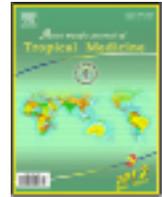




Contents lists available at ScienceDirect

## Asian Pacific Journal of Tropical Medicine

journal homepage: www.elsevier.com/locate/apjtm



Document heading doi:

Antimicrobial activity and essential oils of *Curcuma aeruginosa*, *Curcuma mangga*, and *Zingiber cassumunar* from MalaysiaTg Siti Amirah Tg Kamazeri<sup>1</sup>, Othman Abd Samah<sup>1\*</sup>, Muhammad Taher<sup>2</sup>, Deny Susanti<sup>1</sup>, Haitham Qaralleh<sup>1</sup><sup>1</sup>Department of Biomedical Science, Kulliyah of Science, International Islamic University Malaysia, Jalan Istana, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia<sup>2</sup>Kulliyah of Pharmacy, International Islamic University Malaysia, Jalan Istana, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

## ARTICLE INFO

## Article history:

Received 27 September 2011

Received in revised form 15 December 2011

Accepted 26 December 2012

Available online 20 March 2012

## Keywords:

Antimicrobial

Gas chromatography–mass spectrometry

Essential oil

*Curcuma aeruginosa**Curcuma mangga**Zingiber cassumunar*

## ABSTRACT

**Objective:** To analyze the chemical composition of the essential oils of *Curcuma aeruginosa* (*C. aeruginosa*), *Curcuma mangga* (*C. mangga*), and *Zingiber cassumunar* (*Z. cassumunar*), and study their antimicrobial activity. **Methods:** Essential oils obtained by steam distillation were analyzed by gas chromatography–mass spectrometry (GC–MS). The antimicrobial activity of the essential oils was evaluated against four bacteria: *Bacillus cereus* (*B. cereus*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*); and two fungi: *Candida albicans* (*C. albicans*) and *Cytopoccus neoformans* (*C. neoformans*), using disc–diffusion and broth microdilution methods. **Results:** Cycloisolongifolene, 8,9–dehydro–9–formyl (35.29%) and dihydrocostunolide (22.51%) were the major compounds in *C. aeruginosa* oil; whereas caryophyllene oxide (18.71%) and caryophyllene (12.69%) were the major compounds in *C. mangga* oil; and 2,6,9,9–tetramethyl–2,6,10–cycloundecatrien–1–one (60.77%) and  $\alpha$ –caryophyllene (23.92%) were abundant in *Z. cassumunar* oil. The essential oils displayed varying degrees of antimicrobial activity against all tested microorganisms. *C. mangga* oil had the highest and most broad–spectrum activity by inhibiting all microorganisms tested, with *C. neoformans* being the most sensitive microorganism by having the lowest minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of 0.1  $\mu$  L/mL. *C. aeruginosa* oil showed mild antimicrobial activity, whereas *Z. cassumunar* had very low or weak activity against the tested microorganisms. **Conclusions:** The preliminary results suggest promising antimicrobial properties of *C. mangga* and *C. aeruginosa*, which may be useful for food preservation, pharmaceutical treatment and natural therapies.

## 1. Introduction

Plant oils and extracts have been used for a wide variety of purposes for many thousands of years<sup>[1]</sup>. Recently, the essential oils and various extracts of plants have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of foods from the toxic effects of oxidants. Particularly, the antimicrobial activities of plant oils and

extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies<sup>[2, 3]</sup>.

The revival of interest in herbal medications is due to the perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals<sup>[4]</sup>. There is also an urgent need to search for new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new infectious diseases, as well as the development of resistance to the antibiotics in current clinical use<sup>[5]</sup>. Anwar *et al.* explained that the spread of drug–resistant pathogens is one of the biggest threats to successful treatment of microbial diseases<sup>[6]</sup>. In addition, the consumption of food contaminated with food–borne microorganisms can

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pose a serious threat to human health. The existence of microorganisms causes spoilage and results in reduction of the quality and quantity of processed foods. Not only does this cause major loss to the food industry, it has also increased demands from consumers for fresh and natural foods that are safe for consumption.

Essential oils and/or their components are becoming increasingly popular as natural antimicrobial agents to be used for a wide variety of purposes. At present, essential oils are used by the flavoring industry for flavor enhancement and for their antioxidant effect. However, the potential use of these oils as natural antimicrobial agents has been less explored[7]. While some of the oils used on the basis of their reputed antimicrobial properties have well-documented in vitro activity, there is few published data for many others. Some studies have concentrated exclusively on single purified oil or one microorganism. Even though these data are useful, the reports are not directly comparable due to methodological differences such as choice of plant extract(s), test micro-organism(s) and antimicrobial test method[8].

