Regular Article

Determination of the Marker Diarylheptanoid Phytoestrogens in *Curcuma comosa* Rhizomes and Selected Herbal Medicinal Products by HPLC-DAD

Bancha Yingngam,*[,a] Adelheid Brantner,[b] Damrongsak Jinarat,[a] Rawiwun Kaewamatawong,[a] Wandee Rungseevijitprapa,[a] Apichart Suksamrarn,[c] Pawinee Piyachaturawat,[d] and Ratchanaporn Chokchaisiri[e]

[a] *Department of Pharmaceutical Chemistry and Technology, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University; Ubon Ratchathani 34190, Thailand:* [b] *Department of Pharmacognosy, Institute of Pharmaceutical Sciences, University of Graz; Universitaetsplatz 4/1, A-8010 Graz, Austria:* [c] *Department of Chemistry, Faculty of Science, Ramkhamhaeng University; Bangkok 10240, Thailand:* [d] *Department of Physiology, Faculty of Science, Mahidol University; Bangkok 10400, Thailand: and* [e] *Department of Chemistry, School of Science, University of Phayao; Maeka, Muang, Phayao 56000, Thailand.*

Received June 27, 2017; accepted October 19, 2017; advance publication released online October 26, 2017

A method for quantification of diarylheptanoids in *Curcuma comosa* rhizomes and selected pharmaceutical preparations was established by using HPLC-diode array detector (DAD). The chromatographic separation of three diarylheptanoids [(3*S*)-1-(3,4-dihydroxy-phenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (1), (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (2), and (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol (3)] was performed on a Luna C_{18} analytical column using gradient elution with 0.5% acetic acid in water and acetonitrile with a flow rate of 1 mL/min and a column temperature of 35°C. The calibration curves for the analytes showed good linearity (R^2>0.999), high precision (relative standard deviation (RSD) <2%) and acceptable recovery (98.35–103.90%, RSD <2%). The limit of detection (LOD) and limit of quantification (LOQ) were 0.06–0.22 and 0.18–0.69 μg/mL, respectively. The results of all validated parameters were within the limits according to the International Conference on Harmonization (ICH) Guidelines. The established method was successfully applied for qualitative and quantitative determination of the three constituents in different samples of *C. comosa* and some commercial products in capsules. The simplicity, rapidity, and reliability of the method could be useful for the fingerprint analysis and standardization of diarylheptanoids, which are responsible for the estrogenic activity in raw materials and herbal medicinal products of *C. comosa*.

Key words *Curcuma comosa*; diarylheptanoid; standardization; fingerprint analysis; HPLC; method validation

The use of traditional medicine is still the main sector of medical care worldwide including Thailand.[1–3] One of the most widely used Thai herbs is *Curcuma comosa* Roxb. (Wan Chak Motluk), a plant of the Zingiberaceae family. It has traditionally been used for the treatment of uterine inflammation, postpartum uterine bleeding, premenopausal bleeding, stomachic problems, and tonic being the main uses.[4,5] Currently, many traditional Thai medicines and dietary supplemental products containing *C. comosa* are gained popularity in the market for the treatment of gynecological disorders and health promotion in peri- and postmenopausal women.

Most of the studies on *C. comosa* have dealt with the identification of the ingredients in the plant and the evaluation of the pharmacological activities of diarylheptanoids.[6–20] Pure compounds and plant extracts of *C. comosa* exhibited several pharmacological activities including anti-allergic,[9] anti-inflammatory,[13] anti-melanogenesis,[8] hepatoprotective,[16] bone-sparing[17] and estrogenic effects.[20] Among these pharmacological activities, the estrogen-like property of the *C. comosa* has evoked public interest and led to research studies for its application as a hormone replacement therapy instead of estrogen treatment. A randomized clinical trial assessing the efficacy and safety of the hexane extract of *C. comosa* rhizomes (250 and 500 mg crude extract/tablet) on vasomotor symptoms in peri- and postmenopausal women is ongoing in Thailand.[12]

