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Research article

Isolation and ultra-high performance liquid chromatography analysis of zerumbone and zerumbone epoxide in *Zingiber zerumbet* rhizomes

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Abstract

Importance of the work: *Zingiber zerumbet* (L.) Roscoe ex Sm. is an indigenous plant in Southeast Asia used in numerous ethnomedicinal applications and is of interest for pharmaceutical development.

Objectives: To isolate, optimize the extraction and develop a rapid quantitative analysis of zerumbone and zerumbone epoxide in *Z. zerumbet* rhizomes.

Materials & Methods: Chromatographic isolation of major components in the *Z. zerumbet* rhizomes yielded zerumbone and zerumbone epoxide. Their structures were identified using spectroscopic and spectrometric techniques. An ultra-high performance liquid chromatography (UHPLC) method was developed and validated for the quantitative analysis of zerumbone and zerumbone epoxide in the *Z. zerumbet* rhizomes. Suitable solvents for extracting *Z. zerumbet* were optimized.

Results: The developed UHPLC method demonstrated good sensitivity, linearity, precision and accuracy. Ethanol was considered as a suitable extracting solvent to obtain high yields of bioactive components from *Z. zerumbet* rhizomes. The contents of zerumbone and zerumbone epoxide in the *Z. zerumbet* rhizomes from various locations of Thailand were analyzed based on their UHPLC chromatograms that indicated the homogeneity of *Z. zerumbet* in Thailand.

Main finding: A rapid UHPLC method was developed and validated for quantitative analysis of zerumbone and zerumbone epoxide in *Z. zerumbet* rhizomes. A suitable extracting solvent (methanol) was optimized. The contents were analyzed of the zerumbone and zerumbone epoxide in the *Z. zerumbet* rhizomes from various locations of Thailand.

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Introduction

Zingiber zerumbet (L.) Roscoe ex Sm. is an indigenous herbaceous medicinal plant of Southeast Asia belonging to the Zingiberaceae family that is distributed and has been cultivated in many tropical regions (Wagner and Lorence, 2002; Acevedo-Rodríguez and Strong, 2012). It is naturally found at altitudes of up to 1,200 m in forest margins, brushwood, mixed forests and teak forests and is used as a spice and culinary herb with a large range of medicinal properties. Traditionally, its rhizome is used in various medicinal treatments due to its properties, such as anti-inflammatory, antibacterial, antipyretic, anti-diarrheal, antidiabetics, carminative and diuretic (Haque and Jantan, 2017). In Thailand, *Z. zerumbet* fresh rhizomes are used as an anti-flatulence drug. In India, the rhizomes are used to treat stomachache. The Hawaiians use the compressed rhizomes to treat sores, bruises and wounds and also to relieve head and tooth pain, ringworm and other skin diseases, joint pains or sprains and abdominal pain (Yob et al., 2011).

More than 60 metabolites, mostly polyphenols and terpenoids, have been isolated from *Z. zerumbet* (Haque and Jantan, 2017), with zerumbone, a sesquiterpene with one ring and three double bonds, being the main component (Fig. 2). Characterization of 80% ethanolic *Z. zerumbet* extract using liquid chromatography-tandem mass spectrometry showed many tentative secondary metabolites, including zerumbone, gallic acid, ethyl gallate, catechin, curcumin, kaempferol, kaempferol rhamnoside, kaempferol methyl ether isomer, kaempferol-3-O-(3″,4″-diacetyl) rhamnoside isomers, kaempferol methyl ether, kaempferol glucoside conjugate, bisdesmethoxycurcumin and desmethoxycurcumin (Ghazalee et al., 2019).

