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RESEARCH ARTICLE

Phytochemicals and Antibacterial Activity of *Zingiber zerumbet* Growing in Negeri Sembilan, Malaysia

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ABSTRACT

The present study is aimed at analysing the chemical composition of essential oil and screening the phytochemicals in crude extracts from the rhizomes of *Zingiber zerumbet* (L.) Roscoe ex Sm. (Zingiberaceae) as well as determining their antibacterial activity. The essential oil was extracted from the fresh rhizomes using the hydrodistillation method and analysed by gas chromatography-mass spectrometry (GC-MS). The phytochemicals from the dried rhizomes were extracted by sequential maceration technique using *n*-hexane, ethyl acetate, and methanol, while phytochemical screening was carried out using standard chemical tests. Antibacterial activity was screened using the disc diffusion method. A total of 31 components (94.5%) were detected in the rhizome oil with zerumbone (28.4%), terpinen-4-ol (16.5%) and α-humulene (11.1%) as the main components. Phytochemical screening of the extracts obtained from the dried rhizomes disclosed the existence of steroids, terpenes, quinones, flavonoids, phenols, alkaloids, tannins, coumarins and glycosides. The rhizome oil showed potent antibacterial activity towards *Salmonella typhi* and *Escherichia coli* with inhibition zone diameters of 20.66 and 16.66 mm, respectively. Among the extracts, the methanol extract demonstrated high inhibitory activity against *Bacillus cereus* (14.00 mm), *Staphylococcus aureus* (9.00 mm), and *Salmonella typhi* (10.66 mm), while the ethyl acetate extract gave the biggest inhibition zone on *E. coli* (10.33 mm). The findings from this study indicate that *Z. zerumbet* might be one of a promising source of natural chemicals that can act as antibacterial agents.

Keywords: *Zingiber zerumbet*; Zingiberaceae; essential oil; phytochemicals; antibacterial

1. INTRODUCTION

Zingiber zerumbet (L.) Roscoe ex Sm. or wild ginger is locally known as “Lempoyang”, “Awapuhi”, “Yaiimu”, “Hong Qiu Jiang”, “Jangli adha”, “Ghatian”, “Hiao Dam” and “Zurunbah” (Koga et al., 2016). The plant belongs to the family Zingiberaceae, and grows widely in countries throughout Asia, such as Sri Lanka, Thailand, Nepal, Bangladesh, Laos, Malaysia, India, Indonesia and Southwest China (Murakami et al., 2002; Nag et al., 2013; Tian et al., 2020). *Z. zerumbet* is a perennial tuberous plant that can be found naturally as scattered plants in shady parts of mountain slopes, damp and commonly in deciduous forests (Nair, 2013). The plant is utilised in local traditional medicine for the treatment of various ailments, such as loss of appetite, swelling, worm infestation and as a tonic and stimulant (Nik Norulaini

et al., 2009; Sakinah et al., 2007). Furthermore, people in Indonesia applied *Z. zerumbet* as the main ingredient in 'jamu' manufacturing (Riyanto, 2007). The rhizomes of *Z. zerumbet* have been extensively applied in many studies due to its notable pharmaceutical applications (Tan et al., 2018).

Studies on the essential oils of the rhizomes of *Z. zerumbet* have been conducted by several researchers, and plant samples were collected from various geographical locations including Selangor, Penang, and Sabah, Malaysia. A mixture of volatile compounds, primarily terpenes and terpenoids, were detected in the essential oils, with zerumbone being the predominant compound in the majority of the tested essential oils (Baby et al., 2009; Batubara et al., 2013; Bhuiyan et al., 2009; Chane-Ming et al., 2003; Rana et al., 2012, 2016; Singh et al., 2014; Sulaiman et al., 2010; Suthisut et al., 2011; Tian et al., 2020; Yu et al., 2008). Literature surveys on phytochemical studies have found varied secondary metabolites such as alkaloids, anthraquinones, quinones, glycosides, flavonoids, phenols, tannins, saponins, terpenes and coumarins in rhizomes extracts (Chien et al., 2008; Manonmani and Mehalingam, 2018; Prakash et al., 2011). The essential oils, extracts, and isolated phytochemicals of *Z. zerumbet* have been subjected to many biological activities, resulting in interesting biological effects. Among them are antibacterial, anti-inflammatory, antifungal, antioxidant, cytotoxic, and antidermatophytic activities (Azelan et al., 2015; Jantan et al., 2005; Murakami et al., 2002; 2014; Tian et al., 2020; Yob et al., 2011; Yusmaniar et al., 2015).

