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Original Article

Distribution of phytoestrogenic diarylheptanoids and sesquiterpenoids components in *Curcuma comosa* rhizomes and its related species

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ABSTRACT

Curcuma comosa Roxb., Zingiberaceae, a phytoestrogen-producing herb with vernacularly named "Wan Chak Mod Loog" in Thailand, has been traditionally used for treatment of gynecologic diseases and sold as food supplement in the market. However, similar rhizomes of its related species may lead to the confusion in the uses of this plant. This study was aimed to investigate the phytochemical constituents of different *Curcuma* spp. that used as "Wan Chak Mod Loog". Characteristic major compounds were isolated and identified. Phytochemical analysis of 45 *Curcuma* samples representing *Curcuma* sp., *C. latifolia*, and *C. comosa* were analyzed and compared with their phylogenetic relationship inferred by Amplified Fragment Length Polymorphism analysis. Phytoestrogen diarylheptanoids were found in all samples of *C. comosa* while sesquiterpenoids including hepatoxic zederone were found in *C. latifolia* and *Curcuma* sp. samples.

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Introduction

Curcuma comosa Roxb., an indigenous medicinal herb vernacularly named "Wan Chak Mod Loog" in Thailand, is recognized as a phytoestrogen-producing plant belonging to family Zingiberaceae (Phupatanapong et al., 2001; Soontornchainaksaeng and Jenjittikul, 2010). Its roots have been used for the treatment of fluatalence and gynecologic diseases such as premenstrual syndrome (PMS), abnormal menstruation and uterine pain. This plant is considered as an active constituent in various traditional woman drug preparation in South East Asian countries. It has estrogenic-like activity (Piyachaturawat et al., 1995a,b) and has been extensively used among menopausal women (Winuthayanon et al., 2009a). Various pharmacological activities have also been reported, e.g. anti-lipidemic (Piyachaturawat et al., 1995c, 1999a), choleretic (Suksamrarn et al., 1997), estrogenic (Winuthayanon et al., 2009a,b; Bhukkhai et al., 2012), uterotroic (Piyachaturawat et al., 1995a), growth suppressing on male reproductive organs (Piyachaturawat et al., 1998), male fertility (Piyachaturawat et al., 1999b), plasma cholesterol reduction (Piyachaturawat et al., 1999a), anti-inflammatory (Jantaratnotai et al., 2006; Sodsai et al., 2007), and anti-oxidant effect (Niumsakul et al., 2007). Moreover, in ovariectomized rats with estrogen deficiency, this plant could protect bone loss and accelerate human osteoblast proliferation and differentiation (Tantikanlayaporn et al., 2013a,b; Weerachayaphorn et al., 2011), enhance vascular relaxation (Intapad et al., 2009, 2012), prevent neuron loss and improve learning and memory function (Su et al., 2010, 2011, 2012a,b).

Phytochemical investigation of *C. comosa* revealed the presence of sesquiterpenoids (Xu et al., 2008; Qu et al., 2009) and diarylheptanoids (Suksamrarn et al., 2008). Diarylheptanoids are of interest since they exerted estrogenic-like activity (Suksamrarn et al., 2008; Winuthayanon et al., 2009a,b). However, its *C. comosa* related species (e.g. *C. latifolia* and *C. elata*) available in the market also has similar shape of rhizomes and may lead to the confusion in the uses of this plant. The inconsistency of raw materials hampered the usage, development, and scientific research of these plants. Therefore, a special consideration is needed when purchasing the samples from the market.

In general, sesquiterpenoids may exert a variety of pharmacological properties. However, hexane extract of rhizomes from *C. elata* containing high proportion of sesquiterpene zederone (**5**) caused liver enlargement and scattered white foci over the organs

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at autopsy (Pimkaew et al., 2013). Zederone (**5**) and was found to be hepatotoxic in mice. However, it was also isolated from *C. comosa* (Qu et al., 2009). While there is a very large variation in rhizome morphology of "Wan Chak Mod Loog" in cultivation, the phenotypic plasticity of vegetative parts of "Wan Chak Mod Loog" can lead to the wrong taxonomic assignment (Soontornchainaksaeng and Jenjittikul, 2010). Therefore, this study utilized Amplified Fragment Length Polymorphism (AFLP) markers technique to examine *Curcuma* samples (Keeratinijakal et al., 2010) together with their morphological characteristics and investigated the bioactive constituents of different *Curcuma* species that are used as "Wan Chak Mod Loog".

