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′-trimethoxyxstyryl]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 ± 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 ± 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.

Keywords: anti-inflammatory, curcumin, (E)-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol, Zingiber cassumunar, Zingiberaceae

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′-trimethoxyxstyryl]cyclohex-1-ene (G) (Fig. 1), which are major components in Z. cassumunar rhizomes, showed antioxidant and anti-inflammatory activities (Wanapaumpathmakul 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, Uttaradit, 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 A VP 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 macerated 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, 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
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 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',5'-trimethoxystyryl)cyclohex-1-ene (G), respectively.

**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 guard column (10 mm × 4 mm) (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, 30-60% B; 8-10 min, 45-50% 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 were fitted to a regression equation, linearity, range, limit of detection (LOD).
HPLC analysis of bioactive components in Zingiber cassumunar. Paramapojn et al.

RESULTS AND DISCUSSION

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. Eminent linearities of (A, C and G) were exhibited in the range of 0.47-202, 1.77-103, and 2.10-101 μg/mL, respectively while the LOD was 0.14, 0.53, and 0.63 μg/mL, respectively and the LOQ was 0.47, 1.77, and 2.10 μg/mL, respectively (Table 1). 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).

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.

Table 1 Calibration data of compounds A, C and G including regression equation, correlation coefficient (r²), linear range, limit of detection, limit of quantitation, and standard deviation (in parenthesis) (n=3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression equation</th>
<th>r²</th>
<th>LOD (μg/ml)</th>
<th>LOQ (μg/ml)</th>
<th>Range (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Y = 17045X – 3239.1</td>
<td>0.9998</td>
<td>0.14 (0.02)</td>
<td>0.47 (0.07)</td>
<td>0.47 – 202</td>
</tr>
<tr>
<td>C</td>
<td>Y = 5160.3X – 5917.6</td>
<td>0.9995</td>
<td>0.53 (0.06)</td>
<td>1.77 (0.16)</td>
<td>1.77 – 103</td>
</tr>
<tr>
<td>G</td>
<td>Y = 8203.3X – 17772</td>
<td>0.9992</td>
<td>0.63 (0.11)</td>
<td>2.10 (0.38)</td>
<td>2.10 – 101</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean (%RSD)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td>1999300</td>
<td>199740</td>
<td>1980163 (0.78)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>37252</td>
<td>34215</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34636</td>
<td>34678</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>36119</td>
<td>36104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35326</td>
<td>35326</td>
<td></td>
</tr>
<tr>
<td>Mean (%RSD)</td>
<td>37539 (1.29)</td>
<td>34942 (2.22)</td>
<td>36077 (3.73)</td>
</tr>
</tbody>
</table>

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.

4. Method validation

Eminent linearity 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'''-trimethoxy styryl]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). 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.

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4.1 Linearity

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4.2 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.

4.3 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.

4.4 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-
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 0.27 ± 0.001 to 2.87 ± 0.04% 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 C18 as a stationary phase was a suitable method for quantitative analysis of the active compounds, i.e. (E)-4-(3',4',5'-trimethoxyphenyl)but-3-en-1-ol (A), curcumin (C), and cis-3-(2',4',5'-trimethoxyphenyl)-4-(E-2',3',4',5'-trimethoxy styryl)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 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 quantification 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. cassumunar 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 samples 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',3',4',5'-trimethoxy styryl)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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