Although the pharmacological activities of *C. comosa* have been clearly demonstrated, quality assessment of the diarylheptanoids in *C. comosa* is still rare and needs to be developed for the pharmaceutical industry. The major compound in *C. comosa* rhizomes has been identified as phytoestrogen diarylheptanoids. Up to now, there is not any diarylheptanoid assigned as a chemical marker for the quality control of *C. comosa* in the official Thai Herbal Pharmacopoeia. This is a major disadvantage for the production of herbal medicines from *C. comosa* rhizomes. Suksamrarn *et al.*[5] reported that 14 kinds of the diarylheptanoids were isolated and identified from the plant material. Ideally, all of them should be candidates for the quality control of *C. comosa*. However, minimizing the number of chemical markers may be an alternative choice for this purpose. To the best of our knowledge, three constituents, namely (3*S*)-1-(3,4-dihydroxy-phenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (**1**), (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**2**), and (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol (**3**), are the principle components of the rhizomes (Fig. 1). The diarylheptanoids **1**–**3** are not commercially available and they must be isolated from the plant material. Su *et al.*[4] selected three diarylheptanoids including (6*E*)-1,7-diphenylhept-6-en-3-one (does not appear in the submitted study), (4*E*,6*E*)-1,7-diphenylhepta-4,6-dien-3-ol ("2" of the submitted study), and (6*E*)-1,7-diphenylhept-6-en-3-ol ("3" of the submitted study) as the compounds to be the markers in quality control of *C. comosa*. In the submitted study, compound **1** was selected

* To whom correspondence should be addressed. e-mail: banchaying@yahoo.com

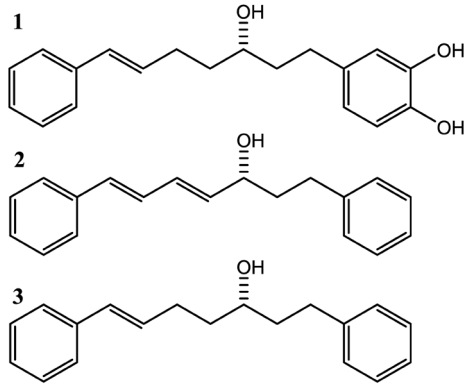

Fig. 1. The Chemical Structure of the Major Diarylheptanoids Selected as Biomarkers for Quality Control of *C. comosa*: (3*S*)-1-(3,4-Dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (**1**), (3*R*)-1,7-Diphenyl-(4*E*,6*E*)-4,6-heptadiene-3-ol (**2**), and (3*S*)-1,7-Diphenyl-(6*E*)-6-hepten-3-ol (**3**)

instead of (6*E*)-1,7-diphenylhept-6-en-3-one because **1**–**3** are most likely the major diarylheptanoids in the plant rhizome responsible for many pharmacological effects both *in vitro* and *in vivo*. Among these constituents, **1** exhibited hypolipidemic activity in hamsters, one of the traditional uses of *C. comosa* in Thailand.[21] Compound **2** was identified as the most potent phytoestrogenic agent.[18–20] In recombinant yeast system, the relative potency of estrogenic activity of **2** and **3** showed 4 and 1% respectively compared to 17β-estradiol.[5] Our unpublished *in silico*-study also found that all compounds exhibited affinity to estrogen receptors and that supported the findings of previous studies in estrogen-like activity of **2** and **3**.[18–20] Meanwhile, other diarylheptanoids isolated previously by Suksamrarn *et al.*[5] are minor components, indicating a lower degree of potential markers. A very small amount in the plant rhizomes was also found for (6*E*)-1,7-diphenylhept-6-en-3-one. *C. comosa* is believed to exert a curative effect *via* the synergistic actions of its multi-diarylheptanoid components. The estrogen-like activities should be related to its major active constituents.[8,9,13,16,17,20] Apart from **2** and **3**, compound **1** still accounted for the overwhelming majority of *C. comosa* and its content was comparable to **3**. Therefore, compounds **1**–**3** were proposed as rational chemical markers to substantiate the diarylheptanoids level in the plant extracts, reflect the relevant pharmacological activities, and allow profiling of the chemical fingerprint of *C. comosa* rhizome. These are the reasons the authors selected **1**–**3** as chemical markers instead the use of other diarylheptanoids. In this regard, a quantitative analysis of the above mentioned three main compounds is necessary and urgently needed to determine the effective quality assessment of the rhizomes.