In the course of identifying the genetic diversity of *C. comosa* and its related species, the samples were collected from different provenances of Thailand (Keeratinijakal et al., 2010). Additional samples were collected and 118 accessions were used to elucidate the phylogenetic relationships. Corresponding plant materials were cultivated under field conditions in the National Corn and Sorghum Research Center of Kasetsart University in the east of Thailand. Major constituents were isolated and identified. After being cultivated in the same condition and harvested in the same age, the chemical constituents phytoestrogen diarylheptanoids and sesquiterpenoids of different *Curcuma* sample were analyzed. The results could identify the most productive plant with high contents of active phytoestrogen constituents with non-toxic compounds. The present paper should provide a basis for further pharmaceutical development of this plant.

Materials and methods

Plant materials

A total of 118 accessions of *Curcuma* spp., locally called "Wan Chak Mod Loog", was collected in our previous study (Keeratinijakal et al., 2010) together with some additional collections from cultivated sites throughout Thailand. All samples were grown at the National Corn and Sorghum Research Center of Kasetsart University, Pakchong, Nakhon-Ratchasima province, Thailand. AFLP analysis was conducted in order to identify and elucidate the phylogenetic relationships. The following characteristics in each accession were observed: plant height, shape of master rhizome, inside color of master rhizome, presence of sessile tubers, presence of red path along the midrib, inflorescence and flower morphology as described in our previous paper (Keeratinijakal et al., 2010).

Chemicals

HPLC grade acetonitrile was purchased from Fisher Scientific (UK). Deionized water was purified by Water Pro PS (Labconco, MO, USA). All reagents were of analytical grade if not stated otherwise.

Extraction and isolation

The rhizomes of *Curcuma* samples were dried in an oven at 60 °C, and pulverized. Each sample was stored in an air tight plastic container and kept in the dry place until used. The successive extraction was done by Soxhlet extractor using hexane, ethyl acetate, and ethanol, respectively. Each extract was filtered and the solvent was evaporated to dryness using rotary evaporator and high vacuum pump. Fractionation was done on column chromatography using solvent mixtures of hexane and ethyl acetate with increasing polarity. Each fraction was monitored with TLC, combined and further purified with column chromatography.

Fresh rhizomes of mixed *Curcuma* sp. and *C. latifolia* (75 kg) yielded 6.5 kg of dried course powder. Successive extraction using Soxhlet extractor yielded the hexane, ethyl acetate, and ethanol extracts of 150.32, 210.75 and 375.69 g, respectively. Hexane extract was subjected to column chromatography (Merck silica gel 60 No. 107734, particle size 0.063–0.20 mm) with solvent mixtures of hexane–ethyl acetate (ratio from 10:0 to 0:10) with increasing polarity, yielding seven fractions (CLH1–CLH7) of 2.35 mg, 1.87 g, 25.98 g, 34.56 g, 12.85 g, 28.14 g, and 47.96 g, respectively. Ethyl acetate extract was subjected to column chromatography with solvent mixtures of hexane–ethyl acetate (ratio from 10:0 to 0:10), and ethyl acetate–methanol (ratio from 10:0 to 0:10), with increasing polarity, yielding five fractions (CLA1–CLA5) of 1.45, 15.68, 35.84, 49.70, and 67.73 g, respectively. Ethanol extract was subjected to column chromatography with solvent mixtures of hexane–ethyl acetate (ratio from 10:0 to 0:10), and ethyl acetate–methanol (ratio from 10:0 to 0:10) with increasing polarity, yielding six fractions (CLE1–CLE6) of 3.45, 26.78, 28.40, 32.50, 69.25, and 56.76 g, respectively. Further purification was done using column chromatography (Merck silica gel 60 No. 107734, particle size 0.063–0.20 mm). Fraction CLH-3 using hexane–ethyl acetate (95:5) as an eluent yielded germacrone (**1**) (20 mg). Fraction CLH-4 using hexane–ethyl acetate (9:1) as an eluent yielded furanodienone (**2**) (35 mg) and curzerenone (**3**) (44 mg). Fraction CLH-4 using hexane–ethyl acetate (85:15) as an eluent yielded curdione (**4**) (75 mg). Fraction CLH-5 using hexane–ethyl acetate (8:2) as an eluent yielded zederone (**5**) (7.8 mg).