In Peninsular Malaysia, 1 200 species of higher plants and 2 000 species in Sabah and Sarawak are reported to have medicinal value and have been used for generations in various traditional health care systems[9]. According to Chen *et al.*, many constituents of zingiberaceous plants have been reported for their biological activities in antifungal, antioxidant, insecticidal, and anti-inflammatory activities[10]. The more common species from this family such as common ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) have been extensively studied and reported for various activities, but studies on many other species are still lacking. Even though there are a number of reported studies on the antimicrobial activity of various *Zingiberaceae* species in Malaysia, studies on the essential oils of different species from a specific area have not been extensively conducted. Hence, this study was conducted to screen the antimicrobial activity of the essential oils of three *Zingiberaceae* species: *C. aeruginosa*, *C. mangga* and *Z. cassumunar*. They are locally available in Pahang, east of Peninsular Malaysia. The chemical compositions of the essential oils were also analyzed by gas chromatography-mass spectrometry (GC-MS).

## 2. Materials and methods

### 2.1. Plant materials

Rhizomes of *C. aeruginosa*, *C. mangga* and *Z. cassumunar* were purchased from a local wet market in Kuantan, Pahang in October 2010. All plants were grown in Kampung Melayu, Jabor, Kuantan. The rhizomes were harvested on the day of purchase and immediately prepared for steam distillation. All species were identified by botanist Dr. Shamsul Khamis (Universiti Putra Malaysia, Malaysia), as well as by comparison with data from literature.

### 2.2. Essential oil extraction

Fresh rhizomes in good condition were thoroughly washed and dried at room temperature. Rhizomes were chopped into small cubes and weighed. A sample of 966 g, 1 806 g, and 1 687 g of *C. mangga*, *C. aeruginosa*, and *Z. cassumunar* rhizomes respectively, were submitted to steam distillation

for a minimum of 6 hours using a Clevenger-type apparatus at atmospheric pressure (101.325 kPa). Distillate (aqueous phase) was extracted with dichloromethane (DCM). The organic phase was dried over anhydrous sodium sulphate, filtered, and the solvent was separated from the oil using the rotary evaporator. Obtained essential oils were stored at 4 °C until used.

### 2.3. GC-MS analysis

The analyses of the volatile compounds were carried out using the PerkinElmer AutoSystem XL gas chromatograph equipped with Elite-5 fused-silica capillary column (inner diameter 30 m × 0.32 m, 0.25 μm film thickness), which was directly coupled to the TurboMass Gold mass spectrometry of the same company (PerkinElmer Inc., Connecticut, USA). The equipments were conditioned and programmed according to the required parameters. The carrier gas was helium at a flow rate of 5 mL/minute. The temperature was set at 50 °C at initial with increment of 4 °C/min until the final temperature of 250 °C was reached. 5 μL of the essential oil were dissolved in 495 μL of solvent (1:100 v/v), and injected into the column automatically. DCM was the solvent used to dissolve the oils of *C. mangga* and *C. aeruginosa*, and absolute ethanol was used to dissolve *Z. cassumunar* oil. The essential oil components were identified by comparing their mass spectra with the National Institute of Standards and Technology (NIST) mass spectral database library, and confirmed by comparison with data published in the literature whenever possible. The composition was reported as relative percentage of the total peak area, using the following calculation:

Relative % of peak area = (Area of the peak/Total peak area) × 100

### 2.4. Antimicrobial screening

#### 2.4.1. Test microorganisms

A panel of six common pathogenic microorganisms was used in the study, which includes two gram-positive bacteria (*Bacillus cereus* ATCC11778 and *Staphylococcus aureus* ATCC25923), two gram-negative bacteria (*Escherichia coli* ATCC35218 and *Pseudomonas aeruginosa* ATCC27853), and two fungi (*Candida albicans* ATCC10231 and *Cryptococcus neoformans* ATCC90112).

#### 2.4.2. Disc-diffusion method

The disc-diffusion method was employed for the determination of antimicrobial activity of the essential oils, according to the methods suggested by the National Community for Clinical Laboratory Standards (NCCLS)[11]. Briefly, a suspension of the tested microorganisms (100 μL of 10<sup>6</sup>–10<sup>8</sup> CFU/mL) was uniformly swabbed on agar plates (Mueller Hinton Agar[MHA] for bacteria and Potato Dextrose Agar[PDA] for fungi) using sterile cotton swabs. Sterile blank discs (6 mm in diameter) were individually impregnated with 15 μL of each pure essential oil and placed onto the inoculated agar plates. The plates were inverted and incubated at 37 °C for 24 hours (for bacteria) or at 30 °C for 48 hours (for fungi). Antimicrobial activity was evaluated by measuring diameter of the resulting zone of inhibition (ZOI) against the tested microorganisms in millimeters. Tetracycline (30 μg/mL) and nystatin (100 μg/mL)

served as positive controls for bacterial and fungal strains respectively. 100% dimethyl sulfoxide (DMSO) (15  $\mu$  L per blank disc) was used as negative control. This assay was performed in duplicate.

