Free radical scavenging activity determination and quantitative analysis of curcuminoids in *Curcuma zedoaria* rhizome extracts by HPLC method

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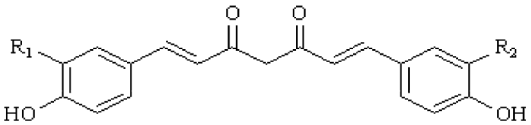
***Curcuma zedoaria* (Zingiberaceae) is a medicinal plant containing antioxidant and anti-inflammatory curcuminoids similar to the well-known *Curcuma longa*. So far, the content of each curcuminoid in the rhizome extract of *C. zedoaria* in Thailand has not been reported. A high-performance liquid chromatography (HPLC) method was developed and validated for quantitative analysis of curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) content in 70% ethanolic extracts of the rhizomes of *C. zedoaria* collected from 10 different locations in Thailand. The method demonstrated good linearity, precision and accuracy. The content of curcumin in all crude extracts was found in the range of 1.46 ± 0.45 to 5.73 ± 0.11 %w/w (average 2.73 ± 1.24 %w/w) whereas the contents of demethoxycurcumin, which is a major component, and of bisdemethoxycurcumin were in the ranges of 3.15 ± 0.15 to 10.98 ± 0.28 %w/w (average 7.37 ± 2.71 %w/w) and 0.49 ± 0.02–2.99 ± 0.20 %w/w (average 1.40 ± 0.82 %w/w) respectively. The highest average total curcuminoids content in the crude extracts was 16.83 ± 0.62 %w/w whereas the lowest content was 6.09 ± 1.79 %w/w. This information will be useful as a guidance for further standardization of *C. zedoaria* raw material and extracts. Free radical scavenging activity of all extracts was determined using 1,1-diphenyl-2-picrylhydrazyl scavenging assay. Values of EC_{50} of all extracts were found in the range of 18.29 ± 0.05 to 40.33 ± 2.24 μg/ml (average 25.71 ± 7.54 μg/ml). Free radical scavenging activity of the separated pure compounds was found in descending order of curcumin > demethoxycurcumin > bisdemethoxycurcumin.**

Keywords: Bisdemethoxycurcumin, *Curcuma zedoaria*, curcumin, demethoxycurcumin, free radical scavenging activity.

CURCUMA ZEDOARIA (Burg) Roscoe belongs to the family Zingiberaceae. In traditional medicine, the tubers of *C. zedoaria* have been used as a carminative, digestive stimulant and for treatment of colds and infections[1]. They also exhibit antibacterial and antifungal activities[2]. The essential oil obtained from the dried rhizome of *C. zedoaria* by steam distillation, demonstrates active antibacterial property[3]. Starch extracted from the plant is used as a diet for infants and convalescent persons due to its cooling and demulcent properties. Curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figure 1) isolated from this plant were reported for antioxidant and anti-inflammatory activities similar to that of *C. longa*[4]. Standardized extracts of *C. longa* rhizomes are popularly used as antioxidants in cosmetics, dietary supplements and other pharmaceutical products[5]. *C. zedoaria* extracts could be used for the same purposes as those of *C. longa*. However, contents of the active ingredients in *C. zedoaria* have not been previously reported. Hence, this study was undertaken using high-performance liquid chromatography (HPLC) method for quantitative analysis of curcuminiods in 70% ethanolic extracts of the rhizomes of *C. zedoaria* collected from 10 different locations in the north, northeast, central and south of Thailand. Free radical scavenging activity of all extracts was determined and compared using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay. The data will be useful as a guidance for further standardization of *C. zedoaria* rhizome extracts and for finding good sources of antioxidant raw materials from this plant for pharmaceutical use.

C. zedoaria rhizomes (age 10–12 months) were purchased from 10 provinces in Thailand during March–April 2005. The samples were identified by comparison with the specimens at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. The voucher specimens (No. WCZ 03200501–03200510) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. Fresh rhizomes were cleaned, sliced, dried in the sun for one week, then dried at 50°C in a hot air oven for 6 h. Dried rhizomes of each sample were cut into small pieces, powdered by an electronic mill and passed through a sieve, mesh no. 60.

