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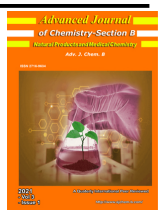

Original Research Article

Study of Phyto- and Physicochemical Analysis, Antimicrobial and Antioxidant Activities of Essential Oil Extract of *Callistemon citrinus* (Curtis) Skeels Leaves

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ABSTRACT

The essential oil was extracted from the leaves of *Callistemon citrinus* (Bottlebrush) by hydro-distillation using a Clevenger apparatus. The component analysis of the oil was carried out by GC-MS technique, which showed eucalyptol (56.47%), α-pinene (18.80%), and D-limonene (9.91%), as the major compounds in *C. citrinus* oil. The physicochemical parameters such as yield percentage, specific gravity, refractive index, optical rotation, acid value, saponification value, and ester value were determined. The antioxidant activities of extracting oils were determined by DPPH assay and the radical scavenging activity (IC_{50}) value was calculated. The remarkable free radical scavenging effect was observed in bottlebrush oil with IC_{50} = 390.73 µg/mL. Bottlebrush oil (50% and 100%) exhibited antimicrobial activity against all the four selected strains of bacteria (*E. coli*, *S. aureus, B. subtilis, S. dysenteriae*) as well as fungi (*Candida albicans)*.

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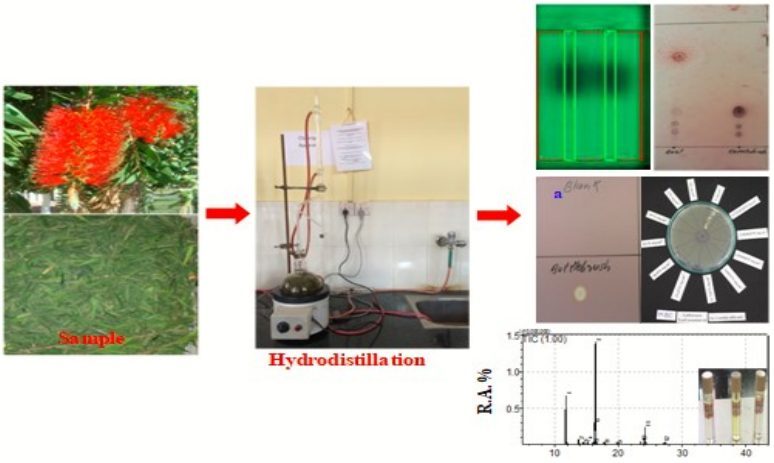
GRAPHICAL ABSTRACT



Introduction

The volatiles from plant materials have been used for various therapeutic applications since ancient medicinal practices and they find various important applications even today [1]. The essential oil derived from different plant parts has been described in religious volumes and find the great value of religious and cultural point of view. Plant materials are being excessively used by traditional practitioners, despite their poor scientific data [1-3].

Essential oils extracted from plants capturing plant's represent one of the most promising herbal medicines which are widely used for the cure of various health ailments like depression, urine associated problems, skin ailments, swollen joints, insomnia, muscular pain, etc. because of the permeability properties on the skin [4-7]. They are a mixture of saturated and unsaturated compounds with colorless pleasant smelling and are applied in various Ayurvedic, allopathic, aromatherapy, including food, cosmetics, and medicinal industries [4]. The essential oil production industry commercially uses several aromatic plant species to extract the high-quality essential oil. The interest is growing in the use of essential oils derived particularly from aromatic plants in the formulation of alternative antimicrobial drugs because of their influential biological activities [8].

Most species of *Callistemon* are known in traditional medicine for their anti-cough behavior and the essential oil derived from it has been used as antimicrobial and antifungal agents [7-11]. *Callistemon citrinus* (Curtis) skeels commonly known as lemon bottlebrush, red bottlebrush, or crimson bottlebrush are flowering plants belonging to the family Myrtaceae [12]. The entire genus is endemic to Australia but widely cultivated in many regions of Asia and America, including Nepal as an ornamental plant [11]. It is a shrub with a height of about 7.5 m tall, bearing beautiful red flowers with dark anthers [13]. They bear attractive narrow foliage with lanceolate (3-6 cm wide and 40-70 mm long) arrangement possessing white papery bark [14]. The entire foliage and flowers pose a pleasant aromatic odor and because of its revitalizing pleasant flavor, its leaves are used as a tea substitute [15]. A good quality essential oil can be extracted from the leaves of *C. citrinus* that are applied in ethnomedicine to treat conditions like gastrointestinal distress, pain,

and infections from bacteria, fungi, viruses, and parasites [16]. Many previous studies reported monoterpenoid, most essentially a Eucalyptol (1,8-Cineole), as a major constituent in the essential oil of *C. citrinus* [17-19]. Eucalyptol, a cyclic ether is an essential ingredient in many modern brands of mouthwash and cough suppressant and is proven to control airway mucus hypersecretion and asthma *via* anti-inflammatory cytokine inhibition. It has been also used as food flavoring agents, and in many cosmetics because of its pleasant smell and fungitoxicity nature [20].

