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Original Article

A High Antibacterial Efficacy of Fruits of *Litsea cubeba* (Lour.) Pers from Nepal. GC-MS and Antioxidative Capacity Analyses

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ABSTRACT

Background: Biological activities of the plant materials may vary with different geographic origin. *Litsea cubeba* fruits are consumed in Nepal as a spice ingredient and are considered to be possessed antibacterial property. **Objectives:** Aims of this work are: to compare phytoconstituents present in litsea oil by GC-MS analysis, and to evaluate antibacterial and antioxidant activities of the volatile oil and the fruit extracts. **Materials and Methods:** Hydrodistillation of *L. cubeba* fruits was performed to obtain litsea oil followed by extractions with boiling methanol. Fractionations of the extract provided hexane, dichloromethane, ethyl acetate and aqueous extracts. The oil and extracts were analysed for the phytochemicals present. They were evaluated by using antibacterial susceptibility, brine-shrimp lethality and antioxidant capacity assays. **Results:** All together 49 compounds (accounting 93.66%) were identified in the litsea oil by GC-MS. Monoterpenes being dominant components, the oil constituted 15.96% of citral (geranial and neral). Other major constituents were capric acid (12.44%), β-caryophyllene oxide (7.69%), linalool (5.96%), eucalyptol (5.13%) and *cis*-β-terpineol (4.22%). Litsea oil, hexane extract and dichloromethane extract displayed very effective antibacterial property. Ethyl acetate extract (LC_{50} = 21.52 µg mL^{-1}) and litsea oil (LC_{50} = 31.62 µg mL^{-1}) were found highly cytotoxic against brine-shrimp nauplii. Ethyl acetate (IC_{50} = 124.57 µg mL^{-1}) and dichloromethane (IC_{50} = 271.08 µg mL^{-1}) extracts displayed a modest DPPH free radical scavenging activity. **Conclusion:** Phytoconstituents present in the *L. cubeba* fruits from Nepal were analysed. Litsea oil and the extracts have displayed high antibacterial and potentially anticarcinogenic activities.

Key words: Antioxidant, Brine-shrimp assay, DPPH assay, GC-MS analysis, Litsea oil, Minimum bactericidal concentration.

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INTRODUCTION

Litsea cubeba (Lour.) Pers (Nepali name: siltimur, family: Lauraceae) is native to East Asia and distributed in East and Central Nepal at 1000-2700 meters altitude.[1] It is a perennial, dioecious tree or shrub, 5-12 meters high, lemongrass scented arils and pepper shaped fruits. It contains amides, alkaloids, steroids, terpenoids, fatty acids and lignans.[2]

Hydrodistillation of *L. cubeba* fruits produces litsea oil, which has lemon like spicy odour and used for manufacturing of citral, vitamins A, E and K, ionone, methylionone and perfumes.[3-5] Its antibacterial,[6,7] antifungal,[8] antioxidant,[9] mosquito repellent[10] and anticancer[11] properties are recently reported.

The composition of *L. cubeba* oil has been reviewed in a few literatures.[12,13] Literature search revealed that the chemical composition of litsea oil varies with different geographic areas and thereby the synergistic or antagonistic effects cause different biological activities.[7,14-17] Several authors have reported that citral (α-citral; geranial and β-citral; neral) is the major constituent in the oil.[7,11,18-21] In Indian litsea oil, Gogoi *et al.* have reported that citral (geranial 51.9% and neral 40.7%) was the major constituent.[18] On the other hand, citronellal (44.8-77.2%) and citronellol (10.9-14.0%) were the major constituents but not citral (geranial 0.2-0.4%) in litsea oil from Assam, India.[22] Litsea oil of Chinese variety contained nerol, β-caryophyllene, 2,6-dimethyl-5-heptenal and 6-methyl-5-hepten-2-ol as major constituents.[22] On the other hand, the major constituents of Tibetian, Chinese litsea oil were limonol (44.2%), linalool (8.8%), eucalyptol (5.4%), elemicin (3.9%), methyl euginol (3.8%), etc.[8] In litsea oil from Jiangxi province of China, Si *et al.*[15] have reported that citral (80.6-82.9%) and limonene (4.1-5.3%) were the major constituents, while the composition was varied with citral (47.9%) and limonene (26.2%) in a report of Wang *et al.*[23] The ethanolic extract of *L. cubeba* fruits from Zhejiang Province, China was analysed by Hu *et al.*, and thirty compounds were identified with citral (59.3%) and limonene (8.5%) as the predominant compounds.[14] Therefore, the composition of litsea oil remarkably varied within India and China. Since Nepal lies between these two countries, we decided to compare the variation in the oil composition from our own product to the global distribution and its affects in the bioactivities. Herein, we report the identification and quantification of volatiles of litsea oil by GC-MS, phytochemical screening of the fruit extracts, and *in vitro* evaluation of antibacterial activity and antioxidant capacity of the oil and extracts.