Numerous pharmacological studies have shown that this plant possesses antioxidant, anti-inflammatory, immunomodulation, antimicrobial, anthelmintic, larvicidal and antihyperlipidemic properties (Singh et al., 2012; Haque and Jantan, 2017). The alcoholic extracts of *Z. zerumbet* demonstrated antinociceptive activity at the peripheral and central levels when assessed using a writhing test (Somchit et al., 2005; Sulaiman et al., 2010; Zakaria et al., 2010; Yob et al., 2011). However, the ethanolic extract of *Z. zerumbet* failed to affect PGE_2-induced paw edema (Somchit and Shukriyah, 2003; Yob et al., 2011). Zerumbone, a major component of *Z. zerumbet* that attenuates the lipopolysaccharide-induced inflammatory response in macrophages, both *in vitro* and *ex vivo*, by suppressing the activation of the ERK-MAPK and NF-κB signaling pathways, as well as blocking the activation of the NLRP3 inflammasome (Su et al., 2021). Zerumbone epoxide inhibited both NF-κB activation and nitric oxide production. However, they were weaker than the inhibition using zerumbone, which may be associated with the change in the molecular geometry (Giang et al., 2009). Methanolic extract of *Z. zerumbet* showed significant antiedema activity in the paw edema test (Zakaria et al., 2010). The ethanolic extract of *Z. zerumbet* showed moderate antiallergic activity compared to other plants (Tewtrakul and Subhadhirasakul, 2007). The methanolic extracts of *Z. zerumbet* and zerumbone showed strong platelet aggregation activity (Jantan et al., 2005, 2008). In addition, the anticancer activity of zerumbone or *Z. zerumbet* extracts has been reported (Sithara et al., 2018; Wani et al., 2018; Jalili-Nik et al., 2022).

Standardization for consistent quality is crucial to ensure the reproducibility of medical treatments. Several techniques have been developed and validated for the quantification of the phytochemical components in *Z. zerumbet*, including high-performance liquid chromatography (HPLC) (Haque et al., 2019), ultra-high performance liquid chromatography (UHPLC) (Ghasemzadeh et al., 2017), headspace-solid phase microextraction-gas chromatography (HS-SPME-GC) (Bhavya et al., 2021), and high-performance thin-layer chromatography (HPTLC) (Dash et al., 2021). Chemical fingerprint for discrimination from related species can be achieved using Fourier-transform infrared spectroscopy (FTIR) spectral analysis (Amponsah et al., 2022) and capillary liquid chromatography (Rafi et al., 2013). However, a more rapid and cost-saving method for routine analysis is needed. Therefore, the current study investigated UHPLC and the developed method was validated for quantification of zerumbone in *Z. zerumbet* rhizome to largely reduce the mobile phase volume consumption with a shorter runtime compared to HPLC. In addition, the effect of extracting solvents on the yields of zerumbone and zerumbone epoxide was investigated. The results of the current study should provide a basis for quality assessment for further pharmaceutical development

Materials and Methods

Chemicals and reagents

HPLC grade methanol was purchased from J.T. Baker, USA. Deionized water was purified by Ultra Clear, Siemen

Water Technologies Corp (Germany). All reagents were of analytical grade unless stated otherwise.

Plant materials

The plant materials were obtained from various localities or purchased from local suppliers in Thailand. Identification was done by observing and comparing the macroscopic properties (aspect, color and smell) with authenticated samples by the authors. The samples were dried at 50 °C using a hot-air oven, cut into small pieces and ground into powder. The samples were stored in an air-tight container at room temperature.

Extraction and isolation of major components

Zerumbone and zerumbone epoxide were isolated from the *Z. zerumbet* rhizome extract. The powdered sample (200 g) was extracted using maceration with occasional shaking with hexane for 72 h (three times) at room temperature. The extract was combined, filtered through Whatman filter paper and dried in a rotary evaporator, which yielded 2.4 g (1.2% weight per weight, w/w). The crude extract was separated based on column chromatography (Merck silica gel 60, 70–230 mesh) with hexane and ethyl acetate mixtures with increasing polarity. Each collected fraction was examined using thin-layer chromatography (TLC, silica get 60 F254) using a ratio of hexane-to-ethyl acetate of 9:1 (volume per volume, v/v) as the mobile phase. Further purification using column chromatography was repeated based on the same process. TLC and UHPLC techniques were used to assess the purity levels. The final elution cleanup was done using a Sephadex LH-20 column using methanol as a solvent, which yielded 139 mg and 45 mg zerumbone epoxide (Fig. 1). The isolated pure compound was structurally elucidated by comparing the melting point, ^{1}H NMR, ^{13}C NMR and mass spectra with reported data. Supporting spectral data were shown in supplementary figures (Figs. S1–S8)