Several works on the exploitation of extracting essential oil from the leaves of *C. citrinus* for its chemical, physicochemical parameters, and antimicrobial activity have already been discussed, yet additional information on percentage yield, physical, and biochemical study of essential oil from its leaves are depicted in this study. Furthermore, the work is also expanded to check antimicrobial and antioxidant activities to realize its numerous restorative behaviors.

Experimental

Plant material

Leaves of *C. citrinus* were collected from Shankhamool of Lalitpur district of Nepal in December 2017. The identification of specimen was carried out at the Department of Plant Resources, National Herbarium, and Plant Laboratory, Godawari, Lalitpur, Nepal.

Chemicals and reagents

All the chemicals used in this experiment were of analytical grade, which was 2,2-diphenyl-1-picrylhydrazyl (DPPH) (St. Louis, USA), ascorbic acid (Merck, Germany), dimethyl sulphoxide (DMSO) (Qualigens, India), and concentrated sulphuric acid (Ranboxy chemicals, India), polysorbate-80, hexane, nutrient agar (NA), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), Muller Hinton agar (MHA), methylene blue, lactose broth (LB) and Sabouraud dextrose broth were from the biological section of Department of Plant Resources, Thapathali, Kathmandu, Nepal.

Oil extraction

Fresh *C. citrinus* leaves were thoroughly washed with water and air-dried at room temperature (24 °C) for 10 days. The air-dried samples were subjected to hydro-distillation in a Clevenger-type apparatus for 6 hours at 50-60 °C, followed by draining off water and collected oil sample. Oil was further accumulated in the vial, dried over Na_2SO_4, and stored in an airtight container at 4 °C.

GC–MS analysis

Chemical constituents of essential oil were analyzed by gas chromatography (Shimadzu GC 2010) at Department of Plant Resources, Thapathali, Kathmandu, having an RTX-5 MS column (30 m×~0.25 mm ×~0.25 μm) using helium as a carrier gas. The sample (1μL) diluted with spectroscopic grade hexane in a ratio 1:10 was injected into the GC inlet maintaining constant column flow 0.68 $mLmin^{-1}$ and purge flow 3 $mLmin^{-1}$ in split mode. The initial column oven temperature was set at 40 ºC and the injection temperature was 250 ºC. The detector gain mode was relative, scanning time was from 4.00 min to 68 min and scan speed was 666 with an m/z range of 40.00-350.00. Relative amounts of detecting compounds were calculated based on GC peak areas. The MS libraries used for comparison were FFNSC1.3 and NIST 11.

Determination of physicochemical parameters

Various physicochemical properties such as an oil percentage (yields), specific gravity, acid value, saponification value, and ester value were determined by standard methods given by Yadav, et *al.,* (2017) [21] and Poudyal *et al.,* (2012) [22]. The refractive index of the oil was determined at 20 °C using Rudolph J357 automatic refractometer and optical rotation was determined using Rudolph Research Analytical Autopol, Automatic Polarimeter at the

Department of Plant Resources, Thapathali, Kathmandu.

Antimicrobial activity

A 50% oil sample was prepared by dissolving 100% oil in 10% aqueous DMSO with 5% v/v polysorbate-80 and the final volume was made up to 10 mL. The antimicrobial activity of both 100% and 50% oil sample were screened against different strains of Gram-positive bacteria; *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 6538P) and Gram-negative bacteria; *Escherichia coli* (ATCC 8739) and *Shigella dysenteriae* (ATCC 13313) and a fungi *Candida albicans* (ATCC 2091) by disc diffusion method. A sterile swab was used to evenly distribute the organisms in a Muller-Hinton agar (MHA) and potato dextrose agar (PDA) as a nutrient medium for bacteria and fungi respectively. Afterward, four sterile 6 mm diameter absorbent blank filter paper disc was placed in inoculated agar plates were 100% (pure), 50% oil sample along with DMSO as negative control were micropipettes. After incubation at 37 ºC (bacteria) and 25 ºC (fungi) for 18 to 24 hours, the diameter of the zone of inhibition (ZOI) was noted for each concentration in the plates [23].

