận nghèo/ gia đình chính sách. Kết quả này tương đồng với nghiên cứu của Trần Thị Hữu năm 2011 với tỷ lệ HGĐ có kinh tế loại nghèo sử dụng nhà tiêu không hợp vệ sinh gấp 3,95 lần so với HGĐ có kinh tế khá hơn. Nghiên cứu này cho thấy có cả mối liên quan giữa tỷ lệ sử dụng nhà tiêu hợp vệ sinh với trình độ học vấn, cụ thể là HGĐ có chủ hộ với trình độ THPT trở nên thì có nhà tiêu hợp vệ sinh về xây dựng và bảo quản cao gấp 2,82 lần so với HGĐ có trình độ học vấn thấp hơn [8]. Cũng phù hợp với kết quả của Dương Chí Nam năm 2014 với tỷ lệ sử dụng nhà tiêu hợp vệ sinh ở HGĐ nghèo/cận nghèo thấp hơn 1,49 lần so với HGĐ khác [9]

KẾT LUẬN

Nghiên cứu này cho thấy các loại nhà tiêu hợp vệ sinh đang được sử dụng tại 5 xã nghiên cứu gồm nhà tiêu tự hoại (72,6%), nhà tiêu khô nổi (19%), nhà tiêu thấm dội nước (7,8%) và nhà tiêu khô chìm (0,6%). Thực trạng sử dụng từng loại nhà tiêu hợp vệ sinh theo QCVN 01:2011/BYT tại các HGĐ đạt thấp (trung bình chung là 58,7%, trong đó 76,2 % đối với nhà tiêu tự hoại; 2,9% đối với nhà tiêu khô nổi; 35,7% đối với nhà tiêu dội nước và 0% đối với nhà tiêu khô chìm. Các yếu tố liên quan đến sử dụng nhà tiêu hợp vệ sinh gồm trình độ học vấn đại diện HGĐ và kinh tế HGĐ.

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NGHIÊN CỨU ẢNH HƯỞNG CỦA 10β-[(2'β-HYDROXY-3'-IMIDAZOL) PROPYL] DEOXOARTEMISININ (32) ĐẾN CHỨC NĂNG GAN CỦA THỎ THỰC NGHIỆM

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TÓM TẮT

Nghiên cứu được tiến hành tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và trường Đại học Y Hà Nội từ tháng 02 - 05 năm 2021 nhằm đánh giá ảnh hưởng của hợp chất 10β-

[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) đến chức năng gan của thỏ thực nghiệm.

Phương pháp: Tiến hành theo hướng dẫn của Bộ Y tế và OECD về thử độc tính bán trường diễn. Hợp chất (32) được dùng bằng đường uống trên thỏ ở 2 mức liều 72 và 216 mg/kg/ngày × 28 ngày liên tiếp. Thử nghiệm tiến hành song song với nhóm chứng. Lấy máu tĩnh mạch tai thỏ để xét nghiệm sinh hóa vào các ngày N0, N14 và N29. Mổ 50% số thỏ ở mỗi lô vào ngày N29 (sau 28 ngày dùng thuốc) và mổ nốt thỏ ở ngày N43 (sau 15 ngày ngừng thuốc) để quan sát đại thể và làm vi thể mô gan. Các chỉ tiêu đánh giá gồm: chỉ số sinh hóa (hàm lượng AST, ALT, protein toàn phần, bilirubin toàn phần), những biến đổi bất thường của hình thái đại thể và vi thể gan thỏ (nếu có).

Kết quả: Hầu hết các chỉ số sinh hóa gan thỏ ở hai lô thử (32) đều không khác biệt có ý nghĩa thống kê so với lô chứng và kết quả tại N14, N29 không khác biệt có ý nghĩa thống kê so với N0 (p > 0,05). Ngoại trừ, ở N14, hàm lượng protein toàn phần ở lô uống (32) liều 72 mg/kg/ngày tăng có ý nghĩa thống kê so với ngày N0 (p < 0,05), tuy nhiên sự khác biệt này không có ý nghĩa thống kê tại N29 (p > 0,05).

Về đại thể, nhu mô gan thỏ ở tất cả các lô tại N29 và N43 đều bình thường, gan mềm, mịn, đồng nhất, đỏ tươi. Ở lô chứng và lô uống (32) liều 72 mg/kg/ngày, cấu trúc vi thể tế bào gan bình thường, không thoái hóa hay hoại tử, bào tương sáng. Có 1/5 thỏ lô chứng và 2/5 thỏ ở mỗi lô dùng thuốc có sung huyết nhẹ ở tĩnh mạch trung tâm và xoang mạch, khác biệt không có ý nghĩa thống kê (p > 0,05). Riêng lô thử (32) liều 216 mg/kg/ngày có 2/6 thỏ (33.33%) có thoái hóa hạt nhẹ ở một số tế bào gan. Tại N43 cấu trúc vi thể gan thỏ ở tất cả các lô đều bình thường.

Kết luận: Hợp chất (32) với liều 72 mg/kg/ngày × 28 ngày liên tiếp bằng đường uống (tương đương liều dùng dự kiến trên người) không ảnh hưởng đến chức năng gan thỏ thí nghiệm. Ở liều 216 mg/kg/ngày × 28 ngày, (32) có xu hướng ảnh hưởng đến tế bào gan của thỏ, nhưng sau 15 ngày ngừng thuốc tế bào gan hồi phục trạng thái bình thường.

Từ khóa: *10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32), thỏ, chức năng gan, chỉ số sinh hóa, AST, ALT, protein toàn phần, bilirubin toàn phần, hình thái đại thể, cấu trúc vi thể.*

SUMMARY

THE EFFECTS OF PROPYL 10β - [(2'β-HYDROXY-3'-IMIDAZOL)]DEOXOARTEMISININ (32) ON RABBIT LIVER FUNCTIONS

This study was conducted at the National Institute of Malariology, Parasitology and Entomology and Hanoi Medical University from February to May, 2021. The compound 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) was treated orally in experimental rabbits in order to examine whether it affected rabbit liver functions or not.

Methods: The Vietnam Ministry of Health's and OECD's guidelines for sub-chronic toxicity testing were applied. The compound (32) was treated orally in two different groups with the dose regimens of 72 and 216 mg/kg/day × 28 consecutive days, respectively. A control group treated orally with solvent was also tested simultaneously. Two milliliters of blood were pulled out from each rabbit's ear vein on day 0 (before testing), day 14 (the middle of testing) and day 29 (after stopping taking 32) in order to test for biochemical parameters including AST, ALT, total bilirubin and total protein. Half of rabbits from each group were operated on day 29 and the others were finished operations on day 43 (after 15 days taking no (32) compound) for general liver observations and microbody structures of liver cells.

Results:

Generally, most of the biochemical parameters of rabbit liver of the two (32)-treated groups were not significantly different from the control group. Besides, those indices on days 14 and 29 did not change significantly compared to before taking the (32) on day 0 (the p values > 0.05). Nevertheless, with the dose of 72 mg/kg/day, rabbits' total protein increased significantly on day 14 comparing to that on day 0 (p < 0.05) and became in normal limits on day 29 (p > 0.05).

Macroscopically, liver parenchyma of all rabbits on days 29 and 43 were normal, soft, smooth, homogeneous and brightly red. Microscopically, most liver cells had no necrosis and degeneration and liver textures were in normal limits with bright cytoplasms. There were 1/5 (20%) rabbits in the control group and 2/5 rabbits (40%) of each treated groups which had mild congestions in the central veins and sinuses on day 29 (p > 0.05) while the others had expected texture. In particular, with the dose of 216 mg/kg/day, 2/6 of rabbits (33.33%) had slight granular degeneration in some hematocytes on day 29 but hepatic microstructures of rabbits were normal on day 43.

Conclusion: The compound (32) dit not affect rabbit's liver functions at the dose regimen of 72 × 28 consecutive days. Nonetheless, at the dose of 216 mg/kg/day × 28 consecutive days, the compound (32) tended to affect rabbit hepatocytes but this adverse drug reaction disappeared after 15 days stopping taking drug.

Keywords: *10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32), rabbit, liver function, biochemical parameter, AST, ALT, total bilirubin, total protein, hepatic parenchyma, macroscopic, microscopic, congestion, granular degeneration.*

ĐẶT VẤN ĐỀ

Sốt rét là bệnh truyền nhiễm do kí sinh trùng *Plasmodium* gây nên, trong số các loài gây bệnh ở người thì *P. falciparum* là loài nguy hiểm hơn cả. Cho đến nay, sốt rét vẫn là một trong những gánh nặng gây áp lực lớn cho nền kinh tế và sức khỏe toàn cầu. Thách thức lớn nhất trong kiểm soát bệnh sốt rét tình trạng kháng thuốc ngày càng lan rộng ở nhiều quốc gia và vùng lãnh thổ. Đáng lo ngại, *P. falciparum* đã ghi nhận đề kháng với hầu hết các thuốc điều trị sốt rét hiện có, đặc biệt là artemisinin (ART) và các dẫn xuất - nhóm thuốc có hiệu lực điều trị KST nhanh và hiệu quả trong những thập kỷ trước [2]. Vì vậy việc nghiên cứu phát triển thuốc sốt rét mới có khả năng chống kháng thuốc và hiệu quả điều trị cao hơn đang là yêu cầu cấp bách được đặt ra với các nhà khoa học.

Viện Hóa học các hợp chất thiên nhiên đã tổng hợp và tinh chế thành công hợp chất 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (dẫn xuất 32). Hợp chất này đã được Viện Sốt rét – KST – CT Trung ương chứng minh hiệu lực *in vitro* tương đương ART [7], có tác dụng chống sốt rét tốt *in vivo* với chủng *P. bergei* kháng cloroquin trên chuột nhắt trắng [8] và không có độc tính cấp đường uống [7],[8]. Các nghiên cứu tiền lâm sàng nói trên cho thấy (32) có triển vọng để điều trị sốt rét. Hiện chưa có nghiên cứu nào theo dõi độc tính dài ngày của (32) trên các cơ quan của động vật thực nghiệm. Nhằm tiếp tục phát triển hợp chất (32), nghiên cứu này được tiến hành nhằm đánh giá ảnh hưởng của (32) đến chức năng gan của thỏ khi cho uống 28 ngày liên tiếp.

ĐỐI TƯỢNG, PHƯƠNG TIỆN VÀ PHƯƠNG PHÁP NGHIÊN CỨU

1. Đối tượng nghiên cứu

Thuốc thử nghiệm: Dẫn xuất 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin **(32)** do Viện Hóa học các hợp chất thiên nhiên cung cấp, độ tinh khiết 99,98%.

Động vật thí nghiệm: Thỏ trưởng thành (*Oryctolagus cuniculus* L.), tổng số 36 con, cân nặng trung bình 2,1± 0,2 kg, 2 tháng tuổi, khỏe mạnh, không phân biệt đực - cái, do Trung tâm nghiên cứu Dê và Thỏ Sơn Tây cung cấp. Với động vật cái phải không mang thai, không nuôi con bú và chưa sinh sản lần nào. Động vật được nuôi ổn định 7 ngày trong điều kiện thí nghiệm trước khi tiến hành nghiên cứu.

2. Phương pháp nghiên cứu

Tiến hành theo hướng dẫn của Bộ Y tế [1] và OECD số 407 [5]. Ảnh hưởng của hợp chất (32) đến chức năng gan của thỏ được đánh giá thông qua việc xác định các chỉ số sinh hóa gan trước, trong và sau dùng thuốc, đồng thời xác định sự biến đổi hình thái vi thể mô gan thỏ ở thời điểm sau 28 ngày dùng thuốc và sau 15 ngày ngưng dùng thuốc.

Thỏ sau khi nuôi ổn định 7 ngày, được chia ngẫu nhiên thành 3 lô, mỗi lô 12 con:

- Lô 1: chứng (n = 12): Uống dung môi gôm arabic 1%, thể tích tương đương liều điều trị thuốc × 28 ngày liên tiếp.
- Lô 2 (n = 12): Uống hỗn dịch (32) liều 72mg/kg/ngày, (tương đương liều dùng dự kiến trên người) × 28 ngày liên tiếp.
- Lô 3 (n = 12): Uống hỗn dịch (32) liều 216mg/kg/ngày, (tương đương gấp 3 lần liều dùng dự kiến trên người) × 28 ngày liên tiếp.

Trước uống thuốc, thỏ được lấy máu tĩnh mạch tai để làm xét nghiệm sinh hóa vào ngày N0. Cho thỏ uống thuốc mỗi ngày một lần vào buổi sáng bằng kim đầu tù, liên tục trong 28 ngày. Các ngày lấy máu tĩnh mạch tai để làm xét nghiệm sinh hóa tiếp theo là N14 (sau khi uống thuốc 2 giờ) và N29. Sau 28 ngày cho uống thuốc liên tục, vào ngày N29, mổ 50% số thỏ ở lô chứng và các lô uống thuốc, lấy các mô gan để làm tiêu bản đánh giá ảnh hưởng của thuốc. Số thỏ còn lại được nuôi bình thường, sau 15 ngày ngừng thuốc, vào ngày N43, mổ hết để đánh giá sự hồi phục của tổ chức gan. Các mẫu tiêu bản gan ở hai đợt được xử lý như nhau.

Phương pháp nghiên cứu mô bệnh học: Sau khi giết động vật thí nghiệm, quan sát tổng thể các mẫu gan một cách sơ bộ, rồi lấy đại diện 5 mẫu gan ở mỗi thỏ tại các vị trí khác nhau. Các mẫu bệnh phẩm được cắt mỏng từ 3-5 mm, cố định trong dung dịch formol 10% trong 48 giờ. Sau đó, khử nước, đúc parafin (khối nến), và cắt mảng 3μm. Nhuộm hematoxylin eosin (HE). Để xác định khả năng dự trữ glycogen của gan,

dùng phương pháp nhuộm periodic acid Schiff (phản ứng PAS).

Các số liệu nghiên cứu được xử lý bằng chương trình Excel 2016 theo phương pháp thống kê y học với cỡ mẫu nhỏ (< 30), sử dụng t-test Student và Fisher's exact test để so sánh các số liệu trước, trong và sau thử nghiệm và so sánh giữa lô dùng thuốc và lô chứng.

KẾT QUẢ NGHIÊN CỨU

Ảnh hưởng của hợp chất (32) đến chức năng gan của thỏ được đánh giá thông qua việc xác định các chỉ số sinh hóa gan trước, trong và sau khi dùng thuốc, đồng thời xác định sự biến đổi hình thái vi thể mô gan thỏ sau khi dùng thuốc và sau khi ngừng thuốc 15 ngày.

Bảng 1. Hàm lượng AST (U/L) trong huyết thanh thỏ tại các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p (N0-N29)*
Lô 1: chứng (n = 12)	22,5 ± 7,96	20,33 ± 8,48	24,42 ± 6,30	> 0,05	*> 0,05*
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	22,00 ± 12,40	16,75 ± 5,12	20,08 ± 7,28	> 0,05	*> 0,05*
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	19,33 ± 6,44	17,92 ± 5,70	21,50 ± 9,79	> 0,05	*> 0,05*
p (1-2), p (1-3), p (2-3)	*> 0,05*	*> 0,05*	*> 0,05*		

Nhận xét: Hàm lượng AST trong huyết thanh thỏ tại N14 và N29 ở các lô uống (32) liều 72 và 216 mg/kg × 28 ngày liên tiếp khác nhau không có ý nghĩa thống kê so với trước khi uống thuốc (N0) và so với lô chứng tại cùng thời điểm tương ứng (các giá trị $p > 0,05$).

Bảng 2. Hàm lượng ALT (U/L) trong huyết thanh thỏ ở các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p (N0-N29)*
Lô 1: chứng (n = 12)	45,17 ± 15,97	51,67 ± 16,72	50,33 ± 7,75	> 0,05	*> 0,05*
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	54,58 ± 9,18	56,75 ± 9,47	49,83 ± 10,65	> 0,05	*> 0,05*
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	49,75 ± 13,01	52,08 ± 8,73	47,75 ± 9,63	> 0,05	*> 0,05*
p (1-2), p (1-3), p (2-3)	*> 0,05*	*> 0,05*	*> 0,05*		

Nhận xét: Hàm lượng ALT ở các lô uống (32) liều 72 và 216 mg/kg/ngày × 28 ngày liên tiếp tại N14 và N29 thay đổi không có ý nghĩa thống kê so với N0 và so với lô chứng tại cùng thời điểm (các giá trị $p > 0,05$).

Bảng 3. Hàm lượng bilirubin toàn phần (µmol/L) trong huyết thanh thỏ tại các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p (N0-N29)*
Lô 1: chứng (n = 12)	5,50 ± 1,57	4,50 ± 1,83	5,25 ± 1,72	> 0,05	*> 0,05*
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	4,58 ± 1,24	4,92 ± 1,08	4,67 ± 1,56	> 0,05	*> 0,05*
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	4,83 ± 1,59	5,25 ± 1,36	5,17 ± 1,34	> 0,05	*> 0,05*
p (1-2), p (1-3), p (2-3)	*> 0,05*	*> 0,05*	*> 0,05*		

Nhận xét: Hàm lượng bilirubin toàn phần trong huyết thanh thỏ ở các lô uống thuốc tại N14 và N29 thay đổi không có ý nghĩa thống kê so với trước khi uống thuốc (N0) và không khác biệt có ý nghĩa thống kê so với lô chứng hay so giữa các lô uống thuốc với nhau tại cùng thời điểm (các giá trị $p > 0,05$).

Bảng 4. Nồng độ protein toàn phần (g/L) trong huyết thanh thỏ ở các thời điểm nghiên cứu

Lô	N0	N14	N29	*p* (N0-N14)	*p* (N0-N29)
Lô 1: chứng (n = 12)	52,91 ± 7,82	56,93 ± 5,58	58,58 ± 6,37	> 0,05	> 0,05
Lô 2: uống (32), 72 mg/kg/ngày × 28 ngày (n = 12)	53,40 ± 3,98	57,59 ± 4,32	56,12 ± 3,60	$p = 0,0217$ (< 0,05)	> 0,05
Lô 3: uống (32), 216 mg/kg/ngày × 28 ngày (n = 12)	53,76 ± 4,55	56,33 ± 5,90	55,77 ± 7,17	> 0,05	> 0,05
p (1-2), *p* (1-3), *p* (2-3)	> 0,05	> 0,05	> 0,05		

Nhận xét: Hàm lượng protein toàn phần của thỏ ở lô uống (32) liều 216 mg/kg/ngày × 28 ngày thay đổi không có ý nghĩa thống kê ở N14 và N29 so với N0 và so với lô chứng tại các thời điểm tương ứng (các giá trị $p > 0,05$). Ở lô uống (32) liều 72 mg/kg/ngày × 28 ngày, hàm lượng

protein tăng có ý nghĩa thống kê ở N14 so với ngày N0 (p = 0,027), nhưng sự khác biệt này không có ý nghĩa thống kê ở N29 (p > 0,05). So với lô chứng và lô dùng thuốc còn lại, hàm lượng protein của lô 2 ở các thời điểm nghiên cứu đều khác nhau không có ý nghĩa thống kê (các giá trị p > 0,05).

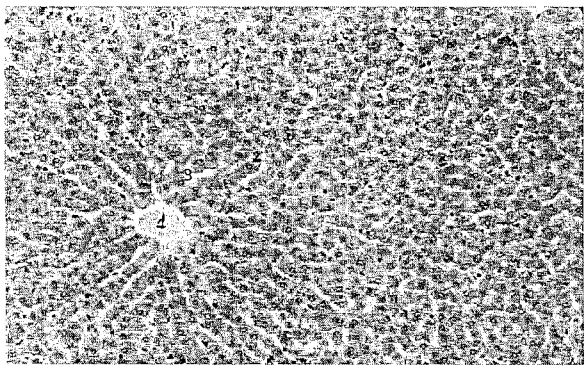
Ở ngày N29, 50% số thỏ được mổ (hình 1) và 50% số thỏ còn lại được nuôi tiếp 15 ngày sau khi ngừng uống thuốc. Ngày N43, mổ tiếp 50% số thỏ còn lại để quan sát đại thể gan. Kết quả cho thấy, không có bất thường về hình thái đại thể gan ở tất cả các lô thí nghiệm.

Hình 1. Mổ thỏ thí nghiệm ở lô uống thuốc (32) liều 216 mg/kg/ngày ×28 ngày

Cấu trúc vi thể gan thỏ (được đánh giá bởi TS. Trần Văn Minh, Trưởng Khoa Giải phẫu bệnh, Bệnh viện Đại học Y Hà Nội) như sau:

Cấu trúc vi thể gan thỏ lô chứng:

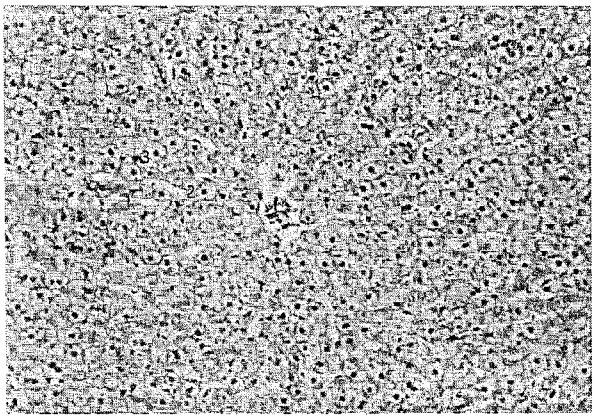
Ở lô chứng, các thỏ được mổ (6 con) có tế bào gan trong giới hạn bình thường, nhu mô gan không có ổ hoại tử, bào tương sáng có các hạt nhỏ đồng đều, 1/6 thỏ có xoang mạch và tĩnh mạch trung tâm sung huyết nhẹ; PAS dương tính, vừa, đồng đều; khoảng cửa không viêm (hình 2).



Hình 2. Gan thỏ lô chứng (HE × 100) ngày N29
1. Tĩnh mạch trung tâm;
2. Tế bào gan: bình thường;
3. Xoang mạch: không sung huyết.

Cấu trúc vi thể gan thỏ uống (32) liều 72 mg/kg/ngày × 28 ngày liên tiếp:

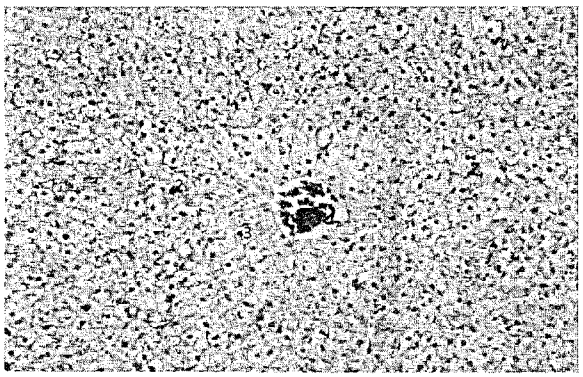
Tế bào gan không thoái hoá hay hoại tử, bào tương sáng, giàu glycogen, PAS dương tính. Có 2/6 thỏ có sung huyết nhẹ các tĩnh mạch trung tâm và xoang mạch (hình 3), các thỏ khác bình thường (không sung huyết xoang mạch).



Hình 3. Gan thỏ uống (32) liều 72 mg/kg/ngày × 28 ngày (HE × 100)
1. Tĩnh mạch trung tâm: có rất ít hồng cầu;
2. Tế bào gan: bình thường; 3. Xoang mạch: sung huyết nhẹ.

Cấu trúc vi thể gan thỏ uống (32) liều 216 mg/kg/ngày × 28 ngày liên tiếp:

Tế bào gan sáng, giàu glycogen, PAS (+++), không có hoại tử tế bào, không sung huyết ở 4/6 thỏ. Tuy nhiên, 2/6 thỏ có sung huyết nhẹ ở tĩnh mạch trung tâm và xoang mạch, thoái hoá hạt nhẹ ở một số tế bào gan (hình 4).



Hình 4. Gan thỏ uống (32) liều 216 mg/kg/ngày × 28 ngày (HE × 100)
1. Tĩnh mạch trung tâm;
2. Xoang mạch: sung huyết;
3. Tế bào gan: thoái hoá hạt nhẹ

Số thỏ được mổ ở ngày N43 cho thấy tế bào gan bình thường, không thoái hóa, PAS dương tính, tĩnh mạch trung tâm và xoang mạch không sung huyết (hình 5).

Hình 5. Gan thỏ uống (32) liều 216 mg/kg/ngày × 28 ngày, sau 15 ngày ngừng thuốc (HE × 250)
1. Tĩnh mạch trung tâm;
2. Xoang mạch;
3. Tế bào gan.

BÀN LUẬN

Trong cơ thể, gan đảm nhận nhiều chức năng quan trọng trong đó có chức năng chuyển hóa và thải trừ các chất. Khi đưa vào cơ thể một liều thuốc lớn hoặc kéo dài có thể làm tổn thương các tế bào gan hay làm suy giảm chức năng gan. Gan chứa rất nhiều enzym làm nhiệm vụ xúc tác các phản ứng chuyển hóa các chất trong tế bào. Khi tế bào gan bị tổn thương, một lượng enzym sẽ được giải phóng vào huyết thanh. Do đó, việc xác định hoạt độ những enzym này trong huyết thanh sẽ giúp đánh giá được các tổn thương của gan, nếu tổn thương càng rộng, càng cấp tính thì lượng enzym giải phóng vào máu càng nhiều. Trái lại, nếu tổn thương mãn tính âm ỉ thì gan chỉ giải phóng một lượng enzym vừa phải vào máu.

Để đánh giá mức độ ảnh hưởng của hợp chất (32) tới chức năng gan thỏ, chúng tôi tiến hành xác định hàm lượng các enzym AST, ALT, nồng độ protein toàn phần, bilirubin toàn phần trong huyết thanh thỏ tại các thời điểm N0 (trước uống thuốc), N14 và N29, đồng thời xác định sự biến đổi hình thái vi thể mô gan thỏ ở N29 và N43 (sau khi ngừng dùng thuốc 15 ngày).

Kết quả nghiên cứu được thể hiện ở các bảng từ 1 đến 4 cho thấy hầu hết các chỉ số như hàm lượng AST, ALT, nồng độ protein toàn phần, bilirubin toàn phần trong huyết thanh thỏ ở các lô thử không có sự khác biệt so với lô chứng và kết quả thu được tại N14, N29 không có sự khác biệt so với N0 ($p > 0,05$). Chỉ có hàm lượng protein của lô thử (32) liều 72 mg/kg/ngày × 28 ngày tại N14 tăng có ý nghĩa thống kê so với ngày N0 ($p = 0,027$), tuy nhiên sự khác biệt này không có ý nghĩa thống kê tại N29 ($p > 0,05$).

Tình trạng tăng hàm lượng protein trong máu ở N14 có ý nghĩa thống kê so với N0 là hiện tượng rối loạn sinh lý thường gặp ở thỏ, tại N29 chỉ số này lại trở về mức bình thường và khác biệt không có ý nghĩa thống kê so với N0, cho thấy hợp chất (32) không làm ảnh hưởng tới khả năng sản xuất protein của gan.

Mổ thỏ, quan sát hình thái đại thể gan ở tất cả các lô thí nghiệm quan sát được ở N29 và N43 đều không ghi nhận bất thường.

Cấu trúc vi thể gan thỏ tại N29 cho thấy: Ở lô chứng cho kết quả bình thường, 1/6 thỏ có xoang mạch và tĩnh mạch trung tâm sung huyết nhẹ. Các lô thử (32) với liều 72 mg/kg/ngày × 28 ngày và 216 mg/kg/ngày × 28 ngày có 2/6 thỏ (33,3 %) có sung huyết nhẹ tĩnh mạch trung tâm và xoang mạch, riêng lô 3 có 2/6 thỏ có thoái hóa hạt nhẹ ở một số tế bào gan.

Vi thể gan thỏ ở cả lô chứng và lô thử đều có xuất hiện tình trạng xuất huyết ở xoang mạch và tĩnh mạch trung tâm. Tuy nhiên số lượng thỏ gặp phải tình trạng này và mức độ tổn thương tăng dần theo liều của hợp chất (32) chứng tỏ hợp chất (32) có thể gây ra các tổn thương nhẹ trên gan mặc dù hoạt độ các enzym do gan tiết ra vẫn trong giới hạn bình thường.

Cấu trúc vi thể gan thỏ tại N43 (15 ngày sau khi ngừng thuốc) ở cả ba lô cho kết quả hoàn toàn bình thường, không có thoái hóa hạt, PAS dương tính, tĩnh mạch trung tâm và xoang mạch không sung huyết.

Nghiên cứu độc tính bán cấp của trifluoromethylhydroartemisinin (BB101) trên khỉ cho kết quả: Liều 4mg/kg và 8 mg/kg × 30 ngày bằng đường tiêm bắp làm giảm rõ hàm lượng SGPT tại các thời điểm N14 và N30 với lô chứng [3]. Một nghiên cứu độc tính bán trường diễn khác trên khỉ của BB134 – dẫn xuất gắn Fluor của artemisinin thì chỉ ra rằng hợp chất BB134 với liều 9 mg/kg/ngày × 28 ngày không làm thay đổi hàm lượng SGOT, SGPT, hàm lượng bilirubin và protein của thỏ [4].

Papiya Bigoniya và cộng sự khi nghiên cứu về độc tính bán trường diễn của Artesunate đã nhận thấy Artesunate làm gia tăng đáng kể SGOT, SGPT, ALP, TC và TG ở liều 8 mg/kg/ngày × 45 ngày. Hình ảnh mô học gan thận cho thấy: Artesunate liều 2mg/kg/ngày, tế bào gan bình thường và có thâm nhiễm viêm nhẹ. Liều 4 mg/kg/ngày có xảy ra sự biến dạng tế bào, tạo các khoảng trống trong tế bào chất. Liều 8mg/kg/ngày, cho thấy trên gan có hoại tử khu trú, sung huyết và viêm nhiễm rộng [6].

Như vậy, hợp chất (32) ở liều 72 mg/kg/ngày × 28 ngày (tương đương liều dùng dự kiến trên người) không ảnh hưởng đến chức năng gan. Ở

mức liều 216 mg/kg/ngày × 28 ngày (liều cao gấp 3 lần liều tương đương dùng dự kiến trên người), (32) có xu hướng làm tổn thương gan ở cấu trúc vi thể, gây thoái hóa hạt nhẹ ở tế bào gan. Tuy nhiên các tổn thương này còn nhẹ và chưa ảnh hưởng tới các chỉ số sinh hóa của gan (AST, ALT, bilirubin và protein trong huyết thanh) và đều phục hồi sau một khoảng thời gian 15 ngày ngừng thuốc. So với các dẫn xuất khác của Artemisinin như BB101, BB134 và Artesunate, hợp chất (32) thể hiện tính an toàn cao và ít ảnh hưởng tới gan của động vật thí nghiệm hơn.

KẾT LUẬN

Đã nghiên cứu ảnh hưởng của 10β-[(2'β-hydroxy-3'-imidazol) propyl] deoxoartemisinin (32) đến chức năng gan của thỏ thực nghiệm với hai liều đường uống 72 và 216 mg/kg/ngày × 28 ngày liên tiếp. Kết quả cho thấy:

Các chỉ số sinh hóa phản ánh chức năng hoạt động của gan (AST, ALT, bilirubin toàn phần, protein toàn phần) ở các lô thử nghiệm đều bình thường, nhìn chung khác biệt không có ý nghĩa thống kê so với lô chứng và giá trị tại N14, N29 không khác biệt so với N0 ($p > 0,05$).

Tuy nhiên, liều 216 mg/kg/ngày × 28 ngày liên tiếp (tương đương liều gấp 3 lần liều dự kiến dùng trên người) có xu hướng ảnh hưởng nhẹ tới cấu trúc vi thể gan thỏ (33,33% thoái hóa hạt nhẹ ở tế bào gan) và sau 15 ngày ngừng thuốc, vi thể gan ở số thỏ này trở về bình thường.

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KẾT QUẢ VÀ MỘT SỐ TAI BIẾN SAU ĐÌNH CHỈ THAI DƯỚI 12 TUẦN TẠI CƠ SỞ CẢM HỘI, BỆNH VIỆN PHỤ SẢN HÀ NỘI NĂM 2020

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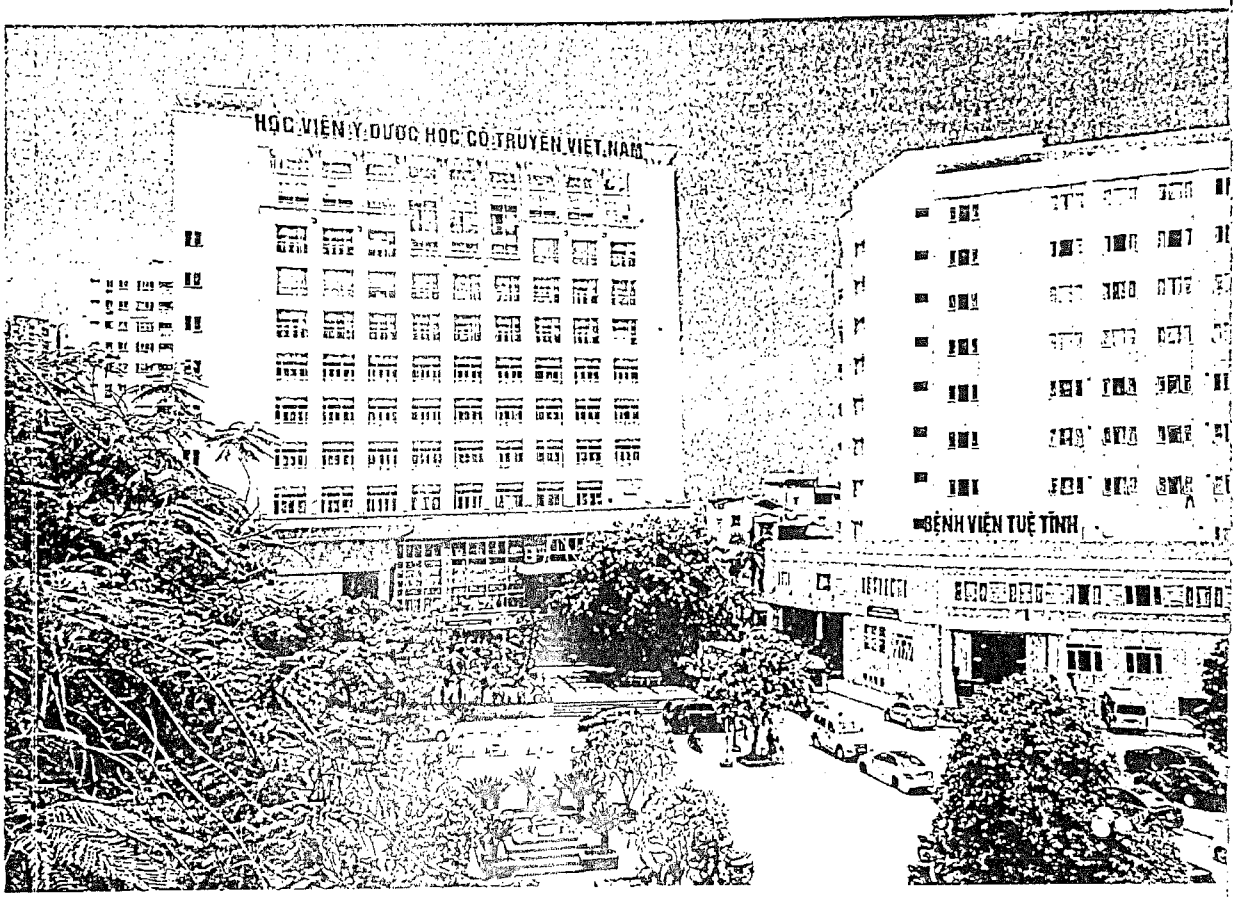

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BÀI NGHIÊN CỨU

Tác dụng *in vivo* và độc tính cấp đường uống của 10β-[(2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32)

IN VIVO EFFECTS AND ORAL ACUTE TOXICITY OF 10β-[(2'β-HIDROXY-3'-IMIDAZOL) PROPYL] DEOXOARTEMISININ (32)

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TÓM TẮT

Nghiên cứu được tiến hành tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương từ tháng 11/2019 đến tháng 5/2020. Hợp chất 10β-[2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32) đã được đánh giá tác dụng diệt ký sinh trùng sốt rét Plasmodium berghei (chủng kháng chloroquin) gây nhiễm ở chuột nhắt trắng theo phương pháp Benazet cải tiến và độc tính cấp đường uống trên chuột nhắt trắng theo hướng dẫn của OECD. Kết quả cho thấy:

Hợp chất (32), với liều 72 mg/kg/ngày x 7 ngày, chỉ có tác dụng ức chế sự phát triển của P. berghei, 100% chuột tái phát và chết sau 28 ngày theo dõi. Ở liều 144 mg/kg/ngày x 7 ngày, hợp chất (32) có khả năng điều trị khỏi 90% số chuột, chỉ 10% chuột tái phát bệnh với mật độ ký sinh trùng thấp và 100% sống sót sau 28 ngày. Với liều 288 mg/kg/ngày x 7 ngày, (32) có tác dụng diệt hoàn toàn P. berghei, điều trị khỏi 100% số chuột sau 28 ngày theo dõi, không có chuột nào tái phát.

Hợp chất (32) không có độc tính cấp, chưa xác định được giá trị LD50 (ngay cả ở liều tối đa đã thử nghiệm là 5.500 mg/kg). Các chuột vẫn hoạt động, ăn uống, bài tiết bình thường sau 7 ngày theo dõi. Mổ quan sát đại thể các cơ quan tim, gan, thận, phổi, bàng quang, ruột của chuột không phát hiện thấy bất thường. Riêng lô chuột uống thuốc liều 5.500 mg/kg, có 3/10 chuột có vài đám sẫm màu nhỏ ở gan, đường kính 2-3 mm.

***Kết luận:** Hợp chất (32) có tác dụng tốt in vivo với chủng P. berghei kháng chloroquin và không có độc tính cấp đường uống.*

***Từ khóa:** 10β-[2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32), tác dụng in vivo, độc tính cấp đường uống, ký sinh trùng sốt rét, Plasmodium berghei kháng chloroquin, tái phát, tỷ lệ điều trị khỏi.*

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ABSTRACT

This study was conducted at National Institue of Malariology, Parasitology and Entomology between November, 2019 and May, 2020. The in vivo effects of compound 10β-[2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32) was assessed on mice that were infected with chloroquine resistant Plasmodium berghei clone, one kind of malaria parasite species, according to the modified Benazet method. Furthermore, this compound was also tested oral acute toxicity on mice following OECD procedure. The results showed that:

At the dose of 72 mg/kg/day x 7 consecutive days, the compound (32) only inhibited Plasmodium berghei's growth in the first week of treatment, then all of these mice (100%) had recrudescence of P. berghei and died after 28 following days. In contrast, mice treated with 144 mg/kg/day x 7 days of (32) survived after 28 following days with the cure rate of 90% and recrudescence rate of 10% at low parasite density. Especially, with the dose regimen of 288 mg/kg/day x 7 consecutive days, the compound (32) had high effect on parasite clearance with 100% of mice had no longer parasites in their blood after 3 days of the treatment. All these mice (100%) were cured and had no recrudescence after 28 days.

The compound (32) had no oral acute toxicity and LD50 value was not found even at the maximum treated dose of 5,500 mg per kg. All mice (100%) had usual action, digestion and excretion on 7 following days. No abnormal signals were found after operating survived mice and observing visually their organs such as hearts, livers, kidneys, lungs, vesicles and intestines except 3/10 mice treated with 5,500 mg/kg had several small red spots on their livers' surface.

Keywords: *10β-[2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32), in vivo effects, oral acute toxicity, malaria parasite, chloroquine resistent Plasmodium berghei, recrudescence, cure rate.*

ĐẶT VẤN ĐỀ

Sốt rét là một trong những bệnh xã hội được y tế cộng đồng rất quan tâm do tỷ lệ mắc và tử vong cao. Mỗi năm, thế giới sử dụng hàng triệu liều thuốc cho dự phòng và điều trị sốt rét. Tại Việt Nam, Chương trình Phòng chống sốt rét Quốc gia đã đạt được nhiều thành tựu đáng kể trong kiểm soát bệnh sốt rét, làm giảm tỷ lệ mắc và tử vong do sốt rét. Tuy nhiên, bệnh vẫn còn diễn biến rất phức tạp với sự di biến động dân cư lớn, mạng lưới y tế chưa ổn định, hạn chế của các nguồn lực và sự gia tăng tình trạng kháng thuốc [2].

Hiện nay, hiện tượng kháng thuốc của ký sinh trùng sốt rét đã lan rộng ở nhiều quốc gia và vùng lãnh thổ, đặc biệt tại các quốc gia trong khu vực Tiểu vùng Sông Mê Kông (GMS), trong đó có Việt Nam. Nhiều thuốc sốt rét đã bị đưa ra khỏi danh sách thuốc sốt rét thiết yếu do bị ký sinh trùng kháng như: Sulfadoxin – pyrimethamin, dẫn xuất artemisinin không còn dùng đơn thuần bằng đường uống,...Trước thực trạng đó, Tổ chức Y tế thế giới đã khuyến cáo các nước tăng cường nghiên cứu phát triển thuốc mới có khả năng chống kháng thuốc hoặc sử dụng phối hợp thuốc trong điều trị [2], [3].

Hợp chất 10β-[2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32) đã được Viện Hóa học các hợp chất thiên nhiên tổng hợp và tinh chế, được Viện Sốt rét – KST –CT Trung ương thử tác dụng *in vitro* và độc tính cấp dạng nguyên liệu thô cho thấy: (32) thể hiện hoạt tính chống sốt rét *in vitro* vượt trội so với artemisinin và arteether; mặt khác, nó bền hơn gấp 10 lần so với artemisinin khi ở trong môi trường acid của dịch dạ dày và tan tốt

nghiệm độc tính cấp. Nghiên cứu này đã tiến hành đánh giá độc tính cấp của 32 trên chuột nhắt trắng bằng đường uống (đúng như đường dùng dự kiến trên người) theo phương pháp OECD [8], [9]. Đây là phương pháp có độ chính xác cao, đặc biệt các số liệu được tính theo chương trình Probit nên kết quả rất đáng tin cậy. Ở tất cả các liều đã thử nghiệm, không có chuột nào tử vong, mổ quan sát đại thể các cơ quan phủ tạng thấy bình thường. Liều cao nhất đã thử nghiệm là 5.500 mg/kg. Theo phân loại GHS (Globally Harmonised Classification System for chemical substances and mixtures: hệ thống phân loại hòa hợp toàn cầu của các chất hóa học và hợp chất), 32 gần như không có độc tính cấp.

Kết quả này cùng với hiệu lực của 32 trên *in vivo* cho thấy, hợp chất 32 có khả năng phát triển tiếp. Tuy nhiên, đây chỉ là những kết quả ban đầu. Để có thể phát triển tiếp thuốc này cần nhiều nghiên cứu tiếp theo, như quy định về nghiên cứu tiền lâm sàng của thuốc dự định dùng trên người của Tổ chức Y tế thế giới.

Các kết quả trên cũng tương tự như nghiên cứu trước đây của Nguyễn Thị Minh Thu và cộng sự [4] về độc tính cấp đường uống của (32) ở dạng nguyên liệu thô. Trong nghiên cứu này, chúng tôi đã thử nghiệm hợp chất (32) ở dạng tinh khiết và có thay đổi một số mức liều thử nghiệm nhằm khẳng định lại tính an toàn của thuốc. Cụ thể, trong nghiên cứu trước, các tác giả đã đánh giá độc tính cấp ở các mức liều 800, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4300, 4500, 4800, 5000 và 5200 mg/kg. Tuy nhiên, trong nghiên cứu này, chúng tôi đã bổ sung thêm 2 mức liều và thay đổi bước nhảy liều giữa các lô. Cụ thể, chúng tôi đã thử nghiệm ở liều 900, 1200, 1600, 2000, 2400, 2850, 3250, 3600, 4000, 4300, 4500, 4800, 5000, 5200, 5500 mg/kg.

Như vậy, qua nghiên cứu đánh giá hiệu lực *in vivo* và độc tính cấp, chúng tôi nhận thấy, hợp chất (32) hầu như không có độc tính cấp, có hiệu lực cao trong điều trị KST *P. berghei* với tỷ lệ điều trị khỏi cao ở các mức liều đã thử nghiệm. Điều này cho thấy, hợp chất (32) rất có triển vọng để nghiên cứu phát triển tiếp.

KẾT LUẬN

Đã nghiên cứu tác dụng *in vivo* trên chuột nhắt trắng nhiễm *Plasmodium berghei* chủng kháng chloroquin và độc tính cấp đường uống của 10β-[2'β-hidroxy-3'-imidazol) propyl] deoxoartemisinin (32). Kết quả cho thấy:

Với liều 72 mg/kg/ngày x 7 ngày, hợp chất (32) chỉ có tác dụng ức chế sự phát triển của *P. berghei*, 100% chuột tái phát và chết sau 28 ngày theo dõi. Ở liều 144 mg/kg/ngày x 7 ngày, hợp chất (32) có khả năng điều trị khỏi 90% số chuột, chỉ 10% chuột tái phát bệnh với mật độ ký sinh trùng thấp và 100% sống sót sau 28 ngày. Với liều 288 mg/kg/ngày x 7 ngày, (32) có tác dụng diệt hoàn toàn *P. berghei*, điều trị khỏi 100% số chuột sau 28 ngày theo dõi, không có chuột nào tái phát.

Hợp chất (32) không có độc tính cấp, chưa xác định được giá trị LD_{50} (ngay cả ở liều tối đa đã thử nghiệm là 5.500 mg/kg).

LỜI CẢM ƠN

Chúng tôi xin trân trọng cảm ơn Viện Hóa học các hợp chất thiên nhiên đã sản xuất và cung cấp mẫu thử nghiệm; trân trọng cảm ơn Chương trình Phòng chống sốt rét Quốc gia đã tài trợ kinh phí cho nghiên cứu. Nghiên cứu có sự tham gia của cán bộ Khoa Nghiên cứu điều trị sốt rét, Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương và sinh viên Lê Thị Hằng (mã 15540100190), Dược 5 K2, Học viện Y Dược học cổ truyền Việt Nam.

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TẠP CHÍ

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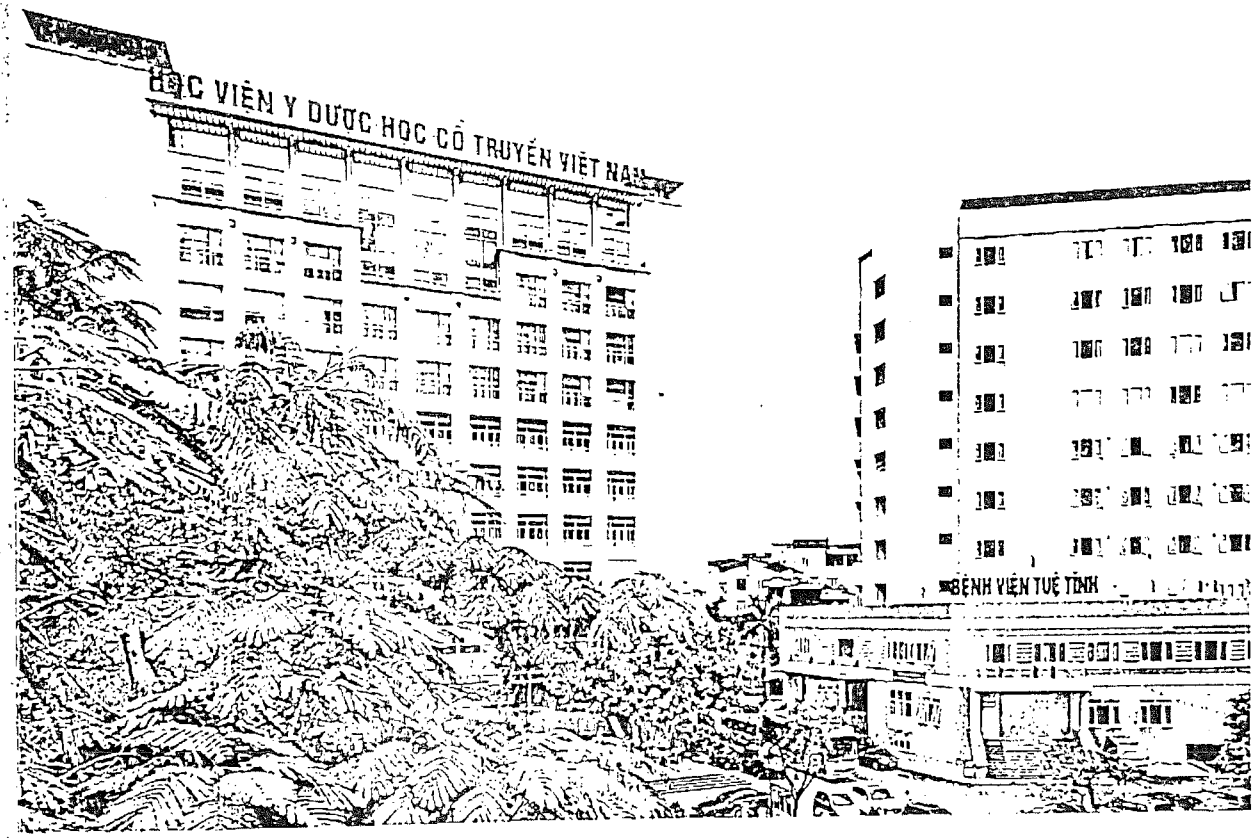

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BÀI NGHIÊN CỨU

BÀI NGHIÊN CỨU

Các biến cố bất lợi của Arterakine và Primaquin ở bệnh nhân sốt rét nhiễm *Plasmodium falciparum* chưa biến chứng tại Bình Phước

ADVERSE EVENTS OF ARTERAKINE AND PRIMAQUINE IN UNCOMPLICATED MALARIA PATIENTS WITH *PLASMODIUM FALCIPARUM* IN BINH PHUOC PROVINCE

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TÓM TẮT

Nghiên cứu được tiến hành từ tháng 4 - 10 năm 2016 tại 2 xã Đăk Nhau và Bù Gia Mập, nơi có tỷ lệ lưu hành sốt rét cao của tỉnh Bình Phước.

Tổng số 61 bệnh nhân được xác định nhiễm sốt rét do Plasmodium falciparum chưa biến chứng đã được lựa chọn và theo dõi đủ lộ trình nghiên cứu với phương pháp theo dõi biến cố thuần tập. Bệnh nhân được điều trị theo phác đồ do Bộ Y tế ban hành: Ngày đầu uống 4 viên Arterakine (40 mg dihydroartemisinin và 320 mg piperaquin phosphat, DHA-PQP), chia 2 lần, mỗi lần 2 viên; ngày 2 và 3, mỗi ngày uống 1 lần 2 viên Arterakine. Ở ngày 3, bệnh nhân được uống thêm 4 viên primaquin diphosphat 13,2 mg, liều duy nhất. Bệnh nhân được theo dõi trong 4 ngày kể từ khi thăm khám lần đầu tiên nhằm đánh giá các triệu chứng xuất hiện trước và sau khi dùng thuốc, đồng thời phân biệt biến cố bất lợi với triệu chứng của bệnh sốt rét.

Kết quả cho thấy, phối hợp Arterakine (DHA-PQP) + primaquin diphosphat gây biến cố bất lợi ở 19 bệnh nhân (31,15% quần thể nghiên cứu) với 68 biến cố bất lợi gồm: Mệt mỏi (18 người, 29,51%), đau đầu (16 người, 26,23%), buồn nôn (15 người, 24,59%), chóng mặt (12 người, 19,67%), nôn (04 người, 6,56%), hoa mắt (02 người, 3,28%) và choáng váng (01 người, 1,64%). Chưa phát hiện thấy biến cố bất lợi nghiêm trọng.

Từ khóa: *Biến cố bất lợi (AEs), Arterakine, dihydroartemisinin - piperaquin phosphat (DHA-PQP), primaquin diphosphat, Plasmodium falciparum.*

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nhân (22,95%) có biểu hiện đau đầu nặng hơn so với ban đầu, phải điều trị bằng paracetamol 15 mg/kg/lần. Sau khi uống paracetamol, triệu chứng đau đầu được cải thiện rõ rệt. Các kết quả trên đây cũng phù hợp với nghiên cứu của Nguyễn Thị Minh Thu và cộng sự (2018) khi nghiên cứu AEs của hai phác đồ Arterakine + primaquin và chloroquin + primaquin trên bệnh nhân sốt rét tại 5 tỉnh của Việt Nam với tỷ lệ mệt mỏi 22 người, 32,35% quần thể nghiên cứu), buồn nôn (20, 29,41%) và đau đầu (19, 27,94%). Tuy nhiên, tỷ lệ bệnh nhân bị chóng mặt ở nghiên cứu nay (12 người, 19,67%) cao hơn so với nghiên cứu trước đó (5 người, 7,35%) [2].

Kết quả trên cũng phù hợp với một số công bố quốc tế trước đó. Denis M.B. và cộng sự (2002), khi nghiên cứu hiệu quả và tính an toàn của thuốc phối hợp DHA-PQP trên bệnh nhân Campuchia mắc sốt rét *P. falciparum* chưa biến chứng đã nhận thấy, biến cố hay gặp là chóng mặt, đau đầu, buồn nôn, nôn, đau bụng và tiêu chảy. Các biến cố ít gặp hơn gồm ngứa, ban da và khô miệng [4]. Myint H.Y. và cộng sự (2007) cũng phát hiện thấy các biến cố tương tự trên thần kinh trung ương và tiêu hóa khi điều trị thuốc DHA-PQP [6]. Trong nghiên cứu này, bệnh nhân nhiễm *P. falciparum* được điều trị bằng DHA-PQP + primaquin diphosphat gặp nhiều hơn các triệu chứng đau đầu, buồn nôn, mệt mỏi, chóng mặt, ít gặp hơn các triệu chứng nôn và hoa mắt. Không bệnh nhân nào bị tiêu chảy hay vấn đề về da và niêm mạc.

Theo một số tác giả, sử dụng primaquin gây chán ăn (24%), đau vùng giữa hoặc dưới dạ dày (32%), nước tiểu sẫm màu (44%) và gây yếu mệt hoặc khó chịu (36%). Primaquin có thể gây tác dụng không mong muốn như: Buồn nôn, đau bụng, ho, chán ăn, hoa mắt, thiếu máu tan huyết ở bệnh nhân thiếu enzym glucose - 6 - phosphat dehydrogenase (G6PD) [7][9][10].

Ở nghiên cứu này, bệnh nhân chủ yếu gặp các biến cố mệt mỏi, buồn nôn, đau đầu, chóng mặt, sau đó là biến cố nôn, hoa mắt và 1 bệnh nhân thấy choáng váng. Không bệnh nhân nào có biểu hiện thiếu máu tan huyết. Kết quả trên phù hợp với công bố tỷ lệ thiếu G6PD rất thấp ở Việt Nam.

Kết quả của nghiên cứu này cũng phù hợp với các báo cáo biến cố bất lợi của phác đồ điều trị sốt rét *P. falciparum* mà Chương trình Phòng chống sốt rét Quốc gia gửi tới Trung tâm thông tin thuốc và phản ứng có hại của thuốc từ tháng 6/2012 đến nay, đồng thời đã góp phần bổ sung dữ liệu về biến cố bất lợi của một số thuốc sốt rét trong cơ sở dữ liệu ADR của Việt Nam.

KẾT KUẬN

Đã xác định được biến cố bất lợi của Arterakine + primaquin diphosphat trên 61 bệnh nhân nhiễm *Plasmodium falciparum* tại tỉnh Bình Phước.

Phối hợp Arterakine (DHA-PQP) + primaquin diphosphat gây biến cố bất lợi ở 19 bệnh nhân (31,15%% quần thể nghiên cứu) gồm: Mệt mỏi (18 người, 29,51%), đau đầu (16 người, 26,23%), buồn nôn (15 người, 24,59%), chóng mặt (12 người, 19,67%), nôn (04 người, 6,56%), hoa mắt (02 người, 3,28%) và choáng váng (01 người, 1,64%).

Chưa phát hiện thấy biến cố bất lợi nghiêm trọng.

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Dietary exposure and health risk characterization of aflatoxin B1, ochratoxin A, fumonisin B1, and zearalenone in food from different provinces in Northern Vietnam

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ABSTRACT

A dietary exposure and health risk assessment of mycotoxins including aflatoxin B1, fumonisin B1, ochratoxin A, and zearalenone was conducted in 3 provinces in Northern Vietnam namely Hanoi, Thanh Hoa, and Ha Giang. Results of the analysis of samples of maize, rice, peanut, and sesame revealed the presence of these mycotoxins in all samples and sampling locations. Aflatoxin B1 was the most frequently detected (19.1%) and widely distributed among different types of samples, whereas the percentage occurrence of fumonisin B1, ochratoxin A, and zearalenone were 11.2, 5.9 and 6.3, respectively. The later three mycotoxins were detected mostly in maize. The exposure to aflatoxin B1 at detected levels could lead to 0.23, 0.65 and 21.0 cases of liver cancer per 100,000 adult people per year in Hanoi, Thanh Hoa and Ha Giang, respectively. The risk assessment also showed the unsafe exposure to ochratoxin A and fumonisin B1 in the highland region where the people consume a large amount of foods derived from maize. In Ha Giang, the mean exposures to fumonisin B1 were lower than its PMTDI (Provisional Maximum Tolerable Daily Intake), however, the 95th percentile values were 1.1–1.9 times of the PMTDI. The mean exposures to ochratoxin A in Ha Giang were about 2.4–3.6 times higher than its PMTWI (Provisional Maximum Tolerable Weekly Intake). There was no risk of fumonisin B1 and ochratoxin A in Hanoi and Thanh Hoa. The dietary exposure to zearalenone was within its PMTDI in all locations. The results pointed out the need for further improvement of the control of these mycotoxins in Vietnam, especially in some highland provinces.

Introduction

Mycotoxins are secondary metabolites produced by moulds, mainly from *Aspergillus*, *Penicillium* and *Fusarium* genera, which are capable of contaminating food and causing harmful effects on human health. Today, the numbers of mycotoxins reach to more than 400 categories (Reddy et al., 2010; Tola & Kebede, 2016). The contamination of mycotoxins in food depends on the presence of moulds, agricultural practice and the conditions of harvesting and storage (Bennett & Klich, 2003). Most mycotoxins are thermostable, which can persist during food processing and cooking (Turner, Subrahmanyam, & Piletsky, 2009). The occurrence of mycotoxins in foods can cause harmful effects ranging from acute intoxication to consequences of chronic exposure (such as carcinogenic, mutagenic, teratogenic ...) in humans and animals (Fung & Clark, 2004; Richard, 2007).

Hot and humid tropical weather in Northern Vietnam is very favorable for the development of mycotoxins. According to Vietnamese regulation for mycotoxins, which followed Codex Alimentarius standards, the concerned mycotoxins in food include aflatoxins (AFs), ochratoxin A (OTA), fumonisin B1 (FB1), deoxynivalenol (DON) and zearalenone (ZEA) (Ministry of Health, 2011; Van Egmond, 2002). Several works have revealed the presence of aflatoxin B1 (AFB1), OTA, FB1, ZEA, citrinin in different kinds of food such as rice, maize, and peanut in Vietnam (Trung, Bailly, Querin, Le Bars, & Guerre, 2001; Nguyen, Tozlovanu, Tran, & Pfohl-Leszkowicz, 2007; Phuong, Thieu, Ogle, & Pettersson, 2015; Huong, Brimer, et al., 2016). So far, there were no reports on the occurrence of DON. Moreover, most studies about mycotoxin contamination in Vietnam focused on mountainous or

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remote rural areas, where the poor storage conditions were most favorable for the development of moulds and the accumulation of mycotoxin (Nguyen et al., 2007; Huong, Brimer, et al., 2016). These results revealed the mycotoxin contamination situation in the most extreme regions of Vietnam, but they were not enough to give a comprehensive understanding of mycotoxin presence in staple food as well as its risk on human health in Vietnam. The majority of Vietnamese people purchase their daily food, including cereals and nuts, the main source of exposure to mycotoxins for human (Reddy et al., 2010), in small retail markets. From basic knowledge about the harmful effect of moulds, most consumers naturally avoid purchasing cereals and nuts with abnormal appearance. Therefore, mycotoxins present in cereals, nuts in retail markets which "apparently safe" (without any abnormal appearance or smell) are the major source for human exposure.

The toxicity of mycotoxins varies depending on the type of mycotoxins. According to International Agency for Research on Cancer (IARC), in terms of carcinogenic hazard, aflatoxin B1 was classified as group 1 (carcinogenic to humans), ochratoxin A and fumonisin B1 were categorized to group 2B (possibly carcinogenic to human) (Anttila et al., 2002; World Health Organization & International Agency for Research on World Health OrganizationInternational Agency for Research on Cancer, 1993). AFB1, the most common aflatoxins, produced mainly by *Aspergillus spp.* Is a hepatotoxic, mutagenic, immunosuppressive and carcinogenic compound. Human exposure to mycotoxins like AFB1 is particularly dangerous for public health in a country with a high rate of hepatitis B virus (HBV) occurrence in population such as Vietnam (Nguyen, 2012), because the risk of liver cancer from exposure to aflatoxin in HBV-positive people was estimated at about 30 times higher than in HBV-negative people (Pitt et al., 2012). Ochratoxin A is another mycotoxin produced by *Aspergillus* and *Penicillium* species. Chronic exposure to OTA is associated with immunotoxic and cytotoxicity effects (Al-Anati & Petzinger, 2006; Fink-Gremmels, Jahn, & Blom, 1995). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the provisional maximum tolerable weekly intake (PMTWI) of OTA at 0.1 μg/kg b.w. (body weight) per week (JECFA, 2004). Fumonisins are a group of mycotoxins produced by *Fusarium* species. FB1, the most common member of fumonisins family, is toxic to the liver and kidney of studied animals (Sharma, Dugyala, & Voss, 1997). Zearalenone is another mycotoxin produced by *Fusarium* species. There are evidence that ZEA can affect the reproductive function (Minervini & Dell'Aquila, 2008). JECFA has set the provisional maximum tolerable daily intake (PMTDI) values for ZEA and FB1 of 0.5 μg/kg b.w. per day and 2 μg/kg b.w. per day respectively (JECFA, 2000; JECFA 2007).

In this study, the exposure to four mycotoxins (AFB1, FB1, OTA, ZEA) has been assessed based on the analysis of rice, maize, peanut and sesame samples in three provinces in Northern Vietnam and the food consumption data for 4 different age groups. The health risk of these mycotoxins from different regions has also been characterized.

2. Materials and methods

2.1. Food sampling

2.1.1. Sampling locations

The study was conducted in three provinces in Northern Vietnam including Hanoi, Thanh Hoa, and Ha Giang. Hanoi is the capital of Vietnam and also the second largest city in the country in terms of population and territory Thanh Hoa is located in the delta region which has the large area of crop production like rice, maize, peanut, and sesame. People in these two provinces, mostly Kinh people, consume rice as the main source of food. Samples were collected in three different districts in each of these two provinces. Meanwhile, sampling areas in Ha Giang, a mountainous province, include five districts of Dong Van, Meo Vac, Vi Xuyen, Yen Minh, and Quan Ba. In these areas, the H'Mong people account for a high proportion, have the habit of using maize as the main food instead of rice compared to other areas. Here, maize was harvested and stored in each family to be used gradually as food. Samples were taken in retail markets of the rural and urban area in these provinces to diversify the source of samples and to take into account the difference among regions in Vietnam. In Vietnam, small retail markets are very similar even between a big city like Hanoi and rural communes; they are typically a cluster of small shops or kiosks. The shops or kiosks selling cereals and nuts are located together in a particular part of markets, usually the driest one, away from a possible source of moisture (kiosks selling fresh fish, meat, vegetable, etc.). In Ha Giang, beside the samples from the retail market, samples from households were also collected, especially maize as this is the main source of food used in this province.

2.1.2. Collection of samples

A total of 606 samples, including 144 rice samples, 189 maize samples, 144 peanut samples, and 129 sesame samples, were randomly collected from October 2016 to September 2017. The numbers of each type of sample from three different locations were incorporated into Table 3. Since maize is the main food in diet of H'Mong people in Ha Giang, the number of maize sample was higher in Ha Giang compared to other provinces. All samples were collected from retailed markets or from household storages which are ready to be cooked. They all had no abnormal appearance under naked-eyes examinations. Each sample was collected at least 1 kg, placed in air-tight sealed sterile plastic bags, encoded and stored at ambient temperature for not more than three days before being transported to National Institute of Food Control for analysis of mycotoxins.

2.2. Analysis of mycotoxins in food samples

2.2.1. Chemicals and reagents

Acetonitrile, methanol, formic acid, magnesium sulfate anhydrous, sodium chloride and C18 sorbent were purchased from Merck KGaA (Darmstadt, Germany). Pure mycotoxin standards of AFB1, OTA, FB1, and ZEA were supplied by Sigma-Aldrich (St. Louis, MO, USA). Chromatography column Cortecs C18 (4.6 × 150 mm, 3.5 μm) and a C18 guard column (3.9 × 5 mm, 2.7 μm) were from Waters (Milford, MA, USA).

2.2.2. Extraction of mycotoxins

The extraction of mycotoxins followed the QuEChERS approach as published by Liu et al. with some modifications (Liu et al., 2014). Each bulk sample (maize, rice, peanut, and sesame) with not less than 0.5 kg was finely grinned in a blender. Two portions of 5 g of homogenized sample was accurately weighed into 50 mL centrifuge tube. The tube was thoroughly mixed with 15 mL of water. Twenty milliliters of acetonitrile containing 1% acetic acid was added into the tube, and it was shaken in a horizontal shaker at 140 rpm in 30 min. A mixture of 8.0 g of anhydrous magnesium sulfate and 2.0 g of sodium chloride was added to the tube, and then the tube was mixed in 1 min before being centrifuged at 6000 rpm in 5 min. One milliliter of supernatant was transferred to a 2 mL centrifuge tube containing 150 mg of anhydrous magnesium sulfate and 100 g of C18 sorbent. The tube was then vortexed in 1 min and centrifuged at 13000 rpm in 1 min. The supernatant was filtered through a 0.2 μm PTFE membrane into the LC-MS/MS vial. All samples were analyzed in duplicate.

2.2.3. LC-MS/MS determination of mycotoxins

The determination of studied mycotoxins was done using an LC-MS/MS system consisting of a UPLC Shimadzu LC20AD-XR (Shimadzu, Kyoto, Japan) and a triple-quadrupole mass spectrometer ABSciex Triple Quad 5500 (Sciex, MA, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was done on a Cortecs C18 column (150 mm × 4.6 mm i.d.; particle size 2.7 μm) and guard column. The mobile phase was a mixture of methanol and

Table 1
MS/MS conditions and retention times of the studied mycotoxins.

Mycotoxins	Retention time	ESI mode	Precursor ion	Product ions	CE (eV)
AFB1	9.8	ESI(+)	313	241	30
				269	30
FB1	9.1	ESI(+)	722	352	45
				334	49
OTA	9.7	ESI(−)	402	358	−24
				167	−35
ZEA	10.7	ESI(−)	317	175	−20
				131	−30

ammonium acetate 10 mM according to the gradient from 20% of methanol (2 min) to 100% of methanol (6 min). The total analysis time was 12 min. Positive ESI was used for AFB1 and FB1 and negative ESI was used for OTA and ZEA. Each mycotoxin was quantified and confirmed by two transitions in multiple reactions monitoring (MRM) mode (Table 1). Matrix-matched calibration curves were used to compensate the matrix effect. The matrix-matched solutions were prepared by diluting the standard solutions with blank matrix extract to get the desired concentrations as indicated in Table 2.

2.4. Method validation and quality control

The method was fully validated with specificity, sensitivity, linearity, repeatability, and recovery. The limit of detection (LOD) and limit of quantification (LOQ) of the mycotoxins were determined from the signal to noise ratios as more than 3:1 and 10:1, respectively (Wenzl, Haedrich, Schaechtele, Robouch, & Stroka, 2016). Blank and spiked samples were used as the quality control samples. The method fulfills the requirements of ISO/IEC 17025 and has been accredited by Vietnam Bureau of Accreditation.

2.3. Exposure assessment

The exposure dose of a mycotoxin from a specific cereal or nut was calculated as followed:

Exposure dose (μg/kg b.w. per day) = Average mycotoxin level (μg/kg) × average food consumption (kg/person per day) × average body weight^{-1} (kg b.w./person).

Since the percentage of censored data (results reported below LOD or LOQ) was higher than 50%, the mycotoxins content in food were done by two scenarios referred to the instruction of the European Food Safety Authority: (1) the lower bound (LB) approach by replacing the results below LOD by zero and results below LOQ by LOD and (2) the upper bound (UB) approach by replacing the results below LOD by LOD and results below LOQ by LOQ (EFSA, 2010).

The data of average food consumption in different regions were calculated based on information collected from a food intake survey conducted by the research group. In each province, two districts were investigated with total number of surveyed family was sixty. The survey focused on the total amount of rice, maize, peanut, and sesame consumed daily. However, since peanut and sesame are often mixed together in a meal, the data for these two foodstuffs were combined and the higher values were selected to be the intakes for both peanut and sesame. All food consumption data were estimated for 4 different age groups which are 3–6 years, 7–11 years, 12–18 years and > 18 years. For the adult group (over 18 years of age), the data was extracted for the whole group and for each gender. Moreover, mean and 95th percentile exposure levels (p95) were calculated to take into account the variability of food consumption data.