Methanol and acetonitrile (HPLC grade) were purchased from Labscan Asia Co. Ltd. (Thailand). Acetic acid was purchased from Merck (Germany). Syringe filters (Nylon, 13 mm, and 0.45 μm pore size) were purchased from Chrom Tech, Inc (USA). DPPH was



Compound	R_1	R_2
Curcumin	OMe	OMe
Demethoxycurcumin	H	OMe
Bisdemethoxycurcumin	H	H

Figure 1. Structures of curcuminoids.

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purchased from Sigma (USA). All other chemicals used were of analytical/HPLC grade, except ethanol which was of commercial grade obtained from the Excise Department, Bangkok, Thailand.

HPLC was performed on a Shimadzu Technologies (Japan) modular model Class VP system consisting of a SCL-10AVP system controller, a SPD-10M 10 AVP diode array detector, two LC-10 AD VP liquid chromatographs and a DGU-12 AM degasser.

Dried powder (500 g) of *C. zedoaria* rhizome (sample 1, purchased from Bangkok) was extracted at room temperature (28–30°C) with hexane (2000 ml) by maceration for 24 h with occasional shaking by hand to extract volatile oil. The extract was filtered and the merc was re-macerated with ethanol (2000 ml) for another 24 h with occasional shaking. The ethanol extract was filtered through Whatman filter paper no. 1 and the filtrate was concentrated by a rotary-evaporator. A crude ethanolic dried extract (75 g), which was black-orange in colour, was obtained. The extract was screened by TLC (silica gel GF_{254}, dichloromethane : methanol :: 95 : 5) and further separated by column chromatography (silica gel 60, 0.063–0.200 mm). The column was eluted with dichloromethane. Fractions containing main compounds were combined. The combined fraction was re-column chromatographed to give crystalline compounds, which were further recrystallized by methanol and yielded three pure compounds. Melting point, ^{1}H-NMR, ^{13}C-NMR of each pure compound were investigated and identified by comparing to the reported data in the reference[6].

The analysis was carried out using a BDS Hypersil C_{18} column (250 mm × 4.6 mm i.d., 5 μm; Thermo Electron Corporation). To protect the integrity of the analytical column, all analyses were performed with a BDS Hypersil C_{18} guard column (10 mm × 4 mm i.d., 5 μm; Thermo Hypersil-Keystone). The mobile phase was 0.25% aqueous acetic acid solution (A) and acetonitrile (B) with gradient concentrations as follows[6,7]: 0–8 min, 50–50% A; 8–10 min, 50–60% A; 10–15 min, 60–60% A; 15–16 min, 60–50% A and 16–20 min, 50–50% A. Total running time was 20 min and the flow rate was 0.8 ml/min. A detector monitored the eluent at 425 nm. The sample injection volume was 5 μl.

The stock solutions of standard compounds (curcumin, demethoxycurcumin and bisdemethoxycurcumin) were prepared as follows: 10 mg of each compound was accurately weighed and placed in a 10 ml volumetric flask. Ethanol (70%) was added and the solution was diluted to volume with the same solvent.

Calibration plots were performed between peak area and concentrations of 4, 25, 50, 75 and 100 μg/ml for curcumin while concentrations of 10, 25, 50, 75 and 100 μg/ml were used for demethoxycurcumin and bisdemethoxycurcumin[8,9].

The precision of the HPLC method was determined by repeatability (intraday) and intermediated precision (interday). Precision was investigated by repeated analysis of standard solutions at 25, 50 and 75 μg/ml for curcumin, demethoxycurcumin and bisdemethoxycurcumin respectively. The intraday variability was performed by the same analyst over one day, while interday precision was carried out by another independent analyst over various days. The precision was expressed as relative standard deviation (%RSD) of the concentrations of these compounds[8,9].

