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PHYSICOCHEMICAL PROPERTIES AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF *LITSEA CUBEBA* PERS. FRUIT

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Abstract

The physicochemical properties of the essential oil (EO) extracted from the fruit of *Litsea cubeba* Pers. were determined, such as pH (5.33±0.15), acid value (8.92±1.02 mg KOH/g EO), saponification value (20.99±2.83 mg KOH/g EO), relative density (0.8836±0.0031), absolute density (0.8820±0.0023 g/mL), and freezing point (-12.67±0.58ºC). The antioxidant capacity was quite low: IC_{50} was not found in this study. In addition, some chemical components were identified using gas chromatography-mass spectrometry (GC/MS) method with some major compounds such as 4-methyl-1,5-heptadiene (26.02%), 1-methoxy-2-butyne (20.05%), and cyclobutane, 1,3-diisopropenyl-, trans (18.06%). The EO was tested for its antibacterial activities against gram-positive (*Staphylococcus aureus* – ATCC 25923, *Bacillus subtilis* – ATCC 11774), and gram-negative (*Escherichia coli* – ATCC 25922), *Salmonella* Enteritidis – ATCC 13076) bacteria. The results showed the highest activity against *B. subtilis* and the lowest activity against both *S.* Enteritidis and *S. aureus*.

Introduction

Litsea cubeba Pers. is a shrub or small deciduous tree, distributed widely in Asian regions including China, Nepal, northeastern India, Indonesia, Laos, Myanmar, Thailand, and Vietnam (SAIKIA et al. 2013) with berry-like spherical fruit. The leaves are staggered, the lanceolate leaflets are 10 cm long, 1.5–2.5 cm wide, thick, the face is green, the gray underside turns black, the edges are intact, and the stems of leaf and veins are clear. In Vietnam, the local citizens exploit the essential oil (EO) from the fruit and leaves of this plant. However, EOs from the leaf are lower in

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quality. Yield of EO from the fruit is 3–5% and contains about 60–75% citral (a mixture of geranial and neral). This component is used for soft drinks and fragrances (SELL 2010).

In addition, *L. cubeba* has been used in traditional Chinese medicine for many centuries. The roots, stems, leaves, and fruits of this plant have been used to treat diseases such as fatigue, headache, muscle aches, and depression. The extract of bark from this tree contains some antioxidants (HWANG et al. 2005) with anti-inflammatory properties (CHOI and HWANG 2004). The EO from *L. cubeba* also has antifungal and antibacterial activity similar to the EOs from other plants (WANG and LIU 2010). Alkaloids, litseacubebic acid, and monoterpene lactones isolated from *L. cubeba* have good antibiotic activity against many bacterial pathogens (FENG et al. 2009). Besides, TAYLOR et al. (2013) pointed out that the EO from the leaves and fruits of *L. cubeba* harvested in northeast India is able to resist *Staphylococcus aureus*, *Listeria monocytogenes, Escherichia coli,* and *Aspergillus niger*. In fact, this EO has many applications in food, medicine, and the cosmetic industry, according to JIANG et al. (2009), *L. cubeba* EO is used as a food additive. It has also been used as a raw material for the manufacture of vitamin A, E, K, ionine, methylionone, and perfumes.

Currently, this plant is widely cultivated in North Vietnam for the extraction of EO. The quality of the EO depends on climate, season, soil, and extraction methods. Although there are many reports on the chemical components of *L. cubeba* EO, until now there are no reports on the chemical components and physicochemical properties of this EO in Vietnam. Hence, the main aim of this study was to clarify some of the issues mentioned above and, in addition, to evaluate its antibacterial activity and antioxidant capacity, and give an overview on the general quality of *L. cubeba* EO.

Materials and Methods

Plant material and EO extraction

Fresh fruit of *L. cubeba* was collected and harvested from Hanoi province (Vietnam). The fresh fruit was distilled by Clevenger-type apparatus. The total mass of sample (fruits), in round-bottom flasks, was about 100 g per extraction time. Firstly, the fruit was completely soaked in water and heated to boiling (fruit/water ratio of 1/10, w/v). Then, the EO was evaporated together with water vapor and finally collected after decantation. The yield of EO obtained was approximately 3% (v/w). The resulting EO was stored at 4ºC until analysis.

Bacteria strains

Antibacterial activity was determined against two gram-positive bacteria such as *B. subtilis* (ATCC 11774), *S. aureus* (ATCC 25923), and two gram-negative bacteria such as *S.* Enteritidis (ATCC 13076), *E. coli* (ATCC 25922). All bacteria strains were purchased from Microbiologics (USA).

Determination of the relative and absolute density

The relative density was evaluated by the ratio between the mass of a given volume of EO at 20°C to the mass of an equal volume of distilled water at 20°C, while the absolute density was determined by ratio of the mass of a given volume of the EO at 20°C to the same volume (*Essential oils…* ISO 279:1998).