The body weight of people was also measured for the studied provinces of different age groups including 3–6 years, 7–11 years, 12–18 years and > 18 years. These figures were also compared to the data from the survey in 2010 (National Institute of Nutrition, 2010).

2.4. Risk characterization

The risk of OTA, ZEA, and FB1 to human health was assessed by direct comparison of exposure dose with their PMTDI (Provisional Maximum Tolerable Daily Intake) of 0.5 μg/kg b.w. per day (for ZEA) and 2.0 μg/kg b.w. per day (for FB1) or with PMTWI (Provisional Maximum Tolerable Weekly Intake) of 0.1 μg/kg b.w. per week (for OTA).

With AFB1, the risk to human health was assessed from the increase of liver cancer risk in the population (the number of case per 100,000 people per year) corresponding to a certain exposure level. To assess the risk of liver cancer from AFB1 exposure, the average potency was calculated based on the prevalence rate of HBV-positive in Vietnamese population and the estimation of liver cancer risk from AFB1 was 0.01 case per year per 100,000 people per 1 ng AFB1 μg/kg b.w. per day on HBV-negative population and 0.3 case per year per 100,000 people per 1 ng AFB1 μg/kg b.w. per day on HBV-positive population (Liu & Wu, 2010). According to a previous study (Nguyen, 2012), the prevalence of HBV infection in Vietnam could be as high as 20% in the general population. Therefore, we took the prevalence rate of 20% to calculate the average potency and the population risk as followed:

Average Potency (case per year per 100,000 people per 1 ng AFB1 μg/kg b.w. per day)

$$= 0.3 \times 0.2 + 0.01 \times 0.8 = 0.068$$

Population Risk (case per year per 100,000 people) = Average Potency × Exposure Dose.

The risk AFB1 was also characterized using margin of exposure (MOE) approach due to its well-established genotoxicity and carcinogenicity. The MOE is the ratio of the lower benchmark dose (BMDL) for the critical effect to the exposure dose (EFSA, 2005). In this study, a $BMDL_{10}$ of 0.17 μg/kg b.w. per day proposed by EFSA was used for MOE calculation (EFSA, 2007). The larger the MOE, the smaller the risk, and a value of lower than 10,000 indicate a human health concern (EFSA, 2005).

2.5. Data analysis

The statistical analysis of food intake and the concentration of mycotoxins in food samples were done using SPSS 16.0. Contamination level of each mycotoxin and the food intake among provinces and types of food were compared by using the independent samples *t*-test. The exposure dose and carcinogenic risk were calculated using Microsoft

Table 2
Summary of method validation results.

Criteria	AFB1	FB1	OTA	ZEA
Specificity	The specific identification of each toxin was assured by means of identification point, ion ratio and by comparing the blank sample, standard sample and spiked sample.			
Linearity	0.3–100 μg/kg	75–2000 μg/kg	1.5–250 μg/kg	1.5–250 μg/kg
Precision and recovery (n = 6 at 3 spiked levels)	RSD = 4.2–12% R% = 86–115%	RSD = 2.4–6.4% R% = 84–106%	RSD = 2.2–12% R% = 89–109%	RSD = 3.2–13% R% = 89–109%
LOD	0.1 μg/kg	25 μg/kg	0.5 μg/kg	0.5 μg/kg
LOQ	0.3 μg/kg	75 μg/kg	1.5 μg/kg	1.5 μg/kg

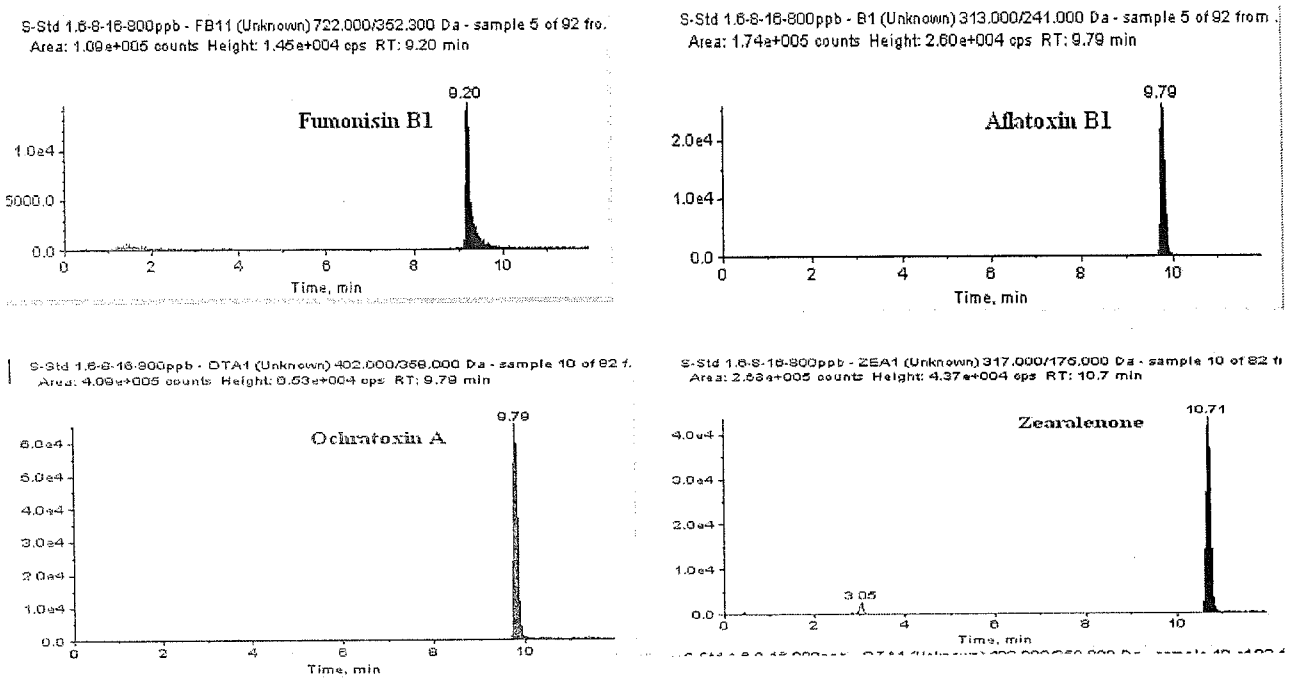

Fig. 1. MRM chromatograms of mycotoxins standard solution (Aflatoxin B1 1.6 ng/mL; Fumonisin B1 80 ng/mL; Ochratoxin A 8 ng/mL and Zearalenone 8 ng/mL).

Excel.

3. Results and discussion

3.1. Occurrence of mycotoxins

The method was fully validated for the matrices analyzed in this study with the results summarized in Table 2 (detailed data not shown). LOD and LOQ of each mycotoxin were identified and further used in the assessment of dietary exposure. The MRM chromatogram of each mycotoxin was presented in Fig. 1. The validation results proved that the method was satisfactory for the intended application.

The mycotoxin analysis results obtained for each type of sample in each sampling location were summarized in Table 3.

In general, AFFB1 was the most detected mycotoxin with 116/606 samples (19.1%) have the concentrations higher than the LOQ. Maize was the highest matrix of AFB1 contamination, with 57/189 samples (30.2%); it was followed by peanuts with 34/144 aflatoxin-contaminated samples (23.6%). AFB1 was also found in rice and sesame sample but with a much lower rate, only 13/144 rice samples (9.0%) and 12/129 sesame samples (9.3%) were detected with AFB1. The contamination rate and average level of AFB1 in maize in this work were lower than those published by some other works in Vietnam (Nguyen et al., 2007; Huong, Brimer, et al., 2016). However, the average concentration of AFB1 in maize in Ha Giang was much higher than those of other provinces. The lower bound and upper bound values of AFB1 in maize in Ha Giang were 66.0 and 66.1 μg/kg, respectively. These figures of Hanoi were 2.56 and 2.62 μg/kg and of Thanh Hoa were 5.31–5.39 μg/kg. The contamination rate and average level of AFB1 in peanut were much lower than those observed in peanut kernel in other works in Vietnam (Tra, Minh, Huong, & Chau, 2004). Again, peanut collected from Ha Giang has a higher average level of AFB1 (16.5 μg/kg) compared to Hanoi (9.2 μg/kg) and Thanh Hoa (4.9 μg/kg). These average values of AFB1 concentration in maize and peanut were higher than the maximum limit in Vietnam (Ministry of Health, 2011). The average levels of AFB1 in rice ranged from 0.4 to 2.0 μg/kg, which were lower than the regulation limit in Vietnam (5 μg/kg) (Ministry of Health, 2011) and lower than those observed in five provinces in central of Vietnam (Nguyen et al., 2007) and in Lao Cai province in the north of Vietnam (Huong, Brimer, et al., 2016). This is the first time the AFB1 concentration in sesame samples in Vietnam was reported but this was relatively low compared to other foods, ranged from 0.37 μg/kg (in Hanoi) to 1.7 μg/kg (in Thanh Hoa).

There were 68 of 606 samples (11.2%) detected with FB1 found mainly in maize in Thanh Hoa and Ha Giang. The average LB and UB concentrations of FB1 in maize were 127 and 129 μg/kg, respectively (in Thanh Hoa) and were 154 and 169 μg/kg, respectively (in Ha Giang), which were higher than those of Hanoi. The average level and contamination rate of FB1 in rice in this study were lower than those observed in Lao Cai province in Vietnam (Huong, Brimer, et al., 2016). OTA was detected mainly in maize and peanut samples (5.9%), and the concentration of OTA in maize in Ha Giang was the highest one. The lower bound and upper bound values of OTA in Ha Giang were 7.44 and 7.87 μg/kg, respectively. Finally, 38 of 606 samples (6.3%) were detected with ZEA. The contamination rates and the average concentration of ZEA in Ha Giang were higher than those in other locations.

Overall, the mycotoxins content in food products in Ha Giang, especially AFB1 in maize, was higher than those in other localities. This may have stemmed from the practice of storing food for a long time in ambient conditions before using of the H'Mong ethnic minority people here. This will contribute to the high risk of mycotoxins on people's health in Ha Giang.

3.2. Assessment of food intake

The intakes of 4 studied foods for Hanoi, Thanh Hoa, and Ha Giang achieved from the recalled survey were summarized in Table 4. The average body weights (kg b.w.) of different age groups in three provinces were integrated into this Table.

From the results, it can be seen that people in Ha Giang used more foods derived from maize than people in other provinces. The average consumption of maize by adults in Ha Giang was 338.4 g/day, about 10 times of that of other provinces. The average consumption of rice in Ha Giang was lower than the other provinces ($p = 0.012$ and 0.016), whereas the difference in rice consumption in Hanoi and Thanh Hoa was not statistically significant ($p = 0.066$). The average value of rice consumption in the studied provinces ranged from 244.4 to 300.5 g/day for adults. The 95th percentile values of consumption reflect the

Table 3
Summary of mycotoxin analysis results.

Sampling location	Type of sample	No. of sample	AFB1 (μg/kg)				FB1 (μg/kg)				OTA (μg/kg)				ZEA (μg/kg)			
			Sample > LOQ	Max	LB mean	UB mean	Sample > LOQ	Max	LB mean	UB mean	Sample > LOQ	Max	LB mean	UB mean	Sample > LOQ	Max	LB mean	UB mean
Hanoi	Maize	40	14	25.0	2.56	2.62	4	215	12.8	35.3	4	12.0	0.908	1.36	2	15.0	0.613	1.09
	Rice	40	4	13.0	0.325	0.423	0	–	0	25	0	–	0	0.5	0	–	0	0.5
	Peanut	40	10	16.0	9.20	9.28	0	–	0	25	2	4.5	0.150	0.625	0	–	0	0.5
	Sesame	40	3	6.6	0.365	0.458	0	–	0	25	0	–	0	0.5	0	–	0	0.5
Thanh Hoa	Maize	49	13	94.5	5.31	5.39	20	1662	127	129	7	44.0	1.95	2.38	7	132	64.5	64.9
	Rice	48	6	93	1.94	2.04	0	–	0	25	0	–	0	0.5	0	–	0	0.5
	Peanut	46	12	159	4.87	4.96	0	–	0	25	3	23	0.565	1.03	0	–	0	0.5
	Sesame	49	4	20	1.65	1.74	0	–	0	25	0	–	0	0.5	0	–	0	0.5
Ha Giang	Maize	100	30	1572	66	66.1	39	1545	154	169	15	126	7.44	7.87	27	212	20.9	21.3
	Rice	56	3	26	0.91	1.01	3	675	15	38.7	0	–	0	0.5	0	–	0	0.5
	Peanut	58	12	362	16.49	16.57	1	545	9.4	34	5	87.5	1.78	2.25	2	53.0	1.4	1.88
	Sesame	40	5	30.0	0.75	0.85	0	–	0	25	0	–	0	0.5	0	–	0	0.5

Note: LB = lower bound (results below LOD were regarded as 0 and those between LOD and LOQ were regarded as LOD).
UB = upper bound (results below LOD were regarded as LOD and those between LOD and LOQ were regarded as LOQ).

degree of variability in research data. In Ha Giang, these values were greater than those in other localities due to the tendency to gradually shift from maize to rice food in a part of the population. There are groups of people who only eat maize based foodstuffs, some others only eat rice based foodstuffs, and some consume both types of food in one meal. Meanwhile, consumption of peanut and sesame was similar among the studied localities. This value was also relative low, indicating that these two products are only side foods on the daily menu.

3.3. Risk characterization

Based on the results of mycotoxins content in food and the food consumption, exposure dose to 4 mycotoxins AFB1, FB1, OTA, and ZEA were calculated for each age group and given in Table 5.

3.3.1. Aflatoxin B1 (AFB1)

In the delta region (Hanoi and Thanh Hoa), among the four types of samples, the average exposure doses to AFB1 from rice and maize were higher than those from peanut and sesame (supplementary data). However, in the highland region, maize was the main source of AFB1 exposure. The highest total exposure dose to AFB1 was found in adults in Ha Giang, with the mean LB was 0.45 μg/kg b.w. per day. The corresponding values of Hanoi and Thanh Hoa were 0.005 and 0.014 μg/kg b.w. per day. It was obvious that the value in mountainous region (Ha Giang) was higher than other locations.

In this study, the exposure doses were estimated in uncooked foods. Several studies had proved that cooking or processing can reduce significantly the level of mycotoxins, including AFB1, in foods. A study in Pakistan estimated an average reduce about 45.0% with AFB1 in rice due to washing and cooking (Majeed et al., 2018). Another study in South Korea estimated a reduction in AFB1 about 31%–36% in rice with ordinary-cooking (Park & Kim, 2006). So the reduction level of AFB1 after cooking at 31% as reported by Park et al. was used to calculate the risk of liver cancer for the population at sampling locations from exposing to AFB1 in rice, maize, peanut, and sesame (Table 6). In which, the mean LB risk of the population ranged from 0.23 to 0.29 cases per 100,000 people per year (in Hanoi) and from 0.65 to 1.28 cases per 100,000 people per year (in Thanh Hoa), depending on the age. These corresponding values in Ha Giang were much higher, varying from 14.26 to 21.03 cases per 100,000 people per year. This came from the habit of storing food, mainly maize, of the people there for a long time before using and their huge consumption of foods derived from maize.

The cancer risk of AFB1 to the adult population in Hanoi and Thanh Hoa (from 0.23 to 0.65 cancer cases per 100,000 people per year) was similar to the studies in Malaysia (from 0.61 to 0.85 cases) (Chin, Abdullah, & Sugita-Konishi, 2012) but higher than the studies in Japan (0.021 cases) (Sakuma et al., 2013), Thailand (0.011 cases) (Panrapee, Phakpoom, Thanapoom, Nampeung, & Warapa, 2016), Hongkong (0.039 cases) (Centre for Food Safety, 2013) and France (0.011 cases) (Sirot, Fremy, & Leblanc, 2013). Comparing to another study in Lao Cai, Vietnam (2.7 cases) (Huong, Brimer, et al., 2016), these results for Hanoi and Thanh Hoa were lower. However, the unusual high risk was recorded in Ha Giang (21 cancer cases per 100,000 people per year), which was equivalent to the risk in some African countries (from 46.5 cases in Gambia to 79.4 cases in Mozambique) (Shephard, 2008). In conclusion, the health hazard caused by AFB1 was more serious from the H'Mong people in Ha Giang, and high intake of AFB1 was still an important factor contributing to the high mortality rate due to liver cancer in Vietnam, at 21,997 deaths on a population of 90,796,000 people in 2014 (WHO, 2014).

Table 7 shows the MOE values for AFB1 exposures for the group of over 18 years of age from the consumption of maize, rice, peanut, and sesame among three studied provinces. The MOE range corresponding to LB average exposure with maize was from 0.56 to 99.9, with rice was from 23.7 to 146, with peanut was from 193 to 726, and highest was with sesame, from 2144 to 7763. The MOE for total four food types

Table 4
Average body weight (kg b.w.) and summary of food intakes (average and percentile 95% - P95) in different age groups (g/day).

Location	Age group	Average weight (kg b.w.)	Rice		Maize		Peanut & Sesame	
			Mean	P95	Mean	P95	Mean	P95
Hanoi	3–6 years	15.5	168.6	224.0	7.9	17.7	2.2	4.1
	7–11 years	26.5	228.9	396.7	26.6	76.5	3.8	6.3
	12–18 years	48.3	267.8	545.2	35.0	80.0	4.4	7.8
	> 18 years	55.2	286.6	514.5	53.2	147.8	4.8	9.6
Thanh Hoa	3–6 years	13.6	165.2	240.0	8.0	40.0	1.1	4.3
	7–11 years	21.5	227.2	342.9	15.0	57.1	2.1	5.7
	12–18 years	40.7	262.4	437.5	28.6	85.7	3.4	8.6
	> 18 years	50.4	300.5	480.0	31.0	85.7	3.9	12.9
Ha Giang	3–6 years	14.4	163.7	325.0	63.6	246.1	1.8	6.7
	7–11 years	26.4	205.0	420.8	152.6	419.2	2.2	8.3
	12–18 years	39.6	208.8	566.7	232.1	554.4	3.0	9.3
	> 18 years	50.5	244.4	691.7	338.6	650.0	3.9	12.5

Table 5
Exposure doses to AFB1, FB1, OTA and ZEA of different age groups in three provinces (ng/kg b.w. per day).

Location	Age group	AFB1				FB1				OTA				ZEA			
		Mean		P95		Mean		P95		Mean		P95		Mean		P95	
		LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB
Hanoi	3–6 years	6.2	7.3	10.2	11.7	6.5	297.0	14.6	414.8	0.5	6.3	1.1	9.1	0.3	6.1	0.7	8.7
	7–11 years	6.7	7.7	14.5	16.2	12.8	258.5	37.0	488.0	0.9	5.8	2.7	11.7	0.6	5.6	1.8	10.9
	12–18 years	4.5	5.1	9.5	10.7	9.3	168.7	21.2	348.7	0.7	3.9	1.5	8.1	0.4	3.7	1.0	7.6
	> 18 years	5.0	5.6	11.5	12.7	12.3	168.2	34.3	336.2	0.9	4.0	2.5	8.5	0.6	3.7	1.6	7.8
Thanh Hoa	3–6 years	27.2	28.5	51.9	54.0	74.7	383.6	373.5	836.4	1.2	7.6	5.9	16.3	37.9	44.3	189.7	200.0
	7–11 years	24.8	26.0	46.8	48.6	88.6	359.1	337.3	754.6	1.4	7.1	5.3	14.7	45.0	50.7	171.3	180.6
	12–18 years	16.8	17.5	33.4	34.7	89.2	256.0	267.4	550.9	1.4	5.0	4.2	10.7	45.3	48.9	135.8	142.2
	> 18 years	13.8	14.4	26.3	27.3	70.3	209.0	194.4	423.2	1.1	4.1	3.1	8.3	35.7	38.7	98.7	103.8
Ha Giang	3–6 years	304.0	305.6	1156	1161	851.9	1194	2975	3789	33.1	40.8	128.0	147.1	92.5	100.1	357.8	376.4
	7–11 years	390.0	391.4	1068	1071	1007.4	1282	2687	3319	43.2	49.6	118.7	133.8	120.9	127.2	332.3	346.9
	12–18 years	392.9	394.1	941.0	943.9	982.4	1199	2373	2934	43.7	49.0	104.6	118.0	122.6	127.7	292.9	305.9
	> 18 years	448.3	449.4	866.1	868.9	1105.9	1325	2190	2720	50.0	55.4	96.2	108.8	140.2	145.4	269.4	281.6

Table 6
Population cancer risk of aflatoxin B1 (case per year per 100.000 people).

Location	Age group	Population cancer risk of AFB1 (case per year per 100.000 people)			
		Mean - LB	Mean – UB	P95 – LB	P95 – UB
Hanoi	3–6 years	0.29	0.34	0.48	0.55
	7–11 years	0.32	0.36	0.68	0.76
	12–18 years	0.21	0.24	0.44	0.50
	> 18 years	0.23	0.26	0.54	0.59
Thanh Hoa	3–6 years	1.28	1.34	2.44	2.53
	7–11 years	1.17	1.22	2.19	2.28
	12–18 years	0.79	0.82	1.57	1.63
	> 18 years	0.65	0.68	1.23	1.28
Ha Giang	3–6 years	14.26	14.34	54.26	54.45
	7–11 years	18.30	18.36	50.10	50.26
	12–18 years	18.44	18.49	44.15	44.29
	> 18 years	21.03	21.09	40.64	40.77

Table 7
Risk characterization for AFB1 from consumption of maize, rice, peanut and sesame in adults among three provinces based on MOE.

Location	Food	Margin of exposure			
		Mean - LB	Mean – UB	P95 – LB	P95 – UB
Hanoi	Maize	99.9	97.6	35.9	35.1
	Rice	146.0	112.2	81.3	62.5
	Peanut	308.0	305.3	154.0	152.7
	Sesame	7763	6186	3881	3093
	Total	49.4	44.3	21.3	19.5
Thanh Hoa	Maize	83.8	82.6	30.3	29.9
	Rice	23.7	22.5	14.8	14.1
	Peanut	726.4	713.3	219.6	215.6
	Sesame	2144	2033	648.2	614.7
	Total	17.9	17.1	9.38	9.03
Ha Giang	Maize	0.56	0.56	0.29	0.29
	Rice	55.9	50.4	19.77	17.8
	Peanut	193.5	192.5	60.36	60.1
	Sesame	4254	3753	2727	1171
	Total	0.55	0.55	0.28	0.28

were lowest in Ha Giang (MOE for average: 0.55; MOE for 95th: 0.28) and highest in Hanoi (MOE for average: 44.3–49.4; MOE for 95th: 19.5–21.3). These values were far lower than the recommended MOE (at 10,000) indicating that the exposure to AFB1 contamination in maize, rice, peanut and sesame was still a non-negligible human health risk in many regions of Vietnam. In Hanoi and Thanh Hoa, AFB1 contamination in rice could pose a higher risk because rice is the major cereal in dietary regime of Vietnamese people. In Ha Giang, on the other hand, the risk of AFB1 coming from maize dominated that from other foods. This can be explained that maize is major daily food for the H'Mong ethnic community in mountainous regions like Ha Giang, with actual consumption level much higher than the average figures.

3.3.2. Fumonisin B1 (FB1), zearalenone (ZEA) and ochratoxin A (OTA)

For FB1, among sampling location, the sum of average exposure

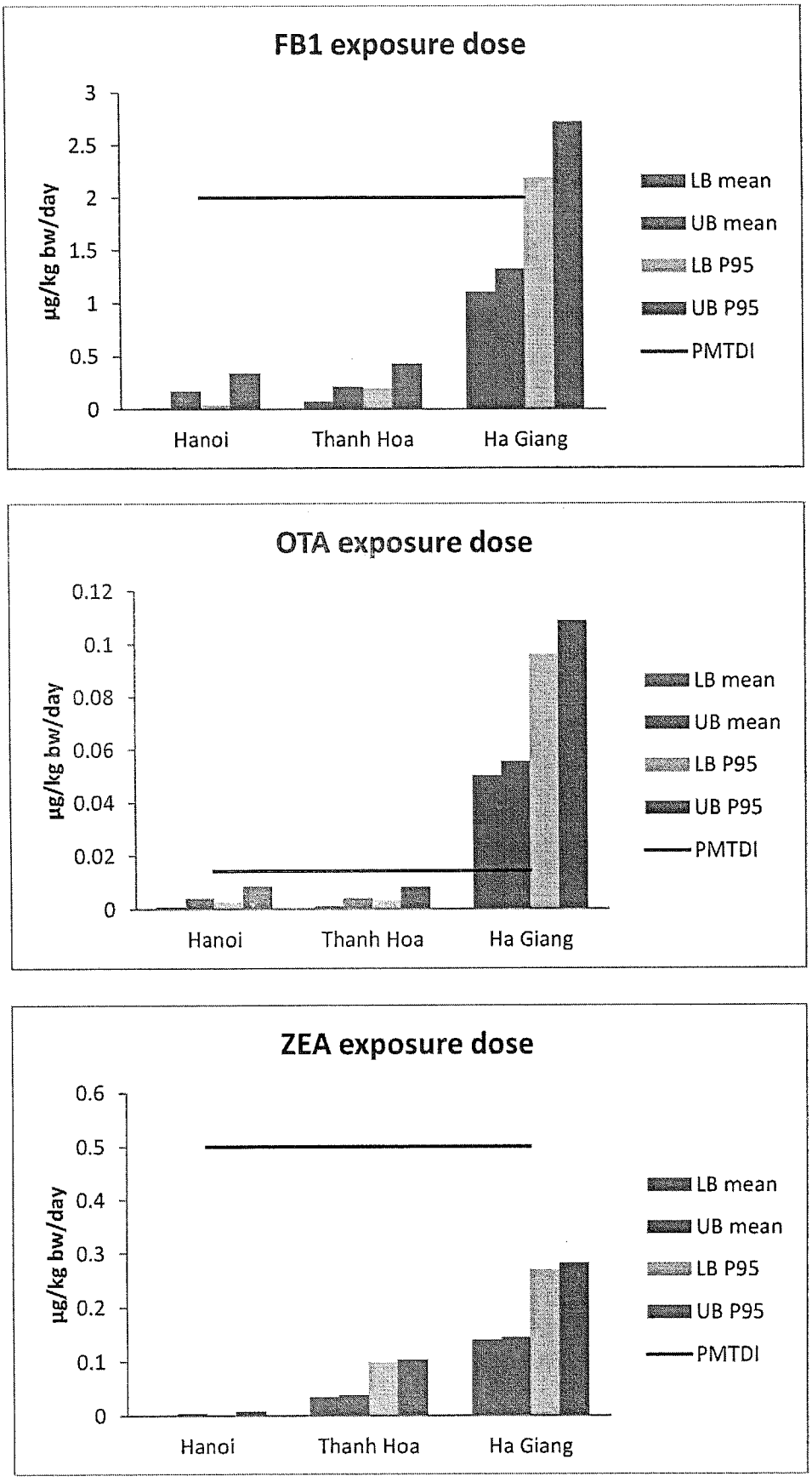

Fig. 2. The exposure dose of adults in Hanoi, Thanh Hoa and Ha Giang to FB1 (a), OTA (b) and ZEA (c) compared to their PMTDIs.

doses to FB1 from all studied sources was highest in Ha Giang (see Table 5 and Fig. 2). The main source of FB1 contamination was from maize. The mean exposure doses of FB1 in all locations were lower than current PMTDI of FB1 (2.0 μg/kg b.w. per day). This result is similar to that of the study of Huong et al. in Lao Cai province (Huong, Brimer, et al., 2016). However, in Ha Giang, the 95th percentile figures for all age groups ranged from 2.2 to 3.7 μg/kg b.w. per day (about 1.1–1.9 times of PMTDI) indicated that there was a risk of FB1 for a part of the population in this province.

For ZEA, it can be seen from Fig. 2 that there are no risk of four mycotoxins in three provinces. Among the four types of samples, the average exposure dose was highest in maize and lowest in sesame. Among sampling locations, the sum of average exposure doses to ZEA from all studied sources was highest in Ha Giang (see Table 5). All mean and 95th percentile values were lower compared to the PMTDI of ZEA (0.5 μg/kg b.w. per day). The highest exposure was recorded in the upper bound 95th percentile for the group of age from 3 to 6 years (0.37 μg/kg b.w. per day). Although the risk of ZEA in this study was low, it was still higher than that of other countries such as France (Sirot et al., 2013) and Iran (Yazdanpanah et al., 2012).

The same trend was also encountered with OTA when the exposure to OTA in Ha Giang was also higher than that of other provinces for all age groups. The average LB exposure dose for adults in Hanoi and Thanh Hoa were 0.0009 and 0.0012 μg/kg b.w. per day, much lower than that in Ha Giang (0.05 μg/kg b.w. per day). From the PMTWI value of OTA (0.1 μg/kg b.w. per week), we can estimate the PMTDI which is only 0.0143 μg/kg b.w. per day. It can be seen that exposure levels in the two delta provinces of Hanoi and Thanh Hoa were lower than that of estimated PMTDI, meanwhile, the average LB exposure level in Ha Giang was about 2.4–3.6 times higher. This indicates a risk of OTA in mountainous people's diets in Ha Giang.

4. Conclusion

The risk assessment of AFB1, FB1, OTA and ZEA in rice, peanut, sesame, and maize revealed the risk of FB1 and OTA in the diet of people in Ha Giang. Exposure to AFB1 from contaminated maize was the most serious health hazard caused by the studied mycotoxins, particularly in aggravating the situation of liver cancer in Vietnam. The obtained results also pointed out the need to improve the control of these mycotoxins in maize, especially in some highland provinces such as Ha Giang. The habit of maize storage for long time in households here should be changed and H'Mong people in the highland should use rice as the main source of food instead of maize.

CRediT authorship contribution statement

Tuan Huu Do: Conceptualization, Methodology, Writing - original draft, Visualization. **Son Cao Tran:** Validation, Data curation, Writing - review & editing, Supervision. **Chi Dinh Le:** Software, Formal analysis. **Ha-Binh Thi Nguyen:** Investigation, Resources. **Phuong-Thao Thi Le:** Investigation. **Hong-Hao Thi Le:** Project administration, Funding acquisition. **Tuyen Danh Le:** Investigation, Formal analysis. **Hung-Thu Thai-Nguyen:** Conceptualization, Supervision.

Acknowledgements

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VIỆN KIỂM NGHIỆM
AN TOÀN VỆ SINH
THỰC PHẨM QUỐC GIA

Số: /BB-KHĐT

CỘNG HOÀ XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập - Tự do - Hạnh phúc

Hà Nội, ngày 19 tháng 12 năm 2014

BIÊN BẢN
Họp Hội đồng nghiệm thu đề tài NCKH cấp cơ sở

Hội đồng KHCN cấp Viện được thành lập theo Quyết định số 520/QĐ - VKNQG ngày 17/12/2014 của Viện trưởng Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia đã tiến hành họp nghiệm thu kết quả đề tài NCKH cấp cơ sở:

"Xác định đồng thời một số độc tố vi nấm trong thực phẩm".

Chủ nhiệm đề tài: ThS. Trần Cao Sơn - CN. Đỗ Thị Thu Hằng

Đơn vị chủ trì: Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia

I. Thời gian: 8h 30 .ngày 19 tháng 12 năm 2014.

II. Địa điểm: Phòng Họp của Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia, số 13 Phan Huy Chú, Hoàn Kiếm, Hà Nội.

III. Thành phần tham gia buổi họp:

1. TS. Lê Thị Hồng Hảo, Phó Viện trưởng, Viện Kiểm nghiệm ATVSTP quốc gia, Chủ tịch hội đồng;
2. PGS. Phạm Gia Huệ, Chuyên gia, Ủy viên phản biện;
3. PGS.TS. Trần Chương Huyến, Trường Đại học quốc gia Hà Nội, Ủy viên phản biện;
4. KS. Phạm Thị Sáng, Viện tiêu chuẩn chất lượng Việt Nam, Ủy viên;
5. ThS. Vũ Thị Thu Hương, Viện Kiểm nghiệm ATVSTP quốc gia, Ủy viên;
6. ThS. Lê Thị Phương Thảo, Viện Kiểm nghiệm ATVSTP quốc gia, Ủy viên;

Thư ký: CN. Đỗ Tất Thành – KS. Vũ Thị Thúy

IV. Nội dung cuộc họp

1. **ThS. Vũ Thị Thu Hương:** Tuyên bố lý do và thông qua quyết định thành lập Hội đồng nghiệm thu.
2. **TS. Lê Thị Hồng Hảo**, Phó Viện trưởng Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia, Chủ tịch hội đồng điều hành phiên họp.

- Phương pháp nghiên cứu dựa vào các bài báo quốc tế, thực hiện trên thiết bị hiện đại tại PTN có hệ thống chuẩn ISO 17025, có tính khoa học và độ tin cậy cao.
- Phương pháp đã thực hiện hoàn thiện phương pháp kiểm nghiệm đồng thời Aflatoxin và thử nghiệm trên nhiều mẫu thực phẩm phù hợp với mục tiêu nghiên cứu đề ra.
- Đề tài giúp giải pháp sử dụng nhiều phương pháp khác nhau, tiết kiệm hóa chất, thời gian. Và là một đề tài triển vọng trong tương lai.
- Tuy nhiên, đề tài còn một số lưu ý sau:

+ Chỉnh sửa một số lỗi chính tả;

+ AOAC: là tên viết tắt của Hiệp hội các cộng đồng phân tích;

+ Cần bổ sung các phụ lục cụ thể bảng kết quả phân tích và thêm sắc đồ mẫu phân tích có phát hiện mycotoxin.

3. CN. Đỗ Thị Thu Hằng: trình bày tóm tắt kết quả nghiên cứu đề tài:

- Điểm qua phần tổng quan;
- Phương pháp phân tích;
- Kết quả nghiên cứu;
- Kết luận

4. Những ý kiến đóng góp của thành viên Hội đồng

4.1. PGS. Phạm Gia Huệ:

- Về hình thức: Đề tài trình bày sáng sủa, danh pháp được sử dụng khá thống nhất và gần sát với qui định của TCVN, tuy nhiên còn một số lỗi đánh máy và một số hình mờ hoặc quá nhỏ, khó đọc để thu nhận thông tin từ đó.

- Về nội dung:

1. Mục tiêu: Vì số lượng mẫu chưa đủ lớn, nên đổi lại là "Áp dụng phương pháp đã xây dựng xác định độc tố trên một số thực phẩm ở Hà Nội năm 2014".

2. Tổng quan: Được trình bày rõ ràng, có tương đối đầy đủ thông tin và các thông tin được chỉ dẫn cụ thể đến tài liệu tham khảo.

3. Phạm vị ứng dụng: Trang 39, kết quả phân tích trên 10 mẫu TPBS cho TE<36 tháng, có đúng là TPBS không hay ML cho độc tố vi nấm cho loại này là bao nhiêu? Nếu thức ăn công thức cho trẻ dưới 36 tháng thì cần xem lại phương pháp có LOD đủ nhỏ hơn ML qui định cho một số độc tố vi nấm trong nhóm thực phẩm này không? Từ đó xác định lại phạm vi ứng dụng của phương pháp.

Nên sửa lại tên đề mục 2.2 (trang 7) là "tình hình nhiễm độc tố vi nấm trong thực phẩm" cho phù hợp với nội dung mục này.

Trang 8 bỏ s trong fumonisin

Trang 10, thêm chú thích các chữ viết tắt DAD, ESI, TOF.

Trang 11, thêm chú thích chữ viết tắt UPLC, PSA.

Trang 18, sửa n thành N (số lần thử nghiệm).

4.2. PGS.TS. Trần Chương Huyến:

- Đề tài đã thực hiện đầy đủ các mục tiêu, nội dung nghiên cứu và các yêu cầu đã đặt ra của đề tài;
- Đề tài đã hoàn thành qui trình chung để chiết đồng thời 8 vi nấm bằng hỗn hợp dung môi acetonitril +1%HCOOH, xử lý dịch chiết bằng hỗn hợp $MgSO_4$ và NaCl, tinh sạch dịch chiết bằng dSPE với hỗn hợp C_{18} +$MgSO_4$.
- Đã nghiên cứu các điều kiện sắc ký cũng như các điều kiện cụ thể về hệ LC-MS/MS đối với 8 vi nấm đã nêu. Các kết quả đạt được về các giá trị LOD, LOQ, RSD, hệ số thu hồi đều khá tốt. Các kết quả đó cho thấy có

thể áp dụng để phân tích đồng thời các vi nấm này bằng phương pháp QuEChERS.

- Các tác giả đã thực hiện phân tích 9 thực phẩm cho thấy tình trạng nhiễm Aflatoxin khá phổ biến, tuy nhiên các số liệu này mới chỉ là bước đầu nhằm mục đích kiểm chứng phương pháp đã đề nghị là chính.
- Về cơ bản đề tài đã hoàn thành tốt các mục tiêu đề ra. Tuy nhiên, để hoàn thiện có thể xay dựng thành quy trình thường qui, nên tạo điều kiện phân tích lặp lại trên một số mẫu và đối chiếu với các phương pháp đã có TCVN.

4.3. ThS. Lê Thị Phương Thảo:

- Đề tài đã thực hiện đầy đủ các mục tiêu, nội dung nghiên cứu và các yêu cầu đã đặt ra của đề tài;
- Phương pháp nghiên cứu thực tế trên mẫu có tính khoa học cao. Kết quả đáng tin cậy.
- Có tính ứng dụng thực tế để phân tích một số độc tố vi nấm trong một số loại thực phẩm.
- Cách diễn giải tính cần thiết xây dựng quy trình phân tích đồng thời nhiều mycotoxin trên đối tượng nào xảy ra và có nguy cơ nhiễm đồng thời nhiều mycotoxin?
- Nền mẫu sử dụng trong nghiên cứu chưa nổi bật đối tượng mẫu nhiễm đồng thời nhiều mycotoxin nên kết quả chí có hiệu quả với đối tượng "ngô hạt". Đối tượng chọn cho xác định OTA và ZEA chưa phù hợp nên 90/90 mẫu đều KPH.
- Một số chỗ còn viết ở dạng văn nói, nên trau chuốt lại.
- Trang 31, xem lại LOD và LOQ của các nền mẫu khác nhau có kết quả khác nhau không.
- Quy trình chiết không có điều chỉnh với các nền mẫu khác nhau?

4.4. KS. Phạm Thị Sáng:

- Đề tài đã thực hiện đầy đủ các mục tiêu, nội dung nghiên cứu và các yêu cầu đã đặt ra của đề tài;
- Phương pháp nghiên cứu thực tế trên mẫu có tính khoa học cao. Kết quả đáng tin cậy.
- Phù hợp với mục tiêu nghiên cứu.
- Đưa ra được phương pháp xác định đồng thời một số độc tố vi nấm bằng LC-MS/Ms trong một số loại thực phẩm.
- Có tính ứng dụng thực tế để phân tích một số độc tố vi nấm trong một số loại thực phẩm.
- Tuy nhiên, cần sửa chữa, bổ sung một số nội dung sau:

  + Một số hình vẽ còn bị mờ và một số lỗi chính tả.

  + Phần kiến nghị nên nêu rõ cần phân tích tiếp trên lượng mẫu đủ lớn, cụ thể là bao nhiêu và có cần kiểm nghiệm liên phòng và nâng cấp

+ Trong báo cáo nên kèm theo phụ lục quy trình phân tích cụ thể.

V. Kết luận:

Sau khi các thành viên hội đồng phát biểu và thảo luận, chủ tịch hội đồng kết luận:

- Hội đồng thống nhất nghiệm thu kết quả đề tài tuy nhiên cần sửa chữa, bổ sung theo ý kiến đóng góp của thành viên Hội đồng.

Cuộc họp kết thúc vào hồi 11 h 00 cùng ngày.

CHỦ TỊCH HỘI ĐỒNG	**THƯ KÝ**
TS. Lê Thị Hồng Hảo	**CN. Đỗ Tất Thành**

BỘ Y TẾ
VIỆN KIỂM NGHIỆM AN TOÀN VỆ SINH THỰC PHẨM QUỐC GIA

Số: 348/QĐ-VKNQG

CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập - Tự do - Hạnh phúc

Hà Nội, ngày 10 tháng 9 năm 2014

QUYẾT ĐỊNH
(Về việc phê duyệt triển khai thực hiện đề tài KH&CN cấp Cơ sở năm 2014)

VIỆN TRƯỞNG
VIỆN KIỂM NGHIỆM AN TOÀN VỆ SINH THỰC PHẨM QUỐC GIA

Căn cứ Quyết định số 376/QĐ-TTg ngày 23/03/2009 của Thủ tướng Chính phủ về việc thành lập Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia trực thuộc Bộ Y tế;

Căn cứ Quyết định số 2456/QĐ-BYT ngày 9/7/2009 của Bộ trưởng Bộ Y tế về việc ban hành Điều lệ tổ chức và hoạt động của Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia, trực thuộc Bộ Y tế;

Căn cứ vào Biên bản họp Hội đồng thẩm định và tư vấn xây dựng thuyết minh đề tài KH&CN cấp Cơ sở ngày 07/ 8 / 2014 theo Quyết định số 243/QĐ-VKNQG ngày 04/ 8/ 2014 của Viện trưởng Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia;

Xét đề nghị của ông Trưởng phòng Khoa học - Đào tạo và Chỉ đạo tuyến,

QUYẾT ĐỊNH:

Điều 1. Triển khai thực hiện đề tài KH&CN cấp Cơ sở năm 2014 "Xác định đồng thời một số mycotoxin trong thực phẩm bằng sắc ký lỏng khối phổ " do **ThS. Trần Cao Sơn** làm chủ nhiệm đề tài.

Kinh phí thực hiện đề tài: *150.000.000 đ (Dự toán chi tiết kèm theo)*

Nguồn kinh phí: Chi thường xuyên - Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia

Thời gian hoàn thành: *06 tháng*

Điều 2. Ông Trần Cao Sơn và nhóm nghiên cứu chịu trách nhiệm triển khai công tác nghiên cứu và báo cáo kết quả về Phòng Khoa học đào tạo & Chỉ đạo tuyến để tổ chức nghiệm thu đúng thời gian đã đăng ký.

Điều 3. Các ông (bà): Trưởng phòng Tổ chức - Hành chính, Trưởng phòng Khoa học Đào tạo và Chỉ đạo tuyến, Phụ trách phòng Tài chính – Kế toán, Ông Trần Cao Sơn và đơn vị liên quan thuộc Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia chịu trách nhiệm thi hành Quyết định này./.

Nơi nhận:
- Như Điều 3;
- Lưu: TCHC, KHĐT.

VIỆN TRƯỞNG

VIỆN KIỂM NGHIỆM AN TOÀN VỆ SINH THỰC PHẨM QUỐC GIA

Phạm Xuân Đà

BỘ Y TẾ
VIỆN KIỂM NGHIỆM
AN TOÀN VỆ SINH
THỰC PHẨM QUỐC GIA
Số: 394 /QĐ-VKNQG

CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập - Tự do - Hạnh phúc

Hà Nội, ngày 11 tháng 6 năm 2018

QUYẾT ĐỊNH
Về việc phê duyệt đề cương đề tài KH&CN cấp Cơ sở năm 2018

VIỆN TRƯỞNG
VIỆN KIỂM NGHIỆM AN TOÀN VỆ SINH THỰC PHẨM QUỐC GIA

Căn cứ Quyết định số 376/QĐ-TTg ngày 23/03/2009 của Thủ tướng Chính phủ về việc thành lập Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia;

Căn cứ Quyết định số 2456/QĐ-BYT ngày 9/7/2009 của Bộ trưởng Bộ Y tế về việc ban hành Điều lệ tổ chức và hoạt động của Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia, trực thuộc Bộ Y tế;

Căn cứ Kế hoạch số 351/KH-VKNQG ngày 6/4/2018 về việc tổ chức triển khai các hoạt động năm 2017 của Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia;

Căn cứ Quyết định số 327/QĐ-VKNQG ngày 28/5/2018 của Viện trưởng Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia về việc thành lập Hội đồng xét duyệt đề cương đề tài NCKH và CN cấp Cơ sở năm 2018;

Căn cứ vào Biên bản họp ngày 8 / 6 / 2018 của Hội đồng xét duyệt đề cương đề tài NCKH&CN cấp Cơ sở năm 2018;

Xét đề nghị của Trưởng phòng Khoa học - Đào tạo và Chỉ đạo tuyến,

QUYẾT ĐỊNH:

Điều 1. Phê duyệt đề cương đề tài nghiên cứu khoa học và công nghệ cấp Cơ sở năm 2018 theo những nội dung sau:

- Tên đề tài: **Nghiên cứu phương pháp sàng lọc chất dị nguyên trong thực phẩm bằng sắc ký lỏng khối phổ 2 lần (LC-MS/MS)**
- Chủ nhiệm đề tài: TS. Trần Cao Sơn, ThS. Nguyễn Thị Hà Bình
- Kinh phí thực hiện đề tài: 70.000.000 đồng

(Bằng chữ: Bảy mươi triệu đồng chẵn).

- Nguồn kinh phí: Quỹ phát triển hoạt động sự nghiệp của Viện.
- Thời gian hoàn thành: 31/12/2018.

Điều 2. Các ông/bà chủ nhiệm đề tài có trách nhiệm:

- Triển khai các nội dung, nhiệm vụ theo đề cương đã được Hội đồng phê duyệt và thực hiện đúng các quy định hiện hành về hoạt động Khoa học công nghệ.

- Báo cáo kết quả triển khai đề tài về Phòng Khoa học đào tạo & Chỉ đạo tuyến và tiến hành tổ chức nghiệm thu đúng tiến độ được phê duyệt.

Điều 3. Quyết định này có hiệu lực kể từ ngày ký.

Điều 4. Các ông/bà Trưởng phòng KHĐT&CĐT, Trưởng phòng TC-KT, Chủ nhiệm đề tài và Trưởng các đơn vị, cá nhân liên quan chịu trách nhiệm thi hành Quyết định này.

Nơi nhận:
- Như Điều 4;
- PVT. Trần Văn Sơn (p/h chỉ đạo);
- Lưu: VT, KHĐT.

VIỆN TRƯỞNG

VIỆN KIỂM NGHIỆM AN TOÀN VỆ SINH THỰC PHẨM QUỐC GIA

Lê Thị Hồng Hảo

VỆ SINH
GIA

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Hindawi

Research Article

Rapid Screening and Quantitative Determination of Illegal Phosphodiesterase Type 5 Inhibitors (PDE-5i) in Herbal Dietary Supplements

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Phosphodiesterase type 5 inhibitors (PDE-5i) are the first-line medication for oral erectile dysfunction, which are used according to the prescription of doctors. However, these substances have been found illegally in supplementary foods. The quality and safety of dietary supplements for enhancing male sexual performance have been questioned, raising the need for continual development of analytical methods. Liquid chromatography coupled with high-resolution mass spectrometry has become one of the most effective methods to identify and measure PDE-5i concentration. In this research, we focused on (i) developing and validating an effective screening and quantitation method for more than 53 PDE-5i in ingredients and supplementary products using LC-Q-Exactive after a simple sample extraction and (ii) assessing PDE-5i content in natural-based supplementary products available in Vietnam market. The extraction method used a small amount of organic solvent, which makes it more environmentally friendly (greener). The developed method has a limit of detection of 0.4 mg/kg, a limit of quantitation of 1.2 mg/kg, recoveries from 80 to 110%, and repeatability lower than 15%. Ninety-two herbal supplementary foods and ingredients used for enhancement of male sexual performance available in Vietnamese markets were collected. Fourteen PDE-5i including conventional and novel analogous were detected and measured in eighteen food supplements and two formulation ingredient samples.

1. Introduction

Phosphodiesterase type 5 (PDE-5) is an enzyme responsible for the breakdown of cyclic guanosine monophosphate (cGMP) in the corporal smooth muscle [1]. Thus, PDE-5i are considered the first-line medication for oral erectile dysfunction (ED) therapies [2]. After the approval of sildenafil, several PDE-5i have been approved and demonstrated well-established efficacy in patients with ED such as tadalafil, vardenafil, avanafil, mirodenafil, undenafil, and lodenafil. Additionally, PDE-5i proved their great potential in the treatment of neuroinflammation, neurodegeneration, cognition (Alzheimer's disease), cancer therapeutics, diabetic peripheral neuropathy, renoprotection, etc. [3]. Although the safety of PDE-5i was proven, the use of PDE-5i has some adverse effects such as ataxia caused by acetildenafil and its analogs and symptoms of giddiness, headache, shortness of breath, and backache [4]. PDE-5i have an interaction effect with other medicines such as nitrates. From 2007, the US FDA announced that a warning of the potential risk of visual and auditory impairment related to nonarteritic anterior ischemic optic neuropathy and sudden sensorineural hearing loss would be added to drug labels of PDE-5i [3]. Seriously, the first known fatal case

related to desmethyl carbodenafil, an unapproved PDE-5i, on a 34-year old male was reported in 2017 [5]. Therefore, the use of PDE-5i in therapy should strictly obey the advice of pharmacists.

PDE-5i, namely sildenafil, tadalafil, vardenafil, and their analogs, have been added in supplementary foods, which were supposed to be made of natural ingredients. The presence of PDE-5i in these supplement foods without labeling is consumer deception. In 2013, J.H. Lee et al. reported more than 46 PDE-5i analogs in various forms of health food products in the online and offline market of Korea [6]. A case study in the Czech market revealed that 10 over 64 natural herbal-based supplements for ED treatment contain both registered or unregistered synthetic PDE-5i [7]. In the summary of the Min-Yong Low research group, Asia reported the highest number of PDE-5i as adulterants in dietary supplements and was followed by Europe and North America [4]. Research of the Malaysian market showed 82% tested unregistered products and 14% of the registered products were adulterated with PDE-5i or their analogs [8]. The regulation of PDE-5i has been complicated because of the increasing number of novel synthetic PDE-5i analogs. Thus, the current situation raises the need for continual development of analytical methods to quickly detect PDE-5i analogs in these products.

The rapid and accurate identification and measurement of popular and unknown PDE-5i have been improved by numerous analytical techniques including high-performance liquid chromatography (HPLC) [9, 10], gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectroscopy [11], vibrational spectroscopy, liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry (LC-FT-ICR-MS), etc. [12]. The most effective approach for the identification of PDE-5i in supplements is HLPC-MS [6, 13, 14], although some publications had been done by HPLC with a UV detector [9] or photodiode array detector [14]. In 2015, the AOAC International published an official method for screening and identification of PDE-5i in dietary ingredients and supplements [15]. The samples were simply extracted with a mixture of solvent (methanol, acetonitrile, water), then diluted, filtered, and analyzed by LC quadrupole-orbital ion trap MS. Identification of targeted and nontargeted analytes was conducted based on retention time, accurate mass, and isotopic pattern of precursors ions and product ions using an in-house database. Recently, Hong et al. have reported a screening and classification method of PDE-5i by GC-MS [16]. Specific common ions according to structural after the trimethylsilyl derivatization characteristics of four PDE-5i classes were found.

The development of high-resolution mass spectrometry (HRMS) techniques has made screening applications more selective than conventional MS techniques. Currently, time-of-flight mass spectrometry (TOF) or Orbitrap mass spectrometry techniques can achieve high mass accuracy (below 5 ppm). In particular, Orbitrap mass spectrometry can perform high-resolution MS/MS allows both the screening of unknown compounds and the quantification of target substances. Therefore, liquid chromatography coupled with HRMS has become one of the most effective methods to identify and measure PDE-5i concentration. Our goals are (i) developing and validating a screening and quantitation method for PDE-5i in supplementary products and (ii) assessing PDE-5i content in natural-based supplementary products and ingredients available in the Vietnam market.

2. Materials and Methods

2.1. Chemicals and Reagents. Fifty-three PDE-5i standards were obtained from Toronto Research Chemicals (Martin Ross Avenue, North York, Ontario, Canada) and LGC Standards (GmbH Mercatorstrasse, Wesel, Germany). Methanol, acetonitrile, n-hexane, and other organic solvents were purchased from Merck (Darmstadt, Germany). Formic acid and ammonium formate were purchased from Sigma-Aldrich (St. Louis, USA). Deionized water (18.2 MegaOhm.cm) was purified using a Milli-Q system (Millipore, Co., Bedford, MA, USA).

Each solid standard was accurately weighed about 10 mg and diluted by 10 mL methanol to obtain a 1000 μg/mL standard solution. The stock solutions were kept in dark bottles at 4°C and to use for 1 year. The working solutions were prepared by diluting the stock solutions with methanol into the concentration of 0.01, 0.1, 0.2, 0.5, and 1 μg/mL.

2.2. Sample Preparation. Ninety-two supplement foods and ingredients specific for the enhancement of male sexual performance were collected in local stories in Vietnam.

Samples in the form of tablets were crushed into a fine powder. In samples in the form of hard-shelled capsules or soft-gel capsules, the capsules had been removed and the inside content only has been homogenized. Each homogeneous sample was weighed 0.10 g in a 15 mL centrifuge tube by an analytical balance. Then, 4.0 mL of acetonitrile: water (1 : 1, v/v) was added and mixed well before being sonicated for 30 minutes. The extract was centrifuged for 5 minutes at a speed of 6000 rpm. The solution was separated from the residue and filtered through a polytetrafluoroethylene filter (0.2 μm) before being injected into a liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS). For the soft-gel capsule samples, 1.0 mL of n-hexane was added to the solution after centrifuging and mixed well to clean the oily components; only the aqueous layer was used for LC-HRMS analysis. For samples containing PDE-5i over the calibration curves, we have to reanalyze with a proper dilution factor.

2.3. Liquid Chromatography and High-Resolution Tandem Mass Spectrometry Condition. The sample solutions were analyzed by the UltiMate 3000 UHPLC system coupled with Q-exactive (Thermo Fisher Scientific Inc., USA). The separation was conducted on Waters BEH C18 (100 mm × 1.7 μm × 2.1 mm) column with an appropriate precolumn at the temperature of 40°C. Mobile phase A was 10 mM ammonium formate and 0.1% formic acid in water; mobile phase B was 10 mM ammonium formate and 0.1% formic acid in acetonitrile: methanol (1 : 1, v/v). The gradient was 0–5 min, 2% B; 5–15 min, 2–40% B;

15–22 min, 40–95% B; 25–26 min, 95–2% B; 26–29 min, 2% B. The injection volume was 10 μL. The flow rate was 0.3 mL/min.

The Q-exactive was equipped with heated electrospray ionization (HESI) source with the following parameters: HESI temperature of 320°C, the capillary temperature of 350°C, spray voltage of 5000 V, sheath gas flow of 30 arbitrary units, the auxiliary gas flow of 10 arbitrary units. The mass spectrometer was operated in the full MS/data-dependent MS/MS mode (full MS-dd-MS/MS) with the following parameters: scan range 200–2000 m/z, resolution 70,000 FWHM (defined for m/z 200; 3 Hz), automatic gain control (AGC) target $1e^6$, maximum inject time 20 ms, and in the dd-MS/MS mode: resolution 17,500 FWHM (defined for m/z 200; 12 Hz), AGC target $1e^5$, isolation window 1 m/z, normalized collision energy 40%, 70%, 100%. Full spectral information was utilized for identification and quantification. For data collection and analysis, the screening PDE-5i process was conducted by Compound discoverer 3.1 software (Thermo Fisher), and the quantitation process was conducted by TraceFinder 4.1 software (Thermo Fisher). Mass spectrometric information, including m/z of precursor and product ions of analytes, was shown in Table 1. It can be seen that the analogs of sildenafil produced the common ions at m/z 283. It is in line with previous studies that the ion is the result of cleavage of the C-S bond and loss of the ethyl group on the ethoxy substituent on the phenyl ring. For the tadalafil group, the ions at m/z 169 (pyridine-indole ring) and 135 were always recorded. The ion at m/z 344 is characterized for vardenafil and its analogs [7, 17]. Thiosildenafil group often produces ion at m/z 299 corresponding to the cleavage of C-N bond and loss of the ethyl group on the ethoxy substituent on the phenyl ring [18].

2.4. Screening and Quantification of Real Samples. Real samples were first screened PDE-5i as the scheme in Figure 1. Most of the detected PDE-5i were listed in our mass spectrometry library unless the new suspected compounds were extracted and purified, and then the structure was determined by infrared spectroscopy. For the quantitative purpose, the concentration of PDE-5i in the samples was calculated by matrix match calibration curves.

3. Results and Discussions

3.1. Optimization of LC-HRMS Condition. First of all, parameters for detecting PDE-5i in Q-exactive mass spectrometer should be set up before further optimization. All PDE-5i have chemical structures suitable for being ionized by electrospray ionization source in positive mode. A 500 μL mixture of 1 μg/mL standard solution of PDE-5i was injected into the Q-exactive mass spectrometer to optimize ionization and detection conditions such as capillary voltage, the temperature of HESI, the temperature of ion transfer tube, S-lens level, maximum injection time, and automatic gain control. After that, the MS/MS data were recorded: full-scan mode for precursor ions and dd-MS^2 (data-dependent MS^2) for product ions (All ion fragmentation-AIF). The normalized collision energy (NCE) was 40, 70, 100%. The MS/MS data were compared with mzCloud Mass Spectral Library (Thermo Fisher Scientific) and the mass accuracy was less than 5 ppm, which meets the requirements of AOAC International. The MS/MS data of fifty-three standard PED-5i for identification was presented in Table 1. For the detection of PDE-5i without standard solutions, MS/MS information (Table S1) in the mzCloud Mass Spectral Library can be used.

PDE-5i are less polar compounds, so they can be analyzed by the C18 base chromatography column. Because of their similar structures, PDE-5i should be separated by a chromatography column that has a small particle size. Therefore, we chose BEH C18 (100 mm × 1.7 μm × 2.1 mm, Waters, Milford, Massachusetts, USA). Commonly, mobile phases for PDE-5i analysis are acetonitrile: water and acetonitrile: methanol (1 : 1, v/v), adding additives such as formic acid, ammonium format, or both of them. We investigated and chose the mobile phase system including mobile phase A: 10 mM ammonium formate and 0.1% formic acid in water, and mobile phase B: 10 mM ammonium format and 0.1% formic acid in acetonitrile: methanol (1 : 1, v/v). The use of both ammonium formate and formic acid additives is important to gain the sensitivity of some PDE-5i and is consistent with AOAC 2015.12 method [15]. Then, the gradient was optimized and lasted 29.0 minutes to separate some isomeric PDE-5i such as Carbodenafil and Noracetildenafil, Benzamidenafil, and Tadalafil. The flow rate was 0.3 mL/min. This slow and long gradient is similar to that of the reference methods published by AOAC International and US USP [19]. The retention time of each analyte was shown in Table 1. Extracted chromatograms of PDE-5i were shown in the supplementary document (Figures S1, S2).

3.2. Optimization of Extraction. Referring to previous studies [7, 20], five extraction solutions were selected to examine extraction efficiency when extracting spiked samples at the concentration of 4 mg/kg in samples. The results of four representative compounds were presented in Figure 2. A one-way ANOVA test was conducted to compare the intensity of four compounds. The results (P value from 1.5E-10-4.9E-6 < 0.05) indicated that signal intensity changed significantly with different solvent extraction, and the mean comparison showed that the mixture of acetonitrile: water (1 : 1, v/v) gave significantly higher intensities of analytes compared to the other tested solvents. Comparing to the mixture of methanol: water (70 : 30, v:v) used by Lee et al. [17], Jeong et al. [21], or methanol used by Ren et al. [22], this method uses less organic solvent for a greener sample preparation. Thus, it was chosen to extract real samples.

For the soft-gel capsule sample, however, we added a second solvent to remove oily components of samples before injecting the extraction into the LC-HRMS system. Three organic solvents including n-hexane, diethyl ether, and ethyl acetate were examined, and the result was shown in Figure 3.

Table 1: Mass spectrometric parameters for identification and retention time of PDE-5i.

No	PDE-5i	Chemical formula	Retention time (min)	Ion type	Precursor ion (m/z)	Product ions (m/z)
1	Mirodenafil	$C_{26}H_{37}N_5O_5S$	7.57	M + H	532.2588	99.09167; 296.13935; 312.13427; 70.06513; 56.04948; 84.0682; 210.06619; 129.10224; 88.07569; 121.03964
2	Noracetildenafil	$C_{24}H_{32}N_6O_3$	13.96	M + H	453.2609	70.06513; 97.07602; 113.10732; 98.08385; 58.06513; 56.04948; 297.1346; 166.09749; 325.12952; 353.16082
3	Desmethyl fondenafil	$C_{23}H_{30}N_6O_3$	15.29	M + H	439.2459	339.14505; 311.11395; 396.20255; 374.42169; 319.71493; 196.38647; 165.01724; 120.37976; 99.0919; 73.11301
4	N-Octylnortadalafil	$C_{29}H_{33}N_3O_4$	15.64	M + H	489.3126	169.07602; 135.04406; 204.08078; 262.08626; 338.22269; 115.05423; 197.07094; 264.10191; 130.06513; 232.07569
5	Acetylvardenafil	$C_{25}H_{34}N_6O_3$	16.01	M + H	467.2765	169.09715; 70.06513; 84.08078; 341.16082; 111.09167; 72.08078; 127.12297; 97.07602; 112.0995; 110.06004
6	Lodenafil carbonate	$C_{47}H_{62}N_{12}O_{11}S_2$	16.07	M + H	1048.642	112.0995; 82.06513; 58.06513; 97.07602; 111.09167; 56.04948; 487.2122; 83.06037; 84.08078; 283.11895
7	Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$	16.13	M + H	483.2714	97.07602; 70.06513; 127.08659; 143.11789; 100.07569; 297.1346; 88.07569; 166.09749; 112.0995; 128.09441
8	Carbodenafil	$C_{24}H_{32}N_6O_3$	16.23	M + H	453.2609	311.11387; 339.14517; 166.09749; 255.12404; 69.04472; 97.07602; 225.07709; 70.06513
9	Acetildenafil	$C_{25}H_{34}N_6O_3$	16.48	M + H	467.2765	111.09167; 97.07602; 70.06513; 84.08078; 72.08078; 127.12297; 112.0995; 297.1346; 56.04948; 166.09749
10	Descarbonsildenafil	$C_{21}H_{30}N_6O_4S$	16.59	M + H	463.2129	418.15475; 311.15069; 432.17177; 406.15494; 361.13279; 344.14795; 283.11908; 238.83536; 192.99106; 175.69979; 151.05383; 125.02768; 87.09227; 72.08158; 58.066
11	Piperiacetildenafil	$C_{24}H_{31}N_5O_3$	16.75	M + H	438.25	98.09643; 70.06513; 297.1346; 55.05423; 166.09749; 341.16082; 69.04472; 325.12952; 86.09643
12	Dimethylacetildenafil	$C_{25}H_{34}N_6O_3$	16.96	M + H	467.2765	84.08078; 127.12297; 112.0995; 111.09167; 70.06513; 297.1346; 58.06513; 166.09749; 325.1659; 410.21867
13	Hydroxyvardenafil	$C_{23}H_{32}N_6O_5S$	17.09	M + H	505.2228	169.09715; 344.14791; 99.09167; 110.06004; 299.11387; 123.09167; 58.06513; 56.04948; 68.01309; 82.06513
14	N-Desethylvardenafil	$C_{21}H_{28}N_6O_4S$	17.10	M + H	461.1966	169.09715; 344.14791; 110.06004; 299.11387; 316.11661; 123.09167; 68.01309; 82.06513; 56.04948
15	Piperazonifil	$C_{25}H_{34}N_6O_4$	17.10	M + H	483.2726	465.26167; 436.22269; 429.52412; 408.22737; 380.20885; 339.1819; 297.13488; 266.45598; 244.12189; 203.11833; 153.1027; 127.08668; 99.0923; 72.08144
16	Vardenafil	$C_{23}H_{32}N_6O_4S$	17.20	M + H	489.2279	169.09715; 344.14791; 110.06004; 299.11387; 72.08078; 123.09167; 70.06513; 376.1074; 68.01309; 113.10732
17	Avanafil	$C_{23}H_{26}ClN_7O_3$	17.33	M + H	484.1858	155.02582; 375.12184; 105.03349; 77.03858; 95.04914; 53.03858; 357.11128; 233.1033; 67.05423; 221.1033
18	Isosildenafil	$C_{22}H_{30}N_6O_4S$	17.35	M + H	475.2122	58.06513; 99.09167; 283.11895; 100.0995; 56.04948; 253.072; 70.06513; 311.15025; 225.07709
19	Hydroxyhomosildenafil	$C_{23}H_{32}N_6O_5S$	17.36	M + H	505.2228	99.09167; 70.06513; 58.06513; 84.0682; 97.07602; 283.11895; 88.07569; 129.10224; 112.0995; 311.15025

Table 1: Continued.

No	PDE-5i	Chemical formula	Retention time (min)	Ion type	Precursor ion (m/z)	Product ions (m/z)
20	N-Desmethylsildenafil	$C_{21}H_{28}N_6O_4S$	17.37	M + H	461.1966	85.07602; 283.11895; 311.15025; 56.04948; 299.10868; 225.07709; 254.07983; 253.072; 377.1278; 344.14791
21	Sildenafil	$C_{22}H_{30}N_6O_4S$	17.39	M + H	475.2122	58.06513; 100.0995; 99.09167; 56.04948; 283.11895; 70.06513; 311.15025; 225.07709; 299.11387
22	Homosildenafil	$C_{23}H_{32}N_6O_4S$	17.47	M + H	489.2279	72.08078; 58.06513; 99.09167; 113.10732; 70.06513; 283.11895; 84.08078; 71.07295; 114.11515; 311.15025
23	Acetaminotadalafil	$C_{23}H_{20}N_4O_5$	17.60	M + H	433.1507	204.08078; 262.08626; 135.04406; 205.0886; 233.08352; 232.07569; 169.07602; 191.07295; 263.09408; 250.08626
24	Aminotadalafil	$C_{21}H_{18}N_4O_4$	17.60	M + H	391.1401	204.08078; 135.04406; 262.08626; 233.08352; 169.07602; 232.07569; 250.08626; 191.07295; 203.07295
25	Sildenafil N-oxide	$C_{22}H_{30}N_6O_5S$	17.60	M + H	491.2071	99.09167; 56.04948; 70.06513; 404.1387; 344.14791; 58.06513; 97.07602; 283.11895; 311.15025; 377.1278
26	Cyclopentylnafil	$C_{26}H_{36}N_6O_4S$	17.70	M + H	529.2592	461.19682; 377.13029; 344.1461; 313.16608; 277.28223; 237.59493; 210.18739; 169.09731; 142.733; 98.09704; 75.59057
27	Dimethylsildenafil	$C_{23}H_{32}N_6O_4S$	17.71	M + H	489.2279	99.09167; 71.07295; 56.04948; 113.10732; 70.06513; 283.11895; 311.15025; 84.08078; 377.1278; 225.07709
28	Nortadalafil	$C_{21}H_{17}N_3O_4$	17.77	M + H	376.1292	204.08078; 262.08626; 135.04406; 233.08352; 232.07569; 169.07602; 191.07295; 254.0924; 250.08626
29	Udenafil	$C_{25}H_{36}N_6O_4S$	17.98	M + H	517.2592	84.08078; 112.11208; 283.11895; 58.06513; 325.1659; 299.11387; 81.06988; 255.12404; 79.05423; 82.06513
30	Benzamidenafil	$C_{19}H_{23}N_3O_6$	18.02	M + H	390.166	151.07536; 107.04914; 135.04406; 91.05423; 79.05423; 105.03349; 90.0464; 136.05188; 65.03858; 93.03349
31	Norneovardenafil	$C_{18}H_{20}N_4O_4$	18.07	M + H	357.1557	169.07602; 110.06004; 329.12443; 328.11661; 123.09167; 68.01309; 300.08531; 55.05423; 82.06513; 95.06037
32	Propoxyphenyl-homohydroxysildenafil	$C_{24}H_{34}N_6O_5S$	18.13	M + H	519.2384	99.09167; 70.06513; 283.11895; 84.0682; 97.07602; 299.11387; 129.10224; 88.07569; 112.0995; 255.12404
33	O-desethyl-o-propyl sildenafil	$C_{23}H_{32}N_6O_4S$	18.14	M + H	489.2285	447.1196; 416.97849; 391.14451; 347.08125; 325.16612; 283.11909; 252.20022; 230.78935; 193.95794; 163.05384; 107.28071; 100.10004; 91.75806; 70.06595; 58.06599
34	2-Hydroxypropyl nortadalafil	$C_{24}H_{23}N_3O_5$	18.20	M + H	434.1711	135.04406; 169.07602; 204.08078; 262.08626; 284.13935; 197.07094; 130.06513; 115.05423; 232.07569; 312.13427
35	Propoxyphenyl aildenafil	$C_{24}H_{34}N_6O_4S$	18.39	M + H	503.2447	461.19514; 391.14362; 347.08197; 325.16609; 283.11925; 256.09442; 189.66799; 159.62481; 137.61562; 113.1077; 99.09223; 91.76256; 71.07376
36	Acetil acid	$C_{18}H_{20}N_4O_4$	18.45	M + H	357.1557	285.1345; 300.08487; 313.16528; 273.23201; 234.78161; 329.12441; 57.55269; 76.77712; 91.7671; 128.3394; 166.09776
37	Tadalafil	$C_{22}H_{19}N_3O_4$	18.69	M + H	390.1448	204.08078; 135.04406; 262.08626; 169.07602; 205.0886; 232.07569; 233.08352; 240.11314; 268.10805; 250.08626

Table 1: Continued.