Various analytical methods have been reported for the quality assessment of *Curcuma* species-related herbal medicinal products worldwide.[22,23] However, the analytical methods used for determining the content of diarylheptanoids in *C. comosa* are limited. Only two isocratic HPLC methods using the combination of *n*-hexane and dichloromethane as eluent and a normal phase column as stationary phase have been described for the simultaneous analysis of **2** and **3** in the hexane extract of *C. comosa*.[4,24] Studies of the HPLC analysis of **1** have not been reported up to now. These methods are limited due to the interfering effects of some chemical constituents that co-eluted when analysis of the samples extracted with other more polar solvents compared to hexane. As a result, an alternative analytical method is necessary. The aim of the present study was to develop and validate a suitable HPLC method for a qualitative and quantitative determination of the three relevant phytoestrogenic diarylheptanoids following the International Conference on Harmonization (ICH) Guidelines.[25] In parallel, the metabolic profiles of the rhizome extracts of different locations in Thailand were analyzed and compared. A reversed HPLC method was chosen for this analysis for its simplicity of process, in which the tested compounds were separated and determined simultaneously. The validated method was subsequently applied for qualitative and quantitative determinations of the three diarylheptanoids in the rhizomes of *C. comosa* and selected commercial herbal medicinal products (capsules).

Experimental

Chemicals Standard references of **1**, **2**, and **3** were isolated from the rhizomes of *C. comosa* according to the method previously described.[5] The purity of these authentic standards evaluated by a photo diode array detector (DAD) was more than 98%. The chemical structures of the obtained diarylheptanoids were confirmed by spectroscopic data analysis. Acetic acid, acetonitrile and methanol were purchased from CARLO ERBA Reagents S.A.S. (Val-de-Reuil, France). All chemicals were of analytical grade.

Plant Materials and Commercial Capsules of *C. comosa* *C. comosa* rhizomes were collected from three different locations in Thailand in March 2015: (1) Kampaengsaen district, Nakhon Pathom province, (2) Phimai district, Nakhon Ratchasima province, and (3) Warinchamrab district, Ubon Ratchathani province. Taxonomic identification of all samples was verified by Dr. B. Yingngam in comparison to the botanical morphology and voucher herbarium specimens (BCY UBU no. 2015030) were deposited at the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand. The samples were dried at 55°C for 72 h in a hot air oven, followed by pulverization with an electronic grinder, and storage at −20°C for further experiments. Additionally, two different commercial brands of product in capsules containing the powder of *C. comosa* rhizomes were purchased from a traditional Thai pharmacy in Ubon Ratchathani province.

HPLC Instrument and Chromatographic Conditions A chromatographic method was used on a Dionex UltiMate 3000 UHPLC system interfaced to a DAD (Dionex, Thermo Fisher Scientific Company, Waltham, MA, U.S.A.). The sample (10 μL) was injected into a security C_{18} guard column (4×3 mm, 5 μm particle size) and connected to a Luna C_{18} analytical column (250×4.6 mm, 5 μm particle size; Phenomenex, Torrance, CA, U.S.A.). The eluents were 0.5% acetic acid in water (solvent A) and acetonitrile (solvent B) with stepwise elution mode. The sample was eluted by applying the following gradient: 0–5 min, 70%B; 5–10 min, 75%B; 10–15 min, 80%B; 15–20 min, 90%B, held 5 min (90%B); and an equilibration period of 10 min before the next injection. Column temperature was set at 35±1°C and the flow rate was 1 mL/min. The UV scan was measured at a wavelength between 200 and 600 nm (detection wavelength at 254 nm for **1** and **3**, and at 280 for **2**). All data acquired were analyzed with Chromeleon software (Dionex, Thermo Fisher Scientific Company, Waltham, MA, U.S.A.).

Method Validation

Linearity

The standard diarylheptanoid mixtures were dissolved in 80% methanol with seven concentrations in the range of 0.5–100 μg/mL. The linearity of the method was studied by injection of the diarylheptanoid solutions into the HPLC in triplicate. The calibration curves were constructed by plotting the average peak area against the amount of standard analyses. The regression equation and coefficient of determination were calculated.

Precision

The precision of the method was determined in terms of intra- and inter-variation on three different days and the results were expressed as percent relative standard deviation (%RSD). The standard diarylheptanoid solutions of the six replicates (10 μg/mL) were analyzed.

Accuracy

Accuracy of the method was evaluated by adding three different levels of the standard diarylheptanoid mixture to the pre-analyzed samples. The recovery of the targeted compounds was calculated by comparing the differences between the spiked amount and the actual amount.

