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A validated HPLC method for the quantitative analysis of evodiamine and rutaecarpine in *Evodia rutaecarpa* (Juss.) Benth

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Abstract

A simple, sensitive and accurate HPLC-DAD method was developed for simultaneous determination of evodiamine and rutaecarpine in *Evodia rutaecarpa* that has been widely used as one of the traditional Vietnamese medicines. The method was carried out by using a Gemini RP C18 column with a gradient solvent system of acetonitrile - methanol - water and a PDA detector (225 nm). The result found that contents of the alkaloids in Evodiae fructus extract could easily be determined within 30 minutes. All calibration curves showed good linear regression (r^2>0.9999) within the concentration ranges of 2 - 100µg/ml for evodiamine and rutaecarpine. The RSDs of intra-day and inter-day were 3.54-4.74% and 1.89- 3.72% for evodiamine and rutaecarpine, respectively. The LOD were 0.0625 and 0.500 µg/ml, and the LOQ were 0.125 and 1.665 µg/ml, for evodiamine and rutaecarpine, respectively. The recovery was 99.8% for evodiamine and 96.4% for rutaecarpine. The validated method was successfully applied for the simultaneous determination of the two chemical constituents in 40 batches of samples collected from different regions in the market. The quantification data of them in Evodiae fructus were 0.017–1.522 g/100g for evodiamine and 0.050 – 1.470 g/100g for rutaecarpine. Hence, the validation procedure confirmed that this technique afforded reliable analysis of these components in complex matrices such as Evodiae fructus extracts. The method proposed was demonstrated to be very useful in guiding chosen herb use due to the relation of fruit maturity degree.

Keyword: *Evodia rutaecarpa* (Juss.) Benth, HPLC-DAD, evodiamine, rutaecarpine, Rutaceae

1. INTRODUCTION

Evodiae fructus is the dried, unripe fruit of *Evodia rutaecarpa* (Juss.) Benth. belonging to the family Rutaceae. It has been widely used as one of the traditional Vietnamese medicines for treatment of gastrointestinal disorders, post-partum hemorrhage and amenorrhea. The two major bioactive alkaloids are evodiamine and rutaecarpine (Fig. 1). Modern pharmacological studies have proved their various activities, such as inhibit corticosterone production, anti-inflammation, antiobesity, cardiotonic, center stimulative, vasodilatatory, antithrombotic and bronchoconstrictive activities[1-4].

Several analytical assays have been reported for determination of evodiamine and rutaecarpine which included thin-layer

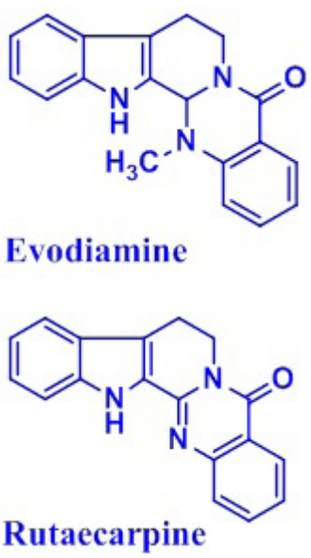

Figure 1. Structures of evodiamine and rutaecarpine

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chromatography[5], liquid chromatography[6], liquid chromatography-tandem mass spectrometry (LC/MS/MS)[7], and capillary electrophoresis[8]. Zhou et al. developed an LC–MS method for simultaneous determination of dehydroevodiamine, 14-formyldihydroru-taecarpine, evodiamine, rutaecarpine and goshuyuamide[11]. Zhao et al. developed an LC–MS/MS determination for dehydroevodiamine, limonin, evodiamine, rutaecarpine. Although the two methods were highly sensitive and selective, their popularities were limited because of the high cost of instrumentation[12]. In general, herbs collected from different regions or at different time are discrepant in the types and quantities of chemical constituents, which influence their therapeutic effects.

Therefore, the aim of this study are to develop a simple, rapid, sensitive, and robust analytical method for quantification of biologically important alkaloids namely evodiamine and rutaecarpine in evodiae fructus, quality evaluation of *Evodia rutaecarpa* (Juss.) Benth. in market. Forty batches of *E. rutaecarpa* were collected and the contents of the two markers were simultaneously determined in our study. The HPLC method developed could be responsible for the quality control of *E. rutaecarpa*. The total contents of evodiamine and rutaecarpine in different samples were analyzed to provide information for using *E. rutaecarpa* reasonably.

