High-performance Liquid Chromatographic Analysis of Selected Bioactive Components in *Zingiber cassumunar* Roxb.

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ABSTRACT

The rhizomes of *Zingiber cassumunar* Roxb. have long been used as a traditional medicine for treatment of asthma, and muscle and joint pain. Major constituents, (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (A), curcumin (C), and *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl]cyclohex-1-ene (G) have been reported to be the active anti-inflammatory constituents in the rhizomes of this plant. A photodiode array HPLC method has been developed and validated for quantitation of the major bioactive compounds in the methanolic extracts of the rhizomes of *Zingiber cassumunar* collected from 14 different locations in the North, North-East, East, Central and South of Thailand. The content of (A) in the dry powdered rhizomes was found in the range of 0.400 ± 0.001 to $1.642 \pm 0.005\%$ w/w while those of (C) and (G) were 0.066 ± 0.001 to 0.265 ± 0.002, and 0.027 ± 0.001 to $0.287 \pm 0.004\%$ w/w, respectively. The proposed HPLC method was found to be accurate, precise, specific and suitable for quantitative analysis of these bioactive constituents. The method can be used for quality control of raw material of *Zingiber cassumunar*, extract and its pharmaceutical products. It can be also applied in quantifying these marker compounds in other drugs. The information of ranges of the contents of bioactive compounds will be useful as a guide for standardization of *Z. cassumunar* powder and its extract, and finding sources of good quality of *Z. cassumunar*.

INTRODUCTION

Zingiber cassumunar Roxb. (Zingiberaceae), commonly known in Thai language as Plai, is indigenous to Thailand and South East Asia. The rhizomes have been used in Thai traditional medicine for treatment of asthma, muscle and joint pain (Panthong *et al*. 1990). They were reported to have antioxidant, anti-inflammatory, insecticidal and uterine relaxant activities (Kanjanapothi *et al*. 1987; Ozaki *et al*. 1991; Han *et al*. 2004). Volatile oil from the rhizome of this plant has been developed as cream and massage oil preparations for relieving muscle pain. (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (A), curcumin (C) and *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl]cyclohex-1-ene (G) (**Fig. 1**), which are major components in *Z. cassumunar* rhizomes, showed antioxidant and anti-inflammatory activities (Wanauppathamkul 2003). Separation and determination of active compounds from this plant were previously achieved by GC (www.essentialoils.org/plai.htm), but so far there has been no report about the content of the active compounds in the rhizome. Thus, this study was undertaken using HPLC for qualitative and quantitative analysis of active components in the methanolic extracts of *Z. cassumunar* rhizomes collected from 14 different locations in the North, North-East, East, Central and South of Thailand.

MATERIALS AND METHODS

Chemicals

Methanol and acetonitrile (HPLC grade) were purchased from Labscan Asia Co., Ltd. (Thailand). Trifluoroacetic acid was obtained from Fluka Chemical Co. (Germany). Syringe filters (Nylon, 13 mm, and 0.45 µm pore size) were supplied by Chrom Tech, Apple Valley, MN, USA. All other chemicals are of analytical grade/HPLC grade.

Plant materials

Fresh rhizomes of *Zingiber cassumunar* were collected from 14 different locations, from the North (Lumphun, Utaradit, Phisanulok, Petchabun), North-East (Udonthani, Loei, Mukdahan), East (Rayong, Chanthaburi), Central (Kanchanaburi, Nakhonpathom, Ratchaburi) and South (Ranong) of Thailand between March and May 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. WZC 03200501-WZC 03200514) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The rhizomes were cleaned, sliced and dried in the sun for 1 week. They were separately powdered by an electronic mill, and passed through a sieve no. 60.

HPLC apparatus

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 chromatograph and a DGU-12 AM degasser.

