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Short communication

Toxicity, antioxidant ability and inhibition of oral pathogens by monoterpene-rich essential oil of *Litsea angulata* Blume

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Abstract

The genus *Litsea* comprises about 136 species worldwide and some species have been used in traditional medicine and as essential oil sources. *Litsea angulata* Blume is distributed throughout Indonesia, Malaysia and New Guinea. This study dealt with the chemical composition, toxicity, antioxidant and in vitro antimicrobial activities of essential oil from *L. angulata* leaves. The essential oil was produced using steam distillation and the chemical components of the oil were analyzed using gas chromatography-mass spectrometry (GC-MS). Inhibitory activity against oral pathogens (*Staphylococcus aureus*, *Streptococcus mutans, Streptococcus sobrinus* and *Candida albicans*) was assayed using the agar well diffusion method. Free radical scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to analyze the antioxidant activity. The toxicity was evaluated using the in vivo brine shrimp lethality test (BSLT). GC-MS analysis revealed the presence of monoterpenoids (85.28%) and the major compounds were β-pinene and cis-verbenol. The essential oil exhibited promising antimicrobial activity against the four test microorganisms, producing zones of inhibition with diameters of 11.44–50.00 mm. The highest inhibitory activity was obtained against *S. mutans* and *S. sobrinus*. The oil exhibited weak DPPH activity with 0–13.96% inhibition in the concentration range 0–50 µg/mL and was not toxic in the BSLT. These results demonstrated that this nontoxic essential oil could be considered as a potential antimicrobial agent against oral pathogens.

Introduction

The use of essential oils for cosmetics, medicines and dental or oral hygiene is of growing interest and the subject of intense modern scientific research because oral diseases are a major health problem, with dental caries and periodontal diseases among the most prevalent, preventable global diseases (Dagli et al., 2015). The use of antioxidants is also becoming more frequent in the medical field as an adjunct to the treatment of oral problems (Sofowora et al., 2013). The search for alternative products continues and natural phytochemicals isolated from plants used as traditional medicines are considered to be promising candidates; for example, the genus *Litsea* comprises roughly 400 species worldwide and some plants have been used in traditional medicine and as essential oil sources (Wang et al., 2016; Kamle et al., 2019). Although there are some reports on the antimicrobial and antioxidant activities of *Litsea* genus extracts (Ho et al., 2011; Choudhury et al., 2013; Su and Ho, 2016; Kuspradini et al., 2018), research into the antimicrobial activity of *Litsea* essential oil on oral pathogens is still limited.

L. angulata is distributed throughout Indonesia (Sumatra, Java, Lesser Sunda Islands, South- and East-Kalimantan, Moluccas),

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Malaysia and New Guinea (Slik, 2009). Based on the International Plant Names Index and World Checklist of Selected Plant Families, another accepted name of *Litsea angulata* Blume are *Tetranthera angulata* (Blume) Nees, *Litsea reinwardtii* Blume ex Meisn., and *Malapoenna angulata* (Blume) Kuntze (Trustees of the Royal Botanic Gardens, 2017). The *L. angulata* seeds have been traditionally used for the treatment of boils and its methanol extracts of *L. angulata* seeds contain chemical compounds such as alkaloids and tannin (Mustikasari and Ariyani, 2010).

To date, there have been no known studies published on *L. angulata* essential oil. Thus, the objective of this study was to chemically characterize the essential oil of the leaves of *L. angulata*: to determine their antimicrobial effects against a panel of oral pathogens, to evaluate their 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and to investigate their potential toxic effects on *Artemia salina*.

Materials and Methods

Plant and chemical materials

L. angulata leaves were collected from the botanical garden of Mulawarman University, East Kalimantan, Indonesia. The leaves were prepared by drying for 1 d. Anhydrous sodium sulfate, glucose and nutrient broth were obtained from Merck (Darmstadt, Germany). DPPH was purchased from Wako Pure Chemical Industries, Ltd (Japan). Other chemicals were obtained from commercially available sources. The plant name has been verified in both http://www.ipni.org and http://apps.kew.org/wcsp and a voucher specimen was deposited in the Dendrology Laboratorium of Mulawarman University.

Steam distillation

The *L. angulata* essential oil (*L. angulata* oil) was collected using a steam distillation method adapted from Kuspradini et al. (2016). The collected material was air-dried at room temperature and subjected to steam distillation, for 4 hr for recovery of the oil. The obtained oils were dried using anhydrous sodium sulfate and weighed.