2. MATERIALS AND METHODS

2.1. Chemical and materials

HPLC-grade acetonitrile and methanol was purchased from Merck Company (Darmstadt, Germany). Water prepared with a Millipore Milli-Q SP water purification system (Millipore, France) was used during sample preparation procedures and HPLC analyses. Evodiamine and rutaecarpine standards were from Sigma- Aldrich. Other reagent solutions were of analytical grade.

Forty batches of samples were collected from different regions in Vietnam market. Voucher specimens were deposited at the Laboratory of *Drug Control and Toxicology*, Can Tho University of Medicine and Pharmacy. Then, they were stored in sealed packages to avoid moisture and light.

2.2. Preparation of standard

Standard stock solutions of evodiamine (0.2 mg/ml) and rutaecarpine (0.2 mg/ml) were prepared in methanol. Working standard solutions containing each of the two compounds were prepared by diluting the stock solutions with mobile phase to suitable volumes of concentration for evodiamine, and rutaecarpine.

2.3. Preparation of sample solutions

Forty batches of Evodiae fructus pulverized into powder, passed through a 0.3 mm sieve (30 meshes) and stored in a desiccator until required for determination. An accurately weighed amount (0.10 g) of each powder sample extracted with 40 ml methanol in an ultrasonic bath for 20 min three times after soaking for 1 h at ambient temperature. The extracted solution was filtrated through 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 was then successively partitioned with water (10 ml each for twice). The CH_2Cl_2 extracts were combined and were carefully evaporated to dryness in vacuum. The dried residue was dissolved in 10 ml of mobile phase and was injected into the HPLC system for quantitative analysis. All the extracts were filtered through a 0.45 μm membrane filter into an HPLC vial and capped.

2.4. Instrumentation and chromatographic conditions

The experiment was carried out by a Hitachi HPLC L-2000 system (Hitachi, Japan) with an L-2130 pump, an L-2200 syringe, an L-2300 temperature control system, an L- 2455 diode- array detector. The chromatographic separation was achieved using a Germini C_{18} column (150 mm; 4.6 μm), detected at 225 nm at room temperature. The mobile phase consisted of acetonitrile (A), methanol (B), water (C). The gradient

program was as follow: 0-20 min, linear gradient 15% A, 45% B with flow rate 1 ml/min; 20-30 min, linear gradient 100% A with flow rate 1.2 ml/min. The volume injected was 10 µl.

2.5. Method validation

For the validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use were followed[9]. The requirement for the drug assay follows these topics: system suitability, specificity, linearity, limits of detection (LOD) and quantification (LOQ), accuracy, precision.

Linearity, limits of detection and quantification

Linearity of the methods was checked using sets of up to six concentration levels. A series solution containing appropriate concentrations of two reference compounds were used for the construction of calibration curves. Limits of detection (LOD) and quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Precision and accuracy

For intra-day variability test, two alkaloids in six sample solutions were determined within one day, while for inter-day variability tests, two concentrations of alkaloids were examined in twice a day on 3 consecutive days. All the results were expressed as relative standard deviations (RSD).

The accuracy of the method was investigated by recovery studies; in particular, samples of powdered evodiae fructus (100.0 mg) were spiked with 1.0 ml aliquot of a solution containing standard compounds in the extraction solvent, at three concentration levels and three samples for each level. The spiked samples were then extracted processed, and quantified in accordance with the methods mentioned above.