No	PDE-5i	Chemical formula	Retention time (min)	Ion type	Precursor ion (m/z)	Product ions (m/z)
38	Depiperazino-thiosildenafil	$C_{17}H_{20}N_4O_4S_2$	18.80	M + H	409.1012	381.06896; 365.03707; 352.03045; 328.13556; 300.10448; 272.07283; 253.43238; 218.38203; 200.90222; 182.07341; 146.98571; 130.30821; 91.75983; 69.50264
39	Mutaprodenafil	$C_{27}H_{35}N_9O_5S_2$	18.95	M + H	630.2282	142.00711; 602.23247; 560.22363; 516.1504; 489.22772; 439.15549; 404.13937; 377.12856; 344.14797; 312.15851; 288.21153; 219.20777; 163.22714; 113.10774; 84.98616
40	Gendenafil	$C_{19}H_{22}N_4O_3$	19.24	M + H	355.1765	327.14517; 285.1346; 298.10604; 256.09548; 311.11387; 69.04472; 120.04439; 154.0611; 313.1659; 166.09749
41	Hydroxychlorodenafil	$C_{19}H_{23}ClN_4O_3$	19.26	M + H	391.1531	313.12952; 285.1346; 363.12184; 256.09548; 120.04439; 69.04472; 166.09883; 78.99452; 327.14517; 255.08765
42	Hydroxythiovardenafil	$C_{23}H_{32}N_6O_4S_2$	19.57	M + H	521.1999	167.06375; 360.12506; 99.09167; 315.09037; 138.02462; 150.10257; 58.06513; 299.09611; 70.06513; 332.09307
43	Chloropretadalafil	$C_{22}H_{19}ClN_2O_5$	19.65	M + H	427.1055	135.04406; 274.08559; 204.08078; 216.08078; 189.06988; 262.08674; 244.0735; 302.08117
44	Chlorodenafil	$C_{19}H_{21}ClN_4O_3$	19.79	M + H	389.1375	361.10619; 285.1346; 311.11387; 154.0611; 166.09749; 69.0573; 256.09548; 76.97887; 165.0183
45	Benzylsildenafil	$C_{28}H_{34}N_6O_4S$	20.01	M + H	551.2435	91.05423; 65.03858; 134.09643; 377.1278
46	Nitrodenafil	$C_{17}H_{19}N_5O_4$	20.09	M + H	358.151	330.11968; 316.11661; 154.0611; 256.09548; 68.0369; 255.08765; 313.11694; 227.09274; 269.1033; 136.05054
47	Pseudovardenafil	$C_{22}H_{29}N_5O_4S$	20.21	M + H	460.2013	169.09715; 110.06004; 344.14791; 299.11387; 123.09167; 284.12678; 68.01309; 82.06513; 55.05423; 95.06037
48	Imidazosagatriazinone	$C_{17}H_{20}N_4O_2$	20.32	M + H	313.1659	285.1346; 256.09548; 120.04439; 68.0369; 255.08765; 241.072; 269.1033; 69.04472; 154.0611; 94.02874
49	Propoxyphenylthio-hydroxyhomosildenafil	$C_{24}H_{34}N_6O_4S_2$	20.55	M + H	535.2156	99.09167; 70.06513; 56.04948; 299.09611; 58.06513; 84.0682; 129.10224; 315.09037; 88.07569; 271.10119
50	Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$	20.55	M + H	505.205	72.08078; 99.09167; 113.10732; 56.04948; 299.09611; 70.06513; 84.08078; 327.12741; 71.07295; 355.15806
51	Hydroxythio-homosildenafil	$C_{23}H_{32}N_6O_4S_2$	20.56	M + H	521.1999	99.09167; 70.06513; 58.06513; 84.0682; 299.09611; 129.10224; 97.07602; 88.07569; 327.12741; 112.0995
52	Norneosildenafil	$C_{22}H_{29}N_5O_4S$	20.64	M + H	460.2013	283.11895; 84.08078; 299.09611; 311.15025; 154.0611; 316.11661; 255.12404; 344.14791; 166.09749
53	Thiosildenafil	$C_{23}H_{32}N_6O_3S_2$	21.47	M + H	505.205	99.09167; 71.07295; 299.09611; 113.10732; 56.04948; 70.06513; 327.12741; 84.08078; 241.0542; 298.08828

We also compared the intensity of analytes by one-way ANOVA test. All three cleaning ways improved the intensity of analytes. The intensity of analytes in oily samples washed by n-hexane was significantly higher than that of the other solvents. Therefore, n-hexane was chosen for cleaning oily samples to reduce unwanted compounds injected into the LC-HRMS system. The use of n-hexane has not been reported before. It helps protect the ion source from fat contamination.

3.3. Method Validation. The developed method had been validated before applied to real samples analysis. The specificity of the method was proved by mass accuracy of precursor ions and productions and comparison between blank samples and standard materials. The validation parameters were showed in Table S2. Calibration curves of PDE-5i were constructed from 10 to 1000 ng/mL, and the regression coefficients were larger than 0.995, and relative standard deviations were less than 15%. The limit of detection and limit of quantification were 0.4

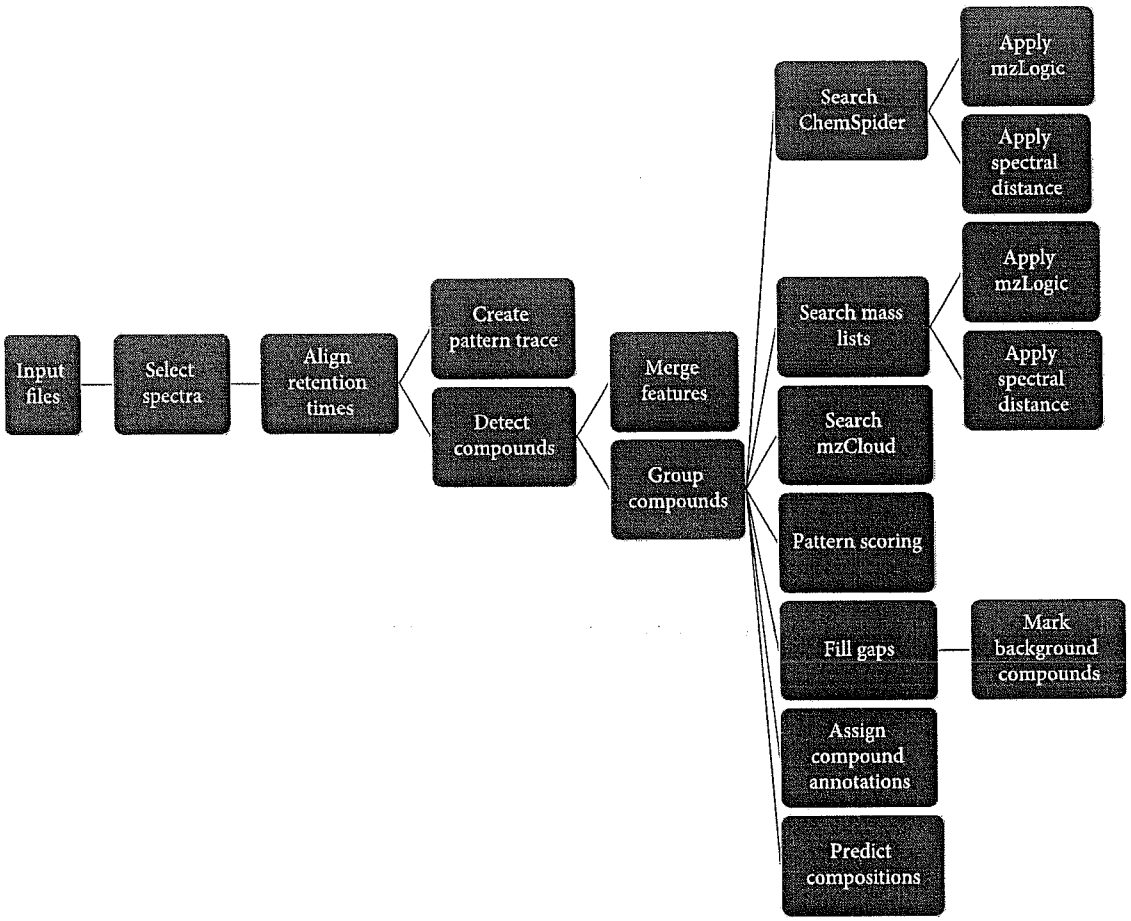

FIGURE 1: Screening workflow for unknown substances.

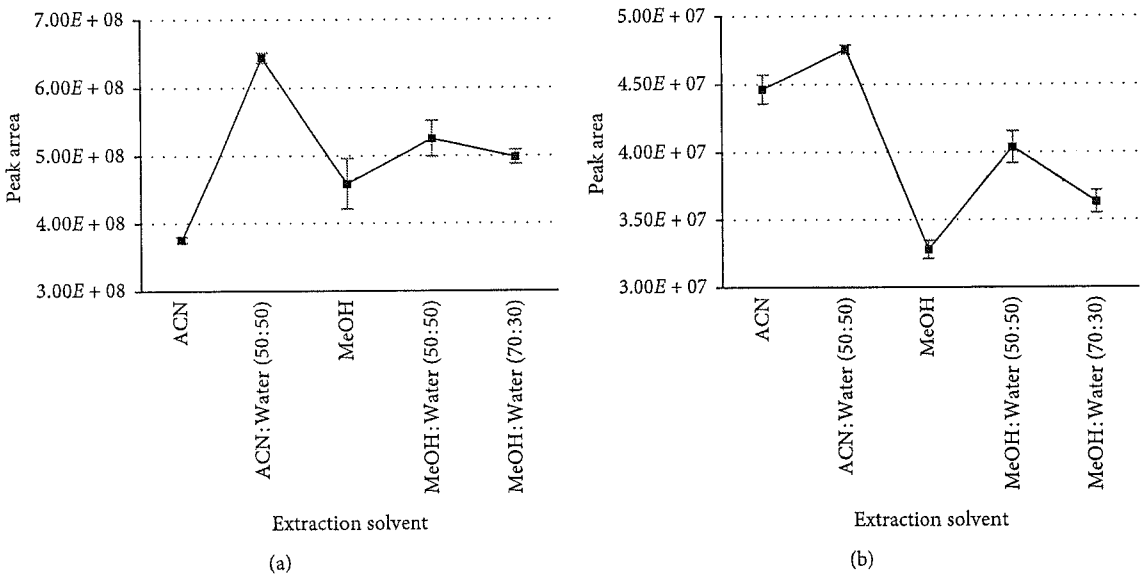

FIGURE 2: Continued.

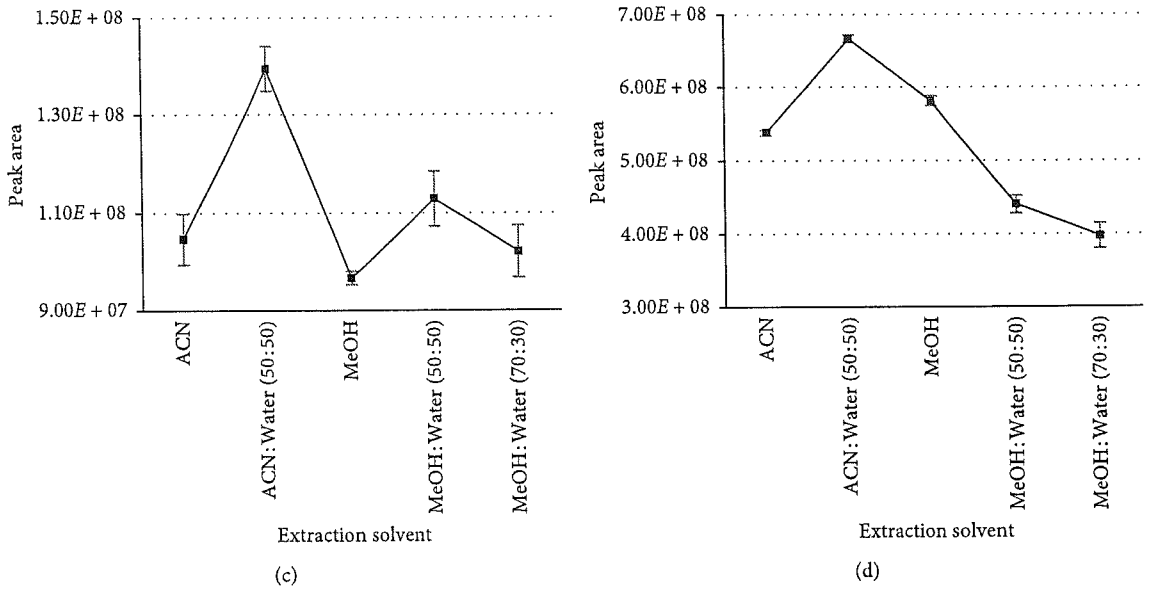

FIGURE 2: Comparison of the intensity of four representative PDE-5i with different extraction solvents. (a) Acetildenafil. (b) Acetaminotadalafil. (c) Hydroxyvardenafil. (d) Avanafil.

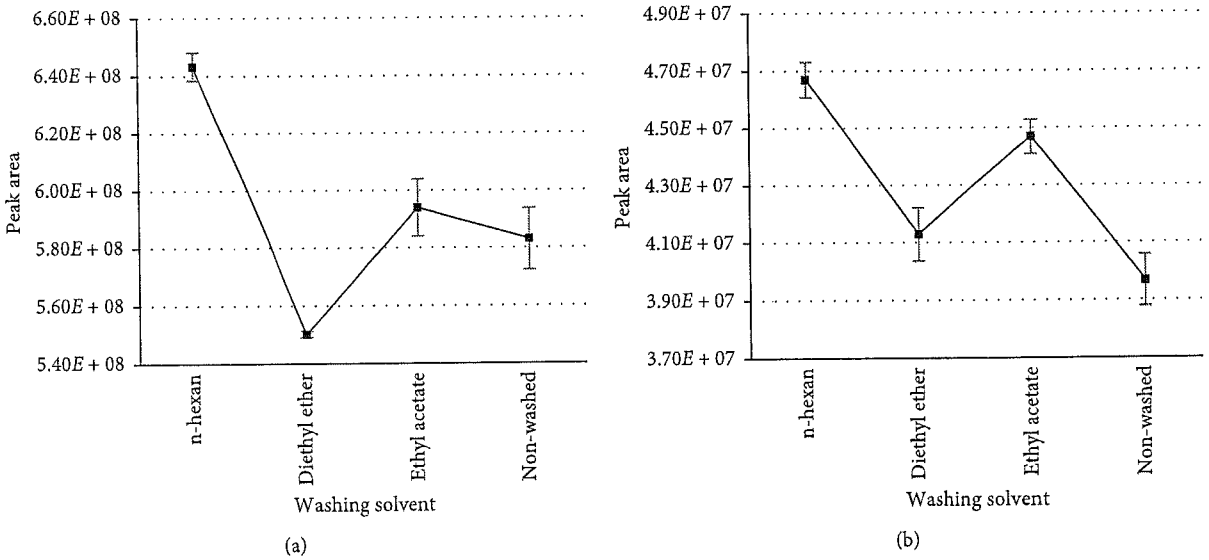

FIGURE 3: Continued.

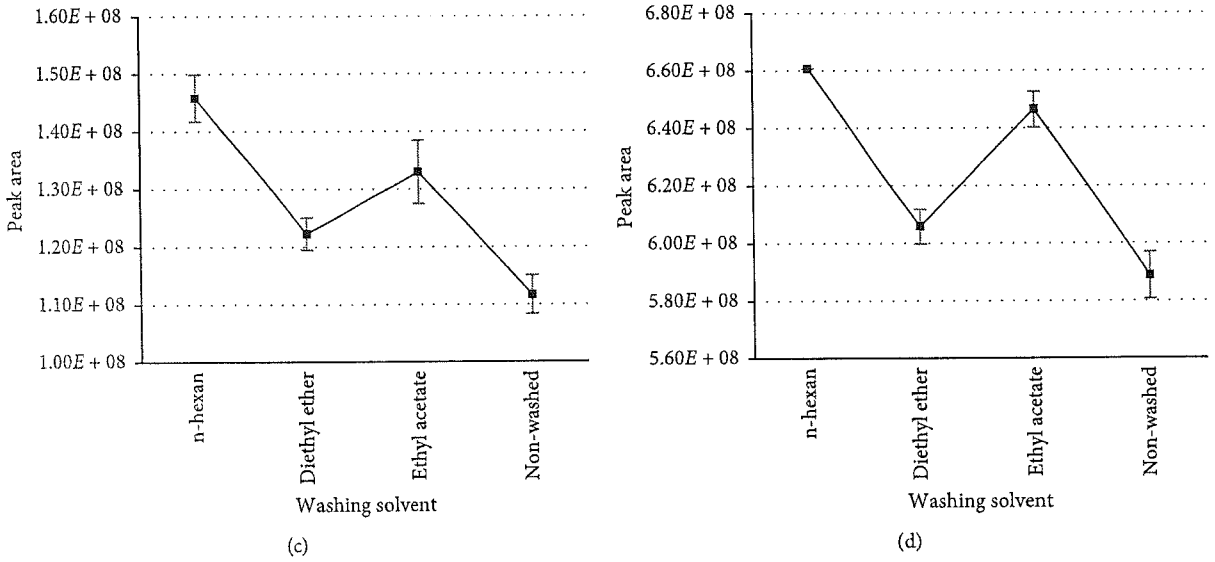

FIGURE 3: Comparison of the intensity of four representative PDE-5i after cleaning with three different solvents. (a) Acetildenafil. (b) Acetaminotadalafil. (c) Hydroxyvardenafil. (d) Avanafil.

and 1.2 mg/kg, respectively. The repeatability and the recovery of the method were evaluated by analyzing spiked tablet samples and soft-gel capsule samples at three levels 30.0, 100.0, 200.0 ng/mL in solution (1.2, 4, 8 mg/kg in samples) and six repetitions. The method met the AOAC International requirement as recoveries were in the range of 80–110%, and the relative standard deviation was from 2.81 to 12.6%. The matrix effect (ME) of the method was assessed by comparing the slope of the calibration curve in solution (A) and one in the matrix (A') as follows:

$$ME = \frac{A - A'}{A} \times 100. \quad (1)$$

All the compounds showed ME less than 10%. Thus, calibration curves in the standard solution can be used to calculate the concentration of PED-5i in real samples.

3.4. Analysis of Real Samples. For screening purposes, 92 real samples were analyzed by the developed method ($n = 3$); the screening process was conducted by Compound Discoverer 3.1 software. The spectrum was compared with the online mzCloud mass spectrometry library, Chemspider library, Mzvault library, and predicted structure. The accuracy of the process was presented by the matching index (>80%) with each library. Among 92 collected samples, twenty samples were detected containing PDE-5i; the others were not detected. In the positive samples, we identified thirteen PDE-5i already existing in the used libraries and one compound nonexisting in the used libraries. This compound was discovered as N-hydroxyethyl dithio-desethyl carbodenafil in a previous study [23]. The number of detected samples and identified PDE-5i was shown in Table 2. Ten of fourteen PDE-5i (71%) were sildenafil analogs, which is higher than the value (62%) reported by Kee et al. [4]. There were three analogs of tadalafil (21%) and only one analog of vardenafil (7.1%).

After screening, positive samples were confirmed and quantified. The concentration of PDE-5i in these samples was calculated by calibration curves and presented in Table 3. It can be seen that most of the positive samples contain one or two PDE-5i at high concentration (>1 mg/g) and some other PDE-5i at low concentration. We suppose that high concentration PDE-5i ingredients were added intentionally to the sample, and the low concentration PDE-5i may be side products in the production of the main PDE-5i ingredients. Nortadalafil, Tadalafil, and Sildenafil were often detected in real samples as the main active compounds. On the other hand, few samples (S 14 and 15) detected some PDE-5i at low concentration (much lower than the dosage using in ED treatment). The origin of PDE-5i in these samples was not clear and needed to be studied further.

Table 2: Result of screening PDE-5i in 92 samples.

PDE-5i	Number of detected samples
Nortadalafil	11
Tadalafil	10
Sildenafil	9
Hydroxyhomosildenafil	6
Hydroxythiohomosildenafil	6
Homosildenafil	6
Thiohomosildenafil	1
Sildenafil N-oxyde	2
Chloropredadalafil	1
Propoxyphenyl-homohydroxysildenafil	1
Propoxyphenylaildenafil	1
Hydroxythiovardenafil	1
Methisosildenafil	1
N-hydroxyethyl dithio-desethyl carbodenafil	2

Table 3: Concentration of PDE-5i detected in real samples.

Sample	PDE-5i	Concentration (mg/g)
S 1	Nortadalafil	50.0 ± 0.13
	Tadalafil	11.6 ± 0.05
	Sildenafil-N-oxide	~0.073
S 2	Nortadalafil	4.41 ± 0.05
	Chloropretadalafil	~0.006
S 3	Sildenafil	22.2 ± 0.11
	Tadalafil	0.39 ± 0.007
	Sildenafil-N-oxide	~0.06
S 4	Propoxyphenylaildenafil	1.02 ± 0.06
	Thiohomosildenafil	0.78 ± 0.005
	Homosildenafil	~0.031
	Methisosildenafil	~0.03
S 5–9	Hydroxyhomosildenafil	1.05–20.1
	Hydroxythiohomosildenafil	0.78–22.5
S 10	Tadalafil	~0.08
S 11	Tadalafil	10.2 ± 0.05
	Chloropretadalafil	5.25 ± 0.06
S 12	Nortadalafil	12.2 ± 0.05
	Chloropretadalafil	0.25 ± 0.06
S 13	Nortadalafil	10.2 ± 0.04
	Chloropretadalafil	5.25 ± 0.10
S 14	Acetil acid	0.13 ± 0.06
S 15	Tadalafil	0.12 ± 0.05
S 16	Sildenafil	5.25 ± 0.11
	Tadalafil	4.77 ± 0.15
	Aminotadalafil	0.52 ± 0.05
	Sildenafil N-oxyde	0.56 ± 0.06
S 17	Nortadalafil	0.52 ± 0.06
	Chloropretadalafil	4.77 ± 0.10
S 18	Nortadalafil	12.1 ± 0.06
M 1	Nortadalafil	170.0 ± 1.22
	Hydroxythiohomosildenafil	226.8 ± 1.36
	Hydroxythiovardenafil	216.8 ± 2.00
M 2	Propoxyphenyl-homohydroxysildenafil	0.13 ± 0.06
	Hydroxyhomo-sildenafil	~0.048
	Hydroxyvardenafil	~0.047

S: supplementary food, M: medicine ingredient, "S 5–9" indicates samples: S 5, S 6, S 7, S 8, S 9.

4. Conclusion

In this study, we have developed and validated a rapid screening and quantitation method using LC-HRMS for more than 53 PDE-5i in ingredients and supplementary products for enhancing male sexual performance. The validation parameters of this method, such as LODs, LOQs, recoveries, and regression coefficients, were acceptable according to the requirement of AOAC for an analytical method. The success of this method demonstrated the utilization of the fragmentation mass spectra library for analytes confirmation. The developed method was applied to analyze 92 natural-based ingredients and supplementary products available in the Vietnam market. We had screened and detected 14 PDE-5i. The results of real samples analysis implied that the manufacturers had deceived customers by not declaring PDE-5i on the label but adding these ingredients in the products. Thus, our study provides a warning on the quality control of supplementary foods to avoid any health risks to the community.

Data Availability

The data used to support the findings of this study are available within the article, the support information in word form, and from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Supplementary information is provided in word form. (*Supplementary Materials*)

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Screening determination of food additives using capillary electrophoresis coupled with contactless conductivity detection: A case study in Vietnam

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ABSTRACT

In this study, a simple and inexpensive method for food analysis is discussed. The approach is based on capillary electrophoresis (CE) instruments with capacitively coupled contactless conductivity detection (C^4D). Following the pilot deployment of in-house fabricated CE-C^4D instruments in Vietnam, a number of food additives in different food matrices, including i) oxalate and citrate in instant noodles, beer and tea drinks, ii) acidulants in beverage, coffee, alcohol and vinegar samples, and iii) artificial sweeteners in jelly and drink samples, were determined using this method. Good agreement between results obtained with CE-C^4D-instruments and those from the confirmation method (HPLC-PDA) was achieved.

1. Introduction

In order to guarantee the safety and good quality of foods, food control is of extreme importance during and after food production in order to assure the safe consumption of foods in compliance with the current and incoming legislation. The food control activities are especially critical in developing countries in general and in Vietnam in particular where food contamination occurs very often whilst the food regulations have not been well established. The scare of food poisoning/contamination has recently hit Vietnam whose markets are flooded with foods containing harmful chemicals. With an alarming surge in cancer rate, many of the cancer cases being linked to toxic or badly treated food, food contamination has become a national problem in Vietnam. In this context, there is an urgent need for robust, efficient and cost-effective analytical methodologies that can be widely used even in the local and decentralized areas and allow screening and/or tracing food contaminants with little requirement of expertise and budget. So far, food analysis has mostly been carried out in central institutes, notably at the National Institute for Food Control (NIFC). Well established methods, for example gas chromatography (GC), high-performance liquid chromatography (HPLC) and ion exchange chromatography (IC) in combination with optical (*i.e.* photodiode array (PDA) or laser induced fluorescence (LIF)) or mass spectrometry (MS) detection have been frequently used for this purpose. However, their high costs for sample analyses and instrumental maintenance, as well as the requirement for skillful and experienced operators render their employment for screening analysis unsuitable to many

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(decentralized) food control centers where only modest financial resources and limited expertise are available. Very often, food samples suspected to contain (overdosed) harmful chemicals are sent to NIFC and there is a delay of at least some days until the analyses are completed. This poses a significant delay for the authority to reach a conclusion. Some quick tests, which are commercially available in the market, however are only limited to some certain chemicals and aim to qualitative identification rather than quantitative determination. The aforementioned issues lead to the desire for a more approachable and affordable alternative for food analysis.

Among different analytical separation techniques used for food analysis, capillary electrophoresis (CE) has been established as a versatile and high-performance tool that provides fast and efficient separations with low consumption of samples, solvents and reagents. This technique relies on the electro-migration of the concerned species through a capillary under an electrical field. The use of CE techniques in food control has been reviewed several times (Acunha, Ibáñez, García-Cañas, Simó, & Cifuentes, 2016; Castro-Puyana, García-Cañas, Simó, & Cifuentes, 2012; García-Cañas, Simó, Castro-Puyana, & Cifuentes, 2014; Herrero, García-Cañas, Simo, & Cifuentes, 2010). Bench-top CE instruments coupled with optical or MS detectors were employed in most of the cases. Nevertheless, the high cost of commercial CE-UV/LIF/MS instruments is still a factor that prevents the widespread use of this electro migration technique in Vietnam. The miniaturized version of CE, *i.e.* microchip electrophoresis has also been used repeatedly for food analyses. Recent applications of microchip electrophoresis in this domain can be found in different reviews (Coltro et al., 2015; Escarpa et al., 2015; Ferey & Delaunay, 2016; Martín, Vilela, & Escarpa, 2012). Although CE and microchip electrophoresis share the same principle of electrokinetic separation, they require much different instrumental setups, methodology development and possess different degrees of robustness and maturity. From our point of view, the suitable option that offers low-cost instrumentation and unsophisticated operation is the employment of a system based on CE with capacitively coupled contactless conductivity detection (C^4D). In this case, ionic species separated by CE are detected based on the difference of their electrical conductivities from that of the background electrolyte (BGE). Fundamental aspects of C^4D can be found in (Brito-Neto, da Silva, Blanes, & do Lago, 2005a,b; Coltro et al., 2012; Kubáň & Hauser, 2004a,b, 2009; Mai & Hauser, 2012; Zemann, 2003). Interesting applications of CE-C^4D in food control can be gleaned from some recent reviews (de Oliveira et al., 2016; Elbashir & Aboul-Enein, 2014; Kuban & Hauser, 2014; Kubáň & Hauser, 2016). Some notable examples include the analysis of saccharides in various matrices, the identification and quantification of monoethyl carbonate in beer, distilled beverage and a carbonated soft drink, the identification of oligosaccharides, the analysis of underivatized fatty acids, and simultaneous determination of biogenic amines in water and hard liquor samples. Very recently, Coelho and Jesus communicated a CE-C^4D method for determination of erythritol, maltitol, xylitol and sorbitol in sugar-free chocolates (Coelho & de Jesus, 2016). Pavlicek and Tuma gave an account of the use of CE-C^4D for determination of stevioside and rebaudioside A in foods and beverages (Pavlíček & Tůma, 2016). Liu et al. employed CE-C^4D coupled with electromembrane extraction for determination of diamine plastic restricted substances (Liu et al., 2016). For CE-C^4D both the separation and detection of ions are based on electronic principles and the method only requires low-pressure fluidic components. This technique therefore offers many advantageous features, notably portable instrumentation for mobile deployment (Greguš, Foret, & Kuban, 2016; Lewis et al., 2013; Schepdael, 2016), high configuration flexibility with multi-channel setups for high throughputs (Duong, Nguyen, Mai, Saiz, & Pham, 2016; Koenka, Mai, Hauser, & Saiz, 2016; Thanh Duc; Mai et al., 2016; Sáiz, Koenka, Mai, Hauser, & García-Ruiz, 2014) and construction of low cost versions (Duong et al., 2015; Nguyen et al., 2015). Nevertheless, the advantageous features of CE-C^4D have not yet been fully exploited for food analysis. Food control applications implemented with CE-UV detection or CE-MS still much outnumbered those carried out with CE-C^4D (Acunha et al., 2016; Castro-Puyana et al., 2012; García-Cañas et al., 2014; Herrero et al., 2010).

Herein, the employment of CE-C^4D for analyses of various compounds in different food matrices in Vietnam, including i) oxalate and citrate in instant noodles, beer and tea drinks, ii) acidulants in beverage, coffee, alcohol and vinegar samples, and iii) artificial sweeteners in jelly and drink samples, is reported. This study is a follow-up of our pioneering work in the topic of food control in Vietnam with the use of CE-C^4D for the determination of beta-agonists in pig feed samples (Nguyen et al., 2014). Some considerations on the effects of sample matrix treatment on the analytical performance using CE-C^4D are discussed. Cross checking data using confirmation methods with HPLC-PDA were also provided to prove the reliability of the analytical data obtained with CE-C^4D.

2. Experimental

2.1. Chemicals and materials

All chemicals were of analytical or reagent grade and purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). Individual stock solutions (10 mmol/L) of formic acid, acetic acid, propionic acid, butyric acid, oxalic acid, citrate sodium, and tartaric acid were used for the preparation of standards of carboxylate-based species. Those of artificial sweeteners were prepared from stock solutions (1000 mg/L) of acesulfame potassium, aspartame, cyclamate sodium and saccharine. Chemicals used for preparation of CE-C^4D buffers include: Tris(hydroxymethyl)aminomethane (Tris), arginine (Arg), acetic acid, histidine (His), 18-crown-6, cetyltrimethylammonium bromide (CTAB), 2-(*N*-morpholino) ethanesulfonic acid (MES), 3-(*N*-Morpholino)propanesulfonic acid (MOPS), *N*-Cyclohexyl-2-aminoethanesulfonic acid (CHES) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS).

Fused silica capillaries of 50–75 μm ID and 365 μm OD were obtained from BGB Analytik AG (Böckten, Switzerland). Before use, the fused silica capillaries were pre-conditioned with 1 M NaOH for 10 min and deionized water for 10 min prior to flushing with the buffer. The capillaries were then used continuously for successive analyses. Deionized water purified using a system from Water Pro (Labconco, Kansas City, MO, USA) was used for the preparation of all solutions and for sample dilution if required. Background electrolyte (BGE) solutions were prepared by addition of the pre-selected acid (Ace, MES, CHES, MOPS or CAPS) into a basic solution containing either His, Arg or Tris at desired concentrations. pH values of solutions were controlled with an HI 2215 Hanna Instruments pH meter (Woonsocket, RI, USA).

2.2. Instrumentation

In-house-made portable CE instruments with manual or semi-automated operation were used for all food analyses. Details on the construction and operation of these CE prototypes can be found in (Kubáň, Nguyen, Macka, Haddad, & Hauser, 2007; Torres, Hauser, Furrer, Brandl, & Mueller, 2013) for the version with manual siphoning injection and in (Nguyen et al., 2014) for the semi-automated single-channel setup. The salient features of these instruments are given in Table 1. Detection was carried out with

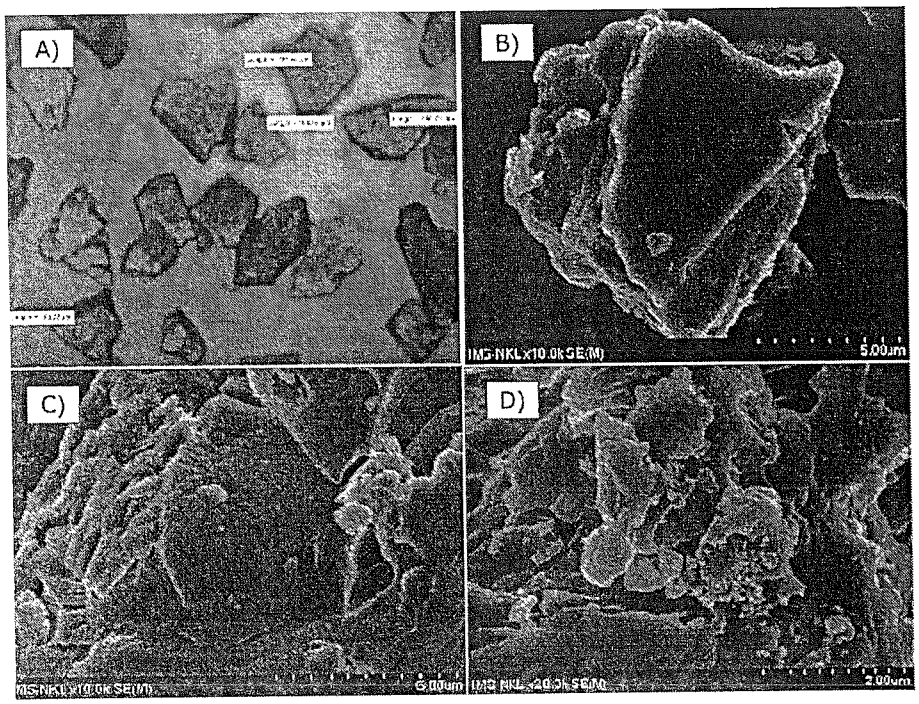
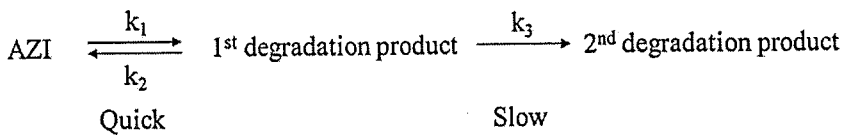


Fig. 5. Images of microparticles prepared by solvent evaporation method taken by (A) optical microscopy and (B–D) scanning electron microscope.

between glycoside and cladinose (2-desoxy) in AZI structure was easily cut out by very low pH acidic media.

To further clarify the degradation process of AZI, the samples with immediate neutralization by 0.2 M phosphate buffer before analyzing by HPLC were compared with samples without neutralization step. Samples which were not immediately neutralized by 0.2 M phosphate buffer had two degradation products (Fig. 6b). Meanwhile, those that neutralized had only one degradation product, i.e. the 2nd degradation product in the chromatogram. The two phases in degradation process of AZI in pH 1.0 included: (1) quick degradation for the first 60 min and (2) very slow degradation or level off for the remaining time. This proved a reversible reaction between AZI and the first degradation product. Besides, reduction in the concentration of the 1st degradation product indicated that it was an intermediate product of degradation process from AZI to the 2nd degradation product. Based on this assumption, the degradation process of AZI could be predicted as follows:

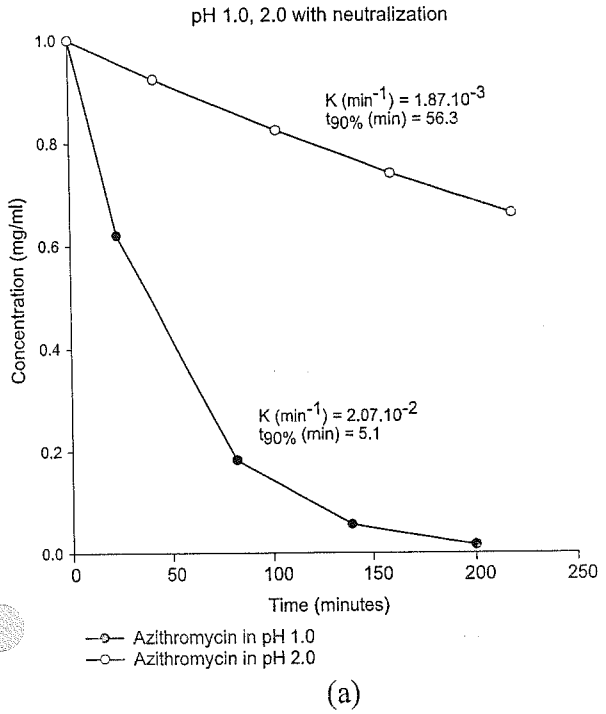


This result showed that neutralization step was quite essential to stop the degradation process and turned the first degradation product into AZI. Consequently, the screening of pH modulators to the composition of tablets, especially basic agents, was crucial to maintain the stability of AZI.

The aim of the screening step was to find out the suitable alkaline agents which kept the pH of the gastric medium around 5.0–6.0. At this pH range, the released amount of AZI in the gastric medium was minimized (Fig. 3b), thus maintaining the stability of AZI in this medium. Furthermore, Curatolo [41] reported that GI side effects of AZI were caused by the binding of AZI to the human gastric antrum motilin receptor which existed primarily in the upper part of GI wall. Thus, minimization of exposure of free drug in the upper GI tract reduced the undesirable effects of AZI [30,41] like intense lingering aftertaste, abdominal pain, diarrhea, nausea, and vomiting.

The effect of the three groups of alkaline agents including soluble alkaline agents (Na_3PO_4, Na_2HPO_4, and NaH_2PO_4), insoluble alkaline agents ($Al(OH)_3$, $Mg(OH)_2$ and $CaCO_3$) and combination of soluble and insoluble alkaline agents ($CaCO_3$: NaH_2PO_4 = 2:1) on modulating gastric medium was investigated (Fig. 7). Insoluble alkaline agents displaying a broad plateau of the titration curve indicated their effectiveness in enhancing and maintaining the pH of the gastric medium at stable levels for a longer time compared to soluble alkaline agents. Especially, only $CaCO_3$ could create a pH range of 5.0–6.0 with an additional amount of hydrochloric acid from 40 to 120 ml. However, right after the dispersion of $CaCO_3$ in gastric medium, the pH was 9.0. In contrast, only one soluble alkaline agent, NaH_2PO_4, created pH of 5.0 right after being dispersed in the gastric medium. Nonetheless, the pH of gastric medium added by NaH_2PO_4 quickly decreased to a risk level (pH 2.0) for the stability of azithromycin.

To create and maintain a pH of 5.0–6.0 right after addition of alkaline agents to gastric medium, a mixture of insoluble and soluble alkaline agents ($CaCO_3$ and NaH_2PO_4 = 2:1) was developed. As shown in Fig. 7, when the ratio of $CaCO_3$: NaH_2PO_4 was 2:1, the pH of the gastric medium was maintained around 5.0–6.0 with the



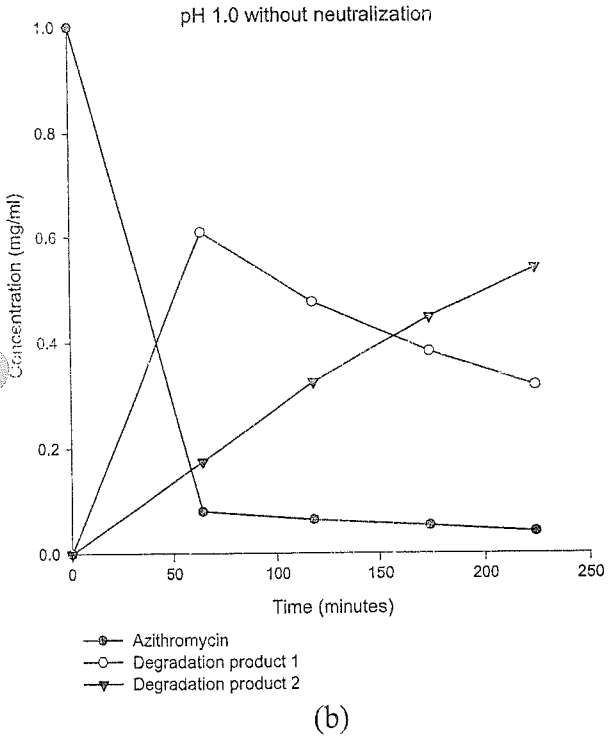


Fig. 6. Stability kinetics of AZI (a) AZI in pH 1.0 and 2.0 with neutralization step; (b) AZI and degradation products in pH 1.0 without neutralization step.

additional amount of hydrochloric acid up to 120 mL. The ability to maintain a pH range of 5.0–6.0 of this mixture depended on the amount of $CaCO_3$ and NaH_2PO_4. When using this couple of alkaline agents, AZI would have a higher chance to prolong the lifetime in a harsh environment, gastric medium, where hydrochloric acid

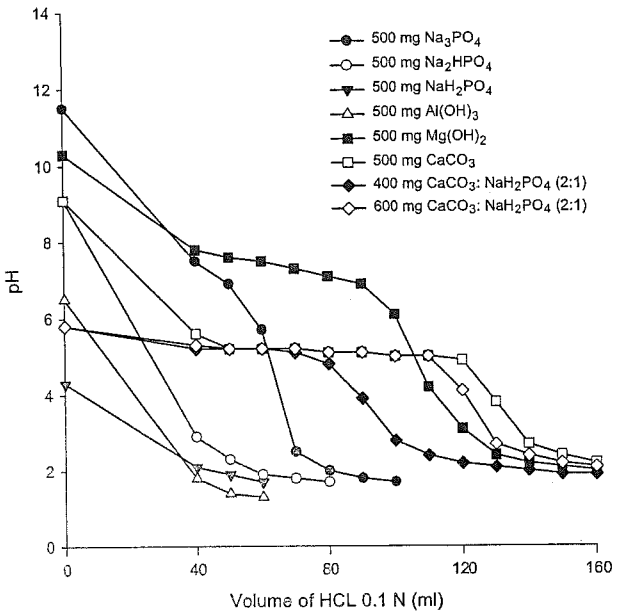


Fig. 7. Ability to neutralize the gastric acid of soluble pH modulators (Na_3PO_4, Na_2HPO_4, NaH_2PO_4), insoluble pH modulators ($Al(OH)_3$, $Mg(OH)_2$, $CaCO_3$) and combination of soluble and insoluble pH modulators ($CaCO_3$: NaH_2PO_4).

was continuously secreted from parietal cells in the stomach. Consequently, these two excipients ($CaCO_3$ and NaH_2PO_4 = 2:1) were used as stabilizers for dispersible tablets.

Dissolution profiles of AZI and 2nd degradation product from microparticles with pH modulators and the reference product (Zithromax oral suspension) in pH 1.2 and 6.8 were displayed in Fig. 8. As shown in Fig. 8a, the concentration of the 2nd degradation product was ignorable, and AZI virtually did not release in the gastric medium. AZI displayed a burst release phase right after changing the dissolution medium from gastric medium (pH 1.2) to intestinal medium (pH 6.8). The addition of a couple of pH modulators ($CaCO_3$ and NaH_2PO_4) enhanced the pH of the gastric medium (hydrochloric acid 0.1 N) to a new pH level (5.0–6.0) thus avoiding the drug contact to the very acidic medium (pH 1–2) and improving the drug stability. Besides, at this new pH level of gastric medium, the matrix using Eudragit L100 as the carrier was insoluble and kept the drug inside. AZI only released from the microparticles when the pH of dissolution medium increased to 6.8 at which Eudragit L100 was completely soluble.

Meanwhile, Fig. 8b showed that Zithromax was quickly hydrolyzed to the 2nd degradation product in pH 1.2. AZI quickly dissolved in pH 1.2 and had the maximum concentration after 5 min because this drug was a weak base drug (pKa = 8.74). However, the drug was immediately decomposed right after exposing to the gastric medium. If the dissolution test of Zithromax in pH 1.2 lasted to 60 or 120 min, AZI would be completely decomposed. Consequently, the dissolution test of Zithromax was only carried out in 30 min in pH 1.2 before changing to pH 6.8. The remaining AZI concentration in pH 6.8 was only around 13.07%. This result proved the pivotal role of the incorporation of the enteric coating polymer (Eudragit L100) as a carrier of microparticles and pH modulators ($CaCO_3$ and NaH_2PO_4) in maintaining the stability of AZI in the gastric medium.

Together with pH modulators, other additives were also screened to prepare the tablets. Based on the preliminary results (disintegration time of tablets and dissolution rate of AZI) obtained from screening steps, optimal microparticles, diluents (Avicel PH 101, lactose or mannitol), lubricants (magnesium stearate, Aerosil, or Talc), stabilizer (NaLS) and pH modulators ($CaCO_3$ and

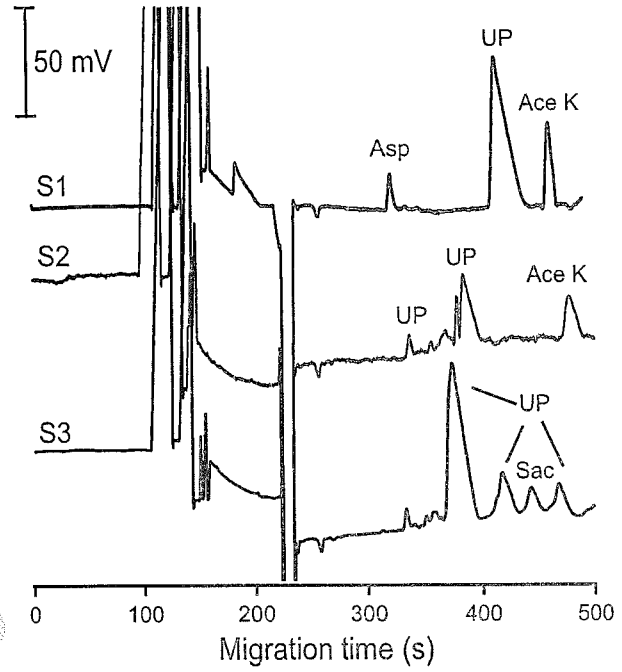


Fig. 3. Electropherograms obtained for determination of artificial sweeteners in various sample matrices. S1: a jelly sample; S2: Huong Chanh beverage sample; S3: the black bean sweet soup sample. UP: unidentified peak. Other conditions as in Fig. 2.

Since these flavoring agents are prepared by chemical synthesis, their presence in food is the cause of extensive consumer mistrust (Kroger, Meister, & Kava, 2006). Some artificial sweeteners, notably aspartame, can cause adverse medical effects (Zygler, Wasik, & Namiesnik, 2009). According to the NIFC, in Vietnam these compounds are very often found in different beverages, tea drinks and sweet soups made from different types of beans. A combination of different artificial sweeteners, notably aspartame, cyclamate, saccharine and acesulfame K is frequently used in order to mask undesired aftertastes, such as bitterness. HPLC has been the method used to control the presence of these compounds in different food stuffs. In an attempt to develop a simple and more cost-effective approach for this purpose, a low-cost and portable CE-C^4D instrument was employed to determine different artificial sweeteners in various food matrices in Vietnam. The electropherogram (*i.e.* the data profile obtained with a CE run) for a standard solution containing aspartame, cyclamate, saccharine and acesulfame K (used as the reference profile) is shown in Fig. 2. The resolutions for the peaks of aspartame - cyclamate, cyclamate - saccharine and saccharine - acesulfame K were 3.7, 2.3 and 1.1, respectively. Calibration curves for aspartame, cyclamate, saccharine and acesulfame K were acquired in the ranges of 20.0–180 mg/L, 5.0–120 mg/L, 5.0–120 mg/mL and 2.5–120 mg/L, respectively. The achieved detection limits were from 0.7 to 5.0 mg/L, which are in the same range as those obtained with CE-C^4D optimized by hydrodynamic pumping (Stojkovic, Mai, & Hauser, 2013). For precision evaluation, 3 replicates were carried out; and the achieved relative standard deviations (RSD %) for migration times and peak areas were less than 0.7% and 3.5%, respectively. Electropherograms obtained with various sample matrices are demonstrated in Fig. 3. The major cations in the sample matrices appear much earlier than the concerned artificial sweeteners in the electropherograms and therefore do not interfere with these targeted species. Under the optimized CE-C^4D conditions, the matrix anions, *e.g.* chloride, either arriving very late at the detector or even not reaching the detection point, do not interfere with the targeted anionic species. Note that some (undesired) unidentified peaks also appeared in these electropherograms. To well distinguish them from those of the concerned analytes, recourse to the standard addition method was needed. The migration times obtained from electropherograms for real samples are sometimes drifted from those observed with the standard solutions. This migration time drift may occur due to modification of the capillary surface when injecting different sample matrices, which consequently leads to changes in the electroosmotic flow during electrophoreses. This can be exacerbated for a non-thermostated CE instrument used in environments with temperature fluctuation from day to day. In this case, peak identification, confirmation and quantification could be realizable using the standard addition method, which is not an operation complication.

To verify the reliability of the data obtained with CE-C^4D, cross-check with the confirmation method (HPLC-PDA) was also carried out for some tested samples. The concentrations of artificial sweeteners in different food matrices revealed by both CE-C^4D and HPLC-PDA were shown in Table 2. The relative errors for the two pairs of data are better than 15%, proving a very good agreement between the results obtained from two methods. The Student's *t*-test was also implemented using the differences between the data obtained with CE-C^4D and those with HPLC. The calculated *t* values for the confidence level of 95% were 4.64 and 1.10 for aspartame and acesulfame K, respectively. These calculated values are smaller than those referenced from the *t* table (12.7 and 2.78 for aspartame and acesulfame K, respectively), meaning that two sets of data obtained with CE-C^4D and HPLC are not significantly different from each other. Acesulfame K was

Table 2
Concentrations of artificial sweeteners in various samples determined with CE-C^4D and with standard reference methods (HPLC-PDA).

Sample	Aspartame			Acesulfame K		
	CE-C4D[a] (ppm)	HPLC[a] (ppm)	D%	CE-C4D (ppm)	HPLC (ppm)	E_R %
Jelly 1	243 ± 17	223 ± 9	9.0	299 ± 8	269 ± 14	+11.2
Jelly 2	301 ± 20	270 ± 18	11.5	278 ± 13	245 ± 7	+13.5
Hương Chanh beverage	ND	ND	–	376 ± 19	336 ± 17	+11.9
Coca Cola zero	ND	ND	–	102 ± 6	110 ± 3	−7.3
Lipton ice tea	ND	ND	–	594 ± 16	600 ± 25	−1.0
Wonderfarm tea	ND	–	–	51.0 ± 1.3	–	–
Energy drink Samurai	ND	–	–	411 ± 11	–	–
Energy drink Number One	43.9 ± 1.2	–	–	ND	–	–
Quang Hanh - Faith beverage	84.3 ± 2.2	–	–	ND	–	–
Herbal tea Dr. Thanh	ND	–	–	73.6 ± 1.9	–	–

E_R % is the relative error calculated from the result obtained with CE-C^4D and that with the standard reference method; $E_R\ \% = \{(R_{CE\text{-}C4D} - R_{reference})/R_{reference}\} \times 100\%$.
ND: not detected.
[a] For each measurement, 3 replicates were carried out for precision evaluation.

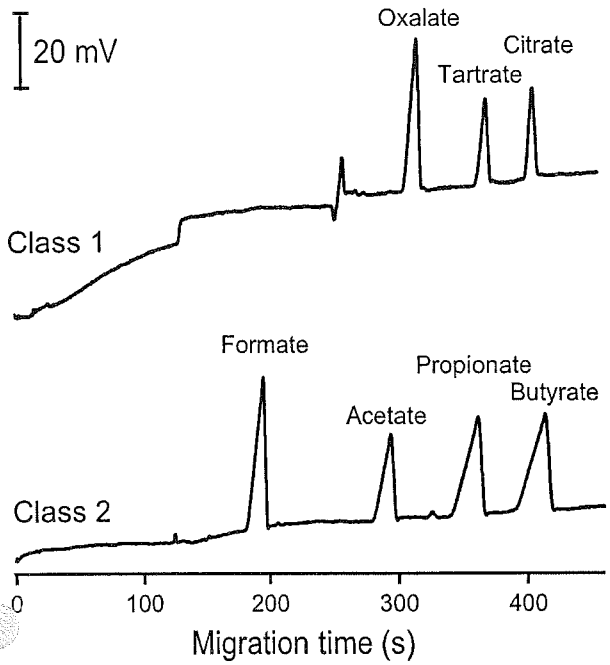


Fig. 4. Electropherograms obtained for two standard solutions containing i) citrate, tartrate and oxalate (class 1) and ii) formate, acetate, propionate and butyrate (class 2). The concentration of each analyte: 500 μM. Conditions for class 1: Electrolyte solution: 50 mM His/30 mM MES and 25 μM CTAB (pH 6.0). Voltage: −15 kV. Sample injection: hydrodynamic (HD) for 20s with the height of 15 cm. Capillary: fused-silica, 75 μm id, Lt = 60 cm (Leff = 50 cm). Conditions for class 2: Electrolyte solution: 30 mM His and 40 mM MES (pH 5.8). Voltage: −18 kV. Sample injection: hydrodynamic (HD) for 20 s with the height of 15 cm. Capillary: fused-silica, 50 μm id, Lt = 50 cm (Leff = 43 cm).

found in many beverages, with the highest concentration of 600 μg/g found in a Lipton ice tea sample. Aspartame and acesulfame K were found in two tested jelly samples with the concentrations in the range of 200–300 μg/g. Saccharine was the edulcorant (111 μg/mL, not shown in Table 2) employed in the black bean sweep soup sample.

3.3. CE-C^4D determination of some acidulants and preservatives in beer, instant noodle, wine, coffee, vinegar and tea samples

Organic acids have been popularly used in a wide variety of foods and beverages in the form of food additives, such as acidulants and preservatives (Carocho, Barreiro, Morales, & Ferreira, 2014). In Vietnam, citric acid and acetic acid are used as synthetic acidulants to add acidity to food stuffs and to replace natural citron juice and vinegar in different beverages and sauces. Citrate and tartrate play the role of antioxidants, thus extending the shelf life of many foodstuffs (Carocho et al., 2014). Other low-molecular-mass organic acids (*i.e.* formic acid, acetic acid, propionic acid and butyric acid) can also be added into food for the antimicrobial purpose. Their presence helps control natural spoilage of food by microorganisms, including pathogenic ones (Carocho et al., 2014). Oxalate is another food additive that has been extensively used in instant noodles. Excessive consumption of these chemicals is harmful to the human body. As in Vietnam instant noodles are much consumed by the population, there is a risk of suffering from gravel due to excessive intake of oxalate. CE determination of organic-acid based additives in food samples were carried out using either i) capillary zone electrophoresis (CZE - the simplest mode of CE) with optical detection (see (Yoshikawa, Saito, & Sakuragawa, 2011) and the references listed therein) and with diamino moiety functionalized silica nanoparticles as a pseudostationary phase (Liu, Ding, & Tang, 2014), or ii) micellar electrokinetic chromatography (Ding et al., 2015; Wu, Li, Zhao, & Ding, 2016). In our case, we demonstrate the potential of CE-C^4D as a straightforward and low-cost solution for control of different carboxylate-based additives in a wide spectrum of food samples. The electropherograms obtained for two standard solutions containing i) citrate, tartrate and oxalate (class 1) and ii) formate, acetate, propionate and butyrate (class 2) are demonstrated in Fig. 4. The resolutions for the peaks of oxalate - tartrate, tartrate - citrate, formate - acetate, acetate - propionate and propionate - butyrate were 3.4, 2.5, 5.0, 2.8 and 1.8 respectively. Note that the peaks of matrix anions, *e.g.* chloride and nitrate, that appear before those of the concerned species in the electropherograms can cause peak overlapping if the concentrations of these interferents are higher than 100 ppm. The performance data are shown in Table 3. LODs obtained for oxalate, tartrate and citrate were 0.22, 0.37 and 0.95 ppm, respectively. The detection limit for oxalate achieved with our purpose-made CE-C^4D instrument is higher than that obtained by Tuma et al. (0.08 ppm) for analysis of low molecular mass organic acids in urine samples (Tůma, Samcová, & Štulík, 2011). The performance variation can be explained by i) the difference in analytical procedures adapted to different matrices (i.e. food samples in our case and urine samples in their case, respectively) and ii) the difference in instrumental setups. The LODs for C1-C4 carboxylates were 0.05, 0.12, 0.15 and 0.17 ppm, respectively. Calibration curves were acquired up to 19.0 ppm for the species in the class 1, and up to 8.7 ppm for those in the class 2.

Determination of oxalate was then carried out in two noodle samples. The obtained electropherograms are shown in Fig. 5. The peaks of oxalate were identified using the standard addition

Table 3
Performance parameters for the determination of class 1 (oxalate, citrate, tatrate) and class 2 (formate, acetate, propionate and butyrate) with the portable CE-C^4D system.

Analyte	Calibration range (ppm)[a]	Coefficient of determination (r^2)	LOD[b] (ppm)	Repeatability of peak area (% RSD, n = 3)	Repeatability of migration time (% RSD, n = 3)
Oxalate	0.66÷8.80	0.9997	0.22	2.48	0.36
Citrate	2.85÷19.0	0.9996	0.95	2.30	0.13
Tartrate	1.11÷14.8	0.9999	0.37	1.00	0.07
Formate	0.23÷4.50	0.9991	0.05	1.23	0.15
Acetate	0.35÷4.72	0.9991	0.12	1.38	0.22
Propionate	0.44÷7.30	0.9992	0.15	0.79	0.07
Butyrate	0.52÷8.70	0.9993	0.17	1.09	0.19

Conditions for class 1: Electrolyte solution: 50 mM His/30 mM MES and 25 μM CTAB (pH 6.0). Voltage: −15 kV. Sample injection: hydrodynamic (HD) for 20s with the height of 15 cm. Capillary: fused-silica, 75 μm id, Lt = 60 cm (Leff = 50 cm).
Conditions for class 2: Electrolyte solution: 30 mM His and 40 mM MES (pH 5.8). Voltage: −18 kV. Sample injection: hydrodynamic (HD) for 20 s with the height of 15 cm. Capillary: fused-silica, 50 μm id, Lt = 50 cm (Leff = 43 cm).
[a] 5 concentrations.
[b] Based on peak heights corresponding to 3 times the baseline noise.

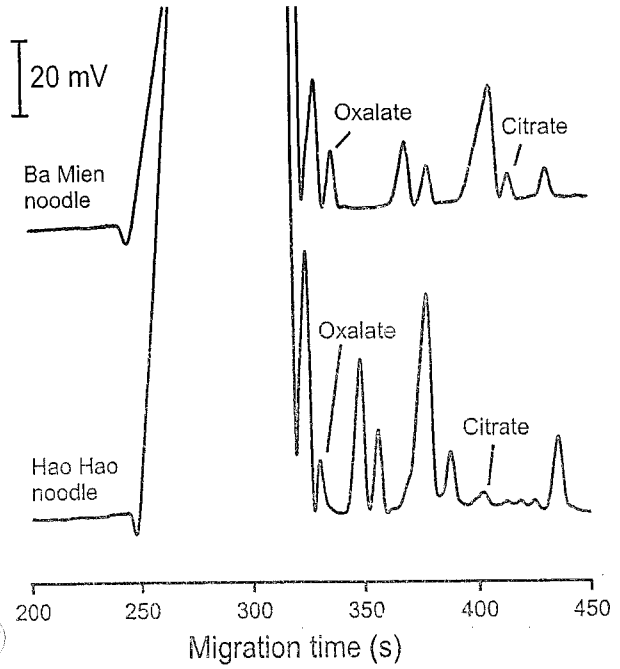


Fig. 5. Electropherograms obtained for determination of oxalate in noodle samples. CE conditions as in Fig. 4. The Hao Hao and Ba Mien noodle samples were respectively diluted 5 and 20 times with deionized water prior to CE-C^4D analyses.

Table 4
Concentrations of oxalate in instant noodle samples determined with CE-C^4D and with standard reference methods (HPLC-PDA).

Sample	CE - C^4D[a] (mg/kg)	HPLC[a] (mg/kg)	E_R %
Hao Hao instant noodle	22.0 ± 2.0	25.2 ± 2.5	−12.7
Ba Mien instant noodle	38.0 ± 4.2	42.0 ± 2.0	−9.5

$E_R\ \% = \{(R_{CE\text{-}C4D} - R_{reference})/R_{reference}\} \times 100\%$.
[a] For each measurement, 3 replicates were carried out for precision evaluation.

method. In the electropherograms also appeared a number of unidentified peaks. Identification of these peaks however was not implemented in the scope of this work. The results obtained with CE-C^4D were cross-checked with those from HPLC-PDA, with relative errors for the two pairs of data smaller than 13% (Table 4). The Student's *t*-test was again carried out based on the differences between the data obtained with CE-C^4D and those with HPLC. The calculated *t* value for the confidence level of 95% was 9.10, which is smaller than the referenced value from the *t* table (12.7). It can therefore be concluded that the data obtained with CE-C^4D match well with those obtained with HPLC. The concentrations of oxalate in these samples were found to be between 31.9 and 177 mg/kg. The comparison to the regulated level/acceptable daily intake (ADI) of oxalate nevertheless was not made as this reference value has not been established for the food control documents in Vietnam. The determination of oxalate as well as citrate with CE-C^4D was then extended to other beer and tea samples (Table 5). Citrate was found in all beer samples whereas no or little amounts of oxalate were detected. Oxalate was detected at higher concentrations (up to 66 μg/g) in some tea samples.

The quantitative analyses of low molecular mass organic anions (formate, acetate, propionate and butyrate) with CE-C^4D were done for a number of beverage, coffee, alcohol and vinegar samples. Electropherograms obtained for these samples are demonstrated in Fig. 6. The obtained results were double-checked with the data from the reference method. Good agreement between the two data

Table 5
Concentrations of oxalate and citrate in beer and tea samples determined with CE-C^4D.[a]

Sample	Oxalate	Citrate
Hà Nội canned beer	ND	94.0 ± 1.0 mg/L
Halida canned beer	ND	230 ± 20 mg/L
Tiger canned beer	ND	170 ± 10 mg/L
Haliken canned beer	ND	120 ± 7 mg/L
Hà Nội bottled beer	21.0 ± 1.0 mg/L	116 ± 8 mg/L
Sài Gòn bottled beer	15.7 ± 0.8 mg/L	104 ± 6 mg/L
Lipton fusion tea	66.0 ± 3.0 mg/kg	0.94 ± 0.05 mg/kg
Sidewalk tea drink	86.0 ± 5.0 mg/L	1170 ± 90 mg/L

ND: not detected.
[a] For each measurement, 3 replicates were carried out for precision evaluation.

sets was again achieved, with the relative errors always better than 17% (see Table 6). The calculated *t* values based on the differences between the data obtained with CE-C^4D and those with HPLC for the confidence level of 95% were 1.32, 0.81 and 1.30 for formate, acetate and propionate, respectively. These calculated values are smaller than those from the *t* table (12.7, 2.45 and 2.57 for formate, acetate and propionate, respectively), meaning that two sets of data obtained with CE-C^4D and HPLC are not significantly different from each other. Formate, acetate and propionate were found in almost all samples whereas butyrate was not detected. Acetate was detected in some samples at concentrations much higher than the regulated value in Vietnam (1500 mg/L). This anion was found at a concentration extremely high in the vinegar sample (more than 29,000 mg/L). The largest relative error (D = +17.1%) obtained for the vinegar sample may be due to the operational error induced

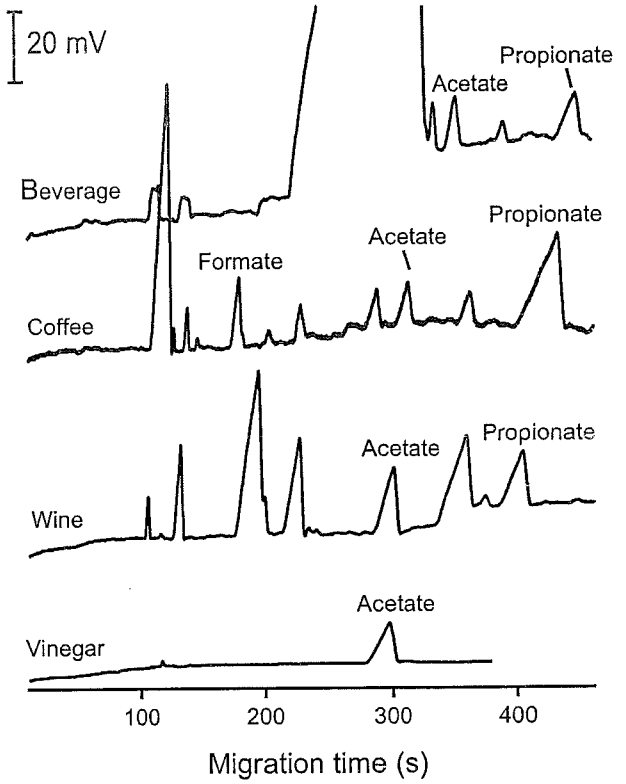


Fig. 6. Electropherograms obtained for determination of small carboxylate ions (formate, acetate, propionate) in orange juice (no dilution), coffee G7 (5 fold dilution), vinegar (500 fold dilution) and wine (Soliera Tempranillo, 20 fold dilution) samples. CE conditions as in Fig. 4.

Table 6
Concentrations of low-molecular-mass organic acids (formic acid, acetic acid and propionic acid) used as food additives in beverage, alcohol, vinegar and coffee samples determined with CE-C^4D and with standard reference methods (HPLC-PDA).

Sample	Formic acid			Acetic acid			Propionic acid		
	CE-C^4D[a]	HPLC[a]	E_R %	CE-C^4D	HPLC	E_R %	CE-C^4D	HPLC	E_R %
Orange juice (mg/l)	ND	ND	–	6130 ± 53	6570 ± 45	−6.7	249 ± 8	261 ± 9	−4.6
C2 beverage (mg/l)	ND	ND	–	1990 ± 17	2390 ± 17	−16.7	448 ± 11	473 ± 12	−5.3
Contre-étiquette alcohol (mg/l)	ND	ND	–	83.9 ± 3.9	90.8 ± 4.5	−7.6	471 ± 12	450 ± 11	+4.7
Soliera Tempranillo alcohol (mg/l)	ND	ND	–	1080 ± 9	1100 ± 8	1.8	851 ± 8	875 ± 9	−2.7
Viet coffee (mg/kg)	7550 ± 66	7710 ± 57	−2.1	1260 ± 10	1320 ± 10	−4.5	4050 ± 36	4120 ± 31	−1.7
G7 coffee (mg/kg)	7440 ± 65	8590 ± 61	−13.4	2320 ± 20	2110 ± 15	+10.0	9490 ± 84	9920 ± 74	−4.3
Thang Long red wine (mg/l)	270 ± 9	–	–	935 ± 8	–	–	41.5 ± 2.8	–	–
Ha Hai vinegar (mg/l)	ND	ND	–	29,400 ± 112	25,000 ± 161	+17.1	ND	ND	–

$E_R\ \% = \{(R_{CE\text{-}C4D} - R_{reference})/R_{reference}\} \times 100\%$.
ND: not detected.
[a] For each measurement, 3 replicates were carried out for precision evaluation.

from several-fold dilution of the sample to make the recorded signal lie within the calibration range.

4. Conclusions

CE-C^4D was found to be a simple and inexpensive solution for analysis of various food additives in different food matrices. This approach does not require costly and sophisticated commercial instrumentation, thus rendering the food control activities feasible even at local laboratories where only modest budget and limited expertise are available. Good agreement between the results obtained with CE-C^4D and those with the reference method was achieved, demonstrating the reliability of the analytical data provided with CE-C^4D. It can be noted from a recent review by Carocho et al. (Carocho et al., 2014) that a number of food adding molecules are charged species with the little or no UV adsorbing feature. These compounds can be seen as preferred candidates for CE-C^4D methods. Extension of the CE-C^4D application spectrum to many other food additives and food quality indicator species is therefore envisaged.

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Pharmacokinetic analysis of levo-tetrahydropalmatine in rabbit plasma by rapid sample preparation and liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid extraction method was developed and validated for levo-tetrahydropalmatine (L-THP) determination in rabbit plasma by liquid chromatography tandem–mass spectrometry (LC–MS/MS). The sample preparation included a single-step acetonitrile extraction and salting out liquid–liquid partitioning from the water in plasma with $MgSO_4$. Berberine was used as internal standard. The mass spectrometry source was negative electrospray ionization. The method showed good performance in the concentration range from 5 to 200 ng mL^{-1}. The limit of quantification (LOQ) was 1 ng mL^{-1}. The method was successfully applied to a pharmacokinetic study in rabbit comparing the two drug formulation of L-THP including the raw material and the self-microemulsifying drug delivery system pellet.

1. Introduction

Levo-tetrahydropalmatine (L-THP), which is called rotundin, is an alkaloid extracted from several different plants (Fig. 1). In Vietnam, it is found in *Stephania rotunda* Lour. [1] and is widely used in the market for the treatment of insomnia, anxiety and stress. Many young Vietnameses attempted suicide by over-dosing with this drug.

