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Original Article

QUANTITATIVE DETERMINATION AND PREPARATIVE ISOLATION OF TWO MAJOR ALKALOIDS FROM THE VIETNAMESE MEDICINAL HERB *EVODIAE FRUCTUS*

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ABSTRACT

Objective: To develop a simple and accurate HPLC-DAD method for simultaneous determination, the content of major components: limonin, evodiamine, and rutaecarpine in *Evodiae fructus* and evaluation the quality of *Evodiae fructus* sold in markets.

Methods: Open column chromatography was used to separate and purify rutaecarpine and evodiamine, the two major alkaloids from *Evodiae fructus* extract as a laboratory standard. Chromatographic separation was achieved using a Germini C_{18} column (150 mm × 4.6 mm I.D., 5 µm), detected at 210 nm. The mobile phase consisted of acetonitrile (A), methanol (B), and water (C). The validated method simultaneously determined alkaloid content in 40 batches of samples collected from markets in different regions of Vietnam.

Results: In one-step purification, our method yielded 326 mg of rutaecarpine and 128 mg of evodiamine from 3.2 g of crude extract, with purities of 98.9 and 98.5%, respectively. The structures of these compounds were identified using ^{1}H NMR and ^{13}C NMR. There was a significant correlation between alkaloid content and fruit size, with a Spearman correlation coefficient of>0.5 ($p<0.001$), and there was a large difference in alkaloid contents between three maturity degrees of the fruit. Open-mouth fruits and fruits with average sizes of 4 to 6 mm had the highest alkaloid contents, whereas closed-mouth fruits had the lowest.

Conclusion: This study provided information on the standardization and quality control of evodiamine and rutaecarpine in *Evodiae fructus*, as well as a foundation for further pharmacological and toxicological studies.

Keywords*:* Isolation, Quality control, Evodiae Fructus, Major alkaloids

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INTRODUCTION

The medicinal plants act as a rich source of natural products. Extraction and characterization of some active components from medicinal plants help in the discovery of new potent drugs [1, 2]. *Evodiae fructus* (Wuzhuyu in China, ngo-thu-du in Vietnam) is the dried, unripe fruit of *Evodia rutaecarpa,* which belongs to the family Rutaceae. It has been widely used in Vietnam as a traditional medicine for gastrointestinal disorders and post-partum hemorrhage and amenorrhea. Three of its major components are limonin, evodiamine, and rutaecarpine (fig. 1). Modern pharmacological studies have proved their various activities, such as anti-inflammatory [3, 4], antiobesity [5, 6], hypotensive, cardiotonic, central stimulative, vasodilatory, antithrombotic, and bronchoconstrictive activities [3, 7, 8].

Interestingly, the degree of maturity of *Evodiae fructus* is known to influence the content of its active ingredients [9]. Even the fruits from the same plant may have different degrees of maturity, and differ in size, color, shape, and smell. To control the quality of this fruit, the Chinese Pharmacopoeia detects its two major alkaloids, namely evodiamine and rutaecarpine. Several analytical assays for determining evodiamine and rutaecarpine contents have been reported, including liquid chromatography-tandem mass spectrometry (LC/MS/MS) [10, 11]. Although the two methods are highly sensitive and selective, their use is limited because of the high cost of their instrumentation. Therefore, this study aimed to develop a simple, rapid, and sensitive analytical method for quantifying biologically important components in *Evodiae fructus*, namely limonin, evodiamine, and rutaecarpine, to evaluate the quality of *Evodiae fructus* sold in markets. In this study, 40 batches of *Evodiae fructus* were collected and the contents of its two major alkaloids were simultaneously determined. Total evodiamine and rutaecarpine contents in different samples were analyzed to provide information on the reasonable use of *Evodiae fructus*. Open column chromatography was conducted to separate and purify of the two major alkaloids from *Evodia rutaecarpa* extract possessing the highest evodiamine and rutaecarpine contents.