3. RESULTS

3.1. Selection of chromatographic conditions

The wavelength for the detection of evodiamine and rutaecarpine in *E. rutaecarpa* (Juss.) Benth. was selected by using photodiode-array detection (DAD). The maximum number and the height of the peak could be obtained and the baseline of chromatogram was stable at 225 nm. Therefore, 225 nm was chosen as detection wavelength. The peak purity of two compounds in the sample was 99.9% obtained from spectrum overlaying graphs of three-point purity detection. The optimization of the chromatographic conditions was performed by using four different columns (Inertsil GL Science ODS C_{18}, Phenomenex Gemini RP-C_{18}, Agilent Zorbax C_8, Phenomenex Gemini RP-C_8) with different compositions of mobile phases ((1) methanol–water system, (2) acetonitrile–water and (3) acetonitrile–methanol- water system) and different ratio of solvent in isocratic mode. The result showed that Phenomenex Gemini RP-C_{18} column could efficiently separate the investigated compounds. It was shown that the resolution was poor with system (1) and analysis time was long with system (2). Good resolution, baseline, sharp and symmetrical peaks were obtained by using system (3). The mobile phase was acetonitrile–methanol-water in the ratio of 15:45:40 (v/v). However, due to polar impurity compounds in extract solution, we shorten analysis time by using gradient mode to elute them. The representative chromatogram of the sample and standard (Fig. 2) could be concluded that evodiamine and rutaecarpine were eluted with highly symmetrical peak under the condition.

3.2. Method validation

System suitability

System suitability was tested by performing six replicate injections and determining theoretical plate number (N), resolution (Rs), and symmetry factor (As) and repeatability (RSD retention time and area)

for the analyte of interest. As summarized in Table 1, the %RSD values of area and retention time were less than 2% indicating the precise analysis of evodiamine and rutaecarpine by this system. All the results showed that the proposed method met the requirement.

Table 1. System suitability parameters of evodiamine and rutaecarpine

Parameter	Evodiamine		Rutaecarpine	
	Mean (n=6)	%RSD	Mean (n=6)	%RSD
Retention time (min)	10.62	0.22	15.39	0.17
Area (mAU)	12183104	1.22	4025445	0.96
Resolution			8.08	
Symmetry factor	1.10		1.09	
Number of theoretical plate	6998		8385	

Specificity

The selectivity was tested by applying the HPLC method to analyze methanol extracts of evodiae fructus. It was evaluated by comparing the retention time of each standard reference compound with that of the peaks obtained by analyzing real extracts. The HPLC method was able to discriminate evodiamine and rutaecarpine alkaloids of Evodiae fructus from the other constituents of the plant material (flavonoids, quinolone alkaloids, etc.). There was no interference with the peaks of evodiamine and rutaecarpine in Evodiae fructus (shown in Fig. 2).