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MATERIALS AND METHODS

Plant material

Fresh fruits of *L. cubeba* (Lour.) Pers were collected from Shantipur, Gulmi District, Western Nepal (altitude 1300 meters, latitude 28°6′ N and longitude 83°24′ E) in June 2012. The plant species was authenticated from National Herbarium and Plant Research Lab, Godawori, Lalitpur, Nepal with a Voucher Specimen Number 19-070/071.

Extraction of the plant material

Hydrodistillation of the dried and coarsely ground fruit sample (500 g) using a Clevenger apparatus (oil lighter than water type) for 4.5 h afforded litsea oil (7 mL, 1.40% v/w), which was dried over anhydrous Na_2SO_4 and stored at 4°C until analysis. The marc remained was extracted with boiling methanol (800 mL × 5 h × 3 times). The combined alcoholic extract was concentrated, diluted with water and then subsequently fractionated with hexane (500 mL × 4), dichloromethane (500 mL × 4) and ethyl acetate (500 mL × 4) followed by concentrations to obtain corresponding hexane, dichloromethane, ethyl acetate and aqueous extracts.

Phytochemical screening

The fruit extracts were subjected to different qualitative tests to identify the presence of various phytochemical constituents.[24,25] Following tests were conducted: Dragendorff's test for alkaloids, Liebermann-Burchard's test for sterols and triterpenes, Shinoda's test for flavonoids, Borntiager's test for anthraquinones, Kedde's test for cardiac glycosides, Molisch's test for polyoses, ferric chloride test for tannins and polyphenols, dinitrobenzoic acid test for lactones, fluorescence test for coumarins, spot test for fatty acids, froth test for saponins, acid test for carotenoids, and base tests for anthocyanosides and anthracenosides.

Gas chromatography-mass spectrometry analysis

Chemical constituent of litsea oil was analysed by GC-MS using an Agilent 7890A GC system coupled with an Agilent 5975 C mass selective detector. The GC column was HP-5MS (5% phenyl methyl siloxane, Agilent 19091S-433, 30 m × 250 µm internal diameter, 0.25 µm film thickness). Helium was used as a carrier gas at flow rate of 1 mL min^{-1}. A 5% v/v sample solution was prepared in methanol and a volume of 0.5 µL was injected using a split mode. The initial column temperature was set at 50°C held for 2 min, ramped at a rate of 3°C min^{-1} to 120°C and held for 2 min, and then increased to 250°C at rate of 15°C min^{-1} (total run time 41 min). The instrument was operated in the electron impact (EI) mode at 70 eV and ion source temperature 230°C in the scan range of 50-1000 amu. Volatile constituents were identified by comparing the mass spectra available in a MS database (NIST 08). The identification was also made by comparing relative retention indices (RI) with the literature.[14-15,22]

Antibacterial susceptibility assay

The litsea oil and fruit extracts were screened for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* (ATCC 25923), *Enterobacter cloacae*, *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14038) and *Serratia marcescens* using the agar well diffusion assay.[26] Briefly, 50 µL of the sample solution in dimethyl sulphoxide (concentration of 50 mg mL^{-1}) was dispensed into the well (6 mm) bored in bacteria stained Mueller Hinton agar plates. Gentamycin (10 µg disc) was used as a positive control. ZOI produced were measured after incubation at 37°C for 24 h.

Microbroth dilution methodology was used to determine MBC values.[27] Briefly, the mixture of nutrient broth agar (1 mL) and 1 mL of the sample solution in dimethyl sulphoxide (concentration of 50 mg mL^{-1}) was serially diluted and then inoculated with bacterial strains (50 µL). After incubation at 37°C for 24 h, a loopful of each bacterial solution was sub-cultured by streaking in nutrient agar plate. After incubation at 37°C for 24 h, the MBC values were determined.

