https://doi.org/10.48048/tis.2023.5212

Antimicrobial and Radical Scavenging Activities of Essential Oils from *Kaempferia larsenii* Sirirugsa

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Received: 14 July 2022, Revised: 31 August 2022, Accepted: 7 September 2022, Published: 17 March 2023

Abstract

Introduction: *K. larsenii* (Zingiberaceae) is an endemic and rare species in Thailand. There have been no previous reports on chemical compositions and biological properties of essential oils from the fresh root and rhizome. This research aims to investigate the chemical compositions, antimicrobial and radical scavenging activities of essential oils from the fresh root and rhizome of *K. larsenii*. Materials and methods: The chemical compositions of hydrodistilled essential oils were analysed by GC-MS technique. The antimicrobial activity was tested by the disc diffusion assay. The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were also evaluated. The radical scavenging activities were evaluated using ABTS, DPPH and hydroxyl radical scavenging activity assay. Results and discussion: Camphene was considered as the main component in root and rhizome essential oils (36.35 and 48.00 %, respectively). The essential oils showed strong antimicrobial activity against *S. aureus*. The rhizome essential oil showed strong DPPH and hydroxyl radical scavenging activities while the root essential oil exhibited strong hydroxyl radical scavenging capacity. Conclusions: The essential oils exhibit strong antimicrobial especially Gram-positive bacteria and antioxidant activity. Therefore, it might be used as a potential source of natural antidiarrheal and antioxidant substances for food and cosmeceutical product industries.

Keywords: ABTS radical scavenging activity, Antimicrobial activity, DPPH radical scavenging activity, essential oil, Hydroxyl radical scavenging activity, *Kaempferia larsenii*, Radical scavenging activity, Zingiberaceae

Introduction

Free radicals are molecules containing an unpaired electron [1]. It was generated in the body by various endogenous and exogenous mechanisms such as byproduct of cell function, enzyme functiom, smoke, air and water pollution [2].

Antioxidants are molecules that inhibit formation of free radicals by several mechanisms such as scavenging free radicals, chelating metal ions, preventing formation of peroxides, breaking the autoxidative chain reactions and reducing localized O_2 concentrations [3,4].

Nowadays, infectious and chronic diseases caused by free radicals such as cardiovascular disease, neurodegenerative disorders and cancer are the main causes of death for the world's population [5]. Several natural products have been studied for their antimicrobial and antioxidant activities to be used as a medicine or a lead compound for drug discovery. Essential oil, a complex mixture of compounds mainly terpenoids, is one of natural compounds which have been reported for their antimicrobial and antioxidant activity [6]. Previously reported showed that essential oils from several plant species ecpecially Zingiberaceous plants possess antimicrobial and antioxidant acitivities. For example, essential oil from rhizome of *Alpinia conchigera* [7], *A. malaccensis* [8], *A. officinarum* [9], *Boesenbergia pulcherrıma* [10], *Curcuma aromatica* [11], *C. longa* [12], *Elettariopsis curtisii* [13] and *Zingiber officinale* [9,14,15] have been reported for their antimicrobial and antioxidant acitivities.

The genus *Kaempferia* (Zingiberaceae) is composed of more than 40 species. The greatest diversity of *Kaempferia* plants occurs in tropical Asia [16]. In Thailand, 31 species of the genus *Kaempferia* were

recorded [17-21]. The rhizome of *Kaempferia* plants was used in ethnomedicinal recipes for treating several diseases such as diarrhea, inflammation, leukorrhea, pneumonia and skin disease [22-27]. In addition, the rhizome essential oils from *K. angustifolia* and *K. galanga* have been reported for antimicrobial and antioxidant activities [28-30].

K. larsenii is an endemic and rare species in Thailand [31]. The rhizome of this plant was used as an ethnomedicine for relieving insect bites and being an antidote [22,32]. However, there have been no previous reports on chemical compositions and biological properties of essential oils of the fresh root and rhizome parts of *K. larsenii*. The present study was thus to investigate the chemical compositions and antimicrobial and radical scavenging activities of essential oils from the fresh root and rhizome of *K. larsenii*.