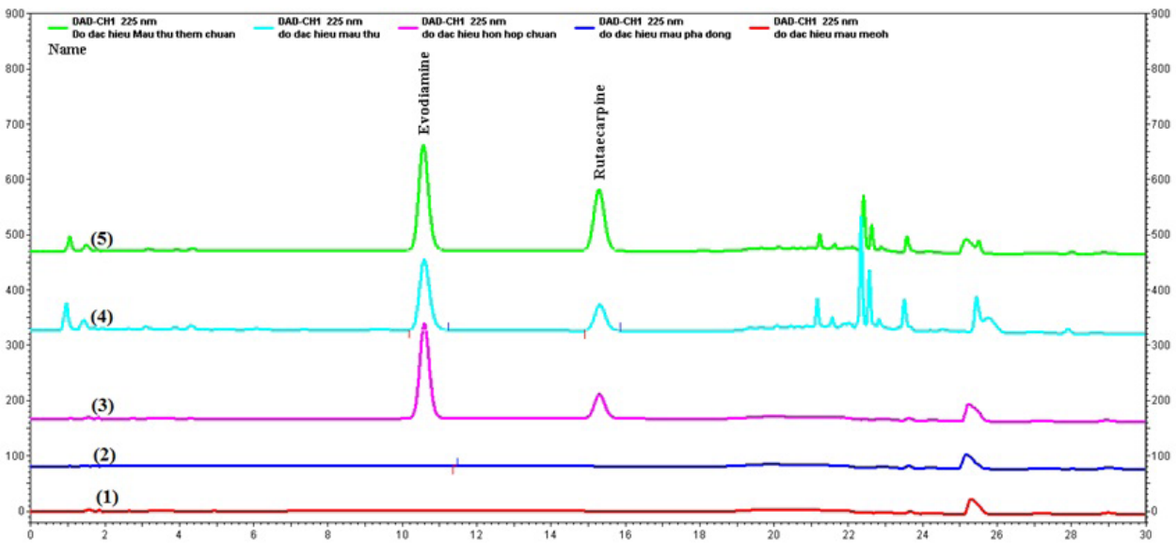

Figure 2. Representative HPLC chromatograms of mixed standards and the extract of *E. rutaecarpa* at 225 nm. (1) dissolving solvent (methanol), (2) mobile phase, (3) mixed standards of the two chemical constituents (4) extract of *E. rutaecarpa*, (5) extract of *E. rutaecarpa* spiked with evodiamine and rutaecarpine standard.

Linearity, limits of detection and quantification

The result for regression equation, and correlation coefficients (r^2) are summarized in Table 2. The LOD were 0.0625 and 0.500 μg/ml, and the LOQ were 0.125 and 1.665 μg/ml, for evodiamine and rutaecarpine, respectively.

Table 2. System suitability parameters of evodiamine and rutaecarpine

Parameter	Evodiamine	Rutaecarpine
Regression equation	y=436845x	y=224034x - 130251
Linearity range (μg/ml)	2 -100	2 - 100
r^2	0.9999	0.9999
LOD (μg/ml)	0.0625	0.5000
LOQ (μg/ml)	0.125	1.665
Precision (intra-day, % RSD)	4.74	3.72
Precision (inter-day, % RSD)	3.54	1.89

Regression curve data for six calibration points is y = ax + b, where y is the ratio between peak area of analytes, x is concentration, a is slope, b is intercept, and r2 is the squared correlation coefficient.

Precision

The RSDs of intra-day and inter-day were 3.54 -4.74% and 1.89- 3.72% for evodiamine and rutaecarpine, respectively (data were shown in Table 2)

Accuracy

Table 3 showed a summary of extraction recovery in Evodiae fructus sample. The developed method had good accuracy with overall recovery was 99.8% for evodiamine and 96.4% for rutaecarpine with RSD less than 5% for the analytes. Considering the results of the recovery test, the method was deemed to be accurate.

Table 3. Recoveries for the assay of the investigated compounds in Evodiae fructus

Analytes	Sample	Concentration (μg/ml)			Recovery (%)	Mean recovery	RSD (%) n=9
		Original	Added	Found			
Evodiamine	S_1[a]	21.71	17.4	38.51	96.55		
	S_2b	21.71	22	44.62	104.14	99.8	3.4
	S_3[c]	21.71	26.2	47.98	100.27		
Rutaecarpine	S_1[a]	18.12	14.4	31.80	95.02		
	S_2[b]	18.12	18.2	36.33	100.07	96.4	4.4
	S_3[c]	18.12	21.8	38.66	94.22		

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).

3.2. Quality evaluation of Evodia rutaecarpa (Juss.) Benth *in the market*

The contents of two alkaloids in 40 commercial samples of evodiae fructus were summarized in Table 4. The amount of them obviously varied among different sample (Fig. 3). Data are expressed as % (grams per 100 gram) of dry weight. The quantification data of them in Evodiae fructus were 0.016–1.470 g/100g for evodiamine and 0.017 – 1.522 g/100g for rutaecarpine

Table 4. Collected information and content of evodiamine and rutaecarpine in *Evodia fruits*