To the best of our knowledge, no study has been found on essential oil, qualitative phytochemical analysis of crude extracts and antibacterial activity of the rhizomes of *Z. zerumbet* growing in Negeri Sembilan, Malaysia. Hence, we would report the chemical composition, phytochemical screening, and antibacterial effect of its essential oil and extracts.

2. MATERIALS AND METHODS

2.1. Plant material

The rhizomes of *Z. zerumbet* (voucher number ID017/2021) was collected in August 2019 from Kampung Lakai, Jelebu, Negeri Sembilan, Malaysia. The plant was determined by a botanist, Dr. Shamsul bin Khamis and the specimen was deposited at UKMB Herbarium, Universiti Kebangsaan Malaysia.

2.2. Extraction and analysis of the essential oil

The fresh rhizome of *Z. zerumbet* (746.6 g) was chopped into small sizes and subjected to hydrodistillation in a Clevenger apparatus for 8 h. The oily layer obtained was separated using liquid-liquid extraction with diethyl ether as the solvent (10 mL × 3 times) and dried over anhydrous magnesium sulphate. The pure essential oil acquired by evaporation at room temperature was then weighed and stored at 4ºC (Jani et al., 2016; Jena et al., 2020). The chemical composition of the essential oil was analysed using an Agilent GC-MS 7890A/5975C Series MSD equipped with a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The essential oil was diluted in diethyl ether and 1 μL of the sample was injected manually. The carrier gas was helium, and the temperatures of the injector and detector were maintained at 250 and 280°C, respectively. The oven temperature was programmed as follows: 60°C initial temperature, held isothermally for 10 min; increased at 3°C/min to 230°C. The electron ionisation (EI) mode was operated at 70eV, the mass range was 50-500 amu, the total scan time was 67.7 min, and the scan rate was set at 2.91 scans/sec. Individual components were identified by matching their mass spectra with the existing HPCH 2205.L and NIST05a.L libraries (Jani et al., 2016).

2.3. Preparation of plant extract

The fresh rhizomes of *Z. zerumbet* were cleaned, cut into small pieces, air-dried at room temperature for 48 h, and ground. Then, the dried rhizomes (254.9 g) were extracted sequentially using *n*-hexane (1.2 L), ethyl acetate (1.2 L) and methanol (1.2 L) at room temperature using an orbital shaker with a speed of 200 rpm. After 3 days, the extracts were filtered and concentrated in a vacuum rotary evaporator to furnish the *n*-hexane, ethyl acetate, and methanol extracts (Alemu et al., 2018; Manonmani and Mehalingam, 2018).

2.4. Phytochemical qualitative analysis

Each extract was tested for the presence of plant secondary metabolites using the following standard methods. The existence of phytochemicals was represented as (+), while their absence was expressed as (-).

Test for flavonoids. A small amount of extract was dissolved in ethanol (2 mL). Then, the ethanolic extract was treated with a few drops of sodium hydroxide solution (2 mL, 2% w/v) to give a concentrated yellow colour. The disappearance of the yellow colour after the addition of a few drops of dilute hydrochloric acid (1% v/v) indicates the presence of flavonoids (Gul et al., 2017).

Test for phenols. Each extract was dissolved in ethanol (2 mL) followed by the addition of distilled water (2 mL) and a few drops of aqueous ferric chloride solution (10% w/v). The appearance of bluish-black or green colour shows the existence of phenols (Gowri and Vasantha, 2010; Khanam et al., 2015).

Test for alkaloids. The extract was dissolved in ethanol (2 mL), followed by the addition of dilute hydrochloric acid (1% v/v). The reaction mixture was filtered and treated with a few drops of Wagner's reagent (iodine in potassium iodide). The formation of a brown or reddish precipitate confirms the presence of alkaloids (Jani et al., 2020).

Test for terpenoids. Each extract (around 1 mg) was dissolved in chloroform (1 mL). Then, concentrated sulfuric acid was carefully added along the side of the test tube. A reddish-brown colouration at the interface represents the existence of terpenoids (Iqbal et al., 2015).