### 2.5. Determination of minimum inhibitory concentration (MIC)

Essential oils that showed significant antimicrobial activity in the disc-diffusion method were chosen for determination of MIC using the broth microdilution method against the same microorganisms, as described by Skočibušić *et al.* with slight modifications<sup>[12]</sup>. Broth microdilutions were performed in Mueller Hinton Broth (MHB), with the exception of the fungal strains (Potato Dextrose Broth[PDB]).

The investigated oil was dissolved in DMSO and diluted to a concentration of 10%. Three-fold dilution of the oil was performed in a 96-well microplate over the range of 0.02  $\mu$  L/mL to 33.30  $\mu$  L/mL. This was achieved by firstly filling all wells to be used with 200  $\mu$  L of media (MHB or PDB). Then, 100  $\mu$  L of 10% essential oil was transferred to the first well and mixed well. Three-fold serial dilution was performed by transferring 100  $\mu$  L of the mixture in the first well into the next consecutive wells, until the end of the row. At the last well, 100  $\mu$  L of the mixture was discharged, so that the total volume of solution in each well was 200  $\mu$  L. Then, 10  $\mu$  L of bacterial or fungi culture was transferred into all wells of the column.

The microplate was incubated at 37 °C for 24 hours (for bacteria) or at 30 °C for 48 hours (for fungi), and microbial growth was determined using a universal microplate reader by interpreting the growth curve in each well. MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The test for all samples, positive control and negative control were performed in duplicate.

### 2.6. Determination of minimum bactericidal or fungicidal concentration (MBC/MFC)

To determine MBC/MFC, all wells that showed no visible growth were sub-cultured on either MHA (for bacteria) or PDA (for fungi) using sterile cotton swabs. The agar plates were then incubated at the appropriate temperature and time. The lowest concentration of oil or antibiotic on the plate that exhibits complete killing of the microorganism was considered as the MBC or MFC. MBC or MFC is defined as the lowest concentration of the essential oil or antibiotic at which inoculated bacteria or fungi were completely killed.

### 2.7. Statistical analysis

Results were reported in the form of mean, standard deviation and percentage value, which was calculated using the Microsoft Excel 2009 software.

## 3. Results

### 3.1. Essential oil yields

Steam distillation of the rhizomes produced different yields of oils from the three plants. All essential oils possess strong and characteristic aromatic fragrances. The rhizomes of

*Z. cassumunar* gave the highest yield of oil (0.30% w/w), followed by *C. aeruginosa* (0.19% w/w) and *C. mangga* (0.12% w/w).

### 3.2. Chemical composition of *C. aeruginosa* essential oil

GC-MS analysis of the *C. aeruginosa* oil has successfully resulted in the identification of 16 compounds in the oil (Table 1). Sesquiterpenes dominated the chemical composition of the oil, as it made up 94.08% of the oil, while the rest of its chemical profile was made up of oxygenated monoterpenes (5.92%). The major compounds were cycloisolongifolene, 8,9-dehydro-9-formyl (35.29%) and dihydrocostunolide (22.51%), which were present in much higher percentages. Other important compounds present in the oil include the sesquiterpenes velleral (10.00%), germacrone (6.50%),  $\beta$ -elemene (4.76%), alloaromadendrene oxide-(2) (4.07%), aromadendrene oxide-(2) (2.40%),  $\alpha$ -bulnesene (2.14%), and eudesma-4(14),11-diene (1.13%). Oxygenated monoterpenes that were present in the oil include eucalyptol (3.98%), L-camphor (1.32%) and isoborneol (0.62%). Trace amounts of caryophyllene,  $\beta$ -cubebene, and xanthinin were also detected.

### 3.3. Chemical composition of *C. mangga* essential oil

About 27 compounds were identified to be present in the *C. mangga* essential oil (Table 2). The oil was in abundance of sesquiterpenes (52.67%) and monoterpenes (31.44%), whereas some diterpenes (15.89%) were also present in small quantities.

The major compounds of the oil were the sesquiterpenes caryophyllene oxide (18.71%) and caryophyllene (12.69%), while 2,6,11,15-tetramethyl-hexadeca-2,6,8,10,14-pentaene (8.06%) was the main diterpene. Other notable constituents were the monoterpene cyclohexane,2-ethenyl-1,dimethyl-3-methylene (7.06%), the oxygenated sesquiterpenes 6-(1-hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one (5.48%) and cis-farnesol (5.14%), the diterpene 1,6,10,14-hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E, E) (4.86%), and monoterpene cyclohexanol, 2-methylene-5-(1-methylethenyl) (4.72%). Small percentages of  $\alpha$ -caryophyllene (2.86%),  $\alpha$ -farnesene (1.84%), longipinane, (E) (1.57%), sabinene (1.08%), and germacrone (0.57%) were also present.