Sample No	Sample code	Source	Distribution channel	Content dry weight, % (g/100g)[1]		
				Evodiamine	Rutaecarpine	Total content
1	A03	Bac Lieu	Retail	0.035 ± 0.0043	0.086 ± 0.0074	0.121 ± 0.0058
2	A14	Ben Tre	Retail	0.219 ± 0.0218	0.168 ± 0.0286	0.387 ± 0.0240
3	A10	Ca Mau	Retail	0.302 ± 0.0115	0.600 ± 0.0014	0.903 ± 0.0097
4	A04	Can Tho	Retail	0.167 ± 0.0066	0.157 ± 0.0064	0.324 ± 0.0067
5	A05	Can Tho	Retail	0.158 ± 0.0071	0.155 ± 0.0081	0.313 ± 0.0070
6	A06	Can Tho	Retail	0.083 ± 0.0053	0.059 ± 0.0026	0.142 ± 0.0037
7	A07	Can Tho	Retail	0.139 ± 0.0021	0.108 ± 0.0033	0.247 ± 0.0027
8	A13	Can Tho	Retail	0.333 ± 0.0117	0.168 ± 0.0097	0.501± 0.0070
9	A23	Can Tho	Retail	0.039 ± 0.0025	0.052 ± 0.0039	0.091 ± 0.0029
10	A24	Can Tho	Retail	0.016 ± 0.0004	0.029 ± 0.0009	0.045 ± 0.0006
11	A02	Dong Thap	Retail	0.120 ± 0.0112	0.108 ± 0.0033	0.228 ± 0.0057
12	A17	Dong Thap	Retail	0.115 ± 0.0021	0.059 ± 0.0039	0.174 ± 0.0047
13	A18	Dong Thap	Retail	0.427 ± 0.0131	0.725 ± 0.0495	1.152 ± 0.0370
14	A19	Ha Noi	Retail	0.103 ± 0.0026	0.064 ± 0.0039	0.168 ± 0.0027
15	A20	Ha Noi	Retail	0.128 ± 0.0112	0.045 ± 0.0009	0.173 ± 0.0180
16	A21	Ha Noi	Retail	0.107 ± 0.0021	0.079 ± 0.0006	0.186 ± 0.0037
17	A22	Ha Noi	Retail	0.172 ± 0.0066	0.161 ± 0.0056	0.333 ± 0.0042
18	A09	Hau Giang	Retail	0.520 ± 0.0043	0.219 ± 0.0026	0.739 ± 0.0031
19	A15	Kien Giang	Retail	0.398 ± 0.0059	0.172 ± 0.0074	0.570 ± 0.0067
20	A08	Soc Trang	Retail	0.117 ± 0.0170	0.094 ± 0.0001	0.211 ± 0.0094
21	A11	Tien Giang	Retail	0.256 ± 0.0100	0.080 ± 0.0007	0.336 ± 0.0170
22	A12	Tien Giang	Retail	0.130 ± 0.0015	0.017 ± 0.0002	0.147 ± 0.0012
23	A25	Tp. HCM	Retail	0.255 ± 0.0218	0.192 ± 0.0114	0.447 ± 0.0150
24	A01	Trà Vinh	Retail	0.070 ± 0.0008	0.072 ± 0.0018	0.142 ± 0.0035
25	A16	Vinh Long	Retail	0.960 ± 0.0396	0.807 ± 0.0495	1.767 ± 0.0480
26	B01	HCM city	Whole sale	0.050 ± 0.0039	0.061 ± 0.0089	0.111 ± 0.0027
27	B03	HCM city	Whole sale	0.220 ± 0.0218	0.117 ± 0.0014	0.337 ± 0.0150
28	B02	HCM city	Whole sale	0.094 ± 0.0053	0.126 ± 0.0019	0.220 ± 0.0023
29	B04	Ha Noi	Whole sale	0.094 ± 0.0028	0.045 ± 0.0009	0.139 ± 0.0027
30	C10	Can Tho	Whole sale	0.220 ± 0.0035	0.180 ± 0.0056	0.400 ± 0.0028
31	C11	Can Tho	Whole sale	0.664 ± 0.0117	0.319 ± 0.0190	0.983 ± 0.0170
32	C07	Ha Noi	Whole sale	0.415 ± 0.0170	0.364 ± 0.0140	0.779 ± 0.0013
33	C08	Ha Noi	Whole sale	0.180 ± 0.0031	0.150 ± 0.0097	0.330 ± 0.0052
34	C09	Hung Yen	Whole sale	0.079 ± 0.0008	0.063 ± 0.0003	0.142 ± 0.0007
35	C01	HCM city	Whole sale	0.360 ± 0.0031	0.157 ± 0.0067	0.517 ± 0.0090
36	C02	HCM city	Whole sale	0.156 ± 0.0097	0.027 ± 0.0007	0.183 ± 0.0084
37	C03	HCM city	Whole sale	1.470 ± 0.0468	1.522 ± 0.0097	2.992 ± 0.0370
38	C04	HCM city	Whole sale	0.230 ± 0.0015	0.060 ± 0.0005	0.290 ± 0.0023
39	C05	HCM city	Whole sale	0.299 ± 0.0048	0.134 ± 0.0053	0.433 ± 0.0036
40	C06	HCM city	Whole sale	0.105 ± 0.0012	0.043 ± 0.0002	0.148 ± 0.0018