Sensitivity

The parameter limit of detection (LOD) and limit of quantification (LOQ) are the minimum concentrations of the substances needed to produce a signal and both can be reliably measured by the analytical method. In this study, the LOD and LOQ values were determined based on the basis of signal to noise ratio as follows: LOD=3.3 σ/s and LOQ=10 σ/s, where σ is the standard deviation (S.D.) of the y-intercept of the regression line and s is the slope of the calibration curve.

Specificity

The specificity of the method was studied by comparing the peak purity of the investigated diarylheptanoids using UV spectra, retention times, and resolutions.

Application of the Method The samples (150 mg) were accurately weighed and exhaustively extracted with 7 mL methanol at 30°C and at a frequency of 45 kHz in an ultrasonic device (ULTRAsonik 57H model, 250 W, C&A Sales Industrial Supplies, U.S.A.) for 30 min. The extraction procedure was repeated three times and the combined extracts were concentrated using a rotary evaporator (Büchi, Flawil, Switzerland). Methanol was added to the residue, adjusted to 10 mL in a volumetric flask and filtered through a 0.22 μm nylon membrane filter. Afterwards, the sample (10 μL) was injected into the HPLC and the validated HPLC method was applied, as described above.

Statistical Analysis The values were expressed as the means±S.D. The data were analyzed by one-way ANOVA, followed by determination of significant difference of the mean values by Tukey's test. p values of 0.05 or less were considered as statistically significant differences between data groups.

Results and Discussion

Chromatography The optimization of the HPLC method for the quality assessment of *C. comosa* is very challenging due to the similarity of the chemical structures of the diarylheptanoid derivatives.[4,5,24] Moreover, no reports about the HPLC analysis of **1** existed in previous literature. Thus, separation of both non-polar and semi-polar diarylheptanoids was carried out using a reverse phase chromatography with stepwise elution instead of those of normal phase column in isocratic mode.[4,24] The effects of various factors were then carefully analyzed to select the optimal chromatographic conditions. Acidification of the mobile phase with acetic acid resulted in a symmetry of the peaks and a column temperature was maintained at 35°C to provide the reproducible peaks. By fixing 0.5% acetic acid in water, a rapid and straightforward separation of the selected three kinds of diarylheptanoids was achieved by using acetonitrile as the mobile phase. Diarylheptanoids **1**–**3** were simultaneously analyzed and all showed a complete separation within 25 min by increasing the solvent strength up to 90% acetonitrile. The retention times of the authentic mixture of compounds **1**–**3** were recorded at 3.99, 8.63, and 9.30 min respectively. This was confirmed by the injection of each standard separately. The UV spectrum of each peak determined with photodiode array detector showed that the three chemical markers absorbed at various UV wavelengths. The wavelength at 254 nm was chosen for detection of **1** and **3**, whereas 280 nm was selected for 2 owing to its higher absorption and better resolution of the peaks. Changing the acetonitrile to methanol contributed to a lower elution capacity of the mobile phase. Compounds **1**, **2**, and **3** eluted at 8.06, 16.48, and 17.75 min, respectively in the presence of methanol. Thus, acetonitrile was selected instead of methanol because of its solvent strength to shorten the analytical time of all the analytes.

Method Validation The developed HPLC method was validated with respect to parameters, including linearity, LOD, LOQ, precision, accuracy and specificity.[25] The calibration curves of the three isolated diarylheptanoids were constructed in the range of 0.5 to 100 μg/mL to display the linear relationship between the quantity and UV absorption intensity. As compiled in Table 1, all the calibration curves show high correction coefficients (R^2>0.999) within the relevant range of the tested diarylheptanoids, indicating good linearity. The LOD and LOQ are defined as the lowest amounts of diarylheptanoids detected in the sample. In this study, the values of LOD for **1**–**3** were found to be 0.06, 0.22, and 0.18 μg/mL, respectively, whereas the values for LOQ were 0.18, 0.69, and 0.54 μg/mL, respectively (Table 1). The sensitivity of the proposed method was comparable to that reported by Su *et al.*[4,24] which stated that the LOD and LOQ for **2** and **3** were 0.10 and 0.30 μg/mL, respectively. However, the co-elution at the same retention time of **2** and **3** was observed in that previous method.[4] On the contrary, the method proposed in the present work provided a good separation between the analytes with a retention time free from interferences. The analytical method showed precision of within-day and between-day %RSD values at lower than 2% that indicated acceptable levels (Table 2). The percent recovery of **1**–**3** ranged from 98.35 to 103.90% (Table 3), demonstrating a satisfactory accuracy. The selectivity of the detection for each tested substance was determined by the retention time. The retention times of the compounds of interest were identical and no co-eluting peaks were observed. As a result, it can be concluded that the developed HPLC method enabled highly accurate analysis due to the analyzed values being in the range of the acceptance criteria.[25]