Some analytical methods have been developed for the determination of L-THP in plasma. HPLC with an UV or DAD detector was predominantly used as a routine method for quantification of L-THP in plasma. Because tetrahydropalmatine is a racemic mixture in herbs, a chiral HPLC method was normally used to separate the two enantiomers [2]. An HPLC-DAD method was developed by Chao-Wu et al. for the determination of L-THP in human plasma using a solvent extraction with ethyl acetate, and reached the limit of quantification (LOQ) of 10 ng mL^{-1} [3]. Zhang et al. proposed a dispersive liquid–liquid microextraction coupled with HPLC-UV method for the determination of tetrahydropalmitine and tetrahydroberberin in rat urine which gave an LOQ of 25 ng mL^{-1} [4]. Yu et al. used UPLC-FLD method and reached the LOQ of 2.5 ng mL^{-1} [5]. Unfortunately, these HPLC methods lack of specificity or sensitivity required for the pharmacokinetic study. In 2009, Ma et al. proposed a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the determination of L-THP, protopine and palmatine in rat plasma [6]. In 2013, Wang et al. also published an LC–MS/MS method for the determination of 4 alkaloids including L-THP in rat plasma [7]. The LC–MS/MS method showed a better performance with the LOQ of 1–2 ng mL^{-1} [6–10]. These methods usually included a solvent extraction with ethyl acetate or acetonitrile without any clean-up step. The extract was then evaporated till dryness, the residue was reconstituted in an appropriate solvent before doing LC–MS/MS analysis. The evaporation step is critical and need to be performed carefully. In 2012, Wang et al. used microdialysis combined with LC–MS/MS which reached the LOQ of 0.1 ng mL^{-1} in rat striatum matrix [11]. However, the method was too complicated to applied in serum or plasma matrix.

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) is a famous methodology which was first published by Anastassiades et al. in 2003 for the determination of pesticide multiresidues in fruits and vegetables [12]. This approach also gives good performance when applied for other compounds in different matrices.

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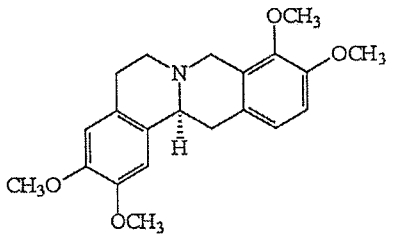
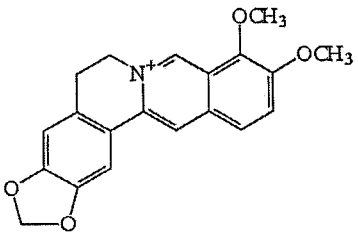
Table 1
The MS/MS transitions and conditions of RTD and IS.

Compound	Retention time (min)	Precursor ion (*m/z*)	Quantification ion (*m/z*)	Collision energy (eV)	Identification ion (*m/z*)	Collision energy (eV)
Rotundin	5.3	356	165	31	176	50
Berberine (IS)	3.2	336	321	25	306	25



Levo-tetrahydropalmatine



Berberine

Fig. 1. The chemical structure of levo-tetrahydropalmatine and berberine.

The method usually consists of two steps. Firstly, the analytes were extracted from aqueous phase into a appropriate solvent such as acetonitrile or ethyl acetate with the help of $MgSO_4$. Secondly, a clean-up step using dispersive solid phase extraction was used to remove inteferences. This paper introduces a modified QuEChERS extraction combined with LC–MS/MS technique for the determination of L-THP in rabbit plasma and its application to a pharmacokinetic study.

2. Experimental

2.1. Materials, reagents and animals

Reference standards of levo-tetrahydropalmatine (L-THP) and berberine chloride (internal standard, IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile was of HPLC grade from Merck KGaA (Darmstadt, Germany). Methanol, ethyl acetate, magnesium sulfate anhydrous, ammonium acetate and sodium chloride all analytical grade were also purchased from Merck KGaA. Distilled water was produced by an Aquatron system (ST15 OSA, UK).

The stock solutions of L-THP and IS were prepared in methanol at the concentration of 100 μg mL^{-1}. The intermediate solutions of the two compounds were prepared by diluting the stock solutions with water to obtain the concentration of 1 μg mL^{-1}. The working solutions were prepared in blank extract at the L-THP concentration from 5 to 200 ng mL^{-1} and IS concentration of 50 ng mL^{-1}.

Animal study was approved by the Local Animal Use Committee. Male rabbits of about 2 kg each were used for the pharmacokinetic study.

The self-microemulsifying drug delivery system (SMEDDS) pellet formulation of L-THP was developed from another research in Hanoi University of Pharmacy.

2.2. Sample preparations

A 500 μL aliquot of the plasma sample was transferred into a 2 mL centrifuge tube. 25 μL of IS solution of 1 μg mL^{-1} were added to the tube. Acetonitrile (0.5 mL) was then added and the tube was shaken by a vortex mixer for 1 min. A mixture of salts (0.2 g of magnesium sulfate anhydrous and 0.05 g of sodium chloride) was gradually added to the tube. After mixing for about 1 min, the tube was centrifuged at the maximum speed (16,000 rcf) for 10 min. The supernatant was filtered through a 0.45 μm PTFE membrane and 5 μL of the filtrate was injected to the LC–MS/MS system.

The blank samples were extracted using the above mentioned procedure. The final extracts were used to prepare matrix-matched calibration solutions by diluting the intermediate standard solution to get the working solutions of concentrations of 5, 20, 50, 100 and 200 ng mL^{-1}.

Table 2
Method repeatability, recovery and the ion ratio at different concentrations.

Concentration added (ng mL^{-1})	RSD%	*R*%	Ion ratio (%)
10	10	89	78.6
50	11	88	79.0
200	4.1	91	78.7

2.3. Instrumentation

An AB Sciex 5500 QQQ mass spectrometer (AB Sciex, USA) coupled with LC-20AD high pressure pumps, a column compartment and an autosampler (Shimadzu, Japan), was used to quantify the analyte. LC separation was obtained by using a Symmetry C18 column (150 × 4.6 mm; 5 μm partical size) and a pre-column (Waters, USA) with a mobile phase composition of 5 mM ammonium acetate and acetonitrile. The gradient program was initially set at 50% acetonitrile for 1 min then increased linearly to 100% acetonitrile over 1 min. After that, the eluent composition was maintained at 100% acetonitrile for 4 min then returned to 50% acetonitrile in 1 min, and re-equilibrated over 3 min. The flow rate was kept constant at 0.5 mL min^{-1}. Total run time was 10 min.

The mass spectrometer was operated in negative ESI mode with the capillary voltage and temperature set at −4500 V and 400 °C, respectively. A Peak Scientific AB-3G gas generator (UK) was used to generate nitrogen which was used as curtain gas and air was used as source gas. MS experiments were carried out in multiple reaction monitoring modes with two transitions for each compound. The higher intensities of the precursor-to-production transition were used for quantification.

2.4. Method validation

The method was validated for specificity, linearity and matrix effect, limits of detection (LOD) and limit of quantification (LOQ), repeatability, and recovery according to guidelines for bioanalytical method validation from the U.S. Food and Drug Administration (FDA) [13] and the EU requirements in EC/657/2002 [14]. The specificity was evaluated by analyzing blank rabbit plasma (4 sources), L-THP and IS standard solutions, and blank plasma spiked with L-THP and IS at the LOQ. The matrix-matched calibration solutions

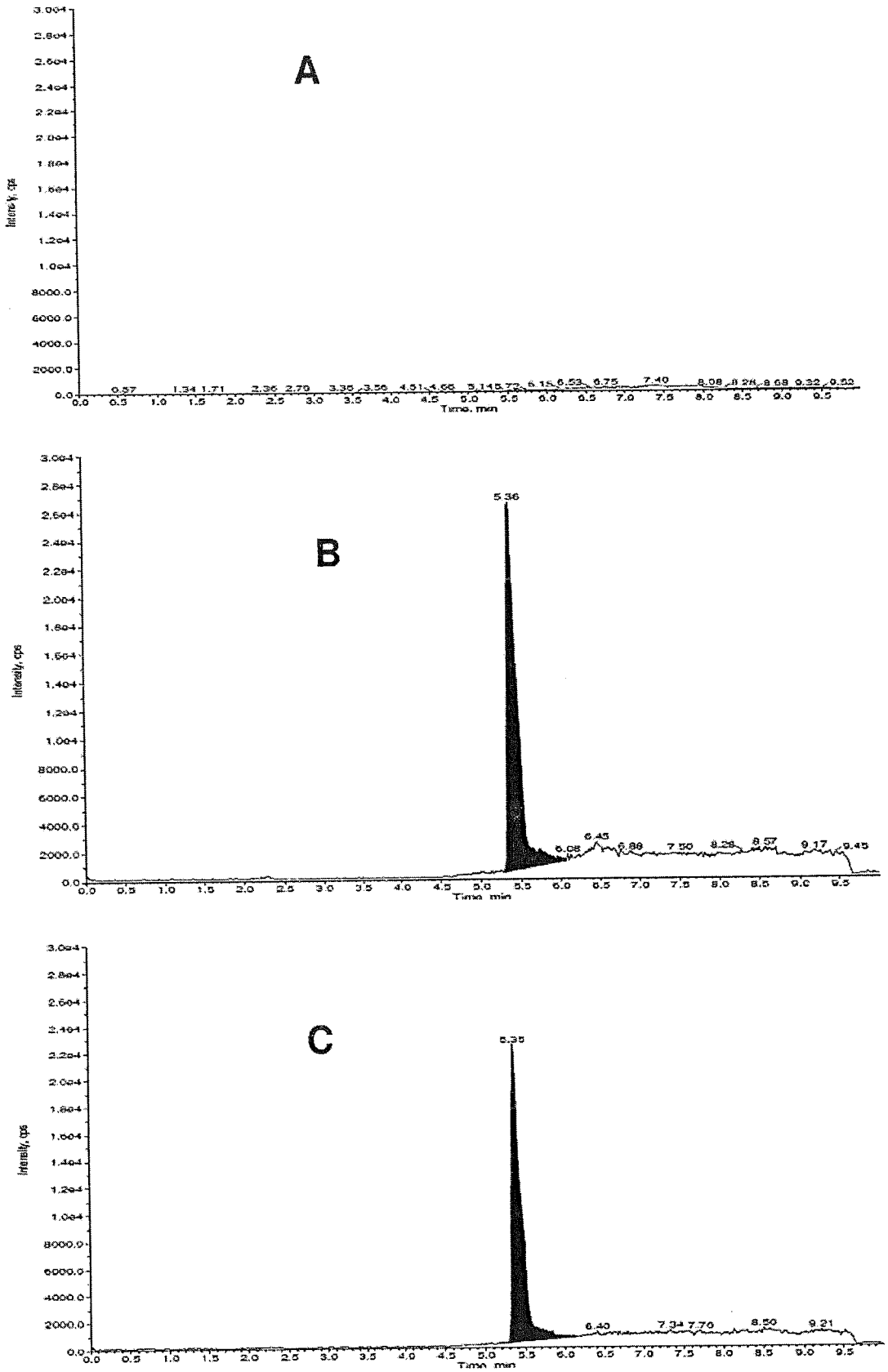


Fig. 2. The chromatogram of (A) blank plasma sample, (B) the quantitative ion and (C) the identification ion of sample spiked at 1 ng mL^{-1} levo-tetrahydropalmatine (LOQ).

from 5 to 200 ng mL^{-1} were analyzed in duplicates to access the linearity. The matrix effects of L-THP with and without use of IS were evaluated by comparing the slopes of matrix-matched calibration curves and those of solvent calibration curves and expressed in percentage. LOD and LOQ were investigated by using signal to noise ratio (S/N) methodology. The blank plasma samples were spiked

Table 3
The calibration curves in solvent and in matrix and the matrix effects of L-THP with and without use of the IS.

Type	Solvent curve	Matrix curve	Matrix effect
Without IS	$y = 17581x + 16074$ ($R^2 = 0.9981$)	$y = 13498x + 14877$ ($R^2 = 0.9979$)	−23%
With IS	$y = 0.0251x + 0.0304$ ($R^2 = 0.9982$)	$y = 0.0226x + 0.0058$ ($R^2 = 0.9980$)	11%

Table 4
Pharmacokinetic parameters of the bioavailability study in rabbits.

Rabbit	Raw material			Self-emulsifying pellet		
	T_{max} (min)	C_{max} (ng mL^{-1})	AUC_{24h} (ng h mL^{-1})	T_{max} (min)	C_{max} (ng mL^{-1})	AUC_{24h} (ng h mL^{-1})
1	15	54.2	31.6	5	108	81.1
2	15	33.3	36.7	5	87.8	34.9
3	15	57.2	36.4	5	94.5	53.4
Average	15	48.2	34.9	5	96.8	56.5
SD	0	13.0	2.84	0	10.2	23.3
Relative bioavailability						**162**

with L-THP at different low concentrations (0,5 and 1 ng mL^{-1}). The concentrations of the sample which gave S/N ratios of 3 and 10 were LOD and LOQ, respectively. All samples were performed in triplicates. The repeatability and recovery were evaluated at three concentration levels (sample concentrations are 10, 50 and 1[illegible] ng mL^{-1}) of spiked samples with 6 replicates per level. The performance criterion for repeatability was below 15% and for recovery was from 80% to 110%.

2.5. Application in a pharmacokinetic study

Two drug forms of L-THP including a self-emulsifying pellet formulation and a raw material were studied in a non-compartmental pharmacokinetic model. Rabbits were divided into 2 groups of three. The two samples were a suspension of L-THP in 0.5% sodium carboxymethyl cellulose (NaCMC) and SMEDDS pellet. The dose of L-THP used in pharmacokinetic study was 1.5 mg kg^{-1}. Blood samples were withdrawn from the ear artery about 2 mL after 0, 5, 15, 30, 60, 120, 240, 360, 480, 720, 1440 min and supplemented with equal amounts of saline containing heparin 50 IU. Plasma was collected by centrifugation of the above blood at 3500 rpm within 10 min and kept it in deep-freezer at −40 °C until the day of analysis.

3. Results and discussions

3.1. Choosing internal standard

Internal standard plays an important role to reduce the error, especially in MS method. The using of internal standard is critical in QuEChERS method because it will eliminate the error come from the volume differences in the sample and the final extract. Since the L-THP isotope was not available, the same-group compounds were preferable. Berberine was chosen in this study because it has the similar chemical structure and properties compared to L-THP. They are both isoquinoline alkaloids, having acetyl groups and a quaternary nitrogen atom (Fig. 1). Moreover, berberine was not naturally found in biosamples. The use of berberine as internal standard for L-THP determination has not been published before.

3.2. Optimization of instrument conditions

The ion transitions for L-THP and IS were chosen and optimized by direct infusion of standard solutions of 100 ng mL^{-1} into the mass spectrometer. The precursor ions were of the most intense masses which were [M + H] for both compounds. Then, the product ions and collision energies were automatically optimized using the system software (Table 1). The ions of higher intensity were used for quantification, the others were used for identification purpose.

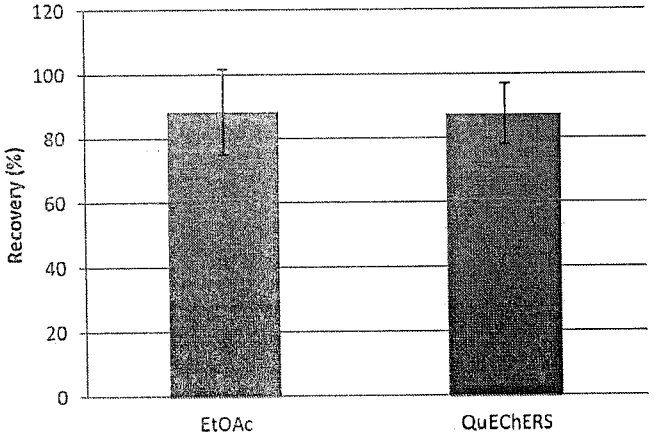


Fig. 3. Comparison of the recoveries of two extraction methods: (1) solvent extraction with ethyl acetate (2) modified QuEChERS method.

From the chromatogram in Fig. 2, both IS and L-THP peaks, which were eluted at 3.2 and 5.3 min respectively, were symmetric and suitable for analysis.

3.3. Investigation of sample preparation

The blank plasma samples (0.5 mL) were spiked with L-THP at the concentration of 50 ng mL^{-1}, then were analysed by two different sample extraction protocols. The first protocol, which was the conventional one, used the solvent extraction with ethyl acetate (5 mL). The ethyl acetate layer was then evaporated under the nitrogen stream at 40 °C. The residue was redissolved in mobile phase and the final solution was filtered through a 0.45 μm membrane filter before being injected into LC–MS/MS system. The second protocol, which is QuEChERS based one, included a single step acetonitrile extraction and salting out using magnesium sulfate and sodium chloride. The detailed steps were shown previously.

The recovery of L-THP obtained from two methods of extraction was compared ($n = 6$). From Fig. 3, the recoveries obtained from two methods were almost the same (88.2 and 87.4%). However, the standard deviation of the recovery from the first method was higher than that from the second one (13.4 compared to 9.5%). The critical step of the first method was the evaporation step which was not needed in the second one. The QuEChERS method was easier and faster, but still gained good performance. The method was then chosen to be further validated.

In this study, the dispersive solid phase extraction clean-up step was not applied due to the fact that the amount of plasma col-

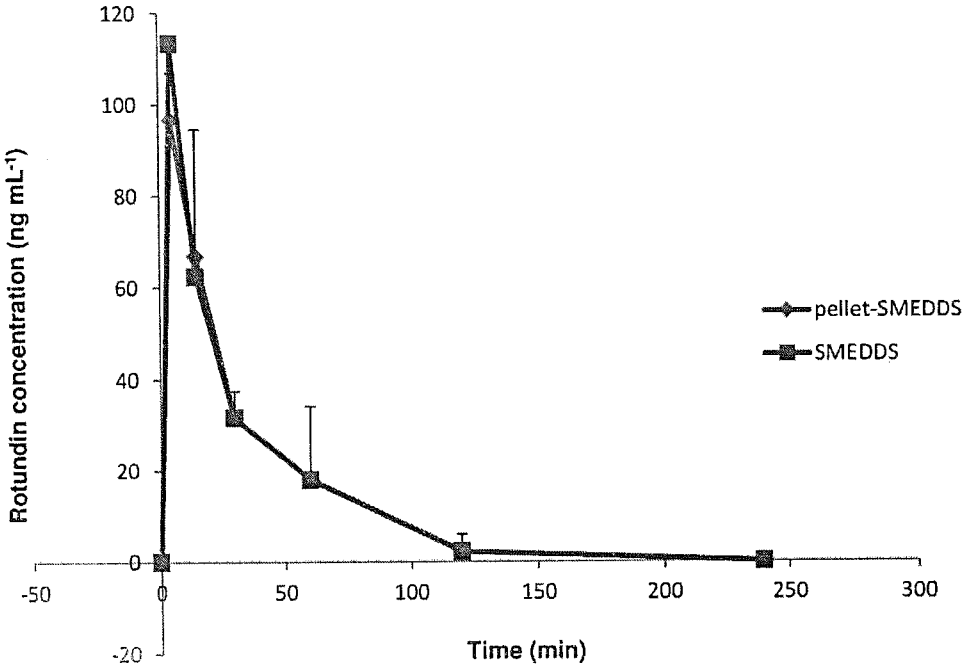


Fig. 4. The area under the plasma concentration–time curve (AUC_{24h}) obtained from the raw material and the pellet SMEDDS formulation.

lected was small. A further clean-up step could be done by using PSA and C18 sorbent. However, this required scaling down to very small amounts of extract and sorbent which could increase the error. Besides, the matrix effect obtained from this protocol was acceptable (shown below); this indicated that the proposed sample preparation was appropriate for the extraction of L-THP in rabbit plasma samples.

3.4. Method validation

The chromatograms of blank rabbit plasma, plasma samples spiked with L-THP and IS are presented in Fig. 2. The retention time was 3.2 for IS and was 5.3 for L-THP. The absence of interference from 4 blank samples indicated that the method was reasonably selective. Besides, the specificity was enhanced by means of mass identification. Two mass transitions gave the number of identification points of 4 that met the requirements of EC/657/2002. The ion ratio of L-THP in the samples was also compared to those of the standards and met the criteria given by EU.

The linearity was checked in the range from 5 to 200 ng mL^{-1}. The response of the standard was linear with the concentration and the coefficient of determination (r^2) was higher than 0.99.

Matrix effect is the effect on an analytical method caused by all other components of the sample except the specific compound to be quantified. A matrix effect higher than 20% must be eliminated or compensated. Matrix effects vary according to the cleanliness of the extracts and the compounds. Table 3 shows the calibration curves of L-THP (with and without normalize response to the IS) in two cases: matrix-matched calibration curve and pure solvent calibration curve. The matrix effect of L-THP without use of IS was −23% indicated that the matrix had some negative effect to the results. The matrix effect of L-THP with use of IS is 11% which was acceptable. However, since the real matrix effect was not in the acceptable range, the matrix-matched calibration curve should be used for the quantitation purpose.

The LOD and LOQ of the method was investigated by analysing the low concentration spiked samples. LOD and LOQ were estimated at 0.3 and 1 ng mL^{-1} in final solution, respectively (0.3 and 1 ng mL^{-1} in sample, respectively). The method LOD was low enough for the determination of L-THP in pharmacokinetic studies in which the L-THP concentration was usually higher than 1 ng mL^{-1}.

The repeatability and recovery obtained with this method are shown in Table 2. The relative standard deviations (RSD%) were lower than 11% and the recoveries (*R%*) were between 88 and 91%. These indicated that this method had an accuracy appropriate for the quantification of L-THP in plasma samples.

3.5. Pharmacokinetic study

The pharmacokinetic parameters are shown in Table 4. The area under the plasma concentration–time curve in 24 h (AUC_{24h}) value from the SMEDDS pellet formulation was about 162% compared to that of raw material (Fig. 4). The maximum concentration obtained from the SMEDDS pellets was as twice as that from the raw material. This indicated that the absorption of the SMEDDS pellets were considerably higher than that of the raw material. Moreover, the time to get maximum concentration from the SMEDDS pellet form was three times faster than that obtained from the raw material. These data suggested that the SMEDDS pellet form has improved both the level and the rate of L-THP absorption resulting in a higher oral bioavailability compared to that of the raw material.

4. Conclusion

A fast and accurate method based on QuEChERS extraction and LC–MS/MS has been developed and validated for the determination of L-THP in rabbit plasma. The method was faster and easier to perform compared to the current LC–MS/MS method. The method was applied to a pharmacokinetic study which indicated that the self-microemulsifying system pellet has improved the oral bioavailability of L-THP.

Acknowledgement

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DETERMINATION OF PESTICIDE MULTI-RESIDUES IN GREEN TEA USING A MODIFIED QUECHERS EXTRACTION AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY TECHNIQUE

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A modified QuEChERS method was developed and validated for determination of pesticide multi-residues in green tea by liquid chromatography tandem mass spectrometry. Lead acetate was first time used together with primary secondary amine and graphite carbon black to eliminate tannin, caffeine, and other pigments in tea and thus reduced the matrix effects. The method was compared to the original QuEChERS method as well as A.O.A.C. QuEChERS method. For accurate quantification, the matrix matched calibration technique was used. The method showed good performance in the concentration range from 0.01 to 1 mg kg^{-1}. All pesticides could be quantified at and lower than 0.01 mg kg^{-1}. Recoveries were from 70 to 120% and repeatabilities were <15% RSD depending on the compounds.
Keywords: pesticide multi-residues, green tea, QuEChERS, liquid chromatography-mass spectrometry

Tea is one of the most favoured beverages worldwide especially in Asian countries. The most tea producing and consuming countries are China, India, Sri Lanka, Kenya, Turkey, Indonesia, and Vietnam (REDIFF.COM, 2012). Pesticides were used in tea farming to control insects, mites, leaf-eating beetles, and caterpillars. Therefore, determination of pesticide residues in tea is an important contribution to a safer tea.

QuEChERS (Quick, easy, cheap, effective, rugged, and safe) is a sample preparation methodology for pesticide multi-residue analysis, which was first published by ANASTASSIADES and co-workers (2003). The method uses a single-step acetonitrile or buffered acetonitrile extraction and salting out liquid-liquid partitioning from the water in the sample with anhydrous magnesium sulphate. Then, the dispersive solid phase extraction (d-SPE) clean-up is done to remove excess water and matrix components with a combination of sorbents including $MgSO_4$ and primary secondary amine (PSA). The final extracts are analysed by mass spectrometry (MS) technique after a liquid or gas chromatographic separation (A.O.A.C., 2010). This method has many advantages. Firstly, it is a multi-residue method, which can be applied to determine hundreds of pesticides in one single procedure. Secondly, the final extract in acetonitrile can be used both for gas chromatography and liquid chromatography. Last but not least, QuEChERS is definitely a cheap, easy, effective, rugged, and safe method like its name (LEHOTAY et al., 2005). For over a decade, QuEChERS has been developing quickly and was accepted by many international organizations (e.g. A.O.A.C. No.

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2007.01, BS EN 15662:2008, etc.) for determination of pesticide residues in fruit and vegetables (BS EN, 2008; A.O.A.C., 2010). The A.O.A.C. method uses acetate buffer as solvent extraction, while EN method uses citrate buffer. The pH value around 4.5 to 5.5 of buffers is favourable with the acidic or basic compounds and matrices. Pesticide multi-class, multi-residue determination relies on the QuEChERS extraction combined to LC-MS/MS and/or GC-MS(/MS) instrumentation could be used to identify a few hundreds of pesticides in one single test (Lehotay et al., 2005; Nguyen et al., 2010; Dai et al., 2011). Its applications have also spread to other matrices like tea, herb, rice, and other grains (Steiniger et al., 2010; Chen et al., 2011; Fan et al. 2013; Guan et al., 2013; Rajski et al., 2013).

Determination of pesticides in tea is somewhat difficult, due to the presence of large amounts of polyphenols, caffeine, and especially tannin (Graham, 1992), which can result in a high matrix effect. A larger number of analytical methods have been published for the determination of pesticide residues in tea. Some methods include the solvent extraction combined to many clean-up techniques, such as solid phase extraction (SPE) or gel permeation chromatography (GPC) (Huang et al., 2007; Cho et al., 2008). These methods, however, are time- and labour-consuming and require large volumes of various kinds of solvents. Recently, QuEChERS method has been applied to extract pesticides from tea (Steiniger et al., 2010; Chen et al., 2011; Lozano et al., 2012; Guan et al., 2013; Rajski et al., 2013; Shoeibi et al., 2013). To deal with the matrix effect problem, many techniques have been tried including a sample dilution, matrix calibration, various types and amounts of sorbent addition, or SPE cleanup (Stahnke et al., 2012; Fan et al., 2013; Guan et al., 2013; Rajski et al., 2013; Wang et al., 2013). However, the high amount of tannin in tea requires a method with a further clean-up step. Until now, the reaction of tannin with lead(II) acetate forming lead(II) tannate is used for the isolation of tannin from green tea.

The aim of this work is the evaluation of lead(II) acetate as a clean-up material for pesticides analysis in tea matrices. This study also included the validation results of a LC-MS/MS technique combined with a modified QuEChERS procedure using lead(II) acetate in the partitioning step for determination of pesticide multi-residues in green tea.

1. Materials and methods

1.1. Chemicals and reagents

All pesticide reference standards, of purity ≥ 95%, were from Dr. Ehrenstorfer (Augsburg, Germany). These pesticides (showed in Table 1) were chosen based on their use in tea crops and were applicable with LC-MS/MS. The application could be generally spread to other pesticides. The internal standard, triphenyl phosphate (TPP), was from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of 1000 μg ml^{-1} were prepared in acetonitrile and stored in dark at –4 °C. The intermediate standard-mix solutions of 0.1, 1, 5, and 10 μg ml^{-1} were diluted from stock solutions in acetonitrile. TPP working solution was of 2 μg ml^{-1}.

Acetonitrile and methanol of HPLC grade were from Merck (Darmstadt, Germany). Acetic acid, anhydrous magnesium sulphate, sodium acetate trihydrate, lead acetate, and sodium chloride were also supplied by Merck. Primary-secondary amine (PSA) and graphite carbon black (GCB) sorbents were obtained from Agilent Technology (USA). Ultra-pure water was obtained by using a SG purification system (Germany).

Table 1. List of pesticides with HPLC retention times and MS/MS conditions

Pesticides	Retention time (min)	Precursor ion (M+H)	Quantification ion (CE, eV)	Confirmation ion (CE, eV)
Acetochlor	10.80	270.0	224 (13)	148 (25)
Aldicarb	8.03	213.0	116 (15)	89 (15)
Atrazine	9.61	216.0	96 (31)	104 (37)
Azoxystrobin	9.83	404.0	372 (19)	344 (31)
Abamectin	13.90	890.5	305 (31)	567.5 (17)
Acetamiprid	6.90	223.0	126 (25)	56 (19)
Carbaryl	9.10	202.0	145 (13)	127 (37)
Carbofuran	8.74	222.0	165 (15)	123 (29)
Carbendazim	5.35	192.0	160 (23)	132 (39)
Carboxin	9.08	236.0	143 (19)	87 (29)
Dichlorvos	8.71	221.0	109 (23)	127 (18)
Dimethoat	7.03	230.0	199 (13)	125 (27)
Edifenphos	11.00	311.0	283 (17)	109 (35)
Emamectin	11.00	886.5	158 (39)	302 (35)
Fenobucarb	10.10	208.0	152 (11)	95 (19)
Imidacloprid	6.32	256.0	209 (21)	175 (27)
Indoxacarb	11.30	528.0	249 (21)	293 (17)
Isoprocarb	9.55	194.0	95 (19)	137 (11)
Methiocarb	10.30	226.0	169 (11)	121 (23)
Methomyl	4.89	163.0	88 (13)	106 (13)
Profenophos	11.90	373.0	303 (23)	345 (17)
Propoxure	8.67	210.0	111 (19)	93 (31)
Terbuconazole	11.10	308.0	125 (39)	151 (31)
Thiamethoxam	5.29	292.0	211 (15)	181 (29)
Trichlorfon	6.97	257.0	109 (23)	221 (15)
TPP (IS)	11.10	327.0	77 (61)	–

Samples were dried Vietnamese green teas (produced from *Camellia sinensis* leaves and flower buds) collected from the market. Blank samples were chosen from the samples in which pesticides had not been detected.

1.2. Instrumentation

An AB Sciex 5500 triple quadrupole mass spectrometer (AB Sciex, USA) coupled with LC-20AD high pressure pumps, column compartment, and autosampler (Shimadzu, Japan) was used to detect and quantify the pesticide residues. LC separation was obtained by using a X-Bridge BEH C18 (150 mm × 2.1 mm, 2.5 µm particle size) and a pre-column BEH C18 (5 mm × 2.1 mm, 1.7 µm) (Waters, USA) with a mobile phase composed of 0.1% (v/v) acetic acid in water (eluent A) and methanol (eluent B). The gradient program was initially set at 25% B in 1 min then increased linearly to 90% B over 8 min. After that, the eluent composition was maintained at 90% B for 4 min, and re-equilibrated over 3 min. The flow rate used was kept constant at 0.7 ml min^{-1}. Total run time was 15 min. The injection volume was 20 µl.

The mass spectrometer was operated in positive ESI mode with capillary voltage and temperature set at 5000 V and 450 °C, respectively. A Peak Scientific AB-3G gas generator (UK) was used to generate N_2 used as curtain gas and air was used as source gas. Curtain gas, collision gas, source gas 1, and source gas 2 were set at 25 psi, 7 psi, 30 psi, and 20 psi, respectively. MS experiments were carried out in multiple reaction monitoring modes with two transitions for each pesticide (Table 1). The higher intensities of the precursor-to-product ion transition were used for quantification; the others were used for confirmation. In addition, the ion ratios were also the criteria for pesticide confirmation.

1.3. Sample preparation

A modified QuEChERS method was applied to extract pesticides in green tea samples. After homogenization, a 3 g portion of sample was weighed in a 50 ml centrifuge tube. Internal standard (TPP) was added to make a sample concentration of 100 μg kg^{-1}. Then, 15 ml of water was added for sample hydration. The tube was carefully shaken by hand for 30 s and let stand for 30 min. After that, 15 ml of acetonitrile were added and the tube samples were shaken vigorously by hand for 1 min. A portion of 6.0 g of anhydrous $MgSO_4$, 1.5 g of NaCl and 1.5 g of $Pb(CH_3COO)_2$ was gradually added and the tubes of samples were tightly capped, shaken as mentioned above, and centrifuged at 6000 r.p.m. (3904×*g*) for 5 min. For clean-up, 1 ml of supernatant was transferred to a 2 ml centrifuge tube containing 150 mg of anhydrous $MgSO_4$, 50 mg of PSA, and 7.5 mg of GCB. The tube was vortexed for 30 s and centrifuged at 12 000 r.p.m. (13 684×*g*) for 1 min. A portion of 0.5 ml of the supernatant was dried under a nitrogen stream at 40 °C. The residue was then reconstituted with 0.5 ml of mobile phase (0.1% acetic acid and acetonitrile, 75:25) and the extract was filtered through a 0.2 μm membrane (Minisart RC 15, Sartorius, Germany) to a LC-MS/MS sample vial. In this procedure, the sample weight was 3 g and the extract volume was 15 ml, so the sample was 5 times diluted.

1.4. Matrix-matched calibration technique

The blank tea sample was extracted with acetonitrile as mentioned above. In the clean-up step, 6 ml of the acetonitrile extract was transferred to a 15 ml centrifuge tube containing 900 mg of anhydrous $MgSO_4$, 300 mg of PSA, and 45 mg of GCB. The final extracts were used to prepare matrix-matched calibration solutions by diluting the intermediate standard-mix solutions to give the final concentrations of 0, 1, 2, 20, 100, and 200 ng ml^{-1} (corresponding to 0, 5, 10, 100, 500, and 1000 μg kg^{-1} in samples). These solutions were used to evaluate the recoveries and to quantify analytes in real samples. Furthermore, a series of standard solutions in solvent (acetonitrile) at the same concentration levels were also prepared for the assessment of matrix effects.

1.5. Method validation

The method was validated for linearity, matrix effects, limits of detection, repeatability, and recovery. To test the linearity, the matrix-matched calibration solutions were analysed. The matrix effects were assessed by comparing the slopes of matrix-matched calibration curves to solvent calibration curves and given in percentage. The repeatability and recovery was evaluated at three concentration levels (sample concentrations were 10, 100, and 1000 μg kg^{-1}) of spiked samples with 6 replicates per level.

2. Results and discussions

2.1. Optimization of LC-MS/MS

The precursor ions, product ions, and collision energy were chosen and optimized by the injection of a pesticide solution of 100 ng ml^{-1} directly into mass spectrometry. The precursor ions were of the highest m/z and most intense ion beam, which were M+H m/z in most cases. Then, the product ions and collision energies were automatically optimized using the system software (Analyst). Other LC and MS parameters were also investigated to obtain peaks of Gaussian shape and the signal to noise ratios (S/N) of the 10 µg kg^{-1} matrix standard with values above 10. A chromatogram of a mixture of matrix pesticide standards is shown in Figure 1.

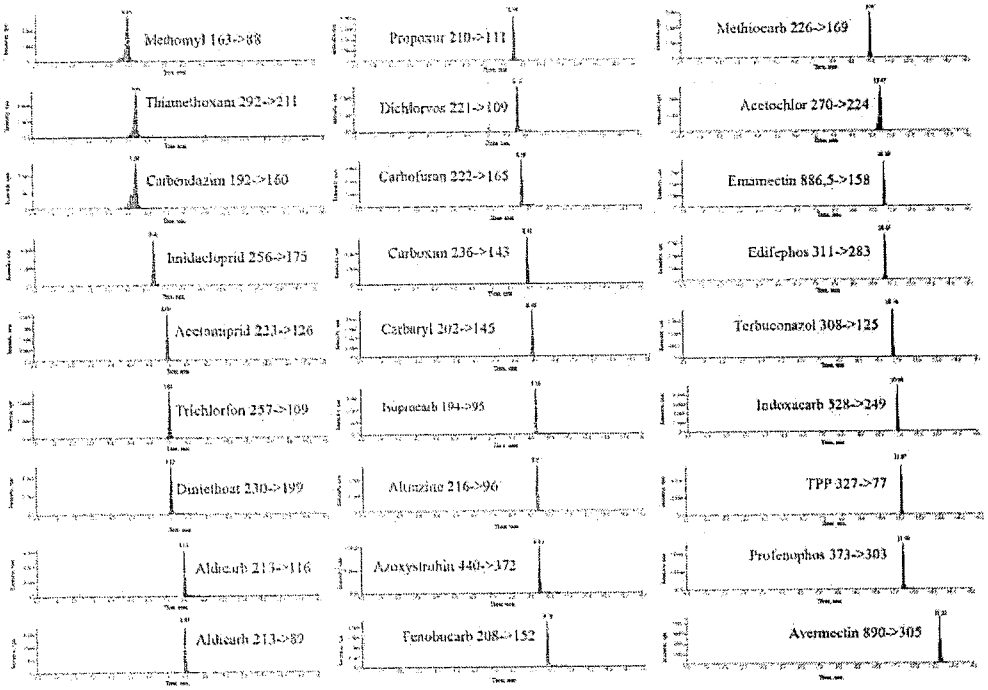

Fig. 1. Exact ions chromatogram of a matrix standard of 25 pesticides and internal standard TPP at the concentration of 100 µg kg^{-1} (Annotations are the names and the transitions of the quantification ions, aldicarb showed two transitions)

Most of the peaks are symmetric except for methomyl, thiamethoxam and carbendazim peaks. These compounds are more polar than others and thus, have strong interaction with residual silanol groups on the packing surface of the LC column. Modifying the mobile phase with a buffer, amine, or ion-pairing reagent is used to improve the peak shape. However, the MS/MS detection could give enough selectivity and sensitivity to gain acceptable accuracy for these three compounds.

2.2. Investigation of sample preparation

The QuEChERS extraction method showed good performances for many types of vegetables and fruit. The method requires a sample moisture content of about 80% or above for maximal pesticide extraction (ANASTASSIADES et al., 2003). Therefore, water was added to dried tea before extraction to hydrate the sample. To get the moisture content of around 80%, the water to sample ratio was set at 5:1 (w/w). The sample then was left for 30 min to ensure a complete hydration without further investigation of the amount of water and the soaking duration (STEINIGER et al., 2010; CHEN et al., 2011; RAJSKI et al., 2013).

Two versions of public QuEChERS method including the original version (ANASTASSIADES et al., 2003) and the A.O.A.C. version (LEHOTAY et al., 2005) were tested and compared to a modified QuEChERS method. The modified one relies on the addition of lead acetate (1.5 g) in the extraction step to adsorb polyphenols, caffeine, and pigments in tea and the addition of GCB in the clean-up step to remove chlorophylls. The extraction efficiencies, the co-extracted materials, and the extract pH values of the three methods were compared. pH values of the extracts obtained from original, A.O.A.C., and modified methods were 5.23, 5.76, and 5.63, respectively. The amount of co-extractives from the initial extraction solvent of the modified method was a half of those of the other methods (Table 2). Therefore, the matrix effects of this method were reduced. Figure 2 shows that the recoveries of eight compounds obtained with the proposed method were higher than the other ones. Most of these compounds are carbamate and organo-phosphorus insecticides. There were no significant differences in recoveries among the three methods for other analytes. After these experiments, we found that the use of lead acetate helped achieve a better cleaning and a higher recovery, overall. Besides, the green tea matrices, having a pH about 6, did not require the addition of a pH buffer.

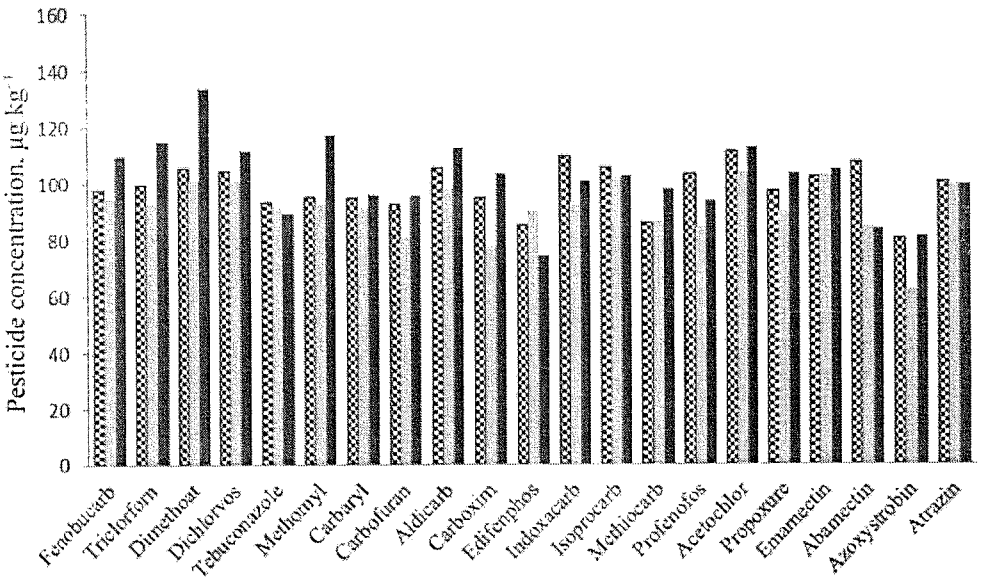

Fig. 2. Comparison of three QuEChERS methods (▩ : the original method; ■ : the AOAC method; ■ : the modified method) for the extraction of pesticide residues in green tea

Table 2. Co-extracted materials and pH values in MeCN or 0.1% acetic acid in MeCN extracts prior to d-SPE step

QuEChERS method	Extraction solvent	Partitioning salts	pH	Co-extracted material (mg ml^{-1})
Original	MeCN	6 g $MgSO_4$ & 1.5 g NaCl	5.23	20.5
AOAC	MeCN (0.1% acetic acid)	6 g $MgSO_4$ & 1.5 g CH_3COONa	5.72	21.3
Modified	MeCN	6 g $MgSO_4$, 1.5 g NaCl & 1.5 g $(CH_3COO)_2Pb$	5.63	10.5

The optimal amount of lead acetate was also investigated. Different amounts of lead acetate including 0.5, 1.0, 1.5, and 2.0 g were used in the extraction step and the recoveries of pesticides derived were compared. The more salt was used the better recovery was obtained. However, the recovery of the samples with above 1.5 g lead acetate decreased, because the pesticide absorption increased and the solubility of lead salt reached a limit. The highest recoveries for most pesticides were obtained when using 1.5 g of lead acetate.

In the dispersive SPE step, GCB was used to eliminate chlorophylls and some of the polyphenols and other pigments. The amount of GCB used was of 7.5 mg ml^{-1} of extract according to previous researches. GCB can absorb some planar pesticides when used at higher concentrations. In this investigation, the use of GCB gave equal or better recoveries for most pesticides (Fig. 3). The higher recoveries were obtained by the reduction of the matrix effect, which was a result of the application of GCB.

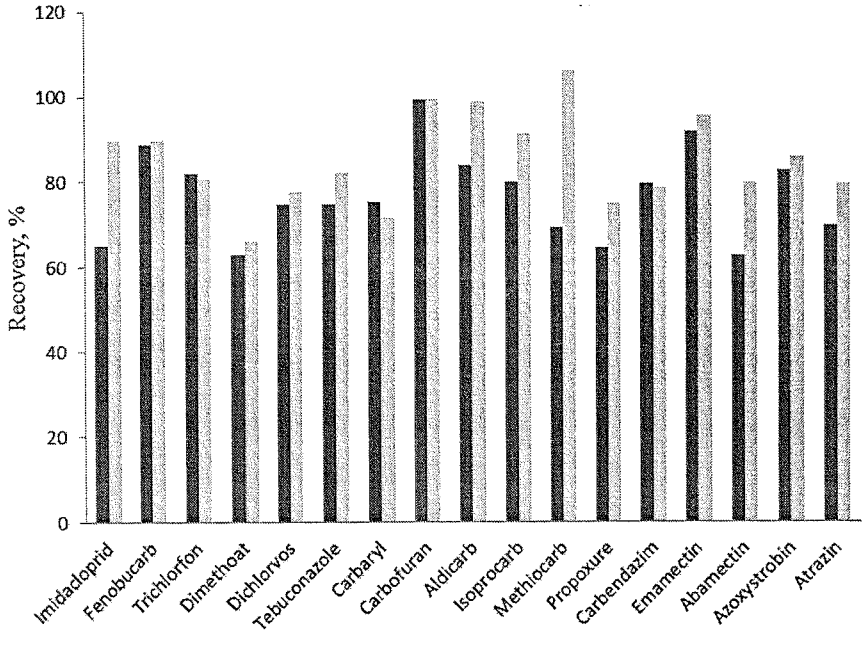

Fig. 3. The recoveries of pesticides obtained from two procedures with using and not using GCB (7.5 mg) in d-SPE step (■ : d-SPE without using GCB and ■ : d-SPE with using GCB)

2.3. Matrix effect assessment

Matrix effect is the effect on an analytical method caused by all other components of the sample beside the specific compound to be quantified. Ion suppression and enhancement are the two causes of the matrix effect. That is why matrix effect is an important criterion in every mass spectrometry method. A matrix effect higher than 20% must be eliminated or compensated. Matrix effects vary according to the cleanliness of the extracts and the compounds.

The matrix effect is the drawback of QuEChERS method especially for complex matrices. Green teas give a very high matrix effect because of the existence of many pigments. This modified QuEChERS method eliminated most of the pigments and with that it could minimize the matrix effect. Figure 4 shows the matrix effect on the analysed pesticides. Most pesticides gave signals of lower intensities in the matrix than in solvents. However, the matrix effects on all analysed pesticides were within ±20%. To eliminate efficiently the effects of matrix, the matrix-matched calibration technique was used.

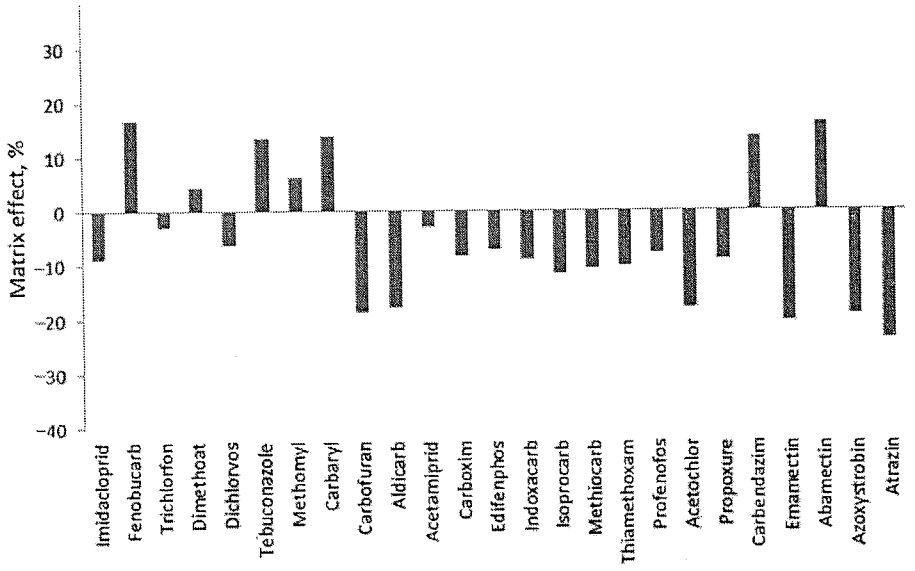

Fig. 4. Green tea matrix effect of pesticides by using modified QuEChERS method

2.4. Method validation

For selectivity, every compound had two signals from two product ions (Table 1). Ion ratios of the compounds in the samples were also compared to that in the standards. The relative ion intensities have to meet the criteria given by EU (EUROPEAN COMMISSION, 2002).

The repeatabilities and recoveries obtained with this method are presented in Table 3. For most pesticides at three levels of concentration, the relative standard deviations (RSD%) were lower than 20% (except for profenofos at 100 μg kg^{-1}) and the recoveries (R%) were between 70–120%. These indicated that this method had accuracy appropriate for the quantification of pesticide multi-residues in green tea.

Table 3. The repeatability and recovery at 3 concentration levels (n=6)

Pesticides	10 μg kg^{-1}		100 μg kg^{-1}		1000 μg kg^{-1}	
	RSD%	R%	RSD%	R%	RSD%	R%
Acetochlor	11	76.9	6.3	90.5	9.3	83.0
Aldicarb	8.4	111	9.0	118	8.1	109
Atrazine	12	91.2	9.6	91.2	4.4	96.7
Azoxystrobin	18	71.1	17	78.5	15	75.3
Abamectin	16	85.4	19	93.9	12	92.0
Acetamiprid	17	89.5	15	94.4	11	91.4
Carbaryl	6.2	87.8	8.8	89.3	7.6	81.9
Carbofuran	9.4	88.9	9.1	89.4	5.9	89.1
Carbendazim	7.6	110	5.4	113	5.0	111
Carboxin	7.5	82.5	7.7	87.6	8.9	86.6
Dichlorvos	16	75.2	13	73.2	9.1	82.2
Dimethoat	5.9	82.0	9.2	75.4	4.4	77.3
Edifenphos	9.6	74.6	7.8	82.3	7.1	83.2
Emamectin	15	98.8	8.0	96.7	18	93.9
Fenobucarb	8.4	84.3	7.2	83.6	7.3	90.5
Imidacloprid	11	97.8	10	95.9	6.1	96.8
Indoxacarb	16	93.3	12	81.9	11	88.4
Isoprocarb	8.2	79.4	7.4	75.8	7.0	73.4
Methiocarb	19	76.0	19	78.6	13	80.3
Methomyl	19	89.9	6.3	94.5	3.1	92.0
Profenophos	17	82.6	23	73.5	19	71.3
Propoxure	5.8	81.9	5.6	85.2	5.4	83.6
Terbuconazole	10	108	11	108	9.9	113
Thiamethoxam	7.5	82.5	8.7	95.5	5.2	86.2
Trichlorfon	10	91.9	6.9	83.2	4.3	81.2

The limit of detection (LOD) and limit of quantification (LOQ) were estimated from the signal to noise (S/N) ratios of the pesticide peaks. All pesticides could be quantified at the concentration of 10 μg kg^{-1} without any concentration steps. This value is acceptable compared to the default maximum residue level (MRL=10 μg kg^{-1}).

The linearity was checked in the range of 1–200 ng ml^{-1}. The response of the matrix matched standard was considered linear when the coefficient of determination (r^2) was equal to or higher than 0.99. For all analysed pesticides, the linearity range was from 2 ng ml^{-1} to 200 ng ml^{-1}. A few pesticides even showed lower sensitivity. Because the sample weight was 3 g and the acetonitrile extract volume was 15 ml, the linearity calculated for samples was from 10 μg kg^{-1} to 100 μg kg^{-1}.

2.5. Analysis of real samples

Once validated, the proposed method was applied to determine 25 pesticides in 20 different dried green tea samples collected in the Hanoi market.

The results are shown in Table 4. Ten of twenty samples were found to be positive to pesticides from different chemical groups, especially imidacloprid and acetamiprid (neonicotinoid insecticides). Most of the pesticide concentrations were of above 10 μg kg^{-1} (default MRL).

Table 4. Pesticide residues found in dried green tea samples and their concentration

Sample ID	Pesticides	Sample conc., μg kg^{-1}	Class
S1	Imidacloprid	10	Neonicotinoid insecticide
	Acetamiprid	154	Neonicotinoid insecticide
S2	Acetamiprid	5.7	Neonicotinoid insecticide
S9	Imidacloprid	5.6	Neonicotinoid insecticide
	Acetamiprid	21	Neonicotinoid insecticide
	Carbendazim	6.4	Benzimidazole fungicide
S10	Imidacloprid	42	Neonicotinoid insecticide
	Acetamiprid	19	Neonicotinoid insecticide
	Thiamethoxam	150	Neonicotinoid insecticide
S11	Imidacloprid	28	Neonicotinoid insecticide
	Acetamiprid	45	Neonicotinoid insecticide
	Fenobucarb	6.7	Carbamate insecticide
S12	Imidacloprid	32	Neonicotinoid insecticide
	Acetamiprid	48	Neonicotinoid insecticide
	Fenobucarb	17	Carbamate insecticide
S13	Emamectin	64	Macrycyclic lactone insecticide
S16	Acetamiprid	16	Neonicotinoid insecticide
S18	Acetamiprid	22	Neonicotinoid insecticide
	Carbofuran	86	Carbamate insecticide
S20	Acetamiprid	19	Neonicotinoid insecticide

3. Conclusions

A modified QuEChERS method was proposed for the determination of pesticide multi-residues in dried green tea. The green tea matrix effects were reduced by the addition of lead acetate in the partition step and the use of GCB in the clean-up step. The validation data show that this method has good accuracy and sensitivity and could be applied for the determination of pesticide multi-residue in green tea samples.

*

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Risk assessment of 3-MCPD esters and glycidyl esters from the formulas for infants and young children up to 36 months of age

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RESEARCH ARTICLE

Risk assessment of 3-MCPD esters and glycidyl esters from the formulas for infants and young children up to 36 months of age

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ABSTRACT

Esters of 2-monochloropropane-1,2-diol (2-MCPD), 3- monochloropropane-1,2-diol (3-MCPD), and glycidol are present in infant formulas, follow-on foods and similar compositions. They arise mainly from the vegetable oil content and may cause harmful effects in consumers. The contents of these substances in formulas were determined indirectly by converting the esters to the free form, followed by derivatization and analysis by gas chromatography-tandem mass spectrometry (GC-MS/MS). The validation results demonstrate that the method had sufficient specificity and adequate accuracy. The limits of detection (LOD) and limits of quantification (LOQ) for each of 2MCPDE, 3MCPDE, and GE were 1.5 and 5 µg/kg, respectively. Formula intake by children up to 36 months of age was surveyed, and the data was used to assess the risks due to 3-MCPD esters (3-MCPDE) and glycidyl esters (GE). The mean exposure dose of 3-MCPDE for different age groups ranged from 0.51 to 1.13 µg/kg bw per day. The corresponding mean GE exposure ranged from 0.031 to 0.069 µg/kg bw per day. Neither mean values nor the percentile 95% values of 3-MCPDE exposure doses exceed the recommended provisional maximum tolerable daily intake (PMTDI).

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Introduction

3-Monochloropropane-1,2-diol esters (3-MCPDE), 2-monochloropropane-1,3-diol esters (2-MCPDE), and glycidyl esters (GE) have recently been identified as contaminants in various kinds of food, mainly from oil composition. These substances are formed during the processing of fatty foods (glycerol or acyl glycerides) at high temperatures in the presence of chloride ions (Zhao et al. 2016). Several studies have revealed the occurrence of 3-MCPDE, 2-MCPDE, and GE in soy sauce, edible oil, smoked meat, smoked fish, potato chips, crackers, French fries, margarine, formula, and so on (Hamlet et al. 2002; Nyman et al. 2003; Zelinková et al. 2009; Weißhaar 2011).

Many published studies have revealed the toxicological effect of these compounds. When entering the body, these esters would be converted to free forms that could be harmful to health. The International Agency for Research on Cancer (IARC) classified glycidol as probably carcinogenic to humans (Group 2 A) (IARC). The European Scientific Committee on Food (SCF) has classified 3-monochloropropane-1,2-diol esters (3-MCPD) as potentially carcinogenic to humans and recommended a Tolerable Daily Intake (TDI) of 2 µg/kg body weight (bw) derived from a lowest-observed-effect level (LOEL) of 1.1 mg/kg bw per day and an uncertainty factor of 500 (European Commission 2001). The Joint FAO/WHO expert committee on Food Additives at the 83rd meeting (2016) has reassessed and proposed a provisional maximum tolerable daily intake (PMTDI) of 4 µg/kg bw for 3-MCPD and 3-MCPD esters (expressed as 3-MCPD equivalent) based on a $BMDL_{10}$ of 0.87 mg/kg bw per day and a 200-fold uncertainty factor (JECFA 2016). Recently, the European Commission updated the maximum levels of the sum of

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3-MCPD and 3-MCPD fatty acid esters (expressed as 3-MCPD) in infant and follow-on formulas at 125 µg/kg (for powder) and 15 µg/kg (for liquid). The values for glycidyl fatty acid esters (expressed as glycidol) are 50 µg/kg (for powder) and 6.0 µg/kg (for liquid) (European Commission 2020). There are limited reports to date on the toxicological effects of 2-MCPD and its esters. According to Becalski et al. (2015) the ratio of 3-MCPDE to 2-MCPDE is consistent, with a mean of 2.6. Although there are data on 2-MCPDE concentrations, it has not been possible to evaluate the risk characterization of these compounds due to the lack of information on their toxicological effects.

Infant and follow-on formulas are usually a homogenized mixture of fats, carbohydrates, proteins, and other micronutrients. The fats in these products are mainly derived from vegetable oils. Most vegetable oils used as ingredients in infant formulas undergo a refining process at high temperatures to remove unwanted components, which could lead to the production of 2-MCPDE, 3-MCPDE, and GE (Hrncirik and van Duijn 2011; Pudel et al. 2011). The significant source of 3-MCPDE and related compounds are known to be due to palm oil refinery processes (Destaillats et al. 2012). Many studies have reported the occurrence of these compounds in formula products (Becalski et al. 2015; Arisseto et al. 2017; Beekman et al. 2019; Beekman et al. 2020; Beekman et al. 2021).

Currently, there are direct and indirect approaches to determining the content of MCPD and glycidyl esters in nutritional formulas. In the first methodology, different fatty acid esters of MCPD and glycidol are separately analyzed by LC-MS (Haines et al. 2011). Concentrations of individual esters can be quantified using this methodology, but several reference standards are required. The second methodology involves hydrolysis of the fatty acid esters to yield the free forms of MCPDs and glycidol, which are subsequently determined by GC-MS after derivatization (Jędrkiewicz et al. 2016; Dubois et al. 2019; Chi et al. 2020; MacMahon et al. 2022). This method is more straightforward and requires a smaller number of reference standards. AOAC International has adopted the indirect method for nutritional formula (Dubois et al. 2019). The method includes a fat extraction step with modified ethyl acetate, followed by solid-phase extraction clean-up. The GEs are converted to 3-monobromopropanediol (3-MBPD) which, together with MCPDEs after hydrolysis in methanolic acid to generate the free MCPDs, are derivatised with phenylboronic acid and analyzed by GC-MS/MS.

In Vietnam, there are some data on 3-MCPD contamination in soy sauce and infant formula (Tam et al. 2019; Chi et al. 2020). 3-MCPDE and 2-MCPDE were detected respectively in 38.9% and 24.7% of samples, (Chi et al. 2020). However, the risk of these substances in people's diet, especially young children, has not been assessed. This study focuses on the occurrence of 2-MCPDE, 3-MCPDE, and GE in infant and follow-on formulas using GC-MS/MS and the risk characterization of these substances in the diet of young children up to 36 months of age in Vietnam.

Materials and methods

Sample collection

Fifty-four samples of 40 infant formulas and 14 follow-on formulas for children under 36 months of age were randomly collected at local markets and stores in Hanoi, Ho Chi Minh City, and five provinces, including Bac Ninh, Hai Duong, Ha Nam, Binh Duong, and Dong Nai. With the desire to obtain the most representative sample for the population, the sampling sites were chosen in different regions of Vietnam where manufacturers and storage sites are located. All major brands of formulas were included in the study. A blank sample of the powder formula, provided by the Laboratory of Quality Assurance, National Institute for Food Control, was used as the method development and validation matrix.

Determination of 2-MCPDE, 3-MCPDE, and GE content

Chemicals and reagents

All reagents, chemicals, and solvents used were of analytical grade. The chemicals, including solvents (diethyl ether, ethyl acetate, *n*-hexane,

methanol, tetrahydrofuran, and toluene) and salts (anhydrous sodium sulfate, sodium hydrogen carbonate), were purchased from Merck Vietnam (Hanoi, Vietnam). The derivatization reagent used was phenylboronic acid, obtained from TRC (Canada). Ultrapure water was obtained from a Milli-Q IQ Water Purification System (Merck). Solid phase extraction columns Bond Elut NH_2 (500 mg, 3 mL) were provided by Agilent Technologies (Singapore).

The reagent for hydrolysis of MCPDEs and GE was a sulfuric acid solution in methanol (1.8 mL sulfuric acid in methanol to 100 mL). The reagent for the transformation of glycidyl esters to 3-monobromopropandiol (3-MBPD) esters was acidic sodium bromide (0.03 g of sodium bromide and 0.5 mL of sulfuric acid in 10 mL water). A saturated solution of sodium hydrogen carbonate was made by mixing 9.6 g of salt in 100 mL water. Phenylboronic acid (PBA) (1.0 g) was dissolved in 25 mL diethyl ether to make the derivatization solution.

The reference standards including 3-chloro-1, 2-propanediol-dipalmitate (PP3MCPD), 2-chloro-1,3-propanediol-dipalmitate (PP2MCPD), glycidyl palmitate (Gly-P) and internal standards 3-chloro-1,2-propanediol-dipalmitate-d5 (PP3MCPD-d5), 2-chloro-1,3-propanediol-dipalmitate (PP2MCPD-d5) and glycidyl palmitate (Gly-P-d5) were purchased from TRC (Canada). The mixed intermediate standards and internal standards solutions were prepared at 1 µg/mL and 5 µg/mL in toluene.

The calibration curve standard solutions from 5 to 1000 ng/mL with an internal standard of 50 ng/mL were made in ethyl acetate. After preparation, each standard solution was hydrolyzed and derivatized similarly to the test sample before being analyzed on GC-MS/MS system. This process was performed to account for the potential incomplete hydrolysis of esters in the samples.

Sample processing

The sample preparation process included four steps: fat extraction, clean-up, acid hydrolysis of the esters into free forms, and PBA derivatization, which was a modification of the AOAC Official Method 2018.03 (Dubois et al. 2019). The homogenized powder sample (0.5 g) or liquid sample (3.0 g) was weighed into a 50 mL polypropylene conical tube. After the addition of 50 µL of 1 µg/mL internal standard solution and 12 mL of water, the tube was mixed thoroughly to homogenize the sample. The fat was extracted two times, each with 12 mL of ethyl acetate for 15 min with the help of the sonication at 50 °C. The residual water was removed by using 10 g of anhydrous sodium sulfate. Ethyl acetate was removed using a nitrogen evaporator at 40 °C. The fat obtained was resuspended in 1 mL of *n*-hexane: ethyl acetate (85:15, v:v) and cleaned up by an NH_2 solid phase extraction column. The eluate was evaporated into dryness, and the cleaned extract was resuspended in 2 mL of tetrahydrofuran. An acidic aqueous solution of sodium bromide (30 µL) was added, and the mixture was incubated at 50 °C for 5 min. After stopping the reaction with sodium hydrogen carbonate solution, the esters were extracted into *n*-hexane (2 mL) and transferred to tetrahydrofuran. The bound forms were then hydrolyzed to free forms using a methanolic sulfuric acid solution at 40 °C for 16 h. The process was terminated with a saturated sodium hydrogen carbonate solution. After removing excess fat by *n*-hexane, free-form MCPD was obtained by triple extraction with ethyl acetate. The sample was derived by PBA solution (4%, w/v) and dried under a nitrogen evaporator at 40 °C. The analytes were dissolved in 1 mL *n*-hexane. After centrifugation at 6000 rpm for 5 min, the extract was transferred to a 1.8 mL vial to be analyzed by GC-MS/MS.

GC-MS/MS determination of 2-MCPD, 3-MCPD, and glycidyl esters

The GC-MS/MS instrument includes a GC 7890 gas chromatograph, an MS 7000B triple quadrupole mass spectrometry, a CTC autosampler, and Mass Hunter software, all from Agilent Technologies (USA).

A DB5MS column (30 m, id 0.25 mm, film thickness: 25 µm 5%-phenyl-methylpolysiloxane) from Phenomenex (USA) was used to separate the compounds. The carrier gas (helium gas 99.99%) rate was 1.0 ml/min. An SSL injector was used in spitless mode with an injector temperature 280 °C. The temperature gradient started at

60 °C for 1 min, then the temperature was increased to 150 °C at the rate of 6 °C/min and maintained at 150 °C for 2 min. The final ramp rate was 40 °C/min to 270 °C and held at 270 °C for 5 min. The injection volume was 1 µL.

The electron ionization source was used in positive mode. The temperature of the transfer line, EI ion source, and triple quadrupoles were controlled at 270 °C, 250 °C, and 150 °C, respectively. Two transitions were selected for each compound, and the collision energy was optimized using the instrument function. The more sensitive transition was used for quantification, while the other was used for confirmation. The MS/MS conditions are presented in Table 1.

Method validation

The specificity, linearity, limit of detection (LOD), the limit of quantification (LOQ), precision, and recovery were evaluated following the guidelines of AOAC International (AOAC International 2012) and AOAC SMPR 2017.017 (AOAC International 2017).

Food consumption survey

The subjects to be investigated are children under 36 months of age living in the provinces and cities where the research team collected infant formula samples. A total of 720 children were surveyed in this study, divided into four groups by age: those under six months, 6–12 months, 12–24 months, and 24–36 months. The average consumption amount of powder formula in kg/person per day was obtained using a 24-hour dietary recall survey. Mothers of the research subjects were interviewed directly by the research team to collect the formula consumption and the formula brands used. All the consumption values were recorded on a powder basis. The amount of ready-to-eat liquid formula was converted into powder formula using a conversion factor of 7.7 from the liquid to powder formula (EFSA Panel on Contaminants in the Food Chain (CONTAM) 2016). The children were weighed to collect the average weight for risk assessment purposes.

Table 1. MS/MS conditions for the studied compounds.

Compound	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (V)
3-MCPD	147	91*	24
	147	105	26
2-MCPD	196	104*	24
	196	91	26
3-MBPD	240	147*	20
	240	105	22
3-MCPD-d5	150	93*	22
	150	106	20
2-MCPD-d5	201	107*	18
	201	93	22
3-MBPD-d5	150	150*	20
	150	93	22

*Quantitative ion

Exposure assessment and risk characterization

The exposure dose (ED, µg/kg bw per day) of 3-MCPDE was calculated using the following formula.

$$ED = \frac{Contamination\ Level\ (\mu g/kg) \times Consumption\ (kg\ per\ day)}{Body\ weight\ (kg\ bw)}$$

In this study, the upper bound scenario was applied, which means the not detected results were replaced by LOD, and the results between LOD and LOQ were replaced by LOQ. The exposure dose of 3-MCPDE was compared with its PMTDI of 4 µg/kg bw to assess the health risk.

Data analysis

The consumption data of the formula were evaluated using SPSS 20.0. The contamination levels of 2-MCPDE, 3-MCPDE, and GE and the exposure dose of 3-MCPDE and GE were computed using Mass Hunter software and Microsoft Excel.

Results and discussion

Method validation data

Specificity

The specificity was evaluated by analyzing the blank sample, spiked samples (with PP2-MCPD, PP-3-MCPD, or Gly-P), and standard solutions at the concentration of 500 ng/mL. The chromatograms (Figure 1) showed that there were no signals at the retention time window of the analytes. The spiked samples gave signals at the retention time similar to those of the standard solutions, with differences in retention time of not more than 5%. The method, therefore, has

appropriate specificity, suitable for 2-MCPDE, 3-MCPDE, and GE analysis in formula matrices.

Linearity

The GC-MS/MS peak area ratio of quantitative ions of 2MCPDE, 3MCPDE, and GE to quantitative ions of internal standards and the concentrations of the analytes were plotted to obtain the calibration curves. In the concentration range from 5 to 1000 ng/mL in solution (the equivalent content on matrices from 10 to 2000 µg/kg), the peak area ratios corresponded to the concentration of the analyte with the coefficient of determination (R^2) higher than 0.999 for all analytes, which met the AOAC International requirements (AOAC International 2012; AOAC International 2017).

Limit of detection (LOD) and limit of quantification (LOQ)

The spiked samples at low concentrations that still give an analyte signal were analyzed six times. Automatic S/N ratios were determined according to the equipment software. The LOD is the level at which $S/N = 3$, and the LOQ is the level at which $S/N = 10$. The LOQ was then re-evaluated by analyzing the spiked samples at LOQ with six replicates to evaluate the repeatability and recovery. The obtained LOD and LOQ for all three analytes were 1.5 and 5 µg/kg, respectively. The LOQs for 2MCPDE, 3MCPDE, and GE were lower than the recommendation of AOAC SMPR 2017.017 (AOAC International 2017).

Precision and recovery

In this study, the blank samples were spiked with esters of analytes at three levels and analyzed six times for each level (Table 2). The recoveries for all analytes ranged from 86.4 to 108%, which fall within the recovery range of 70 and 125% recommended by AOAC SMPR 2017.017. The relative standard deviations (RSD) at three levels for all analytes were lower than the maximum value (22%) of AOAC International (AOAC International 2017), as shown in Table 2.