Fresh rhizomes of mixed *C. comosa* (10 kg) yielded dried course powder of 2 kg. Successive extraction using Soxhlet extractor yielded the hexane, ethyl acetate, and ethanol extracts of 95.30, 150.10 and 275 g, respectively. Hexane extract was subjected to column chromatography (Merck silica gel 60 No. 107734, particle size 0.063–0.20 mm) with solvent mixtures of hexane–ethyl acetate (ratio from 10:0 to 0:10) with increasing polarity, yielding five fractions (CCH1–CCH5) of 2.25 mg, 0.97 g, 30.15 g, 28.45 g, and 10.55 g, respectively. Ethyl acetate extract was subjected to column chromatography with solvent mixtures of hexane–ethyl acetate (ratio from 10:0 to 0:10), and ethyl acetate–methanol (ratio from 10:0 to 0:10) with increasing polarity, yielded five fractions (CCA1–CCA5) of 2.48, 5.46, 20.45, 32.15, and 15.38 g, respectively. Ethanol extract was subjected to column chromatography with solvent mixtures of hexane–ethyl acetate (ratio from 10:0 to 0:10), and ethyl acetate–methanol (ratio from 10:0 to 0:10) with increasing polarity, yielding four fractions (CCE1–CCE5) of 4.45, 27.16, 29.56, and 33.54 g, respectively. Further purification was conducted using column chromatography (Merck silica gel 60 No. 107729, particle size less than 0.063 mm). Fraction CCH-4 using hexane–ethyl acetate (75:25) as an eluent, yielded 1,7-diphenyl-(6E)-6-hepten-3-ol (**6**) (30 mg). Fraction CCE-3 using hexane–ethyl acetate (40:60) as an eluent, yielded 1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**7**) (25 mg).

NMR and MS: Each pure compound was dissolved in 99.98% $CDCl_3$ or in 99.8% methanol (*ca.* 5 mg in 0.7 ml) and transferred into 5 mm NMR sample tube (Promochem, Wesel, Germany). Spectra were recorded by the Bruker Topspin software on a Bruker Avance 400 spectrometer (Bruker, Rheinstetten, Germany). In methanol-d_4 small amounts of methanol-d_1 were used as internal standard for ^1H (δ_H 3.340) and methanol-d_4 for ^{13}C (δ_C 49.86) NMR measurements. In $CDCl_3$ residual $CHCl_3$ (δ_H 7.240) and $CDCl_3$ (δ_C 77.02) were used as internal standards for ^1H and ^{13}C NMR measurements, respectively. Identification was conducted using spectroscopic data and compared with those reported with literatures, i.e. germacrone (**1**) (Yen et al., 2005), furanodienone (**2**) (Hikino et al., 1975), curzerenone (**3**) (Hikino et al., 1968), curdione (**4**) (Hariyama et al., 1991), and zederone (**5**) (Shibuya et al., 1987),

1,7-diphenyl-(6E)-6-hepten-3-ol (**6**) (Suksamrarn et al., 2008), and 1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**7**) (Suksamrarn et al., 2008).

Determination of phytochemical constituents contents in Curcuma *samples*

HPLC was performed on an Agilent 1100 series equipped with a Chemstation software, degasser G1322A, quaternary pump G1322A, thermoautosampler G1329/1330A, column oven G1316A, and diode array detector G1315A. The separation was carried out on a Sorbax reversed-phase C-18 column (250 × 4.6 mm i.d., 5 μm). Mobile phase system was (A) deionized water and (B) acetonitrile. Gradient elution was used by linear increasing of mobile phase compositions from 50% to 90% B in A for 25 min and 90% B in A for 5 min. The column was equilibrated with 50% B in A for 5 min prior each analysis. The flow-rate was set at 1 ml/min at ambient temperature. Injection volume was 10 μl. The detection was monitored at 260 nm.