Test for tannins. The extract was dissolved in ethanol (2 mL) followed by the addition of distilled water (2 mL). Then, the mixture was treated with a few drops of aqueous ferric chloride solution (5% w/v). Dark green precipitate or colouration infers a positive result for tannins (María et al., 2018; Ramos and Bandiola, 2017).

Test for saponins. Each extract was dissolved in ethanol (1 mL) separately followed by the addition of distilled water (5 mL). The mixture was shaken vigorously and the development of frothing, which persists within 5 min, indicates the presence of saponins (Iqbal et al., 2015; Manonmani and Mehalingam, 2018).

Test for coumarins. A small amount of extract was dissolved in ethanol (2 mL). Then, the ethanolic solution was dissolved in hot distilled water, cooled, and divided into two test tubes. The first test tube was the reference, while the second test tube was added with ammonia solution (0.5 mL, 10% v/v). The appearance of yellow fluorescence under UV light (λ_{max}= 365 nm) confirms the presence of coumarins (Benmehdi et al., 2012).

Test for quinones. The extract was dissolved in ethanol (1 mL) followed by the addition of concentrated sulfuric acid (1 mL). The formation of red-coloured solution is indicative of the existence of quinones (Ali et al., 2018).

Test for anthraquinones. Each extract was dissolved in ethanol (2 mL), mixed with chloroform (10 mL), and filtered. Then, ammonia solution (5 mL, 10% v/v) was added to the filtrate and then shaken. Pink or slight red colour on the upper part of the aqueous layer confirmed the presence of anthraquinones (Ayeni et al., 2018).

Test for glycosides. Test 1: Each extract (0.2 g) was mixed with dilute sulphuric acid (5 mL, 1% v/v) and warmed in a water bath. Then, the mixture was filtered. The filtrate was neutralised with sodium hydroxide solutions (5% w/v) and Fehling's solutions A and B (0.1 mL) until it achieved an alkaline condition which was tested with pH paper. Later, the mixture was heated for 2 min in a water bath. Test 2: Each sample extract (0.2 g) was added to distilled water (5 mL) and warmed in a water bath. After boiling, an equal amount of distilled water as sodium hydroxide solution in Test 1 was added. Then, Fehling's solution A and B (0.1 mL) was added to the mixture until it became alkaline (tested with pH paper) and heated for 2 min in a water bath. The presence of glycosides is confirmed if the amount of brick-red precipitate in Test 1 is higher than that in Test 2 (Prasad et al., 2018).

2.5. Antibacterial assay

The antibacterial potency of the essential oil and extracts of *Z. zerumbet* was determined using disc diffusion assay (Bajpai et al., 2009; Chowdhury et al., 2019; Fadli et al., 2012). Two Gram-negative bacteria (*Escherichia coli* (ATCC 25922) and *Salmonella typhi* (ATCC 14028)) as well as two Gram-positive bacteria (*Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 14579) were used as the tested bacterial strains. All bacteria were cultured separately in nutrient broth and incubated at 37ºC for 48 h (Sufian et al., 2013). The bacterial culture was spread evenly over the entire nutrient agar surface by a sterile cotton swab. Each crude extract (200 mg) was dissolved individually in 1% DMSO (1 mL), sonicated, and vortexed to obtain a concentration of 200 mg/mL. Whatman No. 1 sterile paper discs (6 mm diameter) were impregnated with 10 µL of pure essential oil or extract solution (200 mg/mL) and placed on the surface of the inoculated agar. The positive control was streptomycin (10 µg/disc), while the negative control was 1% DMSO. All inoculated plates were inverted and incubated at 37ºC for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around each disc (mm). The experiment was replicated three times and data were reported as means ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Extraction and analysis of the essential oil

Hydrodistillation of fresh rhizomes of *Z. zerumbet* yielded a pale yellow oil of 1.79 g (0.24%). The GC-MS analysis of the oil led to the identification of 31 chemical components representing 94.5% of the total oil (Figure 1). The chemical composition of the oil is tabulated in Table 1. The rhizome oil of *Z. zerumbet* consisted mainly of oxygenated sesquiterpenes (30.9%, seven components) and monoterpene hydrocarbons (30.4%, 12 components) followed by oxygenated monoterpenes (21.2%, nine components) and sesquiterpene hydrocarbons (12.0%, three components).