Accuracy of the method was performed by recovery studies which were carried out by standard addition methods. Different concentration levels of curcumin, demethoxycurcumin and bisdemethoxycurcumin (6, 8 and 10 μg/ml) were added to the extract of *C. zedoaria* containing known amount of the analyte. Three determinations were carried out for each solution. The percentage recovery was calculated by subtracting the values obtained for the control matrix preparation from those samples with the added standards[8,9].

The limit of detection (LOD) and limit of quantification (LOQ) were determined from the calibration curve of each standard. LOD was calculated according to the expression $3\sigma/S$, where σ is the standard deviation of the response and S the slope of the calibration curve. LOQ was established by using the expression $10\sigma/S$[8,9].

For sample preparation, 10 g of each powdered sample was accurately weighed and mixed with 100 ml 70% ethanol as the extracting solvent. The mixture was sonicated for 30 min in an ultrasonic apparatus and the temperature was controlled at 30°C. The supernatant was filtered through Whatman filter paper no. 1. The marc was re-extracted with the same solvent until the extraction was exhausted (the last extract was colourless). The combined extract was concentrated using a rotary evaporator and evaporated to dryness on a boiling water bath until a constant weight was obtained. Ten milligrams of each crude extract was diluted to a 10 ml volume with 70% ethanol. The sample solution was filtered through a 0.45 μm nylon filter. Each sample was processed in triplicate.

A volume (5 μl) of each sample solution was injected in triplicate to the HPLC column and analysed by the proposed method. The concentrations of curcumin, demethoxycurcumin and bisdemethoxycurcumin were calculated using their calibration curves. All extracts were also compared on TLC (silica gel GF_{254}, dichloromethane :: methanol 95 : 5; Figure 2).

The free radical scavenging activity of the extracts was examined by a DPPH scavenging assay[10]. Trolox and quercetin were used as positive standards. Briefly, DPPH was dissolved in methanol (6 mg/100 ml) to make 1.52×10^{-4} M solution. Stock solution in methanol of each sample (5 mg/ml) was diluted with methanol to make a dilution series of 500–1.96 μg/ml. DPPH scavenging reaction was performed when DPPH solution (1.52×10^{-4} M) was added to the sample solution in the

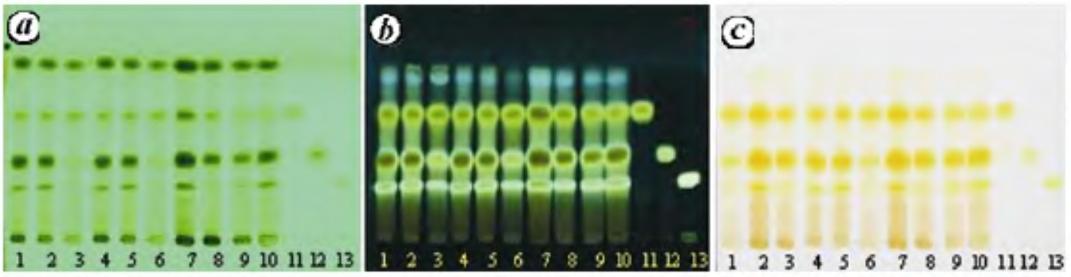

Figure 2. TLC fingerprints of the ethanolic extracts of *C. zedoaria* rhizomes from 10 locations: ***a***, under UV light 254 nm; ***b***, under UV light 366 nm and ***c***, daylight; 1, Bangkok A; 2, Bangkok B; 3, Chiang Mai; 4, Kanchanaburi A; 5, Kanchanaburi B; 6, Loei; 7, Nakornpanom; 8, Nakornpathom; 9, Ratchaburi; 10, Samutsakorn; 11, Curcumin; 12, Demethoxycurcumin and 13, Bisdemethoxycurcumin.