Application of the Method Although isocratic HPLC methods have been previously reported for the determination of some diarylheptanoids in the hexane extract of *C. co-*

Table 1. Summary of Method Validation Results for the Quantification of (**1**), (**2**), and (**3**)

Parameters	Diarylheptanoids		
	(**1**)	(**2**)	(**3**)
Regression equation	$Y=0.3918X+0.3774$	$Y=0.3995X+0.3044$	$Y=0.5577X+0.3053$
Correlation coefficient (R^2)	0.9998	0.9991	0.9992
Linear range (μg/mL)	0.50–100.00	0.50–100.00	0.50–100.00
Number of standards	7	7	7
LOD (μg/mL)	0.06	0.22	0.18
LOQ (μg/mL)	0.18	0.69	0.54

Table 2. Intra-day and Inter-day Precision (%RSD) for the Compounds (**1**)–(**3**)

Compound	Intra-day ($n=6$)			Inter-day ($n=6$)
	Day 1	Day 2	Day 3	
(**1**)	1.68	1.48	0.81	1.27
(**2**)	0.43	1.14	0.15	0.70
(**3**)	0.36	0.11	0.23	0.49

Table 3. Recovery Studies for the Compounds (**1**)–(**3**)

Serial number	Compound	Theoretical (μg/mL)	Found (μg/mL)	Recovery (%)
1	(**1**)	5.75	5.84±0.27	101.57±4.70
	(**2**)	5.89	6.12±0.25	103.90±4.24
	(**3**)	5.76	5.75±0.05	98.35±0.68
2	(**1**)	11.49	11.76±0.53	102.35±4.61
	(**2**)	11.78	11.85±0.07	100.59±0.59
	(**3**)	11.51	11.59±0.12	100.70±1.04
3	(**1**)	16.85	16.61±0.78	98.58±4.62
	(**2**)	17.78	17.84±0.76	100.34±4.27
	(**3**)	16.88	16.99±0.11	100.22±0.38
Average	(**1**)	—	—	100.83
	(**2**)	—	—	101.02
	(**3**)	—	—	99.76

Table 4. The Extraction Recovery of the Selected Diarylheptanoids from Various Solvents

Solvent	Recovery (%)		
	(**1**)	(**2**)	(**3**)
Hexane	2.32±0.20	84.04±0.78	99.09±1.12
Dichloromethane	73.46±3.54	79.64±3.67	83.43±3.32
Chloroform	82.09±4.79	84.84±4.81	87.59±4.05
Ethyl acetate	102.86±3.01	102.04±4.04	101.72±2.77
Ethanol	96.94±0.69	98.32±0.85	98.10±0.63
Methanol	99.99±1.36	100.60±1.64	100.03±1.18
Water	0.19±0.12	0.88±0.33	1.49±0.55

mosa,[4,24] none of them has applicability for the quantification of the diarylheptanoids **1**–**3** in raw drug and herbal medicinal products. Differences between the described methods and the developed technique are the type of eluent used and the analytical column applied. The proposed method provided a good separation of diarylheptanoids, even though they were extracted in solvents with different polarities (hexane, dichloromethane, chloroform, ethyl acetate, methanol, ethanol, water). The rhizomes collected in Kampaengsaen district, Nakhon Pathom province were extracted with the seven solvents mentioned above. Compounds **1**–**3** were present in all extracts with a high resolution. Compound **1** was detected in the hexane extract only in a small amount due to its higher polarity compared to the compounds **2** and **3**. The HPLC profiles observed for the sample extracted with dichloromethane, chloroform, ethyl acetate, ethanol, and methanol were similar. Water showed the lowest extractability concerning the isolated diarylheptanoids because lipophilic compounds tend to not dissolve well in a polar solvent.