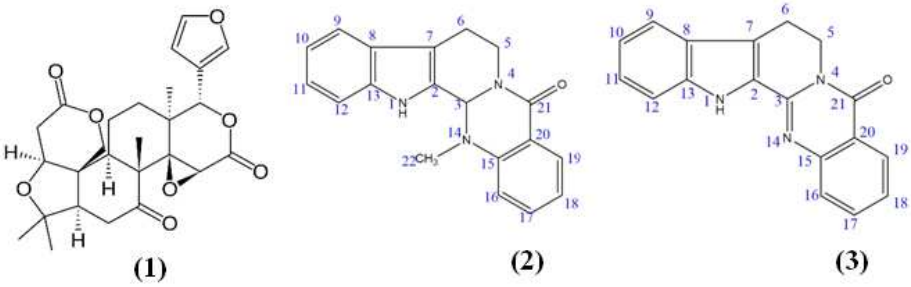

Fig. 1: Structures of limonin (1), evodiamine (2), and rutaecarpine (3)

MATERIALS AND METHODS

Chemical and materials

HPLC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). Limonin (purity 98.47%), evodiamine (purity 99%), and rutaecarpine (purity 99%), were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Forty fruits of *Evodia rutaecarpa* were collected from markets in different regions of Vietnam. The forty voucher specimen of *Evodiae fructus* was identified at Botany Department of Can Tho University of Medicine and Pharmacy and deposited at the Laboratory of *Drug Control and Toxicology.* The specimens were stored in sealed packages to avoid exposure to moisture and light.

Preparation of standard

Standard stock solutions of limonin (1 mg/ml), evodiamine (0.2 mg/ml), and rutaecarpine (0.5 mg/ml) were prepared in acetonitrile. The standard working solution of each compound was prepared by diluting the stock solution with a mobile phase to suitable volumes of concentration.

Preparation of sample solutions

Forty batches of *Evodiae fructus* were pulverized into a powder, passed through a 0.3 mm sieve (30 meshes), and stored in a desiccator until use. Each powdered sample was accurately weighed (0.10 g), soaked in 80% ethanol for 10 min, and then extracted three times with 40 ml 80% ethanol in an ultrasonic bath for 20 min. The extracted solution was filtered through an analytical filter paper and then evaporated to dryness by rotary vaporization under reduced pressure. The residue was suspended in 20 ml of CH_2Cl_2 and then successively partitioned twice with water (10 ml each). The CH_2Cl_2 extracts were combined and carefully evaporated to dryness in a vacuum. The dried residue was dissolved in 10 ml of mobile phase and injected into the HPLC system for quantitative analysis. All extracts were filtered through a 0.45 µm membrane filter into an HPLC vial and capped.

Instrumentation and chromatographic conditions

The experiment was carried out by a Hitachi HPLC L-2000 system (Hitachi, Japan) equipped with an L-2130 pump, L-2200 syringe, L-2300 temperature control system, and L-2455 diode-array detector. Chromatographic separation was achieved using a Germini C_{18} column (150 mm × 4.6 mm I.D., 5 µm), detected at 210 nm. The mobile phase consisted of acetonitrile (A), methanol (B), and water (C). The gradient elution program was as follows: 0-18 min, linear gradient 40% A and 5% B with a flow rate of 1 ml/min; 18-30 min, linear gradient 100% A with a flow rate of 1.2 ml/min. The injection volume was 10 µl.

NMR spectroscopy was performed with a Brucker Advance III (500 MHz; Brucker, Germany) operating at a probe temperature of 23±1 °C. Tetramethyl silane (TMS) was used as reference (δ = 0.00 ppm). To mix the contents of the NMR tube, a Maxi Mix II mixer (Barnstead/Thermolyne, USA) was used. Spectra were recorded at 500 MHz with $CDCl_3$ (99.8 atom% D) containing 0.05% (v/v) TMS as an internal standard.

Method validation

The proposed method was validated under the guidance of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005) [12]. The assays required to validate the method are as follows: system suitability, selectivity, linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, and precision assays.

Isolation and purification of two major alkaloids in *Evodiae fructus*

Preparation of crude sample

After alkaloid contents were determined in the 40 batches of *Evodiae fructus,* fruits possessing the highest alkaloid contents were chosen for extraction, isolation, and purification of evodiamine and rutaecarpine. Each compound was isolated using open column chromatography. Each fraction was manually collected according to the thin layer of the chromatogram and evaporated under reduced pressure. The residues were dissolved in methanol for the subsequent HPLC analysis.

HPLC analysis and identification of each fraction

The crude extract and all fractions were analyzed by HPLC using a Germini C_{18} column (150 mm × 4.6 mm I.D., 5 µm). Acetonitrile-methanol-water (15:45:40) was used as a mobile phase in isocratic mode, with a flow rate of 1.0 ml/min. The effluents were monitored at 225 nm by a photodiode array detector. The identification of each fraction was performed by ^{1}H NMR and ^{13}C NMR. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance III (500 MHz; Brucker, Germany).