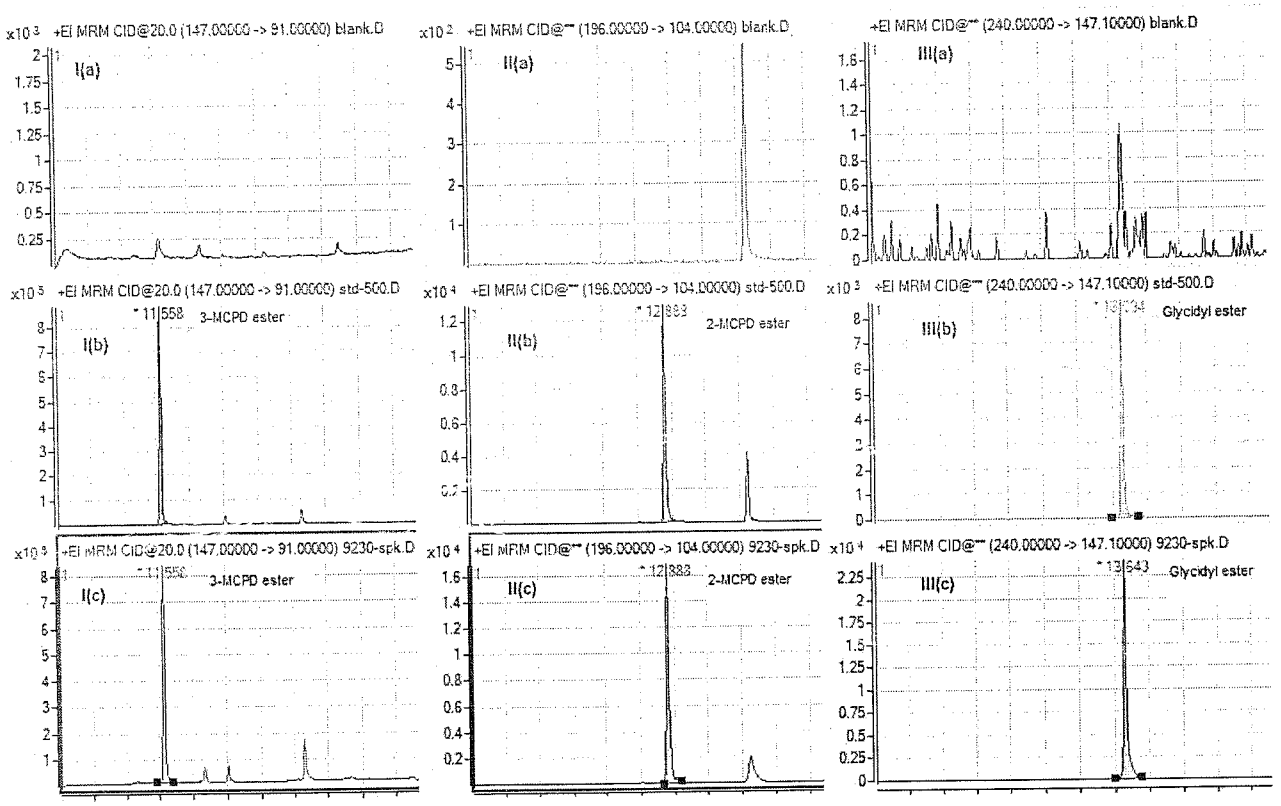


Figure 1. Chromatograms of 3-MCPDE (I), 2-MCPDE (II), and GE (III) in blank sample (a), standard solution (b), and spiked sample (c).

Level of 2-MCPDE, 3-MCPDE, and GE in formula samples

The levels of 2-MCPDE, 3-MCPDE, and GE calculated in the powder formulas are summarized in Table 3. 3-MCPDE was detected in all 54 formula samples with concentrations ranging from 7.3 to 536 µg/kg, with an average value of 124 µg/kg (± 132.0 µg/kg). Fourteen samples had a concentration exceeding the EU recommendation maximum level of 125 µg/kg (European Commission 2020). The level of 2-MCPDE was lower, with an average amount of 46.6 µg/kg (± 45.0 µg/kg) found in 47 samples. GE was detected in 13 of 54 samples, of which seven samples had a concentration lower than LOQ and six samples had an average content of 24.6 µg/kg (range from 5.6 to 105 µg/kg). Only one sample had a value exceeding the EU recommendation maximum level of 50 µg/kg (European Commission 2020).

The mean level of 3-MCPDE in the formula obtained is comparable to those reported in Brazil (mean 150 µg/kg, range from ND to 600 µg/kg; Arisseto et al. 2017) or in the United States (range from 35 to 630 µg/kg; Beekman et al. 2020). However, it is higher than some data published recently, such as the data reported for formula purchased in Denmark, where average levels of 2-MCPDEs, 3-MCPDEs, and GEs were 18.0, 49.8, and 11.5 µg/kg, respectively (Nguyen and Fromberg 2020). According to Beekman et al. (2021) the level of MCPDE and GE in the German formula sampled in 2019 has decreased almost two times compared to those in 2015. The percentage of samples with detectable MCPDE in our study was 100%, higher than the published results with similar LOD (Nguyen and Fromberg 2020; Beekman et al. 2021). The detection rate of GE, in contrast, was lower, at only 24% of samples, of which only six out of fifty-four samples had concentrations greater than the LOQ.

3-MCPDE and GE are present in the formula due to the addition of refined vegetable oil, highest in products with palm oil. In this study, unfortunately, the information on the details of fat source and other compositions of the formulas was not collected. Nevertheless, the status of 3-MCPDE and GE contamination in infant and follow-on formulas is fit for the risk assessment purpose and indicates that maximum limits of these compounds should be implemented in Vietnam.

Table 2. Recovery and repeatability results of 2MCPDE, 3MCPDE, GE ($n = 6$).

Analyte	Level µg/kg	Repeatability, RSD (%)	Recovery, R (%)
2-MCPDE	25	3.3	86.4–94.2
	125	3.1	93.9–102
	500	2.9	96.4–104
3-MCPDs	25	1.1	86.4–90.0
	125	2.7	93.5–101
	500	2.1	99.4–104
GE	15	4.8	98.1–108
	50	2.0	100.1–106
	150	3.4	93.2–103

Food consumption data

The consumption of formula by the four groups of children under 36 months of age is presented in Table 4. The results show that 85.1% of the children had used infant formula within three months before the surveying time. Children from 6 to 12 months old were the group with the highest percentage of using powdered formula, at 94.7%. Surprisingly, the rate of children using formula daily was lower in the group of children higher than 12 months of age. This could be explained that in some rural places in Vietnam, parents start to train their children to eat rice porridge from 12 months or even from 6 months of age. The average daily intake of powdered formula for all age groups was 60.3 grams. This value is lowest in infants under six months of age because they are also fed breast milk. For the older groups, the daily intakes ranged from 60 to 66 g, which is lower than the recommended

Table 3. The concentration of 2-MCPDE, 3-MCPDE, and GE in the formula samples (calculated on the powder basis).

Analyte	Number of samples	Samples with content > LOQ	Average (µg/kg)	Standard deviation (µg/kg)	Lowest detected content (µg/kg)	Highest detected content (µg/kg)	Upper bound value (µg/kg)
2-MCPDE	54	47	46.6	45.0	8.8	179	–
3-MCPDE	54	54	124	132	7.3	536	124
GE	54	13	24.6	27.0	5.6	105	7.6

Table 4. Daily infant formula consumption by age groups.

Children group	Body weight (kg)	Number of children	Children with daily use		Daily intake (g per day)[a]	
			n	%	Mean (g)	P95 (g)
Younger than 6 months	5.52	90	76	84.4	50.3	84.6
From 6 months to 12 months	8.82	189	179	94.7	65.9	112
From 12 months to 24 months	11.22	241	209	86.7	65.4	155
From 24 months to 36 months	14.43	200	149	74.5	59.6	121
Total (Up to 36 months)	10.86	720	613	85.1	60.3	155

[a]Values calculated on a powder basis.

Table 5. Exposure dose to 3-MCPDE and GE in the formula of different age groups.

Children group	3-MCPDE exposure dose (μg/kg bw/day)		GE exposure dose (μg/kg bw/day)	
	Mean	P95	Mean	P95
Younger than 6 months	1.13	1.90	0.069	0.116
From 6 months to 12 months	0.93	1.57	0.057	0.096
From 12 months to 24 months	0.72	1.72	0.044	0.105
From 24 months to 36 months	0.51	1.04	0.031	0.064
Up to 36 months	0.69	1.77	0.042	0.108

consumption of formula (National Health and Medical Research Council 2012). The mean and percentile 95% (P95) values reported in Table 5 were used in risk characterization.

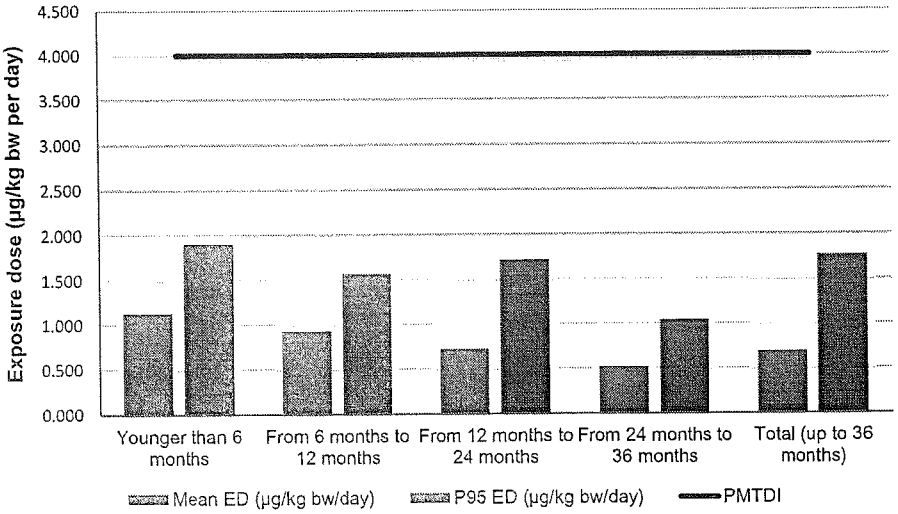
Exposure assessment and risk characterization

Table 5 shows the calculated exposure dose for both the mean and the 95% percentile. The mean daily intake of 3-MCPDE from the formula ranged from the lowest of 0.61 μg/kg bw per day in children from 24 to 36 months to the highest of 1.13 μg/kg bw per day in children younger than six months. The corresponding P95 values ranged from 1.04 to 1.90 μg/kg bw per day. All these values were lower than the PMTDI for 3-MCPDE at 4.0 μg/kg bw per day (JECFA 2016). From Figure 2, it is obvious that the exposure doses are far lower than the recommended level, even in the worse scenario.

Table 6 compares exposure doses of 3-MCPDE and GE with different groups of children. For children aged 0 to 6 months, the exposure dose of 3-MCPDE in this study was significantly lower, with an average value of only about 20–50% of the exposure published in the studies of Spungen et al. and Arisseto et al. in the US and Brazil (Arisseto et al. 2017; Spungen et al. 2018). However, the exposure dose of 3-MCPDE was slightly higher than that found in recent Danish and Chinese studies (Nguyen and Fromberg 2020; Li et al. 2022). For children of older age, the results of this study showed higher dose exposure values than studies conducted in China. Even so, these values are still much lower than the PMTDI established by JECFA (Figure 2). For GE, the mean exposures ranged from 0.31 to 0.69 μg/kg bw per day, with the highest values occurring in children aged 0 to 6 months. These values are significantly lower than those published in Brazil (Arisseto et al. 2017) and the US (Spungen et al. 2018) in 2017 but are close to some recently published results in Denmark (Nguyen and Fromberg 2020) and China (Li et al. 2022; Zhang et al. 2023).

According to the food consumption results, the 3-MCPDE level in infant formula that could result in an exposure dose higher than its PMTDI (4 mg/kg bw per day) for infants under 6 months of age is 439 μg/kg. As shown in Table S1, the 3-MCPDE levels in different brands were markedly different, ranging from low (brand B21) to very high (brand B10, B12). Among the 54 formula samples tested, 3 samples from 2 brands have 3-MCPDE levels higher than 439 μg/kg. Due to the fact that children are often given a specific brand of formula for a long time, this can pose a health risk to them.

The results obtained from this study express only the risk of 3-MCPDE and GE from formula for young children. The exposure doses of 3-MCPDE and GE for different groups of children under 36 months of age were compared with PMTDI obtained from the benchmark dose approach. The carcinogenic risk of these substances needs to be further assessed based on the



ED – Exposure dose; PMTDI – Provisional Maximum Tolerable Daily Intake

Figure 2. Exposure dose of 3-MCPDE from formula (µg/kg bw per day) for children of different age groups and the PMTDI.

Table 6. Comparison of the exposure dose of 3-MCPDE and GE from the formula in different age groups of children.

Countries	Age group (months)	3-MCPDE mean exposure dose (µg/kg bw/day)	GE mean exposure dose (µg/kg bw/day)	Reference, year
United States	0–6	1–14	1–3	Spungen et al. 2018
Brazil	0–5	2.49	3.65	Arisseto et al. 2017
	6–11	1.05	1.54	
Denmark	0–6	0.30–1.14	0.08–0.31	Nguyen and Fromberg 2020
China	0–6	2.34	0.37	Li et al. 2022
	6–12	0.26	0.12	
	12–24	0.24	0.08	
China	0–6	0.56	0.16	Zhang et al. 2023
	6–12	0.21	0.08	
	12–36	0.13	0.05	
	36–72	0.04	0.01	
Vietnam	0–6	1.13	0.07	This study
	6–12	0.93	0.06	
	12–24	0.72	0.04	
	24–36	0.51	0.03	

assessment of particular cancer in the population. Moreover, children older than 6 months will consume other kinds of food in addition to formulas. Although the formula is the main source of 3-MCPDE and GE contaminations in children, the addition from other food should also be evaluated in the future.

Conclusions

A method for the simultaneous determination of 2-MCPDE, 3-MCPDE, and GE in the infant and follow-on formulas based on AOAC Official Method 2018.03 was modified, validated, and applied for the determination of these substances in formula samples collected in Vietnam markets. Exposure to 3-MCPDE has not exceeded the PMTDI recommended by ESFA. Exposure levels of both 3-MCPDE and GE for children aged 0–6 months were similar to that of some recent studies but higher for other age groups.

Disclosure statement

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Chuyên mục

Bài nghiên cứu

Trích dẫn bài báo

Nguyễn, T. M. L., Nguyễn, V. Q., Trần, Đức H., Nguyễn, T. N. Q., & Nguyễn, H. N. (2023). Đánh giá ảnh hưởng của bài thuốc "Thái bình HV" lên các chỉ số sinh hóa và mô bệnh học của động vật thực nghiệm. *Tạp Chí Y Dược cổ truyền Việt Nam*, *42*(1), 9-15. https://doi.org/10.60117/vjmap.v42i1.96

Thêm định dạng trích dẫn

QR bài báo

Đánh giá ảnh hưởng của bài thuốc "Thái bình HV" lên các chỉ số sinh hóa và mô bệnh học của động vật thực nghiệm

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[3] Học viện Quân y

Tóm tắt

Mục tiêu: *Đánh giá ảnh hưởng của bài thuốc "Thái bình HV" lên các chỉ số sinh hóa máu đánh giá chức năng gan, thận và hình ảnh mô bệnh học gan, lách, thận.*

Phương pháp: *Chuột cống trắng được cho uống "Thái bình HV" liều 11,90g/kg/ngày và 35,70g/kg/ngày, hàng ngày trong 30 ngày. Xét nghiệm các chỉ số sinh hoá máu ALT, AST, billlirubin TP, albumin, cholesterol TP, creatinin tại 3 thời điểm: trước uống thuốc, sau 15 ngày và sau 30 ngày uống thuốc. Hình ảnh đại thể và vi thể gan, lách, thận chuột được đánh giá khi kết thúc thí nghiệm.*

Kết quả: *Không có sự khác biệt có ý nghĩa thống kê khi so sánh các chỉ số sinh hóa máu giữa các lô với nhau trong cùng thời điểm cũng như trong từng lô giữa các thời điểm đánh giá. Hình ảnh đại thể và mô bệnh học của gan, lách, thận bình thường.*

Kết luận: *Bài thuốc "Thái bình HV" không ảnh hưởng lên chức năng gan, thận và mô bệnh học gan, lách thận ở các mức liều dùng 11,90g/kg/ngày và 35,70g/kg/ngày và thời gian sử dụng 30 ngày trong nghiên cứu thực nghiệm trên chuột cống trắng.*

Từ khóa

"Thái bình HV", sinh hóa máu, hình ảnh mô bệnh học.

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Chuyên mục

Bài nghiên cứu

Trích dẫn bài báo

Trần, T. H. N., & Nguyễn, V. Q. (2023). Xác định một số yếu tố ảnh hưởng đến chi phí kinh doanh của nhà thuốc tại một số quận trên địa bàn Hà Nội năm 2021. *Tạp Chí Y Dược cổ truyền Việt Nam*, *49*(2), 72-80. https://doi.org/10.60117/vjmap.v49i2.31

Thêm định dạng trích dẫn

QR bài báo

Xác định một số yếu tố ảnh hưởng đến chi phí kinh doanh của nhà thuốc tại một số quận trên địa bàn Hà Nội năm 2021

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[1] *Học viện Y-Dược học cổ truyền Việt Nam*

Tóm tắt

Mục tiêu: Xác định rõ một số yếu tố ảnh hưởng đến chi phí kinh doanh tại nhà thuốc nhằm giúp các nhà thuốc thấy được các yếu tố tác động đến hoạt động kinh doanh của nhà thuốc.

Đối tượng và phương pháp nghiên cứu: Bằng phương pháp mô tả cắt ngang kết hợp nghiên cứu định lượng, sử dụng mô hình giải thiết với 31 biến trong 6 yếu tố ảnh hưởng, phân tích nhân tố EFA từ 209 nhà thuốc tại 4 quận Đống Đa, Hai Bà Trưng, Thanh Xuân và Hà Đông.

Kết quả nghiên cứu: Khảo sát 209 nhà thuốc thuộc 4 quận Đống Đa, Hai Bà Trưng, Thanh Xuân, Hà Đông thuộc thành phố Hà Nội cho thấy từ mô hình giả thiết đề xuất đưa ra mô hình giải thiết sau pilot, trên cơ sở đó xác định lại 6 yếu tố và 31 biến. Từ đánh giá độ tin cậy thang đo qua hệ số Cronbach α sẽ tiến hành phân tích yếu tố (EFA) kiểm định giá trị thang đo qua một số tiêu chuẩn: Kiểm định Bartlett: Sig ≤ 0,05. Kaiser - Meyer - Olkin (KMO): 0,5 < KMO < 1.

Kết luận: Đã xác định được 5 yếu tố với 26 biến quan sát ảnh hưởng trực tiếp tới chi phí kinh doanh của nhà thuốc bao gồm: Hoạt động nội bộ của chủ quan nhà thuốc, khả năng cạnh tranh của nhà thuốc đối thủ, quyền thương lượng của nhà cung cấp, áp dụng các văn bản quy phạm pháp luật và quản lý nhà nước về đảm bảo kỹ thuật.

Từ khóa

Chi phí, nhà thuốc, Hà Nội

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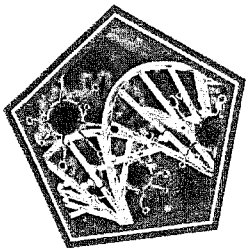

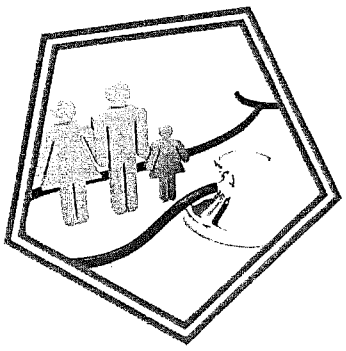

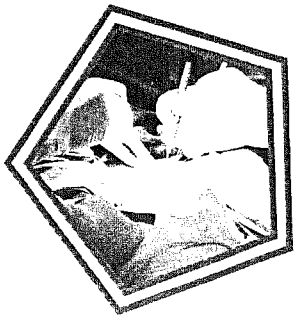

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GASTROPROTECTIVE EFFECT OF VIEN KHOI TIM CAPSULES ON INDOMETHACIN-INDUCED GASTRIC ULCERS IN RATS

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Available anti-ulcer drugs unveil partial effectiveness and numerous adverse reactions. Plants offer an alternative strategy in the search for new drugs in the therapy and prevention of peptic ulceration. The present study investigated the possible protective effect of the herbal formulation Vien Khoi Tim (VKT) on indomethacin-induced gastric mucosal damage in rats. VKT was tested at two doses (1.44 & 0.48 capsules/kg/d po) ten days before the indomethacin single-dose challenge (40 mg/kg po). Animals were sacrificed six hours after indomethacin administration, and gastric tissues were collected for gross observation and histopathological analyses. The results revealed that the administration of indomethacin caused evident gastric mucosal damage with morphological and histological manifestations. VKT pretreatment tended to avert the rise in lesion numbers, reduce the ulcer index, and improve the severity of bleeding streaks and epithelial sloughing in gastric mucosa on the macroscopic examination compared to the model group. It is worth noting that no ulcerative lesions were observed in the gastric tissues of rats receiving VKT upon microscopic examination. Our results indicated that Vien Khoi Tim capsules might possess a protective role against indomethacin-induced gastric ulcers. Additional research is needed to better understand the mechanism by which Vien Khoi Tim capsules exert their gastroprotective effect.

Keywords: Vien Khoi Tim, ulcer, indomethacin, rat.

I. INTRODUCTION

Peptic ulcer disease (PUD) is one of the most common diseases seen worldwide. PUD is defined as damage to the mucosa of the upper gastrointestinal tract due to acid-peptic digestion leading to the formation of an ulcer that extends beyond the muscularis mucosa into the submucosa.[1] *Helicobacter pylori* infection and the use of nonsteroidal anti-inflammatory drugs (NSAIDs) are the most important causes of PUD.[1] Thanks to their effectiveness in reducing pain and inflammation, NSAIDs are among the most commonly used drugs, confirming their place on the WHO Model List of Essential Medicines.[2] NSAIDs are considered to not only cause stomach damage, but through varied mechanisms, they slow down the healing process of ulcers.[3] NSAID use is responsible for about half of all ulcer perforations, occurring most commonly in older patients taking aspirin or other NSAIDs for cardiovascular or joint disease.[4] Therefore, preventing gastric ulcers caused by NSAIDs is extremely important for both medical professionals and researchers. Natural herbs and their phytoconstituents with potent antioxidant, anti-inflammatory, and antiapoptotic effects may offer good gastrointestinal protection.

Previously, indomethacin (IND) was more

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likely to cause stomach damage than common NSAIDs.[5] Hence, in this study, we aimed to mimic this condition using an indomethacin-induced gastric ulcer model to evaluate the possible protective effect of Vien Khoi Tim capsules against NSAID-associated peptic ulcers. Functional food Vien Khoi Tim (abbreviated as VKT) is a product derived from five medicinal herbs, including *Ardisia silvestris* Pitard, *Pseuderanthemum palatiferum* (leaves), *Lactuca indica*, *Curcuma longa*, and *Glycyrrhiza uralensis*, which is distributed by Bavieco Joint Stock Company. *Ardisia silvestris* Pitard, a precious medicinal herb widely used in folk medicine, is the main medicinal herb of VKT. According to traditional medicine, *Ardisia silvestris* Pitard contains the principal ingredients tannins, glucosides, saponins, alkaloids, fats, carotenes, flavonoids, which have anti-inflammatory effects, astringent ulcers, heal scars and reduce the increase in stomach acid.[6,7] Additionally, *Pseuderanthemum palatiferum* and *Curcuma longa* have also demonstrated anti-gastric ulcer activity in some studies.[8,9]

Considering the beneficial properties of the medicinal ingredients in VKT capsules, we tried to test the anti-ulcer effect of these polyherbal capsules on indomethacin-induced gastric ulcers in rats.

II. MATERIALS AND METHODS

Vien Khoi Tim capsules

Vien Khoi Tim is produced by CVI Pharmaceutical Joint Stock Company and distributed by Bavieco Joint Stock Company. Each hard capsule contains Bavieco Khoi Tim extract equivalent to 495 mg of crude herbal mixture extracted from 5 herbal ingredients including *Ardisia silvestris* Pitard (1400 mg), *Pseuderanthemum palatiferum* (leaves) (560 mg), *Lactuca indica* (300 mg), *Curcuma longa* (280 mg), and *Glycyrrhiza uralensis* (140 mg).

The predicted human dose of VKT was 2-3 capsules twice daily. VKT was suspended in distilled water and administered to the rats by oral gavage at dose levels of 1.44 capsules or 712.8 mg herbal extract (high dose) and 0.48 capsules or 237.6 mg herbal extract (low dose) per kg b.w/day (*based on the conversion from an equivalent dose of 4 capsules/day and 12 capsules/day for patients in the clinic, respectively).* Dose formulations were prepared fresh daily before administration. The intra-gastric gavage procedure was performed without anesthesia by gently holding the animal to immobilize the head and keep the animal in an upright position while inserting the gavage needle along the side of the mouth.

Chemicals and Reagents

Indomethacin (IND) (Indomethacin 25 mg tablets; Kwality Pharmaceutical, India); Misoprostol (MIS) (Misoprostol STELLA 200 mcg tablets, STELLAPHARM, Vietnam) were obtained.

Induction of gastric ulcer

Gastric ulcers were induced in animals according to the procedure described by Guzmán-Gómez O et al.[10] Briefly, ulcers were induced by a single oral dose of IND (40 mg/kg body weight). Animals were food deprived but had free access to water 18 hours before ulceration. Varying degrees of ulceration manifested six hours after indomethacin administration.

Animal grouping and treatment

The experiment used adult *Wistar* rats weighing 180-250 grams. The animals were acquainted with the laboratory for 7 days before the start of the experiment at normal room temperature (22°C) under appropriate conditions. The study was conducted from July to August 2023 at the Department of

Pharmacology, Hanoi Medical University. All animal procedures were performed under the recommendations for the proper care and use of laboratory animals.

Fifty-five rats were arbitrarily into five groups of eleven rats each. Group 1 (normal control) animals received only distilled water. Rats in Group 2 (ulcerated control) were given only IND. Animals in group 3 were administered IND after pretreatment with MIS (50 μg/kg b.w.). Groups 4 and 5 comprised ulcerated rats pretreated with VKT (1.44 capsules/kg b.w.), and VKT (0.48 capsules/kg b.w.) respectively. Treatments with the reference drug and test capsules lasted for 10 days prior to IND administration. These were orally administered once daily using gastric gavage with ad libitum provision of food and water throughout the experimental period. All rat groups (except for Group 1) were gavaged 40 mg/kg IND one hour after the last application of the test item.

Gastric lesion evaluation

Six hours after giving IND, the animals were sacrificed by cervical dislocation. The abdomens were opened, and the stomachs were excised from the esophagus (close to the cardia) to the small intestine (3 cm from the pylorus). The gastric pouches were opened along the greater curvature, rinsed with cold normal saline, blotted dry between filter papers, and stapled flat on a polystyrene foam board to examine gross lesions. Each gastric cavity was thoroughly examined under a 10× magnifying glass and the degree of ulceration was graded as follows:[10]

- 0, no lesions (normal stomach); 0.5, hyperemia (red coloration);
- 1, hemorrhagic spots;
- 2, 1–5 small ulcers;
- 3, many small ulcers;
- 4, many small and large ulcers;
- 5, stomach full of ulcers with perforations.

The ulcer index (UI) for each animal is the sum of the macroscopic score. The protective index (PI) was calculated as the following: (ulcer index of the ulcerated group − ulcer index of treated group × 100)/ulcer index of the ulcerated group.

For evaluation of histopathological changes, gastric tissues of 4 animals in each group were fixed in formalin 10%, sectioned into 4-6 μm slices, and stained with hematoxylin and eosin (H&E). Histological features included the following parameters: depth of tissue erosion, depth of ulcerative lesions, and presence of hemorrhage, inflammation, and apoptosis. Micro-assessment score evaluated according to the description of Simões S et al.[12] and modified is presented in Table 1. Representative histological images of each group were photographed digitally at 100x.

Table 1. Microscopic score evaluation

	Score 0	Score 1	Score 2	Score 3
Depth of the erosion	No erosion	Up to 1/3 of total mucosa depth	Up to 2/3 of total mucosa depth	Total mucosa
Depth of the ulceration	No ulceration	Limited to the muscularis mucosae	Beyond the muscularis mucosae, limited to the submucosa	Deep into the muscle layer

	Score 0	Score 1	Score 2	Score 3
Hemorrhage	No hemorrhage	Focal	Mild	Severe
Inflammation	No inflammation	Light	Mild	Severe
Apoptosis	No apoptosis	Light	Mild	Severe

Statistical analysis

The data were processed in Microsoft Excel and analyzed using IBM SPSS Statistics software. The results were expressed as the Mean ± Standard Deviation (SD) and presented in tables, graphs, and images. Statistical differences between the groups were determined using the Chi-square test and Mann-Whitney U test. A p-value less than 0.05 was regarded as significant.

III. RESULTS

1. Effects of VKT capsules pretreatment on macroscopic lesions

Table 2. Effects of VKT on the mean number of lesions

Group	n	Ulcer ratio	Lesion numbers (± SD)
Normal	11	0/11	0
Indomethacin	11	11/11	9.00 ± 2.41
Misoprostol + Indomethacin	11	10/11	6.55 ± 2.88
High dose VKT + Indomethacin	10	9/10	8.50 ± 3.21
Low dose VKT + Indomethacin	11	11/11	8.27 ± 2.05

Table 3. Effects of VKT on the ulcer index and protective index

Group	n	Ulcer index (± SD)	Protective index (%)
Indomethacin	11	4.50 ± 0.63	---
Misoprostol + Indomethacin	11	3.18 ± 1.27**	29.29
High dose VKT + Indomethacin	10	4.00 ± 1.43	11.11
Low dose VKT + Indomethacin	11	4.32 ± 0.84	4.04

***p < 0.01 as compared with ulcerated control (Mann-Whitney U test)*

The gastric tissues of control animals had normal gross morphology (Figure 1A). Animals administered IND showed prominent mucosal folds and severe erosions, while marked ulcers admixed with hemorrhages were observed in the gastric mucosa (Figure 1B), with the highest average lesion numbers coupled with the uppermost ulcer index (Table 2, 3). Macroscopic examination of the stomachs of the MIS (50 μg/kg) pretreated group revealed mild edema in the serosa, whereas mild erosions and bleeding in the gastric mucosa were observed. Based on macroscopic findings, the calculated ulcer index was significantly lower in the IND + MIS ($p < 0.01$) compared to the IND group (Table 3). These data verified the protective

effect of misoprostol on gastric ulceration. Macroscopically, the administration of VKT (1.44 and 0.48 capsules/kg) tenderly reduced the cruelty of visible lesions: milder edema in the serosa, slighter erosions in the mucosa, and bloody streaks less severe. (Figure 1D, 1E). Corresponding to gross observations, VKT at both doses tended to reduce the number of lesions, and calculated ulcer index compared to the ulcerated control, however, the difference was not statistically significant ($p>0.05$) (Table 2, 3).

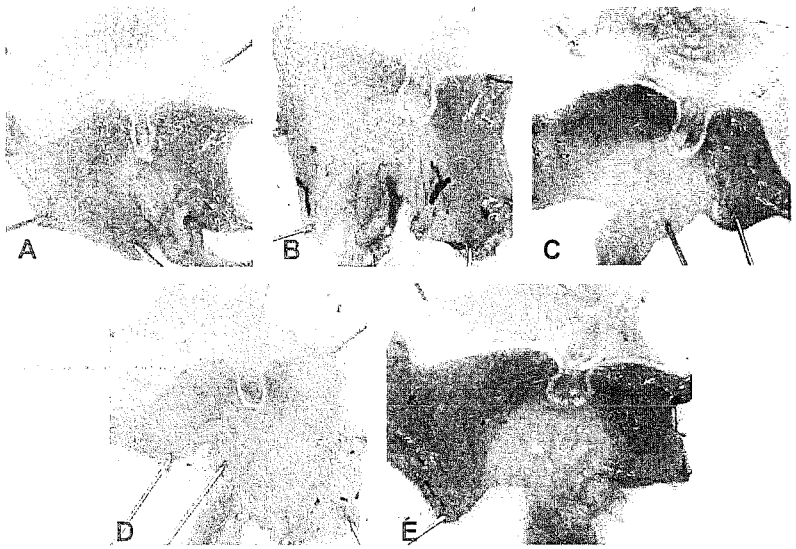

Figure 1. Gastric mucosa appearance in stomachs of the normal control group (A), indomethacin-induced ulcer group (B), misoprostol-treated group (C), high-dose VKT-treated group (D), and low-dose VKT-treated group (E).

2. Effects of VKT capsules pretreatment on microscopic lesions

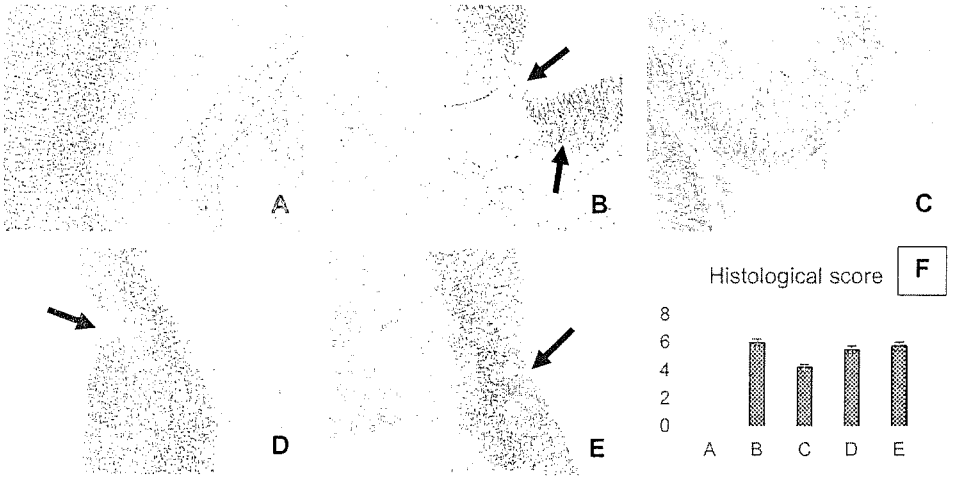

Figure 2. Histopathological changes of gastric tissue stained with hematoxylin and eosin at ×100 magnification. (A) Stomach from the normal control group. (B) Stomach from the IND-treated group. (C) Stomach from IND + MIS (50 μg/kg). (D) Stomach from IND + VKT (1.44 capsules/kg). (E) Stomach from IND + VKT (0.48 capsules/kg). (F) Histological score (n = 4). The data are presented as mean ± SD

Regarding histopathological examination as shown in Fig. 2A, the control group showed normal gastric histological architecture of the mucosa, submucosa, muscularis mucosa, and serosa. However, indomethacin-exposed rats showed reduced mucosal thickness with desquamation of the epithelial lining associated with focal ulceration and necrosis, as well as infiltrated with many inflammatory cells into stromal tissues (arrow), manifested quantitatively by the highest evaluation of histological score (Fig. 2B). Group pretreated with misoprostol (Fig. 2C) showed milder destruction of the lining epithelium of the mucosa with scattered erosions up to 1/3 of the thickness of the epithelium, reduced inflammatory cell infiltration, as quantified by lower histological score. Commendably, both doses of VKT resulted in some positive histological changes (Fig. 2D, E), with no observable ulceration and less severe epithelial sloughing (erosion up to 2/3 of the total mucosal depth), however, many inflammatory cell infiltrates in the mucosa and submucosa layer were still detected (arrow). Respectively, the micro-assessment scores in the rats' stomachs that received the test capsules tended to decrease, although these values presented no significant difference compared to the ulcerated control group.

III. DISCUSSIONS

Preventing or treating gastric ulcers is a major challenge for health authorities nowadays. With inherent side effects and the high cost of synthetic drugs, the exploitation of plant-based products that are considered non-toxic, effective, and affordable will be most suitable for preventing and treating stomach ulcers. This study was carried out to explore the possible gastroprotective effect of VKT hard capsules containing a mixture of five herbal extracts versus indomethacin-induced gastric ulcers in rats.

Many animal models have been used to induce gastric ulcers, in which the NSAID-induced gastric ulcer model is considered one of the most common gastric ulcer models.[12,13] NSAIDs are known to cause ulcers by inhibiting prostaglandin synthetase in the cyclooxygenase pathway. Prostaglandins are found in many tissues including the stomach, where they play a vital protective role through stimulating bicarbonate and mucus secretion, maintaining mucosal blood flow, and regulating regeneration and repair. Thus, inhibition of prostaglandin synthesis by NSAIDs leads to increased susceptibility to mucosal damage and subsequent gastric ulceration.[13] In the present study, indomethacin exposure resulted in severe morphological lesions with extremely hemorrhagic ulcerated mucosal layer, the highest gastric lesion numbers coupled with the uppermost ulcer index, as well as negative histopathological changes in the rat stomach with destructed lining epithelium of mucosal layer and excessive inflammatory cells infiltration in the mucosa and submucosal layers. As a synthetic analog of prostaglandin E1, misoprostol, in the role of a reference drug, has been shown to protect the animal stomachs exposed to indomethacin, as demonstrated by a reduction in the mean number of macroscopic gastric lesions, significantly scaling down the ulcer index with a protection index of 29.29% (Table 2, 3). Correlating with the degree of visible injuries, marked improvements in microscopic inspections were observed in the misoprostol-treated rat stomachs, quantified by lower histopathological scores, compared to the ulcerated group.

VKT pretreatment at doses (0.48 and 1.44 capsules/kg) showed limited injuries relative to the indomethacin-exposed group. Macroscopically, the administration of VKT at tested dosages tenderly reduced the gastric

lesion severity with milder edema in the serosa, slighter erosions in the mucosa, and bloody streaks less severe (Figure 1D, 1E). Microscopically, although the inflammation has not improved, less extreme epithelial sloughing and especially no ulcerative lesions were recognized in the VKT groups. Quantitively, both macro- and microscopic scores of VKT groups were lower than those of IND-treated animals, however, the statistical evaluation did not exhibit a significant in the treated groups in comparison to ulcerated control groups.

The above findings suggested the potential attenuation of gastric affronts of indomethacin by administration of VKT capsules at 0.48 and 1.44 capsules/kg b.w regimens. The exact mechanism of gastric protection of VKT has been not yet elucidated. Generally, the protection afforded by VKT capsules against indomethacin-induced gastric ulcers may be related to the beneficial pharmaceutical properties of the medicinal herbs contained in Bavieco Khoi Tim extract, mainly the role of *Ardisia silvestris* Pitard, *Pseuderanthemum palatiferum* (leaves), and *Curcuma longa*.

The anti-gastric ulcer activity of *Payawanorn* (*Pseuderanthemum palatiferum*) water extract (PPE) was evaluated using three gastric ulcer models:

(1) ethanol/hydrochloric acid (EtOH/HCl),

(2) restraint water immersion stress, and

(3) indomethacin. This study showed that the group treated with PPE reduced gastric volume but had no effect on gastric pH, total acidity, or rate of gastric acid secretion.

This suggests that the anti-gastric secretion effect is unlikely to be an anti-gastric ulcer effect of PPE. Meanwhile, PPE significantly promoted gastric mucus content in the EtOH/HCl model, which suggests that the main gastroprotective activity of PPE is related to gastric mucus protection.[8] The protective effect of the mucus barrier of PPE is consistent with the mechanism of causing ulcers due to reduced mucus secretion related to prostaglandins of indomethacin.

Constituents of *Curcuma longa* have also shown gastroprotective effects through stimulation of gastric wall mucus in a study conducted by Rafathullah S and coworkers. These authors reported that turmeric extract not only increased the gastric wall mucus significantly but also restored the non-protein sulfhydryl (NP-SH) content in the glandular stomachs of the rats.[14] It may thus be beneficial in protecting the gastric mucosa from irritants. Besides the effect of enhancing shielding factors, the findings from the study by Kim DC et al. suggested that *C. longa* extract specifically inhibits gastric acid secretion, an aggressive factor, by competitively blocking histamine H(2) receptors.[9]

Alongside its prostaglandin synthesis inhibition, there are studies suggesting that indomethacin causes gastric injury in rats by inducing the reactive oxygen species (ROS) level.[13] It has been reported that compounds with anti-inflammatory and antioxidant properties can prevent indomethacin-induced gastric mucosal damage in vivo models. *Ardisia silvestris* Pitard, the main medicinal herb of VKT, contains the principal ingredients tannins, glucosides, saponins, alkaloids, fats, carotenes, and flavonoids, which have anti-inflammatory effects, astringent ulcers, heal scars and reduce the increase in stomach acid.[6,7] These benefits may be related to the ability to scavenge free radicals and exert potent antioxidant properties confirmed using ABTS, DPPH, FRAP, and CUPRAC assays in previous studies.[6,15] Based on the IC_{50} values (46 µg/mL and 13 µg/mL) of *A. silvestris* ethanol extract (As-EE) for DPPH and ABTS, Huang L et al. assumed that this plant may have higher

antioxidant activity than other plants such as *Malus baccata*, *Canarium subulatum*, *Licania macrocarpa*, *Atriplex halimus* and *Euphorbia Resinifera*, with IC_{50} values ranging from 50 to 200 µg/mL. The rutin and quercetin contents in As-EE were calculated to be 0.53 and 0.03%, respectively, using the standard area curves of these compounds. These results proposed that the antioxidant properties of As-EE may be beneficial as the main pharmacological activities and that rutin and quercetin may be considered active ingredients in As-EE.[15] Not only *A. silvestris*, but the remaining herbal ingredients of the tested capsule also showed antioxidant activity in many studies.[17,18,19,20]

IV. CONCLUSIONS

From the above discussion, it can be suggested that treatment with Vien Khoi Tim hard capsules at 0.48 and 1.44 capsules/kg b.w regimens can improve the gastric damage caused by indomethacin in Wistar rats. This finding could lead to strategies for treating gastric ulcers using more novel therapies based on herbal-derived preparations. Additional research is needed to better understand the mechanism by which Vien Khoi Tim capsules exert their gastric protective effect.

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BÀI NGHIÊN CỨU

Nghiên cứu độc tính của dịch chiết húng quế (*Ocimum Basilicum* L.) đến điện tâm đồ ở thỏ thực nghiệm

STUDY ON TOXICOLOGY OF BASIL (*OCIMUM BASILICUM* L.) EXTRACT ON EXPERIMENTAL RABBITS' ELECTROCARDIOGRAMS

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TÓM TẮT

Mục tiêu: *Đánh giá ảnh hưởng của dịch chiết nước Húng quế (Ocimum basilicum L.) đến điện tim của thỏ thí nghiệm với liều đường uống liên tục 28 ngày.*

Đối tượng và Phương pháp: *Tiến hành theo hướng dẫn của Bộ Y tế và OECD về thử độc tính bán trường diễn. Dịch chiết nước húng quế được dùng bằng đường uống trên thỏ ở 2 mức liều 0,6 và 1,8 g/kg/ngày x 28 ngày liên tiếp. Thử nghiệm tiến hành song song với nhóm chứng. Ghi điện tim thỏ ở các ngày N0 (trước khi dùng thuốc), N14 và N29. Điện tim thỏ được ghi ở 12 đạo trình (3 đạo trình song cực chi, 3 đạo trình đơn cực chi, 6 đạo trình đơn cực ngực). Các chỉ tiêu đánh giá gồm: nhịp tim, sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT, và các dấu hiệu khác (nếu có).*

Kết quả: *Dịch chiết húng quế ở cả hai mức liều 0,6 và 1,8 g/kg/ngày x 28 ngày liên tiếp không làm thay đổi có ý nghĩa thống kê nhịp tim và các sóng điện tim (sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT) tại các thời điểm trước uống thuốc (ngày 0), ngày 14 và ngày 29 (sau đợt dùng thuốc). Các chỉ số trên giữa lô dùng thuốc và lô chứng cũng khác biệt không có ý nghĩa thống kê (các giá trị p > 0,05).*

Kết luận: *Như vậy, dịch chiết húng quế ở liều 0,6 và 1,8 g/kg/ngày x 28 ngày liên tiếp an toàn với tim thỏ thí nghiệm.*

Từ khóa: Húng quế, Ocimum basilicum L., dịch chiết nước, điện tâm đồ, nhịp tim, các sóng điện tim.

SUMMARY

Objective: *To assess the effects of aqua basil extract (Ocimum basilicum L.) on experimental rabbits' electrocardiograms with a continuous oral regimen of 28 days.*

Subject and Methods: *The Vietnam Ministry of Health's and OECD's guidelines for sub-chronic toxicity testing were applied. The aqua basil extract was treated orally in two different groups with dose regimens of 0.6 and 1.8 g/kg/day x 28 consecutive days, respectively. A control group treated orally with distilled water was also tested simultaneously.*

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The rabbit's electrocardiograms were recorded on days N0 (before taking samples), N14 and N29 (after finishing the treatment) in 12 leads (3 leads bipolar limb, 3 leads unipolar limb, 6 leads thoracic). Evaluation indicators include heart rate, P wave, PQ interval, QRS complex, T wave, QT interval, and other signs (if any).

***Results:** With the two dose regimens of 0,6 and 1,8 g per kg per day for 28 consecutive days, the aqua basil extract did not significantly change rabbits' heartbeats and cardiovascular waves (such as P, QP, QRS, T and QT) through study days (D0, D14 and D29). These indices did not differ significantly among the treated and control groups (the P values > 0.05).*

***Conclusion:** The aqua basil extract (at the two oral doses of 0,6 and 1,8 g/kg per day for 28 consecutive days) was safe in rabbits' cardiovascular systems.*

***Keywords:** Basil, Ocimum basilicum L., aqua basil extract, electrocardiograms, heartbeats, and cardiovascular waves.*

ĐẶT VẤN ĐỀ

Từ lâu, cây húng quế (*Ocimum basilicum* L.) đã nổi tiếng là cây thuốc quý và được sử dụng trong y học cổ truyền của nhiều nước như Ấn Độ, Iran, Ả rập, Hy Lạp, ... Toàn bộ phần trên mặt đất của cây húng quế đều được dùng làm thuốc, với mục đích chữa bệnh khác nhau. Lá húng quế được dùng làm thuốc bổ và thuốc trừ giun. Trà húng quế (dùng nóng) để trị buồn nôn, đầy hơi, chứng kiết lỵ. Tinh dầu húng quế rất có tác dụng làm giảm mệt mỏi về tinh thần, trị cảm lạnh, viêm mũi, chống co thắt, và là thuốc ưu tiên dùng để trị ong đốt và rắn cắn [1]. Húng quế rất hiệu quả trong điều trị bệnh tim, bệnh về máu, bệnh bạch bì... Nước ép húng quế làm giảm chứng đau. Dịch chiết húng quế dùng để trị chứng đau đầu, đau do bệnh gout, cải thiện chức năng đường tiêu hóa, nhuận tràng nhẹ và làm nước súc miệng để chữa hơi thở hôi. Ngoài ra còn có tác dụng giảm đau khi sinh đẻ [1]. Húng quế còn có tác dụng kháng khuẩn tốt [2],[3]. Ở Việt Nam, húng quế từ lâu đã được dùng chủ yếu làm gia vị cho các món ăn (lá và ngọn) hoặc để uống cho mát (hạt é).

Với nhiều tác dụng như đã kể trên, một số nghiên cứu trên thế giới và trong nước đã tiến hành nhằm phát triển thuốc từ dược liệu này. Các nghiên cứu ngoài nước cho thấy, húng quế được dung nạp tốt, hầu như rất ít tác dụng không mong muốn khi sử dụng liên tục tới 13 tuần. Tuy nhiên, sử dụng quá liều húng quế hoặc lạm dụng trong thời gian dài có thể gây khó thở, thở gấp, ho hoặc tiểu ra máu; hạ đường huyết, làm loãng máu hoặc chậm quá trình đông máu; ung thư niêm mạc gan; có thể làm co thắt cổ tử cung với phụ nữ mang thai, gây biến chứng trong khi sinh [1]. Chưa có công trình nghiên cứu nào công bố về ảnh hưởng của dịch chiết húng quế đến điện tim. Đặc biệt, các nghiên cứu về tính an toàn của húng quế ở Việt Nam hầu như chưa có.

Vì vậy, để phát triển thuốc có nguồn gốc từ dược liệu này, nghiên cứu được tiến hành nhằm đánh giá ảnh hưởng của dịch chiết húng quế tới điện tim của thỏ thí nghiệm với liều bán trường diễn 28 ngày.

VẬT LIỆU VÀ PHƯƠNG PHÁP

Thời gian và địa điểm nghiên cứu

Nghiên cứu được tiến hành từ tháng 5 - 7 năm 2021, tại Viện Sốt rét - Ký sinh trùng - Côn trùng Trung ương.

Đối tượng và vật liệu nghiên cứu

Mẫu nghiên cứu

Phần trên mặt đất của cây húng quế còn tươi được thu hái từ tháng 5-6/2021 tại Yên Xá, Tân

Triều, Thanh Trì, Hà Nội. Mẫu sau thu hái được rửa sạch, thái nhỏ và được chiết nóng với nước như sau. Lấy 1 kg húng quế, thêm 2 lít nước, đun nhỏ lửa trong 30 phút. Sau đó lọc lấy dịch chiết. Thêm tiếp 1 lít nước vào và đun nhỏ lửa tiếp trong 30 phút nữa. Sau đó lọc lấy dịch chiết lần 2. Gộp dịch chiết lần 1 và lần 2 lại, cô cách thủy cho tới khi được dịch chiết tỷ lệ 1: 1 (100 g/100 ml). Từ dịch chiết này, cô cách thủy tiếp để được các dịch chiết với các nồng độ khác nhau dùng trong thử nghiệm.

Động vật dùng trong nghiên cứu

Thỏ trưởng thành (*Oryctolagus cuniculus* L.), tổng số 36 con, cân nặng trung bình 2,1± 0,2 kg, 2 tháng tuổi, khỏe mạnh, không phân biệt đực - cái, do Trung tâm nghiên cứu Dê và Thỏ Sơn Tây cung cấp. Với động vật cái phải không mang thai, không nuôi con bú và chưa sinh sản lần nào. Động vật được nuôi ổn định 7 ngày trong điều kiện thí nghiệm trước khi tiến hành nghiên cứu.

Thiết bị và dụng cụ dùng trong nghiên cứu

- Máy cất nước hai lần Aquatron (hãng Bibby sterilin, Anh).
- Cân Sauter, độ chính xác d = 0,1 mg.
- Máy điện tim ECG - 2150 Nihon Kohden (Nhật).
- Kim đầu tù cho động vật uống.
- Cốc thủy tinh có chia vạch, bơm kim tiêm 5 ml.

Phương pháp tiến hành

Tiến hành theo hướng dẫn của Bộ Y tế và OECD [4],[5]. Sau khi nuôi ổn định 1 tuần, thỏ được đánh dấu, cân trọng lượng và chia ngẫu nhiên vào 3 lô:

- Lô 1 (n = 11): chứng, uống nước cất 2 lần, thể tích tương đương liều điều trị thuốc x 28 ngày liên tiếp.
- Lô 2 (n = 11): uống dịch chiết nước húng quế liều 0,6 g/kg/ngày, (tương đương liều dùng dự kiến trên người), × 28 ngày liên tiếp.
- Lô 3 (n = 12): uống dịch chiết nước húng quế liều 1,8 g/kg/ngày, (tương đương gấp 3 lần liều dùng dự kiến trên người), × 28 ngày liên tiếp.

Trước uống thuốc, thỏ được ghi điện tim vào ngày N0. Cho thỏ uống thuốc mỗi ngày một lần vào buổi sáng bằng sonde dạ dày, liên tục trong 28 ngày. Các ngày ghi điện tim tiếp theo là N14 (sau khi uống thuốc 2 giờ) và N29.

**** Phương pháp ghi điện tim thỏ:***

Các thỏ lần lượt được buộc cố định trên bàn gỗ theo tư thế nằm ngửa, 4 chi được giữ cố định ở 4 góc bàn. Cắm các điện cực vào chi và quanh ngực thỏ theo quy định. Để thỏ nằm yên trong 2 phút. Nhấn nút máy ghi điện tim. Điện tim thỏ được ghi ở 12 đạo trình (3 đạo trình song cực chi, 3 đạo trình đơn cực chi, 6 đạo trình đơn cực ngực). Ghi điện tim khi thỏ nằm yên, so sánh sự biến đổi các sóng điện tim của thỏ trước và sau khi dùng thuốc, và giữa các lô dùng thuốc so với lô chứng. [6]

Chỉ tiêu đánh giá

Các chỉ tiêu đánh giá điện tim thỏ (Electrocardiogram ECG) gồm nhịp tim (lần/phút), sóng P [biên độ (mv), thời khoảng (giây)], khoảng PQ (giây), phức bộ QRS [biên độ (mv), thời khoảng (giây)], sóng T [biên độ (mv), thời khoảng (giây)], khoảng QT (giây), các dấu hiệu khác (nếu có).

Xử lý số liệu

Số liệu được biểu thị bằng trị số trung bình ± độ lệch chuẩn (M ± SD). Các số liệu được xử lý bằng chương trình Excel 2016 theo phương pháp thống kê y học với cỡ mẫu nhỏ (< 30), sử dụng t-test Student và Fisher's exact test để so sánh các số liệu trước, trong và sau thử nghiệm và so sánh giữa lô dùng thuốc và lô chứng.

KẾT QUẢ NGHIÊN CỨU

Ảnh hưởng của dịch chiết húng quế đến điện tim thỏ được đánh giá thông qua xác định nhịp tim và các sóng điện tim của thỏ ở lô chứng và các lô uống dịch chiết húng quế, và được thể hiện ở các bảng sau.

Bảng 1. Ảnh hưởng của dịch chiết húng quế đến nhịp tim thỏ (n = 11)

Lô	Nhịp tim (lần/phút) tại các thời điểm			p (N0-N14)	p (N0-N29)
	N0	N14	N29		
Lô 1: chứng (n = 11)	225,8 ± 35,5	220,0 ± 25,5	235,9 ± 36,9	> 0,05	> 0,05
Lô 2: uống dịch chiết húng quế, 0,6 g/kg/ngày × 28 ngày (n = 11)	220,3 ± 26,5	234,6 ± 27,4	231,8 ± 32,7	> 0,05	> 0,05
Lô 3: uống dịch chiết húng quế, 1,8 g/kg/ngày × 28 ngày (n = 11)	210,0 ± 29,2	213,6 ± 34,1	210,6 ± 30,5	> 0,05	> 0,05
p (1-2), p (1-3), p (2-3)	> 0,05	> 0,05	> 0,05		

Tại các ngày N14 và N29, nhịp tim thỏ ở các lô uống dịch chiết húng quế liều 0,6 và 1,8 g/kg thay đổi không có ý nghĩa thống kê so với thời điểm ban đầu (N0), cũng như so với lô chứng tại các thời điểm tương ứng (các giá trị p > 0,05).

Bảng 2. Các chỉ số sóng điện tim của thỏ ở lô chứng (n = 10)

Sóng điện tim		Thời điểm nghiên cứu (ngày)			p (N0-N14)	p (N0-29)
		N0	N14	N29		
Sóng P	Mv	0,082 ± 0,03	0,065 ± 0,03	0,074 ± 0,04	> 0,05	> 0,05
	Giây	0,036 ± 0,008	0,031 ± 0,01	0,029 ± 0,01	> 0,05	> 0,05
Khoảng PQ (giây)		0,058 ± 0,014	0,062 ± 0,017	0,058 ± 0,017	> 0,05	> 0,05
Phức bộ QRS	mv	0,382 ± 0,13	0,373 ± 0,135	0,355 ± 0,10	> 0,05	> 0,05
	giây	0,029 ± 0,01	0,031 ± 0,01	0,031 ± 0,01	> 0,05	> 0,05
Sóng T	mv	0,186 ± 0,06	0,223 ± 0,06	0,191 ± 0,05	> 0,05	> 0,05
	giây	0,06 ± 0,013	0,064 ± 0,02	0,062 ± 0,01	> 0,05	> 0,05
Khoảng QT (giây)		0,135 ± 0,02	0,136 ± 0,01	0,138 ± 0,01	> 0,05	> 0,05

Ở ngày N14 và N29, các chỉ số sóng điện tim thỏ (sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT) ở lô chứng thay đổi không có ý nghĩa thống kê so với N0 (trước uống thuốc), các giá trị $p > 0,05$.

Bảng 3. Các chỉ số sóng điện tim thỏ ở lô uống dịch chiết húng quế liều 0,6 g/kg/ngày × 28 ngày liên tiếp (n = 11)

Sóng điện tim		Thời điểm nghiên cứu (ngày)			p (N0-N14)	p (N0-29)
		N0	N14	N29		
Sóng P	mv	0,091 ± 0,02	0,086 ± 0,02	0,086 ± 0,01	> 0,05	> 0,05
	giây	0,036 ± 0,012	0,029 ± 0,01	0,035 ± 0,009	> 0,05	> 0,05
Khoảng PQ (giây)		0,064 ± 0,012	0,051 ± 0,014	0,058 ± 0,014	> 0,05	> 0,05

Phức bộ QRS	mv	0,473 ± 0,006	0,418 ± 0,133	0,418 ± 0,08	> 0,05	> 0,05
	giây	0,035 ± 0,01	0,029 ± 0,01	0,027 ± 0,01	> 0,05	> 0,05
Sóng T	mv	0,205 ± 0,06	0,191 ± 0,06	0,223 ± 0,06	> 0,05	> 0,05
	giây	0,067 ± 0,01	0,064 ± 0,02	0,064 ± 0,01	> 0,05	> 0,05
Khoảng QT (giây)		0,142 ± 0,01	0,136 ± 0,01	0,133 ± 0,01	> 0,05	> 0,05

Bảng 3 cho thấy, ở N14 và N29, các chỉ số sóng điện tim thỏ (sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT) ở lô uống dịch chiết húng quế liều 0,6 g/kg/ngày × 28 ngày liên tiếp thay đổi không có ý nghĩa thống kê so với N0 (các giá trị p > 0,05).

Bảng 4. Các chỉ số sóng điện tim thỏ ở lô uống dịch chiết húng quế liều 1,8 g/kg/ngày × 28 ngày liên tiếp (n = 11)

Sóng điện tim		Thời điểm nghiên cứu (ngày)			p (N0-N14)	p (N0-29)
		N0	N14	N29		
Sóng P	mv	0,100 ± 0,04	0,086 ± 0,02	0,091 ± 0,02	> 0,05	> 0,05
	giây	0,038 ± 0,011	0,035 ± 0,009	0,027 ± 0,01	> 0,05	> 0,05
Khoảng PQ (giây)		0,064 ± 0,015	0,069 ± 0,014	0,067 ± 0,01	> 0,05	> 0,05
Phức bộ QRS	mv	0,445 ± 0,100	0,373 ± 0,047	0,355 ± 0,12	> 0,05	> 0,05
	giây	0,031 ± 0,01	0,035 ± 0,01	0,029 ± 0,01	> 0,05	> 0,05
Sóng T	mv	0,218 ± 0,06	0,182 ± 0,05	0,173 ± 0,05	> 0,05	> 0,05
	giây	0,067 ± 0,01	0,067 ± 0,01	0,060 ± 0,02	> 0,05	> 0,05
Khoảng QT (giây)		0,145 ± 0,02	0,149 ± 0,02	0,140 ± 0,02	> 0,05	> 0,05

Các chỉ số sóng điện tim thỏ (sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT) ở ngày N14 và N29 của lô uống dịch chiết húng quế liều 1,8 g/kg/ngày x 28 ngày thay đổi không có ý nghĩa thống kê so với N0 (trước uống thuốc), $p > 0,05$.

Các chỉ số sóng điện tim thỏ ở lô chứng và các lô uống dịch chiết húng quế tại cùng thời điểm N0, N14 và N29 khác biệt không có ý nghĩa thống kê (các giá trị $p > 0,05$).

BÀN LUẬN

Việc nghiên cứu ảnh hưởng của thuốc đến chức năng tim mạch của động vật là một trong số các nghiên cứu thường được tiến hành, đặc biệt với những thuốc dự định dùng dài ngày trên người. Các nghiên cứu ảnh hưởng của thuốc đến tim mạch của động vật gồm ảnh hưởng của thuốc đến sự thay đổi huyết áp, đến sự co hay giãn mạch, đến nhịp tim và các sóng điện tim. Trong nghiên cứu này, ảnh hưởng của dịch chiết húng quế đến điện tim thỏ được đánh giá thông qua sự thay đổi của nhịp tim và các sóng điện tim của thỏ ở lô chứng và các lô uống dịch chiết húng quế tại các thời điểm nghiên cứu (ngày 0 - trước khi dùng thuốc, ngày 14 - giữa đợt dùng thuốc và ngày 29 - sau khi kết thúc dùng mẫu thử).

Kết quả cho thấy, trước khi dùng thuốc (N0), nhịp tim và các sóng điện tim ở lô chứng và các lô dùng thuốc khác nhau không có ý nghĩa thống kê (p > 0,05). Điều này cho thấy, các thỏ được lựa chọn vào nghiên cứu có chỉ số sinh học tương đối đồng đều giữa các lô.

Sau khi cho thỏ uống dịch chiết húng quế với liều 0,6 g/kg/ngày × 28 ngày (tương đương liều dùng điều trị ở người) và 1,8 g/kg/ngày × 28 ngày liên tiếp, dịch chiết húng quế không làm thay đổi có ý nghĩa thống kê các chỉ số nghiên cứu ở ngày N14 và N29 so với thời điểm ban đầu (N0), cũng như giữa các lô uống thuốc so với lô chứng (các giá trị p > 0,05). Các chỉ số như: nhịp tim, các sóng điện tim (sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT) ở ngày N14 và N28 thay đổi không có ý nghĩa thống kê so với N0 và so với lô chứng tại cùng thời điểm nghiên cứu (p > 0,05). Điều này chứng tỏ, dịch chiết húng quế ở liều tương đương liều dùng điều trị trên người và gấp 3 liều dùng trên người an toàn với tim mạch thỏ, không gây ảnh hưởng có ý nghĩa thống kê đến điện tim của thỏ thực nghiệm.

Cho đến nay, rất ít nghiên cứu về ảnh hưởng của dịch chiết húng quế đến chức năng tim mạch của động vật hay người. Tuy nhiên, một số nghiên cứu ở châu Phi đã cho thấy húng quế có tác dụng cân bằng hormone cortison, cải thiện chức năng của não, tim, gan và có tác dụng chống đông máu [1],[7]. Điều này cũng phù hợp với kết quả nghiên cứu trên của chúng tôi.

Như vậy, các kết quả nghiên cứu ban đầu cho thấy, ở liều 0,6 và 1,8 g/kg/ngày × 28 ngày liên tiếp, dịch chiết nước húng quế không ảnh hưởng đến chức năng hoạt động của tim thỏ thí nghiệm.

KẾT LUẬN

Đã nghiên cứu ảnh hưởng của dịch chiết nước húng quế đến điện tim của thỏ thí nghiệm với liều đường uống 0,6 và 1,8 g/kg/ngày × 28 ngày liên tiếp. Kết quả cho thấy:

Các chỉ số như nhịp tim, sóng P, khoảng PQ, phức bộ QRS, sóng T, khoảng QT của thỏ ở các lô dùng thuốc khác biệt không có ý nghĩa thống kê so với lô chứng tại các thời điểm N0, N14 và N29; không có sự khác biệt có ý nghĩa ở các ngày N14 và N29 so với N0 (các giá trị $p > 0,05$). Dịch chiết nước húng quế không ảnh hưởng đến chức năng hoạt động của tim thỏ thí nghiệm.

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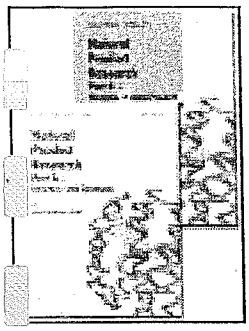

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Bioactive compounds from the aerial parts of *Hypericum sampsonii*

Dung Nguyen Viet, Vinh Le Ba, Thuan Nguyen Duy, Van Anh Pham Thi, Han Tran Thi, Viet Cuong Le Canh, Giang Bach Long, Young Ho Kim & Hoang Le Tuan Anh

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SHORT COMMUNICATION

Bioactive compounds from the aerial parts of *Hypericum sampsonii*

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ABSTRACT

Hypericum sampsonii is an important medicinal plant used in Vietnam to treat many diseases such as backache, burns, diarrhea, and swelling. In order to study the chemical constituents in the aerial parts of *H. sampsonii*, five compounds, including two xanthones (**1**–**2**), and three benzophenones (**3**–**5**) were isolated from the aerial parts of the *H. sampsonii* with various chromatographic separations. Their chemical structures were established on the basis of spectroscopic data such as 1D- and 2D-NMR, HR-ESI-MS. Their anti-inflammatory activities were investigated by measuring nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 cells. Moreover, the DPPH radical scavenging was evaluated. As the obtained results, compound **5** showed the strongest inhibitory activity against LPS-stimulated NO production in RAW264.7 cells with IC_{50} value ranging from $2.00 \pm 0.34\,\mu M$.

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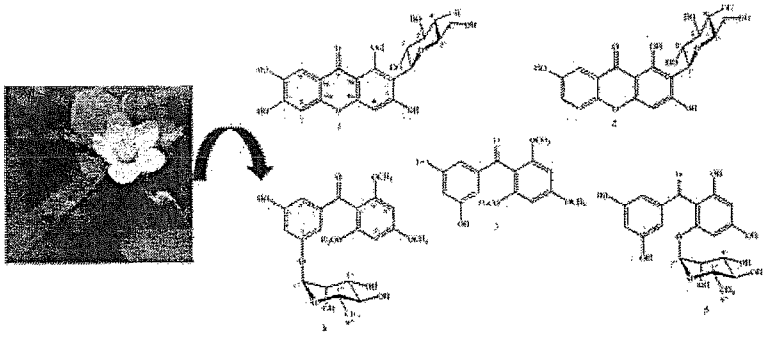

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1. Introduction

Hypericum sampsonii Hance (Hypericaceae) is a traditional Vietnamese medicine that the leaves is used to treat many diseases such as backache, burns diarrhea, and swelling (Don et al. 2004). The extract of plant has been found to have various pharmacological effects such as anti-inflammatory, antioxidant, and cytotoxic activities (Xin et al. 2012). The major chemical constituents of *H. sampsonii* are xanthones, anthraquinones, benzophenones, polyprenilated phloroglucinols, phenolics, and flavonoids (Caprioli et al. 2016; Ferretti et al. 2005; Hong et al. 2004; Nabavi et al. 2018; Zhu et al. 2015; Zorzetto et al. 2015). Especially, hyperisampsins A, and D which were isolated from the methanol extract *H. sampsonii* showed highly potent anti-HIV activities (Zhu et al. 2014). To increase our knowledge of the chemical constituents and biological activities of Vietnamese medicinal plants, we report herein five compounds (**1–5**) from the methanol extract of *H. sampsonii*, as well as evaluation of their antioxidant and anti-inflammatory activities.

2. Results and discussion

Fractionation and purification of the ethyl acetate (EtOAc) fraction from *H. sampsonii* led to isolation of two xanthones mangiferin (**1**), neolancerin (**2**), and three benzophenones (**3 – 5**), namely sampsine A (**3**), sampsine B (**4**), and petiolin F (**5**) (Figure 1). These compounds were determined by means of the spectroscopic methods (1 D, 2D-NMR, and HR ESI-MS) and comparison with reported values. To the best of our knowledge, compound **5** was the first time isolated from *H. sampsonii*. This is the first study of the chemical constituents and biological activities of this species from Vietnamese.

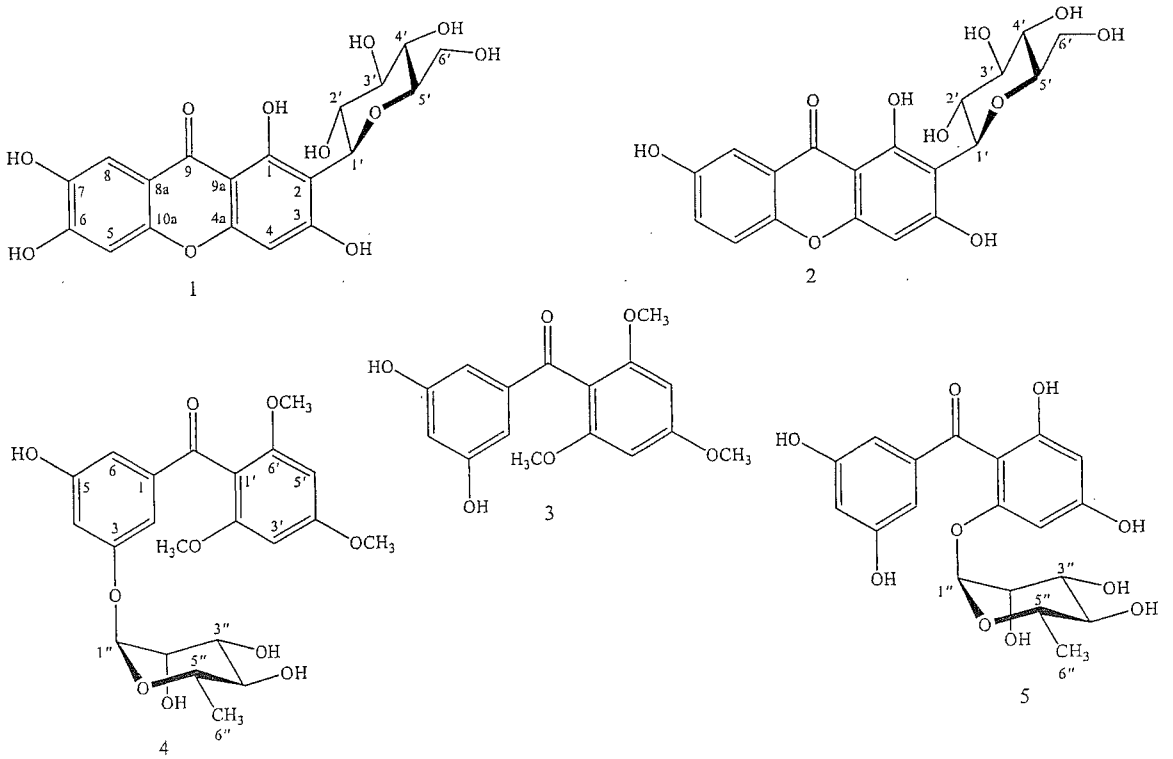

Figure 1. The structures of compounds isolated from *H. sampsonii*.