Separation and identification of major chemical constituents from *Z. cassumunar* rhizome

The dried powder of *Z. cassumunar* rhizome (500 g) was extracted by maceration with hexane (2000 ml) for 24 hrs. The extract was filtered and the marc was remacerated with hexane (3 × 2000 ml). The combined extract was concentrated by a rotary evaporator at 60°C. The crude hexane extract (24.9 g) obtained as orange oil, which was screened by TLC (silica gel GF_{254}, hexane: dichloromethane: ethyl acetate 5: 1: 2), indicated the main compounds as shown in **Fig. 2**. The extract was further separated by column chromatography (Silica gel 60, 0.063-0.200 mm). The column was eluted with a hexane-ethyl acetate gradient system and the eluates
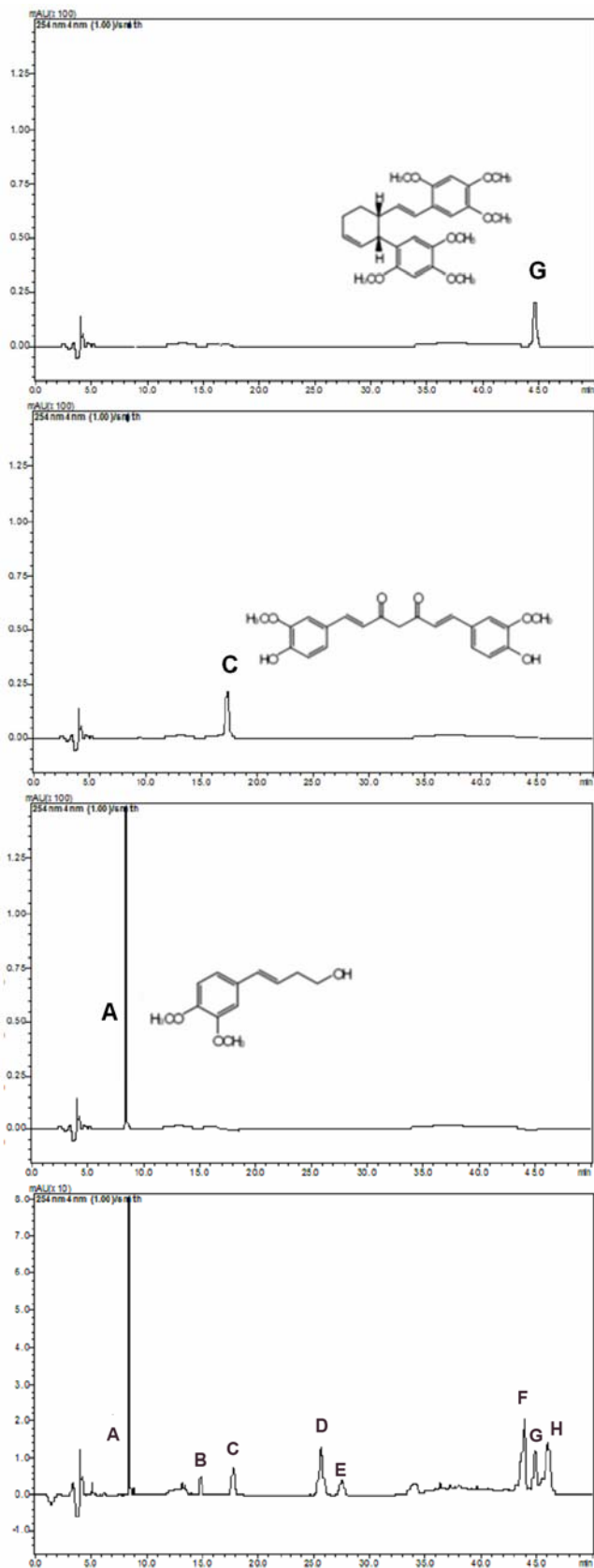