Materials and methods

Chemical

Mueller-Hinton agar, Mueller Hinton broth and Tryptic Soy Broth were purchased from Difco, USA; 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *L*-Ascorbic acid, dimethyl sulfoxide (DMSO), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), gentamicin, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Sodium salicylate, triphenyltetrazolium chloride, Vancomycin from Sigma Aldrich (USA); sodium chloride from Ajax Finechem Pty Limited (Australia); ferrous sulfate from Kemaus (Australia); Hydrogen peroxide from Chem supply (Australia); Potassium persulfate from AppliChem (Germany); Methanol from Merck (Germany).

Plant material

The 1 year old plant sample was collected from Ubon Ratchathani province, Thailand in July 2019 and identified by comparison with herbarium specimens at the Suanluang Rama IX Herbarium (voucher no. SL 006733). The voucher specimen of the plant sample was deposited at Suanluang Rama IX Royal Botanic garden, Bangkok, Thailand (voucher no. SL 009427).

Isolation of essential oils [33]

The essential oil from each part of the ground plant sample (300 g) was extracted by hydrodistillation (sample: Distilled water ratio 1:3) for 3 h using Clevenger apparatus (V.N. Supply, Thailand). The essential oils were collected and stored at 4 °C in an air-tight vial.

Gas chromatography-mass spectrometry analysis [34]

The chemical compositions of essential oils were identified by an Agilent 7890A gas chromatography (Agilent Technologies, Inc., USA.) connected with 5975C inert XL EI/CI MAD and Triple-Axis detector. The GC column was DB-5 MS capillary column (30 m×0.25 mm; film thickness 0.25 μm). Helium at a flow rate 1 mL/min) was used as carrier gas. The oven temperature was programmed from 60 to 240 °C at rate of 3 °C/min. The temperature of GC injector and GC-MSD interface were set at 180 and 290 °C, respectively. Mass spectra were recorded at 70 eV (electron impact ionization mode), in a range from m/z 40 - 650 at a scanning rate of 2.42 amu/s.

Identification of essential oil components

The chemical compositions of root and rhizome essential oils were identified by the comparison of their mass fragmentation pattern with Adams Essential Oil Mass Spectral library and NIST05 Mass Spectral library. The contents of chemical compositions were determined based on the relative peak area measurement.

Antimicrobial activity assays

Bacterial strains

Antimicrobial activity testing of the essential oils was evaluated against standard strains including Gram-positive bacteria; i.e. *Staphylococcus aureus* ATCC 6538, and 2 species of Gram-negative bacteria; *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 27853. All bacteria strains were obtained from National Nanotechnology Center, National Science and Technology Development Agency, Thailand.

Disc diffusion assay [35]

The zone of inhibition of the essential oils was determined by disc diffusion assay. Each microbial strain was newly inoculated on Tryptic Soy Broth medium and incubated overnight at 37 °C. After that, the microbial cultures were approximately diluted with 0.85 % sterile normal saline solution to give McFarland 0.5 turbidity. The inoculum cultures were spread on Mueller-Hinton agar plate with sterile cotton swab and left until drying. Sterilized paper discs of diameter 6 mm were impregnated with 20 µl of essential oil solution (300 µg/mL) and placed on the inoculated agar with each tested bacteria. Those plates were left to stand at room temperature for 30 min to allow the diffusion of the essential oils and then they were incubated overnight at 37 °C. The zone of inhibition against the tested bacteria was measured. Standard antibiotic discs including vancomycin (30 µg/disc) and gentamicin (10 µg/disc) were use as positive control.

Determination of minimum inhibitory concentration (MIC) [36]

The minimum inhibitory concentration (MIC) of the essential oils was determined by micro dilution method in a 96-well plate. The both of essential oils were diluted in twofold series in 5 % v/v dimethyl sulfoxide (DMSO) to get the final concentrations in a range of 0.6 - 300 µg/mL. The different concentrations of essential oils (20 µl), Mueller Hinton broth (80 µl) and each inoculum bacteria with McFarland 1 standard turbidity (100 µl) were added in each well. The well plate was incubated overnight at 37 °C. After that, 0.5 % aqueous solution of triphenyltetrazolium chloride (10 µl) was added and incubated at 37 °C for 30 min. The viable bacterial showed pink/red color. The lowest concentrations of both of essential oils exhibiting no viable bacterial were recorded as their MIC value. Vancomycin and gentamicin (0.6 - 300 µg/mL) were use as positive control.