Brine-shrimp lethality assay

The brine-shrimp assay was used to evaluate cytotoxicity.[28] Briefly, freshly hatched ten brine-shrimp (*Artemia salina*, procured from San Francisco Bay Brand Inc., USA) nauplii in triplicates were exposed to the sample in 10, 100 and 1,000 µg mL^{-1} dose levels in artificial sea water (total volume 5 mL) under warmth. After 24 h, the number of survivors was counted. The percentage death and LC_{50} values were computed.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was used to evaluate antioxidant capacity.[29] Briefly, overnight stirring of 3.9 mg of DPPH (Sigma-Aldrich) in methanol (100 mL) at 0°C gave DPPH free radical (DPPH•) solution. Working solutions of litsea oil and fruit extracts were prepared in methanol (concentrations 5, 50, 100, 250 and 500 µg mL^{-1}). Distilled water was used as a solvent to prepare working solutions of the aqueous extract. To 0.5 mL of the working solution was added 2.5 mL of DPPH• solution. A control was prepared by mixing methanol or distilled water (0.5 mL) and DPPH• solution (2.5 mL). These solutions were vortexed, kept at room temperature in the dark for 30 min and then absorbance was measured at 517 nm using a 6715 UV/Vis Spectrophotometer JENWAY. The inhibition percentage was calculated as:

Scavenging activity (% of inhibition) = (1 – Asample/Acontrol) × 100 (1)

where, Asample and Acontrol are the absorbance values of the reaction mixture with and without sample, respectively. IC_{50} values were calculated. A standard linear curve was constructed using methanolic solutions of gallic acid with known concentrations of 5, 25, 50 and 75 µg mL^{-1}. The DPPH value obtained was considered the slope of the linear curve derived from the constructed graph and expressed in gallic acid equivalence (GAE).

RESULTS

Extraction

Litsea oil was obtained in 1.4% v/w yield by hydrodistillation of the dried fruits of *L. cubeba* as yellow oil. Subsequently, the marc was extracted with boiling methanol, concentrated and then diluted with water. Fractionation using hexane, dichloromethane and ethyl acetate followed by concentration afforded hexane extract (19.60 g, 3.92%), dichloromethane extract (2.76 g, 0.55%), ethyl acetate extract (1.63 g, 0.33%) and aqueous extract (58.61 g, 11.72%).

Phytochemical analysis

Although the chemical tests were essentially qualitative, phytochemical screening of the extracts of *L. cubeba* fruits revealed the presence of alkaloids, steroids, terpenoids, polyoses, polyphenols, coumarins, fatty acids, saponins and carotenoids.

GC-MS analysis of litsea oil led to identify 49 compounds accounting 93.66% of the oil and the data are presented in Table 1.

Biological assays

The results of antibacterial susceptibility assay of litsea oil along with four different extracts against a total of eight Gram-positive and Gram-negative bacteria are depicted in Table 2. The results of the brine-shrimp and DPPH assays are summarized in Table 3.

Table 1: Chemical composition of litsea oil of *L. cubeba* fruits.

RI[a]	Compound	%	RI[a]	Compound	%
3.286	3-methyl-2-butenal; prenal	0.08	16.639	borneol	0.12
6.790	α-thujene	0.30	17.207	4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol; terpinen-4-ol	2.06
6.948	α-pinene	0.97			
7.443	camphene	0.07	17.459	1-(2-methylphenyl)-ethanone	0.29
8.474	β-pinene	3.66	17.828	α-terpineol	0.99
9.158	6-methyl-5-hepten-2-one; sulcatone	4.59	18.007	dihydronopol	0.66
9.326	6-methyl-5-hepten-2-ol ; sulcatol	0.13	20.395	neral (β-citral)	7.23
10.115	(+)-4-carene	0.13	20.479	1-methyl-2-acetyl-3-(1-methylethenyl)-cyclopentane	1.24
10.431	*p*-cymene	3.55			
10.550	*o*-cymene	1.24	20.721	3-methyl-6-(1-methylethyl)-2-cyclohexen-1-one; piperitone	0.30
10.768	eucalyptol	5.13			
11.123	hexylene glycol	0.36	21.784	geranial (α-citral)	8.73
11.736	2,6-dimethyl-5-heptenal; bergamal	0.46	23.794	methyl 3,7-dimethylocta-2,6-dienoate; methyl geranate	2.45
11.853	γ-terpinene	0.33			
12.419	*cis*-β-terpineol	4.22	29.097	n-decanoic acid; capric acid	12.44
12.609	*trans*-linaloloxide	1.99	30.202	isocaryophyllene	0.65
13.293	*cis*-linaloloxide	2.81	30.530	methyl 10-methylundecanoate	1.31
13.987	linalool	5.96	30.549	methyl dodecanoate; methyl laurate	2.25
14.282	tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2H-pyran; rose oxide	0.14	31.464	caryophyllene oxide	7.69
			32.695	dodecanoic acid; lauric acid	0.29
14.741	3,6-dimethyl-2-octanone	0.47	33.432	tetradecanoic acid; myristic acid	0.93
14.882	α-campholenal	0.52	33.842	1-heptatriacotanol	0.08
15.197	2,6-dimethyl-5-heptenol; melonol	0.14	34.474	2,10-dimethyl-9-undecenol	0.64
15.376	isopinocarveol	0.56	34.642	9-hexadecenoic acid; palmitelaidic acid	0.17
15.765	isopulegol	0.89	34.821	n-hexadecanoic acid; palmitic acid	1.56
16.207	3,7-dimethyl-6-octenal; β-citronellal	2.26	35.926	*cis*-13-octadecenoic acid	0.14
16.334	5-isopropyl-bicyclo[3.1.0]hexan-2-one; Sabina ketone	0.36	36.557	1α,3α,4β,6α-4,7,7-trimethyl-bicyclo[4.1.0]heptan-3-ol; 4-caranol	0.12