Calculation

According to the Chinese Pharmacopoeia, a high-quality *Evodiae fructus* should contain at least 0.15% of total evodiamine and rutaecarpine contents. The total content of each compound was calculated by the following formula:

Content (%) of analyte $= (C*V*D)/10000W$

Where:

C = the concentration (mg/l) of an analyte in a test solution,

D = dilution factor, if any,

V = the final make-up volume (ml) of the test solution,

W = the weight (g) of the sample used to prepare the test solution.

RESULTS

Optimization of chromatographic conditions

Limonin, evodiamine, and rutaecarpine were identified by their retention times (t_R) and by co-injection with standards. The wavelength used to detect limonin, evodiamine, and rutaecarpine in *Evodiae fructus* was selected by using photodiode-array detection (DAD). The maximum number and height of the three peaks were obtained and the baseline of the chromatogram was stable at 210 nm. Therefore, 210 nm was chosen as a detection wavelength. The peak purity of the three compounds in the samples was 99.9%, which was the spectrum overlaying the graphs of three-point purity detection. Optimization of chromatographic conditions was performed by using a Gemini RP-C_{18} with different compositions of mobile phases [methanol-water (1), acetonitrile-water (2), and acetonitrile-methanol-water (3) systems] and a different ratio of solvents in isocratic mode. The results showed that with system 1, limonin was eluted much more rapidly (2-3 min) than evodiamine and rutaecarpine (50 min).

In contrast, elution with system 2 produced a good resolution of the three components but with short analysis time (under 8 min), which proves inconvenient for herbal matrices. Good resolution, baseline, sharp and symmetrical peaks, and favorable retention time were obtained by using system 3. The mobile phase was acetonitrile–methanol-water in the ratio of 40:5:55, and the retention times of limonin, evodiamine, and rutaecarpine were 8.1, 12.5, and 15.5 min, respectively. However, because of the polar impurity of compounds in the extract solution, we shortened the analysis time by using gradient elution. The representative chromatogram of the sample and standard (fig. 2) showed that limonin, evodiamine, and rutaecarpine were eluted with highly symmetrical peaks under the conditions. The analysis time was 30 min.

Method validation

System suitability

System suitability was tested by performing six replicate injections and determining the theoretical plate number (N), resolution (Rs), symmetry factor (As), and repeatability (RSD of retention time and area) of the analyte of interest. The % RSD values of area and retention time were less than 2%, indicating the precise analysis of limonin, evodiamine, and rutaecarpine by this system. All results showed that the proposed method met the requirements.

Selectivity

Method selectivity was tested by using HPLC to compare the retention time of each standard reference compound with that of the peaks of *Evodiae fructus* extract. The HPLC method was able to distinguish limonin, evodiamine, and rutaecarpine from other constituents in *Evodiae fructus* (flavonoids, quinolone alkaloids, etc.). There was no interference with the peaks of limonin, evodiamine, and rutaecarpine in *Evodiae fructus*. Therefore, the peak purity of the three compounds in the sample was 99.9%, as obtained from the spectrum overlaying the graphs of three-point purity detection (fig. 3).