Zerumbone (28.4%), terpinen-4-ol (16.5%), and α-humulene (11.1%) were the major components in the oil. The other 24 components were only present in less than 3.6% except for sabinene (7.9%), β-pinene (6.3%), γ-terpinene (6.2%) and α-terpinene (3.6%). The major components in the present *Z. zerumbet* rhizome oil were almost similar to several previous reports, but the amount varies. In this study, the percentage of zerumbone was a bit lower than that of a in the essential oils from Selangor, Penang, and Sabah, Malaysia, as well as from other regions, such as Vietnam, France, Bangladesh, India and China (Baby et al., 2009; Batubara et al., 2013; Bhuiyan et al., 2009; Chane-Ming et al., 2003; Madegowda et al., 2016; Malek et al., 2005; Padalia et al., 2018; Rana et al., 2012, 2016; Singh et al., 2014; Sulaiman et al., 2010; Tian et al., 2020). However, the amount of zerumbone in the current oil was higher than the

amount of essential oils from Thailand and Bihar, India (Srivastava et al., 2000; Suthisut et al., 2011). Previously, it was reported that the content of zerumbone in the *Z. zerumbet* rhizome oils from different localities were ranged from 8.1% to 84.8% (Tian et al., 2020).

Apart from zerumbone, another major component, α-humulene, was also reported to be a significant component in the essential oils of *Z. zerumbet* from Bangladesh, France, India, China and Malaysia. Furthermore, terpinen-4-ol, which was identified in a considerable amount in this study, was previously detected in low concentrations in oils from other localities (Baby et al., 2009; Bhuiyan et al., 2009; Chane-Ming et al., 2003; Malek et al., 2005; Rana et al., 2012, 2016; Srivastava et al., 2000; Sulaiman et al., 2010; Tian et al., 2020). The qualitative and quantitative composition of the essential oils may be influenced by several factors, such as soil composition, environment, temperature, and adaptive process to some particular and local ecological conditions (Arruda et al., 2012; Fejer et al. 2018).

Table 1. Chemical composition of *Z. zerumbet* rhizome oil

No.	Component	Molecular formula	Rt (min)	Percentage
Monoterpene hydrocarbons				
1	α-Thujene	$C_{10}H_{16}$	7.00	0.7
2	α-Pinene	$C_{10}H_{16}$	7.30	2.1
3	Camphene	$C_{10}H_{16}$	8.03	0.1
4	Sabinene	$C_{10}H_{16}$	9.56	7.9
5	β-Pinene	$C_{10}H_{16}$	9.72	6.3
6	Myrcene	$C_{10}H_{16}$	10.79	0.7
7	α-Phellandrene	$C_{10}H_{16}$	11.59	0.4
8	α-Terpinene	$C_{10}H_{16}$	12.44	3.6
9	*ρ*-Cymene	$C_{10}H_{14}$	13.01	0.4
10	(*E*)-β-Ocimene	$C_{10}H_{16}$	14.71	0.7
11	γ-Terpinene	$C_{10}H_{16}$	15.33	6.2
12	α-Terpinolene	$C_{10}H_{16}$	17.15	1.3
Oxygenated monoterpenes				
14	1,8-Cineole	$C_{10}H_{18}O$	13.35	2.4
15	*cis*-Sabinene hydrate	$C_{10}H_{18}O$	15.98	0.1
16	Linalool	$C_{10}H_{18}O$	18.23	0.1
17	*cis*-Menth-2-en-1-ol	$C_{10}H_{18}O$	19.38	0.5
18	*trans*-Menth-2-en-1-ol	$C_{10}H_{18}O$	20.53	0.3
19	Terpinen-4-ol	$C_{10}H_{18}O$	22.82	16.5
20	α-Terpineol	$C_{10}H_{18}O$	23.57	1.0
21	*cis*-Piperitol	$C_{10}H_{18}O$	24.52	0.1
22	Bornyl acetate	$C_{12}H_{20}O_2$	28.29	0.2
Sesquiterpene hydrocarbons				
22	β-Elemene	$C_{15}H_{24}$	33.57	0.1
23	β-Caryophyllene	$C_{15}H_{24}$	34.77	0.8
24	α-Humulene	$C_{15}H_{24}$	36.39	11.1
Oxygenated sesquiterpenes				
25	Elemol	$C_{15}H_{26}O$	40.71	0.3
26	Caryophyllene oxide	$C_{15}H_{24}O$	41.96	0.5
27	Humulene epoxide II	$C_{15}H_{24}O$	43.06	0.7
28	γ-Eudesmol	$C_{15}H_{26}O$	44.05	0.3
29	β-Eudesmol	$C_{15}H_{26}O$	44.82	0.3
30	α-Eudesmol	$C_{15}H_{26}O$	44.93	0.4
31	Zerumbone	$C_{15}H_{22}O$	48.16	28.4
	Total			**94.5**