All of isolated compounds were evaluated for their DPPH radical-scavenging ability by following the previously described protocol (Vinh et al. 2008). Among these, compound **1** exhibited the strongest antioxidant effect with an IC_{50} value of $35.48 \pm 1.23\,\mu M$. Moreover, the inhibitory effects of all isolated compounds on LPS-induced NO production in RAW264.7 cells were investigated. As the shown in the results, compounds **3**, **4**, and **5** demonstrated significantly NO inhibitory activity against NO production inhibitory activity with an IC_{50} values $2.40 \pm 0.69\,\mu M$, $2.29 \pm 0.12\,\mu M$, and $2.00 \pm 0.34\,\mu M$, respectively and relative to the positive control, cadamonin (IC_{50} values $1.41 \pm 0.03\,\mu M$). The results indicate that benzophenones constituents have greater anti-inflammatory activity than does xanthones constituents. Due to the potential antioxidant effect and anti-inflammatory of **1**, **3**, **4**, and **5**, these compounds could be useful potential agents for treatments of inflammation-related diseases such as allergy, arthritis, dermatitis, cancer, and the others diseases.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Medical Science

Pain relief effect of TT knee remedy on knee osteoporosis

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ABSTRACT

Objective: Evaluate the analgesic effect of TT knee remedy on patients with knee osteoarthritis. Method: prospective clinical trial study, comparing before and after controlled study. Results: TT knee remedy has pain relief effect on patients with knee osteoarthritis, this effect appears after seven days (VAS score decreases by 30.19%) and gradually lasts to 28 days (decreases by

(decreases by 76.34%). The analgesic effect of "TT knee" in patients with knee osteodegeneraion was better than in the control group using glucosamine 1500mg ($p < 0.001$).

Funding: This study did not receive any funding.

Conflict of Interest: The authors declare that they have no conflict of interest.

Informed consent: Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this manuscript.

Ethical approval for study protocol: The study was approved by the Medical Ethics Committee of Tue Tinh Hospital (ethical approval code: 09/TTH).

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Medical Science

Evaluate the effects of the Duong Cot HV remedy combined with electro-acupuncture on patients with low back pain due to spinal degeneration

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ABSTRACT

Objective: Evaluate the effects of the Duong Cot HV remedy combined with electro acupuncture on patients with low back pain due to spinal degeneration. *Method*: Randomized control trial study. *Results*: Taking Duong Cot HV combined with electric acupuncture is

be maintained for how long? "Duong cot HV" remedy works when used independently? Continue to extend the duration of treatment; the results will be better or not? We need more in-depth studies to confirm.

5. CONCLUSION

Through the research results, we come to the following conclusion:"Duong cot HV" combined with electro-acupuncture work to improve the symptoms of patients with low back pain due to spinal degeneration: reduce pain sensation according to VAS, increase spinal motor skills. This effect tends to be better than the control group using electro-acupuncture in combination with "San bi tang"; however, the difference is not statistically significant after 20 days of treatment.

Funding: This study did not receive any funding.

Conflict of Interest: The authors declare that they have no conflict of interest.

Informed consent: Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this manuscript.

Ethical approval: The study was approved by the Medical Ethics Committee of Traditional Hospital (ethical approval code: 16/TH).

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Nghiên cứu độc tính bán trường diễn của cao mềm tỏi đen Hà Giang trên thực nghiệm

STUDY THE SUB-CHRONIC TOXICITY OF HA GIANG ALLIUM SATIVUM EXTRACT ON EXPERIMENT

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TÓM TẮT

Nghiên cứu được tiến hành nhằm đánh giá độc tính bán trường diễn của cao mềm Tỏi đen Hà Giang theo đường uống trên động vật thực nghiệm. Nghiên cứu độc tính bán trường diễn được tiến hành theo hướng dẫn của WHO, chuột cống được uống liên tục cao mềm Tỏi đen với mức liều 0,4 g/kg/ngày và 2,0 g/kg/ngày trong vòng 4 tuần liên tục. Kết quả nghiên cứu cho thấy cao mềm Tỏi đen khi dùng đường uống liều 0,4 g/kg/ngày và 2,0 g/kg/ngày liên tục trong 4 tuần không ảnh hưởng đến tình trạng chung, thể trọng, các chỉ số huyết học, chức năng gan, thận và mô bệnh học gan, thận trên chuột cống trắng. Như vậy, cao mềm Tỏi đen không gây độc tính bán trường diễn trên chuột cống thực nghiệm.

Từ khóa: *Tỏi đen, bán trường diễn, chuột cống.*

SUMMARY

Objectives: *The research evaluated the sub-chronic toxicity of Ha Giang black garlic (Allium Sativum) extract through oral administration in animal experiment. The sub-chronic toxicity study was conducted by the recommendation of WHO in rats with oral doses of 0.4 g/kg/day and 2.0 g/kg/day in 4 consecutive weeks. As a result, oral administration of black garlic extract at the doses of 0.4 g/kg/day and 2.0 g/kg/day had no effect on general condition, body weight, hematological parameters, hepato-renal functions and histopathological structures of liver and kidney. In conclusion, black garlic extract did not appear to produce sub-chronic toxicity in rats.*

Keywords: black garlic, sub-chronic toxicity, rat.

ĐẶT VẤN ĐỀ

Ngày nay, việc nghiên cứu các thuốc có nguồn gốc từ tự nhiên đã và đang ngày càng phát triển mạnh mẽ. Chúng thường có ưu điểm hơn về giá thành cũng như an toàn hơn cho người sử dụng.[1] Tỏi là một vị dược liệu được sử dụng từ lâu để phòng và điều trị các tình trạng nhiễm trùng, ung thư, và các bệnh lý rối loạn miễn dịch khác.[2,3] Tỏi

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Ngày phản biện: 3/2/2021

Ngày chấp nhận đăng: 4/2/2021

vi thể gan thận là chỉ số bắt buộc khi đánh giá độc tính bán trường diễn. Ngoài ra, xét nghiệm vi thể còn là tiêu chuẩn vàng để đánh giá tổn thương 2 cơ quan chính chịu trách nhiệm chuyển hóa và thải trừ thuốc là gan và thận.[7] Trên tất cả chuột nghiên cứu, không quan sát thấy có thay đổi bệnh lý nào về mặt đại thể của các cơ quan. Kết quả giải phẫu bệnh cho thấy cao mềm Tỏi đen Hà Giang cả 2 liều khi dùng đường uống trên chuột cống liên tục trong 4 tuần không làm thay đổi hình ảnh mô bệnh học gan và thận so với lô chứng sinh học.

Kết quả nghiên cứu của chúng tôi cũng phù hợp với kết quả nghiên cứu tính an toàn của Tỏi đen đã được công bố. Theo Vũ Bình Dương và cộng sự (2015), sau khi cho thỏ uống dịch chiết Tỏi đen ở 2 mức liều 2 g/kg/ngày và 4 g/kg/ngày liên tục trong 42 ngày không ảnh hưởng đến sự phát triển trọng lượng, các chỉ số điện tim, huyết học, sinh hóa và mô bệnh học gan, lách và thận của thỏ.[10] Nghiên cứu độc tính cấp cho thấy Tỏi đen có tính an toàn cao khi dùng đến liều 10 g/kg/ngày trên chuột nhắt trắng không biểu hiện độc tính, không có chuột chết, không làm thay đổi tình trạng toàn thân, số lượng tế bào máu và chức năng gan, thận của chuột.

KẾT LUẬN

Cao mềm Tỏi đen Hà Giang khi dùng đường uống trong 4 tuần liên tục với 2 mức liều 0,4 g/kg/ngày và liều cao gấp 5 lần (2,0 g/kg/ngày) không gây độc tính bán trường diễn trên chuột cống trắng thông qua không ảnh hưởng đến tình trạng chung, thể trọng, chức năng của hệ tạo máu và chức năng gan, thận chuột cống.

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Nghiên cứu độc tính cấp và bán trường diễn của cao lỏng Actiso trên thực nghiệm

STUDY OF SEMI-PERMANENT TOXICITY OF ACTISO EXTRACT ON EXPERIMENT

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TÓM TẮT

Nghiên cứu được tiến hành nhằm đánh giá độc tính bán trường diễn của cao lỏng Actiso theo đường uống trên động vật thực nghiệm. Nghiên cứu độc tính bán trường diễn theo hướng dẫn của WHO, chuột cống được uống liên tục cao lỏng Actiso với mức liều 0,2 g/kg/ngày và 1,0 g/kg/ngày trong vòng 4 tuần liên tục. Kết quả cho thấy cao lỏng Actiso khi dùng đường uống liều 0,2 g/kg/ngày và 1,0 g/kg/ngày liên tục trong 4 tuần không ảnh hưởng đến tình trạng chung, thể trọng, các chỉ số huyết học, chức năng gan, thận và mô bệnh học gan, thận trên chuột cống trắng. Như vậy cao lỏng Actiso không gây độc tính bán trường diễn trên động vật thực nghiệm.

Từ khóa: Actiso, bán trường diễn, động vật thực nghiệm.

SUMMARY

Objectives: The research evaluated the sub-chronic toxicities of artichoke (Cynara scolymus L.) liquid extract through oral administration in experimental animals.

Methods: The sub-chronic toxicity study was conducted by the recommendation of WHO in rats with oral doses of 0.2 g/kg/day and 1.0 g/kg/day in 4 consecutive weeks. As a result, oral administration of Cynara scolymus L. liquid extract at the doses of 0.2 g/kg/day and 1.0 g/kg/day had no effect on general condition, body weight, hepato-renal functions and histopathological structures of liver and kidney. In conclusion, Cynara scolymus L. extract does not appear to produce sub-chronic toxicities in experimental animals.

Key words: artichoke (Cynara scolymus L.), sub-chronic toxicity, experimental animals.

ĐẶT VẤN ĐỀ

Ngày nay, việc nghiên cứu các thuốc có nguồn gốc tự nhiên đã và đang ngày càng phát triển mạnh mẽ. Chúng thường có ưu điểm hơn về giá thành cũng như an toàn hơn cho người sử dụng [1].

Actiso được sử dụng từ lâu đời trong điều trị các bệnh rối loạn mỡ máu, đái tháo đường,... [2]. Actiso là loài thực vật có chứa các chất chống

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phần, cholesterol toàn phần và albumin trong máu [6]. Kết quả cho thấy sau 2 tuần và 4 tuần uống cao lỏng Actiso, các chỉ số hoạt độ AST, ALT, nồng độ bilirubin toàn phần, cholesterol toàn phần và albumin trong máu không thay đổi có ý nghĩa so với lô chứng và so sánh giữa hai thời điểm trước và sau khi uống thuốc thử.

Thận là cơ quan bài tiết của cơ thể, nhu mô thận rất dễ tổn thương bởi các chất nội sinh và ngoại sinh. Vì vậy, khi đưa thuốc vào cơ thể thuốc có thể gây tổn thương thận, từ đó ảnh hưởng đến chức năng thận [7]. Creatinin là thành phần đạm trong máu ổn định nhất, hầu như không phụ thuộc vào chế độ ăn hoặc những thay đổi sinh lý mà chỉ phục thuộc vào khả năng đào thải của thận. Khi cầu thận bị tổn thương, nồng độ creatinin máu tăng sớm hơn ure. Do vậy, creatinin máu là chỉ tiêu tin cậy và quan trọng hơn ure máu, nên hiện nay dùng để đánh giá và theo dõi chức năng thận [1]. Kết quả nghiên cứu cho thấy nồng độ creatinin trong máu chuột sau dùng cao lỏng Actiso không có sự thay đổi khác biệt với lô chứng và so sánh giữa hai thời điểm trước và sau khi uống thuốc thử.

Ảnh hưởng của cao lỏng Tỏi đen lên cấu trúc đại thể và vi thể

Theo hướng dẫn của WHO, giải phẫu đại thể và vi thể gan thận là chỉ số bắt buộc khi đánh giá độc tính bán trường diễn. Ngoài ra, xét nghiệm vi thể còn là tiêu chuẩn vàng để đánh giá tổn thương 2 cơ quan chính chịu trách nhiệm chuyển hóa và thải trừ thuốc là gan và thận [5]. Trên tất cả chuột nghiên cứu, không quan sát thấy có thay đổi bệnh lý nào về mặt đại thể của các cơ quan. Kết quả giải phẫu bệnh cho thấy cao lỏng Actiso cả 2 liều khi dùng đường uống trên chuột cống liên tục trong 4 tuần không làm thay đổi hình ảnh mô bệnh học gan và thận so với lô chứng sinh học.

KẾT LUẬN

Cao lỏng Actiso khi dùng đường uống trong 4 tuần liên tục với 2 mức liều 0,2 g/kg/ngày và liều cao gấp 5 lần (1,0 g/kg/ngày) không gây độc tính bán trường diễn trên chuột cống trắng thông qua không ảnh hưởng đến tình trạng chung, thể trọng, chức năng của hệ tạo máu và chức năng gan, thận chuột cống.

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Nghiên cứu độc tính bán trường diễn của Bạch phụ thang trên thực nghiệm

STUDY THE SUB-CHRONIC TOXICITY OF "BACH PHU THANG" ON EXPERIMENT

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TÓM TẮT

Nghiên cứu được tiến hành nhằm đánh giá độc tính bán trường diễn của Bạch phụ thang theo đường uống trên động vật thực nghiệm. Nghiên cứu độc tính bán trường diễn được tiến hành theo hướng dẫn của WHO, chuột cống được uống liên tục Bạch phụ thang với mức liều 11,4 g/kg/ngày và 22,8 g/kg/ngày trong vòng 4 tuần liên tục. Kết quả nghiên cứu cho thấy Bạch phụ thang khi dùng đường uống liều 11,4 g/kg/ngày và 22,8 g/kg/ngày liên tục trong 4 tuần không ảnh hưởng đến tình trạng chung, thể trọng, các chỉ số huyết học, chức năng gan, thận và mô bệnh học gan, thận trên chuột cống trắng. Như vậy, Bạch phụ thang không gây độc tính bán trường diễn trên chuột cống thực nghiệm.

Từ khóa: Bạch phụ thang, bán trường diễn, chuột cống.

SUMMARY

Objectives: The research evaluated the sub-chronic toxicity of "Bach phu thang" through oral administration in animal experiment. The sub-chronic toxicity study was conducted by the recommendation of WHO in rats with oral doses of 11.4 g/kg/day and 22.8 g/kg/day in 4 consecutive weeks. As a result, oral administration of "Bach phu thang" at the doses of 11.4 g/kg/day and 22.8 g/kg/day had no effect on general condition, body weight, hematological parameters, hepato-renal functions and histopathological structures of liver and kidney. In conclusion, "Bach phu thang" did not appear to produce sub-chronic toxicity in rats.

Keywords: Bach phu thang, sub-chronic toxicity, rats.

ĐẶT VẤN ĐỀ

Ngày nay, việc nghiên cứu các thuốc có nguồn gốc từ tự nhiên đã và đang ngày càng phát triển mạnh mẽ. Chúng thường có ưu điểm hơn về giá thành cũng như an toàn hơn cho người sử dụng.[1]

Nghiên cứu độc tính là một bước rất quan trọng trong nghiên cứu phát triển thuốc. Thuốc muốn được sử dụng thì phải đảm bảo an toàn và có hiệu

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Ngày chấp nhận đăng: 10/2/2021

còn là tiêu chuẩn vàng để đánh giá tổn thương 2 cơ quan chính chịu trách nhiệm chuyển hóa và thải trừ thuốc là gan và thận.[5] Trên tất cả chuột nghiên cứu, không quan sát thấy có thay đổi bệnh lý nào về mặt đại thể của các cơ quan. Kết quả giải phẫu bệnh cho thấy Bạch phụ thang cả 2 liều khi dùng đường uống trên chuột cống liên tục trong 4 tuần không làm thay đổi hình ảnh mô bệnh học gan và thận so với lô chứng sinh học.

Kết quả nghiên cứu của chúng tôi cũng phù hợp với kết quả nghiên cứu tính an toàn của một số thành phần trong Bạch phụ thang đã được công bố. Theo Cha SB và cộng sự (2021), sau khi cho chuột cống uống Hoài sơn ở các mức liều 800, 2000 và 5000 mg/kg/ngày liên tục trong 13 tuần không ảnh hưởng đến trọng lượng chuột, các chỉ số phân tích nước tiểu, huyết học, sinh hóa và mô bệnh học các cơ quan của chuột cống. Tại nghiên cứu của chúng tôi, Hoài sơn đang được sử dụng ở liều 1,2 g/kg/ngày, thấp hơn gấp 4,2 lần so với liều cao nhất được sử dụng trong nghiên cứu của Cha SB[8]. Theo nghiên cứu của LIU Jia và cộng sự (2021), Thục địa (*Rehmania glutinosa* Libosch) ở các mức liều 1,67 g/kg/ngày, 8,33 g/kg/ngày và 16,7 g/kg/ngày cũng được chứng minh không thể hiện độc tính bán trường diễn khi uống liên tục trong 30 ngày. Nghiên cứu của chúng tôi đang sử dụng Thục địa với liều 1,2 g/kg/ngày, thấp hơn 13,9 lần so với liều cao nhất sử dụng trong nghiên cứu của LIU Jia.[9]

KẾT LUẬN

Bạch phụ thang khi dùng đường uống trong 4 tuần liên tục với 2 mức liều 11,4 g/kg/ngày và liều cao gấp 2 lần (22,8 g/kg/ngày) không gây độc tính bán trường diễn trên chuột cống trắng thông qua không ảnh hưởng đến tình trạng chung, thể trọng, chức năng của hệ tạo máu và chức năng gan, thận chuột cống.

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Nghiên cứu ảnh hưởng của viên nang Đông trùng hạ thảo Banikha lên chức năng gan, thận chuột cống thực nghiệm

EVALUATION OF THE INFLUENCE OF DONG TRUNG HA THAO BANIKHA CAPSULES ON LIVER AND KIDNEY FUNCTION IN EXPERIMENTAL RATS

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[3]Công ty Cổ phần Dược thảo Thiên Phúc
[4]Học viện Y Dược học cổ truyền Việt Nam

TÓM TẮT

Mục tiêu: Đánh giá ảnh hưởng của viên nang Đông trùng hạ thảo Banikha theo đường uống lên chức năng gan, thận chuột cống thực nghiệm.

Phương pháp nghiên cứu: Chuột cống trắng được uống liên tục viên nang Đông trùng hạ thảo Banikha với mức liều 0,39 g/kg/ngày và 1,16 g/kg/ngày trong vòng 4 tuần. Trước lúc uống thuốc, sau 2 tuần và 4 tuần uống thuốc, chuột được đánh giá chức năng gan và chức năng thận. Giải phẫu bệnh gan, thận được đánh giá khi kết thúc nghiên cứu ở tuần thứ 4.

Kết quả và bàn luận: Kết quả nghiên cứu cho thấy viên nang Đông trùng hạ thảo Banikha liều 0,39 g/kg/ngày và 1,16 g/kg/ngày dùng đường uống liên tục trong 4 tuần không ảnh hưởng đến hoạt độ AST, hoạt độ ALT, nồng độ bilirubin toàn phần, cholesterol toàn phần, albumin, creatinin trong máu và hình ảnh mô bệnh học gan, thận trên chuột cống trắng ở cả 2 mức liều.

Từ khóa: Viên nang Đông trùng hạ thảo Banikha, chức năng gan, chức năng thận, chuột cống.

SUMMARY

Objectives: *The research evaluated the influence of Dong trung ha thao Banikha capsules on liver and kidney function of experimental rats.*

Subjects and methods: *Rats was given continuous oral administration of Dong trung ha thao BANIKHA capsules at doses of 0.39 g/kg and 1.16 g/kg during 4 weeks. Hepato-renal toxicity was assessed before the*

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Ảnh hưởng của viên nang Đông trùng hạ thảo Banikha lên hình thái và cấu trúc vi thể gan, thận của chuột cống thực nghiệm

Theo hướng dẫn của WHO, giải phẫu đại thể và vi thể gan thận là chỉ số bắt buộc khi đánh giá độc tính bán trường diễn [3]. Ngoài ra, xét nghiệm vi thể còn là tiêu chuẩn vàng để đánh giá tổn thương 2 cơ quan chính chịu trách nhiệm chuyển hóa và thải trừ thuốc là gan và thận [1]. Trên tất cả chuột nghiên cứu, không quan sát thấy có thay đổi bệnh lý nào về mặt đại thể của các cơ quan. Kết quả giải phẫu bệnh cho thấy viên nang Đông trùng hạ thảo Banikha cả 2 liều khi dùng đường uống trên chuột cống liên tục trong 4 tuần không làm thay đổi hình ảnh mô bệnh học gan và thận so với lô chứng sinh học.

Trên thế giới cũng như ở Việt Nam cho đến nay chưa công bố công trình nghiên cứu về độc tính của sự phối hợp các thành phần trong viên nang Đông trùng hạ thảo Banikha. Tuy nhiên, các nghiên cứu độc tính của từng thành phần riêng rẽ đã được nhiều tác giả thực hiện và kết quả cho thấy không xuất hiện độc tính khi dùng các vị dược liệu này trên động vật thực nghiệm [5],[6],[7].

KẾT LUẬN

Viên nang Đông trùng hạ thảo Banikha liều 0,39 g/kg/ngày (tương đương với liều điều trị dự kiến trên lâm sàng) và 1,16 g/kg/ngày (gấp 3 lần liều tương đương với liều điều trị dự kiến trên lâm sàng) dùng đường uống liên tục trong 4 tuần không ảnh hưởng đến hoạt độ AST, ALT, nồng độ bilirubin toàn phần, cholesterol toàn phần, albumin, creatinin trong máu và không làm thay đổi hình ảnh mô bệnh học gan, thận trên chuột cống trắng ở cả 2 mức liều.

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SAGE journals

Biodiversity and potential of Vietnamese medicinal plants - Short Communication

Chemical Constituents and Neuroprotective Activity of *Hypericum hookerianum*

Vu Duy Hong[1], Van Tuan Vu[2], Baskar Selvaraj[3], Nguyen Manh Tuyen[4], Nguyen Minh Khoi[1], Nguyen Duy Thuan[5], Phuong Thien Thuong[6], and Jae Wook Lee[3]

Objective/Background The *Hypericum hookerianum* is widely distributed in Asian countries and prevalently used in traditional herbal medicines for its significant antioxidant, antipyretic, and anticancer properties. To date, very limited reports are available regarding the phytochemical profile, and there are no reports that have studied the neuroprotective potentials of this plant.

Experimental Methods The aerial parts of *H. hookerianum* were collected from the Sapa mountainous district, Lao Cai province of Vietnam. The extracts were prepared using methanol, n-butanol, ethyl acetate, and chloroform and fractionated by normal-phase silica gel column chromatography to isolate compounds. The purity of the isolated compounds was analyzed by liquid chromatography combined with mass spectrometry. The structures were studied using nuclear magnetic resonance spectroscopy. All the isolates were evaluated for their neuroprotective activity against the neurotoxicity induced by glutamate in HT-22 hippocampal cells and 6-hydroxydopamine (6-OHDA) in SH-SY5Y neuroblastoma cells. The fluorescent dye calcein-AM image assay was used to measure the cell viability.

Results Bioassay-guided fractionation of the aerial parts of *H. hookerianum* led to the isolation of 20 previously reported compounds (**1-20**), including 3 flavonoids (**1-3**), 2 xanthones (**4 and 5**), 1 chalcone (**6**), 4 lignans (**7-0**), 1 chromone-C-glycoside (**11**), 1 cinnamic acid derivative (**12**), 7 phloroglucinols (**13-9**), and 1 stilbene (**20**). The structures of the compounds were determined by spectroscopic methods and compared with the reported data. The extracts and isolated compounds showed neuroprotection in both cell models. The compounds 4-hydroxy-2,6,4′-trimethoxydihydrochalcone (6, $EC_{50} = 1.48$ μM) and sesamin (10, $EC_{50} = 2.85$ μM) exhibited significant neuroprotective effects in HT-22 and SH-SY5Y cells, respectively.

Conclusion This is the first report on the neuroprotection of extracts and isolated compounds from *H. hookerianum*. The results provide information for further studies to develop products for the prevention and treatment of neurological disorders.

Keywords
Hypericum hookerianum, 4-hydroxy-26, 4′-trimethoxydihydrochalcone, sesamin, neuroprotective activity

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BÀI NGHIÊN CỨU

Nghiên cứu độc tính cấp và ảnh hưởng lên các chỉ số huyết học của Cao khô thanh nhiệt trên thực nghiệm

STUDY ON THE ACUTE AND THE EFFECTS ON THE HEMATOLOGICAL INDEXES OF CAO KHO THANH NHIET POWDER ON EXPERIMENT

Hoàng Trọng Tuấn [1], Nguyễn Duy Thuần [1], Nguyễn Phương Hiền [1], Lê Thu Trang [1], Nguyễn Thị Thu Thủy [2], Nguyễn Hoàng Ngân [3]

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TÓM TẮT

Mục tiêu: *Nghiên cứu độc tính cấp và ảnh hưởng lên các chỉ số huyết học của Cao khô thanh nhiệt trên thực nghiệm.*

Đối tượng và phương pháp: *Nghiên cứu độc tính cấp của Cao khô thanh nhiệt trên chuột nhắt trắng bằng đường uống theo phương pháp thử nghiệm giới hạn áp dụng cho mẫu thử có nguồn gốc từ thực vật ít độc tính, theo hướng dẫn thử nghiệm tiền lâm sàng và lâm sàng thuốc đông y, thuốc từ dược liệu của Bộ Y tế, đánh giá ảnh hưởng lên các chỉ số huyết học trên chuột cống trắng được tiến hành theo hướng dẫn của WHO theo đường uống.*

Kết quả: *Độc tính cấp: liều cao nhất 10g/kg (tương đương gấp khoảng 11 lần liều tối đa trên lâm sàng) không gây chết chuột, chưa xác định LD_{50} theo đường uống. Ảnh hưởng lên các chỉ số huyết học: Cao khô thanh nhiệt ở cả 2 liều 525 mg/kg/24h (tương đương liều trên lâm sàng) và 1575 mg/kg/24h (gấp 3 liều lâm sàng) đều không gây ảnh hưởng đến các chỉ số huyết học của chuột cống trắng sau 30 ngày và 60 ngày uống thuốc.*

Kết luận: *Cao khô thanh nhiệt không gây độc tính cấp trên chuột nhắt trắng uống đến liều 10,0g/kg/24h, không gây ảnh hưởng đến các chỉ số huyết học khi cho chuột cống trắng uống liều 525 mg/kg/24h và liều 1575 mg/kg/24h liên tục trong 60 ngày.*

Từ khoá: *Cao khô thanh nhiệt, độc tính cấp, chỉ số huyết học.*

SUMMARY

Objective: *To study the acute toxicity and the effects on the hematological indexes of Cao kho thanh nhiet powder on experiment.*

Subject and methods: *The acute toxicity of Cao kho thanh nhiet powder was determined by using Litchfield- Wilcoxon method, the effects on hematological indexes were determined by using*

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đổi, đặc biệt thường làm giảm số lượng các tế bào máu.

Huyết sắc tố trong hồng cầu có nhiệm vụ tiếp nhận oxy khi qua phổi và nhường lại lượng oxy đó cho các tế bào khi qua các mao mạch. Định lượng huyết sắc tố cho biết rõ chức năng của hồng cầu. Thể tích trung bình hồng cầu phản ánh đặc điểm của tình trạng thiếu máu [7]. Hematocrit là tỷ lệ % giữa khối hồng cầu và máu toàn phần. Nếu thuốc làm thay đổi số lượng hồng cầu hoặc làm mất nước hay ứ nước trong tế bào máu thì chỉ số này sẽ thay đổi.

Vì vậy, các xét nghiệm về số lượng hồng cầu, thể tích trung bình hồng cầu, hàm lượng hemoglobin, hematocrit, số lượng bạch cầu, công thức bạch cầu và số lượng tiểu cầu của chuột thí nghiệm được xác định. Kết quả từ bảng 2 đều cho thấy các chỉ số trên của chuột ở cả hai lô trị đều không có thay đổi có ý nghĩa thống kê ($p > 0,05$) so với trước khi sử dụng Cao khô thanh nhiệt và so với lô chứng ở cùng thời điểm. Như vậy, Cao khô thanh nhiệt không gây ảnh hưởng đến chức năng tạo máu của chuột nghiên cứu.

KẾT LUẬN

Chưa xác định được LD50 của Cao khô thanh nhiệt theo đường uống trên chuột nhắt trắng. Với mức liều cao nhất cho chuột uống trong 24h là 10,0g/kg thể trọng không xuất hiện độc tính cấp.

Cao khô thanh nhiệt ở cả 2 liều 525 mg/kg/24h (tương đương liều trên lâm sàng) và 1575 mg/kg/24h (liều gấp 3) đều không gây ảnh hưởng đến thể trạng, các chỉ số tạo máu của chuột cống trắng sau 30 ngày và 60 ngày uống thuốc.

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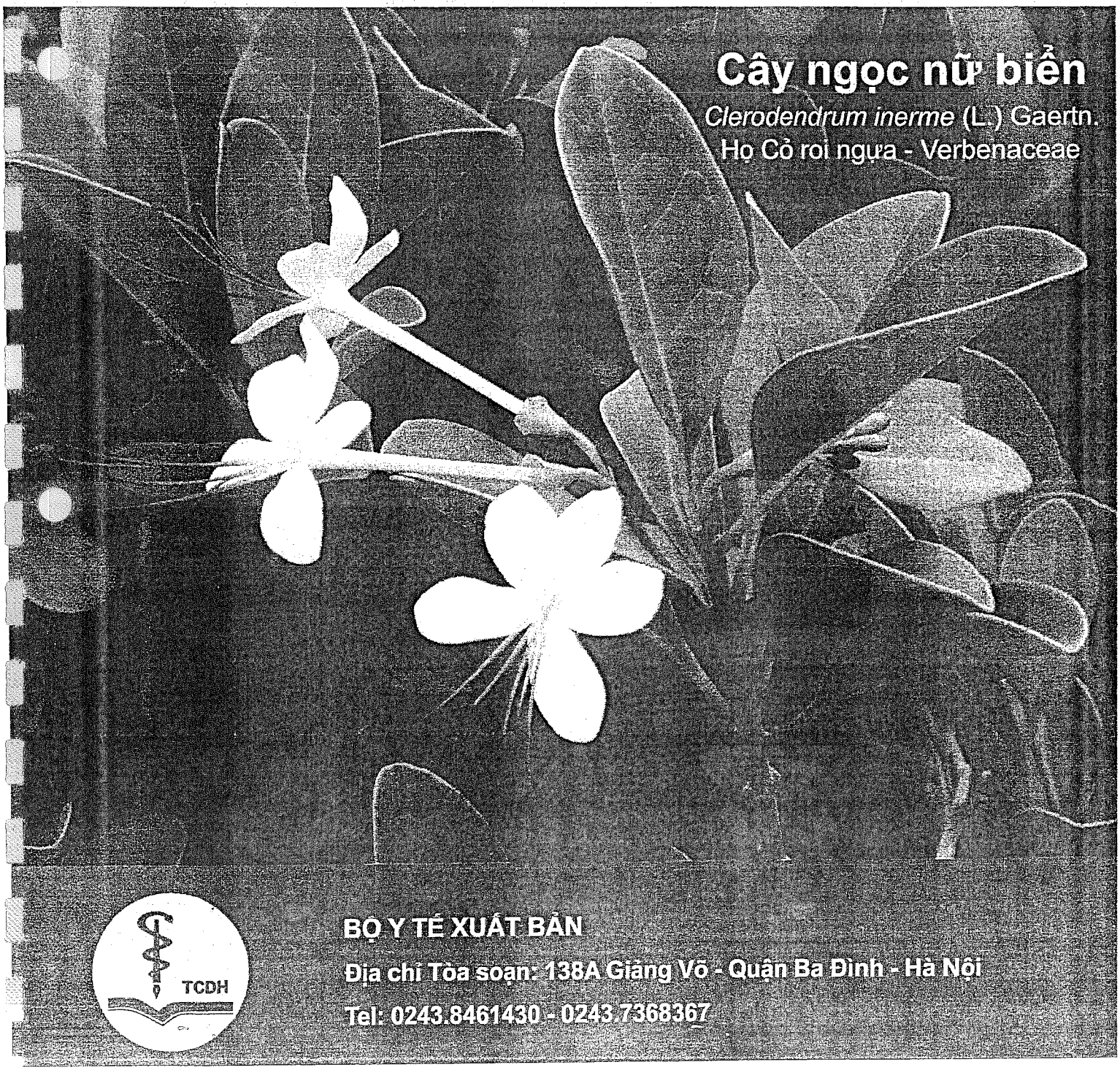

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CYTOTOXIC ALKALOIDS FROM *Stephania dielsiana*

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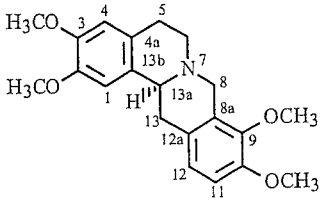
The genus *Stephania* belongs to the family Menispermaceae, widely distributed in Africa, India, Southeast Asia, and the northern and eastern parts of Australia. They comprise more than 60 species, in which several *Stephania* genus were used as folk remedies [1]. Many alkaloids with interesting pharmacological activity were isolated from this genus [2]. *Stephania dielsiana* Y. C. Wu is a medicinal plant, which has been used in Vietnamese traditional medicine as an analgesic and sedative and for the treatment of neuralgia, stomachache, and arthritis. There are some reports on the chemical constituents of the tubers of this species [3–5]. Some isolated compounds showed antimicrobial, hypotensive, and anticarcinogenic activities [6, 7]. In this study, seven alkaloids, tetrahydropalmatine (**1**), stephanine (**2**), crebanine (**3**), *O*-methylbulbocapnine (**4**), oxostephanine (**5**), palmatine (**6**), and thailandine (**7**), were isolated from the leaves of this plant. The structures of these alkaloids were elucidated by spectroscopic methods, including MS, PMR, ^{13}C NMR, HSQC, and HMBC and compared with the reported references. Among them, compounds **4**, **6**, and **7** were isolated for the first time from this plant.

Some alkaloids from *Stephania* were investigated for their cytotoxic activities against some kinds of mammalian cancer cell lines such as KB, HepG2, MRC-5, MCFGLC4, GLC4/Adr, K562, and K562/Adr [2, 8–10]. Herein, the isolated alkaloids **1–7** were tested for their cytotoxic activities against human breast cancer cell line (BT474) and human colon cancer cell line (HCT116). Docetaxel was used as positive control [11]. Testing was carried out in triplicate for each compound and concentration. Then the results were analyzed and processed statistically. The IC_{50} values are listed in Table 1. All tested compounds exhibited antiproliferative activities against two cancer cells tested. Compounds **2** and **3** demonstrated potent cytotoxicity against BT474 cell with IC_{50} of 1.55 and 1.58 μg/mL, respectively. Compound **7** showed strong cytotoxicity against both cell lines (IC_{50} = 1.89 and 2.76 μg/mL, respectively).



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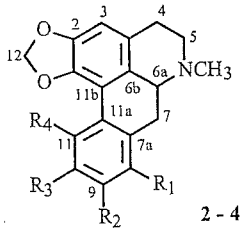

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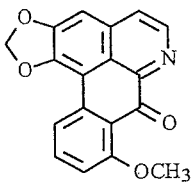
2: $R_1 = OCH_3$, $R_2 = R_3 = R_4 = H$
3: $R_1 = R_2 = OCH_3$, $R_3 = R_4 = H$
4: $R_1 = R_2 = H$, $R_3 = R_4 = OCH_3$



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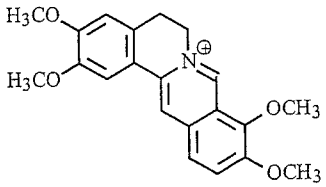

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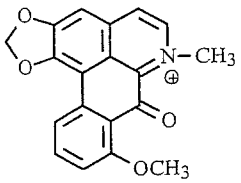

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Original Article

DEVELOPMENT OF ANTI-WRINKLE CREAM FROM *PUERARIA CANDOLLEI* VAR. *MIRIFICA* (AIRY SHAW AND SUVAT.) NIYOMDHAM, "KWAO KRUA KAO" FOR MENOPAUSAL WOMEN

PANEE SIRISA-ARD[1*], NICHAKAN PEERAKAM[1,2], NGUYEN QUOC HUY[3], TRAN VAN ON[3], PHUNG TUAN LONG[4], AEKKHALUCK INTHARUKSA[1]

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ABSTRACT

Objective: The aim of this study was to incorporate *Peraria candollei* var. *mirifica* extract into the cream, to evaluate the physical properties and to conduct the skin tests in participants.

Methods: *Pueraria candollei* var. *mirifica* was extracted with 95% ethanol to obtain crude pueraria extract (PCM). Crude PCM was developed as an anti-wrinkle PCM cream (B) intended for menopausal women. PCM cream was evaluated for stability of pH and viscosity, primary skin irritation, wrinkle reduction and moisturizing as well as customer satisfaction. Cream base (A) and cream purchased from the market (C) were used for comparison. ANOVA post hoc Turkey was used to analyze the variance ($p<0.05$) of the mean comparisons between groups by cluster analysis.

Results: The PCM cream appeared as white color, pH was 6.80, and viscosity was 4.069±0.01 Pa. s, as well as physical characteristic and texture, were acceptable and no irritating reaction. PCM cream exhibited a similar level of moisturizer as cream A and C. The PCM cream revealed an ability to decrease the wrinkle surface and wrinkle volume after applied for 7 and 14 d that shows the activity of this product performed from the PCM extract. Satisfaction of PCM cream showed good acceptance.

Conclusion: These results suggest that PCM cream has the ability to reduce skin wrinkles. It is a good product for postmenopausal women and may also be of benefit for the general population for protection skin wrinkle.

Keywords: *Peraria candollei* var. *mirifica*, Anti-skin wrinkle cream, Menopausal woman

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INTRODUCTION

As is well-known, skin wrinkling is a problem for women. It has become obvious especially on women going through the period of menopause. In Thailand, one of the medicinal plants that have been used for treating this symptom is called KwaoKrua Kao well known as *Pueraria candollei* var. *mirifica* (PCM). The tuberous root of this plant was used as a local remedy for menopause-related vasomotor symptoms for centuries [1]. Suntara [2] mentioned PCM in a pamphlet written as a source of rejuvenation and good health and also stated PCM acted as the "Fountain of youth" for aged men and women when used for anti-skin wrinkle, an increase of hair growth and recovered black hair, to help with memory loss etc. [2], all of these functions probably related that PCM is the folk medicine that contained the substances of female hormone [3]. Much research determined the active compounds from PCM and reported that phytoestrogens such as miroestrol, deoxymiroestrol and isomiroestrol as well as the isoflavonoids comprised pureraria, daidzin, genistin, daidzein and genistein, etc. contained in this plant [4-6, 16]. These compounds were tested for biological activities in many studies. The results found that miroestrol was produced from the oxidation of deoxymiroestrol [5] and both compounds showed an activity of estrogenic properties in ovariectomized rats [7] and also performed to enhance the effects of toremifene on MCF-7 human breast cancer cells [5]. Furthermore, miroestrol and deoxymiroestrol presented potentiality similar with estradiol [8-9] and both compounds are highly active phytoestrogens [10]. For the isoflavonoids, puerarin and daidzein showed the same level of antioxidant activity with α-tocopherol [11] and the isoflavone, genistein, and daidzein (isolated from soybean) showed stronger antioxidant activity than isoflavone glycosides, daidzin and genistin [12]. However, the isoflavonoids were reported as the major components of PCM [13] and the amount of these compounds related to factors such as sub-species, cultivation area, harvesting period, etc. [14]. Therefore the number of active ingredients in PCM was recommended to use as standardization for crude PCM extract [1] before the development of the products. Moreover, PCM was developed into products for skin such as breast creams, eye gel, body gel, day and night cream and cataplasm/patches [15-16].

Some research reported that the skin moisture levels were improved by 39% after 24 h and retained 26% improvement after 3 w when tested with ABS *Pueraira mirifica* extract PF compared with the untreated control and also showed moisturization 37% better after 24 hr and 7% better after 3 w when compared with the base cream [17]. Suwanvesh *et al.* (2017) examined the effect of PCM gel on vaginal health in postmenopausal women for 12-weeks of treatment and found that PCM gel showed to be efficacy and safety for the treatment of vulvovaginal atrophy [18]. Our previous research focused on determining the quantity of phytoestrogen compounds and the isoflavonoids as standardization for crude extracts before the development of the products. The study discovered that crude extract of PCM obtained from 95% ethanol extraction showed the highest quantity of phytoestrogens and isoflavonoids [unpublished]. Therefore the aim of this study was to develop the PCM as an anti-wrinkle PCM cream for postmenopausal women.

MATERIALS AND METHODS

Plant material and chemicals

Tuberous root of PCM was collected from a controlled farm in Nakhon Prathom Province, Thailand. Voucher specimen No. CMU023231 was identified and kept at Herbarium of Faculty of Pharmacy, Chiang Mai University, Thailand. Chemical ingredients and solvent used for preparation the PCM cream are pharmaceutical grade were purchased from Union Sciences Co. Ltd., Thailand.

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Isoflavonoids and Phytoestrogens from Pueraria candollei var. mirifica Related with Appropriate Ratios of Ethanol Extraction

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Isoflavonoids and Phytoestrogens from *Pueraria candollei* var. *mirifica* Related with Appropriate Ratios of Ethanol Extraction

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In the folk wisdom of Thailand, chemical compounds from *Pueraria candollei* var. *mirifica* play an important role similar to hormones for post-menopausal women. This study aims to determine the appropriate ratio of ethanol to be used for isoflavonoids and phytoestrogens extraction from *Pueraria candollei* var. *mirifica*, subsequently, the development as an obstetric cream. *Pueraria candollei* var. *mirifica* collected from Thailand and Vietnam were extracted with ethanol (50, 75 and 95 % v/v). The 95 % of ethanolic extract (S095) contained higher quantities of isoflavonoids and phytoestrogens than other ethanolic extracts. The amount of individual isoflavonoids (mg/g extract) were puerarin (5.012), daidzin (2.278), daidzein (1.886), genistin (0.620) and genistein (0.437) which were analyzed by HPLC. The phytoestrogens (mg/g extract) tested by indirect competitive enzyme-linked immunosorbent assay were miroestrol (1.581) and deoxymiroestrol (0.397). In conclusion, 95 % ethanol revealed the highest potential extraction of isoflavonoids and phytoestrogens for use as active ingredients in the development of obstetric creams for post-menopausal women.

Keywords: *Pueraria mirifica*, Isoflavonoids, Puerarin, Miroestrol, Deoxymiroestrol.

INTRODUCTION

Pueraria candollei var. *mirifica* (Air Shaw & Suvat.) Niyomdham is a member of the family Leguminosae and is well known in Thailand as Kwao Krua Khaw or white Kwao Krua. This plant is usually found in the northern part of Thailand [1]. *Pueraria candollei* var. *mirifica* has been mentioned as a rejuvenation elixir and a source of good health by Luang Anusan Sunthorn in 1931 [2]. Since ancient times this plant has been used by native Thai people as folk medicine for women to treat menopausal syndrome and to increase sexual desire [3]. Previous studies have reported this medicinal herb provided benefits such as rejuvenating properties [1], to prevent osteoporosis in elderly hypogonadism subjects [4] and also showed estrogenic activity [3]. The tuberous roots of the plant contain active chemical constituents of phytoestrogen compounds such as miroestrol, deoxymiroestrol and isomiroestrol as well as isoflavonoids [5-7]. Miroestrol has similar structure as endogenous estrogen that is found in the human body and having estrogenic-link properties [5,8,9] and also showed function of estrogenic properties in ovariectomized rats [10]. Miroestrol and deoxymiroestrol were reported to enhance the effects of toremifene on MCF-7 human breast cancer cells [6]. The pharmacological activity indicated that miroestrol and deoxymiroestrol are potentially similar to estradiol [11,12]. It was suggested that miroestrol was produced from the oxidation of deoxymiroestrol [6]. Both compounds are highly active phytoestrogens [13]. Miroestrol was estimated to have activity 0.25×10^{-1} times similar to 17β-estradiol in the rat virginal cornification assay [14]. Furthermore the isoflavonoids characterized in *Pueraria candollei* var. *mirifica* such as puerarin, daidzin and genistin are isoflavone glycosides and also daidzein and genistein are isoflavones [1,7,15,16] as shown in Fig. 1. They are used as a standardization for active compounds in *Pueraria candollei* var. *mirifica* [16] and also showed *in vitro* activities of antioxidant, antimutagenic, anticarcino-

TABLE-4
QUANTITY OF MIROESTROL AND DEOXYMIROESTROL IN *Pueraria candollei* var. *mirifica* EXTRACTS

Sample	Miroestrol (mg/g extract)		Deoxymiroestrol (mg/g extract)	
	Mean ± SD	% RSD	Mean ± SD	% RSD
S050 (extracted by using 50 % ethanol)	0.293 ± 16.30	5.57	0.055 ± 4.34	7.92
S075 (extracted by using 75 % ethanol)	0.318 ± 20.66	6.50	0.105 ± 7.42	7.04
S095 (extracted by using 95 % ethanol)	1.581 ± 98.62	6.24	0.397 ± 15.01	3.78
V050 (extracted by using 50 % ethanol)	0.026 ± 2.480	9.66	0.011 ± 0.44	4.10
V075 (extracted by using 75 % ethanol)	0.227 ± 25.82	11.39	0.185 ± 19.05	10.28
V095 (extracted by using 95 % ethanol)	1.378 ± 49.19	3.57	0.220 ± 13.57	6.16

S = Sample from Thailand, V = Sample from Vietnam

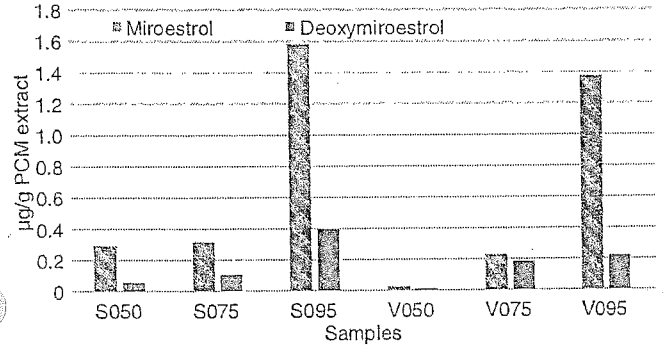

Fig. 3. Quantity of miroestrol and deoxymiroestrol from *Pueraria candollei* var. *mirifica* from Thailand and Vietnam with different ratios of solvent

Pueraria candollei var. *mirifica* for quantity control of crude drug as well as crude extract and it will be beneficial for suppliers and consumers before being used to develop as supplements and cosmetic products.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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Original Research Article

Investigation of the estrogenic activity of *Pueraria candollei* variety *mirifica* extract on rats

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ABSTRACT

Background: *Pueraria candollei* variety *mirifica* (PM) has been widely used as ingredient in many rejuvenating products. In this study, we aimed to assess the estrogenic activity of PM extract grown in Vietnam.
Methods: Estrogenic activity of PM extract was estimated on immature female rats by using uterotrophic method to measure the weight of the reproductive organs. Estrogenic activity of PM extract also was investigated in mature female ovariectomized rats by evaluating the vaginal cells growth, reproductive organs weight, serum estradiol concentration.
Results: Our results showed that PM extract at doses of 100 mg/kg, 200 mg/kg had increased the reproductive organs weight in immature rats and female ovariectomized rats. In addition, PM extract had increased the serum estradiol concentration and the vaginal cells growth by increasing the percentage of keratinocytes in female ovariectomized rats.
Conclusions: Our results showed that PM extract has strong estrogenic activity in rats.

Keywords: Estrogenic activity, *Pueraria mirifica*, Uterotropic assay, Vaginal cytology assay, Ovariectomized rats

INTRODUCTION

Phytoestrogen is a plant estrogen proven effective, less unwanted effects than estrogen in complementary therapies for women with estrogen deficiency. Therefore, the finding for phytoestrogen-rich medicinal plants is a new direction for scientists. *Pueraria candollei* variety *mirifica* (Shaw and Suvat) Niyomdham (PM) is widely distributed in the highlands of northern Thailand, Myanmar and Vietnam which has been identified in a high-phytoestrogen component.[1] Some phytochemicals compounds have been identified in PM extract such as miroestrol, deoxymiroestrol, puerarin, daidzin, genistin, daidzein, genistein and isoflavonoid.[2,3] Miroestrol was the first phytoestrogen isolated from PM, which has the strong estrogenic potency.[1] In traditional medicine, PM has been used for a long time with the purpose of improving tonic, beauty for women. Pharmacological studies have shown that PM has anti-osteoporotic effects, anti-collagenase, anti-elastase and antioxidant and also improving estrogenic activity.[4-7] This study was carried out to evaluating the improving estrogenic activity of the

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Study the therapeutic effect of capsule CTHepaB on nude mice carrying human hepatocellular carcinoma cells infected with hepatitis B virus

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ABSTRACT

Objective: To assess the effect the therapeutic effect of CTHepaB capsules on nude mice carrying human hepatocellular carcinoma cells (HCC) infected with hepatitis B virus (HBV). *Method:* Nude mice after HCC transplant Hep3B infected with HBV on the right thigh, appeared a tumor on the thigh on the 14th day of transplantation, had an average size of 74,6 ± 14,3 mm3), randomly divided into 2 groups of 15 rats each and randomly divided into 3 lots (5 mice / lot): in the control group, drink 0.9% NaCl solution; Treatment, oral dose of CTHepaB 0.96g / kg / 24h; Reference batch, oral dose of 5FU 10mg / kg / 24h.Give the rats the drug according to the above division for 4 weeks. Group 1 used to evaluate the effect of research drugs on tumor size, survival / death rate; Average lifetime of the mouse. Group 2, used for splenectomy to evaluate the density of some immune cells and the HBV-DNA quantification of tumor cells. *Results*: CTHepaB capsules reduced the average tumor size compared to the control group, equivalent to that of the group using 5FU at dose of 10mg/kg/24h; Limiting the death rate of mice at the evaluation points 70, 76, 85, 90 days after taking the drug compared to the control group and on 85, 90 days, compared with the reference group; increases average survival time of mice; Increased number of NK cells, Macrophage cells and DC cells in mouse spleen; Reduces the amount of HBV-DNA in mouse tumor cells. *Conclusion*: CTHepaB capsule with dose of 0.96g/kg/24h has good therapeutic effect, in nude mice carrying HCC infected with HBV.

Keywords: CTHepaB, hepatocellular carcinoma, hepatitis B virus, hepatitis B virus-infected hepatocellular carcinoma cell transplant model

Ethical approval

The study was approved by the Medical Ethics Committee of National Hospital of Traditional Medicine (ethical approval code: 34/IBR-NHTM).

Data and materials availability

All data associated with this study are present in the paper.

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Effectiveness of auricular acupressure and breathing exercises for smoking cessation

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ABSTRACT

Objective: To determine the effectiveness of auricular acupressure and breathing exercises in smoking cessation treatment and monitor their adverse drug reaction (ADR). *Methods:* We prospectively analyzed 60 patients in National Hospital of Traditional Medicine who were addicted to smoking between June 2020 and September 2020. This is a case-control study. Patients were enrolled into two groups: a case group, which was treated with auricular acupressure and breathing exercises (n=30); a control group which was only treated with auricular acupressure (n=30). The results between 2 groups after 28 days of treatment are compared based on many criterias which includes Symptoms of withdrawal syndrome, Mood and Physical Symptoms Scale (MPSS) and breathe carbon monoxide concentrations. *Results*: After 28 days of treatment, in the study group 63.3% of the cases were very good, 10% were good, 26.7% were ineffective; in the control group 46.7% of the cases were very good, 20% were good, 33.3 % were ineffective. *Conclusion*: Auricular acupressure combined with breathing exercises is better in improving symptoms of withdrawal syndrome (shortness, irritability, insomnia, cravings) and decreasing levels of CO in patients' breath after smoking cessation than cases using auricular acupressure only.

Keywords: Smoking cessation, withdrawal syndrome, auricular acupressure, breathing exercises.

1. INTRODUCTION

Tobacco is one of the biggest public health threats the world have faced. Tobacco kills more than 8 million people each year. More than 7 million of those deaths are the result of direct tobacco use while around 1.2 million are the results of non-smokers being exposed to second-hand smoke (St Claire et al., 2020). In Vietnam, according to the Global Adult Tobacco Survey in 2015, 22.5% of the population over 15 years old are smoking cigarettes, which are the equivalent of 15.6 million people. There are more than 7,000 chemicals in tobacco smoke, in which at least 250 are known to be harmful, including

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5. CONCLUSION

The method of auricular acupressure combined with breathing exercises brings better results than using only auricular acupressure. During the study, the auricular acupressure combined with breathing exercises did not have adverse effects on the clinical and some subclinical indicators.

Contribution of the authors

All authors have contributed equally to this work. All authors read and approved the final manuscript and agreed to publish this manuscript.

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Conflict of Interest

The authors declare that there is no conflicts of interests.

Ethical approval

The study was approved by the Medical Ethics Committee of National Hospital of Traditional Medicine (ethical approval code: 32/IBR-NHTM).

Data and materials availability

All data associated with this study are present in the paper.

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Protective role of TD0014 against sodium valproate-induced reproductive toxicity in male wistar rats

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ABSTRACT

Sodium valproate (VPA), a common treatment of epilepsy and other diseases, is known to have severe toxic effects on testis both in experimental animals and in humans. This study was carried out to assess the protective activities of the TD0014 against sodium valproate (SVP)-induced reproductive toxicity in male rats. Animals were treated with TD0014 at the dose of 1.8 g/kg/day and 5.4 g/kg/day, and co-administered with SVP (500 mg/kg) for 7 weeks before all reproductive parameters were determined. The results showed all doses of TD0014 significantly protected the decrease testicular weight and testosterone level in SVP rats. TD0014 significantly improved the decrease sperm count and sperm motility in SVP treated rats. Moreover, testicular histology of TD0014 + SVP groups showed declining of testicular histopathologies as compared to SVP group. Therefore, it seems that TD0014 can prevent testicular and spermatozoal damage in male rats induced with SVP. The higher protective effect was seen with TD0014 at 5.4 g/kg dose.

Keywords: TD0014, sodium valproate, male rat

1. INTRODUCTION

Infertility is one of the major health problems in life, which affects 8–12% of couples worldwide. Of all infertility cases, approximately 40–50% is due to "male factor" infertility (McNamara, 2011). One of the male factor infertility is male hypogonadism, which is characterized by a deficiency in testosterone – a critical hormone for sexual, cognitive, and body function and development. Clinically low testosterone levels can lead to the absence of secondary sex characteristics, infertility, muscle wasting, and other abnormalities. In individuals who also present with clinical signs and symptoms, clinical guidelines recommend treatment with testosterone replacement therapy

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Tổng hợp và thử tác dụng ức chế enzym glutaminyl cyclase của một số dẫn chất piperazin/piperidin của *N*-(4-methoxy-3-aminoalkyloxy-phenyl)thiourea mới

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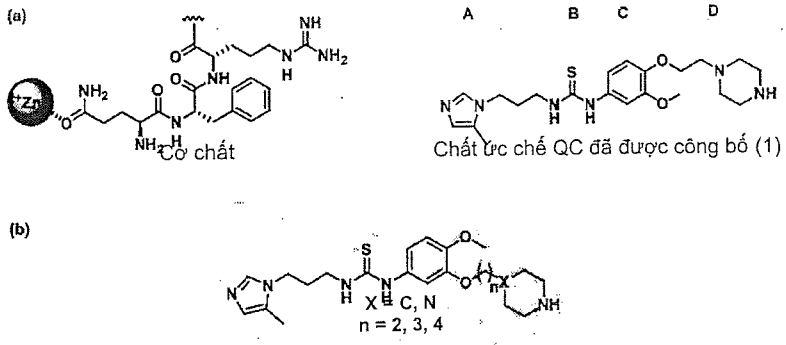
Summary

A new piperazine/piperidine series of N-(4-methoxy-3-aminoalkyloxy-phenyl)thiourea were synthesized and evaluated for glutaminyl cyclase (GC) inhibition by fluorometrical assay. The compounds were obtained via 4 or 5 synthetic steps with well-known reactions. Six synthesized compounds (24 – 29) exhibited potential glutaminyl cyclase (GC) inhibitory activity (IC_{50} values of 7.3 – 18.4 nM).

Keywords: *N-(5-Methyl-1H-imidazol-1-yl)propyl, piperazine, piperidine, thiourea, inhibition, glutaminyl cyclase.*

Đặt vấn đề

Bệnh Alzheimer là dạng phổ biến nhất, chiếm khoảng 60-80% trường hợp của hội chứng suy giảm trí nhớ. Các nhà khoa học chưa khẳng định được chắc chắn nguyên nhân gây nên sự hủy hoại tế bào và tổn hại mô trong não bị bệnh Alzheimer, nhưng các mảng xơ (amyloid beta-Aβ) và các đám xơ rối (tau) được cho là những nghi phạm chính [1-3].



Hình 1. *(a) Cấu trúc của cơ chất, chất ức chế QC đã được công bố 1; (b) cấu trúc chung của các dẫn chất mới được thiết kế*

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Phân tích ABC-VEN ... *(Tiếp theo trang 51)*

hoặc tối cần. Có 19 thuốc và 21 vị thuốc thuộc các nhóm AV, AE, BV tuy là các thuốc và vị thuốc đắt tiền nhưng cũng không được để xảy ra tình trạng hết hàng với các thuốc và vị thuốc thuộc nhóm này vì đó là các thuốc hoặc vị thuốc tối cần hoặc thiết yếu. Để ngăn chặn tình trạng bị tồn đọng các nhóm thuốc và vị thuốc này cần kiểm soát các thuốc và vị thuốc thuộc nhóm này chặt chẽ ở mức thấp. Với các thuốc và vị thuốc nhóm CV là nhóm thuốc có giá trị thấp nhưng có tầm quan trọng cao. Các thuốc và vị thuốc nhóm CV có thể mua sắm 1 lần/1 năm và tồn trữ trong kho vì không tốn nhiều chi phí và sẽ giúp giảm chi phí vận chuyển của các thuốc và vị thuốc nhóm này. Các thuốc nhóm AN (6 thuốc chiếm 5,2% về giá trị) và vị thuốc (6 vị chiếm 3,6% về giá trị) nhóm AN cần phải được giám sát chặt chẽ việc kê đơn. Chỉ tiến hành mua sắm và sử dụng sau khi đánh giá cẩn thận về nhu cầu sử dụng. Việc sử dụng hợp lý các thuốc hoặc vị thuốc thuộc nhóm này, thậm chí loại bỏ các thuốc và vị thuốc thuộc nhóm AN nếu có thể, sẽ giúp tiết kiệm chi phí đáng kể mà không ảnh hưởng đến hoạt động chăm sóc người bệnh. Những vị thuốc và thuốc nhóm II có thể đặt mua 1 hoặc 2 lần/năm, giúp tiết kiệm chi phí đặt hàng và giảm bớt hoạt động quản lý, chi phí vận chuyển sẽ ở mức vừa phải và cũng không làm tồn đọng đáng kể.

Kết luận

Sử dụng phương pháp phân tích ma trận ABC-VEN cho thấy:

Về cơ cấu thuốc YHCT sử dụng tại Viện Y học cổ truyền Quân đội: Thuốc E ở các hạng đều có tỷ lệ giá trị và số khoản cao. Về giá trị nhóm AE chiếm 66,1%; nhóm BE 9,5%; nhóm CE 3,3%. Nhóm AE gồm 14 nhóm dược lý, thuốc bổ khí có giá trị sử dụng cao nhất chiếm 22,8%. Vị thuốc nhóm N ở hạng A chiếm 3,6% về giá trị.

Về cơ cấu thuốc thành phẩm: Nhóm thuốc thành phẩm AE chiếm 51,5% về giá trị; nhóm BE 4,1% ; nhóm CE 3,4%. Nhóm thuốc thành phẩm N hạng A chiếm 5,2% về giá trị; hạng B 4,2%; hạng C 1,5%. Thuốc chữa bệnh về âm về huyết trong nhóm AE chiếm tỉ lệ lớn nhất về giá trị 51,1%, Huyết phủ trục ứ hoàn chiếm 50,9% giá trị. Thuốc thành phẩm nhóm AN gồm 6 thuốc đều được mua sẵn từ các công ty bên ngoài, có giá trị gần 1,39 tỷ đồng.

Kiến nghị: Bệnh viện cần định kỳ rà soát danh mục thuốc sử dụng bằng công cụ phân tích ABC- VEN để có thể sử dụng tốt nhất nguồn ngân sách; xác định mức độ ưu tiên trong mua sắm và sử dụng thuốc, giám sát chặt chẽ các thuốc thuộc nhóm quan trọng (AV, BV, CV) và loại bỏ đi các thuốc không cần thiết (nhóm AN).

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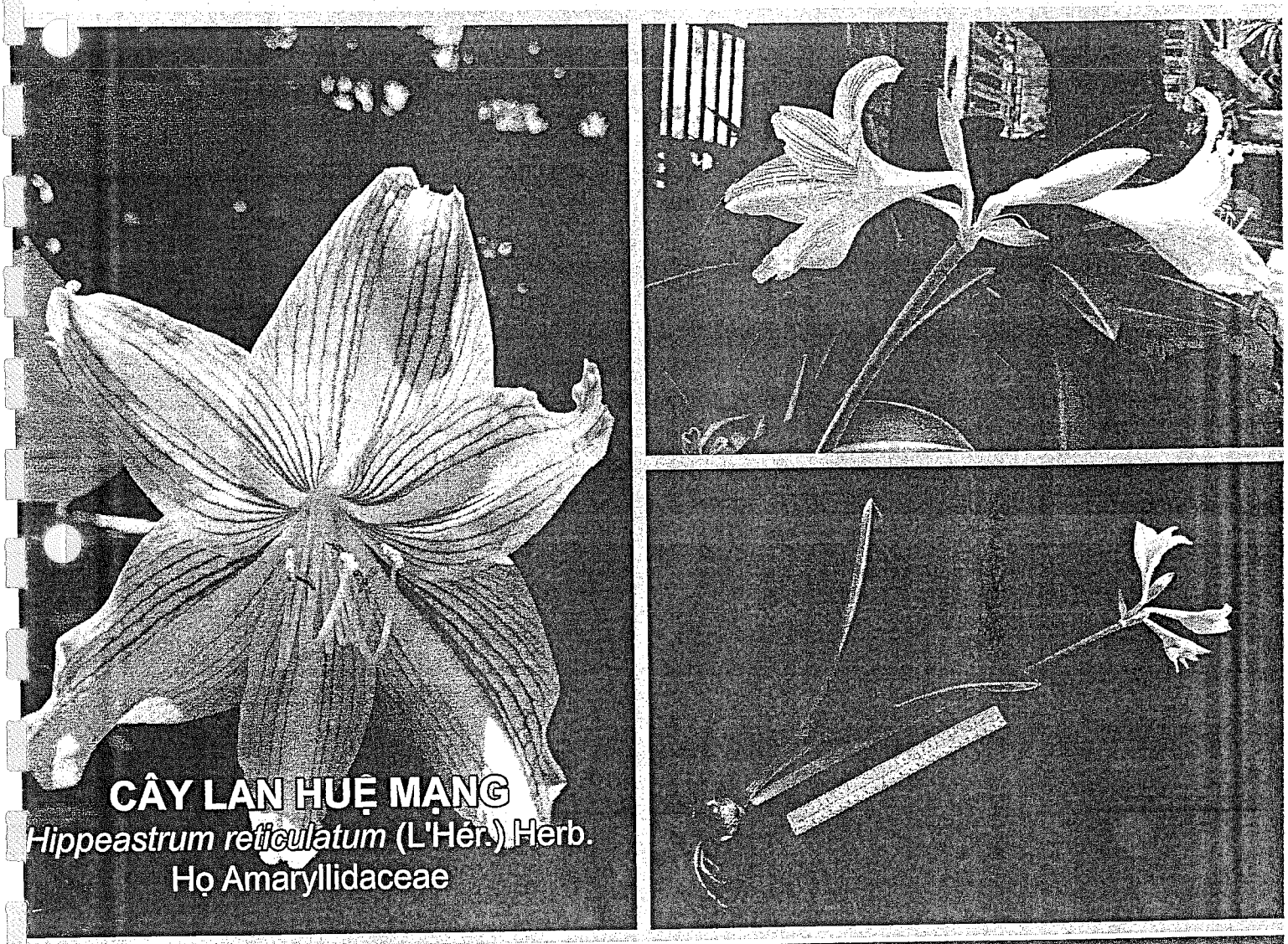

CÂY LAN HUỆ MẠNG
Hippeastrum reticulatum (L'Hér.) Herb.
Họ Amaryllidaceae

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CONTENTS

RESEARCH - TECHNIQUES

Mục lục

cả 3 chất (**4a, 4b, 4d**) đều có log P cao hơn **4c** và SAHA, cho phép làm tăng khả năng đi qua màng tế bào. Ngoài ra, rất có thể sự cồng kềnh của hai nhóm OCH_3 gắn vào khung quinazolin cũng gây cản trở cho khả năng tác động của **4c** trên các tế bào ung thư. Hơn nữa, không loại trừ khả năng tác dụng trên tế bào ung thư của **4b, 4c** còn dựa trên cơ chế khác nữa ngoài ức chế HDAC.

Kết luận

Trong phần nghiên cứu này, chúng tôi đã tổng hợp được thêm 3 dẫn chất *N*- hydroxybenzamid mới mang khung quinazolin. Kết quả thử hoạt tính sinh học cho thấy cả 4 chất có tác dụng ức chế HDAC với IC_{50} = 0,17-0,88 µM và có độc tính tế bào với 3 dòng tế bào ung thư là SW620 (IC_{50} = 1,43-5,45 µM), PC3 (IC_{50} = 0,65-6,72 µM) và NCI-H23 (IC_{50} = 2,51-9,00 µM). Các kết quả này cho thấy việc nghiên cứu tiếp tục để phát triển dãy chất này là có nhiều triển vọng.

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3. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.00 for Windows: (2015), GraphPad Software, La Jolla California USA www.graphpad.com.

(Ngày nhận bài: 04/04/2019 - Ngày phản biện: 12/05/2019 - Ngày duyệt đăng: 03/06/2019)

Tổng hợp và thử tác dụng ức chế enzym glutaminyl cyclase của một số dẫn chất pyrimidin-2-ylamino của *N*-(5-methyl-1*H*-imidazol-1-yl)propyl-*N'*-(3-methoxy-4-alkyloxyphenyl)thiourea

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Summary

Five new pyrimidin-2-ylamino derivatives of N-(5-methyl-1H-imidazol-1-yl)propyl-N'-(3-methoxy-4-alkyloxyphenyl) thiourea were synthesized by 5-step synthetic pathway with well-known chemical reactions. The obtained derivatives were evaluated for glutaminyl cyclase (QC) inhibition by fluorometrical assay, and all (15 – 19) showed the potential activity (IC_{50} values of 5.7 - 39.4 nM).

Keywords: *Pyrimidin-2-ylamino, N-(5-methyl-1H-imidazol-1-yl)propyl, thiourea, inhibition, glutaminyl cyclase.*

Đặt vấn đề

Bệnh Alzheimer được biết đến là một trong những bệnh suy giảm trí nhớ nghiêm trọng nhất, ảnh hưởng tới hàng triệu người trên thế giới. Ước tính mỗi năm có thêm 10 triệu người mắc phải căn bệnh này. Các nhà khoa học dự đoán chi phí cho điều trị và chăm sóc bệnh nhân Alzheimer có thể lên tới 2000 tỷ đô la Mỹ vào năm 2030 [1]. Các giả thuyết chỉ ra rằng nguyên nhân gây nên bệnh Alzheimer có thể do sự tạo thành các mảng amyloid và các đám rối sợi thần kinh (tau) [2]. Do vậy, các nghiên cứu gần đây tập trung vào các đích phân tử có tác dụng giảm sự hình thành và kết tụ tạo mảng amyloid [3]. Một trong những đích tác dụng phân tử đang được chú ý hiện nay cho nghiên cứu phát triển thuốc điều trị Alzheimer là glutaminyl cyclase (EC 2.5.2.3, QC). QC xúc tác cho quá trình đóng vòng các glutaminyl đầu gắn với nitơ thành acid pyroglutamic. Việc ức chế enzym QC sẽ ngăn cản quá trình tạo ra các acid pyroglutamic, do đó làm giảm sự tạo thành các mảng amyloid. Vì vậy, nghiên cứu các chất ức chế enzym QC mở ra một hướng điều trị mới, nhiều triển vọng cho bệnh Alzheimer [4].