Table 1. Recovery test of compounds in the extract of *C. zedoaria* rhizomes

Compound	Spiked concentration (μg/ml)	Detected concentration (μg/ml)	Recovery (%) (mean ± SD)	RSD (%)
Curcumin	6	6.00	99.82 ± 0.97	0.77
	8	8.04	101.07 ± 1.10	
	10	10.07	101.22 ± 1.48	
Average			100.70 ± 0.77	
Demethoxycurcumin	6	6.05	102.46 ± 2.48	1.66
	8	8.13	103.15 ± 1.10	
	10	10.00	99.94 ± 2.02	
Average			101.85 ± 1.69	
Bisdemethoxycurcumin	6	6.04	101.79 ± 2.76	0.63
	8	8.09	102.23 ± 0.04	
	10	10.06	100.97 ± 2.31	
Average			101.67 ± 0.64	

RSD = Relative standard deviation.

same volume (750 μl). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm by a spectrophotometer using methanol as a blank. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percentage of DPPH decolouration of the samples was calculated according to the formula:

$$\% \text{ inhibition} = \frac{(A_c - A_s)}{A_c} \times 100,$$

where A_c is absorbance of control; A_s the absorbance of sample.

EC_{50} value (a concentration at 50% inhibition) was determined from the curve between percentage inhibition and concentration. All determinations were done in triplicate and the average of EC_{50} value was then calculated.

By column chromatography, three pure compounds were isolated from the crude ethanolic extract of *C. zedoaria* rhizomes. During identification, ^{1}H-NMR and ^{13}C-NMR spectra of each compound were found equivalent to the spectra reported in the reference[5]. These compounds showed melting points at 183–184°C, 168–169°C and 224–225°C, and were identified as curcumin, demethoxycurcumin and bisdemethoxycurcumin respectively (Figure 1)[6].

HPLC method was validated for its linearity, accuracy, precision, limit of detection and limit of quantification. The method is specific as curcumin, demethoxycurcumin and bisdemethoxycurcumin were well resolved from each other without interference from other components in the samples. Good linear relationships of curcumin, demethoxycurcumin and bisdemethoxycurcumin were obtained within the concentration ranges of 4–100, 10–100 and 10–100 μg/ml respectively, whereas regressive coefficients (r^2) were 0.9992, 0.9998 and 0.9993 respectively. Accuracy was determined by percentage recovery. The estimation of curcumin, demethoxycurcumin and bisdemethoxycurcumin in the solution after spiking with 6, 8 and 10 μg/ml of each standard afforded average recoveries of 100.70, 101.85 and 101.67% respectively as shown in Table 1. The %RSD precision for intraday analysis of curcumin, demethoxycurcumin and bisdemethoxycurcumin ranged

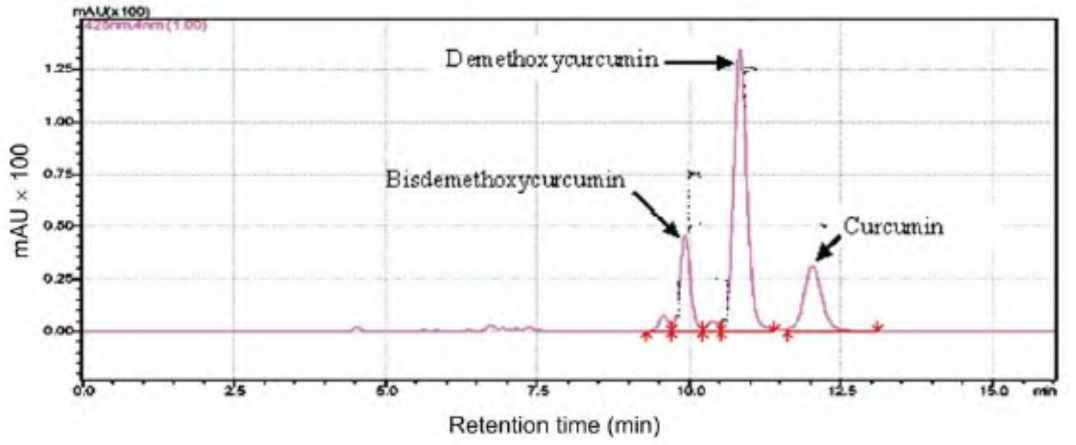

Figure 3. HPLC fingerprints of the ethanolic extracts of *C. zedoaria* rhizome from different locations showing three main peaks of demethoxycurcumin as a major constituent, curcumin and bisdemethoxycurcumin as minors.