Determination of minimum bactericidal concentration (MBC) [36]

The inoculum cultures from wells that exhibited no viable bacterial (5 µl) were spread on Muller Hinton agar and incubated overnight at 37 °C. The lowest concentration of both of essential oils without any bacterial growth was recorded as their MBC value. Vancomycin and gentamicin (0.6 - 300 µg/mL) were use as positive control.

Radical scavenging activity assays

Sample preparation

Each essential oil sample was diluted in methanol to afford the concentrations in a range of 1 - 300 µg/mL.

***2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activity assay* [37]**

$ABTS^+$ solution was produced by the reaction between 7 mM ABTS (5 mL) and 2.45 mM potassium persulfate (5 mL) and kept in the dark at room temperature for 16 h. After that, the reaction mixture was diluted with distilled water to obtain an absorbance of 1.00 at 734 nm. The essential oil (200 µl) was mix with freshly prepared $ABTS^+$ solution (200 µl). The reaction mixture was left at room temperature for 6 min. The assay was performed in a 96-well plate and a microplate reader was used to measure absorbance at 734 nm. Trolox® was used as a positive control. The percentage of ABTS radical scavenging was calculated as follows:

$$\text{Percent scavenging} = [(A_0-A_1)/A_0]\times 100 \quad (1)$$

where A_0 is the absorbance of the control (without the sample) and A_1 is the absorbance of the sample.

***2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay* [38]**

The essential oil (200 µl) was mixed with 0.2 mM DPPH in methanol (200 µl). The reaction mixture was kept in the dark at room temperature for 30 min. The assay was performed in a 96-well plate and a microplate reader was used to measure absorbance at 517 nm. *L*-ascorbic acid was used as a positive control. The percentage of DPPH radical scavenging was calculated as Eq. (1).

***Hydroxyl radical scavenging activity assay* [38]**

The essential oil (200 µl) was mix with 1.5 mM ferrous sulfate (200 µl), 6 mM hydrogen peroxide (140 µl) and 20 mM sodium salicylate (20 µl). The reaction mixture was kept at 37 °C for 1 h. The assay was performed in a 96-well plate and a microplate reader was used to measure absorbance at 562 nm.

L-ascorbic acid was used as a positive control. The percentage of hydroxyl radical scavenging was calculated as follows:
Percent scavenging = $[(A_1-A_2)/A_0]\times100$

where A_0 is the absorbance of the control, A_1 is the absorbance of the sample and A_2 is the absorbance without sodium salicylate.

Statistical analysis

All the assays were carried out in triplicate. The experimental results were reported as mean ± SD. Data analyses were performed using the SPSS software version 26 (SPSS Inc.; Chicago, IL, USA). One-way analysis of variance (ANOVA) and Duncan's multiple range test were used for multiple comparisons ($p < 0.05$). The half maximal effective concentration (EC_{50}) was calculated from linear equation of graph plotted between percent scavenging vs concentration.

Results and discussion

Essential oil composition

The essential oils from root and rhizome of *K. larsenii* gave yellowish colour and characteristic odor. Their percent yields were 0.22 and 0.26 % v/w, respectively. Twenty-three compounds comprising 97.83 were characterized from the root essential oil whereas 36 compounds comprising 98.02 % were characterized from the rhizome essential oil. **Table 1** showed that monoterpene hydrocarbons represented by camphene was the main component in the root and rhizome essential oils (36.35 and 48.00 %, respectively). The results were different from previous reports. Theanphong *et al.* reported that epi-13-manool (21.93 %), caryophyllene oxide (12.66 %) and camphene (9.71 %) were 3 major components of rhizome essential oil from *K. larsenii* [39]. However, the variation in chemical compositions of essential oil might be affected by age of plant sample, extraction methods, geographical sources, environmental conditions, genetic factors and evolution [40-42].

Table 1 Essential oil compositions of the fresh roots and rhizomes of *K. Larsenii*.