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Fig. 2 TLC-fingerprints of the hexane extract of *Zingiber cassumunar* rhizome, (sample from, Ratchaburi province, silica gel GF₂₅₄, hexane: dichloromethane: ethyl acetate 5: 1: 2). Lane 1 = *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl]cyclohex-1-ene (G), Lane 2 = curcumin (C), Lane 3 = (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (A), Lane 4 = crude extract; A'. observed under UV 254 nm, B'. observed under UV 366 nm, C'. sprayed with 10% sulfuric acid and observed under UV 366 nm, D'. sprayed with 10% sulfuric acid and observed under white light.

on a silica gel column to give five fractions HR1-HR5. Fraction HR3 was rechromatographed and gave compound A (250 mg). The residual powder from hexane extraction was air dried and extracted with ethyl acetate (2000 ml) yielding ethyl acetate crude extract (20.5 g) which was further chromatographed on a silica gel column, eluted with hexane-ethyl acetate gradient system to give eight fractions (EA1-EA8). Fraction EA6 contained compound C which was recrystallized from ethanol to give orange crystals (150 mg). Compounds C and G were recrystallized to give pure compounds with melting points 186-188 and 133-135°C, respectively. The NMR spectral data of compound A, which is oil characteristic, was identified by comparing with the reported NMR spectral data and was indicated as (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (Amatayakul *et al.* 1979; Kuroyanagi *et al*. 1980). Nuclear magnetic resonances of compounds C and G were confirmed by ^{1}H, ^{13}C and 2D-NMR spectra. The NMR spectral data and the melting points of each compound were compared to reported data and identified as curcumin and *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl] cyclohex-1-ene, respectively (Amatayakul *et al.* 1979; Kuroyanagi *et al*. 1980; Jitoe *et al*. 1992; Masuda and Jitoe 1994; Masuda and Jitoe 1995; Nualkaew *et al*. 2004).

HPLC conditions

The chromatographic conditions were developed and optimized using a mixture of standard compounds and the extracts of *Z. cassumunar*. Several stationary phases (C₈, C₁₈ and cyano) were initially screened, and the best resolution was obtained with a C₁₈ column. The analysis was carried out using a BDS Hypersil C₁₈ column (250 mm × 4.6 mm i.d., 5 μm) (Thermo Electron Corp.). To protect the integrity of the analytical column, all analyses were performed with a BDS Hypersil C₁₈ guard column (10 mm × 4 mm i.d., 5 μm) (Thermo Hypersil-keystone). The mobile phase was 0.5% aqueous trifluoroacetic acid solution (A) and acetonitrile (B) with gradient concentrations as follows: 0-8 min, 30-45% B; 8-10 min, 45-60% B; 10-11.5 min, 60-45% B; 11.5-30 min, 45-45% B; 30-35 min, 45-70% B; 35-40 min, 70-70% B; 40-45 min, 70-55% B; 45-50 min, 55-30% B. The total running time was 50 min and a flow rate was 0.8 mL/min. The detector was monitored for the eluent at 254 nm while the sample injection volume was 5 μL.

Calibration curves

Stock solutions of standard compounds A, C and G were prepared by dissolving each compound in methanol to obtain a concentration of 10 mg/ml. Six addition calibration levels were prepared by diluting this solution 1:1 with methanol. The calibration data (regression equation, linearity, range, limit of detection (LOD),

Fig. 1 HPLC fingerprints of methanolic extract of the rhizome of *Zingiber cassumunar*; A = (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol, B, D, E = unknown, C = curcumin, F = *cis*-3-(3′,4′-dimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl]cyclohex-1-ene, G = *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl] cyclohex-1-ene, H = *cis*-3-(3′,4′-dimethoxyphenyl)-4-[(*E*)-3‴,4‴-dimethoxystyryl]cyclohex-1-ene.