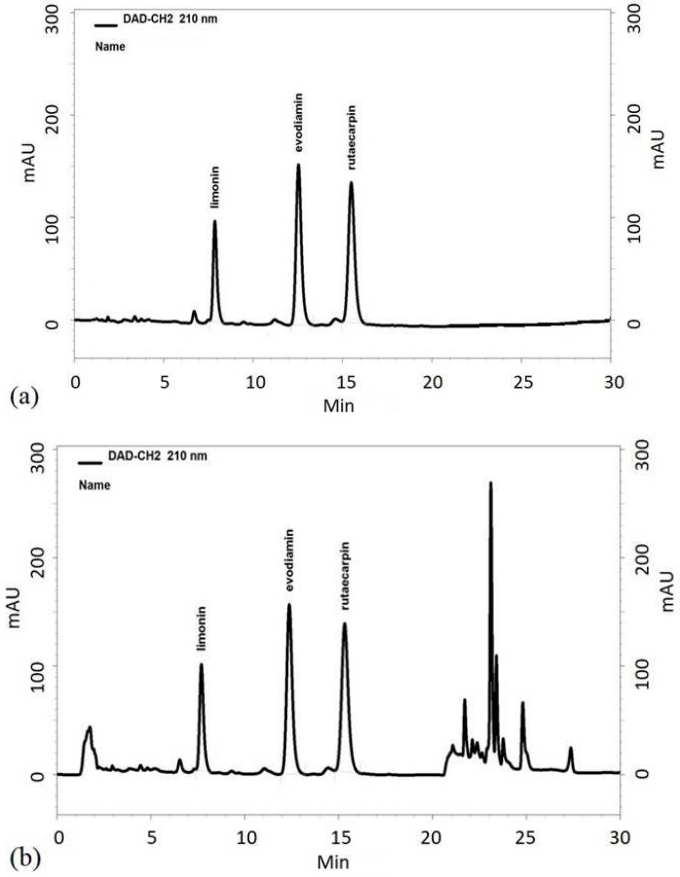

Fig. 2: Representative HPLC chromatograms of mixed standards (a) and *Evodiae fructus* extract (b) at 210 nm

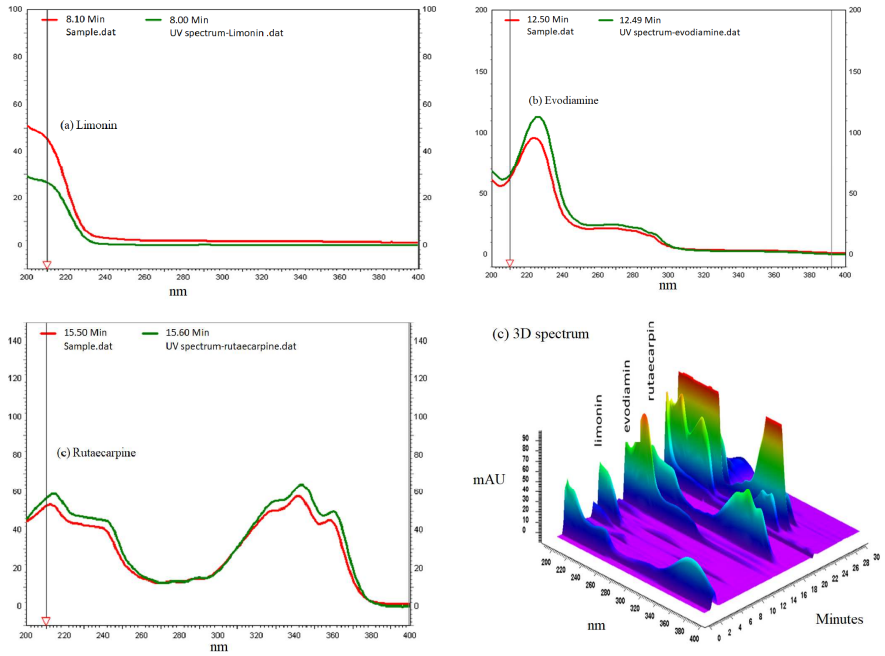

Fig. 3: UV-vis spectrum between standard and extract at their retention time: (a) limonin, (b) evodiamine, (c) rutaecarpine, and (d) 3 D spectrum of *Evodiae fructus* extraction

Precision, linearity, limits of detection, and limits of quantification

The results of the regression equation and squared correlation coefficients (r^2) are summarized in table 1. The LOD of the three constituents was 0.0625 µg/ml. The LOQ of limonin and rutaecarpine was 0.125 µg/ml, whereas that of evodiamine was 0.2 µg/ml. The RSDs of intra-day and inter-day were 4.81–6.07, 2.15–6.79, and 6.01-6.13% for limonin, evodiamine, and rutaecarpine, respectively.

Table 1: Linear regression data, LOD and LOQ, precision of the HPLC method for determination of limonin, evodiamine and rutaecarpine

Parameter	Limonin	Evodiamine	Rutaecarpine
Regression equation[a]	y = 51262x-177671	y = 268015x-109314	y = 324051x-875002
Linearity range (µg/ml), n=8	5-200	5-100	5-200
r^2	0.9977	0.9959	0.9967
LOD (µg/ml), n=3	0.0625	0.0625	0.0625
LOQ (µg/ml), n=3	0.125	0.2	0.125
Precision (intra-day, % RSD, n=6)	4.81	2.15	6.01
Precision (inter-day, % RSD, n=3)	6.07	6.79	6.13

a= y is the concentration of the analyte (µg/ml), and x is the peak area, n=number of determination, % RSD = % Relative standard deviation

Accuracy

Table 2 shows a summary of extraction recovery in *Evodiae fructus* samples. The developed method had good accuracy with an overall recovery of 102.73, 101.58, and 103.34% for limonin, evodiamine, and rutaecarpine, respectively, with % RSD of less than 5% for the analytes. Considering the results of the recovery test, the method was deemed to be accurate.