Determination of the freezing temperature

According to *Essential oils…* ISO 1041:1973, 5 mL of EO was added to the test-tube. Then, the test-tube was put into the freezing container. The temperature in the freezing container was decreased slowly until the EO appeared to crystallize and the freezing temperature recorded.

Determination of acid value (AV)

The acid value was determined by the titration method. The EO (1 g) was dissolved in 5 mL of 96 % ethanol and about three drops of 1% phenolphthalein solution. The mixture was titrated with 0.1 N KOH until the solution turned pink.

$$\mathrm{AV}=\frac{V_{\mathrm{KOH}} \cdot 0.1 \cdot 56.1}{\text{mass of essential oil}}$$

Determination of saponification value (SV)

The EO (1.5 g) was put into a glass flask (250 mL) and 25 mL of 0.5 M ethanolic KOH added. The mixture was heated for 1 h under reflux and then 25 mL of deionized water and five drops of 1% phenolphthalein solution added. The solution was titrated with 0.5 M HCl until the solution turned colorless.

$$\mathrm{SV}=\frac{\left(V_{\mathrm{blank}}-V_{\mathrm{sample}}\right) \cdot 56.1 \cdot 0.5}{\text{mass of essential oil}}$$

Determination of antioxidant activity

Determination of the antioxidant activity of the EO to scavenge DPPH free radicals was as described by KIRBY and SCHMIDT (1997) with some small modifications. The EO was dissolved in ethanol to achieve the various concentrations (1, 2, 4, 8, 16, 32, and 64 mg/mL). Then, 50 µL of the solution was mixed with 1950 µL of an ethanolic solution of DPPH ($6 \cdot 10^{-5}$ M). The solution was kept in the dark for 30 min at room temperature (OUIS and HARIRI 2018). Antioxidant activity was recorded by monitoring the decrease in absorbance at 517 nm against a control consisting of DPPH in ethanolic solution ($6 \cdot 10^{-5}$ M) and the antioxidant activity was described using the following expression:

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \cdot 100$$

Determination of antibacterial activity

Antibacterial activity was determined by the paper disk diffusion method for antibiotic susceptibility testing (BAUER et al. 1966) with some slight modifications. At first, 100 µL of bacterial suspension (0.5 McFarland standard, approximately $1.5 \cdot 10^8$ cfu/mL) was spread on MHA medium (Mueller-Hinton agar) by a sterile hockey stick. Then, the sterile paper disks of 6 mm diameter were impregnated with the selected EO (10 µL), while gentamicin (10 µg/disk) were used as positive controls to compare the antibacterial activity of the EO, and 20% dimethylsulfoxide (DMSO) solution (10 µL/disk) was used as negative control. These dishes were incubated for 24 h at 37ºC and the diameter of the inhibition zones were expressed in mm including the disk diameter of 6 mm.

Analysis of EO by GC–MS

A volume of 1 µl of EO was injected into a gas chromatograph (Agilent HP 6890 N, USA) equipped with a quadrupole mass analyzer (Agilent HP 5972, USA) in the electron impact ionization (EI) mode (70 eV), split/splitless injector. A capillary column (HP-5 ms, 30 m × 0.25 mm × 0.25 µm, Agilent Technologies, USA) was used with helium as a carrier gas at a constant flow of 1 mL/min. Temperatures: injector = 280ºC, column = 60ºC (5 min), then 15ºC/min to 300ºC (5 min).

Data analysis

Experimental results were analyzed by one-way analysis of variance (ANOVA), and significant differences among the means from triplicate analyses at ($p < 0.05$) were determined by Fisher's least significant difference (LSD) procedure using Statgraphics software (Centurion XV). The values obtained were expressed in the form of a mean±standard deviation (SD).

Results and Discussion

Determination of physicochemical properties of EO

Table 1 shows that the magnitude of the pH of *L. cubeba* fruit EO was recorded in the acidic range (pH = 5.33). This result is in agreement with the EO of *Ceratonia siliqua* seeds (pH = 5.2) and higher than EO of *C. siliqua* pulp (pH = 4.3) (OUIS and HARIRI 2018). This can be explained by the pH value depending on the various main components of the EO.