Antioxidant activity

The free radical scavenging activity of the *C. citrinus* essential oil (EO) was measured by recording the extent of bleaching of the purple-colored DPPH solution to yellow. Samples were prepared by dissolving 40 mg oil to 1 mL methanol. Different concentrations (100, 200, and 300 µg/mL) of methanol solutions of each extract were prepared by the serial dilution of the stock solution of the respective extract. To each methanol solution of extract 2.5 mL, DPPH solution and 0.5 mL methanol were added. A control was prepared by mixing 1 mL distilled water and 0.5 mL methanol in 2.5 mL DPPH solution. After 30 min of incubation at room temperature, the absorbance was taken against a blank at 517 nm using a spectrophotometer [17]. DPPH free radical scavenging activity with the reduction in absorbance of the sample was taken as a measure of their antioxidant activity IC_{50}, which represented the concentration of the EO that caused 50% neutralization of DPPH radicals, was calculated from the calibration curve plotting between percentage inhibition of DDPH radicals and concentration of sample [10,18].

$$\text{Inhibition (\%)} = [(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}}] \times 100$$

Where, A_{Control} = absorbance of the control,

A_{Sample} = absorbance of the sample at 517 nm.

Results and Discussion

Component analysis

The volatile oil obtained by hydro-distillation of *C. Citrinus* leaves gave a total of 1% of the yield on a dry weight basis. The GC-MS analysis of *C. citrinus* identified thirteen components where eucalyptol (1,8-cineole) (56.47%), α-pinene (18.80%), D-limonene (9.91%), and α-terpenol (7.13%) were found as major components and out of which eucalyptol (1,8-cineole) was present in higher percentage [19,24,25]. The gas chromatogram and relative concentrations of the volatile components identified are presented in Figure 1 and Table 1.

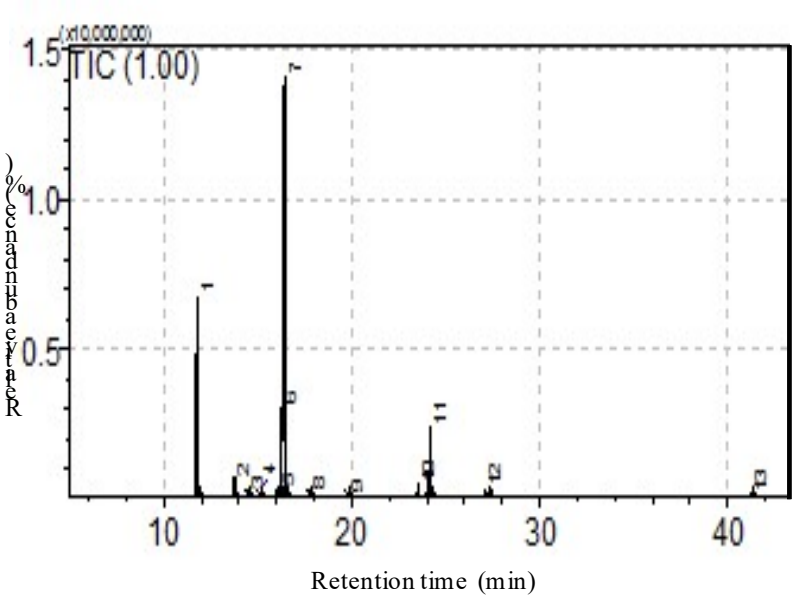


Fig. 1: Gas chromatogram of essential oil from *C. citrinus* leaves

Comparing results with those of Oydeji *et al.,* (2009), *C. citrinus* from South Africa revealed a greater percentage of 1,8-cineole (61.2%) and a lesser percentage of α-pinene (13.4%) as the major constituents compared to 56.47% of 1,8-cineole and 18.80% of α-pinene of this study from *C. citrinus* [14]. The presence of eucalyptol as a major component revealed its encouraging effect on the lung function parameters, whether for the common cold or continual obstructive pulmonary ailment [13]. The determination of these bioactive compounds in essential oil leaf extract of *C. citrinus* possesses synergistic effects which may be defined for the therapeutic benefits of this plant.

Table 1: Chemical composition of essential oils from *C. citrinus leaves*

S.N.	Name of compound	Retention time	Area (%)
1	α-pinene	11.775	18.80
2	β-pinene	13.754	1.81
3	Myrcene	14.456	0.62
4	γ-terpinene	15.059	0.41
5	Para cymene	16.066	0.99
6	D-Limonene	16.272	9.91
7	Eucalyptol	16.427	56.47
8	Terpinene-γ	17.738	0.60
9	Linalool	19.742	0.55
10	Terpinen-4-ol	23.518	1.28
11	α-terpineol	24.157	7.13
12	(R)-lavandulyl acetate	27.113	0.95
13	β-humulene	41.174	0.49

Similarly, essential oil of *C. citrinus* from Ethiopia reported by Aweke and Yeshanew, (2016) contained fifteen major compounds 76.9 % of eucalyptol and 15.3% of α-terpenol compared to only 56.47% and 7.13% of eucalyptol and α-terpenol from this investigated oil of *C. citrinus* [8]. This difference in composition of components may be attributed to different geographical locations, climatic conditions, and time of sample harvesting which largely determines the composition of essential oil.