Trong nghiên cứu trước đây, chúng tôi nhận thấy rằng dẫn chất pyrimidin của *N*-(5-methyl-1*H*-imidazol-1-yl)propyl-*N'*-(3-methoxy-alkyloxyphenyl)thiourea **(1)** (hình 1a) có tác dụng ức chế QC tốt với IC_{50} = 21,3 nM [5].

với các dẫn chất (bromoalkyl)phthalimid trong môi trường kiềm (K_2CO_3) ở 100 °C trong vòng 1 giờ để tạo ra các ether **3 - 5** tương ứng. Các ether **3 - 5** đồng thời là các nitro thơm sẽ được khử hóa bằng khí hydro với xúc tác là 10 % paladi trong carbon thu được các dẫn chất amin thơm **6 - 8** với hiệu suất cao. Phản ứng cộng hợp tạo thiourea giữa các amin thơm **6 - 8** với 3-(5-methyl-1*H*-imidazol-1-yl)propan-1-amin [2], có sự tham gia của chất xúc tác đặc hiệu TCDI trong môi trường kiềm (TEA) cho các chất **9 - 11**. Các thiourea **9 - 11** được tách loại nhóm bảo vệ phthalimid bằng hydrazin monohydrat để thu được các amin bậc nhất **12 - 14**. Các amin **12 - 14** tiếp tục tham gia phản ứng thế ái nhân với 2-cloropyrimidin hoặc 2-cloro-5-fluoropyrimidin để thu được các chất mục tiêu tương ứng **15 - 19**.

Các chất mục tiêu (**15 - 19**) được tinh chế bằng sắc ký lớp mỏng điều chế; Kết quả chấm sắc ký lớp mỏng của các chất khảo sát trên 3 hệ dung môi khác nhau (MeOH/DCM = 1/9, 1/12; 1/15) đều cho một vết gọn, rõ; Nhiệt độ nóng chảy của các chất mục tiêu đều có khoảng dao động từ 1 đến 2 độ, điều này chứng tỏ các chất tổng hợp được đều tinh khiết. Trên phổ ***^{1}H-NMR*** của các chất thấy xuất hiện các tín hiệu đặc trưng của vòng methylimidazol, vòng phenyl, vòng pyrimidin, mạch alkyl (ethyl, propyl, butyl), cũng như tín hiệu đặc trưng của nhóm chức thiourea, nhóm chức amin bậc 2. Trên phổ ***^{13}C-NMR*** của các chất thấy xuất hiện các tín hiệu đặc trưng của carbon trong vòng imidazol, vòng phenyl, vòng pyrimidin; Carbon của nhóm methyl gắn vào vòng imidazol (9,9 ppm), nhóm methoxy (57,3 pm), ... Phổ ***MS*** của các chất đều cho pic giả phân tử m/z $[M+H]^+$. Như vậy, qua dữ liệu phổ ***^{1}H-NMR***, ***^{13}C-NMR*** và phổ ***MS*** cho phép xác định cấu trúc của các chất mục tiêu là đúng như dự kiến.

Các chất sau khi khẳng định cấu trúc được đánh giá tác dụng ức chế QC bằng phương pháp huỳnh quang [6]. Kết quả trình bày ở bảng 1 cho thấy cả 5 chất tổng hợp được đều có tác dụng ức chế QC tốt, với IC_{50} từ 5,7 đến 39,4 nM. So với tác dụng ức chế QC của dẫn chất pyrimidin 1 đã được công bố (hình 1, IC_{50} = 21,3 nM) [5], 4/5 chất (**16 - 19**) thể hiện hoạt tính ức chế QC tốt hơn 1. Kết quả này cho thấy việc thay thế nhân pyrimidin-2-alkyl ở phần D-region của 1 bằng nhân pyrimidin-2-ylamino có thể đã góp phần làm tăng hoạt tính của các dẫn chất tạo thành. Thêm vào đó, các chất với cầu nối C-region và D-region là mạch 3 carbon (**17**, **19** với giá trị IC_{50} tương ứng là 5,7 và 17,5 nM) giúp cho chất tạo thành có cấu trúc phù hợp, có thể gắn kết chặt chẽ vào trung tâm hoạt động của enzym hơn so với các chất có mạch nối 1 hoặc 2 carbon (**15**, **16**, **18** với giá trị IC_{50} tương ứng là 39,4; 17,5 và 18,5 nM). Việc gắn thêm nhóm thế -F vào vị trí số 5 trên vòng pyrimidin dường như không ảnh hưởng nhiều tới tác dụng của các chất được tạo thành (chất **16** có IC_{50} = 17,5 nM trong khi đó chất **18** có gắn 5-F có IC_{50} = 18,5 nM).

Kết luận

Chúng tôi đã tổng hợp thành công 5 dẫn chất pyrimidin-2-ylamino của *N*-(5-methyl-1*H*-imidazol-1-yl)propyl-*N'*-(3-methoxy-4-alkyloxyphenyl)thiourea (**15 - 19**) qua 5 bước, bằng các phản ứng hóa học thông thường như: Williamson, *N*-alkyl hóa, khử hóa, cộng hợp thiourea, tách loại nhóm bảo vệ phthalimid. Cấu trúc của các chất tổng hợp đã được khẳng định bằng các phương pháp ***^{1}H-NMR***, ***^{13}C-NMR***, ***MS***. Các chất tổng hợp được có tác dụng ức chế enzym QC với IC_{50} từ 5,7 đến 39,4 nM.

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RESEARCH - TECHNIQUES

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Tổng hợp một số hợp chất thioure mang bộ khung piperazin và piperidin hướng ức chế glutaminyl cyclase trong phòng và điều trị Alzheimer

Trần Phương Thảo [1*], Hoàng Văn Hải [2], Trần Thị Thu Hiền [3]

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Summary

Four thioure derivatives of piperazine and piperidine were synthesized, assayed for glutaminyl cyclase (QC) inhibitory activity and studied on their structure-activity relationships (SAR). Of these four synthesized compounds, three had the IC_{50} values below 10 nM.

Keywords: *Alzheimer's disease, Glutaminyl cyclase, QC inhibitors*

Đặt vấn đề

Alzheimer là một chứng mất trí nhớ phổ biến với đặc trưng bởi việc mất dần các noron và synap trong vỏ não và một số vùng dưới não. Bệnh đặc trưng bởi sự xuất hiện của các mảng amyloid [1] và đám rối sợi thần kinh trong giai đoạn muộn của bệnh [2]. Các nghiên cứu gần đây chủ yếu tập trung vào các đích phân tử các tác dụng giảm quá trình hình thành và kết tụ mảng bám amyloid [3]. Trong giai đoạn đầu của quá trình tạo mảng amyloid, peptid amyloid beta bị chia cắt thành các phần nhỏ bởi aminopeptidase và đóng vòng nội phân tử bởi glutaminyl cyclase

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Đặc điểm hình thái... *(Tiếp theo trang 70)*

Bột thân rễ và rễ có màu vàng, mùi hơi hắc, vị đắng. Quan sát dưới kính hiển vi có các đặc điểm sau: Mảnh bần (4) gồm các tế bào thành dày; Các tinh thể calci oxalat (2,3); Mảnh mô mềm (1), Mảnh mạch vạch và mảnh mạch mạng (5,6). Tế bào cứng (5) thành dày đứng riêng lẻ hay tụ lại thành từng đám 4 - 5 tế bào. Mảnh mạch mạng (6) (hình 4).

Phân bố

Bát giác liên mọc nhiều ở núi cao, chỗ ẩm mát, ven suối Lai Châu, Lào Cai, Hà Giang, Vĩnh Phúc, Hoà Bình, Ninh Bình, Hà Nội. Cây tái sinh chủ yếu tự nhiên từ hạt. Thân rễ cũng có khả năng phân nhánh, hàng năm phần trên mặt đất tàn lụi, đồng thời tạo thành một "đốt củ" ở thân rễ. Căn cứ vào số đốt củ trên trục chính của thân rễ, có thể ước tính được tuổi của cây [7].

Hiện nay, do bị khai thác quá mức nên cây đã trở nên cực hiếm, rất nguy cấp, đã được ghi trong Sách Đỏ Việt Nam và cần được quan tâm nghiên cứu bảo tồn [6,8].

Kết luận

Căn cứ vào đặc điểm hình thái, cơ quan dinh dưỡng và cơ quan sinh sản đã xác định loài bát giác liên thu hái ở Vườn Quốc gia Ba Vì, Hà Nội có tên khoa học là *Podophyllum tonkinense* Gagn., thuộc họ Hoàng liên (Berberidaceae). Đây là lần đầu tiên các đặc điểm hình thái cấu tạo giải phẫu của loài *Podophyllum tonkinense* Gagn. được mô tả đầy đủ ở Việt Nam. Kết quảnghiên cứu góp phần đảm bảo tính đúng của cây thuốc, dược liệu và xây dựng tiêu chuẩn chất lượng dược liệu bát giác liên sử dụng làm thuốc ở nước ta.

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In vitro and *in silico* determination of glutaminyl cyclase inhibitors†

Phuong-Thao Tran, ‡*[a] Van-Hai Hoang,‡[b] Jeewoo Lee,[c] Tran Thi Thu Hien,[d] Nguyen Thanh Tung [e] and Son Tung Ngo *[fg]

Alzheimer's disease (AD) is the most common form of neurodegenerative disease currently. It is widely accepted that AD is characterized by the self-assembly of amyloid beta (Aβ) peptides. The human glutaminyl cyclase (hQC) enzyme is characterized by association with Aβ peptide generation. The development of hQC inhibitors could prevent the self-aggregation of Aβ peptides, resulting in impeding AD. Utilizing structural knowledge of the hQC substrates and known hQC inhibitors, new heterocyclic and peptidomimetic derivatives were synthesized and were able to inhibit the hQC enzyme. The inhibiting abilities of these compounds were evaluated using a fluorometric assay. The binding mechanism at the atomic level was estimated using molecular docking, free energy perturbation, and quantum chemical calculation methods. The predicted log(BBB) and human intestinal absorption values indicated that these compounds are able to permeate the blood–brain barrier and be well-absorbed through the gastrointestinal tract. Overall, 5,6-dimethoxy-*N*-(3-(5-methyl-1*H*-imidazol-1-yl)propyl)-1*H*-benzo[*d*]imidazol-2-amine (1_2) was indicated as a potential drug for AD treatment.

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Introduction

Alzheimer's disease (AD) is known to be one of the most critical types of dementia, affecting several million people worldwide. There are *ca.* 10 million patients arising annually.[1] The financial cost of treatment and Medicare for AD patients is rising rapidly.[1] AD is strongly associated with the self-aggregation of amyloid beta (Aβ) peptides,[2,3] which are a heterogeneous mixture of peptides having different solubility, stability, and biological and toxic properties.[3,4] These peptides are generated from the single-pass transmembrane amyloid precursor protein (APP) by several proteases at different sites. Several species of Aβ peptides have been observed, whereas $Aβ_{42}$ and $Aβ_{40}$ form the majority.[2] The self-assembly of Aβ peptides produces several products including random coils, oligomers, photofibrils, fibrils, and plaques.[5–8] This process results in the impairment of memory function and the loss of neurons and leads to synaptic dysfunction.[9–11]

Pyroglutamate Aβ peptides (AβpE) were found in the self-aggregation of Aβ peptides and act as initiators for Aβ accumulation. In fact, AβpE are only found in AD brains and constitute approximately 50% of the total Aβ.[12] Their formation is a multistep process requiring the loss of two or ten amino acids to expose the *N*-terminal glutamate at the third or eleventh position, followed by intramolecular dehydration of the exposed glutamate. The Aβ formation process results in the loss of three or six charges for Aβ3pE or Aβ11pE, respectively, leading not only to higher hydrophobicity but also to more rapid forming of β-sheet structures and, thus, greater stability and aggregation

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† Electronic supplementary information (ESI) available: Include Scheme 3, chemistry, additional computational approach. Additional figures involve the input of quantum chemical calculation to estimate detail of coordination link and force constant between Zn(II) and its ligands, the input of quantum chemical calculation to estimate atomic charges of the hQC active site including Zn(II) *via* RESP approach, the time dependence of RMSD of soluble complex over mimic time, the distribution of coordination links between Zn(II) and its ligands, the modeling of the quantum chemical calculation the interaction energy between compound 1_2 and the hQC active site including Zn(II), and the modeling of the quantum chemical calculation the interaction energy between compound 1_2 and the hQC active site without Zn(II). Additional table about comparison of molecular docking results of available inhibitors on hQC using Autodock Vina and Autodock4.2, the force constant between Zn(II) and its ligands, detail of available compounds, and the binding free energy of inhibitors 2_x to hQC enzyme. Additional data of ^{1}H NMR & MS spectra of the compounds. See DOI: 10.1039/c9ra05763c

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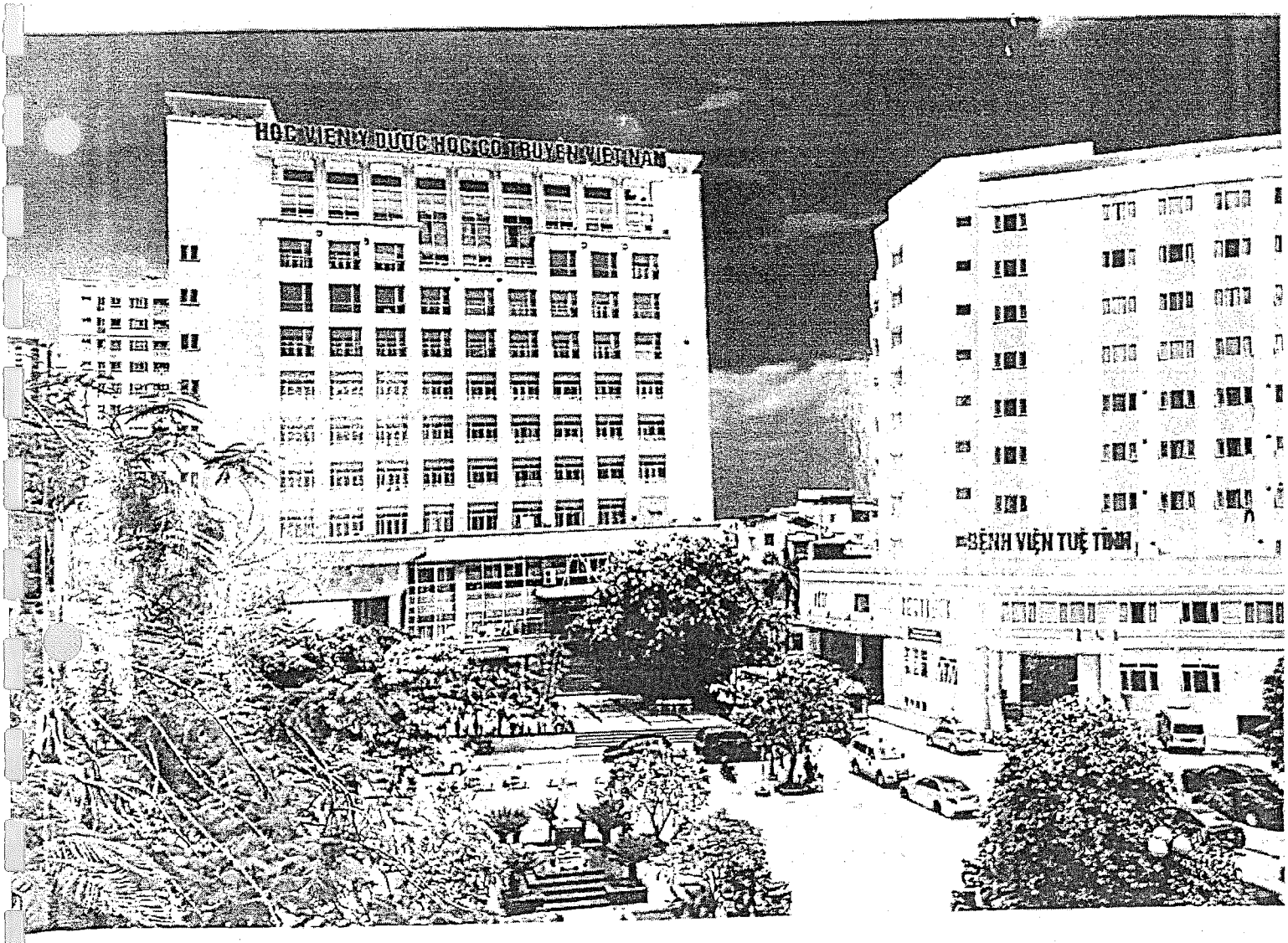

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Định lượng alkaloid trong phần trên mặt đất của cây củ dòm *(Stephania dielsiana Y.C.Wu)* bằng sắc ký lỏng hiệu năng cao

DETERMINING THE AMOUNT OF ALKALOID IN THE UPPER PART OF STEPHANIA DIELSIANA Y.C.WU BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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TÓM TẮT

Oxostephanin là một trong những thành phần chính của cây củ dòm – Stephania dielsiana Y.C.Wu, đã được chiết xuất, phân lập và định lượng bằng HPLC. Oxostephanin được trong dược liệu chiết xuất bằng methanol. Phương pháp định lượng có độ đúng và độ chính xác cao. Đường tuyến tính được đánh giá trong khoảng 3,125 – 100 µg/ml với hệ số tương quan 0,9993. Phương pháp này phù hợp để xác định oxostephanin trong cây củ dòm và áp dụng hiệu quả trong tối ưu hóa chiết xuất, phân lập oxostephanin từ S. dielsiana.

SUMMARY

Oxostephanin, as one of the active component from Stephania dielsiana Y.C.Wu, was isolated and analyzed by HPLC. Oxostephanin was extracted using methanol. The proposed method was fully validated proving acceptable prescision and accuracy. A good linearity was observed in concentration range of 3,125 – 100µg/ml with a correlation coefficient of 0,9993. The method was suitable for determination of oxostephanin in Stephania dielsiana, and effectively applicable to following oxostephanin concent in optimzing the isolation and purification of oxostephanin from S. dielsiana.

Keywords: Stephania dielsiana, oxostephanin.

ĐẶT VẤN ĐỀ

Củ dòm (*Stephania dielsiana* Y.C.Wu, họ Tiết dê – Menispermaceae)[1] là cây thân leo nhỏ, cao 2 – 3m. Rễ phình thành củ. Cành non nâu nhạt, khi già nâu xám. Cây có nhựa màu đỏ. Củ dòm được dân tộc Dao ở Ba Vì dùng để chữa đau dạ dày, phong thấp. Năm 2009, Nguyễn Quốc Huy, Phạm Thanh Kỳ, Nguyễn Mai Hương[3] đã công bố oxostephanin gây trên 3 dòng ung thư gan, ung thư phổi và ung thư màng tim. Năm 2010, Nguyễn Quốc Huy[2] đã công bố phân lập được 3 chất từ cây củ dòm là L-tetrahydropalmatin, dehydrocrebain

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Bảng 4. Kết quả khảo sát độ lặp lại

STT	Khối lượng (g)	Diện tích pic (mAU.s)	Hàm lượng (%)
1	0,5001	1424964	0,281
2	0,5002	1444767	0,285
3	0,5007	1475847	0,291
4	0,5001	1465971	0,289
5	0,5003	1447025	0,286
6	0,5001	1414563	0,279
Trung bình hàm lượng (%)			0,285
RSD (%)			1,605

Độ chính xác trung gian

Tiến hành phân tích 6 mẫu thử độc lập vào các ngày khác nhau, xác định hàm lượng oxostephanin. Kết quả ghi trong bảng 5.

Bảng 5. Kết quả độ chính xác trung gian

STT	Khối lượng (g)	Diện tích pic (mAU.s)	Hàm lượng (%)
1	0,5001	1424964	0,281
2	0,5002	1444767	0,285
3	0,5005	1447125	0,280
4	0,5001	1414563	0,280
5	0,4999	1414231	0,280
6	0,5001	1414565	0,286
Trung bình hàm lượng (%)			0,282
RSD (%)			0,977

Áp dụng phương pháp HPLC đã thẩm định để định lượng oxostephanin trong một số mẫu

Kết quả trình bày trong bảng 6.

Bảng 6. Kết quả định lượng oxostephanin trong các mẫu thân, lá cây cù dòm thu hái ở Ba Vì (Hà Nội) và Quản Bạ (Hà Giang)

Mẫu thu hái	Hàm lượng (%)
Lá thu hái ở Quản Bạ	0,32
Thân thu hái ở Quản Bạ	0,04
Lá thu hái ở Ba Vì	0,34
Thân thu hái ở Ba Vì	0,08

KẾT LUẬN

Trong quá trình nghiên cứu phương pháp chiết xuất, việc định tính và định lượng các hoạt chất là không thể thiếu để đánh giá được hiệu suất chiết, từ đó định hướng cải thiện và nâng cao hiệu suất chiết. Vì vậy, việc xây dựng quy trình phân tích phù hợp với điều kiện có sẵn là hết sức quan trọng. Trong nghiên cứu này, đề tài đã xây dựng phương pháp định lượng oxostephanin đơn giản có độ đúng và độ chính xác cao. Phương pháp đã áp dụng để định lượng oxostephanin trong một số mẫu thân lá củ dòm với hàm lượng oxostephanin trong lá cao hơn ở thân đồng thời có sự khác biệt không lớn về hàm lượng oxostephanin giữa 2 vùng trồng dược liệu.

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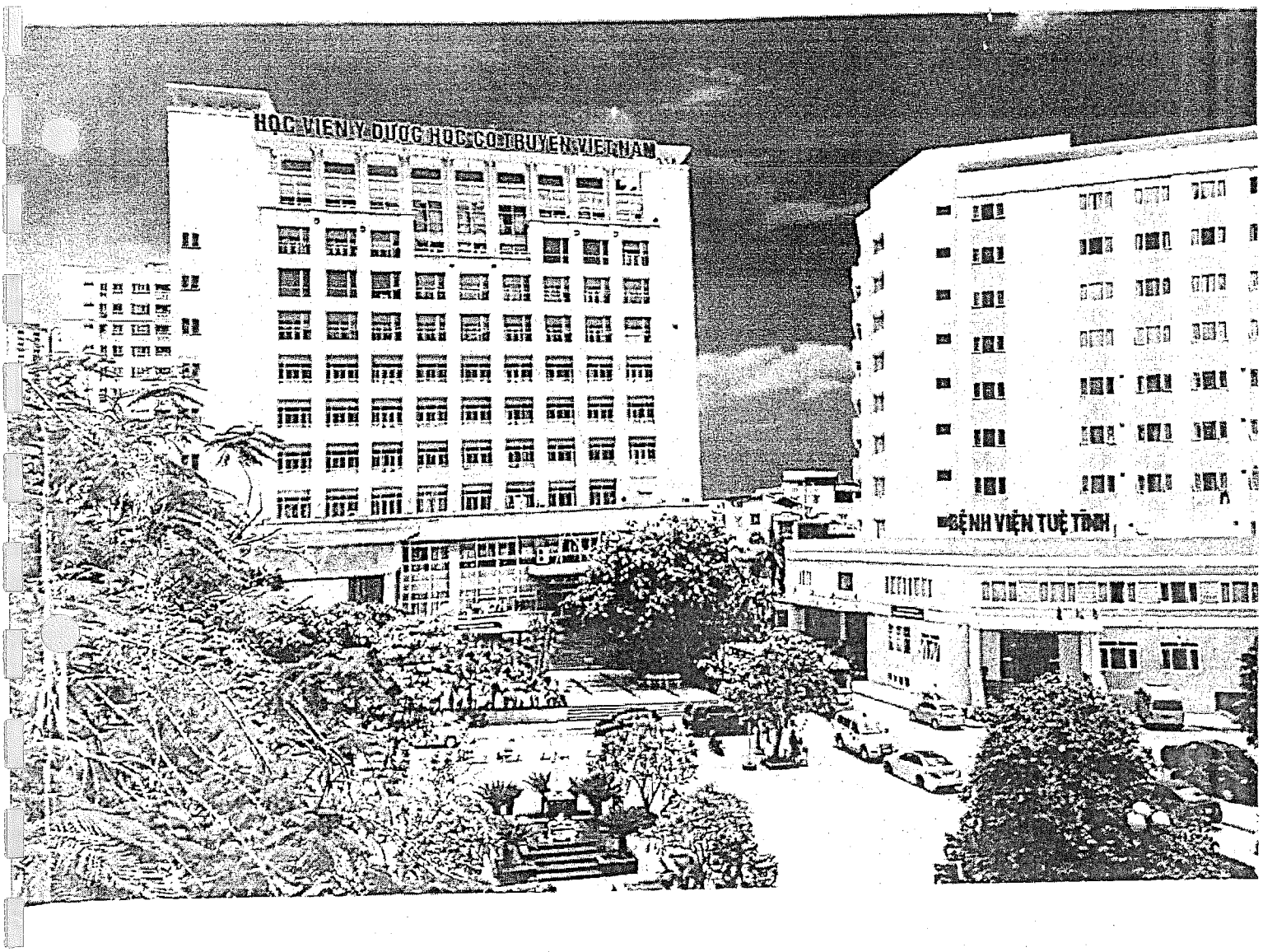

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BÀI NGHIÊN CỨU

Nghiên cứu định lượng rutin trong một số dạng chế biến của nụ hòe *(Flos Styphnolobii japonici)* bằng phương pháp HPLC

RESEARCH ON THE AMOUNT OF RUTIN IN SOME FORRM OF PROCESSING FLOS STYPHNOLOBII JAPONICI BY HPLC METHOD

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TÓM TẮT

Nghiên cứu đã định lượng rutin trong nụ hòe bằng hệ thống HPLC SHIMADZU với pha động là methanol – acid acetic 1% (32:68). Các chỉ tiêu thẩm định bao gồm: Độ đặc hiệu, tính thích hợp của hệ thống, khoảng tuyến tính và đường chuẩn, độ đúng, độ chính xác đều phù hợp với yêu cầu của Bộ Y tế. Do vậy, phương pháp này có khả năng ứng dụng để định lượng rutin trong một số dạng chế biến của nụ hòe.

SUMMARY

Research has quantified rutin in Flos Styphnolobii japonici by HPLC SHIMADZU system with mobile phase is methanol - 1% acetic acid (32:68). The evaluation criteria include: specificity, system suitability, linearity, range, accuracy and precision are consistent with the requirements of the Ministry of Health. Therefore, this method can be applied to quantify rutin in some form of processing Flos Styphnolobii japonici.

Keywords: *Flos Styphnolobii japonici, rutin, HPLC.*

ĐẶT VẤN ĐỀ

Nụ hòe là một vị thuốc y học cổ truyền rất phổ biến tại Việt Nam. Từ xa xưa, nụ hòe đã được sử dụng dưới các dạng sống, sao vàng, sao cháy, cao dược liệu,... để làm thuốc cầm máu, hạ huyết áp và điều trị bệnh đau thắt động mạch vành [4]. Thành phần quan trọng của nụ hòe là các flavonoid, trong đó rutin là flavonoid chính. Rutin có tác dụng làm bền và giảm tính thấm của mao mạch, làm tăng sự bền vững của hồng cầu, làm giảm trương lực cơ

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hóa học theo "Hướng dẫn của ASEAN về thẩm định quy trình phân tích" và hoàn toàn phù hợp để đánh giá hàm lượng rutin trong các dạng chế biến của nụ hòe.

Nghiên cứu đã đánh giá được hàm lượng rutin trong nụ hòe sống là 28,15%, đạt yêu cầu theo Dược điển Việt Nam V (hàm lượng rutin không ít hơn 20,0% tính theo dược liệu khô kiệt) [1]. Việc nghiên cứu định lượng rutin trong nụ hòe cũng đã được tiến hành trong một số đề tài trước đó. Bằng phương pháp cân thu được hàm lượng rutin trong nụ hòe khoảng 30,63%, song phương pháp này thường gặp sai số. Cũng có nghiên cứu đã tiến hành định lượng rutin trong nụ hòe sống bằng phương pháp HPLC thu được hàm lượng rutin trong nụ hòe sống là 28,78%. Tuy nhiên, phương pháp này sử dụng dung môi pha động là tetrahydrofuran là một hợp chất hữu cơ dị vòng, có thể tạo thành các peroxid có khả năng bắt nổ cao khi tiếp xúc với oxy và ánh sáng. Trong khi đó, đề tài sử dụng dung môi pha động là methanol: acid acetic, là các hóa chất ít có nguy cơ cháy nổ và thông dụng hơn trong các phòng thí nghiệm. Do vậy, phương pháp dùng trong đề tài có khả năng ứng dụng nhiều trong các phòng phân tích và trung tâm kiểm nghiệm. Nghiên cứu đã tiến hành đánh giá hàm lượng rutin trong nụ hòe sao vàng, kết quả thu được là 22,65% phù hợp với một nghiên cứu khác đã tiến hành bán định lượng rutin trong nụ hòe sao vàng bằng phương pháp HPLC với hàm lượng là 22,73%. Hiện nay, chưa có nghiên cứu nào đánh giá hàm lượng rutin trong các cao chiết từ nụ hòe sao vàng bằng phương pháp HPLC, đề tài đã tiến hành định lượng và thu được kết quả hàm lượng rutin trong các cao chiết từ nụ hòe sao vàng bằng dung môi ethanol 30% và 70% lần lượt là 30,35% và 37,03%.

KẾT LUẬN

Nghiên cứu đã tiến hành thẩm định phương pháp sắc ký lỏng hiệu năng cao dùng để định lượng rutin với các tiêu chí về: độ đặc hiệu, tính thích hợp của hệ thống, khoảng tuyến tính và đường chuẩn, độ đúng, độ chính xác. Đã đánh giá hàm lượng rutin trong trong một số dạng chế biến của nụ hòe:

- Hàm lượng rutin trong nụ hòe sống là: 28,15%.
- Hàm lượng rutin trong nụ hòe sao vàng là: 22,65%.
- Hàm lượng rutin trong cao chiết từ nụ hòe sao vàng bằng dung môi ethanol 30% là: 30,35%.
- Hàm lượng rutin trong cao chiết từ nụ hòe sao vàng bằng dung môi ethanol 70% là: 37,03%.

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CÂY KIM NGÂN
Lonicera japonica Thunb.
Họ Kim ngân - Caprifoliaceae

Photo by Nguyễn Hoàng Tuấn

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(Ngày nhận bài: 22/12/2019 - Ngày phản biện: 02/01/2020 - Ngày duyệt đăng: 06/3/2020)

Sàng lọc mảnh liên kết với ion kẽm trong nghiên cứu phát triển thuốc mới ức chế glutaminyl cyclase hướng điều trị bệnh Alzheimer

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Summary

As the zinc ion is a cofactor of enzyme glutaminyl cyclase playing an important role in the catalytic action of the enzyme, and on the other hand, all of glutaminyl cyclase inhibitors have been designed from fragments that bind to zinc ion, but so far, the only imidazole, benzimidazole, and phenol have been used as zinc-binding fragments of glutaminyl cyclase inhibitors, ... to find out new zinc-binding groups of glutaminyl cyclase inhibitors, in this study 32 zinc-binding fragments were selected, synthesized and estimated the glutaminyl cyclase inhibitory activity. Of these, two fragments (2b, 7b) showed good IC_{50} values and ligand efficiency and as such, promising for design and development of novel QC inhibitors.

Keywords: *Zinc binding, glutaminyl cyclase, inhibitors, IC_{50}.*

Đặt vấn đề

Alzheimer là căn bệnh thường gặp ở người cao tuổi với biểu hiện đặc trưng về suy giảm trí nhớ, suy nghĩ, hành vi, cùng các tổn thương không hồi phục não bộ. Bệnh ảnh hưởng nghiêm trọng tới chất lượng cuộc sống của người bệnh và cả bản thân người chăm sóc bệnh nhân khi phải trải qua những cảm xúc căng thẳng trầm trọng có thể dẫn tới trầm cảm.

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Định lượng paeoniflorin trong cao đặc tiêu dao bằng sắc ký lỏng hiệu năng cao

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Summary

An HPLC method was proposed for quantification of paeoniflorin in the viscous aqueous estracts (Extractum spisum) from the herbal remedy of "Tieu dao" (which is composes of: Radix Bupleuri; Radix Angelicae sinensis; Radix Paeoniae lactiflorae; Rhizoma Atractylodis macrocephlae; Poria; 6-Radix et Rhizoma Glycyrrhizae; Herba Menthae and Rhizoma Zingiberis Recens by hot aquaeous extraction. The chromatography was established as: Column - C_{18} (250 x 4.6 mm; 5 µm); Detector - UV (230 nm); Mobile phase - acetonitrile: 0.1 % phosphoric acid solution (14:86, v/v); Flow rate - 1.0 ml/min; Column temperature - 40 °C; Injection volume - 10 µl. The method was validated for the specificity (RSD of peak area = 0.33; RSD of t_R = 0.46); linearity (R = 0.9999) and accuracy (recovery: 97.22 % - 100.04 %). As for practical application, the tested samples showed the content of paeoniflorin ranging at 1.15 ± 0.07 %.

Keywords: *Tieu dao, Extractum spisum, paeoniflorin, HPLC.*

Đặt vấn đề

Phương thuốc tiêu dao gồm các vị thuốc: Sài hồ, đương quy, bạch thược, bạch truật, bạch linh, cam thảo, bạc hà và sinh khương có công năng sơ can kiện tỳ, dưỡng huyết, điều kinh; dùng trong trường hợp can uất huyết hư gây đau hai bên sườn, đau đầu, mờ mắt, chán ăn, chướng bụng, tiêu chảy mạn tính; tinh thần mệt mỏi, trầm cảm; hồi hộp, bứt rứt; kinh nguyệt không đều, đau bụng kinh, bầu vú căng tức; phụ nữ tiền mãn kinh có cơn bốc hoả, huyết áp dao động; hội chứng tắt dục ở nam và nữ; viêm gan virus, viêm túi mật mạn tính, sỏi mật... [1]. Cao đặc được bào chế từ phương thuốc này là bán thành phẩm để bào chế một số sản phẩm như thuốc cốm, viên nang ... Việc nghiên cứu tiêu chuẩn hoá và xác định được hàm lượng hoạt chất trong cao đặc là cần thiết. Paeoniflorin là một hợp chất monoterpenoid glycosid và là hoạt chất chính của bạch thược, có tác dụng chống viêm, điều hòa miễn dịch [2], hạ lipid huyết [3], bảo vệ thần kinh [4], chống loãng xương [5], chống ung thư [6],... Một số tài liệu đã công bố phương pháp định lượng paeoniflorin trong dược liệu bạch thược [7,8] và trong một số dạng bào chế phương thuốc tiêu dao như thuốc cốm, viên nang, viên hoàn, viên nén [8], nhưng chưa có nghiên cứu định lượng chất này trong cao đặc phương thuốc. Nghiên cứu này được thực hiện nhằm xây dựng phương pháp định lượng bằng sắc ký lỏng hiệu năng cao và xác định hàm lượng paeoniflorin trong cao đặc phương thuốc tiêu dao làm căn cứ để xây dựng tiêu chuẩn chất lượng của cao này.

Nguyên liệu và phương pháp

Nguyên liệu

Các vị thuốc được cung cấp bởi Công ty cổ phần Dược phẩm VCP: Sài hồ, đương quy, bạch thược, bạch truật, bạch linh, cam thảo, bạc hà đạt Dược điển Việt Nam V [7], sinh khương đạt Dược điển Trung Quốc 2015 [8].

Cao đặc phương thuốc tiêu dao: Các vị thuốc được chiết bằng nước nóng, cô đến thể chất cao đặc (độ ẩm < 20%).

Cao đặc placebo: Chuẩn bị như cao đặc phương thuốc tiêu dao, bỏ bạch thược.

Hoá chất, chất chuẩn

Chất chuẩn paeoniflorin ($C_{23}H_{28}O_{11}$): hàm lượng: 99,30 % (Chengdu Must Bio-Technology Co. Ltd., Lot No. MUST-17031901).

Hoá chất, dung môi: Methanol, acetonitril, acid phosphoric đạt tiêu chuẩn tinh khiết phân tích dùng cho ***HPLC*** (Merck), nước cất 2 lần.

Thiết bị, dụng cụ

Máy sắc ký lỏng hiệu năng cao (***HPLC***) Shimadzu bao gồm: Bơm LC-30AD, detector mảng diod (DAD)

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của: [(4',5,7-trihydroxyflavon 8-C-glucosid (vitexin) (**CE1**), 3',4',5,7-tetrahydroxyflavon (luteolin) (**CE2**), 4',5,7-trihydroxyflavon (apigenin) (**CE3**), luteolin 6-C-β-D-glucosid (isoorientin) (**CE4**), apigenin 6-C-β-D-glucosid (isovitexin) (**CE5**)]. Các hợp chất này lần đầu tiên được phân lập được từ cây môn nước (*Colocasia esculenta* (L.) Schott) ở Việt Nam.

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Nghiên cứu tác dụng hạ huyết áp trên thực nghiệm của một số dạng chế biến theo y học cổ truyền từ nụ hòe (*Styphnolobium japonicum* (L.) Schott)

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Summary

The blood pressure lowering effect of Flos Styphnolobii japonici imaturi (Styphnolobium japonicum (L.) Schott) was experimentally evaluated in cortisone-induced hypertensive white rats. 90 white rats were randomly divided into 9 lots. The rats in the control and model lot were treated with saline; those of positive lot were administered hydroclothrothiazid, each of the 6 trial lots was orally administered by one of the 6 types of extracts from 3 kinds of material - fresh, stir-fried and carnonizing stir-fried one; and each was extracted with two different solvents - 30% and 70% ethanol. Experimentally, all the tested dry extracts decreased the blood pressure and exhibted no influence on heart rate. Dry ethanol extracts of Flos Styphnolobii japonici imaturi (extracted with 30% ethanol) significantly improved systolic, diastolic and average blood pressures in comparison with those indexes in model and positive lots.

Keywords: *Blood pressure lowering effect, flos styphnolobii japonici.*

đang phát triển, bệnh có xu hướng ngày càng gia tăng và trở thành một vấn đề sức khỏe cộng đồng nghiêm trọng. Để kiểm soát huyết áp, bệnh nhân phải sử dụng thuốc hàng ngày và suốt đời. Xu hướng mới hiện nay là sử dụng các sản phẩm có nguồn gốc từ dược liệu để có thể kiểm soát huyết áp trong thời gian dài mà ít gây ra tác dụng không mong muốn.

Hòe là một dược liệu được sử dụng lâu đời trong y học cổ truyền với nhiều công dụng, có tác dụng tốt trên tim mạch và được sử dụng nhiều với tác dụng bình can hạ áp. Hiện nay cả trên thế giới và Việt Nam đều chưa có nghiên cứu về tác dụng hạ huyết áp của hòe trên thực nghiệm.

Đề tài này đã thực hiện đánh giá tác dụng hạ áp của các cao chiết từ nụ hòe trên chuột cống chủng *Wistar* được gây tăng huyết áp bằng cortison acetat liều 2,5 mg/kg và uống nước muối NaCl 1%. Kết quả thu được cho thấy cả 6 loại cao chiết thu được từ nụ hòe đều cho tác dụng hạ áp, trong đó cao chiết từ nụ hòe sao vàng bằng dung môi ethanol 30% làm giảm rõ rệt huyết áp tâm thu, huyết áp tâm trương, huyết áp trung bình (giảm lần lượt là 29,90%, 20,38% và 24,14%) so với lô mô hình. So với lô chứng dương uống hydrochlorothiazid, cao chiết từ nụ hòe sao vàng bằng dung môi ethanol 30% (SV-T1B) cũng cho thấy tác dụng hạ huyết áp tương đương. Kết quả nghiên cứu cũng cho thấy, các cao chiết từ nụ hòe không làm ảnh hưởng đến nhịp tim chuột, vì vậy có thể sử dụng lâu dài để điều trị tăng huyết áp.

Kết quả nghiên cứu cũng cho thấy các cao chiết từ nụ hòe bằng dung môi ethanol 30% cho tác dụng hạ huyết áp tốt hơn so với các cao chiết từ dung môi ethanol 70%. Dung môi ethanol 30% có nhiều đặc tính giống nước hơn dung môi ethanol 70%, vậy việc dùng nước để chiết xuất nụ hòe theo kinh nghiệm dân gian là tương đối phù hợp. Tuy nhiên, việc dùng nước chiết xuất có nhiều nhược điểm như dịch chiết thu được lẫn nhiều tạp chất khác nhau, khó bào chế cao, cao khó bảo quản nên đề tài dùng ethanol thay thế để khắc phục các nhược điểm đó.

Kết luận

Các cao chiết từ nụ hòe liều tương đương 1,2 g dược liệu/kg/ngày đều cho thấy tác dụng hạ huyết áp tâm thu, huyết áp tâm trương, huyết áp trung bình. Trong đó, mẫu cao chiết từ nụ hòe sao vàng bằng dung môi ethanol 30% (SV – T1B) cho tác dụng hạ huyết áp tâm thu, huyết áp tâm trương, huyết áp trung bình tốt nhất với các tỷ lệ tương ứng là 29,90%, 20,38% và 24,14% so với lô mô hình.

Các cao chiết từ nụ hòe liều tương đương 1,2 g dược liệu/kg/ngày không làm ảnh hưởng đến nhịp tim chuột.

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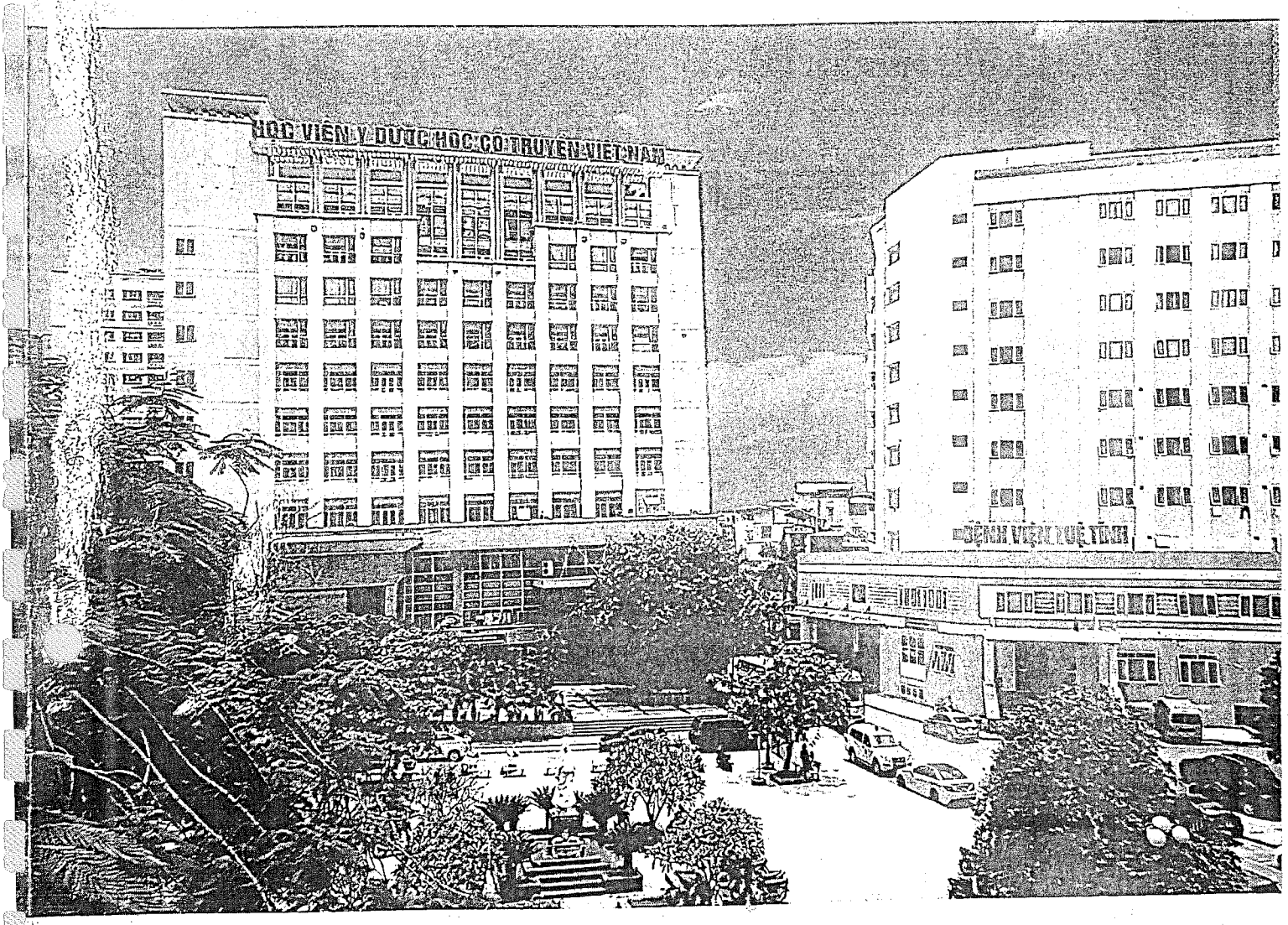

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Nghiên cứu xác định nhóm chất chuẩn có tác dụng sinh học liên quan tới điều chỉnh rối loạn lipid trong bài thuốc "Hạ mỡ NK"

DETERMINATION OF QUALITY COMPONENTS OF A POLYHERBAL FORMULATION HAMONK FOR DYSLIPIDEMIA

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TÓM TẮT

Bài thuốc Hạ mỡ NK là bài thuốc do nguyên viện trưởng Học viện Y dược học cổ truyền Tuệ Tĩnh sưu tầm và phát triển gồm hòe hoa, lá sen, trần bì, ngưu tất, rễ cỏ tranh, và hạ khô thảo có hiệu quả cao trong điều trị chứng rối loạn lipid trên lâm sàng. Hàm lượng tổng flavonoid theo quercetin, tổng saponin tính theo acid oleanoic và tổng polysaccharid theo D-Glucose trong cao chiết nước sắc của bài thuốc lần lượt là 1,11; 0,56; và 8,75 % dựa trên phương pháp định lượng UV-VIS. Thiết kế thí nghiệm giai thừa 2^3 được thiết lập nhằm biến thiên hàm lượng các nhóm chất trên trong cao chiết theo yếu tố thời gian, nhiệt độ và tỷ lệ rắn/lỏng. Sau khi phân tích tương quan hàm lượng các chất và giá trị IC_{50} thử hoạt tính chống oxy hoá DPPH của các cao chiết trong thí nghiệm giai thừa, nhóm flavonoid được xác định là chất đối chiếu sinh học có liên hệ tới tác dụng điều chỉnh lipid của bài thuốc này. Kỹ thuật trong nghiên cứu này có thể áp dụng để xác định chất đánh dấu hóa học lý tưởng trong dược liệu hay bài thuốc y dược cổ truyền một cách nhanh chóng, rẻ tiền và dễ thực hiện mà không cần phải tách chiết hay tinh khiết từng chất.

Từ khóa: Hạ mỡ NK, nhóm chất đánh dấu hoá học, chất đánh dấu sinh học, factorial experimental design.

ABSTRACT

HAMONK as a polyherbal formulation including S. japonicum, N. nucifera, Citrus spp, A. bidentata, I. cylindrica, and P. vulgaris by Prof. Nguyen Kieu's development has been used in clinical practice for dyslipidemia. The conventional water extraction of this formulation had 1.1, 0.56, and 8.75 % of chemical markers as total

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hóa học của flavonoid. Với cấu trúc hệ vòng thơm, flavonoid đã được chứng minh có thể ức chế các gốc tự do theo cơ chế chuyển nguyên tử, cơ chế chuyển proton – mất electron hay cơ chế mất proton – chuyển electron. Trên các mô hình *in vitro, in vivo* flavonoid cũng được quan sát thấy khả năng quét gốc tự do với IC_{50} tương đối thấp.

Chú ý sâu hơn về kết quả, hệ số tương quan trong phân tích này không ngụ ý về quan hệ nhân quả (dự đoán) tức là hàm lượng flavonoid tăng sẽ làm tăng giá trị IC_{50} mà chỉ phản ánh mức độ quan trọng mối liên hệ giữa hai giá trị so sánh. Cũng trong giới hạn của phương pháp này, giữa các nhóm chất hóa học sẽ coi như không có tương tác hiệp đồng hay ức chế ảnh hưởng tới giá trị IC_{50}

Thêm nữa, hàm lượng tổng flavonoid trong cao chiết không phải làm cao nhất nếu so với polysaccharide. Điều này chỉ ra thực tế nhiều khi chất chứa hàm lượng lớn lại không quyết định đến tính chất dược lý của bài thuốc dược liệu. Và nếu so sánh với các kỹ thuật gần đây về tìm kiếm chất đánh dấu sinh học trong bài thuốc cổ truyền hoặc dược liệu như Ligand fishing [13], hay Knockout Extract [14] đề cập trong phần đặt vấn đề, rõ ràng kỹ thuật trong nghiên cứu này có độ chính xác thấp hơn nhưng rẻ tiền, đơn giản và nhanh chóng thu đạt kết quả hơn.

KẾT LUẬN

Qua nghiên cứu này, các điều kiện chiết xuất của bài thuốc *hạ mỡ NK* gồm nhiệt độ, thời gian, và tỷ lệ rắn/lỏng thay đổi theo quy luật của thiết kế 2^3 đã tạo ra 8 cao chứa hàm lượng tổng flavonoid, saponin và polysaccharide khác nhau. Kết quả sau khi phân tích tương quan cho thấy nhóm flavonoid có tương quan mạnh tới tác dụng chống ôxy hóa DPPH của bài thuốc với hệ số Pearson bằng 0,65 ($p<0,1$). Từ đó, chỉ có tổng flavonoid trong 3 nhóm chất nghiên cứu được chọn là nhóm chất đánh dấu hóa học của bài hạ mỡ NK dựa trên phân tích thống kê. Kỹ thuật trong nghiên cứu này đơn giản, rẻ tiền và dễ dàng mở rộng ứng dụng cho các nghiên cứu khác về bài thuốc đa dược liệu.

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Design, synthesis and bioevaluation of novel 6-substituted aminoindazole derivatives as anticancer agents†

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In the present study, a series of 6-substituted aminoindazole derivatives were designed, synthesized, and evaluated for bio-activities. The compounds were initially designed as indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors based on the structural feature of five IDO1 inhibitors, which are currently on clinical trials, and the important anticancer activity of the indazole scaffold. One of them, compound *N*-(4-fluorobenzyl)-1,3-dimethyl-1*H*-indazol-6-amine (36), exhibited a potent anti-proliferative activity with an IC_{50} value of 0.4 ± 0.3 μM in human colorectal cancer cells (HCT116). This compound also remarkably suppressed the IDO1 protein expression. In the cell-cycle studies, the suppressive activity of compound 36 in HCT116 cells was related to the G2/M cell cycle arrest. Altogether, the current findings demonstrate that compound 36 would be promising for further development as a potential anticancer agent.

Introduction

Immunotherapy is a type of cancer treatment that exposes cancer cells and/or enhances the immune system to fight cancer cells along with chemotherapy, radiation and surgery. Although these approaches elicit advanced benefits cancer patients, drug resistance has become one of the big challenges in cancer treatment.[1–6] The resistance mechanisms are still under debate, but most scientists support the immunosuppressive tumor microenvironment concept.[7–10]

Indoleamine 2,3-dioxygenase 1 (IDO1) is one of the heme-containing enzymes involved in the immune system suppression process.[11] It catalyzes the oxidative ring-opening of tryptophan: the first and rate-limiting step of the kynurenine pathway.[12] IDO1 suppresses the immune system *via* the kynurenine pathway by two mechanisms: (1) tryptophan depletion and (2) toxicity of the metabolites in the kynurenine pathway. Tryptophan depletion inhibits T-cell proliferation and induces cell cycle arrest and the apoptosis of T lymphocytes, while tryptophan metabolites promote the activity of regulatory T cells, a types of T cell inhibits the maturation and cytotoxicity of a T cell.[12–14] IDO1 is an overexpression in different types of cancer cells and tumors, such as prostate, colorectum, pancreas, cervix, stomach, ovary and lung, and helps these cancers escape the immune system.[15,16] Prior studies have reported that high IDO1 expression could be related to inadequate outcome of chemotherapy, radiotherapy[17,18] and other immunotherapies.[10]

During the last decades, thousands of compounds have been identified as IDO1 inhibitors for cancer immunotherapy, but none of these was approved and brought to the market. Indoximod (D1MT, 1), PF-0684003 (2), novaximod (3), BMS-986205 (4), and epacadostat (5) (Fig. 1A) are five compounds

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EFFECTS OF HAMO NK HARD CAPSULE ON SERUM LIPID PROFILES IN DYSLIPIDEMIA EXPERIMENTAL ANIMALS

Pham Thuy Phuong[1], Pham Quoc Binh[1], Dinh Thi Hong Minh[1], Tran Thi Thu Hien[1], Nguyen Trong Thong[3], Pham Thi Van Anh[2] and Dang Thi Thu Hien[2,✉]

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[2]Hanoi Medical University,
[3]Phenikaa University

Dyslipidemia is a major risk factor for cardiovascular disease. Polyherbal formulation is a traditional therapeutic strategy used to treat dyslipidemia over many years of tradition. The aim of this study was designed to evaluate the effects of Hamo NK hard capsule on endogenous dyslipidemia and exogenous dyslipidemia experimental animal model. In endogenous hyperlipidemia model, mice were previously treated by Hamo NK hard capsule, and intraperitoneally injected by poloxamer - 407 to induce hyperlipidemia. Rats were oral administration of oil - cholesterol mixture and Hamo NK for 4 consecutive weeks (exogenous dyslipidemia). Parameters of serum lipid were determined. Hamo NK ameliorated the elevation of serum total cholesterol, Non - HDL - cholesterol at the daily dose of 1.5g/kg b.w ($p < 0.05$). Also, there was no signicant difference in increase on high - density lipoprotein cholesterol levels and decrease triglyceride levels between the groups. Hamo NK at two doses of 0.25g/kg b.w and 0.75g/kg b.w significantly reduced serum LDL - C levels compared to the cholesterol control group. Hamo NK hard capsule affected on serum lipid modulations in dyslipidemia models.

Keywords: Hamo NK, dyslipidemia, serum lipid levels.

I. INTRODUCTION

Polyherbal formulation is a traditional therapeutic strategy that takes advantage of the combination of several medicinal herbs to achieve enhanced therapeutic effects against a disease.[1] The use of herbal medicines is globally increasing tremendously and about 8% of the world population rely on it for some part of their primary healthcare.[2] Dyslipidemia refers to excess status of fatty substances including cholesterol, triglyceride and decreased high - density lipoprotein cholesterol (HDL - C) in the bloodstream.[3] Besides, dyslipidemia is a notable risk factor for the development of cardiovascular disease (CVD), which is the main cause of mortality worldwide, one of the most important risk factors for cardiovascular diseases (CVD) such as atherosclerosis, myocardial infarction, and cerebral vascular accidents.

At present, statins, such as simvastatin, atorvastatin, rosuvastatin...are the most commonly used lipid - lowering drugs, as they efficiently reduce plasma lipids; however, they also present a number of undesirable side effects, such as hepatotoxicity, rhabdomyolysis and skeletal muscle injury, which have limited their usage.[4] Therefore, it is necessary to identify and develop effective, and natural agents that may be valuable in regulating lipid metabolism. In recent years, traditional Vietnamese medicine has attracted greater attention in metabolic syndrome treatments, and has become a common therapy for controlling

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BÀI NGHIÊN CỨU

Nghiên cứu áp dụng phương pháp tinh chế hệ dung môi cồn muối làm giàu saponin từ ngưu tất

STUDY OF APPLYING SALT ALCOHOL SOLVENT PURIFICATION METHOD ENRICHED FROM "*ACHYRANTHES ASPERA* L."

Nguyễn Thị Khánh Huyền[1], Phạm Thủy Phương[1], Trần Thị Thu Hiền[1]
Hoàng Lê Sơn[2], Trần Minh Ngọc[2], Phạm Quốc Bình[1]
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TÓM TẮT

Điều kiện tinh chế saponin từ ngưu tất (Achyranthes bidentata Blume*) bằng phương pháp hệ hai pha cồn muối $(NH4)_2SO_4$ được xác định dựa vào thiết kế thí nghiệm đáp ứng bề mặt (RSM). Saponin toàn phần theo acid oleanolic định lượng bằng đo UV-Vis dựa trên phản ứng Rosenthaler. Kết quả cho thấy tại điều kiện tối ưu gồm khối lượng EtOH 34.4g, $(NH4)_2SO_4$ 18g và khối lượng nước 42.5g, saponin tập trung chủ yếu tại lớp trên của hai pha với khả năng thu hồi lên tới 83.79%. Áp dụng điều kiện này, cao ngưu tất ban đầu với hàm lượng saponin 7.5% được tinh chế tăng gấp đôi 15.5% (g/g). Trong khi đó, hàm lượng saponin trong cao khi áp dụng phương pháp cổ điển hai pha không đồng tan n-buntanol/H_2O chỉ khoảng 13.3%. Phương pháp này cho thấy hiệu quả cao trong việc làm giàu saponin, dễ nâng cấp quy mô, và dung môi thân thiện với môi trường.*

ABSTRACT

The conditions of purification of saponin from Achyranthes bidentata Blume *by the aqueous two-phase system of salt alcohol $(NH4)_2SO_4$ were determined based on the design of response surface nnethodology (RSM). Total saponin according to oleanolic acid quantified by UV-Vis measurement based on Rosenthaler reaction. The results showed that at the optimal condition including EtOH 34.4g, $(NH4)_2SO_4$ 18g and 42.5g water, saponin concentrated mainly in the top phase of two phases with recovery up to 83.79%. Applying this condition, the original sage with a refined 7.5% saponin content doubled 15.5% (g/g). Meanwhile, the concentration of saponin when applying the classical two-phase method of n-buntanol/H_2O is only about 13.3%. This method has been shown to be highly effective in enriching saponin, easy to scale up, and environmentally friendly solvents.*

ĐẶT VẤN ĐỀ

Ngưu tất với tên khoa học *Achyranthes bidentata* Blume (Họ Amaranthaceae) được phân phối rộng rãi ở các nước châu Á như Hàn Quốc, Nhật Bản và Trung Quốc. Từ lâu rễ ngưu tất đã được sử dụng trong các bài thuốc cổ truyền như một vị thuốc bổ,

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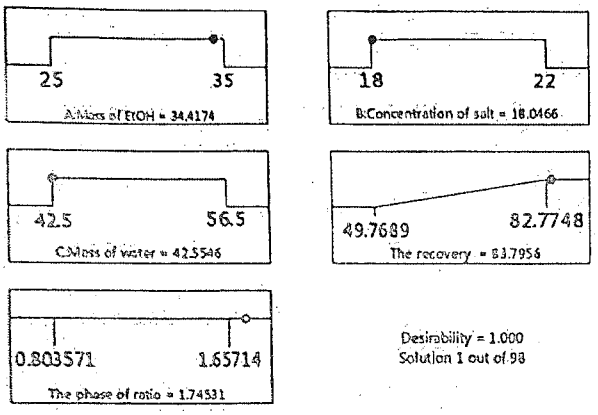

Hình 4. Điều kiện hệ dung môi tối ưu bằng RSM

Kết quả cho thấy tại điều kiện: $(NH4)_2SO_4$ 18g, EtOH 34.4g và nước 42.5g cho năng suất thu hồi cao nhất lên tới 82.77%. Phân tích ANOVA cũng cho thấy rằng năng suất thu hồi R là giá trị đáng tin cậy vì có giá trị p= 0.0303 < 0.05.

Tiến hành làm giàu saponin từ ngưu tất bằng hệ dung môi cồn muối đã được tối ưu kết quả thu được hàm lượng saponin trong cao lên tới 15.5%.

Thu hồi thành phần tạo pha

Pha trên được tách ra, đem cô quay thu được dung môi thu hồi là EtOH. Pha dưới đem tủa cồn tuyệt đối với các tỷ lệ $H = V_{cồn}/V_{pha\ dưới}$ từ 2 – 10 lần. Kết quả cho thấy tại H = 6 cho hiệu suất thu hồi muối lớn nhất đạt khoảng 94%.

KẾT LUẬN

Kết quả cho thấy ATPS hiệu quả trong việc làm giàu saponin từ ngưu tất. Tại điều kiện tối ưu gồm khối lượng EtOH 34.4g, $(NH4)_2SO_4$ 18g và khối lượng nước 42.5g, saponin tập trung chủ yếu tại lớp trên của hai pha với khả năng thu hồi lên tới 83.79%. Áp dụng điều kiện này, cao ngưu tất ban đầu với hàm lượng saponin 7.5% được tinh chế tăng gấp đôi 15.5% (g/g). Trong khi đó, hàm lượng saponin trong cao khi áp dụng phương pháp cổ điển hai pha không đồng tan n-butanol/H_2O chỉ khoảng 13.3%. Phương pháp này cho thấy hiệu quả cao trong việc làm giàu saponin, dễ nâng cấp quy mô, và dung môi thân thiện với môi trường.

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Xây dựng phương pháp định lượng và theo dõi độ ổn định của oxostephanin trong thân lá cây củ dòm (*Stephania Dielsiana* Y. C. Wu) trong thời gian bảo quản

METHOD FOR THE QUANTIFICATION AND MONITORING OF THE STABILITY OF OXOSTEPHANIN IN THE STEMS AND LEAVES OF *STEPHANIA DIELSIANA* Y. C. WU IN THE STORAGE TIME

Trần Thị Thu Hiền[1], Nguyễn Quốc Huy[1], Hoàng Lê Sơn[2]
Lê Thị Kim Vân[2], Đào Thị Diễm[1], Phạm Đoàn Anh Ninh[3]
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[3]Trường Cao đẳng Quân y 2 - Quân khu 7

TÓM TẮT

Phương pháp định lượng oxostephanin trong thân lá củ dòm (Stephania dielsiana Y.C.Wu) bằng phương pháp đo quang UV-Vis tại bước sóng hấp thụ cực đại 414nm. Phương pháp xây dựng đã được thẩm định, các chỉ tiêu đạt yêu cầu, đảm bảo phương pháp có thể áp dụng định lượng oxostephanin trong thân lá củ dòm.

Đã áp dụng phương pháp định lượng để theo dõi độ ổn định của oxostephanin trong quá trình bảo quản, sơ chế. Hàm lượng oxostephanin có sự thay đổi khi tiến hành sấy ở các nhiệt độ sấy khác nhau và trong thời gian bảo quản khác nhau. Kết quả cho thấy nhiệt độ sấy tối đa khuyến cáo là 60°C và dược liệu sau khi thu hái nên được chiết ngay hoặc tối đa khoảng 4 tuần bảo quản.

Từ khóa: Độ ổn định, Stephania dielsiana Y.C. Wu, UV-Vis.

SUMMARY

Quantitative method of oxostephanin in the stems and leaves of S.dielsiana by UV-Vis photometric method at the maximum absorption wavelength of 414 nm. The method has been validated, the criteria are satisfactory, ensuring that the method can be quantitatively applied to oxostephanin in the stems and leaves.

Quantitative method has been applied to monitor the stability of oxostephanin during storage and preliminary processing. The oxostephanin content changed when drying at different drying temperatures and during different storage times. The results show that the maximum recommended drying temperature is 60°C and the medicinal herbs should be extracted immediately or stored for up to 4 weeks

***Keywords:** độ ổn định, Stephania dielsiana Y.C. Wu, UV-Vis.*

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Bảng 7. Kết quả khảo sát sự thay đổi hàm lượng oxostephanin trong thân, lá củ dòm trong thời gian bảo quản

Thời gian bảo quản (tuần)	Hàm ẩm (%)	Khối lượng cân (g)	Độ hấp thụ A	Nồng độ (µg/ml)	Hàm lượng oxostephanin (%)	Tỷ lệ giảm hàm lượng oxostephanin (%)
1	3,769	1,0013	0,498	49,840	0,052	0
2	3,768	1,0015	0,490	49,168	0,051	1,92
4	3,669	1,0015	0,475	47,907	0,050	3,84
6	3,665	1,0018	0,419	43,204	0,045	13,46
8	3,650	1,0016	0,315	34,463	0,036	30,77
16	3,558	1,0012	0,159	21,354	0,022	57,69

Trong quá trình bảo quản dược liệu củ dòm ở điều kiện thường trong khoảng thời gian 16 tuần, nhận thấy hàm lượng oxostephanin đo được có giá trị giảm dần. Do vậy, dược liệu sau khi thu hái nên được chiết ngay hoặc tối đa khoảng 4 tuần bảo quản.

KẾT LUẬN

Phương pháp định lượng bằng phương pháp đp quang UV-Vis đã được xây dựng để định lượng hàm lượng oxostephanin trong thân, lá cây củ dòm.

Phương pháp xây dựng đã được thẩm định, các chỉ tiêu đạt yêu cầu, đảm bảo phương pháp có thể áp dụng định lượng oxostephanin trong thân, lá củ dòm.

Đã áp dụng phương pháp định lượng để theo dõi độ ổn định của oxostephanin trong quá trình bảo quản, sơ chế. Hàm lượng oxostephanin có sự thay đổi khi tiến hành sấy ở các nhiệt độ sấy khác nhau và trong thời gian bảo quản khác nhau. Kết quả cho thấy nhiệt độ sấy tối đa khuyến cáo là 60°C và dược liệu sau khi thu hái nên được chiết ngay hoặc tối đa khoảng 4 tuần bảo quản.

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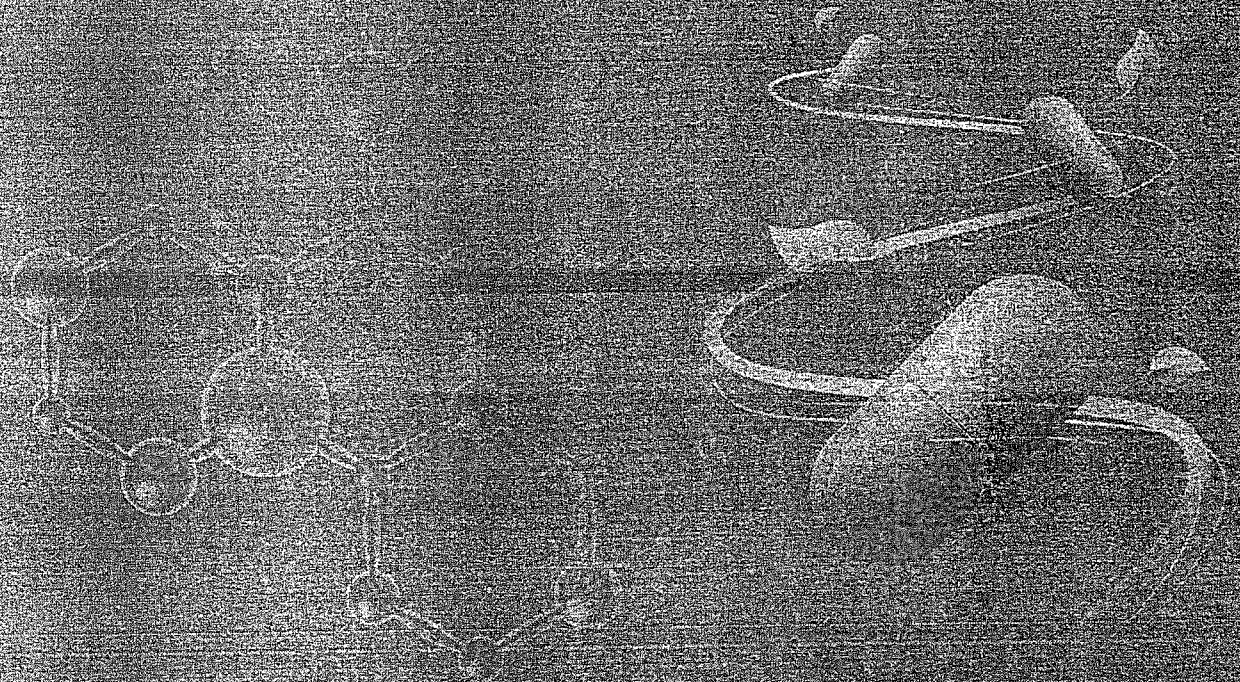

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45 **Đánh giá tác dụng của phương pháp xoa bóp bấm huyệt điều trị thoái hóa cột sống cổ tại trung tâm y tế huyện Tam Bình, tỉnh Vĩnh Long**

Evaluate the effect of acupuncture massage method in cervical spondylosis treatment at Tam Binh medical center, Vinh Long province.

[1]Mai Hồng Cẩm, [2]Trương Thị Ngọc Lan
[1]Trung tâm Y tế huyện Tam Bình
[2]Viện Y Dược dân tộc Thành phố Hồ Chí Minh

52 **Nguyên nhân chưa kiểm soát được đường huyết ở người bệnh đái tháo đường type 2 điều trị ngoại trú tại Bệnh viện Bạch Mai năm 2021**

Cause of uncontrolled glycemic outcomes in outpatient at Bach Mai hospital in 2021

Phan Thị Ngọc Anh[1], Nguyễn Thị Tuyến[2]
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59 **Đánh giá tác dụng điều trị bệnh Parkinson của các hợp chất trong cây Câu Đằng (Uncaria Rhynchophylla) bằng phương pháp docking phân tử**

Evaluation of the therapeutic effect on parkinson's disease of compounds from uncaria rhynchophylla by molecular docking method

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67 **Đánh giá thực trạng giao tiếp của điều dưỡng, kỹ thuật viên và nữ hộ sinh đối với người bệnh tại Bệnh viện Tuệ Tĩnh**

Assessment of communication status of nutritional, techniques and midwives for patients at tue tinh hospital

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73 **Nghiên cứu định lượng Mangostin trong vỏ quả Măng cụt (Pericarpium Garciniiae Mangostanae) bằng phương pháp quang phổ UV-VIS.**

Quatitative reseaches of mangostin in mangosteen (pericarpium garciniiae mangostanae) fruit rind by (uv- vis) spectrophotometry.