Table 2: Recoveries for the assay of the investigated compounds in *Evodiae fructus*

Analytes	Sample	Concentration (µg/ml)			Recovery	Mean recovery	RSD (%)
		Original	Added	Found	(%)	n=9	n=9
Limonin	S_1[a]	56.11	50	106.68	101.14		
	S_2[b]	56.11	60	118.36	103.76	102.73±5.12	4.98
	S_3[c]	56.11	70	128.42	103.30		
	S_1[a]	19.87	15	34.88	100.06		
Evodiamine	S_2[b]	19.87	20	39.86	99.94	101.58±4.51	4.44
	S_3[c]	19.87	25	46.05	104.73		
	S_1[a]	20.64	15	36.01	102.46		
Rutaecarpine	S_2[b]	20.64	20	41.09	102.27	103.34±3.91	3.78
	S_3[c]	20.64	25	46.96	105.30		

Recovery (%) = ((found−original)/added) ×100., [a]The samples added known amounts of standards at low level (80% of the known amounts)., [b]The samples added known amounts of standards at medium level (same as the known amounts)., [c]The samples added known amounts of standards at high level (120% of the known amounts), *% RSD = % Relative standard deviation*

Quality evaluation of *Evodiae fructus* in markets

Evodiamine and rutaecarpine contents in 40 samples of *Evodiae fructus* are summarized in table 3 and 4. Data are expressed as % (grams per 100 gram) of dry weight. The results showed a relationship between alkaloid contents and fruit size (fig. 4). The contents of the two major alkaloids were also significantly correlated with maturity degrees, as shown in fig. 5.

Table 3: Correlations between alkaloid contents and fruit size

Fruit size (mm)	N	Mean of alkaloids content (%)	Test
2	9	0.1744	
3	9	0.2024	Spearman parameter r=0.597
4	3	0.4179	p<0.001
5	6	0.9539	
6	8	0.7214	
7	3	0.3624	
8	2	0.3124	
Total	40	0.44629	

N= number of samples

Table 4: Correlations between alkaloid contents and maturity degree

Maturity degree	N	Content		Kruskal wallis test χ^2. p
		mean±SD	Median	
Closed mouth fruit	12	0.18716±1156.4	0.1445	χ^2= 11.1
Open-mouth fruit	19	0.65173±7075.9	0.3875	p = 0.004
Large mouth fruit	9	0.3581±2036.7	0.2892	
Total	40	0.44629±5351.4	3013	

N= number of samples, SD=Standard deviation

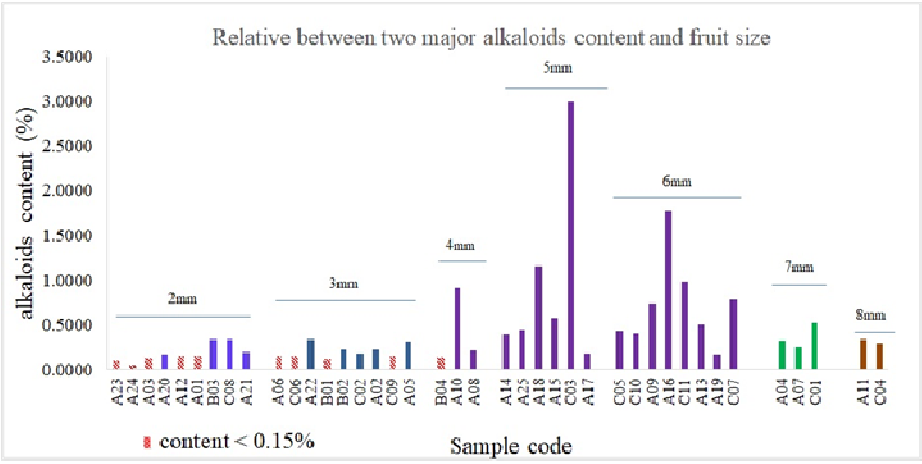

Fig. 4: Correlation of alkaloids content of *Evodiae fructus* samples and their fruit size