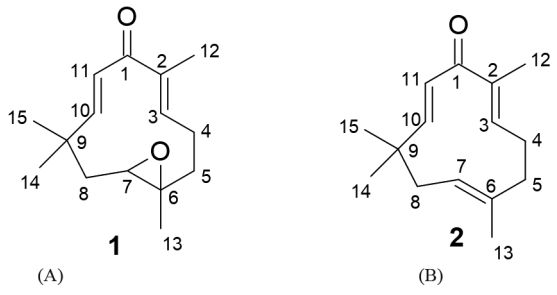

Fig. 1 Chemical structures: (A) zerumbone epoxide; (B) zerumbone

The *Zerumbone epoxide* [1, (4*E*,7*E*)-1,5,9,9-tetramethyl-12-oxabicyclo[9.1.0]dodeca-4,7-dien-6-one] was characterized as: white powder,mp 88.5−90.5 °C (lit. 96.0−96.5 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.12–6.04 (m, 3 x 1H, *H-3*, *H-10*, and *H-11* of vinyl proton), 2.72 (d, *J*=11.1 Hz, 1H, *H-8*), 2.47-2.36 (m, 2H, *H-5*), 2.28-2.23 (m, 1H, *H-4*), 1.90 (d, *J*=14.0 Hz, 1H, *H-8*), 1.82 (s, 3H, *CH_3-12*), 1.42 (dd, *J* = 13.9 Hz, 11.3 Hz, 1H, *H-7*), 1.35-1.28 (m, 1H, *H-4*), 1.26 (s, 3H, *CH_3-13*), 1.19 (s, 3H, *CH_3-14*), 1.05 (s, 3H, *CH_3-15*). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm) = 202.9, 159.5, 147.7, 139.4, 128.2, 62.8, 61.4, 42.6, 38.2, 35.9, 29.7, 24.6, 24.0, 15.6, 12.1. FT-IR (ATR): ν (cm^{-1}) = 3028, 3002, 2961, 2930, 2869, 1654, 1639, 1455, 1385, 1365, 1301, 1261, 1200, 1166, 1118, 1060, 970, 882, 840, 822, 764, 702, 628. Q-Orbitrap HRMS: m/z $[M+Na]^+$, calculated for $C_{15}H_{22}O_2Na$ = 257.1517 versus found = 257.1512.

The *Zerumbone* [2, (2*E*,6*E*,10*E*)-2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one] was characterized as: white powder, mp 64.7−65.9 °C (lit. 66.0-66.5°C); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 6.01-5.98 (m, 1H, *H-3*), 5.96 (d, *J*=16.4 Hz, 1H, *H-11*), 5.84 (d, *J*=16.4 Hz, 1H, *H-10*), 5.25-5.21 (m, 1H, *H-7*), 2.48-2.16 and 1.89-1.86 (m, 1H, 2H, 2H, 1H, *H-4*, *H-5*, *H-8*), 1.78 (s, 3H, *CH_3-12*), 1.52 (s, 3H, *CH_3-13*), 1.18 (s, 3H, *CH_3-14*), 1.05 (s, 3H, *CH_3-15*). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm) = 204.3, 160. 7, 148.8, 137.9, 136.2, 127.1, 124.9, 42.4, 39.4, 37.8, 29.4, 24.4, 24.1, 15.2, 11.7. FT-IR (ATR): ν (cm^{-1}) = 3025, 2963, 2919, 2857, 1652, 1638, 1455, 1429, 1385, 1363, 1262, 1183, 1103, 964, 907, 827, 697, 628. Q-Orbitrap HRMS: m/z $[M+H]^+$, calcd for $C_{15}H_{23}O$: 219.1749, found: 219.1741; $[M+Na]^+$, calculated for $C_{15}H_{22}ONa$ = 241.1568 versus found = 241.1558.