Table 2. Free radical scavenging activity and contents of curcumin, demethoxycurcumin and bisdemethoxycurcumin in ethanolic extracts of *C. zedoaria* rhizomes from various locations

Province	Drug extraction ratio	Curcumin %w/w*	Demethoxy-curcumin %w/w*	Bisdemethoxy-curcumin %w/w*	Total curcuminoids (%w/w)*	EC_{50} (μg/ml)*
1. Bangkok A (Vetchapong)	5 : 1	1.46 ± 0.45	4.17 ± 1.20	0.47 ± 0.14	6.09 ± 1.79	37.33 ± 0.53
2. Bangkok B (Chao-krom-per)	4 : 1	3.18 ± 0.28	10.98 ± 0.28	1.86 ± 0.03	16.02 ± 0.59	18.29 ± 0.05
3. Chiang Mai	7 : 1	1.55 ± 0.26	4.92 ± 0.81	0.60 ± 0.10	7.07 ± 1.17	25.10 ± 0.10
4. Kanchanaburi A (Sai-yoke)	4 : 1	2.46 ± 0.29	8.65 ± 0.54	1.60 ± 0.08	12.71 ± 0.91	22.07 ± 0.29
5. Kanchanaburi B (Thong pha)	5 : 1	3.45 ± 0.02	10.39 ± 0.40	2.99 ± 0.20	16.83 ± 0.62	21.14 ± 0.13
6. Loei	6 : 1	5.73 ± 0.11	3.15 ± 0.15	0.59 ± 0.02	9.47 ± 0.27	40.33 ± 2.24
7. Nakormpanom	5 : 1	2.76 ± 0.03	8.21 ± 0.14	1.67 ± 0.01	12.65 ± 0.17	28.87 ± 0.70
8. Nakormpathom	5 : 1	1.89 ± 0.10	5.39 ± 0.21	0.49 ± 0.02	7.78 ± 0.33	22.74 ± 0.12
9. Ratchaburi	5 : 1	2.98 ± 0.34	9.42 ± 0.67	1.66 ± 0.10	14.05 ± 1.10	19.44 ± 0.16
10. Samutsakorn	2 : 1	1.82 ± 0.22	8.41 ± 1.03	2.04 ± 0.27	12.27 ± 1.52	21.78 ± 0.16
Average	5 : 1	2.73 ± 1.24	7.37 ± 2.71	1.40 ± 0.82	11.49 ± 3.74	25.71 ± 7.54
Curcumin						7.89 ± 0.04
Demethoxycurcumin						9.52 ± 0.23
Bisdemethoxycurcumin						149.09 ± 0.46
Standard Trolox						5.26 ± 0.06
Standard Quercetin						2.18 ± 0.02

*Expressed as mean ± SD (n = 3).

from 0.14 to 0.35, 0.18 to 0.26 and 0.04 to 1.96% respectively, whereas the precision for interday of the three compounds ranged from 0.43 to 1.32, 0.22 to 1.01 and 0.64 to 3.87% respectively. The LOD of curcumin, demethoxycurcumin and bisdemethoxycurcumin was 0.14, 0.33 and 0.21 μg/ml respectively. The LOQ of curcumin, demethoxycurcumin and bisdemethoxycurcumin was 0.47, 1.11 and 0.72 μg/ml respectively.