Chemical compositions	m/z	KI*	Content [%]	
			Root	Rhizome
Monoterpene hydrocarbons				
Tricyclene	136	926	1.23	0.81
α-Pinine	136	939	9.55	15.09
Camphene	136	954	36.35	48.00
β-Pinine	136	979	2.04	6.63
Myrcene	136	990	-	0.75
3-Carene	136	1,011	1.06	-
Limonene	136	1,029	3.63	5.11
γ-Terpinene	136	1,059	-	1.11
α-Terpinolene	136	1,088	-	0.30
Oxygenated monoterpenes				
1,8-Cineloe	154	1,031	7.78	4.10
Linalool	154	1,096	-	0.54
Camphor	152	1,146	5.01	7.05
Borneol	154	1,169	6.57	0.72
Terpinen-4-ol	154	1,177	0.95	0.54
α-Terpineol	154	1,188	-	0.33

Chemical compositions	m/z	KI*	Content [%]	
			Root	Rhizome
Neral	152	1,238	-	0.06
Geraniol	154	1,252	-	0.50
Geranial	152	1,267	-	0.07
Bornyl acetate	196	1,288	2.25	0.05
Geranyl acetate	196	1,381	-	0.06
Sesquiterpene hydrocarbons				
α-Gurjunene	204	1,409	6.49	1.36
Caryophyllene	204	1,419	1.48	0.77
γ-Elemene	204	1,436	-	0.05
Humulene	204	1,454	0.69	0.19
Alloaromadendrene	204	1,461	1.13	0.28
γ-Gurjunene	204	1,477	0.58	0.16
β-Chamigrene	204	1,477	-	0.06
γ-Muurolene	204	1,479	-	0.06
γ-Selinene	204	1,484	-	0.06
Valencene	204	1,496	0.57	-
δ-Cadinene	204	1,523	0.65	0.13
Oxygenated sesquiterpenes				
Palustrol	222	1,568	-	0.14
Caryophyllene oxide	220	1,583	3.48	1.14
Globulol	222	1,590	0.87	0.12
Ledol	222	1,602	1.41	0.27
Isolongifolanone	220	1,613	-	0.67
α-Cadinol	222	1,654	3.42	0.55
Alloaromadendrene oxide	220	1,702	0.64	-
trans-Farnesol	222	1,715	-	0.04
Non terpenoid compounds				
Pseudocumene	120	1,023	-	0.19
Total identified (%)			**97.83**	**98.02**
% Yield (%v/w)			**0.22**	**0.26**

*The Kovats index is determined relative to n-alkanes (C6 - C24) on a DB-5 MS column
-Not detect

Antimicrobial activity assays

The antimicrobial activity of the root and rhizome essential oils was investigated by disc diffusion assay. Their MIC was evaluated by micro dilution method. The zone of inhibition, MIC and MBC values of both of the essential oils and positive controls are showed in **Tables 2** and **3**. The root and rhizome essential oils exhibited strong antimicrobial activity against Gram-positive bacteria; *S. aureus* ATCC 6538, with inhibition zones of 38.6 ± 0.71 and 40.0 ± 1.05 mm, respectively. As show in **Table 3**, the MIC and MBC values of both essential oils against *S. aureus* ATCC 6538 were 2.34 µg/mL as same as

vancomycin (positive control). In contrast, both of the essential oils exhibited weak antimicrobial activity against Gram-negative bacteria; *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853 with MIC 4.69 and 9.38 μg/mL, respectively. The MBC of both essential oils against *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853 were 9.38 and 18.75 μg/mL, respectively. The results were similar with the previously reports. The rhizome essential oil of *K. angustifolia* (10 % essential oil in 0.05 % tween80) show moderate activity against *S. aureus* ATCC 29213 with inhibition zones of 10.63 ± 0.23 mm while it is no inhibitory actiivity against *E. coli* ATCC 25922 [15]. In addition, the inhibition zone of essential oil from rhizome of *K. galanga* (10 μl) against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were 12 and 8 mm, respectively [43]. Previous study showed that terpenoid compounds such as thymol, menthol and geraniol exhibited strong antimicrobial activity [44,45]. As seen in **Table 2**, the root essential oil show higher antimicrobial activity than rhizome essential oil. The root essential oil contain high oxygenated monoterpenes and sesquiterpenes (28.90 %). Therefore, it might be show strong antimicrobial activity. A toxic effect by denatureing proteins on cell membrane is one of important mechanism of antibaterial activity [46,47]. In this study, the essential oils show weak activity against Gram-negative bateria might be due to the complex and thick outer lipopolysaccharide membrane of Gram-negative bateria [48].

Table 2 The zone of inhibition of essential oils from the fresh roots and rhizomes of *K. larsenii*.