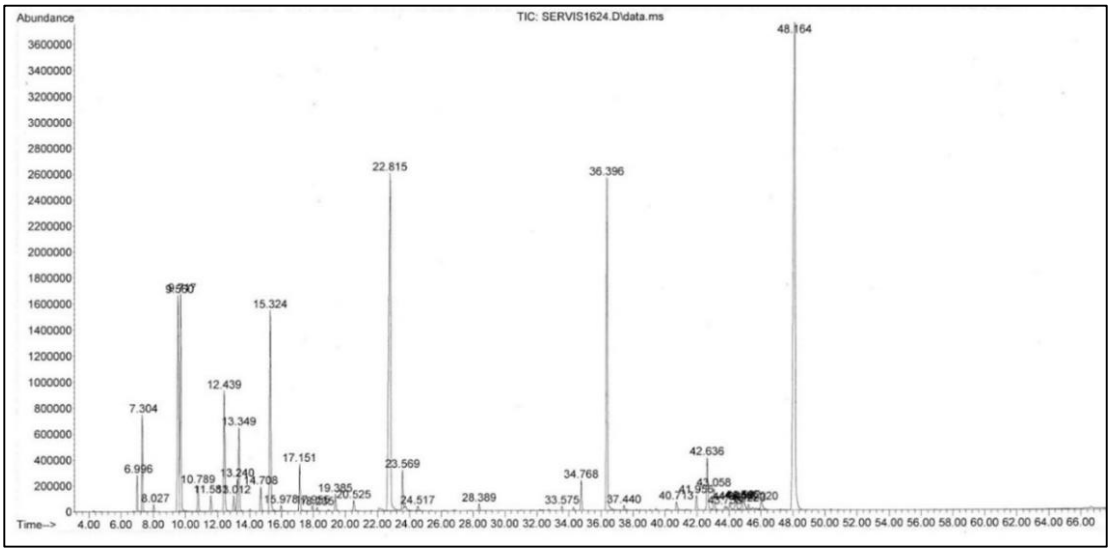

Figure 1. GC chromatogram of *Z. zerumbet* rhizomes oil

3.2. Extraction and screening of phytochemicals

Extraction is an important step to recover bioactive compounds. Preparation of crude extracts from plant materials is an initial step for the purification and identification of chemical constituents in a plant (Koparde and Magdum, 2017). Sequential maceration of the dried rhizomes of *Z. zerumbet* using *n*-hexane, ethyl acetate, and methanol produced extracts in the range of 0.83 to 3.69% (Table 2) with the methanol extract (3.69%) resulted in the highest extraction yield, followed by *n*-hexane extract (1.43%) and ethyl acetate extract (0.83%).

These results indicated that the highly polar solvent was the best selection to obtain the maximum extraction yield (Abarca-vargas et al., 2016). On top of that, the present findings also showed that most phytochemicals present in the rhizomes of *Z. zerumbet* were polar compounds (Manonmani and Mehalingam, 2018). The qualitative screening of the phytochemicals in the *n*-hexane, ethyl acetate, and methanol extracts of the rhizomes of *Z. zerumbet* revealed the presence of steroids and terpenes in all extracts (Table 2). Concurrently, flavonoids, phenols, alkaloids, tannins, and coumarins were present in both ethyl acetate and methanol extracts, while quinones existed in the *n*-hexane and ethyl acetate extracts. Remarkably, glycosides were only detected in the methanol extract, while saponins and anthraquinones were absent in all extracts. Based on the phytochemical screening results, it can be concluded that the ethyl acetate and methanol extracts of *Z. zerumbet* are good sources of different classes of phytochemicals. In comparison, the rhizome extracts of *Z. zerumbet* from India also gave identical results, differing only in the absence of saponins as in this study (Manonmani and Mehalingam, 2018; Prakash et al., 2011).