Identification of essential oil compounds

The volatile compounds in the essential oil were analyzed using gas chromatography (GC) in a GC 2010 equipped with a GC-MS QP2010 system (Shimadzu, Japan). A fused silica capillary SH-Rxi-5Sil MS column (30 m × 0.25 mm, 0.25 µm internal diameter) was used with helium as the carrier gas (1.51 mL/min). The temperature program was from 70°C to 280°C at 25.71°C/min. The injector temperature was set at 250°C. The injection mode was a split ratio of 200:1 and the inlet pressure was 98.3 kPa. Mass spectra were recorded at 0.89 kV with the mass range 40–400 m/z. The constituents of the oils were identified based on the retention index (RI) determined by using a homologous series of n-alkanes (C10–C20) injected under the same chromatographic conditions as the samples and fragmentation models of mass spectra. Both data were compared to those in the NIST mass spectral data system libraries and NIST Standard Reference Database Number 69 (https://webbook.nist.gov/chemistry/).

Toxicity assay

Toxicity activity was determined using the BSLT method, according to the method of Mirzaei et al. (2013). A sample of 20 mg of extract was diluted with 2 mL ethanol. Using a pipette, 250 μL, 125 μL and 50 µL diluted extract were transferred and evaporated in test tubes. Brine shrimp eggs (*Artemia salina*) were hatched in artificial seawater. After 48 hr incubation at room temperature (25–29°C), nauplii (larvae) were collected using a pipette and prepared for the assay. Ten active brine shrimp nauplii (larvae) of *Artemia salina* were placed in each test tube and diluted with sea water to a volume of 5 mL. The plant extracts were tested at concentrations of 100 $\mu g/$mL, 250 $\mu g/$mL and 500 µg/mL. The number of dead brine shrimps was then recorded 24 hr after subjecting each brine shrimp in each of the solutions. Survivors were counted after 24 hr and the percentages of lethality at each concentration (%M) were calculated according to Abbot (1925) formula as shown in Equation 1:

$$\%M = [(me - mb) / (10 - mb)] \times 100 \quad (1)$$

where, me = the number of dead shrimps in the sample and mb = the number of dead shrimps in the blank.

The values of the concentration killing 50% of the brine shrimp larvae (LC50) were obtained from the best-fit line plotted concentration versus the percentage lethality. An LC50 value greater than 100 μg/mL is considered to represent a nontoxic compound or extract (Moshi et al., 2010). The test was carried out three times and the LC50 value was reported as mean ± SD.

DPPH radical scavenging activity

The determination of antioxidant activity was adapted from the DPPH radical scavenging activity method of Siatka and Kašparová (2010). Samples of the stock solution (3mg/mL) were diluted to a dilution series (four different concentrations) using methanol. An aliquot (500 μL) was mixed with a methanolic solution of DPPH (500 μL). The final concentrations of samples were 6.25, 12.5, 25 and 50 µg/mL. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control. Ascorbic acid was used as the positive control.

The absorbance was measured at 517 nm against methanol as a blank. The mixtures were incubated at room temperature in the dark for 20 min. The absorbance was measured at 517 nm against methanol as a blank. The percentage of DPPH scavenging was calculated as: DPPH radical scavenging activity (%) = [(Absorbance of negative control - Absorbance of the sample) / Absorbance of negative control] × 100. All experiments were repeated three times (Rashid et al., 2018).

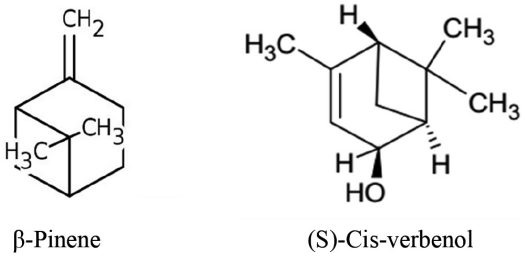
Antimicrobial activities

The agar well diffusion method was modified from Kuspradini et al. (2016) and used to evaluate the antimicrobial activity of the essential oil against three bacteria (*S. aureus, S. mutans and S. sobrinus*) and one fungus (*C. albicans*). *S. aureus, S. mutans, S. sobrinus* and *C. albicans* are all pathogens that can cause oral disease. The agar plate surface was inoculated by spreading a microbial inoculum over the entire agar surface. Samples of 20 µL of pure *L. angulata* oil (100%) and two dilutions (ten-fold serial dilutions) at 10% and 1% in 40% ethanol were tested. Agar plates were then incubated under suitable conditions, depending upon the test microorganism. In this test, the antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. An amount of 40% ethanol and an antibiotic standard (10µg/well; chlorhexidine for bacteria and chloramphenicol for the fungus) was used as negative and positive controls, respectively. The zone of inhibition (ZI), representing a region of depressed growth of microorganisms was measured and the activity index (AI) for each extract was calculated using: AI = (ZI of the sample) / (ZI of the positive control). Antibacterial activity was obtained by measuring the diameter of the zone of inhibition of triplicate samples (Ashraf et al., 2015).