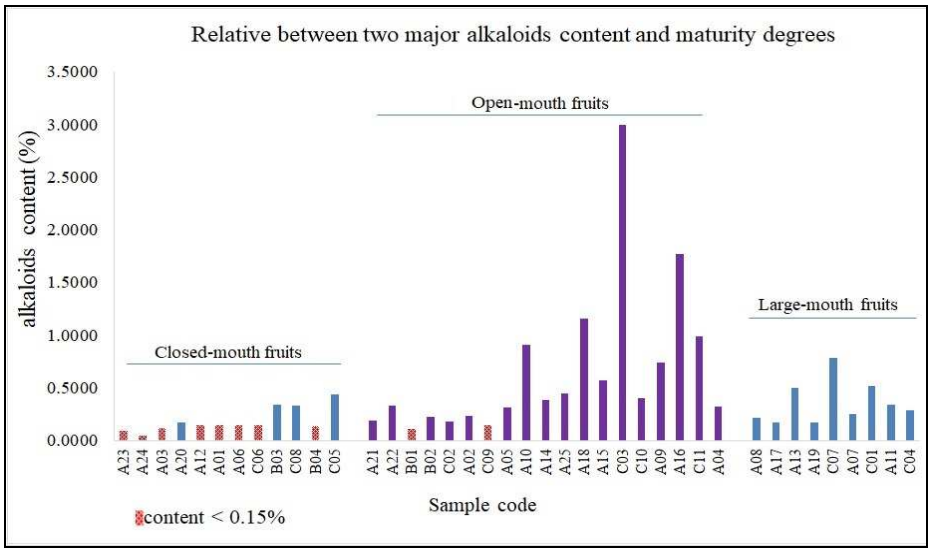

Fig. 5: Correlation of alkaloids content of *Evodiae fructus* samples and their maturity degrees

Isolation and purification of rutaecarpine and evodiamine

Open column chromatography and purification conditions

A series of experiments were performed to optimize the mobile phase solvent system for the proposed column separation method. Chloroform-methanol, chloroform-ethyl acetate, and *n*-hexane-ethyl acetate were tested as mobile phase solvents. When ethyl acetate-methanol was used, the target compounds mainly co-elute. By using chloroform-ethyl acetate, the elution was improved, but target compounds and nonpolar impurities were not separated. Thus, chloroform-methanol and chloroform-ethyl acetate were unsuitable to separate and purify the alkaloids in *Evodiae fructus*. In contrast, a mixture of *n*-hexane and ethyl acetate improved the fraction separation. The first fraction was purified by washing with cool n-hexane and a pure second fraction was obtained through precipitation with chloroform.

Two kinds of alkaloids were obtained in the one-step purification, yielding 326 mg rutaecarpine (**I**) and 128 mg of evodiamine (**II**) from 3.2 g crude sample. The purities of the two compounds were 98.9 and 98.5% respectively, as determined by HPLC. The chromatograms and UV spectra of these compounds are shown in fig. 6.

The structural identification

The chemical structure of each fraction was identified according to its ^{1}H NMR, ^{13}C NMR and ESI-MS data:

Fraction (I): ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 9.51 (br s. 1H. NH); 8.32 (d. 1H; J=7; H19); 7.70 (m. 1H. H17); 7.63 (m. 1H; H16); 7.42 (m. 1H; H9); 7.36 (m. 1H; H12); 7.31 (m. 1H; H18); 7.25 (s. 1H; H11); 7.17 (m. 1H; H10); 4.59 (t. 2H; H5); 3.23 (t. 2H; H6). ^{13}C-NMR (125 MHz, $CDCl_3$) δ ppm: 127.2 (C-2), 145.4 (C-3), 41.1 (C-5), 19.7 (C-6), 118.4 (C-7), 125.6 (C-8), 120.6 (C-9), 120.1 (C-10), 125.6 (C-11), 112.1 (C-12), 138.3 (C-13), 147.5 (C-15), 126.2 (C-16), 134.3 (C-17), 127.1 (C-18), 126.6 (C-19), 121.1 (C-20), 162.0 (C-21). ESI-MS m/z 288.1131 calculated for $C_{18}H_{13}N_3O$. According to the data obtained by Liu *et al.*, fraction I corresponded to rutaecarpine [14].