### 3.4. Chemical composition of *Z. cassumunar* essential oil

GC-MS analysis of the *Z. cassumunar* essential oil has successfully identified the presence of 15 compounds (Table 3), which consisted mainly of sesquiterpenes (98.78%) and small amounts of monoterpenes (1.22%). The sesquiterpenes dominating the oil were 2,6,9,9-tetramethyl-2,6,10-cycloundecatrien-1-one (60.77%) and  $\alpha$ -caryophyllene (23.92%). Caryophyllene oxide made up 6.44% of the oil, while the rest of the components were only present in small individual abundances or trace amounts.

### 3.5. Antimicrobial activity

The antimicrobial activity of the essential oils against the microorganisms employed was qualitatively and quantitatively assessed by the presence or absence of

inhibition zones, diameter of zone of inhibition, MIC and MBC/MFC values.

### 3.5.1. Disc-diffusion assay

In general, the three *Zingiberaceae* essential oils displayed varying degrees of antimicrobial activity against the tested microorganisms. Results of the disc-diffusion assay (Table

4) indicated that *C. mangga* essential oil had the strongest activity, whilst *C. aeruginosa* had moderate activity, and *Z. cassumunar* showed no activity against most of the microorganisms tested. *S. aureus* was the most sensitive bacteria since it was inhibited by all essential oils, and *P. aeruginosa* was the most resistant bacteria since the oils showed either weak or no inhibition towards it.

**Table 1**

Essential oil composition of *C. aeruginosa*.

Peak	RT	Compound Name	Formula	%
1	11.57	Eucalyptol	C <sub>10</sub> H <sub>18</sub> O	3.98
2	15.72	L-camphor	C <sub>10</sub> H <sub>16</sub> O	1.32
3	16.31	Isoborneol	C <sub>10</sub> H <sub>18</sub> O	0.62
4	24.00	β-Elemene	C <sub>15</sub> H <sub>24</sub>	4.76
5	24.90	Caryophyllene	C <sub>15</sub> H <sub>24</sub>	1.01
6	26.07	β-Farnesene	C <sub>15</sub> H <sub>24</sub>	2.65
7	26.82	β-Cubebene	C <sub>15</sub> H <sub>24</sub>	0.92
8	27.05	Eudesma-4(14),11-diene	C <sub>15</sub> H <sub>24</sub>	1.13
9	27.26	α-Bulnesene	C <sub>15</sub> H <sub>24</sub>	2.14
10	30.46	Cycloisolongifolene, 8,9-dehydro-9-formyl-	C <sub>16</sub> H <sub>22</sub> O	35.29
11	32.96	Germacrone	C <sub>15</sub> H <sub>22</sub> O	6.50
12	33.74	Alloaromadendrene oxide-(2)	C <sub>15</sub> H <sub>24</sub> O	4.07
13	34.24	Dihydrocostunolide	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	4.28
14	34.40	Dihydrocostunolide	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	6.89
15	35.23	Dihydrocostunolide	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	11.34
16	35.52	Velleral	C <sub>15</sub> H <sub>20</sub> O <sub>2</sub>	10.00
17	36.77	Aromadendrene oxide-(2)	C <sub>15</sub> H <sub>24</sub> O	2.40
18	40.51	Xanthinin	C <sub>17</sub> H <sub>22</sub> O <sub>5</sub>	0.69

RT = retention time

**Table 2**

Essential oil composition of *C. mangga*.

Peak	RT	Compound Name	Formula	%
1	10.09	Sabinene	C <sub>10</sub> H <sub>16</sub>	1.08
2	10.57	trans-Chrysanthenyl Acetate	C <sub>12</sub> H <sub>18</sub> O <sub>2</sub>	0.62
3	13.11	1,3,8-p-Menthatriene	C <sub>10</sub> H <sub>14</sub>	0.28
4	16.19	α-Terpeneol acetate	C <sub>12</sub> H <sub>20</sub> O <sub>2</sub>	3.45
5	19.69	(2,4,6-Trimethylcyclohexyl) methanol	C <sub>10</sub> H <sub>20</sub> O	0.92
6	19.98	endo-1,5,6,7-Tetramethylbicyclo[3.2.0]hept-6-en-3-ol	C <sub>11</sub> H <sub>18</sub> O	3.59
7	20.09	Longipinane, (E)-	C <sub>15</sub> H <sub>26</sub>	1.57
8	22.39	5-Isopropenyl-1,2-dimethylcyclohex-2-enol	C <sub>11</sub> H <sub>18</sub> O	0.69
9	24.66	Caryophyllene	C <sub>15</sub> H <sub>24</sub>	12.69
10	25.24	Cyclohexane,2-ethenyl-1,dimethyl-3-methylene	C <sub>11</sub> H <sub>18</sub>	7.06
11	25.83	α-Caryophyllene / Humulene	C <sub>15</sub> H <sub>24</sub>	2.86
12	27.13	Cyclohexanol, 2-methylene-5-(1-methylethenyl)-	C <sub>10</sub> H <sub>16</sub> O	4.72
13	27.68	2-Isopropylidene-3-methylhexa-3,5-dienal	C <sub>10</sub> H <sub>14</sub> O	3.71
14	28.51	3-Methyl-2-methylene-3-butenyl 2-methylacrylate	C <sub>10</sub> H <sub>14</sub> O <sub>2</sub>	0.94
15	29.78	Caryophyllene oxide	C <sub>15</sub> H <sub>24</sub> O	18.71
16	31.18	But-3-enal, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexenyl)-	C <sub>14</sub> H <sub>22</sub> O	2.67
17	31.40	2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol	C <sub>14</sub> H <sub>24</sub> O	1.71
18	32.37	Alloaromadendrene oxide-(1)	C <sub>15</sub> H <sub>24</sub> O	1.79
19	32.93	Germacrone	C <sub>15</sub> H <sub>22</sub> O	0.57
20	35.78	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	5.48
21	38.42	Pentadecanoic acid, 14-methyl-, methyl ester	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	1.12
22	39.63	2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene	C <sub>20</sub> H <sub>32</sub>	8.06
23	40.49	1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-	C <sub>20</sub> H <sub>34</sub> O	4.86
24	41.35	α-Farnesene	C <sub>15</sub> H <sub>24</sub>	1.84
25	42.24	(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene	C <sub>20</sub> H <sub>32</sub>	2.97
26	42.96	cis-Farnesol	C <sub>15</sub> H <sub>26</sub> O	5.14
27	48.55	1-Formyl-2,2-dimethyl-3-trans-(3-methyl-but-2-enyl)-6-methylidene-cyclohexane	C <sub>15</sub> H <sub>24</sub> O	0.90