Table 1

Physicochemical properties of *L. cubeba* EO

Physicochemical properties	Value
pH	5.33±0.15
Relative density	0.8836±0.0031
Absolute density [g/mL]	0.8820±0.0023
Freezing point [ºC]	–12.67±0.58
Acid value [mg KOH/g EO]	8.92±1.02
Saponification value [mg KOH/g EO]	20.99±2.83

The absolute density of *L. cubeba* fruit EO is also higher than that of coriander EO (0.8737 g/mL) (PORTER and LAMMERINK 1994), *C. siliqua* pulp EO (0.833 g/mL), and lower than that of *C. siliqua* seeds EO (0.910 g/mL) (OUIS and HARIRI 2018). The different temperatures and the components of initial material caused the differences in the relative and absolute density. PORTER and LAMMERINK (1994) believed that the absolute density of all EOs decreased as temperatures increased, whereas PORNPUNYAPAT et al. (2011) noticed that an increase in the distilling temperatures can lead to an increase in the relative density for EO of *Aquilaria crassna* wood. The freezing point of the *L. cubeba* fruit EO is higher than that of the cocoa beans EO (–16ºC) (BAINBRIDGE and DAVIES 1912) and lower than that of *Eucalyptus camaldulensis* leaves EO (0ºC) (ABDUL-MAJEED et al. 2013). In fact, all EOs have a freezing point; some EOs need quite low tem-

peratures to freeze, some EOs will freeze in a refrigerator and others can even be solid or crystalline at room temperature. This strongly depends on the main component of the EO.

The acid and saponification values of *L. cubeba* fruit EO are 8.92 mg KOH/g EO and 20.99 mg KOH/g EO, respectively. The acid value of this study is higher than that of the EO of *C. siliqua* seeds EO (2.2 mg KOH/g EO), and lower than that of *Plectranthus amboinicus* Lour. leaves (10.35 mg KOH/g EO), while the saponification value is lower than that of *P. amboinicus* Lour. leaves EO (54.72 mg KOH/g EO) and *C. siliqua* seeds EO (37.2 mg KOH/g EO) (THUYEN et al. 2012; OUIS and HARIRI 2018). The acid value depends on the extraction method and the freshness of the initial material and increases with increasing time preservation because EOs are oxidized and the esters are hydrolyzed to release the free acid.

Determination of antioxidant activity of EO

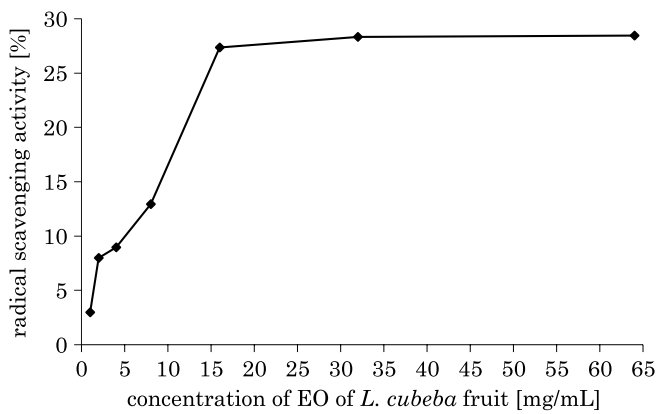

Fig. 1. Radical scavenging activity of *L. cubeba* fruit EO

The appearance of yellow color due to bleaching the purple color of the DPPH attested to the positive antioxidant activity of EO. The percentage inhibition increased with increasing concentration from 1 to 64 mg/mL. Figure 1 shows the percentage inhibition of the EO, but the IC_{50} values could not be determined in this study because the antioxidant activity of *L. cubeba* fruit EO is quite low (< 30%). The antioxidant activity of different EOs depends directly on the concentrations used. However, it is lower than that of studies from the different materials; for instance, the EOs of *Piper betle* L. (IC_{50} = 3.6 µg/mL) (PRAKASH et al. 2010) or of *C. siliqua* pulp and seeds (IC_{50} = 7.8 and 31.25 µg/mL, respectively) (OUIS and HARIRI

2018). Compared with the similar material, *L. cubeba* fruit EO collected from Nepal and China possessed the high antioxidant activity (IC_{50}=1629 and 850 μg/mL, respectively) (BAJRACHARYA and PRATIGYA 2019; BI et al. 2017). This finding revealed that the antioxidant activity of the EO was affected by the initial material, production region, and chemical compositions.

Determination of the chemical compositions of EO

GC–MS analysis of the *L. cubeba* fruit EO revealed 17 different components, which made up approximately 100% of the EO (Table 2). The major components included 4-methyl-1,5-heptadiene (26.02%); 1-methoxy-2-butyne (20.05%); cyclobutane, 1,3-diisopropenyl-, trans (18.06%); 2,7-dimethyl-2,6-octadien-1-ol (9.98%); cyclobutanone, 2-methyl-2-oxiranyl (7.11%) and 2-methyl-6-methylene-2-octene (5.76%). Some components belonged to the terpene group and its derivatives such as 2-methyl-6-methylene-2-octene; 2,7-dimethyl-2,6-octadien-1-ol, and *α*-terpineol, which made up a significant proportion of the EO and other small groups (Table 2). These bioactive compounds have an important role in the antibacterial and antioxidant activity of the EO.