were combined into 10 fractions (H1–H10). Fraction H6 gave a crystalline compound, which was recrystallized from ethanol to give compound G (180 mg). Fraction H7 was rechromatographed
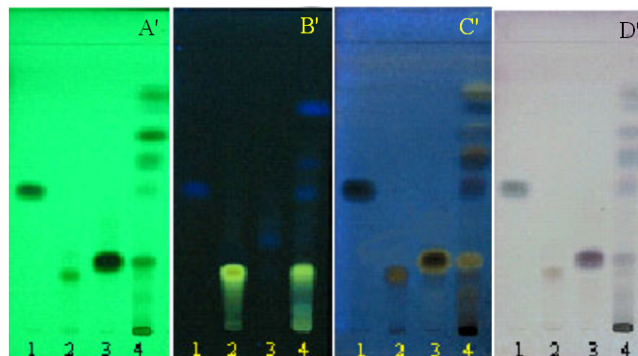
Table 1 Calibration data of compounds A, C and G, including regression equation, correlation coefficient (r^2), linear range, limit of detection, limit of quantitation, and standard deviation (in parenthesis) (n=3).

Compound	Regression equation	r^2	LOD (µg/ml)	LOQ (µg/ml)	Range (µg/ml)
A	Y = 17045X – 3239.1	0.9998	0.14 (0.02)	0.47 (0.07)	0.47 – 202
C	Y = 5160.3X – 5917.6	0.9995	0.53 (0.06)	1.77 (0.16)	1.77 – 103
G	Y = 8203.3X – 17772	0.9992	0.63 (0.11)	2.10 (0.38)	2.10 – 101

Table 2 Intra- and inter-day precisions of the HPLC method using sample ZC-13; results are based on peak area and % relative standard deviation (in parenthesis) (n=5).

Compound	Intra-day			Inter-day
	Day 1	**Day 2**	**Day 3**	
A	1969530	1999300	1987470	
	1975355	1995509	1956369	
	1988621	1956566	1980456	
	1978010	1992744	1953211	
	1990258	1998396	1980644	
Mean (%RSD)	1980355 (0.45)	1988503 (0.91)	1971630 (0.79)	1980163 (0.78)
C	37036	37252	34215	
	38083	34636	34678	
	37222	34899	35326	
	37328	36119	36104	
	38026	35839	34387	
Mean (%RSD)	37539 (1.29)	35749 (2.92)	34942 (2.22)	36077 (3.73)
G	110055	110211	109009	
	111401	112009	111785	
	110364	112877	109387	
	115322	112112	111898	
	116633	112556	109161	
Mean (%RSD)	112755 (2.68)	111953 (0.92)	110248 (1.33)	111652 (1.94)

and limit of quantification (LOQ)) are shown in **Table 1**. Triplicate injections were performed for each standard solution. The calibration curve of each reference standard was obtained by plotting the peak area versus the concentrations of the standard.

Method validation

Extraction efficacy of each sample was investigated by comparing peak areas of the same sample which was extracted with different sonication times and volumes of methanol. Peak purity was assured by evaluating available DAD data using the respective "peak purity" option within the Chemstation software (threshold value was set to 980). In all samples, the identified peaks were found to be sharp and free from co-eluting compounds.

Accuracy was determined by spiking the sample from Ratchaburi province with three concentrations of standard compounds (low, 5 µg/ml; medium, 25 µg/ml; and high, 50 µg/ml) and assaying these samples under optimized conditions. The obtained results were compared with those of the unspiked sample. Repeatability of the method was assured by observing relative standard deviations of multiple injections of the same sample solution. Precision of the method was evaluated by running five replicate samples prepared independently on day 1; the same procedure was repeated on two more days (ICH 1996).

Sample preparation for quantitative analysis

The finely powdered plant material (10 mg) was extracted three times with 3 ml of methanol by sonication for 10 min (each), at ambient temperature and centrifuged at 3,000 rpm for 5 min. The supernatant extracts were combined in a 10 ml volumetric flask which was adjusted to the final volume with methanol. Prior to injection, all solutions were filtered through a 0.45 µm nylon membrane filter. Each sample solution was assayed in triplicate.