Sample	Zone of inhibition (mm)		
	S. aureus	***E. coli***	***P. aeruginosa***
Root essential oil	40.0 ± 1.05	36.7 ± 0.87	28.0 ± 0.72
Rhizome essential oil	38.6 ± 0.71	34.2 ± 0.53	22.1 ± 0.51
Vancomycin	21.6 ± 0.66	-	-
Gentamicin	-	20.2 ± 0.47	25.2 ± 0.64

Each value is reported as a means ± standard deviation (n = 3)
-The experiments are not tested.

Table 3 The MIC and MBC values of essential oils from the fresh roots and rhizomes of *K. larsenii*.

Sample	MIC (μg/mL)			MBC (μg/mL)		
	S. aureus	***E. coli***	***P. aeruginosa***	***S. aureus***	***E. coli***	***P. aeruginosa***
Root essential oil	2.34	4.69	9.38	2.34	9.38	18.75
Rhizome essential oil	2.34	4.69	9.38	2.34	9.38	18.75
Vancomycin	2.34	-	-	2.34	-	-
Gentamicin	-	2.34	4.69	-	2.34	4.69

-The experiments are not tested.

Radical scavenging activity assays

The radical scavenging activities of the root and rhizome essential oils were studied by 3 various methods including ABTS, DPPH and hydroxyl radical scavenging assays. The root and rhizome essential oils exhibited strong hydroxyl radical scavenging activity with insignificantly different EC_{50} values as compared with positive control (**Table 4**). In addition, the EC_{50} value of the rhizome essential oil was not significantly different from that *L*-ascorbic acid for scavenging DPPH radicals. However, both of the essential oils showed less ABTS radical scavenging activity as compared with Trolox®. The EC_{50} values of the essential oils and positive controls are shown in **Table 4**. The results were in agreement with the previous reports. The rhizome essential oil of *K. galanga* possessed high antioxidant properties against DPPH radicals with IC_{50} 7.93 μg/mL [49]. In addition, the root and rhizome essential oil of *K. angustifolia* exhibited strong DPPH and OH radical scavenging activity with EC_{50} 9.14 ± 1.27 and 10.89 ± 1.64 μg/mL for DPPH radical scavenging activity and 24.66 ± 3.48 and 29.33 ± 4.25 μg/mL for

OH radical scavenging activity [50]. Several previously research indicated that momoterpenoids exhibited high free radical scavenging activity [51,52]. Therefore, the strong antioxidant activity of root and rhizome essential oils may be resulted from the hight content of monoterpenes in essential oils together with synergistic effect of their own chemical compositions [53].

Table 4 The EC_{50} values of essential oils from the fresh roots and rhizomes of *K. larsenii*.

Sample	EC_{50} (μg/mL)**		
	ABTS radical scavenging activity assay	**DPPH radical scavenging activity assay**	**OH˙ radical scavenging activity assay**
Root essential oil	20.04 ± 0.62[a]	21.26 ± 0.37[a]	27.74 ± 0.55[a]
Rhizome essential oil	19.47 ± 0.55[a]	19.93 ± 0.44[b]	27.56 ± 0.43[a]
Positive control	13.08 ± 0.36[b]	19.12 ± 0.36[b]	26.82 ± 0.48[a]

**Means ± SD followed by the same letter for each experiment, within a column, are not significantly different ($p < 0.05$).

Conclusions

In this study, the chemical compositions and biological activities including antimicrobial and radical scavenging activities of essential oils from the fresh root and rhizome of *K. larsenii* were reported. The results showed that camphene, a monoterpene hydrocarbon, was the main compound in the root and rhizome essential oils. The root and rhizome essential oils of *K. larsenii* exhibited strong antimicrobial activity against *S. aureus* that causes diarrhea. In addition, both of the essential oils exhibited strong antioxidant activities. Therefore, it might be concluded that the essential oils of the fresh root and rhizome of *K. larsenii* might be a potential source of natural antidiarrheal and antioxidant substances for food and cosmeceutical product industries. However, *K. larsenii* is an endemic and rare species in Thailand, and therefore information on chemical compositions and biological activities are very scarce. Further *in vitro* and *in situ* propagations are necessary to be carried out.

Acknowledgements

This research project was financial supported by the Agricultural Research Development Agency (Public Organization) or "ARDA" (Grant no. CRP62050310510) and the Research Institute of Rangsit University, Thailand (Grant no. 3/2562).

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