Results

Yield and chemical composition

The steam distillation process of the leaves of *L. angulata* resulted in the formation of 0.9% (weight per weight) and had a light-yellowish color with a fruit-like aroma. Thirty compounds of the essential oil were characterized being oxygenated monoterpenes and monoterpene hydrocarbons as the major class of compounds. Four of the components were monoterpene hydrocarbons (22.4%), 22 were oxygenated monoterpenes (62.42%), 2 were sesquiterpene hydrocarbons (3.42%) and 2 were other components (11.3%). The main compounds were characterized as: (+)-β pinene (18.19%) and cis-verbenol (11.10%) as shown in Fig. (1). The monoterpenes (85.28%) were the dominant groups in the *L. angulata* essential oil (Table 1).



β-Pinene (S)-Cis-verbenol

Fig. 1 Chemical structure of main compounds of oil from *L. angulata* leaves

Toxicity activity

Toxicity tests were conducted to determine the level of toxicity of the essential oil against larvae shrimp *A. salina.* The test results showed that the *L. angulata* oil at different concentration levels had an impact on mortality and larval toxicity as shown in Table 2. This study showed that essential oil of *L. angulata* had no lethal implication on brine shrimp larvae at concentrations of 250 µg/mL and 500 µg/mL, but showed high mortality rates at the high concentration 1,000 µg/mL. reaching 83.3% after 24 hr of treatment. The *L. angulata* essential oil in this study had an LC50 value of 784.24 µg/mL.

Antioxidant activity

The DPPH radical scavenging activity of *L. angulata* are shown in Table 3. The essential oil showed an ability to scavenge the DPPH radical. However, the activity was much lower than that of reference standards. The essential oil of *L. angulata* showed a concentration-dependent rise in DPPH scavenging up to a specific concentration and declined thereafter (Table 3). The *L. angulata* oil was able to reduce the radical DPPH without reaching 50% DPPH radical inhibition. The DPPH activity was 6.14–13.72% for a concentration range of 6.25–100 µg/mL.

Antimicrobial activity

The antimicrobial activity of *L. angulata* oil was tested against three bacteria (*S. mutans, S. sobrinus* and *S. aureus*) and one fungus (*Candida albicans*) based on the zone of inhibition and activity index values. The growth of all tested microorganisms was inhibited by the pure essential oil samples and a decrease in this antimicrobial activity was observed following dilution of the oil in 40% ethanol. The inhibitory activities of both the oil and of standard antibiotics on the growth of oral pathogens are shown in Table 4. *L. angulata* oil inhibited the growth of all the tested oral microbial pathogens in a dose-dependent manner and exhibited equal or better activity than the positive or antibiotic controls. The maximum inhibitory effect was observed with the pure essential oil (20 µL, 100% concentration). Of the four microorganisms tested, *S. sobrinus* and *S. mutans* were the most sensitive and the inhibition zone produced by *L. angulata* oil against these pathogens was almost 50 mm. *L. angulata* oil also exhibited high inhibitory activity against *S. aureus* (42.67 mm diameter zone) and *C. albicans* (34.22 mm diameter zone). The AI values of *L. angulata* oil against all pathogens were higher than one (1.36–2.74), between 0.46 and 1.29 and lower than one (0.24–0.48) at concentrations of 100%, 10% and 1%, respectively. The highest AI value was observed with *S. sobrinus* (2.74) and the lowest with *C. albicans* (0.24). AI values greater than 1 indicate that the essential oil exhibited more inhibitory activity than the antibiotic controls, which are the most commonly used clinical treatments for the infections caused by these oral pathogens.