RESULTS AND DISCUSSION

Chromatographic optimization

The HPLC-DAD was used for quantitative analysis. The appropriate gradient program was used for a good separation and acceleration to analyze the marker compounds in *Z. cassumunar* rhizome. The mobile phase used was the mix-ture of 0.5% aqueous trifluoroacetic acid (A) and acetonitrile (B) with gradient concentrations of B to maintain reproducibility.

Method validation

Linearity

Eminent linearities of (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (A), curcumin (C) and *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2′′′,4′′′,5′′′-trimethoxystyryl]cyclohex-1-ene (G) were exhibited in the range of 0.47-202, 1.77-103, and 2.10-101 µg/mL, and were confirmed by the correlation coefficient of 0.9998, 0.9995, and 0.9992, respectively (**Table 1**).

Precision

The results of repeatability studies and intermediate precision are presented in **Table 2**. Precision of the method showed a relative standard deviation (% RSD) not more than 2.92% for repeatability, and 3.73% for intermediate precision.

Accuracy

The results were expressed as percent recoveries of the particular components in the samples. **Table 3** shows the overall percent recoveries of the three compounds which were obtained by a relationship between the amount of added standard and the amount detected.

Limit of detection and limit of quantification

The LOD of compounds A, C, and G was 0.14, 0.53, and 0.63 µg/mL, respectively while the LOQ was 0.47, 1.77, and 2.10 µg/mL, respectively (**Table 1**).

Quantitative analysis of samples

A recovery rate up to 100% indicates exhaustive extraction of samples. **Table 4** shows that the dried powdered samples of *Z. cassumunar* contained compound A in the range per-

Table 3 Accuracy of the HPLC method, based on recovery (low, medium and high spiking) of sample ZC-13 (n = 3).

Compound	Spiking	Theoretical, µg/ml (Mean ± SD)	Found, µg/ml (Mean ± SD)	% Recovery (Mean ± SD)	% RSD
A	Low	10.37 ± 0.23	9.97 ± 0.20	96.14 ± 1.82	1.42
	Medium	20.74 ± 0.34	20.46 ± 0.41	98.65 ± 0.78	
	High	41.48 ± 0.97	39.99 ± 0.98	96.41 ± 1.94	
C	Low	10.20 ± 0.24	10.46 ± 0.22	102.55 ± 1.91	3.09
	Medium	20.40 ± 0.43	20.74 ± 0.39	101.67 ± 1.05	
	High	40.80 ± 0.81	39.49 ± 0.61	96.79 ± 1.67	
G	Low	50.13 ± 0.84	50.81 ± 1.04	101.36 ± 1.32	0.95
	Medium	100.26 ± 0.96	100.11 ± 1.34	99.85 ± 0.77	
	High	200.52 ± 3.13	199.74 ± 2.88	99.61 ± 1.23	

Table 4 Variation of active components in dry powdered rhizomes of *Z. cassumunar* collected from 14 locations in Thailand determined by HPLC.

Sample	Content of compound (%w/w)[*]		
	A	C	G
ZC-1 (N-Lumphun)	0.811 ± 0.002	0.156 ± 0.002	[**]n.d.
ZC-2 (N-Utaradit)	0.638 ± 0.003	0.066 ± 0.001	0.137 ± 0.002
ZC-3 (N-Phisanulok)	0.801 ± 0.003	0.171 ± 0.003	0.130 ± 0.003
ZC-4 (N-Petchabun)	0.884 ± 0.004	0.160 ± 0.002	0.126 ± 0.001
ZC-5 (NE-Udonthani)	0.538 ± 0.002	0.161 ± 0.004	0.117 ± 0.003
ZC-6 (NE- Loei)	0.400 ± 0.001	0.129 ± 0.001	0.183 ± 0.001
ZC-7 (NE-Mukdahan)	0.664 ± 0.001	0.149 ± 0.001	0.027 ± 0.001
ZC-8 (E-Rayong)	1.461 ± 0.013	[**]n.d.	0.287 ± 0.004
ZC-9 (E-Chanthaburi)	1.642 ± 0.005	0.067 ± 0.001	0.238 ± 0.002
ZC10 (C-Kanchanaburi 1)	0.721 ± 0.001	0.133 ± 0.003	0.106 ± 0.003
ZC-11 (C- Kanchanaburi 2)	1.005 ± 0.003	0.100 ± 0.002	0.089 ± 0.003
ZC-12 (C- Nakhonpathom)	0.615 ± 0.002	0.070 ± 0.001	0.048 ± 0.002
ZC-13 (C- Ratchaburi)	1.455 ± 0.006	0.103 ± 0.002	0.143 ± 0.002
ZC-14 (S- Ranong)	0.903 ± 0.002	0.265 ± 0.002	0.138 ± 0.001