*C. mangga* essential oil showed a broad-spectrum activity as it was the only oil that successfully inhibited the growth of all tested microorganisms. For this oil, the gram-positive bacteria were more sensitive towards it compared to the gram-negative bacteria, as bigger ZOI was produced. The two fungi *C. albicans* and *C. neoformans* were also highly susceptible towards the *C. mangga oil*, with ZOI  $\geq 10$  mm (strongly inhibitory). Overall, *C. albicans* was the most sensitive microorganism as the largest ZOI ( $14.8 \pm 0.4$  mm) was produced, and this was followed by *S. aureus* ( $13.5 \pm 0.7$  mm). Meanwhile, *P. aeruginosa* was only slightly susceptible towards the *C. mangga oil* as the ZOI was the smallest ( $7.0 \pm 0.0$  mm).

### 3.5.2. MIC and MBC/MFC of *C. mangga*

Essential oils that showed significant ZOI ( $\geq 10$  mm) against tested microorganisms in the disc-diffusion assay

were chosen for further determination of the MIC and MBC/MFC, using the broth microdilution assay. Since only the *C. mangga* essential oil showed significant ZOI of  $\geq 10$  mm, it is the only oil that was further investigated. The microorganisms that showed high sensitivity towards the *C. mangga oil* were the gram-positive bacteria (*B. cereus* and *S. aureus*), and the fungi (*C. albicans* and *C. neoformans*).

MIC and MBC/MFC results (Table 5) indicate that the *C. mangga* oil had different levels of activity against the microorganisms. The inhibitory properties of the oil were observed within a range of concentrations from 0.02 to 33.30  $\mu$  L/mL. In liquid medium the essential oil was active against all the gram-positive bacteria and fungi. It is noted that the MIC and MBC/MFC value of the *C. mangga oil* was the same, which suggests that the oil is both inhibitory and bactericidal or fungicidal at a single concentration.

**Table 3**

Essential oil composition of *Z. cassumunar*.

Peak	RT	Compound Name	Formula	%
1	9.33	$\beta$ -Myrcene	C <sub>10</sub> H <sub>16</sub>	0.11
2	9.85	1-Isopropyl-4-methylbicyclo[3.1.0]hex-2-ene	C <sub>10</sub> H <sub>16</sub>	0.12
3	9.91	3-Carene	C <sub>10</sub> H <sub>16</sub>	0.05
4	10.48	o-Cymene	C <sub>10</sub> H <sub>14</sub>	0.30
5	10.63	D-Limonene	C <sub>10</sub> H <sub>16</sub>	0.06
6	10.73	Eucalyptol	C <sub>10</sub> H <sub>18</sub> O	0.04
7	11.66	$\gamma$ -Terpinene	C <sub>10</sub> H <sub>16</sub>	0.02
8	23.98	Caryophyllene	C <sub>15</sub> H <sub>24</sub>	2.03
9	25.29	$\alpha$ -Caryophyllene	C <sub>15</sub> H <sub>24</sub>	23.92
10	27.63	1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)	C <sub>14</sub> H <sub>22</sub> O	0.52
11	29.00	Caryophyllene oxide	C <sub>15</sub> H <sub>24</sub> O	1.34
12	29.57	Caryophyllene oxide	C <sub>15</sub> H <sub>24</sub> O	4.79
13	29.88	1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	C <sub>15</sub> H <sub>24</sub> O	3.32
14	30.46	Caryophyllene oxide	C <sub>15</sub> H <sub>24</sub> O	0.33
15	30.74	4,6,6-Trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)]octane	C <sub>15</sub> H <sub>22</sub> O	0.76
16	31.40	$\gamma$ -Gurjunenepoxide-(1)	C <sub>15</sub> H <sub>24</sub> O	1.54
17	33.95	2,6,9,9-Tetramethyl-2,6,10-cycloundecatrien-1-one	C <sub>15</sub> H <sub>22</sub> O	60.77

**Table 4**

Antimicrobial activity of the three Zingiberaceae plants essential oils by the disc-diffusion method.