Fraction (I): ^{1}H-NMR (500 MHz, $CDCl_3$) *δ ppm*: 8,24 (br *s*, 1H, NH); 8,12 (*dd*, 1H; *J=6.5*; H_{19}); 7,59 (*d*, 1H; *J=7.5;* H_9); 7,49 (*m*, 1H, H_{17}); 7,41 (*d*, 1H, *J=8;* H_{12}); 7,25 (*m*, 2H; H_{11}, H_{16}); 7,19 (*m*, 2H; H_{10}, H_{18}); 5,92 (*s*, 1H; H_3); 4,87 (*m*, 1H; H_5); 3,29 (*m*, 2H; H_5); 2,96 (*m*, 2H; H_6); 2,505 (*s*, 3H; N-CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$) δ ppm: 129.0 (C-2), 68.8 (C-3), 39.5 (C-5), 20.1 (C-6), 113.7 (C-7), 126.3 (C-8), 118.9 (C-9), 123.1 (C-10), 124.1 (C-11), 111.3 (C-12), 136.7 (C-13), 150.6 (C-15), 122.4 (C-16), 133.1 (C-17), 123.8 (C-18), 128.2 (C-19), 120.0 (C-20), 164.7 (C-21), 37.2 (N CH3). ESI-MS m/z 303.1464 calculated for $C_{19}H_{17}N_3O$. In accordance with the data obtained by Liu *et al.*, fraction II corresponded to evodiamine [14].

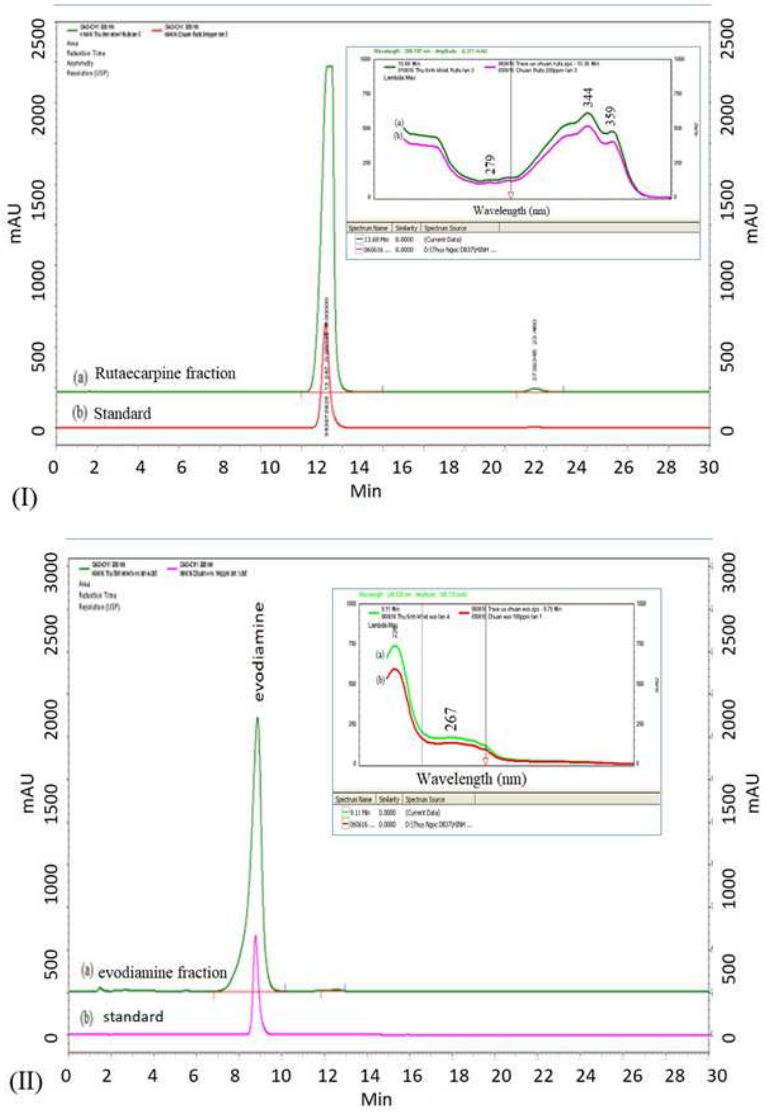

Fig. 6: HPLC purity test of two fractions collected from *Evodiae fructus* extraction, fraction (I): rutaecarpine, fraction (II) evodiamine