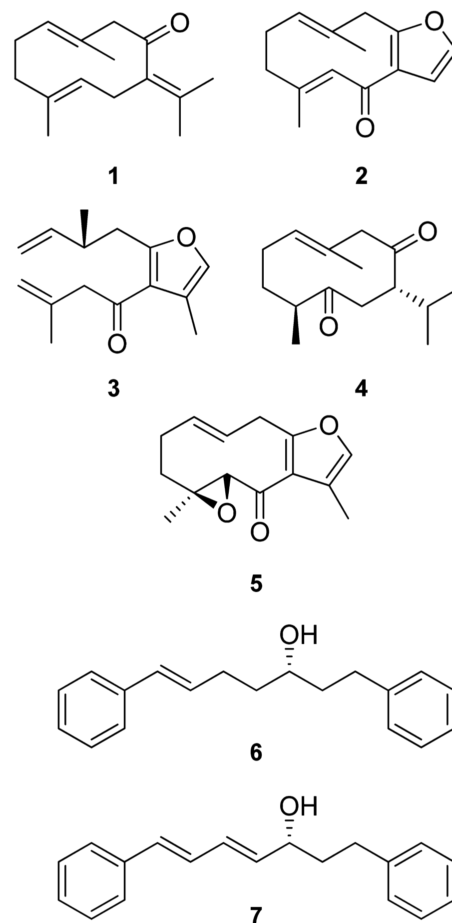
Each *Curcuma* sample was prepared from three rhizomes cultivated in the same field condition and age at National Corn and Sorghum Research Center. The rhizomes were sliced, dried in 60 °C hot air oven for 20 h, ground, and passed through a sieve (60 mesh). Each sample was accurately weighed (10 g) and successively extracted with hexane and ethanol (200 ml each) for 6 h using Soxhlet extractor. The extract was filtered and evaporated to dryness using rotary evaporator. The sample solution was prepared by accurately weighing each extract and dissolving in methanol. Prior to the HPLC injection, each solution was filtered through a 0.45 μm nylon membrane filter.

Stock solutions of standard compounds **1**–**7** were prepared by accurately weighing and dissolving the compounds in acetonitrile to obtain the final concentration of 1000 μg/ml. Working solutions of standard compounds were obtained by diluting the stock standard solutions with acetonitrile to achieve the desired concentrations. Calibration curves were constructed from the peak area versus the amount of the standards by least square regression across the range of 50–1000 μg/ml.

Results and discussion

Rhizomes of 118 *Curcuma* samples were collected from various localities of Thailand. Identification was done using the amplified fragment length polymorphism (AFLP) markers and their morphological characteristics as described in our previous study (Keeratinijakal et al., 2010). Phylogenetic tree illustrating the relationship among 118 accessions as inferred by AFLP analysis was shown in Fig. 1. The samples were classified into four major clusters, i.e. *Curcuma* spp. (Cluster I), *C. latifolia* (Cluster II), *C. elata* (Cluster III), and *C. comosa* (Cluster IV). The clustering of the accessions based on genetic similarity did not correlate with the region of origin of the samples (Keeratinijakal et al., 2010).

In the course of phytochemical analysis of *Curcuma* samples, 45 selected samples representing *Curcuma* sp., *C. latifolia*, and *C. comosa* were cultivated at the National Corn and Sorghum Research Center, Thailand. All samples were grown at the same condition and harvested at the age of 8 months. *C. elata*, a known plant containing toxic compound zederone (**5**) (Pimkaew et al., 2013), has been neglected due to its low potential on further development. Characteristic major compounds were isolated, and their structures elucidated by NMR and MS analyses. They have been identified as germacrone (**1**), furanodienone (**2**), curzerenone (**3**), curdione (**4**), zederone (**5**), 1,7-diphehyl-(6*E*)-6-hepten-3-ol (**6**), and 1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**7**). Compounds **1–5** are classified as sesquiterpenoids while compounds **6–7** are diarylheptanoids.



Comparative analysis of the major components was done using high-performance liquid chromatography (HPLC) technique coupled with diode array detector (DAD). UV-spectra obtained from DAD could confirm the specificity of analytes. On the basis of these findings, two clear-cut phytochemical accumulation trends could be distinguished (Fig. 2). Diarylheptanoid phytoestrogens **6–7** were found in all samples of *C. comosa* (cluster IV) but not detected in *C. latifolia* and *Curcuma* sp. (cluster I–III). Sesquiterpenoids **1–5** were found in *C. latifolia* and *Curcuma* spp. (cluster I–III) but not detected in *C. comosa* (cluster IV). A known toxic compound zederone (**5**) was found in all samples of *C. latifolia* and *Curcuma* spp. but not detected in *C. comosa*. Compound 1,7-diphenyl-(6*E*)-6-hepten-3-one (D2) has not been analyzed in this study. However, it has been found together with compounds **6–7** in various samples of *C. comosa* (Suksamrarn et al., 2008).