Ultra-high performance liquid chromatography apparatus and conditions

UHPLC was performed on a Vanquish UHPLC system (Thermo Fisher Scientific Inc.) equipped with a Thermo Scientific Vanquish- Binary Pump F, a Thermo Scientific Vanquish-Split Sampler FT, a Thermo Scientific Vanquish-Column Compartment H and a Thermo Scientific Vanquish-Diode Array Detector FG. The separation was done on a BDS Hypersil C18 column (50 mm × 2.1 mm with internal diameter of 2.4 µm). The mobile phases were (A) 0.1% formic acid in water and (B) methanol. A mobile phase time program was set up with linear gradient elution from 40% B to 85% B in A for 4 min and then 100% B for 1 min. Before each injection, the column was equilibrated with 40% B in A for 2 min.

The column temperature was controlled at 25°C with a constant flow rate of 1 mL/min. Diode array detection was set at a wavelength of 254 nm. The injection volume setting was 2 µL for all samples and standards.

Stock and working solutions of standard compounds

Stock solutions of zerumbone and zerumbone epoxide were prepared at a concentration of 1,000 µg/mL by dissolving each accurately weighed standard compound in methanol. Appropriate dilution of the stock solutions with methanol to obtain the desired concentration was used to prepare working standard solutions.

Optimization of extracting solvent

The optimal extracting solvent was investigated using different solvents (water, methanol, ethanol, acetonitrile, tetrahydrofuran, mixtures of water and methanol, and mixtures of water and ethanol at different ratios. A *Z. zerumbet* sample (40 mg) was accurately weighed and separately extracted with 2 mL of each solvent using sonication at ambient temperature. Each extract was prepared in triplicate and analyzed by the proposed method. The solvent yielding the highest content of zerumbone in the extract was chosen as the appropriate solvent for extraction.

Sample preparation

Each *Z. zerumbet* rhizome triplicate samples was accurately weighed and extracted with ethanol at a concentration of 20 mg/mL in an ultrasonic bath for 30 min. Each solution was passed through a 0.22 µm nylon membrane filter prior to injection.

Method validation

The developed method was validated according to the guideline of ICH (International Conference on Harmonization,1996/2005). The method validation parameters were linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

Linearity

The linearity relationship between each analyte concentration and the response was evaluated across eight known concentrations in the range 3.9–500 µg/mL. Each standard concentration was prepared and analyzed in triplicate. The calibration curves were plotted using the peak area versus the concentration of the standard. Regression lines were calculated using the least squares method.

Precision

Repeatability and intermediate precision were evaluated for method precision. Intra- and inter-day precision were measured by analyzing 50 µg/mL standard solution. The intra-day precision (representing repeatability of the method) was determined seven times within 1 d, while the inter-day precision (representing intermediate precision) was determined for three consecutive days. Then, the percentage relative standard deviation (%RSD) was calculated and used to indicate the precision.

Accuracy

A spiking study was used to evaluate the accuracy of the method. Three different levels of standard mixtures of pre-analyzed standard solution were spiked to the known concentration sample extract. Spiked samples were prepared in triplicate for each concentration. The results from measurements on unspiked and spiked samples were evaluated. The recovery was calculated as: Recovery (%) = 100 × (Amount found – Original amount) / Amount spiked.

Limit of detection and limit of quantitation

Determination of the signal-to-noise ratio was performed by comparing measured signals from samples with known low concentrations of analytes with noise situated around the peak of interest. A minimum concentration that produced a signal-to-noise ratio of 3:1 was considered for the LOD and of 10:1 for the LOQ.