In order to compare the extraction efficiency of methanol with the above mentioned solvents, the extraction efficiency of each solvent on the recovery of the analytes was studied. Table 4 shows the extraction recovery of the target diarylheptanoids from the plant material and the results were be ranked from the highest to the lowest as methanol≈ethyl ac etate≈ethanol>chloroform>dichloromethane>hexane>water. The mean recovery for the diarylheptanoids **1**–**3** in methanol was in the range of 99.99–100.60%, indicating the accuracy of the method. Moreover, sonicating the sample in methanol for 30 min with the extraction process repeated three times was found to be the optimized condition. This was confirmed by there being no content of the diarylheptanoids in the remaining matrix after repeated extraction using methanol. So, methanol was chosen as the solvent for the extraction of diarylheptanoids from the rhizomes. The sample solutions obtained after filtration could be analyzed by HPLC without further pretreatments.

C. comosa drug samples from various origins were extracted and placed in methanol and analyzed to demonstrate the applicability of the developed HPLC method. Figure 2 represents the metabolic profiles of *C. comos*a rhizomes collected from three different locations in Thailand and two commercial capsules. Concerning the qualitative analysis, a slight variation was observed in the HPLC profiles of all drug samples. Thirteen peaks were found in each sample. Among them, 3 peaks with high intensities in the chromatograms were chosen as the 'characteristic fingerprint peaks' of the rhizomes. Peak 1, 2, and 3 were identified as compound **1**–**3**, respectively, by comparing their retention times and UV spectra with those of standard compounds. This is the first report for the HPLC fingerprint and the quantitative analysis of the three major components in *C. comosa*. Concerning the importance of chromatographic fingerprints, the European Medicines Agency (EMEA),[26] the China State Food and Drug Administration[27] and the U.S. Food and Drug Administration (USFDA)[28] recommend this tool to assess the quality of markers and unknown components in the herbal medicinal products due to its

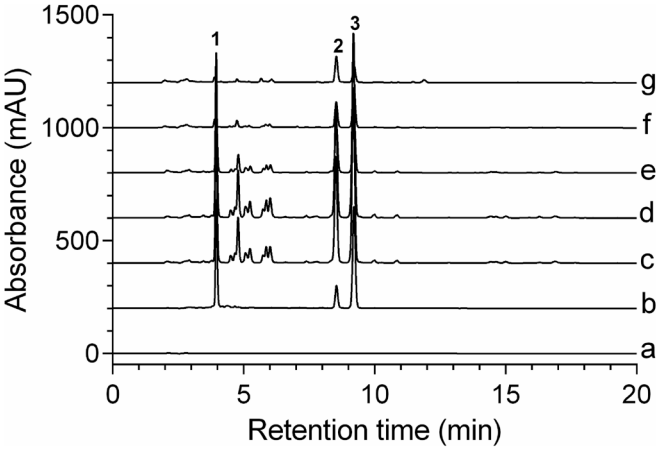

Fig. 2. Overlay of Metabolic HPLC Profiles of *C. comosa* Samples at 254 nm; (a) Blank Solution; (b) Diarylheptanoid Standard Solution; Samples Collected from (c) Kampaengsaen District, Nakhon Pathom Province, (d) Phimai District, Nakhon Ratchasima Province, (e) Warinchamrab District, Ubon Ratchathani Province; (f) Commercial Capsule No. 1, and (g) Commercial Capsule No. 2

Table 5. Amount of the Compounds (**1**)–(**3**) in Dried Powder of *C. comosa* Rhizomes Collected from Three Locations in Thailand and of Two Commercial Gelatin Capsules

Samples	Diarylheptanoid contents (mg/g)		
	(**1**)	(**2**)	(**3**)
Dried powder of *C. comosa* rhizomes			
Nakhon Prathom province	18.35±0.29	64.38±1.14	22.81±0.46
Nakhon Ratchasima province	19.61±0.35	67.61±0.83	24.38±0.31
Ubon Ratchathani province	8.89±0.36	31.55±1.36	8.82±0.47
Commercial capsules			
Product no. 1	0.51±0.23	11.26±3.89	0.34±0.07
Product no. 2	0.33±0.01	2.93±0.58	0.24±0.06

convenience and efficiency.