Sesquiterpenoids **1**–**5** were not found in *C. comosa* in our study. Since "Wan Chak Mod Loog" in Thailand can be assigned into various *Curcuma* species, taxonomic identification should be carefully done due to the variation in morphology depending on cultivation and geographical difference (Soontornchainaksaeng and Jenjittikul, 2010). However, several cultivated plants in Thailand were mistaken for similar plant species such as *Curcuma* sp. or *C. latifolia* instead of *C. comosa* which contains the toxic component zederone (**5**). The recommendation on using correct plant materials for specific medicinal purposes and large-scale production should be made.

Despite the comprehensive overview of various phytochemical components in the extract. The semi-quantification of each isolated compound (**1**–**7**) has been done. The calibrations curves of compound **1**–**7** proved that the developed method were linear across the concentration range of 50–1000 μg/ml with a good correlation coefficient ($r^2 > 0.987$) (Table 1). The contents of compounds

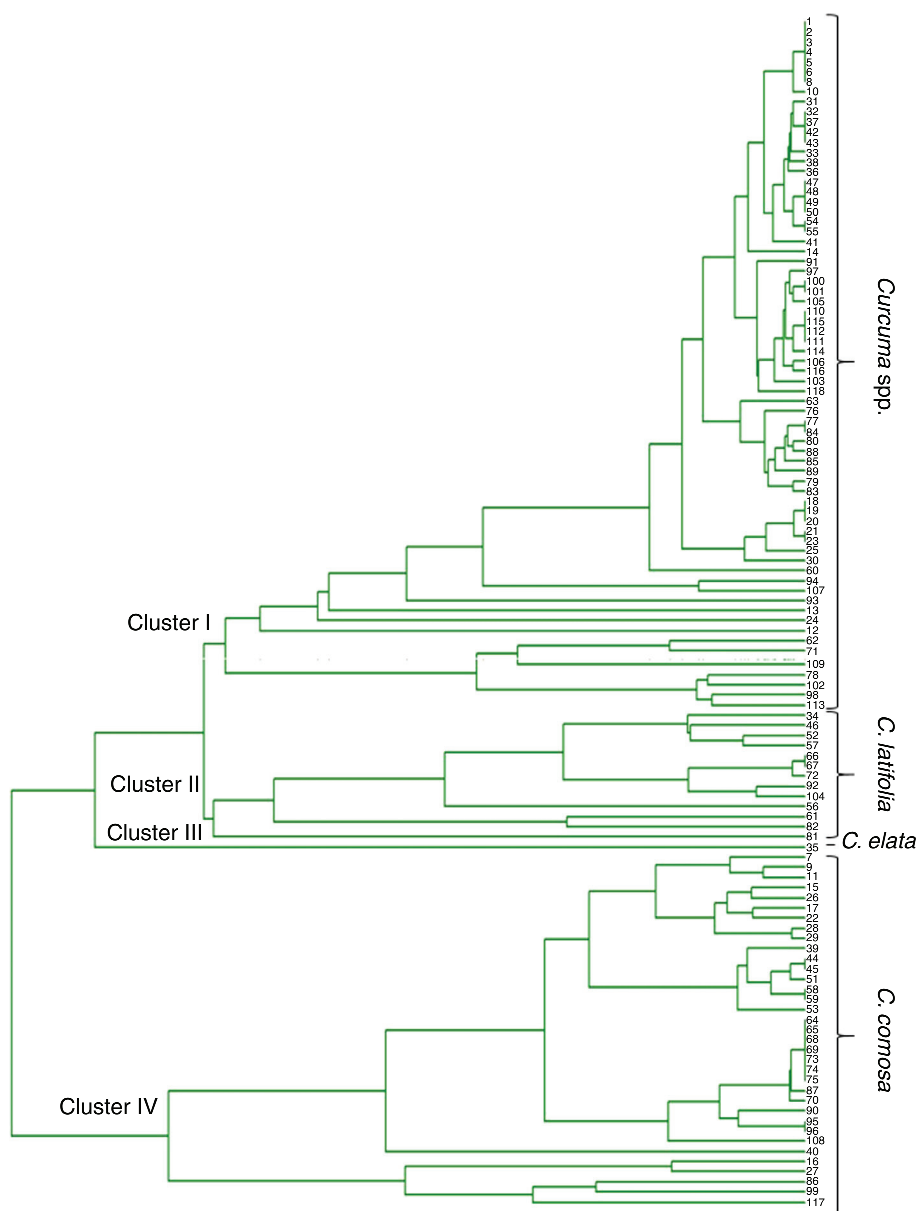


Fig. 1. Phylogenetic tree illustrating the relationship among 118 accessions as inferred by AFLP analysis.