[a]Retention indices on a HP-5MS (Agilent 19091S-433, 30 m × 250 μm internal diameter, 0.25 μm film thickness).

Table 2: Antibacterial activity of the volatile oil and extracts of *L. cubeba* fruits.

Pathogenic bacteria used	ZOI in mm (MBC value in μg mL^{-1})					
	Litsea oil	HE	DE	EE	AE	Gentamycin
Bacillus subtilis	17 (390.6)	15 (195.3)	16 (1562.5)	–	–	33
Staphylococcus aureus	19 (6.1)	17 (6.1)	17 (3125.0)	9 (25.0)	–	30
Enterobacter cloacae	19 (48.8)	22 (6.1)	12 (195.3)	13 (3125.0)	–	23
Escherichia coli	17 (0.38)	15 (24.4)	15 (12500.0)	–	–	23
Klebsiella pneumonia	19 (12.2)	22 (48.8)	16 (3125.0)	–	11 (6250.0)	30
Pseudomonas aeruginosa	12 (12.2)	–	–	–	–	32
Salmonella typhimurium	17 (48.8)	16 (195.3)	13 (97.6)	–	–	30
Serratia marcescens	10 (195.3)	–	–	–	–	30

Notes: HE = Hexane extract, DE = Dichloromethane extract, EE = Ethyl acetate extract, AE = Aqueous extract, (–) sign = no ZOI was observed.

Table 3: Antioxidant activity of the volatile oil and extracts of *L. cubeba* fruits.

Samples	Brine-shrimp lethality LC_{50} (μg mL^{-1})	DPPH scavenging activity IC_{50} (μg mL^{-1})	DPPH value (mg GAE g^{-1} dried plant material)
Litsea oil	31.62	1628.85	Not calculated
Hexane extract	130.31	1511.33	Not calculated
Dichloromethane extract	348.33	271.08	0.52
Ethyl acetate extract	21.52	124.57	0.47
Aqueous extract	1000.00	426.74	5.30
Gallic acid	-	8.56	-

DISCUSSION

Citral and limonene were reported as major constituents in litsea oil.[7,11,14,18-20,22,23] Being citral as major constituent, litsea oil from Nepal constituted neral (7.23%) and geranial (8.73%) (Table 1). Other major components present in the oil were capric acid (12.44%), caryophyllene oxide (7.69%), linalool (5.96%), eucalyptol (5.13%), *cis*-β-terpineol (4.22%), 6-methyl-5-hepten-2-one (4.59%), β-pinene (3.66%), *p*-cymene (3.55%), etc. Limonene was not detected in the volatile oil. To the best of our knowledge, 3-methyl-2-butenal, hexylene glycol, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2*H*-pyran, 1-(2-methylphenyl)-ethanone, dihydronopol, 1-methyl-2-acetyl-3-(1-methylethenyl)-cyclopentane, 1α,3α,4β,6α-4,7,7-trimethyl-bicyclo[4.1.0]heptan-3-ol and 4-caranol were identified for the first time in litsea oil.