Microorganisms		Zone of inhibition of (mm)			Positive control
		<i>C. aeruginosa</i>	<i>C. mangga</i>	<i>Z. cassumunar</i>	
Gram-positive	<i>S. aureus</i>	7.0 $\pm$ 0.0	10.0 $\pm$ 0.0	-	17.0 $\pm$ 0.0
	<i>B. cereus</i>	9.3 $\pm$ 0.4	13.5 $\pm$ 0.7	8.5 $\pm$ 0.0	22.5 $\pm$ 0.7
Gram-negative	<i>P. aeruginosa</i>	7.5 $\pm$ 0.0	9.0 $\pm$ 0.0	-	23.0 $\pm$ 0.0
	<i>E. coli</i>	-	7.0 $\pm$ 0.0	7.5 $\pm$ 0.0	13.3 $\pm$ 0.4
Fungi	<i>C. neoformans</i>	8.8 $\pm$ 0.4	14.8 $\pm$ 0.4	-	18.0 $\pm$ 0.0
	<i>C. albicans</i>	7.0 $\pm$ 0.0	10.3 $\pm$ 0.4	-	10.3 $\pm$ 0.4

Data expressed as mean of duplicate  $\pm$  standard deviation (SD) including the disc diameter (6 mm), All values were rounded to one decimal place, '-' indicates no inhibition, Positive control: tetracycline or nystatin.

**Table 5**

Antimicrobial activity of *C. mangga* essential oil against sensitive microorganisms by the broth microdilution method.

Microorganisms		<i>C. mangga</i> essential oil		Positive control	
		MIC ( $\mu$ L/mL)	MBC/MFC ( $\mu$ L/mL)	MIC (mg/mL)	MBC/MFC (mg/mL)
Gram-positive	<i>B. cereus</i>	11.1	11.1	0.002	0.12
	<i>S. aureus</i>	1.2	1.2	0.010	0.12
Fungi	<i>C. albicans</i>	3.7	3.7	0.120	0.37
	<i>C. neoformans</i>	0.1	0.1	0.370	0.37

Positive control: Tetracycline or nystatin

Maximum activity of the *C. mangga* essential oil was observed against the fungi *C. neoformans*, which had the lowest MIC and MFC value of 0.1  $\mu$  L/mL. This is followed by *S. aureus* (1.2  $\mu$  L/mL) and *C. albicans* (3.7  $\mu$  L/mL). *B. cereus* needed the highest MIC and MBC value, which was 11.1  $\mu$  L/mL. This suggests that it was the least susceptible microorganism in this assay, as it needed a higher oil concentration before it can be inhibited and completely killed.

#### 4. Discussions

The findings on the major components of this *C. aeruginosa* oil were different from previous reports in the literature. Zwaving & Bos reported that the essential oils of *C. aeruginosa* rhizomes from Indonesia and India contain high percentages of curcumanolides A, B (11.4%), curcumenol (9.9%), dehydrocurdione (9.4%), and isocurcumenol (8.5%)<sup>[13]</sup>. Jarikasem *et al.* found that the rhizomes from Thailand contain curzerenone (41.63%) and eucalyptol (9.64%) as the major compound, but eucalyptol only represented a small percentage of the rhizome in this study while curzerenone was not detected<sup>[14]</sup>.

The major chemical constituents of *C. mangga* essential oil in this study were different from that reported by Wong *et al.*, whose essential oil from a different area in Malaysia consisted mainly of monoterpenes instead of sesquiterpenes<sup>[15]</sup>. Myrcene (78.68%) was the majoritary compound in their sample, with small traces of (E)- $\beta$ -ocimene (5.1%),  $\beta$ -pinene (3.7%), and pinene (2.9%).

Overall, the majoritary compounds of the *Z. cassumunar* in this study were also different from those reported in literature data. *Z. cassumunar* from the northern and eastern parts of Thailand contained sabinene, terpinen-4-ol, and (E)-1(3, 4-dimethylphenyl) butadiene (DMPBD) as the main component of the rhizome essential oil<sup>[16]</sup>. The presence of  $\beta$ -myrcene and  $\gamma$ -terpinene was also reported, which were also identified in the essential oil in this study but in much lower or trace amounts. In Bangladesh, Md. Nazrul *et al.* reported the rhizome essential oil to contain triquinacene 1,4-bis (methoxy) (26.47%), (Z)-ocimene (21.97%) and terpinen-4-ol (18.45%) as the major compounds, none of which were identified to be present in this study<sup>[17]</sup>.