Table 1 Composition of essential oils of *Litsea angulata* leaves

No.	Compound[a]	Area (%)				RI[b]	RI[c]
		MH	MO	SH	OT		
1	Camphene	1.87	–	–	–	953	–
2	(+)-β Pinene	18.19	–	–	–	980	1000
3	o-Cymene	0.42	–	–	–	1020	1043
4	d-Limonene	2.38	–	–	–	1032	1049
5	Eucalyptol (1,8-cineole)	–	1.38	–	–	1033	1054
6	(+)-Limonenoxid 1	–	3.78	–	–	1116	1135
7	(+)-Fenchol	–	0.55	–	–	1117	1144
8	α-Campholene aldehyde	–	3.54	–	–	1125	1149
9	cis-Verbenol	–	11.1	–	–	1133	1169
10	Pinocarvone	–	2.09	–	–	1165	1189
11	(±)-Myrtenol	–	8.87	–	–	1191	1225
12	cis-Verbenone	–	3.58	–	–	1205	1240
13	(E)-Carveol	–	1.98	–	–	1217	1246
14	(+)-(S)-Carvone	–	0.76	–	–	1240	1277
15	α-Limonene diepoxide	–	0.49	–	–	1294	1293
16	(2E)-3-Pentyl-2,4-pentadien-1-ol	–	0.92	–	–	–	1298
17	cis-Pinonic acid	–	1.58	–	–	–	1243
18	Limonene dioxide 1	–	1.21	–	–	–	1306
19	(+)-Limonene oxide, cis-	–	7.52	–	–	1142	1326
20	(+)-Verbenol	–	7.83	–	–	1144	1333
22	(1s,2s,3r,5s)-(+)-Pinanediol	–	0.54	–	–	1313	1357
23	Limonene-1,2-diol	–	0.99	–	–	1343	1367
24	Trans-sobrerol	–	1.81	–	–	1382	1413
25	Isogeraniol	–	1.05	–	–	1273	1434
26	Campholaldehyde	–	0.81	–	–	1582	1494
27	Caryophyllene oxide	–	–	3.04	–	1592	1615
28	Humulene oxide	–	–	0.38	–	1608	1649
29	Stigmast-5-en-3-ol, oleate	–	–	–	1.80	–	2004
30	(±)-α Tocopherol	–	–	–	9.50	3142	–
	TOTAL	22.86	62.4	3.42	11.3		

No. = number; MH = monoterpene hydrocarbon; MO = oxygenated monoterpene; SH = sesquiterpene hydrocarbon; OT = other.

[a] Compounds listed in order of elution.

[b] RI: retention indices from NIST Standard Reference Database Number 69 literature (non-polar HP-5 MS column).

[c] RI: relative retention indices calculated against n-alkanes (SH-Rxi-5Sil MS column).

Table 2 Mortality rate and toxicity of different concentrations of *L. angulata* leaves oil using brine shrimp lethality testing

Concentration (µg/mL)	Mortality ± SD (%)	LC50 (µg/mL)
1,000	83.33 ± 15.28	784.24
500	0.00 ± 0.00	
250	0.00 ± 0.00	
0	0.00 ± 0.00	

LC50 = concentration that kills 50% of brine shrimp larvae.

Table 3 DPPH free radical scavenging activity of different concentrations of *Litsea angulata* essential oil and of ascorbic acid solution

Concentration (µg/mL)	DPPH free radical scavenging activity (%)	
	Litsea angulata oil	Ascorbic acid
50	8.30 ± 0.63	95.91 ± 0.21
25	10.59 ± 1.10	94.95 ± 0.63
12.5	13.96 ± 1.27	95.31 ± 0.55
6.25	13.72 ± 0.36	95.31 ± 0.36

DPPH = 1,1-diphenyl-2-picrylhydrazyl.

Values are mean ± SD.

Table 4 Inhibition zone and activity index of different essential oil concentrations of *Litsea angulata* and antibiotic control

Sample	Concentration	*C. albicans*		*S. aureus*		*S. mutans*		*S. sobrinus*	
		ZI	AI	ZI	AI	ZI	AI	ZI	AI
Positive control[a]	100 µg/mL	25.11 ± 0.19	1.00	16.00 ± 0.00	1.00	20.33 ± 1.76	1.00	18.22 ± 0.77	1.00
L. angulata oil (20 µL)	100%	34.22 ± 5.98	1.36	42.67 ± 4.98	2.67	50.00 ± 0.00	2.46	50.00 ± 0.00	2.74
	10%	11.44 ± 0.51	0.46	20.56 ± 0.51	1.29	23.56 ± 1.39	1.16	16.22 ± 1.84	0.89
	1%	6.00 ± 0.00	0.24	6.00 ± 0.00	0.38	6.00 ± 0.00	0.30	8.78 ± 0.69	0.48

ZI = zone of inhibition diameter (mm); AI= activity index.

[a] Antibiotic (chlorhexidine for bacteria: *S. aureus*, *S. mutans,* and *S. sobrinus*) and chloramphenicol for the fungus: *C. albicans*).