[1]Data are expressed as mean ± SD. For each sample n=3

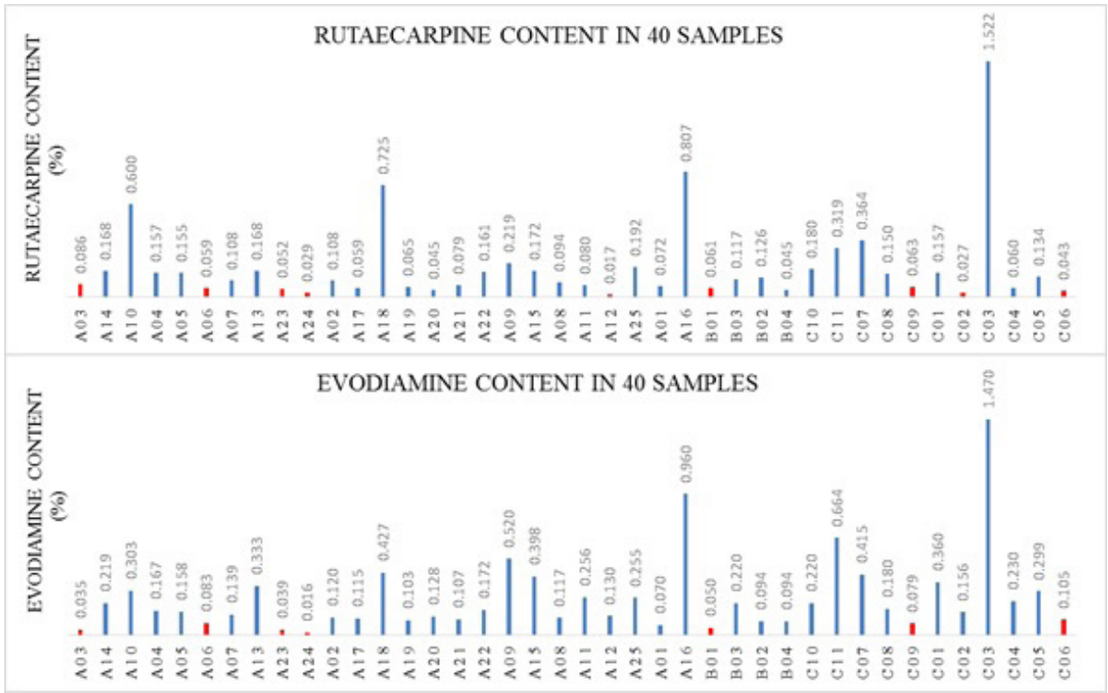

Figure 3. Total alkaloid content of *Evodiae fructus*

4. DISCUSSION

4.1. Method validation

Contents of the alkaloids in Evodiae fructus extract could easily be determined within 30 minutes. All calibration curves showed good linear regression (r^2>0.9999) within the concentration ranges of 2 - 100 µg/ml for evodiamine and rutaecarpine. The RSDs of intra-day and inter-day were 3.54 -4.74% and 1.89- 3.72% for evodiamine and rutaecarpine, respectively. The LOD were 0.0625 and 0.500 µg/ml, and the LOQ were 0.125 and 1.665µg/ml, for evodiamine and rutaecarpine, respectively. The recovery was 99.8% for evodiamine and 96.4% for rutaecarpine.

Zhao et al. developed an LC method for the determination of dehydroevodiamine, wuchuyuamide-I, 5-hydroxyrutaecarpine, 14-formyldihydrorutaecarpine, evodiamine and rutaecarpine, but it took a long analysis time of 70 min and complicated mobile phase consisted of methanol, acetonitrile and phosphoric acid–triethylamine–buffer solution was used[10].