Caryophyllene (C<sub>15</sub>H<sub>24</sub>), which is a bicyclic sesquiterpene, was found to be the only common compound present in all three species investigated in this study. It was present in small amounts in all three essential oils, with the highest percentage in *C. mangga* oil (12.69%), followed by *Z. cassumunar* (2.03%) and *C. aeruginosa* (1.01%). Caryophyllene is a constituent of many other essential oils, including cloves, hemp, and rosemary.

All essential oils in this study were found to have different chemical compositions compared to literature data of the same oils from other geographical locations. Variations in the chemical composition of distilled essential oils is known to differ considerably not only due to the existence of different subspecies, but might also be attributed to other

factors such as: varied agro climatic condition (climatic, seasonal, geographic) of the regions, stage of maturity, adaptive metabolism of plants, distillation conditions, the plant part analyzed and some other factors<sup>[6]</sup>.

Philip *et al.* reported that the methanol and ethyl acetate extract of *C. mangga* rhizome showed remarked sensitivity towards *P. aeruginosa* (ZOI of 13.0 mm) at concentration 500 mg/mL<sup>[18]</sup>. However, none of the *C. mangga* extracts in their study were capable of inhibiting the growth of *E. coli*, which were mildly inhibited by the essential oil in this study. The gram-positive bacteria in their study were generally reported to be more susceptible towards *C. mangga* extracts compared to the gram-negative bacteria, and this is in agreement with the findings in the present study.

Meanwhile, *C. aeruginosa* essential oil were found to have moderate activity, as it showed moderate inhibition towards the microorganisms, but failed to inhibit the growth of *P. aeruginosa*. *S. aureus* was the most sensitive microorganism (9.3  $\pm$  0.4 mm), whilst *P. aeruginosa* was resistant towards the oil. *C. aeruginosa* oil had moderate activity against the fungi *C. albicans* (8.8  $\pm$  0.4 mm), and inhibition against *C. neoformans* was weak (7.0  $\pm$  0.0 mm).

On the other hand, *Z. cassumunar* essential oil only displayed weak or no antimicrobial activity. It failed to inhibit the growth of the gram-positive *B. cereus*, Gram-negative *E. coli*, as well as the two fungi, *C. albicans* and *C. neoformans*. It has been reported that the DCM and methanol extracts of *Z. cassumunar* rhizome and root were also inactive against *E. coli* and *C. albicans*<sup>[19]</sup>. However, the *Z. cassumunar* oil in the present study was slightly inhibitory towards *P. aeruginosa* (7.5  $\pm$  0.0 mm), which was resistant or slightly resistant to the other essential oils. This oil also displayed moderate activity against the gram-positive *S. aureus* (8.5  $\pm$  0.0 mm).

Based on the screening of antimicrobial activity using the disc-diffusion method, it was found that *P. aeruginosa* was the most resistant bacteria towards the essential oils studied. Skočibušić *et al.* explained that the gram-negative *P. aeruginosa* is known to have high level of intrinsic resistance to virtually all known antimicrobials and antibiotics<sup>[12]</sup>. This is due to a combination of a very restrictive outer membrane barrier, which is highly resistant even to synthetic drugs. Overall, gram-positive bacteria were also more susceptible to the *Zingiberaceae* essential oils tested in this study when compared to gram-negative bacteria. Most studies agree that essential oils are generally slightly more active against gram-positive than gram-negative bacteria. It is expected that gram-negative organisms are less susceptible to the action of antibacterials since they possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering<sup>[12]</sup>.

The positive controls used in this assay were tetracycline and nystatin for the bacteria and fungi respectively. All bacteria were susceptible to the inhibitory action of tetracycline, with ZOI ranging from 13.3 - 23.0 mm. Both fungi were also susceptible to nystatin, with ZOI ranging

from 10.3 – 18.0 mm. The presence of ZOI in positive controls indicated that all the microorganisms used in the study were sensitive towards antimicrobial agents.

In comparison with the standard antibiotics tetracycline and nystatin, the essential oils showed promising antimicrobial activity, especially the oils of *C. mangga* and *C. aeruginosa*. According to Matasyoh *et al.*, even though the concentrations of the essential oils used might be different than those of the standard antibiotics, they showed marked antimicrobial activity, as evidenced by their ZOI[20–26]. This can be explained in terms of the fact that the active components of the oil comprise only a fraction of the oil used. Therefore, the concentration of the active components could be much lower than those in the standard antibiotic. It is important to note that, if the active components were isolated and purified, they would probably show higher antimicrobial activity than those observed in this study.

It should also be taken into account that the comparatively weak inhibition found in this study might be influenced by the fact that inhibition area depends on the ability of the antimicrobial compound to diffuse uniformly through the agar. Different inhibition results might be obtained if alternative methods were applied.