Lương Thị Lan, Bùi Khánh Chi, Lê Thị Thu Hà, Trần Thị Thu Hiền
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Nghiên cứu định lượng Mangostin trong vỏ quả Măng cụt (Pericarpium Garciniiae Mangostanae) bằng phương pháp quang phổ UV-VIS.

QUATITATIVE RESEACHES OF MANGOSTIN IN MANGOSTEEN (PERICARPIUM GARCINIIAE MANGOSTANAE) FRUIT RIND BY (UV- VIS) SPECTROPHOTOMETRY.

Lương Thị Lan, Bùi Khánh Chi, Lê Thị Thu Hà, Trần Thị Thu Hiền
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TÓM TẮT

Mục tiêu: *Xây dựng và thẩm định phương pháp định lượng mangostin trong vỏ quả Măng cụt (Pericarpium Garciniiae mangostanae) bằng quang phổ UV – Vis.*

Phương pháp nghiên cứu: *Định lượng mangostin bằng phương pháp UV-Vis dựa vào khả năng hấp thụ ánh sáng từ ngoại của mangostin và tiến hành thẩm định phương pháp theo tiêu chuẩn AOAC năm 2016.*

Kết quả nghiên cứu: *Xác định được hàm lượng mangostin trong vỏ Măng cụt là 9,82 ± 0,23% trong điều kiện chiết xuất tối ưu là: dung môi là ethanol, chiết bằng phương pháp siêu âm ở 60□C, trong thời gian 60 phút, tỷ lệ dược liệu/dung môi: 1/20 (g/ml), chiết 3 lần và tiến hành loại tạp tanin bằng gelatin tỷ lệ 7,5g/l. Tiến hành thẩm định phương pháp đã đáp ứng được các yêu cầu của một phương pháp phân tích: trong khoảng nồng độ đã khảo sát (2, 4, 8, 16, 20 µg/ml) có R^2= 0,9992; LOD= 0,1187 µg/ml; LOQ= 0,3955 µg/ml; RSD < 2,7% và độ thu hồi trong khoảng 97 – 103%.*

Từ khóa: *Mangostin, Măng cụt, UV-Vis*

SUMMARY

Objectives: *To vaildate a quantiative anlaysis procedure of mangostin in mangosteen fruit rind using UV-Vis methods.*

Research method: *Mangostin quantification by UV-VIS method based on mangostin's ability to absorb ultraviolet light and conduct method appraisal according to AOAC standards in 2016.*

Results: *Determining the mangostin content in mangosteen shells is 9.82 ± 0.23% under optimal extract conditions: Ethanol solvent, extracted by ultrasound method at 60□C, during the time 60 minutes, the ratio of material-solvent: 1/20 (g/ml), extracted 3 times and proceeded with tannin map with gelatin ratio of 7.5g/l. Conducting the appraisal of the method that meets the requirements of an analytical method: In the surveyed concentration range (2, 4, 8, 16, 20 µg/ml), R^2 = 0.9992; LOD = 0.1187 µg/ml; LOQ = 0.3955 µg/ml; RSD <2.7% and the recall in the rangle of 97 - 103%.*

Keywords: *mangostin, mangosteen, UV-Vis.*

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trong vỏ quả Măng cụt bằng phương pháp đo quang. Đây là phương pháp đơn giản, dễ thực hiện, giá thành không cao. Tuy vậy, phương pháp vẫn được chứng minh phù hợp với việc phân tích các chất có hàm lượng nhỏ. Kết quả nghiên cứu thu được hàm lượng mangostin là 9,82 ±0,23% tương đương với nghiên cứu của W.Pothitirat anhd W. Gristsanapan thực hiện thu được hàm lượng α-mangostin trong dịch chiết ethanol 95% nằm trong khoảng 8,36 ±0,17% đến 10,04 ±0,33 % khi định lượng bằng phương pháp HPLC [4].

Lựa chọn dung môi chiết xuất, năm 2016 Abdalrahim F. A. Aisha và cộng sự [5] đã thực hiện thu được toluen là dung môi chiết xuất thu được hàm lượng thu được hàm lượng xanthon cao nhất, tiếp đó là ethanol và cuối cùng là methanol. Tuy nhiên, do toluen là chất khá nguy hiểm và hàm lượng xanthon trong toluen và methanol không chênh lệch quá lớn. Nên nghiên cứu chọn ethanol 70% - "dung môi xanh" trong chiết xuất, thân thiện với môi trường và rẻ tiền làm dung môi chiết xuất.

Nghiên cứu cũng tiến hành phương pháp loại tạp tanin, chất gây nhiều sai số trong quá trình định lượng mà các nghiên cứu trước đó chưa làm.

Thẩm định phương pháp

Kết quả thẩm định cho thấy phương pháp đã đáp ứng được các yêu cầu của một phương pháp phân tích.

Kết quả thẩm định cho thấy phương pháp đã đáp ứng được các yêu cầu của một phương

pháp phân tích.

- Độ tuyến tính: Trong khoảng nồng độ đã khảo sát (2- 20 µg/ml) có R^2=0,9992 chứng tỏ sự tương quan tuyến tính giữa nồng độ của mangostin và độ hấp thụ khá chặt chẽ.

- Giới hạn phát hiện (LOD= 0,1187 µg/ml) và giới hạn định lượng (LOQ= 0,3955 µg/ml) khá thấp vì vậy có thể dùng để đánh giá sự có mặt và hàm lượng mangostin ở nồng độ rất nhỏ trong sản phẩm từ vỏ Măng cụt.

- Độ chính xác: được kiểm tra đạt yêu cầu AOAC với RSD< 2,7%, với độ lặp lại trong ngày và khác ngày đều có RSD < 2%.

- Độ đúng: được kiểm tra với tỉ lệ tìm lại trung bình=100,48% trong khoảng nồng độ tuyến tính đạt yêu cầu AOAC.

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Dual roles of oxostephanine as an Aurora kinase inhibitor and angiogenesis suppressor

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Abstract. The Aurora kinases, including Aurora A, B and C, play critical roles in cell division. They have been found overexpressed in a number of types of cancer and may thus be potential targets in cancer therapy. Several Aurora kinase inhibitors have been identified and developed. Some of these have been used in clinical trials and have exhibited certain efficacy in cancer treatment. However, none of these has yet been applied clinically due to the poor outcomes. Oxostephanine is an aporphine alkaloid isolated from several plants of the genus *Stephania*. This compound has been reported to inhibit Aurora kinase activity in kinase assays and in cancer cells. The present study aimed to investigate the real-time effects of oxostephanine extracted from *Stephania dielsiana* Y.C. Wu leaves on the growth of an ovarian cancer cell line (OVCAR-8, human ovarian carcinoma); these effects were compared to those of the well-known Aurora kinase inhibitor, VX-680. The effects of oxostephanine on stromal cells, as well as endothelial cells were also examined. The results demonstrated that oxostephanine was an Aurora kinase inhibitor through the prevention of histone H3 phosphorylation at serine 10, the mislocalization of Aurora B and the induction of aneuploidy. Moreover, this substance was selectively cytotoxic to human umbilical vein endothelial cells (hUVECs), whereas it was less cytotoxic to human fibroblasts and umbilical cord-derived mesenchymal stem cells. In addition, this compound significantly attenuated the migration and tube formation ability of hUVECs. Taken together, the present study demonstrates that oxostephanine plays dual roles in inhibiting Aurora kinase activity and angiogenesis. Thus, it may have potential for use as a drug in cancer treatment.

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Key words: Aurora kinases, Aurora kinase inhibitor, ovary cancer cell line, angiogenesis, endothelial cells, growth factors

Introduction

The Aurora kinases, including Aurora A, B and C, are serine/threonine kinases that play a central role in regulating cell division and multiple signaling pathways. Aurora A functions in the formation of a typical bipolar spindle (1), the maturation of centrosomes, which is necessary for G2/M transition (2), and the formation and stimulation of the cyclin B-CDK1 complex (3). Moreover, Aurora A helps to increase both size and microtubule-nucleating capacity just before mitotic entry (3). Aurora B plays a function in the chromosome biorientation on the mitotic spindle. It mediates the attachment of the microtubule to the kinetochores and regulates the spindle assembly checkpoint (SAC) (4,5). The improper attachment of kinetochores promotes Aurora B to recruit and phosphorylate its substrates at the kinetochores to depolymerize the uncorrected attachment, allowing other microtubules to capture the unattached kinetochores. The inhibition of Aurora B can impair the chromosome arrangement at the mitotic spindle equator (6).

Furthermore, Aurora B phosphorylates histone H3 at the serine 10 (H3S10ph) residue at the beginning of the prophase and leads to a peak in H3S10ph at the prometaphase and metaphase. This phosphorylation contributes to the active chromosome conformation at the entry of mitosis (7). Other studies have reported that H3S10ph may involve chromosome condensation and Aurora B recruitment to the centromere (8,9). Most notably, Aurora B is the only enzymatic member of the chromosomal passenger protein complex (CPC). All members of CPC share the co-localization during mitosis: They concentrate in the kinetochore

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Cytotoxic effects of aporphine alkaloids from the stems and leaves of *Stephania dielsiana* Y.C.Wu

Tran Thi Thu Hien, Vinh Le Ba, Nguyen Quoc Huy, Nguyen Phuong Thao, Seo Young Yang & Le Thi Kim Van

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Cytotoxic effects of aporphine alkaloids from the stems and leaves of *Stephania dielsiana* Y.C.Wu

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ABSTRACT

Phytochemical studies of the stems and leaves of *Stephania dielsiana* Y.C.Wu yielded two new aporphine alkaloids (**1** and **5**), along with six known alkaloids (**2**–**4** and **6**–**8**). Their structures were characterised based on analyses of spectroscopic data, including one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy and high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS). The cytotoxic activities of the isolated compounds against a small panel of tumour cell lines were assessed by MTS assay. Interestingly, compound **2** exhibited particularly strong cytotoxic activities against HepG2, MCF7 and OVCAR8 cancer cell lines, with IC_{50} values of 3.20±0.18, 3.10±0.06 and 3.40±0.007 μM, respectively. Furthermore, molecular docking simulations were carried out to explore the interactions and binding mechanisms of the most active compound (compound **2**) with proteins. Our results contribute to understanding the secondary metabolites produced by *S. dielsiana* and provide a scientific rationale for further investigations of cytotoxicity of this valuable medicinal plant.

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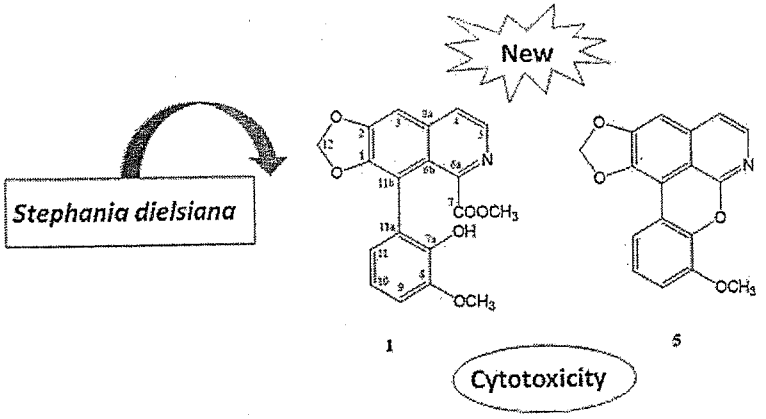

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MỘT SỐ HỢP CHẤT STEROID, TERPENOID VÀ ACID PHENOLIC PHÂN LẬP TỪ CÂY NHO RỪNG

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Tóm tắt

Năm hợp chất đã được phân lập từ phân đoạn *n*-hexan và ethyl acetat của cao chiết ethanol phần trên mặt đất cây nho rừng (*Vitis heyneana* Roem. & Schult.) bằng các phương pháp sắc ký. Trên cơ sở so sánh các đặc trưng lý hóa và các phổ NMR và MS kết hợp với các tài liệu tham khảo, cấu trúc hóa học của các hợp chất được xác định gồm dehydrocostus lacton (1), *β*-sitosterol (2), daucosterol (3), acid betulinic (4) và acid protocatechuic (5). Trong đó, hợp chất (1) lần đầu tiên được phân lập từ chi *Vitis*, hợp chất 4 và 5 lần đầu tiên được báo cáo từ loài *V. heyneana*.

Từ khóa: *Vitis heyneana, Dehydrocostus lacton, Acid betulinic, Acid protocatechuic.*

Summary

Steroids, Terpenoids and Phenolic Acid Isolated from *Vitis heyneana* Roem. & Schult.

Five compounds were isolated from the *n*-hexane and ethyl acetate fractions of the ethanol extract from the aerial parts of *Vitis heyneana* Roem. & Schult. by using various chromatographic methods. Based on physical and chemical properties and NMR and MS spectra, and by comparing to those of literature data, the chemical structures of the compounds were determined as dehydrocostus lactone (1), *β*-sitosterol (2), daucosterol (3), betulinic acid (4) and protocatechuic acid (5). Among them, compound 1 was isolated for the first time from the genus *Vitis*, and compounds 4 and 5 were reported from *V. heyneana* for the first time.

Keywords: *Vitis heyneana*, Dehydrocostus lactone, Betulinic acid, Protocatechuic acid.

1. Đặt vấn đề

Nho rừng hay còn gọi là nho lông, nho năm góc có tên khoa học là *Vitis heyneana* Roem. & Schult. tên đồng nghĩa *V. pentagona* Diels and Gilg, *V. quinquangularis* Rehder, thuộc họ Nho (Vitaceae). Nho rừng thường mọc hoang trên vùng núi đá vôi thuộc các tỉnh như Cao Bằng, Lạng Sơn, Quảng Ninh, Ninh Bình và Ninh Thuận [1]. Ở Campuchia, rễ nho rừng được dùng để ăn với trầu, dùng trị viêm phế quản, cũng dùng làm thuốc lợi tiểu hay phối hợp với rễ dứa (hãm hoặc sắc) để trị bệnh lậu [1]. Ở Trung Quốc, vỏ rễ nho rừng được dùng trị kinh nguyệt không đều và bạch đới, dùng ngoài trị đòn ngã tổn thương, gân cốt tê đau. Toàn cây dùng trị bệnh sởi, lá dùng trị bệnh lỵ và mụn nhọt sưng lở [1]. Cho đến nay, trên thế giới có rất ít nghiên cứu về nho rừng. Một số công bố về hóa học cho thấy 14 hợp chất bao gồm 7 hợp chất stilbenoid, 2 hợp chất cycloartan triterpenoid và cycloartan nortriterpenoid và 5 hợp chất megastigman đã được phân lập từ loài nho rừng *V. heyneana* [2],[3],[4],[5]. Tiếp nối các nghiên cứu trước về các hợp chất stilbenoid của phần trên mặt đất cây nho rừng ở Việt Nam [6],[7], trong báo cáo này, chúng tôi tiếp tục công bố kết quả phân lập và xác định cấu trúc của 5 hợp chất khác thuộc các nhóm steroid, terpenoid và acid phenolic từ phần trên mặt đất cây nho rừng.

2. Nguyên liệu và phương pháp nghiên cứu

2.1. Nguyên liệu

Mẫu nghiên cứu là phần trên mặt đất cây nho rừng mọc tự nhiên tại xã Bản Mế, huyện Si Ma Cai, tỉnh Lào Cai, được thu hái vào tháng 9/2016. Mẫu nghiên cứu được TS. Nguyễn Thế Cường - Viện Sinh thái và Tài nguyên sinh vật giám định tên khoa học là Vitis heyneana Roem. & Schult. họ Nho (Vitaceae), mang số hiệu TL07 và được lưu tại Phòng Tiêu bản Thực vật, Viện Sinh thái và Tài nguyên sinh vật, Hà Nội.

2.2. Dung môi, hóa chất

Các dung môi dùng trong chiết xuất, phân lập như ethanol (EtOH), methanol (MeOH), *n*-hexan, dicloromethan (DCM), ethyl acetat (EtOAc), aceton đều đạt tiêu chuẩn công nghiệp và được chưng cất lại trước khi dùng. Pha tĩnh dùng trong sắc ký cột là *silica gel* pha thường (0.040 - 0.063 mm, Merck), pha đảo RP-C_{18} (30 - 50 µm, FuJisilisa Chemical Ltd). Bản mỏng tráng sẵn DC-Alufolien 60 F_{254} (Merck) (*silica gel*, 0.25 mm) và bản mỏng pha đảo RP-18 F_{254S} (Merck, 0.25 mm). Phát hiện chất bằng đèn tử ngoại ở hai bước sóng 254 nm và 366 nm hoặc dùng thuốc thử là dung dịch H_2SO_4 10% trong EtOH 96% hơ nóng để phát hiện vết chất.

2.3. Thiết bị, dụng cụ

Các thiết bị dùng trong chiết xuất và phân lập bao gồm: Máy cất quay Rotavapor R-220, Rotavapor R-200 (Büchi); tủ sấy Memmert,

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SÀNG LỌC CÁC HỢP CHẤT ỨC CHẾ ENZYM α-GLUCOSIDASE CỦA CÂY XẤU HỔ TRÊN MÔ HÌNH *IN SILICO*

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Tóm tắt

Đái tháo đường có đặc trưng là tăng đường huyết do sự rối loạn bài tiết insulin từ tế bào β của tuyến tụy, hoạt động của insulin hoặc cả hai. Enzym α-glucosidase là một đích phân tử quan trọng trong điều trị bệnh đái tháo đường typ 2. Cây xấu hổ (*Mimosa pudica* Linn.) được chứng minh có tác dụng trong điều trị bệnh đái tháo đường. Trong nghiên cứu này, chúng tôi đánh giá khả năng ức chế enzym α- glucosidase của các hợp chất trong cây xấu hổ trên mô hình sàng lọc ảo. Dựa trên các công bố trước đây chúng tôi thu thập được 53 hợp chất và đánh giá tác dụng ức chế enzym α- glucosidase trên mô hình *in silico*. Kết quả cho thấy 7 hợp chất có khả năng ức chế cao nhất và cao hơn chất chứng dương acarbose bao gồm: rutin; 9-

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BÀI NGHIÊN CỨU

BÀI NGHIÊN CỨU

Xây dựng quy trình bào chế vị thuốc thần khúc (Massa medicata fermentata)

PROCESS FOR MAKING UP MASSA MEDICATA FERMENTATA MEDICINE

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TÓM TẮT

Mục tiêu: *Xây dựng quy trình bào chế vị thuốc thần khúc (Massa medicata fermentata) nhằm tăng tác dụng của vị thuốc này thông qua việc tối ưu hóa hoạt độ enzym amylase.*

Đối tượng và phương pháp: *Đối tượng nghiên cứu là thần khúc được lên men từ những nguyên liệu và quy trình khác nhau. Tiến hành thay đổi các thông số trong quy trình bào chế Thần Khúc, bao gồm công thức, nhiệt độ lên men, độ ẩm lên men, thời gian lên men, sau đó đánh giá hoạt độ enzym amylase để tìm ra công thức và quy trình tối ưu.*

Kết quả: *Công thức và quy trình sản xuất thần khúc cho hoạt độ enzym amylase cao nhất như sau: Công thức gồm 500g bột gạo, 5g nghệ, 5g lá dâu, 5g ngải cứu, 5g ké đầu ngựa. Điều kiện lên men: nhiệt độ 40°C, độ ẩm 75%, thời gian 4 ngày. Sản phẩm thần khúc thu được có tỉ lệ hoạt độ enzym amylase 53,62 ± 2,19 %.*

Kết luận: *Nghiên cứu đã xây dựng được quy trình bào chế thần khúc tối ưu hóa hoạt độ enzym amylase.*

Từ khóa: *Thần khúc, Massa medicata fermentata, quy trình, amylase.*

SUMMARY

Objective: *To develop a process to prepare the Shenqu (Massa medicata fermentata) to enhance its effect by optimizing amylase enzyme activity.*

Subjects and methods: *The object of this study is Shenqu product which is fermented from different materials and processes. Change the parameters in the preparation process, including recipe and temperature, humidity, time of fermentation, then evaluate amylase enzyme activity to find optimal formula and process.*

Results: *The formula and production process of Shenqu for the highest amylase enzyme activity is as follows: The recipe includes 500g of rice flour, 5g of turmerie (Rhizoma Curcumae longae), 5g of red mulberry leaves (Folium Mori albae), 5g of mugwort (Folium Artemisiae), 5g of common cocklebur (Fructus Xanthii); Fermentation conditions: temperature 40°C, humidity 75%, time 4 days. The obtained product has amylase activity rate of 53.62 ± 2.19%.*

Conclusion: *This study has built a process of preparing Shenqu to optimize amylase enzyme activity.*

Key words: *Shenqu, Massa medicata fermentata, production process.*

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ĐẶT VẤN ĐỀ

Thần Khúc (*Massa medicata fermentata*) là vị thuốc cổ truyền đã được sử dụng từ lâu, có tác dụng tiêu thực, hành khí, kiện tỳ, dưỡng vị. Thần khúc được tạo nên từ quá trình lên men tự nhiên gồm nhiều vị thuốc phối hợp với bột mì hoặc bột gạo [3]. Tuy nhiên, qua rà soát tài liệu, chúng tôi nhận thấy có nhiều quy trình chế biến thần khúc khác nhau, dẫn đến việc khó khăn trong việc sử dụng và sản xuất vị thuốc này. Do vậy, trong nghiên cứu này, chúng tôi xây dựng quy trình bào chế vị thuốc Thần khúc nhằm nâng cao tác dụng của vị thuốc này thông qua việc tối ưu hóa hoạt độ enzym amylase.

NGUYÊN LIỆU VÀ PHƯƠNG PHÁP NGHIÊN CỨU

Nguyên liệu nghiên cứu

Nguyên liệu bao gồm các thành phần được trình bày ở bảng 1. Công thức thành phần và hàm lượng được chúng tôi lựa chọn dựa trên các tài liệu [2], [3], [4].

Bảng 1. Các công thức được sử dụng làm nguyên liệu nghiên cứu.

Công thức 1 [3]	Công thức 2 [4]	Công thức 3 [2]
500g Bột mì	500g Bột gạo	500g Cám lúa mì
20g Bột đậu đỏ	5g Bột nghệ	250g Bột mì
20g Bột hạnh nhân	5g Lá dâu	10g Bột đậu đỏ
5g Bột ké đầu ngựa	5g Ngải cứu	10g Bột hạnh nhân
5g Nghệ răm	5g Bột ké đầu ngựa	50g Nghệ răm
5g Lá thanh hao		50g Bột ké đầu ngựa
		50g Lá thanh hao

Mẫu đối chiếu là vị thuốc Thần khúc của Nhà thuốc gia truyền Phúc Minh Đường (Nghi Xuân, Hà Tĩnh)

Phương pháp nghiên cứu:

Tiến hành thay đổi các thông số trong quy trình bào chế Thần Khúc, bao gồm công thức, nhiệt độ lên men, độ ẩm lên men, thời gian lên men để thu được sản phẩm có hoạt độ enzym amylase cao nhất.

Phương pháp xác định hoạt độ enzym amylase [5]: Cân chính xác khoảng 5g mẫu thử (Thần khúc). Xay nhỏ rồi cho vào bình định mức 100ml, cho dung dịch đệm phosphate pH =4,9. Thêm nước vừa đủ 100 ml. Đem đi siêu âm. Lọc thu lấy dịch chiết. Lấy 1ml dịch chiết, thêm 1 ml tinh bột gạo 1%, 0,5 ml dung dịch NaCl 3% rồi đem ủ ở 50oC trong 30 phút. Sau đó, thêm 1ml dung dịch HCl 1N, 5,5 ml nước cất và 0,05 ml dung dịch Lugol. Lắc đều hỗn hợp rồi đem đo quang ở bước song 620 nm. Tiến hành song song với mẫu trắng.

Hoạt độ enzym amylase được tính theo công thức sau:

$$HđA = \frac{(OD_0 - OD_t)}{OD_t \times t} \times C \times L$$

Trong đó: Hđ A: hoạt độ enzym amylase; OD_0: Mật độ quang của ống chuẩn.; Od_t: Mật độ quang của ống thử; C: lượng tinh bột ban đầu tham gia phản ứng (mg); t: thời gian phản ứng (30 phút); L: hệ số pha loãng mẫu enzym.

KẾT QUẢ

Khảo sát công thức

Tiến hành bào chế 3 mẫu Thần khúc theo công

thức tại Bảng 1 trong điều kiện nhiệt độ 35°C, độ ẩm 80%, thời gian: 4 ngày. Kết quả cho thấy, công thức số 2 cho hoạt độ enzym amylase cao nhất. Kết quả chi tiết được trình bày tại Bảng 2.

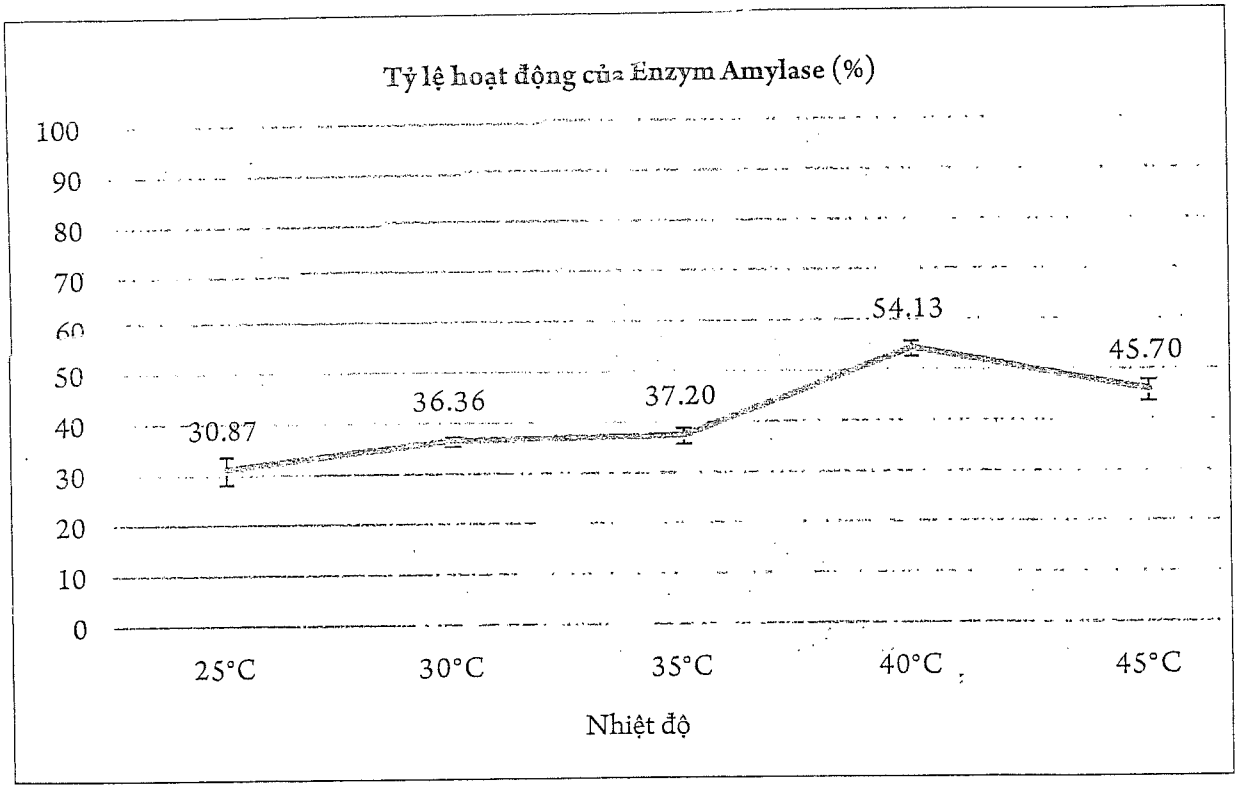
Bảng 2. Kết quả khảo sát các công thức

Công thức	1	2	3
Tỷ lệ hoạt độ của enzym amylase (%)	33,47 ± 3,24	62,92 ± 2,03	50,19 ± 3,62

Số liệu được trình bày dưới dạng M ± SD, với n = 3.

Khảo sát nhiệt độ lên men

Chúng tôi tiến hành bào chế thần khúc theo công thức số 2, với các điều kiện: độ ẩm 80%, thời gian 4 ngày và nhiệt độ lên men thay đổi từ 25°C-45°C. Kết quả cho thấy, tại mức nhiệt độ 40°C, sản phẩm cho hoạt độ enzym amylase cao nhất, đạt 54,13 ± 1,53%. Kết quả khảo sát hoạt độ enzym tại các nhiệt độ lên men được trình bày tại **Biểu đồ 1.**

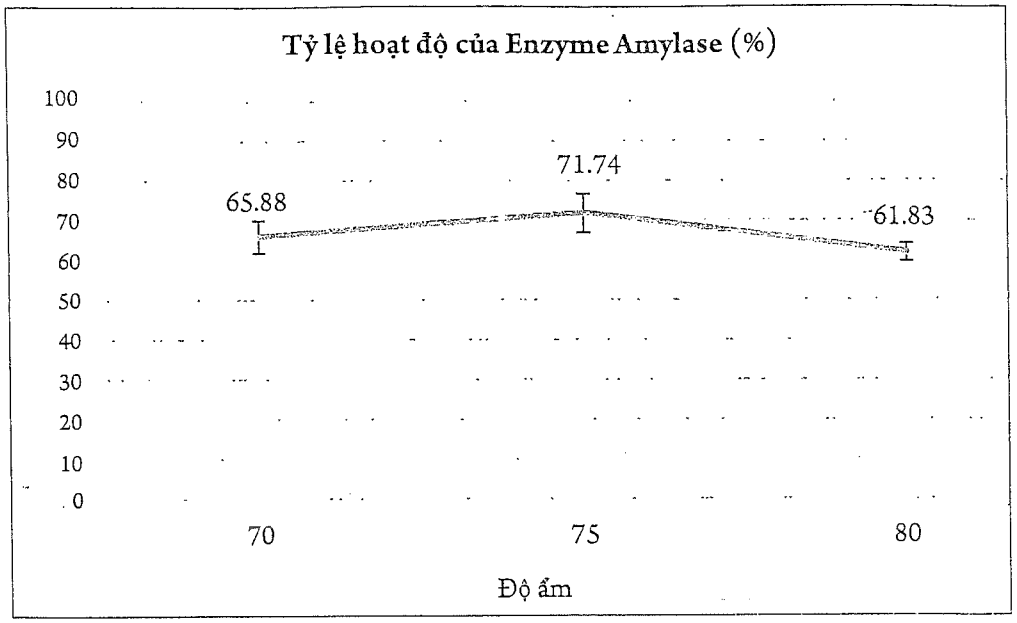


Số liệu được trình bày dưới dạng M ± SD, với n = 3.

Biểu đồ 1. Kết quả khảo sát hoạt độ của enzym amylase của sản phẩm tại các nhiệt độ lên men.

Khảo sát độ ẩm lên men

Tương tự như trên, chúng tôi bào chế thần khúc theo công thức 2, cố định nhiệt độ tại 40°C, thời gian 4 ngày, và thay đổi thông số độ ẩm từ 70-80%. Kết quả cho thấy, tại độ ẩm 75%, sản phẩm có hoạt độ enzym cao nhất, đạt 71,74 ± 4,91 %. Kết quả khảo sát hoạt độ enzym tại các độ ẩm được trình bày tại **Biểu đồ 2.**

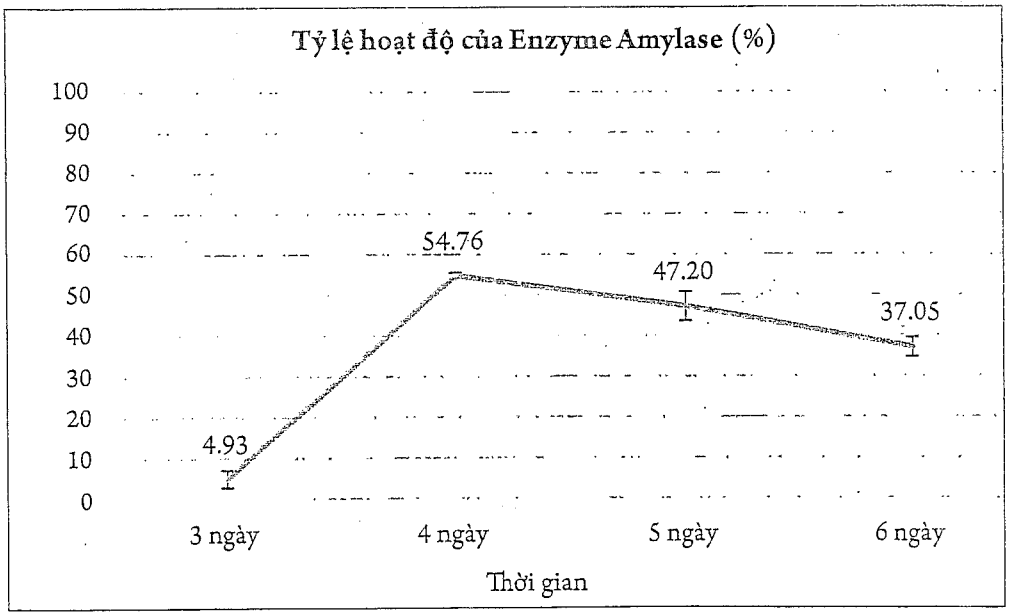


Số liệu được trình bày dưới dạng M ± SD, với n = 3.

Biểu đồ 2. Kết quả khảo sát hoạt độ của enzym amylase của sản phẩm tại các độ ẩm

Khảo sát thời gian lên men

Chúng tôi cố định nhiệt độ tại 40°C, độ ẩm 75%, và khảo sát hoạt độ enzym của mẫu thử được ủ từ 3-6 ngày. Kết quả cho thấy hoạt độ enzym cao nhất tại mẫu được lên men 4 ngày, đạt 54,76 ± 0,70%, sau đó giảm dần. Kết quả khảo sát hoạt độ enzym amylase của các mẫu lên men sau 3-6 ngày được trình bày tại **Biểu đồ 3**.



Số liệu được trình bày dưới dạng M ± SD, với n = 3.

Biểu đồ 3. Kết quả khảo sát hoạt độ enzym amylase trong mẫu nghiên cứu được lên men với thời gian khác nhau

So sánh hoạt tính enzym của mẫu nghiên cứu với sản phẩm thần khúc đang lưu hành trên thị trường

Chúng tôi tiến hành bào chế thần khúc theo các thông số đã khảo sát được: công thức số 2, nhiệt độ 40°C, độ ẩm 75%, thời gian 4 ngày. Sau đó sản phẩm được tiến hành so sánh hoạt độ enzym amylase với mẫu thần khúc đối chiếu (được mua trên thị trường). Kết quả cho thấy, mẫu nghiên cứu có hoạt độ cao hơn đáng kể, đạt 53,62 ± 2,19 %, so với mẫu đối chiếu chỉ đạt 41,20 ± 2,16 ($P < 0,01$).

BÀN LUẬN

Thần Khúc là vị thuốc cổ truyền đã được sử dụng từ lâu có tác dụng tiêu thực, hành khí, kiện tỳ, dưỡng vị [4]. Một số nghiên cứu gần đây trên thế giới cho thấy vị thuốc Thần Khúc chứa thành phần chính là enzym amylase. Đây là enzym có vai trò xúc tác quá trình thủy phân thức ăn có nguồn gốc tinh bột thành các dextrin và phân tử đường mạch ngắn, giúp ruột non dễ dàng hấp thu [1]. Điều này phù hợp với tác dụng của Thần khúc trong Y học cổ truyền. Do vậy chúng tôi lựa chọn enzym amylase làm chất chỉ dấu để khảo sát quy trình bào chế thần khúc.

Qua quá trình rà soát tài liệu, chúng tôi nhận thấy có khá nhiều công thức thần khúc khác nhau, được bào chế theo quy trình khác nhau. Điều này gây khó khăn trong việc sử dụng và chế biến thần khúc. Trong nghiên cứu này, chúng tôi đã xây dựng được quy trình bào chế thần khúc với 4 thông số chính, bao gồm: công thức, nhiệt độ, độ ẩm, thời gian. Sản phẩm thu được có tỷ lệ hoạt độ enzym cao hơn đáng kể so với mẫu đối chiếu ($P < 0,01$).

KẾT LUẬN

Trong nghiên cứu này, chúng tôi đã xây dựng được quy trình bào chế thần khúc như sau: Công thức gồm 500g bột gạo, 5g nghệ, 5g lá dâu, 5g ngải cứu, 5g ké đầu ngựa. Điều kiện lên men: nhiệt độ 40°C, độ ẩm 75%, thời gian 4 ngày. Sản phẩm thần khúc thu được có tỷ lệ hoạt độ enzym amylase 53,62 ± 2,19 %.

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Số 02 (21)
2019

MỤC LỤC SỐ 02(21) - 2019

BÀI NGHIÊN CỨU

Đánh giá công dụng hỗ trợ điều trị mất ngủ của viên Tâm não Thông Tuệ trên người bệnh mắc mất ngủ do thiếu máu não

EVALUATE THE SUPPORTIVE EFFECT IN SLEEPLESSNESS TREATMENT OF "TAM NAO THONG TUE" CAPSULE ON PATIENTS WITH SLEEPLESSNESS CAUSED BY CEREBRAL ANAEMIA

Nguyễn Văn Quân

Học viện Y Dược học cổ truyền Việt Nam

TÓM TẮT

Thiếu máu não là một bệnh ngày càng trẻ hóa. Người thiếu máu não mắc tới 6/7 triệu chứng đau đầu, hoa mắt, ù tai, buôn nôn, giảm trí nhớ, tê bì chân tay, mất ngủ, tâm lý bất an. Trong đó người mất ngủ do thiếu máu não làm giảm chất lượng cuộc sống

Tâm não Thông Tuệ có tác dụng nhanh sau 2 tuần. Sau 4 tuần sử dụng bằng viên Tâm não Thông Tuệ, có tới 96,1% giảm triệu chứng mất ngủ, tăng thời gian ngủ từ 4,17 + 1,34 (giờ) lên 7,01 + 0,66 (giờ) sau 28 ngày can thiệp, Giảm tần suất tỉnh giấc trong giấc ngủ đạt 96,1%

- Có 92,2% nhận xét Tâm não Thông Tuệ là thuốc hữu ích cho người cao tuổi bị thiếu máu não.

Từ khóa: *Thiếu máu não, Tâm não Thông Tuệ.*

SUMMARY

Cerebral anaemia is rejuvenating disease. Persons who have the cerebral anaemia also got 6/7 symptoms such as headache, dizziness, tinnitus, nausea, absent minded, limb numbness, sleeplessness and unsafe psychology. In which persons who have sleeplessness because of cerebral anaemia bring to reduction of the quality of life.

Tam nao Thong tue has the fast effectiveness after 2 week treatment. After 4 week treatment of Tam nao Thong Tue tablet, 96.1% of patient reduced the sleeplessness symtoms. While the sleeping time increased from 4.17+1.34 hours to 7.01 + 0.66 hours after 28 day treatment. Moreover, the reduction of frequency of awakeness during sleeping also achieved 96.1%.

There is 92.2% of comments that Tam nao Thong tue is the useful medicine for elderly persons who got cerebral anaemia.

Key word: *Cerebral anaemia, Tam nao Thong Tue.*

Ngày nhận bài: 20/2/2019

Ngày phản biện: 28/2/2019

Ngày chấp nhận đăng: 28/2/2019

- Viên tâm não Thông Tuệ giúp giảm triệu chứng mất ngủ đến 96,1% và tăng thời gian ngủ từ 4.17 + 1.34 (giờ) lên 7.01 + 0.66 (giờ) sau 28 ngày can thiệp, Giảm tần suất tỉnh giấc trong giấc ngủ đạt 96.1%

- Có 92.2% nhận xét Viên Tâm não Thông Tuệ hữu ích cho người mất ngủ do thiếu máu não

Kiến nghị

- Tiếp tục nghiên cứu chất lượng giấc ngủ và chất lượng cuộc sống người bệnh sau 4 tuần sử dụng viên Tâm não Thông Tuệ ở các thời điểm 2 tháng và 3 tháng.

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Số 05 (18)
2018

MỤC LỤC SỐ 05(18) - 2018

BÀI NGHIÊN CỨU

Đánh giá kỹ năng thực hành tư vấn của nhân viên quầy thuốc trên địa bàn thành phố Hải Phòng năm 2017

EVALUATE THE CONSULTANCY SKILL OF DRUGSTORE STAFFS IN HAI PHONG CITY IN 2017

Nguyễn Văn Quân[1], Đỗ Thị Hồng Sâm[1], Lê Thị Hiền[1]
Bùi Thị Ngoan[1], Bùi Thị Hảo[1], Phạm Thị Thu Giang[2]
[1]Học viện Y Dược học cổ truyền Việt Nam
[2]Đại học Công nghiệp Hà Nội

TÓM TẮT

Hải Phòng là thành phố triển khai sớm về thực hành tốt nhà thuốc GPP và chuẩn hóa quầy thuốc. Kết quả nghiên cứu cho thấy. Hầu hết các chỉ số kỹ năng về câu hỏi, lời khuyên và tư vấn của các nhân viên bán hàng cho người mua thuốc là thấp, chỉ số cao nhất về triệu chứng bệnh là 66,9% và thấp nhất dị ứng với thuốc chiếm 6,3%. Chỉ có kỹ năng tư vấn thuốc chiếm tỉ lệ cao, chỉ số thấp nhất là 90,7% trừ chỉ số về tác dụng bất lợi của thuốc đạt yêu cầu chỉ chiếm 22,4%.

SUMMARY

Hai Phong City has early implemented GPP practices for pharmacy and standardised the drugstores. The research result shows that most skill indicators of questions, advices & consultancy from chemists to the medicine buyers are low. The highest indicators of illness signs is 69.9% while the lowest one of medicine allergy is accounted for 6.3%. Only indicator for skills of advices are high, the lowest one is 90.7% except the adverse effectiveness of medicine with accepted requirement is only 22.4%.

Keyword: *Quầy thuốc;, Kỹ năng thực hành (staff's practice); Hải Phòng.*

ĐẶT VẤN ĐỀ

Ngành dược Việt Nam phát triển vượt bậc trong sản xuất, lưu thông cung ứng thuốc cho cộng đồng từ khi tiến hành đổi mới kinh tế năm 1986[1]. GPP là một trong 5 tiêu chuẩn thực hành tốt nhà thuốc của qui trình đảm bảo chất lượng: từ sản xuất, kiểm tra chất lượng, tồn trữ bảo quản, lưu thông phân phối đến tay người bệnh. Tiêu chuẩn này được Tổ

Ngày nhận bài: 14/9/2018
Ngày phản biện: 21/9/2018
Ngày chấp nhận đăng: 24/9/2018

4	Tổng số ngày dùng thuốc	816	90,7	60	6,7	24	2,6
5	Tác dụng bất lợi của thuốc khi sử dụng và cách xử lý khi gặp phải	202	22,4	155	17,2	543	60,4
6	Tư vấn thay thế thuốc theo đơn (39 đơn)	33	84,6	6	15,4	0	0

Nhận xét: Kỹ năng hướng dẫn sử dụng thuốc là kỹ năng rất quan trọng có ảnh hưởng trực tiếp tới việc sử dụng thuốc an toàn, hiệu quả của bệnh nhân. Kỹ năng này đòi hỏi nhân viên nhà thuốc phải hiểu biết, tận tình, chu đáo đối với khách hàng [4]. Kết quả tại Bảng 4 cho thấy:

- Số khách hàng nhận được đầy đủ nội dung tư vấn và hiểu được trong các chỉ tiêu là rất cao, tối thiểu là 90,7%, riêng chỉ số về tư vấn tác dụng bất lợi của thuốc và cách xử lý còn thấp mới đạt 22,4% 9 (mặc dù chỉ số tư vấn về tác dụng bất lợi tổng cộng là 22,4 + 17,2 = 39,6%). Như vậy, chỉ số này cần phải cải thiện để tránh rủi ro cho người sử dụng thuốc. Kết quả này nhìn chung cao hơn kết quả nghiên cứu của Lương Văn Bảo [4].

Có 15,4% số đơn thuốc bị thay thế thuốc chưa được quầy thuốc lý giải rõ cho khách hàng.

KẾT LUẬN

- Nghề nghiệp và trình độ người mua thuốc phản ánh dân trí ở một thành phố công nghiệp là Hải Phòng.
- Thời gian khách hàng dược tư vấn tại quầy thuốc trên 5 phút chiếm 63,0%.
- Hầu hết số lời khuyên và số câu hỏi khách hàng nhận được từ người bán thuốc là thấp, chỉ số cao nhất về triệu chứng bệnh là 66,9% và thấp nhất dị ứng với thuốc chiếm 6,3%.
- Chỉ có kỹ năng tư vấn thuốc chiếm tỉ lệ cao, chỉ số thấp nhất là 90,7% trừ chỉ số về tác dụng bất lợi của thuốc đạt yêu cầu chỉ chiếm 22,4%.

KIẾN NGHỊ

Sở y tế Hải Phòng định kỳ tổ chức đào tạo liên tục về kỹ năng bán hàng và tư vấn sử dụng thuốc cho các quầy thuốc, kết hợp với thanh tra kiểm tra và ban hành thông tin nhà thuốc, quầy thuốc trong thành phố.

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BÀI NGHIÊN CỨU

Đánh giá ảnh hưởng của bài thuốc "Thái bình HV" lên các chỉ số sinh hóa và mô bệnh học của động vật thực nghiệm

EVALUATE THE EFFECT OF "THAI BINH HV" REMEDY ON THE BLOOD BIOCHEMICAL AND HISTOPATHOLOGICAL IMAGE ON EXPERIMENTAL ANIMAL

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TÓM TẮT

Mục tiêu: *Đánh giá ảnh hưởng của bài thuốc "Thái bình HV" lên các chỉ số sinh hóa máu đánh giá chức năng gan, thận và hình ảnh mô bệnh học gan, lách, thận.*

Phương pháp: *Chuột cống trắng được cho uống "Thái bình HV" liều 11,90g/kg/ngày và 35,70g/kg/ngày, hàng ngày trong 30 ngày. Xét nghiệm các chỉ số sinh hoá máu ALT, AST, billlirubin TP, albumin, cholesterol TP, creatinin tại 3 thời điểm: trước uống thuốc, sau 15 ngày và sau 30 ngày uống thuốc. Hình ảnh đại thể và vi thể gan, lách, thận chuột được đánh giá khi kết thúc thí nghiệm.*

Kết quả: *Không có sự khác biệt có ý nghĩa thống kê khi so sánh các chỉ số sinh hóa máu giữa các lô với nhau trong cùng thời điểm cũng như trong từng lô giữa các thời điểm đánh giá. Hình ảnh đại thể và mô bệnh học của gan, lách, thận bình thường.*

Kết luận: *Bài thuốc "Thái bình HV" không ảnh hưởng lên chức năng gan, thận và mô bệnh học gan, lách thận ở các mức liều dùng 11,90g/kg/ngày và 35,70g/kg/ngày và thời gian sử dụng 30 ngày trong nghiên cứu thực nghiệm trên chuột cống trắng.*

Từ khóa: *"Thái bình HV", sinh hóa máu, hình ảnh mô bệnh học.*

ABSTRACT

Objective: *To evaluate the effect of "Thai binh HV" remedy on the blood biochemical indices to assess liver and kidney function and histopathological images of liver, spleen, and kidney.*

Methods: *Wistar rats were given "Thai binh HV" at doses of 11.90 g/kg/day and 35.70 g/kg/day, daily*

Ngày nhận bài: 6/01/2022

Ngày phản biện: 10/01/2022

Ngày chấp nhận đăng: 10/2/2022

BÀN LUẬN

Đánh giá các chỉ tiêu sinh hóa

Các chỉ tiêu sinh hóa đánh giá ảnh hưởng lên sự huỷ hoại tế bào gan (AST, ALT), chức năng gan (albumin, cholesterol TP, Bilirubin TP), chức năng thận (albumin, creatinin), cho phép khảo sát những ảnh hưởng lên chức năng của gan và thận là các cơ quan quan trọng trong chuyển hóa, thải trừ thuốc [5, 6]. Các chỉ số sinh hóa đã khảo sát đều không có thay đổi khác biệt so với lô chứng sinh lý cũng như so với trước khi dùng thuốc.

Đánh giá kết quả mô bệnh học

Hình ảnh mô bệnh học (cả đại thể và vi thể) của gan, lách, thận cho phép đánh giá ảnh hưởng lên cấu trúc của các cơ quan chính liên quan đến chuyển hóa, thải trừ thuốc [5]. Gan là cơ quan chính chuyển hoá thuốc của cơ thể, thận là cơ quan chính thải trừ thuốc của cơ thể. Lách có cấu trúc gần giống như một hạch bạch huyết lớn, nó hoạt động chủ yếu như là một bộ lọc máu, do đó những biến đổi trong cơ thể đặc biệt các ảnh hưởng gây độc đến các tế bào hồng cầu và hệ bạch huyết nếu có đều dễ dàng được nhận thấy ở lách. Kết quả nghiên cứu cho thấy hình ảnh mô bệnh học gan, lách, thận của các chuột nghiên cứu đều bình thường, cho thấy tính an toàn của bài thuốc "Thái bình HV".

Kết quả đánh giá trên các chỉ tiêu sinh hoá và mô bệnh học động vật thực nghiệm khi cho chuột uống dịch chiết bài thuốc "Thái bình HV" trong thời gian 90 ngày cho thấy bài thuốc an toàn trên các chỉ tiêu đánh giá. Với dự kiến điều trị kéo dài nhằm phát huy hiệu quả dự phòng cũng như hồi phục tổn thương đột quỵ não của bài thuốc y học cổ truyền, độc tính của bài thuốc được đánh giá trong thời gian 30 ngày giúp khẳng định được tính an toàn của bài thuốc khi sử dụng trên người trong thời gian trên 01 tháng [5,6].

KẾT LUẬN

Từ các kết quả thu được trên thực nghiệm, chúng tôi kết luận: Trên các lô chuột uống "Thái bình HV" ở các mức liều 11,90 g/kg/24h và liều 35,70 g/kg/24h liên tục trong 30 ngày cho thấy:

- Không làm thay đổi các chỉ tiêu sinh hóa máu bao gồm nồng độ men gan AST, ALT, bilirubin toàn phần, cholesterol toàn phần, creatinin, albumin huyết tương.
- Không gây tổn thương mô bệnh học gan, lách, thận.

Như vậy "Thái bình HV" an toàn với các mức liều dùng và thời gian sử dụng trong nghiên cứu thực nghiệm trên chuột cống trắng.

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TẠP CHÍ

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MỤC LỤC SỐ 01(26) - 2020

BÀI NGHIÊN CỨU

Đánh giá tác dụng giảm đau của bài thuốc "Thái Bình HV"trên động vật thực nghiệm

STUDY ON ANALGESTIC EFFECT OF THE EXTRACT FROM "THAI BINH HV" PRESCRIPTION

Trần Đức Hữu[1], Nguyễn Thị Như Quý[1], Nguyễn Văn Quân[1], Nguyễn Hoàng Ngân[2]

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TÓM TẮT

Mục tiêu: *Đánh giá tác dụng giảm đau của cao lỏng "Thái Bình HV" trên động vật thực nghiệm.*

Đối tượng và phương pháp nghiên cứu: *Nghiên cứu thực nghiệm có đối chứng, mô tả cắt ngang. Đánh giá tác dụng giảm đau trung ương của cao lỏng Thái Bình HV trên mô hình gây đau bằng phiến nóng (Hotplate) và tác dụng giảm đau ngoại vi trên mô hình gây đau quặn.*

Kết quả: *Cao lỏng Thái Bình HV ở cả 2 mức liều dùng trên chuột nhắt trắng: Có tác dụng giảm đau tốt khi thử theo phương pháp "mâm nóng" (Hotplate) và phương pháp gây đau quặn bằng acid acetic (phương pháp Koster).*

Từ khóa: *Thái bình HV, Giảm đau, Thực nghiệm.*

SUMMARY

"Thai Binh HV" is a popular anti-rheumatoid arthritis remedy that Vietnamese has been using for a long time. The experimental study aimed to explore the analgesic activity of "Thai Binh HV" extract using hotplates, and acetic acid induced writhing models in mice. Results demonstrated that "Thai Binh HV" remedy exhibited analgesic activity in all tested models for analgesia.

Keywords: *Thai Binh HV, Analgestic, experimental animal.*

ĐẶT VẤN ĐỀ

Viêm đa khớp dạng thấp (Rheumatoid arthritis) là một bệnh thường gặp trong các bệnh xương khớp mạn tính. Đây là bệnh mang tính xã hội vì tỉ lệ mắc bệnh cao, diễn biến kéo dài, có thể gây ra hậu quả nặng nề như tàn phế. Tỉ lệ mắc bệnh chung: Khoảng 0,3-1% dân số thế giới [1], riêng ở nước ta có khoảng 0,52% dân số mắc bệnh này, tập trung 80% ở độ tuổi trung niên, người già.

Theo Y học hiện đại, tình trạng đau và sưng viêm trong viêm đa khớp dạng thấp được điều trị bằng các loại thuốc chống thấp khớp hoặc thuốc giảm đau. Các loại thuốc chữa đau xương khớp thường có tác dụng nhanh chóng, giảm đau kịp

Ngày nhận bài: 2/12/2019

Ngày phản biện: 3/12/2019

Ngày chấp nhận đăng: 4/12/2019

Nhận xét: Kết quả ở bảng 3 cho thấy:

- So với lô chứng, số cơn đau quặn trong cả 25 phút sau tiêm acid acetic ở cả 2 lô dùng Thái Bình HV liều 1, liều 2 và lô dùng thuốc tham chiếu Aspegic đều nhỏ hơn có ý nghĩa thống kê với $p < 0,05$. Tính toán ở trong khoảng thời gian 25 phút này, tỉ lệ phần trăm làm giảm số cơn đau quặn ở lô dùng Aspegic liều 180 mg/kg/ngày, và các lô dùng Thái Bình HV cả 2 mức liều 20,40 mg/kg/ngày và 40,80 mg/kg/ngày, lần lượt là 33,25 %; 27,23 %; và 34,29 %.

- So với lô tham chiếu dùng Aspegic liều 180 mg/kg/ngày, số cơn đau quặn trong cả 25 phút sau tiêm acid acetic ở các lô dùng Thái Bình HV cả 2 mức liều (20,40 mg/kg/ngày và 40,80 mg/kg/ngày) không có sự khác biệt có ý nghĩa thống kê ($p_{3,4-2} > 0,05$).

- So với ở lô dùng Thái Bình HV liều thấp, ở lô dùng Thái Bình HV liều cao có số cơn đau quặn trong cả 25 phút sau tiêm acid acetic ít hơn, tuy nhiên sự khác biệt chưa có ý nghĩa thống kê ($p_{3-4} > 0,05$). Kết quả này tương tự như kết quả tác dụng giảm đau của bài thuốc Thiên cốt đan của trong nghiên cứu của Phạm Tuấn Thanh [9] và bài thuốc KNC trong nghiên cứu của Nguyễn Thị Ngọc [8]

KẾT LUẬN

Cao lỏng Thái Bình HV (4,08g/kg/ngày và 8,16g/kg/ngày) có tác dụng giảm đau rõ rệt khi thử trên mô hình Hotplate

Cao lỏng Thái Bình HV (4,08g/kg/ngày và 8,16g/kg/ngày) có tác dụng giảm đau rõ rệt trên khi thử theo phương pháp Koster, làm số cơn đau quặn giảm hơn so với lô chứng sinh lý, với $p < 0,01$. Tác dụng này tương đương với khi dùng Aspegic liều 180mg/kg/ngày.

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TẠP CHÍ

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BÀI NGHIÊN CỨU

KHOA HỌC THƯỜNG THỨC

Đánh giá kỹ năng giao tiếp và năng lực chuyên môn của nhân viên nhà thuốc trên địa bàn Thành phố Đà Nẵng năm 2017

EVALUATING THE COMMUNICATION SKILL AND PROFESSIONAL CAPACITY OF THE PHARMACY STAFFS AT DA NANG CITY IN 2017

Nguyễn Thị Xuân Thủy [1], Nguyễn Văn Quân [2], Nguyễn Vân Anh [2]

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[2]Học viện Y Dược học cổ truyền Việt Nam

TÓM TẮT

Đà Nẵng là thành phố triển khai sớm về thực hành tốt nhà thuốc GPP từ năm 2011. Kết quả nghiên cứu cho thấy: - 100% phụ trách chuyên môn và nhân viên nhà thuốc đảm bảo trình độ chuyên môn của nhà thuốc đạt nguyên tắc, tiêu chuẩn GPP. Hầu hết các chỉ số về thái độ, kỹ năng giao tiếp và năng lực chuyên môn của các nhân viên bán hàng cho người mua thuốc SCT (sau can thiệp) thay đổi khác biệt so với TCT (trước can thiệp) (p<0,05).

SUMMARY

Da Nang city has early implemented the Good Pharmacy Practice (GPP) for 2011. The research showed that: 100% of professional staff and drugstore employees satisfy with GPP principles and standards. Most of indicators related to behavior, communication skills and professional competence of the sale staff who distribute the products to the after intervention customers show the remarkable differences compared to before intervention (p<0.05).

Keyword: *Thực hành tốt nhà thuốc; Good Pharmacy Practice (GPP), Thái độ, kỹ năng thực hành (staff's practice), năng lực chuyên môn (competence); Đà Nẵng;*

ĐẶT VẤN ĐỀ

Dược cộng đồng (Community pharmacy) là hoạt động chăm sóc thuốc cho người dân thông qua hệ thống các nhà thuốc và cơ sở bán lẻ thuốc được thực hiện bởi người dược sĩ và nhân viên cơ sở bán lẻ thuốc đóng vai trò quan trọng [7]. Tại nhiều quốc gia, trong đó có Việt Nam, Dược cộng đồng chính là cơ sở y tế quan trọng trong chăm sóc sức khỏe ban đầu cho người dân trong cộng đồng.

Trong hội nghị FID (2012) cho thấy người

Ngày nhận bài: 20/4/2018

Ngày phản biện: 18/5/2018

Ngày chấp nhận đăng: 26/5/2018

- NCT: Các chỉ số TCT so với SCT 01 tháng rất khác biệt nhau (p<0,01) và khác hẳn so với NĐC chỉ khác nhau ở mức p<0,05, và so với SCT 07 tháng khác biệt nhau (p<0,01). Tuy nhiên giữa SCT 01 tháng và 07 tháng, các chỉ số đều khác nhau ở mức không có ý nghĩa thống kê (p>0,05).

- So sánh các chỉ số SCT 1 tháng với SCT 07 tháng không khác biệt nhau (p>0,05). Điều này cho thấy năng lực chuyên môn của nhân viên nhà thuốc tương đối ổn định và kết quả kéo dài đến sau 06 tháng. Tất nhiên có sai sót không đáng kể do 6 nhà thuốc thay đổi một nhân viên bán hàng.

- So sánh NĐC với NCT: TCT các chỉ số là tương đương (p>0,05), SCT1 tháng, các chỉ số rất khác nhau (p<0,01), SCT 07 tháng có 3/6 chỉ số khác biệt nhau (p<0,05). Ở đây thấy rõ sự thay đổi ngày càng sâu ở NĐC so với NCT nhờ nhận thức của nhân viên bán hàng thông qua nhu cầu tư vấn tất yếu của khách hàng về nâng cao chất lượng dịch vụ của nhà thuốc.

KẾT LUẬN

- 100% phụ trách chuyên môn và nhân viên nhà thuốc đảm bảo trình độ chuyên môn của nhà thuốc đạt nguyên tắc, tiêu chuẩn GPP

- Có sự cải thiện ở các chỉ số trong NCT sau khi can thiệp (p<0,01) và mang tính ổn định kéo dài 7 tháng nghiên cứu (p>0,05) cả về thái độ và kỹ năng giao tiếp với năng lực chuyên môn của nhà thuốc.

KIẾN NGHỊ

Sở Y tế Đà Nẵng tổ chức củng cố mạng lưới nhà thuốc đạt GPP thân thiện với cộng đồng đảm bảo đạt mục tiêu chăm sóc sức khỏe người dân thành phố và khách du lịch tốt nhất.

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MỤC LỤC SỐ ĐẶC BIỆT 20/11

BÀI NGHIÊN CỨU

Đánh giá thực trạng chất lượng thông tin quảng cáo thực phẩm bảo vệ sức khỏe từ thảo dược trên internet

EVALUATE THE STATUS OF ADVERTISED INFORMATION ABOUT FOOD SUPLEMENT FORM HERB ON INTERNET

Nguyễn Thị Thơm, Nguyễn Văn Quân

Học viện Y Dược học cổ truyền Việt Nam

TÓM TẮT

Thông tin thực phẩm bảo vệ sức khỏe là vô cùng cần thiết cho doanh nghiệp trong kinh doanh (DN), cho người tiêu dùng và trong công tác quản lí nhà nước. Kết quả nghiên cứu: Có 33.3% số DN đã đăng kí quảng cáo cho sản phẩm với 12 nhóm chức năng trên trang Website. Số lượng Website không phải của DN cao khác biệt với số lượng của trang Website DN ($p < 0.05$); Số lượng thông tin trung bình mỗi sản phẩm là 9.1 + 3. Thông tin "tên sản phẩm" đạt 100%, 3 TT về "công dụng", "cách dùng" và "liều dùng" cùng chiếm 94.6%. Thông tin chiếm tỉ lệ thấp nhất là "địa chỉ về nhà sản xuất" chiếm 40.5%; Có đến 6/10 thông tin bị sai lệch chủ yếu về "công dụng", "đối tượng sử dụng", "liều dùng"... Số lượng thông tin sai lệch chiếm 50.2% trong tổng số lượng thông tin.

SUMMARY

Information about food supplement plays an important role in the business activities of companies, customers 's buying behavior, and is necessary for law-makers. Research shows that 33.3% of companies which were license to advertise their food supplements on websites with 12 groups depending on their uses. Moreover, the number of companies 's websites was significantly higher than others ($p < 0.05$); Average amount of information of each product was pretty high with 9.1+3. In particular, information about the name of product reached 100% and both benefit, direction for use and dosage occupied 94.6%. By contrast, address of manufacturer accounted for the smallest proportion with 40.5%. 6 in 10 types of information in this research were infringed mainly about benefits, users, dosage of food supplement. The amount of inaccurate information was 50.2% which was slightly higher than correct information.

ĐẶT VẤN ĐỀ

Từ năm 1990, Việt Nam đi theo hướng xây dựng nền kinh tế thị trường và cơ bản hoàn thành vào năm 2018. Từ đây thị trường Việt Nam xuất hiện nhiều dòng sản phẩm mới như dòng Thực phẩm bảo vệ sức khỏe (TPBVSK) phát triển

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Ngày phản biện: 13/10/2019

Ngày chấp nhận đăng: 14/10/2019

trung vào nhóm bệnh phổ biến, hay gặp như tiêu hóa, chuyển hóa và thận - tiết niệu, ung thư; Hệ thống Website quảng cáo (QC) sản phẩm rất đa dạng gồm QC trên Website chính của doanh nghiệp (DN), Website đại lí, Website khác. Số lượng Website không phải của DN cao khác biệt với số lượng của trang Website DN ($p < 0.05$); Số lượng thông tin trung bình mỗi sản phẩm tương đối cao 9.1 + 3, giúp truyền tải thông tin sản phẩm ngày càng rõ ràng hơn đến người tiêu dùng.

- Trên sản phẩm đang lưu hành đều có đủ thông tin bắt buộc theo quy chế. Thông tin (TT) bắt buộc của 37 sản phẩm trên Website doanh nghiệp không đầy đủ. Chỉ có thông tin "tên sản phẩm" đạt 100%, 3 TT về "công dụng", "cách dùng" và "liều dùng" chiếm 94.6%. Thông tin chiếm tỉ lệ thấp nhất là "địa chỉ về nhà sản xuất" chiếm 40.5%; Vẫn còn tình trạng thông tin quảng cáo chưa chuẩn: thông tin TPBVSK sai lệch chưa đúng các quy chế thông tin quảng cáo hiện hành. Có đến 6/10 thông tin bị sai lệch chủ yếu về "công dụng", "đối tượng sử dụng", "liều dùng"... mà nghiêm trọng nhất là thông tin "công dụng" (17/35 sản phẩm sai phạm). Doanh nghiệp dược phẩm Á Âu chia sản phẩm theo hàng ETC và OTC là không đúng quy định với thực phẩm; Số lượng thông tin sai lệch lớn hơn so với số lượng thông tin đúng, chiếm 50.2%. Tất cả sản phẩm nghiên cứu đều có sự sai lệch so với Website doanh nghiệp, sai nhiều nhất là 15/16 thông tin, chủ yếu là thông tin thừa và sai thông tin về "công dụng", "đối tượng sử dụng".

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BÀI NGHIÊN CỨU

Phân tích một số yếu tố liên quan đến chi phí kinh doanh tại một số nhà thuốc trên địa bàn quận Đống Đa thành phố Hà Nội năm 2020

ANALYSIS OF SOME FACTORS RELATED TO BUSINESS COSTS AT SOME DRUGSTORES AT DONG DA DISTRICT, HANOI IN 2020

Nguyễn Văn Quân, Phạm Thị Bích Phương

Học viện Y Dược học cổ truyền Việt Nam

TÓM TẮT

Hoạt động nhà thuốc là hoạt động kinh doanh. Chi phí kinh doanh của nhà thuốc bị ảnh hưởng bởi các yếu tố pháp luật, kinh tế, hành chính, đối thủ cạnh tranh, nhà cung cấp và chính nhà thuốc. Kết quả nghiên cứu tại 45 nhà thuốc thuộc quận Hà Bà Trưng, thành phố Hà Nội cho thấy có nhiều yếu tố ảnh hưởng tích cực đến chi phí như Quản lý giá thuốc (chiếm 79,7% số mẫu nghiên cứu), Danh mục thuốc kê đơn (85,1%), thu hồi thuốc (75,9%); thuế (88,9%); tiền nộp phạt (94,5%), Nhà thuốc đặt ở các vị trí (90,7% -96,3%), Đầu tư các mặt hàng phù hợp với mô hình bệnh tật tại khu vực (92,6%). Các yếu tố ảnh hưởng tiêu cực gồm không kê đơn thực phẩm bảo vệ sức khỏe và mỹ phẩm trên đơn thuốc (81,5%), tăng giá thuốc đột xuất từ nhà cung cấp (90,7%), thuê địa điểm (68,5%), đầu tư các mặt hàng và tài sản cố định không phù hợp (83,3%), quy mô vốn của nhà thuốc (92,6%). Tất cả 5 yếu tố thuộc đối thủ cạnh tranh đều gây tiêu cực đến chi phí của nhà thuốc.

Từ khóa: *Chi phí, nhà thuốc, Đống Đa.*

SUMMARY

Drugstores are business activities. Their expense is affected by elements such as law, local economic, administrative work, competitors, suppliers and their own operation. The study about how these elements have effect on 45 drugstores in Hai Ba Trung District, Hanoi shows that there are either positive and negative factors. On the one hand, the positive ones which play a role in drugstrores' expense reduction including price controls on pharmaceutical (79.7%), prescription drug category (85.1%), drug recall (75.9%), tax (88.9%), penalty fee (94.5%), positions (90.7%-96.3%), suitable drugs with local disease pattern (92.6%). On the other hand, the negative ones comprise removing supplements and cosmetics against prescriptions (81.5%), the sudden price increase of suppliers (90.7%), rent (68.5%), unnecessary fixed assets (83.3%), amount of capital of drugstores (92.6%). It can be seen that all five elements in competitors cause drugstore's expense growth.

Keywords: *Expense, Drugstore, Dong Da.*

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Ngày phản biện: 30/7/2021

Ngày chấp nhận đăng: 10/8/2021

Quy mô vốn của nhà thuốc gây rủi ro chiếm từ 68,5% đến 92,6%. Nhà thuốc có thâm niên hoạt động ít năm dễ gây rủi ro chiếm tới 85,2%; Có tới 68,5% nhà thuốc khẳng định bị ảnh hưởng tiêu cực khi phải thuê mặt bằng. Nhà thuốc đặt ở trung tâm thành phố; trong khu dân cư; gần chợ, siêu thị và gần bệnh viện ảnh hưởng tích cực chiếm từ 90,7% đến 96,3%; Khi đầu tư các mặt hàng và tài sản cố định không phù hợp dễ gây rủi ro chiếm 83,3% và 61,1%. Nhà thuốc không thay đổi nhân viên và khả năng khai thác nguồn hàng tốt ảnh hưởng có lợi chiếm 100%.

Kiến nghị

Kiến nghị với các nhà thuốc trên địa bàn quận Đống Đa, Hà Nội như sau: Đầu tư nghiên cứu về các yếu tố: Qui mô vốn kinh doanh, khả năng khai thác nguồn hàng, đầu tư trang thiết bị, các mặt hàng trong nhà thuốc trong những năm đầu hoạt động cho phù hợp; Chú trọng việc tự kiểm tra, đánh giá và huấn luyện, đào tạo nhân viên nhà thuốc; Lập kế hoạch các phương án kinh doanh để giảm các chi phí bất hợp lý. Đồng thời chuẩn bị các phương án, giải pháp để phòng rủi ro xảy ra.

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