The contents of the three diarylheptanoid markers in drug samples from the three different locations in Thailand and the two commercial capsules are summarized in Table 5. Compounds **1**–**3** were the most dominant constituents and accounted for 27, 30, and 40%, referring to the total peak area. In all the analyses of the raw drug samples, the amount of diarylheptanoids resulted in order of **2**>**3**≈**1**. The results of this study concerning the amounts of **2** and **3** as major diarylheptanoids in the rhizomes were consistent with a previous report.[4] It could be noticed that the content of the diarylheptanoids in the plant material was varying in regard to the different locations of collection. Different parameters such as collection time, place, temperature, and cultivation environment may influence the amount of markers. However, it is still impossible to define a minimum content of diarylheptanoids in the raw drugs to meet the requirement of acceptable quality. This was attributed to a limitation in the number of investigated samples.

It was noteworthy that two brands of commercial capsules of *C. comosa* powder analyzed in this study showed relatively low levels of **1**–**3** per dosage form taken (500 mg plant powder per capsule, 1–2 capsules two times a day). The content of diarylheptanoids in sample no. 1 was 0.51±0.23, 11.26±3.89, and 0.34±0.07 mg/g powder for **1**–**3**, respectively. A similar result was also found in sample no. 2. This was probably due to the influence of some pharmaceutical excipients added into the formulation, such as microcrystalline cellulose for the manufacturing processes. As reported earlier, declarations in amounts of **1**–**3** in traditional medicine are not yet recommended by the Thai Food and Drug Administration. Also, the diarylheptanoids **1**–**3** are not commercially available as analytical standards. However, the proposed HPLC method was found to be a precise, powerful method, and feasible for both qualitative and quantitative evaluation of the drug material.

Conclusion

A simple, rapid, specific, and accurate HPLC method for the simultaneous quantification of the diarylheptanoid**s 1**–**3** in the rhizomes and capsules of *C. comosa* was developed and validated. The proposed method was successfully applied for the analysis of the three makers in the plant material collected at three different locations in Thailand and two herbal medicinal products. The method provides a short-time analytical process that is suitable for large numbers of samples to be analyzed. This method also exhibits great potential for confirming the authenticity of the samples. Therefore, the proposed HPLC method may serve as an alternative way to guide the fingerprint analysis of diarylheptanoids and the quality assessment of phytoestrogenic markers in *C. comosa* rhizomes. This is an important pre-requisite for the quality assessment for the pharmaceutical industry manufacturing herbal medicinal products derived from *C. comosa* rhizomes.

Acknowledgments Research reported in this publication was supported by the ASEAN-European Academic University Network (ASEA-UNINET). Support from the Thailand Research Fund (DBG 5980003) is gratefully acknowledged.

Conflict of Interest The authors declare no conflict of interest.

References

1) Chotchoungchatchai S., Saralamp P., Jenjittikul T., Pornsiripongse S., Prathanturarug S., *J. Ethnopharmacol.*, **141**, 193–205 (2012).
2) Chang Y. X., Yan D., Chen L., Ding X., Qi J., Kang L., Zhang B., Yu B., *Chem. Pharm. Bull.*, **57**, 586–590 (2009).
3) Maarisit W., Yamazaki H., Abdjul D. B., Takahashi O., Kirikoshi R., Namikoshi M., *Chem. Pharm. Bull.*, **65**, 586–588 (2017).
4) Su J., Sripanidkulchai K., Suksamrarn A., Hu Y., Piyachuturawat P., Sripanidkulchai B., *J. Nat. Med.*, **66**, 468–475 (2012).
5) Suksamrarn A., Ponglikitmongkol M., Wongkrajang K., Chindaduang A., Kittidanairak S., Jankam A., Yingyongnarongkul B., Kittipanumat N., Chokchaisiri R., Khetkam P., Piyachaturawat P., *Bioorg. Med. Chem.*, **16**, 6891–6902 (2008).
6) Jantaratnotai N., Utaisincharoen P., Piyachaturawat P., Chongthammakun S., Sanvarinda Y., *Life Sci.*, **78**, 571–577 (2006).
7) Kaewamatawong R. J. Sci. Technol., *Ubon Ratchathani University*, **19**, 19–21 (2017).
8) Matsumoto T., Nakamura S., Nakashima S., Yoshikawa M., Fujimoto K., Ohta T., Morita A., Yasui R., Kashiwazaki E., Matsuda H., *Bioorg. Med. Chem. Lett.*, **23**, 5178–5181 (2013).
9) Matsumoto T., Nakamura S., Fujimoto K., Ohta T., Ogawa K., Yoshikawa M., Onishi E., Fukaya M., Matsuda H., *J. Nat. Med.*, **69**, 142–147 (2015).
10) Piyachaturawat P., Ercharuporn S., Suksamrarn A., *J. Pharmacogn.*, **33**, 334–338 (1995).