The susceptibility of the microorganisms in this assay was different to that observed in the disc–diffusion assay. In the disc–diffusion assay, the susceptibility of the microorganism was *C. albicans* > *S. aureus* > *C. neoformans* > *B. cereus*, with *C. albicans* being the most susceptible microorganism. However, in the broth microdilution assay, the susceptibility was as follows: *C. neoformans* > *S. aureus* > *C. albicans* > *B. cereus*, with *C. neoformans* being the most susceptible. These differences occurred due to the variation in diffusibility of the essential oil compounds in the disc–diffusion assay. Rios *et al.* also observed similar inconsistencies between disc–diffusion assay and MIC determination, and concluded that the disc–diffusion method is not reliable enough to be used as a definitive method for determining the strength of antimicrobial activity[27]. The determination of MIC and MBC/MFC is regarded as a more precise evaluation of antimicrobial property, since those determinations are more sensitive than the agar disk diffusion assay.

A wide variety of essential oils are known to possess antimicrobial properties and in many cases this activity is due to the presence of active constituents, mainly attributable to isoprenes such as monoterpenes, sesquiterpenes and related alcohols, other hydrocarbons and phenols[28]. In this study, the essential oils of all three Zingiberaceae species were found to be abundant in sesquiterpenes and monoterpenes. Sesquiterpenes are the major compounds in all three oils, with cycloisolongifolene, 8,9–dehydro–9–formyl and dihydrocostunolide as the major compounds in *C. aeruginosa* oil; caryophyllene oxide and caryophyllene in *C. mangga* oil; as well as 2,6,9,9–tetramethyl–2,6,10–cycloundecatrien–1–one and  $\alpha$ –caryophyllene in *Z. cassumunar* oil. It can be deduced that there is a relationship between the chemical components of the essential oils and the antimicrobial

activity.

Berger mentioned that the lipophilic character of their hydrocarbon skeleton and hydrophilic character of their functional groups are of main importance in the antimicrobial activity of essential oil components[28]. Essential oils with high levels of sesquiterpenes have been reported to exhibit antifungal and antibacterial activity. In addition, caryophyllene oxide has been reported to display slight antibacterial activity and was inhibitory to the growth of several pathogenic fungi. Caryophyllene oxide was the major compound of the *C. mangga* essential oil in this study, and the high antimicrobial activity exhibited by the oil may be contributed to its presence. Meanwhile, caryophyllene was detected in all essential oils in this study but in varying quantities.

Even though major components are usually responsible for the antimicrobial activity of many essential oils, some studies have found that whole essential oils have higher antimicrobial activity compared to the combination of the major isolated components. This indicates that minor components are also critical to the activity, probably by producing a synergistic effect[28]. The antimicrobial activity displayed by the *C. mangga* and *C. aeruginosa* essential oils in this study might possibly be attributed to the synergistic effects of the compounds present in the oils.

However, Berger further explained that antagonism effects of the compounds present in essential oils might also affect its activity[28]. This might explain the inactivity of the *Z. cassumunar* essential oil in this study against most of the tested microorganisms. The individual components of the oil might have had antagonistic effects on each other, hence reducing its antimicrobial efficacy. The modes of action of terpenes in antimicrobial activity are complex and in some cases still unknown. Considering the large number of different groups of chemical compounds present in essential oils, it is assumed that their antimicrobial properties are most likely not attributable to only one specific mechanism.

Steam distillation had successfully yielded 0.19%, 0.12%, and 0.30% of the *C. aeruginosa*, *C. mangga*, and *Z. cassumunar* essential oil respectively. The oils were found to have different fragrances and appearance. GC–MS analyses identified a total of 58 compounds in the three Zingiberaceae species, with 16 compounds belonging to *C. aeruginosa*, 27 compounds from *C. mangga*, and 15 compounds from *Z. cassumunar*. It was found that the essential oils were rich in sesquiterpenes and monoterpenes.

In terms of antimicrobial activity, *C. mangga* essential oil displayed the highest and most broad–spectrum activity amongst the three essential oils, by successfully inhibiting the growth of all bacteria and fungi. *C. aeruginosa* displayed mild antimicrobial activity and inhibited all microorganisms except the gram–negative *P. aeruginosa*. *Z. cassumunar* oil had the least activity as it only showed weak inhibition of *S. aureus* and *P. aeruginosa*. The results of this study suggest that the essential oils of *C. mangga* and *C. aeruginosa* have potent antimicrobial properties that may be useful in many applications, such as food preservation, pharmaceuticals, and natural therapies. However, further phytochemical

and pharmacological studies are necessary to isolate the bioactive compound(s) and study their mechanisms of action.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

The authors are extremely grateful to the Kulliyah of Science, International Islamic University Malaysia for providing financial support and research facilities to accomplish this study, and Dr. Shamsul Khamis of Universiti Putra Malaysia for plant identification.

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