1–7 in *Curcuma* samples were shown in Table 2. However, the method has not been validated and further optimization to maximize the separation and speed for routine analysis is in progress. The co-chromatographically quantitative analyzes could enable the comparison of each sample. Amongst the sample study, *C. comosa* variety 29 gave highest content of 1,7-diphenyl-(6*E*)-6-hepten-3-ol (**6**) and variety 15 gave highest content of 1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**7**) without detectable toxic sesquiterpenoids **1**–**5**.

"Wan Chak Mod Loog" in the different cultivars of Thailand has been assigned to different *C. comosa*, *C. elata*, *C. latifolia*, and *Curcuma* sp. (Soontornchainaksaeng and Jenjittikul, 2010; Keeratinijakal et al., 2010). They have been used as food supplement and Thai traditional medicine for treatment of abnormality of the uterus and ovarian hormone deficit. The present study demonstrates the phytoestrogenic diarylheptanoids and sesquiterpenoids-containing species which could shed some light on the correct uses of these plants. Our results could be used as a

Table 1
Calibation curves of compounds (**1**)–(**7**).

Compounds	Equation	Correlation coefficient (r^2)
Germacrone (**1**)	$Y = 5.6843X + 100.1$	0.9991
Furanodienone (**2**)	$Y = 5.1361X + 159.99$	0.9993
Curzerenone (**3**)	$Y = 5.1648X + 160.06$	0.9990
Curdione (**4**)	$Y = 0.454X - 5.6349$	0.9998
Zederone (**5**)	$Y = 4.116X + 286.19$	0.9873
1,7-Diphenyl-(6*E*)-6-hepten-3-ol (**6**)	$Y = 4.3517X + 133.89$	0.9971
1,7-Diphenyl-(4*E*,6*E*)-6-heptadien-3-ol (**7**)	$Y = 5.0334X + 94.865$	0.9989

Table 2
Percentage of compounds **1–7** in *Curcuma* spp.

Species	Sample No.	Content (mg/g)						
		1	2	3	4	5	6	7
C. comosa	7	–	–	–	–	–	10.5321	11.0490
	9	–	–	–	–	–	13.5346	12.1291
	11	–	–	–	–	–	11.4672	8.7025
	15	–	–	–	–	–	13.4491	16.1910
	16	–	–	–	–	–	13.7456	11.7699
	17	–	–	–	–	–	12.6640	10.5897
	22	–	–	–	–	–	10.9100	13.0040
	26	–	–	–	–	–	9.4777	13.9868
	27	–	–	–	–	–	10.8922	13.7833
	28	–	–	–	–	–	11.8533	10.4879
	29	–	–	–	–	–	17.8707	13.9429
	39	–	–	–	–	–	11.8671	12.7361
	40	–	–	–	–	–	13.9232	13.7214
	45	–	–	–	–	–	10.8725	12.8123
	51	–	–	–	–	–	10.4936	11.7267
	53	–	–	–	–	–	7.7451	6.9547
	58	–	–	–	–	–	8.9132	9.6968
	65	–	–	–	–	–	12.7290	11.3671
	87	–	–	–	–	–	10.4617	9.6654
	90	–	–	–	–	–	9.4295	6.6308
	96	–	–	–	–	–	10.1766	7.9710
	99	–	–	–	–	–	12.2783	13.3476
	108	–	–	–	–	–	11.4772	10.4794
	117	–	–	–	–	–	12.3013	10.6693
C. latifolia	104	2.8098	0.0096	0.0772	0.0807	11.5061	–	–
Curcuma sp.	13	1.7880	–	–	–	13.2415	–	–
	14	0.8305	–	–	–	10.6561	–	–
	21	3.4354	–	–	–	9.4357	–	–
	24	0.2527	–	–	–	2.5481	–	–
	25	5.7551	–	–	–	7.9040	–	–
	30	3.5471	–	–	–	5.7558	–	–
	47	2.2337	–	–	–	11.3616	–	–
	60	1.4745	–	–	–	13.2356	–	–
	63	2.6914	–	–	–	10.4765	–	–
	77	5.8431	–	0.0906	0.0799	14.5061	–	–
	88	3.6186	0.0078	0.0821	0.3193	13.1272	–	–