TLC and HPLC fingerprints of all the extracts of *C. zedoaria* showed a similar pattern of which demethoxycurcumin was the major component (Figures 2 and 3). The contents of curcumin in all 70% ethanol extracts were in the range of 1.46 ± 0.45 to 5.73 ± 0.11 %w/w (average 2.73 ± 1.24 %w/w) whereas the contents of demethoxycurcumin and bisdemethoxycurcumin were in the ranges of 3.15 ± 0.15 to 10.98 ± 0.28 %w/w (average 7.37 ± 2.71 %w/w) and 0.47 ± 0.14 to 2.99 ± 0.20 %w/w (average 1.40 ± 0.82 %w/w) respectively (Table 2). Total curcuminoid contents of all extracts were in the range of 6.09 ± 1.79 to 16.83 ± 0.62 %w/w (average 11.49 ± 3.74 %w/w). The average EC_{50} of all ethanol extracts was 25.71 ± 7.54 μg/ml as shown in Table 2. A sample each from Bangkok (sample B) and Ratchaburi province exhibited the highest DPPH scavenging activity (lower than 20 μg/ml). When compared to the ethanol extract of *C. longa* which contains total curcuminoids 20.90 %w/w, comprising curcumin (11.03 %w/w) > bisdemethoxycurcumin (7.35 %w/w) > demethoxycurcumin (2.66 %w/w)[11], the extract of *C. zedoaria* contained about half amount of total curcuminoids of *C. longa* while EC_{50} of the ethanol extract of *C. zedoaria* is nearly the same as EC_{50} of the *C. longa* extract (23.01 μg/ml; ref. 12).

EC_{50} value of each curcuminoid was found to be increasing in the order: curcumin (7.89 ± 0.04 μg/ml) < demethoxycurcumin (9.52 ± 0.23 μg/ml) < bisdemethoxycurcumin (149.09 ± 0.46 μg/ml) and this order is the same as that for *C. longa*[12].

The developed HPLC method was sensitive, precise, and accurate for detection and quantification of curcumin, demethoxycurcumin and bisdemethoxycurcumin in 70% alcoholic extracts of the rhizomes of *C. zedoaria*. TLC and HPLC fingerprints of the extracts from different locations showed a similar pattern, of which demethoxycurcumin was a major component. The average content of demethoxycurcumin in all extracts was 7.37 ± 2.71 %w/w. Curcumin was the second major constituent, whereas bisdemethoxycurcumin was the minor one. This result is different from a previous report of *C. longa* extract in which curcumin is a major component and demethoxycurcumin is a minor one[11]. The HPLC method appeared to be a recommended method for quantitative analysis of the active compounds in *C. zedoaria* extracts and their pharmaceutical preparations. For free radical scavenging activity, EC_{50} values were in accordance with the contents of curcumin and demethoxycurcumin in the extracts. EC_{50} value of curcumin (7.89 μg/ml) is lower than those of demethoxycurcumin (9.52 μg/ml) and bisdemethoxycurcumin (149.09 μg/ml; Table 2). The results are the same as those reported in the case of *C. longa*[12].

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Relation between sedimentary layer thickness and fundamental frequency of the *H*/*V* spectra for Bangalore city

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Soil amplification and topographical effects play a major role in earthquake damage to civil structures. Study of sedimentary layer thickness and behaviour under stress cycles is crucial for earthquake hazard analysis. Borehole logs for Bangalore are used to benchmark the relation between sedimentary thickness and resonant frequency of the soil layer for this region. Microtremor measurements are carried out at the locations where borehole drilling was done and the frequencies corresponding to spectral peaks of the *H*/*V* ratio are estimated, where *H* and *V* denote the horizontal and vertical spectral component of the microtremor displacement respectively. The thicknesses of the soil layer (*D*), obtained from borehole logs and the soil layer resonant frequencies (f_r) determined from the *H*/*V* spectral peaks are used to obtain a regression relation between them. The regression relation obtained is given by $D = (58.29 \pm 8.8) \times f_r^{-(0.95 \pm 0.1)}$. Using the data from the work of Divya *et al.*, a similar regression relation is plotted. The results obtained are

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