Results and Discussion

To obtain an overview of the characteristic compound profiles, dried rhizome powder samples of *Z. zerumbet* rhizomes were extracted using methanol, followed by UHPLC analyses. The major components from *Z. zerumbet* rhizome were isolated using column chromatographic techniques, yielding zerumbone epoxide (1) and zerumbone (2). Characterization of the isolated compounds was done by comparing the melting point, ^{1}H NMR, ^{13}C NMR and mass spectra with reported data (Kitayama et al., 1999, 2001).

The UHPLC method was developed for the quantitative analysis of the major components (zerumbone and zerumbone epoxide) in the *Z. zerumbet* rhizome. A broadly used reversed-phase C-18 column was chosen. The mobile phase composition was optimized. Methanol, which is more readily available and cheaper than acetonitrile, was used as the mobile phase with 0.1% formic acid in water. A gradient system was used because of the wide range of polarities in the analyzed components. From the various mobile phases trialed, the proposed system produced symmetrical peaks and provided the most efficient separation and speed. The representative chromatograms of the optimized condition are shown in Fig. 2. A wavelength of 254 nm was used for detection. Peak purity was investigated using the DAD and there was no indication of co-elution or impurities.

The developed method was validated according to the ICH guidelines (International Conference on Harmonisation, 1996/2005) to ensure that the method was suitable for its intended purpose. The method validation parameters (linearity, precision, accuracy, LOD and LOQ) were analyzed. Linearity relationship was evaluated across the range 3.9–500 µg/mL. The calibration curve of the peak areas versus the concentrations of zerumbone and zerumbone epoxide provided an excellent linear relationship, as shown by the coefficient of determination (R^2) in Table 1. The repeatability of method showed that the percentage of relative standard deviation was lower than 1%, while the measurement of intermediate precision based

Table 1 Method validation parameters for quantification of zerumbone epoxide and zerumbone

Parameter	Result	
	Zerumbone epoxide	Zerumbone
Regression equation	Y = 0.837X + 0.1441	Y = 0.08X + 0.1393
Coefficient of determination	0.9999	0.9999
Linear range (µg/mL)	3.9–500	3.9–500
LOD (µg/mL)	0.03	0.03
LOQ (µg/mL)	0.1	0.1

LOD = limit of detection; LOQ = limit of quantitation;
X is the concentration of standard in µg/mL; Y is the peak area at 254 nm