[*]expressed as mean ± SD (n = 3) [**]n.d. not detectable
N = North, NE = North-East, C = Central, S = South

formed in positive ESI mode, and all compounds were unambiguously identified by their molecular peaks of 0.400 ± 0.001 to 1.642 ± 0.005% w/w while the contents of compounds C and G were 0.066 ± 0.001 to 0.265 ± 0.002 and 0.027 ± 0.001 to 0.287 ± 0.004% w/w, respectively. By iontrap mass spectrometer, ionization was $[M-H_2O]^+$ for A and $[M+H]^+$ for C and G.

HPLC technique using reverse phase C_{18} as a stationary phase was a suitable method for quantitative analysis of the active compounds, i.e. (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol (A), curcumin (C), and *cis*-3-(2′,4′,5′-trimethoxy-phenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl]cyclohex-1-ene (G) in the methanolic extract of the rhizome of *Z. cassumunar*. The method showed precisions with relative standard deviations (RSD) not more than 2.92% for repeatability and 3.73% for intermediate precision. Compared to other chromatographic analysis methods such as TLC-densitometry and UV-spectrophotometry, the magnitude of deviation found in the proposed HPLC method is quite small. The HPLC method is simple, precise, accurate and valuable for quantitative analysis of these components in *Z. cassumunar* raw material and its extracts. Although UV-spectrophotometric method allows a rapid and economical quantitation of total curcuminoids in turmeric extracts but it can not analyse each curcuminoid component (Pothitirat and Gritsanapan 2004). While TLC-densitometry can determine each component in crude curcuminoids (Pothitirat and Gritsanapan 2005), consumes less time, and a large number of samples can be screened in parallel, but the precision and sensitivity of the method is lower than those of the HPLC (Pothitirat and Gritsanapan 2008).

The maximum content of curcumin was found in the South sample, while the minimum content was found in the East samples. This shows that *Z. cassumuna*r grown in the South of Thailand, where the sea is nearby, rain is found all year, and the weather is not too warm and not too cool, contains high content of curcumin, as same as *Curcuma longa* reported in the previous study (Pothitirat and Gritsanapan 2006). The high content of the main component, (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol, was found in the East samples, while the low content was found in the sam-

ples from the North-East where the climate is warm and dry in Summer and cool in Winter. For *cis*-3-(2′,4′,5′-trimethoxyphenyl)-4-[(*E*)-2‴,4‴,5‴-trimethoxystyryl] cyclohex-1-ene, the maximum content was also detected in the East samples, but the minimum content was found in the samples from the northern part where the climate is cooler than the other parts in all seasons.

CONCLUSIONS

The proposed HPLC method is precise, accurate and appropriate for quantitative analysis of major components in *Z. cassumunar* raw material and its extracts. This is the first report on variation of bioactive components in *Z. cassumunar* determined by the HPLC method. The results will be useful for qualitative and quantitative analysis of these active compounds in *Z. cassumunar* powder, extracts and its pharmaceutical preparations. Also, it will be useful for further standardization of raw materials and the extracts from this plant used for pharmaceutical production, and for finding sources of good quality of *Z. cassumunar*.

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