Table 2. Yield and phytochemical screening of *Z. zerumbet* rhizome extracts

Item	***n*-Hexane**	**Ethyl Acetate**	**MeOH**	**Item**	***n*-Hexane**	**Ethyl Acetate**	**MeOH**
Yield (%)	1.43	0.83	3.69	Tannins	-	+	+
Phytochemical				Saponins	-	-	-
Flavonoids	-	+	+	Glycosides	-	-	+
Phenols	-	+	+	Coumarins	-	+	+
Alkaloids	-	+	+	Quinones	+	+	-
Steroids	+	+	+	Anthra-quinones	-	-	-
Terpenes	+	+	+				

+: present; -: absent

3.3. Antibacterial activity

The essential oil and extracts of the rhizomes of *Z. zerumbet* were assessed for their antibacterial activity against four different bacteria (i.e., *E. coli*, *S. typhi*, *S. aureus* and *B. cereus*) using the disc diffusion method. The results of the activity are presented in Table 3. The rhizome oil of *Z. zerumbet* inhibited the growth of all tested bacteria, with the inhibition zones ranging between 13.33 to 20.66 mm. The highest inhibition zone was against *S. typhi* (20.66 mm), while the lowest inhibition zone was observed for *B. cereus* and *S. aureus* (13.33 mm each). Interestingly, the inhibition zone of *S. typhi* was larger than the inhibition zone generated by the positive control, streptomycin (15.33 mm) when tested with the same bacteria. These results were in line with the previous study by Yusmaniar et al., (2015) that reported similar findings on antibacterial activity against *S. typhi*.

Table 3. Antibacterial activity of the essential oil and extracts of *Z. zerumbet*

Sample	Inhibition zone diameter (mm)[b]			
	B. cereus	***S. aureus***	***E. coli***	***S. typhi***
Essential oil	13.33±0.58	13.33±0.58	16.66±0.58	20.66±1.15
n-Hexane extract	10.66±0.58	6.33±0.58	9.00±0.00	-
Ethyl acetate extract	9.66±0.58	8.66±0.58	10.66±0.58	9.66±0.58
Methanol extract	14.00±1.00	9.00±1.00	10.33±0.58	10.66±1.15
Streptomycin[c]	20.33±0.58	19.33±0.58	20.00±0.00	15.33±0.58

[a]Data represent mean ± standard deviation of three replicate experiments; [b]Inhibition zone diameter (mm) including diameter of disc 6 mm; [c]Positive control; - = inactive

At a concentration of 200 mg/mL, the zones of inhibition produced by the *n*-hexane, ethyl acetate, and methanol extracts were in the range of 6.33–10.66 mm, 8.66–10.66 mm and 9.00–14.00 mm, respectively, against all bacteria, except for *S. typhi*. The growth of *S. typhi* was uninhibited by the *n*-hexane extract. Amongst the extracts, the methanol extract possessed the highest antibacterial activity against all tested bacteria except for *E. coli*, which was more sensitive against the ethyl acetate extract (10.66 mm). The methanol extract gave the highest inhibition zones against *B. cereus*, *S. aureus*, and *S. typhi* with inhibition zones of 14.00, 9.00, and 10.33 mm, respectively. The antibacterial activity of the active extracts might be due to the presence of flavonoids, phenols, alkaloids, terpenes, tannins, and coumarins that are known to have antibacterial properties (Anulika et al., 2016; Ghasemzadeh et al., 2016; Kabera et al., 2014; Kader et al., 2011; Tiwari et al., 2011).

4. CONCLUSION

The essential oil of rhizomes of *Z. zerumbet* is comprised of various volatile components with oxygenated sesquiterpenes (30.9%) and monoterpene hydrocarbons (30.4%) being the major contributors. Meanwhile, the results of the phytochemical screening of the rhizome extracts revealed that the extracts contained varieties of phytochemicals that offer remarkable medicinal importance. Regarding the antibacterial activity, it was found that the rhizome essential oil of *Z. zerumbet* and its extracts exhibited antibacterial effect against most of the tested bacteria. Thus, it can be concluded that *Z. zerumbet* can be a valuable source of bioactive compounds for the treatment of bacterial infections. However, further studies on the mode of action are necessary to understand the mechanism responsible for the antibacterial action.

Declaration of Interest
The authors declare that there is no conflict of interest.

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