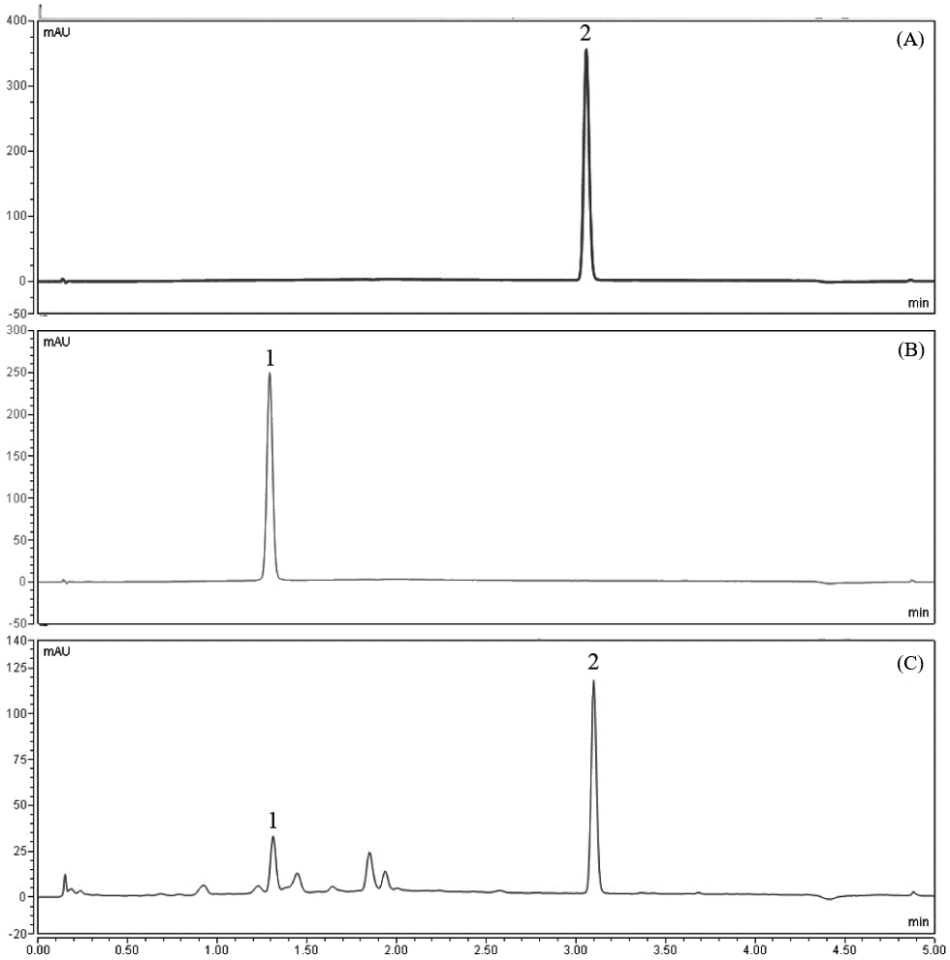

Fig. 2 Representative chromatograms (254 nm) of: (A) zerumbone standard; (B) zerumbone epoxide standard; (C) crude *Z. zerumbet* rhizome extract, where peak identification numbers are 1 = zerumbone epoxide and 2 = zerumbone

on analysis using the same analyte on three different days provided a percentage of relative standard deviation less than 2% (Table 2). These results showed that the method has a good precision. A spiking study was used to evaluate the accuracy of the method which was represented by the recovery values. The results showed that the recovery values of zerumbone epoxide and zerumbone were in the range 100.90–101.78% (average 101.27%) and 99.02–99.87% (average 99.53%), respectively, as shown in Table 3. The LOD and LOQ values were 0.03 and 0.1 µg/mL, respectively (Table 1).

Various extracting solvents (water, methanol, ethanol, acetonitrile, tetrahydrofuran, mixture of water and methanol, and mixtures of water and ethanol at different ratios) were studied to determine the optimum for highest yield of active components. Because it was not possible to predict the extraction efficiency as a model was not available, optimization of the extraction of this plant was based on experimentation. Sonication was used as the extraction method because of its simplicity and being fast and compatible with various solvents. After UHPLC analysis, the yields of zerumbone and zerumbone epoxide from the different extracting solvents were quantified (Table 4). The highest zerumbone yield was achieved with tetrahydrofuran followed by ethanol, whereas methanol produced the highest amount of zerumbone epoxide. The results from using the sonication method were different from the microwave-assisted method of Ghasemzadeh et al. (2017) in which the solvent was optimized to an ethanol-to-water ratio (44:56, v/v). The difference in the yields of zerumbone using these methods might have been due to the temperature during the extraction process. Standardized *Z. zerumbet* extraction was performed Ghazalee et al. (2019), Haque et al. (2019) and Akhtar et al. (2019) using 80% ethanol as the extracting solvent. However, the yield from ethanol was higher yield than for an ethanol-to-water ratio of 8:2 (v/v). Thus, ethanol was chosen as a suitable extracting solvent. Notably, it is less hazardous than tetrahydrofuran and methanol, as well as being cheap and suitable for industrial extraction upscaling.

The *Z. zerumbet* dried rhizomes obtained from various locations in Thailand were extracted using ethanol and quantified for their zerumbone and zerumbone epoxide contents using the proposed method. The results are shown in Table 5.