11) Piyachaturawat P., Chai-ngam N., Chuncharunee A., Komaratat P., Suksamrarn A., *Eur. J. Pharmacol.*, **387**, 221–227 (2000).
12) Sophonsritsuk A., 2014, "A randomized clinical trial assessing efficacy and safety of *Curcuma comosa* extract on vasomotor symptoms (efficacy and safety of *Curcuma comosa*) (study ID: TCTR20140818001), Thai Clinical Trials Registry.": ‹http://www.clinicaltrials.in.th.› accessed 4 April 2017.
13) Sornkaewa N., Lin Y., Wang F., Zhang G., Chokchaisiri R., Zhang A., Wongkrajang K., Suebsakwong P., Piyachaturawat P., Suksamrarn A., *Nat. Prod. Commun.*, **10**, 89–93 (2015).
14) Su J., Sripanidkulchai K., Wyss J. M., Sripanidkulchai B., *J. Ethnopharmacol.*, **130**, 70–75 (2010).
15) Su J., Sripanidkulchai B., Sripanidkulchai K., Piyachaturawat P., Wara-Aswapati N., *J. Nat. Med.*, **65**, 57–62 (2011).
16) Suksen K., Charaslertrangsi T., Noonin C., Jariyawat S., Devakul Na Ayutthaya W., Suksamrarn A., Tuchinda P., Piyachaturawat P., *Pharm. Biol.*, **54**, 853–862 (2016).
17) Tantikanlayaporn D., Wichit P., Weerachayaphorn J., Chairoungdua A., Chuncharunee A., Suksamrarn A., Piyachaturawat P., *PLOS ONE*, **8**, e78739 (2013).
18) Winuthayanon W., Piyachaturawat P., Suksamrarn A., Ponglikitmongkol M., Arao Y., Hewitt S. C., Korach K. S., *Environ. Health Perspect.*, **117**, 1155–1161 (2009a).
19) Winuthayanon W., Suksen K., Boonchird C., Chuncharunee A., Ponglikitmongkol M., Suksamrarn A., Piyachaturawat P., *J. Agric. Food Chem.*, **57**, 840–845 (2009b).
20) Winuthayanon W., Piyachaturawat P., Suksamrarn A., Burns K. A., Arao Y., Hewitt S. C., Pedersen L. C., Korach K. S., *Environ. Health Perspect.*, **121**, 433–439 (2013).
21) Suksamrarn A., Eiamong S., Piyachaturawat P., Charoenpiboonsin J., *Phytochemistry*, **36**, 1505–1508 (1994).
22) Tanaka K., Kuba Y., Ina A., Watanabe H., Komatsu K., *Chem. Pharm. Bull.*, **56**, 936–940 (2008).
23) Maehara S., Ikeda M., Haraguchi H., Kitamura C., Nagoe T., Ohashi K., Shibuya H., *Chem. Pharm. Bull.*, **59**, 1042–1044 (2011).
24) Su J., Sripanidkulchai K., Hu Y., Chaiittianan R., Sripanidkulchai B., *AAPS PharmSciTech*, **14**, 1055–1062 (2013).
25) International Conference on Harmonization "ICH (Q2) R1: Validation of Analytical Procedures: Test and Methodology," ICH, Geneva, Switzerland (2005).
26) EMEA, "Guidance on Quality of Herbal Medicinal Products/Traditional Herbal Medicinal Products." European Medicines Agency, 2006.
27) Drug Administration Bureau of China, "Guidance for Experimental Research on HPLC Fingerprint of Traditional Chinese Injections, (Draft)," Beijing, 2002.
28) US Food and Drug Administration, "Guidance for Industry Botanical Drug Products," Rockville, 2004.