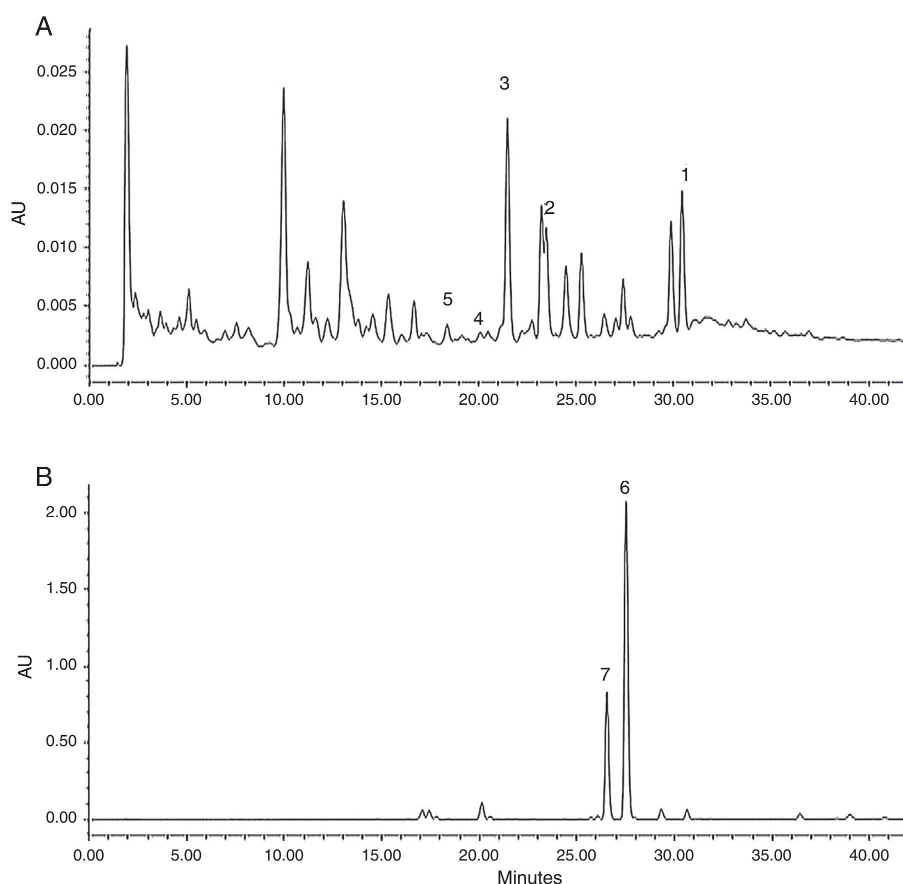


Fig. 2. HPLC chromatographic fingerprint of (A) *Curcuma latifolia* extract (sample No. 104) and (B) *C. comosa* extract (sample No. 44). Compounds identification: **1** (retention time, t_R 30.7 min) germacrone; **2** (t_R 23.4 min) furanodienone; **3** (t_R 21.2 min) curzerenone; **4** (t_R 19.8 min) curdione; **5** (t_R 18.4 min) zederone; **6** (t_R 22.7 min) 1,7-diphenyl-(6*E*)-6-hepten-3-ol; **7** (t_R 21.8 min) 1,7-diphenyl-(4*E*,6*E*)-6-heptadien-3-ol. HPLC condition: Agilent 1100 series, column: Sorbax RP-C_{18} (250 × 4.6 mm i.d., 5 μm), mobile phase: (A) water and (B) acetonitrile, gradient elution system: 50–90% (B) in (A) for 25 min and 90% (B) in (A) for 5 min, flow rate 1 ml/min, ambient temperature, injection volume 10 μl, detection 260 nm.

guideline for consumers as well as farmers who must use the right cultivars for the right medicinal purposes. Moreover, *C. comosa* variety 29 and 15 provided the highest diarylheptanoids contents among the other samples which could be used for breeding and further development as pharmaceutical products.

Authors' contributions

VK contribution included collecting samples, designing and performing laboratory work, analyzing the results, and supervision of the laboratory work. SK contribution included analyzing the results and preparing the paper. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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