Table 2

Chemical composition of *L. cubeba* fruit EO

Compound	Rt. [min.]	[%]
4-Methyl-1,5-heptadiene	7.851	26.02
1-Methoxy-2-butyne	7.584	20.05
Cyclobutane, 1,3-diisopropenyl-, trans	5.540	18.06
2,7-Dimethyl-2,6-octadien-1-ol	7.484	9.98
Cyclobutanone, 2-methyl-2-oxiranyl	6.750	7.11
2-Methyl-6-methylene-2-octene	7.732	5.76
5-Methoxy-1-pentene	5.004	3.99
2-Propenamide	6.244	3.52
2-Heptyne	6.978	1.42
α-terpineol	4.280	0.76
Butanedinitrile	4.856	0.67
2,5-Dimethyl-2-hexanol	7.088	0.63
6-Methylene bicyclo [3.1.0] hexane	4.806	0.61
2-Furanmethanamine	8.476	0.44
Methyl isocyanoacetate	8.238	0.4
2,5-Octadiene	6.820	0.39
Butanedinitrile	8.843	0.20

However, some previous studies have reported that the prime components of *L. cubeba* EO in Tipet (China) were limonol (44.2%); *β*-linalool (8.8%); and 1,8-cineole (5.4%) (YANG et al. 2010), while the EO of this material from India consisted of sabinene (58.52%); *α*-pinene (12.59%); and 1.8-cineole (12.66%) (SAIKIA et al. 2013). These findings differ from our results. However, some chemical components of this study are similar to those of *L. cubeba* EO in the study of WANG and LIU (2010), such as terpenoid groups. Changes in chemical composition have been recorded in most EOs due to the age of the plant, genes, time of harvesting, extraction method, and geographical and ecological conditions. Therefore, the percentage of the main constituents of *L. cubeba* fruit EO should be mentioned if it is applied as a food additive.

Determination of antimicrobial activity of EO

According to the results in Table 3, it can be observed that the *L. cubeba* fruit EO shows positive antibacterial activity for four strains of bacteria including two gram-positive bacteria (*S. aureus, B. subtilis*) and two gram-negative (*E. coli, S.* Enteritidis). The inhibitor zones of positive control are in order of susceptibility: *S. aureus* < *S.* Enteritidis < *E. coli* < *B. subtilis*. The EO shows the highest antimicrobial activity against *B. subtilis* (inhibition zone 44.33 mm) and the lowest antibacterial activity against *S. aureus* (inhibition zone 14.33 mm). The sensitivity of EOs is classified by the diameter of the inhibition zone. Those of *S.* Enteritidis, *S. aureus*, and *E. coli* were considered "very sensitive" with diameters of 15–19 mm, while that of *B. subtilis* was considered "extremely sensitive" with a diameter of > 20 mm (PONCE et al. 2003). The presence of terpenoids in an EO can strongly inhibit bacteria because their site of action appears to be at the phospholipid bilayer, caused by biochemical mechanisms catalyzed by the phospholipid bilayers of the cell. These processes include the inhibition of phosphorylation steps, electron transport, protein translocation, other enzyme-dependent reactions and microbial oxygen uptake (KNOBLOCH et al. 1986). In addition, the bacterial inhibition also depends on the structure of the terpenoids. However, until now the actual structure-activity relationships of terpenoids are not well understood (GRIFFIN et al. 1999). Compared with other previous studies on disk diffusion assay, *L. cubeba* fruit EO from northeast India also inhibited the grown of *B. cereus* and *S. aureus*, whereas it did not show any appreciable inhibition toward *B. subtilis* (GOGOI et al. 2018). *L. cubeba* fruit were collected from southern regions of China and the EO revealed antibacterial activity against all six bacteria tested: *B. subtilis, E. coli, E. faecalis,*

M. albicans, P. aeruginosa, and S. aureus (WANG and LIU 2010). This proved that the antibacterial activity strongly depends on the chemical composition of the EO.

Table 3

Inhibition zones of *L. cubeba* fruit EO for some bacteria

Microorganism	Diameter of the inhibition zones [mm]
S. Enteritidis	14.67 ± 0.58^a
E. coli	17.67 ± 0.58^b
B. subtilis	44.33 ± 0.58^c
S. aureus	14.33 ± 0.58^a

Different lowercase letters in the same column denote significant difference ($p < 0.05$) with respect to the type of microorganism.

Conclusion

The 17 main chemical components in the *L. cubeba* fruit EO were identified by GC-MS. Further, the *L. cubeba* fruit EO showed marked in vitro antimicrobial activities against *S.* Enteritidis, *E. coli*, *B. subtilis* and *S. aureus*. In addition, the data demonstrated antioxidant activity and determined some physicochemical properties of the *L. cubeba* fruit EO. This is a cheap source of bioactive compounds with high potential as green and natural additives in the food and cosmetics industries.

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