Litsea oil showed a higher potentiality for antibacterial activity with ZOI values ranging 10-19 mm and MBC values ranging 0.38-390.6 μg mL^{-1} for the Gram-positive and Gram-negative bacteria tested (Table 2). When we compared the antibacterial activity of litsea oil to that of Chinese variety reported by Wang and Liu,[7] the oil used in present study was found more effective against *S. aureus* (MBC = 6.1 *versus* MIC = 200 μg mL^{-1}), *E. coli* (MBC = 0.38 *versus* MIC = 500 μg mL^{-1}) and *P. aeruginosa* (MBC = 12.2 *versus* MIC = 620 μg mL^{-1}). The presence of significant amounts of fatty acids and linalool could be responsible for a high antibacterial efficacy of the volatile oil from Nepal. The antibacterial activity of fatty acids and linalool is reported.[30,31] The hexane and dichloromethane extracts were also found effective except for two strains of bacteria used *viz. S. marcescens* and *P. aeruginosa*. The ethyl acetate extract showed antibacterial activity against *S. aureus* (ZOI = 9 mm, MBC = 25.0 μg mL^{-1}) and *E. cloacae* (ZOI = 13 mm, MBC = 3125.0 μg mL^{-1}). The aqueous extract showed poor activity compared to other extracts and could inhibit the growth of *K. pneumonia* (ZOI = 11 mm, MBC = 6250.0 μg mL^{-1}). The ethyl acetate and aqueous extracts were completely ineffective against *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. typhimurium* and *S. marcescens* upon loading of 2.5 mg dose level.

The brine-shrimp assay is reasonably reliable for screening of bioactivity of the plant materials despite inadequate to evaluate the anticancer property. All the isolates exhibited LC_{50} = <1000 μg mL^{-1} and therefore were considered pharmacologically active. Litsea oil (LC_{50} = 31.62 μg mL^{-1}) and the ethyl acetate extract (LC_{50} = 21.52 μg mL^{-1}) showed very high cytotoxicity, both hexane (LC_{50} = 130.31 μg mL^{-1}) and dichloromethane (LC_{50} = 348.33 μg mL^{-1}) extracts were moderately cytotoxic, and the aqueous extract has displayed marginal cytotoxicity (LC_{50} = 1000.00 μg mL^{-1}). Recently, we[32] and another group[33] have reported the brine-shrimp lethality assay of litsea oil.

To the best of our knowledge, DPPH free radical scavenging activity of *L. cubeba* is rarely investigated.[9,34] The ethyl acetate and dichloromethane extracts displayed a modest antioxidant activity with IC_{50} values of 124.57 and 271.08 μg mL^{-1}, respectively (Table 3). The aqueous extract exhibited a weak antioxidant activity (IC_{50} = 426.74 μg mL^{-1}). Although litsea oil and the hexane extract were cytotoxic against brine-shrimp nauplii; however, they were poor in scavenging of DPPH free radical. The aqueous extract possesses a high quantity of antioxidants as GAE due to its higher extractive value (for dichloromethane extract = 0.55%, ethyl acetate extract = 0.33% and aqueous extract = 11.72%).

CONCLUSION

In conclusion, litsea oil of *L. cubeba* fruits from Nepal was analyzed by GC-MS and forty-nine components were identified. Besides citral; capric acid, caryophyllene oxide, linalool, eucalyptol, *cis*-β-terpineol, 6-methyl-5-hepten-2-one, β-pinene, *p*-cymene, etc. were abundantly deposited in the oil showing genuineness of the product. Litsea oil and other extracts (particularly hexane and dichloromethane extracts) displayed a high antibacterial activity. The study of brine-shrimp and DPPH assays of the oil and extracts indicated that the plant material constituted of potential anticarcinogenic agents.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

DPPH: 2,2-diphenyl-1-picrylhydrazyl; GC-MS: gas chromatography-mass spectrometry; IC_{50}: 50% inhibition concentration; LC_{50}: lethal concentration for 50% mortality; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; ZOI: zone of inhibition.

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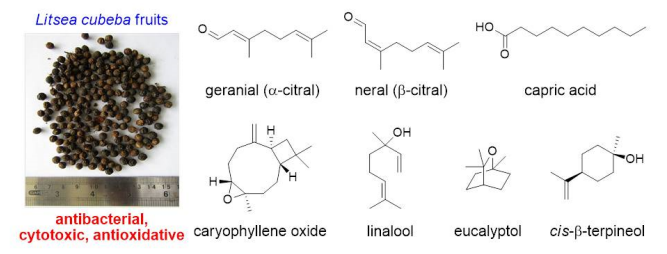
GRAPHICAL ABSTRACT



SUMMARY

- This work showed litesa oil from the fruits of *Litsea cubeba* (Lour.) Pers from Nepal abundantly constituted citral, capric acid, caryophyllene oxide, linalool, eucalyptol, *cis*-β-terpineol, etc.
- Litsea oil and the fruits extracts have displayed high antibacterial and potentially anticarcinogenic activities using antibacterial susceptibility, brine-shrimp lethality and DPPH assays.

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