Table 2 Intraday and Interday precision (percentage relative standard deviation) of zerumbone epoxide and zerumbone

Compound	Intraday			Interday
	Day 1	Day 2	Day 3	
Zerumbone epoxide	0.15	0.11	0.57	0.97
Zerumbone	0.19	0.10	0.37	0.91

Table 3 Recovery study of zerumbone epoxide and zerumbone

Level	Compound	Theoretical[a] (µg/mL)	Found[b] (µg/mL)	Recovery[b] (%)
1	Zerumbone epoxide	24.01	24.43±0.38	101.78±1.57
	Zerumbone	55.86	55.70±1.00	99.71±1.80
2	Zerumbone epoxide	33.71	34.01±0.12	100.90±0.36
	Zerumbone	78.12	77.36±0.13	99.02±0.17
3	Zerumbone epoxide	46.09	46.61±0.14	101.13±0.31
	Zerumbone	103.78	103.64±0.33	99.87±0.32
Average	Zerumbone epoxide			101.27
	Zerumbone			99.53

[a]Theoretical value is the amount calculated by original amount plus amount spiked

[b]Expressed as mean ± SD (n = 3)

Table 4 Zerumbone epoxide and zerumbone contents from various extracting solvents

Extracting solvents	Extraction yield (% weight per weight)[a]	
	Zerumbone epoxide	Zerumbone
Water	0.1086±0.0018	0.0067±0.0052
Methanol	0.1494±0.0038	0.3064±0.0103
Ethanol	0.1391±0.0041	0.3157±0.0118
Acetonitrile	0.1212±0.0066	0.2816±0.0075
Tetrahydrofuran	0.0727±0.0015	0.3242±0.0028
Methanol-to-water ratio (5:5, v/v)	0.1317±0.0069	0.1937±0.0026
Ethanol-to-water ratio (8:2, v/v)	0.1215±0.0045	0.2085±0.0109
Ethanol-to-water ratio (5:5, v/v)	0.1192±0.0069	0.2080±0.0210
Ethanol-to-water ratio (2:8, v/v)	0.1085±0.0014	0.0800±0.0119

v/v = volume per volume

[a]Expressed as mean ± SD (n = 3)

Table 5 Bioactive contents of Z. zerumbet rhizomes collected from various locations in Thailand

Location	Content (% weight per weight)[a]	
	Zerumbone epoxide	Zerumbone
Phuket	0.0710 ± 0.0020	2.3767 ± 0.0516
Bangkok (sample 1)	0.0237 ± 0.0016	0.7023 ± 0.0576
Bangkok (sample 2)	0.1160 ± 0.0070	2.2081 ± 0.1067
Bangkok (sample 3)	0.1563 ± 0.0020	0.5459 ± 0.0219
Nongkhai	0.0535 ± 0.0016	1.9685 ± 0.0902
Nakhonpathom (sample 1)	0.0372 ± 0.0004	2.1166 ± 0.1248
Nakhonpathom (sample 2)	0.0775 ± 0.0015	1.1632 ± 0.0243
Average	0.0765 ± 0.0462	1.5830 ± 0.7618

[a]Expressed as mean ± SD (n = 3)

The zerumbone epoxide and zerumbone contents in the *Z. zerumbet* rhizomes were in the ranges 0.02–0.16% w/w (average 0.08% w/w) and 0.55–.38% w/w (average 1.58% w/w), respectively. The UHPLC chromatogram showed similar chromatographic fingerprint patterns, indicating the homogeneity of *Z. zerumbet* in Thailand. Thus, the UHPLC chromatogram could be used for identification and characterization of *Z. zerumbet* crude drug and phytopharmaceutical preparations.

In conclusion, the UHPLC method was developed and validated for the quantitative analysis of zerumbone and zerumbone epoxide extracted from *Z. zerumbet* rhizomes. The developed method had a much shorter runtime and used less solvent compared to the traditional HPLC method. The proposed method could be used for quality assessment and standardization of *Z. zerumbet* raw materials and extract.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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