DISCUSSION

Contents of the three major components in *Evodiae fructus* extract could easily be determined within 30 min. All calibration curves showed good linear regression (r^2>0.99), with RSDs of intra-day and inter-day of approximately 6%. The overall recovery of the analytes was in the range of 101.58 to 103.34% with % RSD of less than 5%; thus, the proposed method was deemed to be precise and accurate. Zhao *et al.* developed an LC method for determining dehydroevodiamine, wuchuyuamide-I, 5-hydroxyrutaecarpine, 14-formyldihydrorutaecarpine, evodiamine, and rutaecarpine contents; however, the method requires a long analysis time of 70 min and complicated mobile phase consisting of methanol, acetonitrile, and phosphoric acid-triethylamine-buffer solution [13]. In this study, we conducted a simple chromatography using popular solvents commonly available in laboratories, which required only a short time (30 min) to measure the amount of the three major components in *Evodiae fructus*.

The validated method was successfully applied to simultaneously determine the contents of the two major alkaloids of *Evodiae fructus* in 40 samples collected from markets in different regions of Vietnam. As shown in table 3, alkaloid concentrations were the highest in the open-mouth fruits, followed by those in the large-mouth fruits and closed-mouth fruits, with χ^2= 11.1 and p = 0.004, as analyzed by the Kruskal-Wallis test. Closed-mouth fruits showed low contents of the two major alkaloids. In this group, 8 out of 12 samples showed evodiamine and rutaecarpine contents of less than 0.15%. In contrast, in open-mouth fruits, 19 samples showed high evodiamine and rutaecarpine contents. For instance, sample C03 showed total evodiamine and rutaecarpine contents of up to 3%. Interestingly, in large mouth-fruits (similar to open-mouth fruits except that their ovaries are split completely into five compartments), 9 samples showed uniformly high evodiamine and rutaecarpine contents. Similarly, as shown in table 4 and fig. 6, the highest alkaloid concentrations were observed in the fruit group with an average size of 4 to 6 mm, followed by those with big sizes of 7 to 8 mm and small sizes of 1 to 3 mm. The differences were significant, with a Spearman correlation coefficient of r>0.5 and p<0.001. The results were useful as a guide for choosing *Evodiae fructus* base on the fruit's degree of maturity.

In a one-step purification of 3.2 g crude extract, we obtained 326 and 128 mg of rutaecarpine (98.9% purity) and evodiamine (98.5% purity), respectively. Liu *et al.* used high-speed counter-current chromatography with a two-phase solvent system to isolate and purify five alkaloids, including 18 mg of evodiamine and 9 mg of rutaecarpine (purity of 98.4%), from 6.2 g crude sample [14]. In comparison with our results, we can conclude that our method was effective and economical. It can be implied that these pure alkaloids can be used as a standard to control the quality of *Evodiae fructus* in Vietnam. Currently, the Vietnamese Pharmacopoeia uses total oil

content to evaluate the quality of this fruit. Our results provided an option to use natural standard compounds, namely evodiamine and rutaecarpine, to control the quality of *Evodiae fructus* in the market.

CONCLUSION

In summary, this study described the development, validation, and application of an HPLC method for determining limonin, evodiamine, and rutaecarpine contents in *Evodiae fructus* using Germini C_{18} column. The relatively simple sample preparation, together with the short HPLC run time (30 min), proved that the present method was useful for routine quantitative analysis and quality control of limonin, evodiamine, and rutaecarpine, the major components in *Evodiae fructus*. This method was satisfactory in terms of accuracy, precision, sensitivity, and reproducibility. The method was successfully applied to analyze 40 batches of samples collected from markets, and the results showed that alkaloid contents were related to fruit size and fruit maturity degree. The highest alkaloid concentrations were observed in fruits with an average size of 4 to 6 mm, and open-mouth fruits. The proposed method was shown to be useful as a guide for choosing *Evodiae fructus* base on its maturity degree. Next, fruits possessing the highest alkaloid contents were chosen for isolation and yielded 326 mg of rutaecarpine and 128 mg of evodiamine from 3.2 g crude sample. The purity was 98.9 and 98.5%, respectively, as determined by HPLC. This study sheds light on the standardization and quality control of evodiamine and rutaecarpine.

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AUTHORS CONTRIBUTIONS

N. Van. T. Nguyen, X. Dao T. Nguyenperformed the experiments, K. Ngan H. Nguyen and T. Kien Nguyen analyzed the data; N. Van. T. Nguyen and K. Ngan H. Nguyen wrote and edited the paper; M. Phuong Nguyen gave suggestion, Kyeong-Ho Kim supervised the project.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

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