Discussion

The volatile constituents and biological activity of essential oil *L. angulata* were investigated. The monoterpene (85.28%) were the dominant forming groups of *L. angulata* oil. The major groups reported in the leaf of *Litsea* spp., such as *L. resinosa, L. gracilipes, L. sessilis, L. megacarpa* and *L. ferestrata* are alcohols (39.47%), sesquiterpenoids (79.7%), aldehydes/ketones (67.10%) and sesquiterpenes (83.82%), respectively (Salleh et al., 2016). A comparative analysis of the studied oils of *L. angulata* with previous data indicated that oils were dominated by monoterpenes as seen in *L. cubeba* and *L. laevigata* (Muhammed et al., 2008; Son et al., 2014). The *L. angulata* oil has a fruit-like aroma, which might have been due to the β-pinene, since according to Farhad et al. (2014) the volatiles liberated from ripe mangos include β-pinene, which is also commonly found in a variety of herbs and spices (El-Zaeddi et al., 2016; Kuete, 2017).

In the current study, *L. angulata* oil showed no substantial toxicity since the brine shrimp test results indicated LC50 values above 100 µg/mL (784.24 µg/mL), which suggested that the oil is effectively non-toxic, since extract values with an LC50 value greater than 100µg/mL showed no substantial toxicity against brine shrimp while the LC50 values below 20 µg/mL have potency as anticancer compounds (Moshi et al., 2010). The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity but a finer level of discrimination for anticancer activity is required for the human cancer cell panel which in most cases correlates reasonably well with cytotoxicity and anti-tumor properties (Krishnaraju et al., 2005; Alali et al., 2006). The results suggested that *L. angulata* oil poses no threat of acute toxicity and might not be toxic to humans.

The present study showed that the essential extract from *L. angulata* leaves was a weak antioxidant at concentrations in the range 6.25–50 µg/mL. The highest scavenging was observed at 12.5 µg/mL with only 13.96 % inhibition. The weak antioxidant activity of *L. angulata* oil might be related to its high monoterpene content (non-phenolic compounds) and the presence of β-pinene and cis-verbenol as the main components. The essential oil of *Cinnamodendron dinisii* Schwacke, *Siparuna guianensis* Aublet and *Etlingera fimbriobracteata*, also had very poor free radical scavenging activity due to their high content of β-pinene (Andrade et al., 2013; Ud-daula et al., 2016). According to Choi et al. (2010), (S)-cis-verbenol reacts with DPPH radicals. This was supported in the current study by the poor activity of the *L. angulata* oil, probably due to the weak ability of the β-pinene and cis-verbenol as main components to scavenge DPPH free radicals. Even though tocopherol was found in *L. angulata* oil, it could be suggested that the components in the *L. angulata* oil had an antagonistic reaction and neutralized the antioxidant effects of tocopherol in the oil.

Plant-derived essential oils containing monoterpenoids have been used as antimicrobials and antifungals (Garcia et al., 2008; Marchese et al., 2017; Tardugno et al. 2017). As shown by the diameters of the ZIs and the magnitudes of the AIs, *L. angulata* oil was most potent against *S. mutans* and *S. sobrinus*, which both cause dental caries. Thus *L. angulata* oil appeared to have medicinal potential and could be used as an antimicrobial against oral pathogens. The strong antimicrobial activity exhibited by *L. angulata* oil could be accounted for by the presence of β-pinene, a monoterpene compound that has been reported to have potent antimicrobial activity (Leite et al., 2007; da Silva et al., 2012), that is due to its ability to diffuse into and damage the cell membranes of microorganism (Sikkema et al., 1995).

In conclusion, this study reported on the essential oils extracted from *L. angulata* leaves from East Kalimantan, Indonesia. This extract was nontoxic and had good antimicrobial properties against four oral pathogens causing dental caries (*S. sobrinus, S. mutans, S. aureus* and *C. albicans*). The observed antimicrobial activity of *Litsea angulata* may be attributed to its high relatively high content of oxygenated monoterpenes (62.4%) in the essential oil. Comparing the antimicrobial activities (against *S. aureus*) of the essential oils from other species of Litsea such as *Litsea laevigata* Nees. (Muhammed et al., 2008), *Litsea elliptica* Blume and *Litsea resinosa* Blume (Wong et al., 2014), the oil from *L. angulata* leaves was superior. In addition, it is important to note that in previous studies, several plants such as *Boswellia socotrana*, sage, rosemary and lavender that had large amounts of oxygenated monoterpenes showed remarkable antimicrobial activity with poor antioxidant activity (Mothana et al., 2011; Odak et al., 2015). Thus, this nontoxic essential oil deserves further investigation to isolate it major compounds and to evaluate its potential as an alternative natural oral treatment due to its strong antimicrobial activity. It is believed that this is the first report on the chemical content and bioactivity of essential oil from *L. angulata* leaves.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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