4.2. Quality evaluation of Evodia rutaecarpa (Juss.) Benth *in the market*

The validated method was successfully applied for the simultaneous determination of the two chemical constituents in 40 batches of samples collected from different regions in market. The quantification data of them in Evodiae fructus were 0.016–1.470 g/100g for evodiamine and 0.017 – 1.522 g/100g for rutaecarpine. With refer to the Chinese Pharmacopoeia[13], the total contents of evodiamine and rutaecarpine in qualified Evodiae fructus samples should reach at least 0.15% (0.15 g/100g) or it would not be used as the raw material and is regarded as substandard herb. Based on this definition, sample code A01, A03, A06, A12, A23, A24, B01, B04, C06 and C09 should not be put into production, which causes serious waste of the herbs. Among them sample code A01, A03, A06, A12, A23 and A24 were from retail channel and sample code B01, B04, C06 and C09 were from wholesale channel.

In accordance with Chuang *et al*, by comparing the chromatographic profiles of *E.*

rutaecarpa and *E. officinalis* samples, it is difficult to clearly distinguish between the two species in terms of their constituents[14]. The degree of maturity of Evodiae fructus is known to influence the content of the active ingredients. Even the fruits from the same plant may have different degrees of maturity, and differ in size, color, shape, and smell. Mature or so-called open-mouth fruits (i. e., fruits of big size, black color, strong aroma, and ovaries that have been split into five compartments) have high contents of active compounds, while immature or so-called closed-mouth fruits (i. e., small round fruits of small sizes, with low or no smell, and closed ovaries) have low contents. This observation is consistent with the results of our study (shown in Fig. 4)

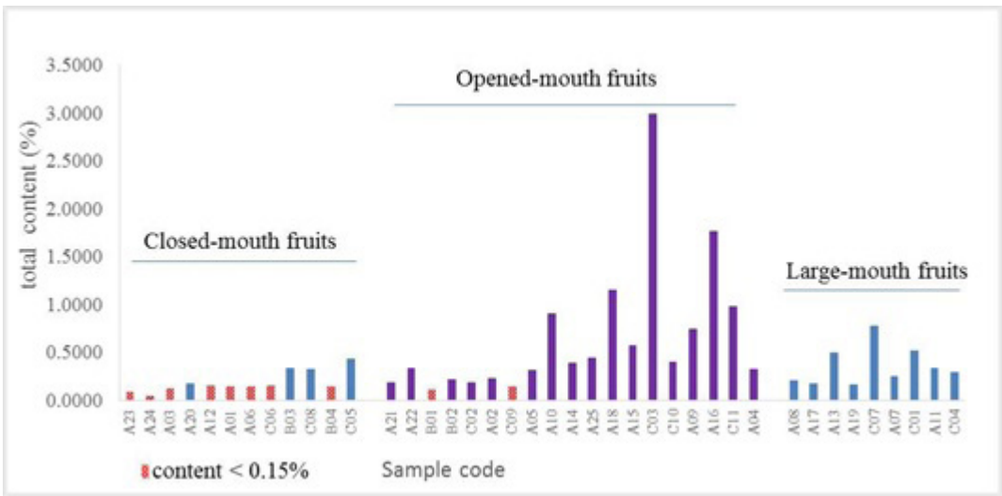

Figure 4. Correlation of total alkaloid content of Evodiae fructus samples and their degrees of maturity

Regarding to closed-mouth fruits groups, displayed very small content of two major alkaloids. In this group, there are 8 per 12 samples have low content of evodiamine and rutaecarpine that less than 0.15%. Concerning open-mouth fruits, there are 19 samples with very high content of evodiamine and rutaecarpine, such as sample C03 with total content of evodiamine and rutaecarpine up to 3%. Interestingly, with large mouth-fruits (similar to open-mouth fruits but ovaries that have been split into five compartments completely) there are 9 samples showed high amount of active constituents uniformly.

5. CONCLUSION

The method was simple, precise, and economical in terms of time and solvent usage. The validation procedure confirmed that this technique afforded reliable analysis of evodiamine and rutaecarpine in complex matrices such as Evodiae fructus extracts. The results showed that the content of evodiamine and rutaecarpine was closely related to the degree of maturity of the fruits.

6. ACKNOWLEDGEMENTS

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