Physicochemical parameters

Hydro-distillation of fresh leaves of *C. citrinus* gave a yield of 1% (w/w) volatile oil on the dry weight basis. The oil was characterized by a strong odor, colorless appearance, and soluble in organic solvents. Other physicochemical parameters such as refractive index, specific gravity, specific optical rotation, acid value, saponification value, and ester values of the oil were also determined by standard protocol and presented in Table 2. The physicochemical parameters help to determine the physical nature and chemical properties of the oil. The percentage yield of essential oil is high (1%) and is a profit from its commercialization in allopathy and aromatherapy and novel drug production as it has proved to have many health benefits. The specific gravity and refractive index values are close to each other. The oil is dextrorotatory at 20 °C. The saponification value and the acid number are less for essential oil indicating the presence of less amount of fatty acids and free acids [22]. The lesser value of acid value is of the utmost importance as it reveals less deterioration or rancidity of the oil.

Other than percentage yield all other physicochemical values were found to be less than the previous study by Awake and Yeshanew, (2016) to our study [8]. The variation in the acid, saponification, and ester value may be attributed to the rate of hydrolysis and oxidation of ester linkages present in the essential oil which is directly related to the storage time of essential oil.

Table 2: Physicochemical parameters of essential oils from leaves of *C. citrinus*

Parameters	Values
Percentage yield	1%
Specific gravity (20 °C)	0.91
Refractive index (20 °C)	1.42
Optical rotation (20 °C)	2.10
Acid value	1.02
Saponification value	1.86
Ester value	0.84

Antimicrobial activity

The antibacterial activity of the oil was carried out using the agar disc diffusion method. The zone of inhibition revealed by 100%, 50 % of an essential oil sample against selected both gram (+ve) and gram (-ve) species of the test bacteria and fungi are represented in figure 3. The highest antibacterial activity was observed on *S. aureus* (13.2 mm) followed by *B. subtilis* (10.4 mm), *E. coli* (9.9 mm), and the last response was shown by *S. dysenteriae* (9.2 mm) at the tested dose of 100 % of oil concentration. A similar trend was observed in 50% of the oil concentration of the bacteria tested, which is presented in the bar diagram (figure 2). Similarly, fungi (*Candida albicans)* 10.8 mm of ZOI at 50% of concentration and complete ZOI at 100% of concentration were recorded.

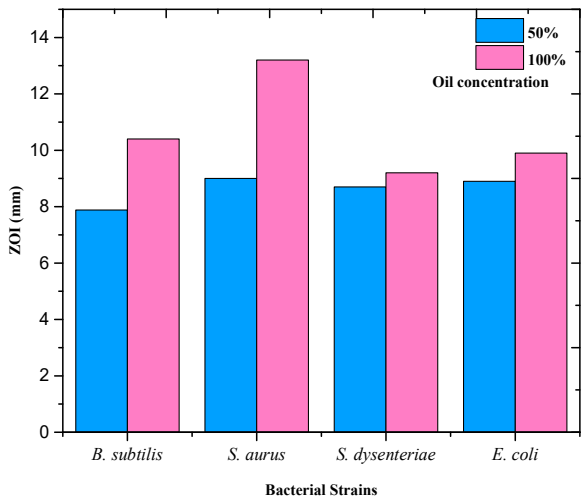


Fig. 2: Bar diagram for the zone of inhibition by 50% and 100% concentrations of *C. citrinus* oil against selected bacterial strains

Both gram-positive and gram-negative bacteria were inhibited by *C. citrinus* although gram-positive bacteria were found to be more susceptible. The antibacterial activity demonstrated in the essential oil of this plant material could be attributed to the presence of 1,8-cineole (Eucalyptol) and α-terpineol along with D-limonene that are found to be major constituents of the oil [20,26,27]. Several previous studies reported that the presence of these constituents has antimicrobial activities yet, trace constituents could also play a role in the antibacterial effect of the oil [16,24]. The good inhibitory effect for a gram-positive bacterium (*S. aureus*) represents that the essential oil extracts can be used to cure lower respiratory tract infection, ventilator-assisted pneumonia, and osteomyelitis as *S. aureus* are responsible to cause those diseases [15].

This study and previous studies exhibited that the *C. citrinus* leaf extracts contain antibacterial components and support the use of *Callistemon spp.* to protect against contamination caused by bacteria and fungi [28].

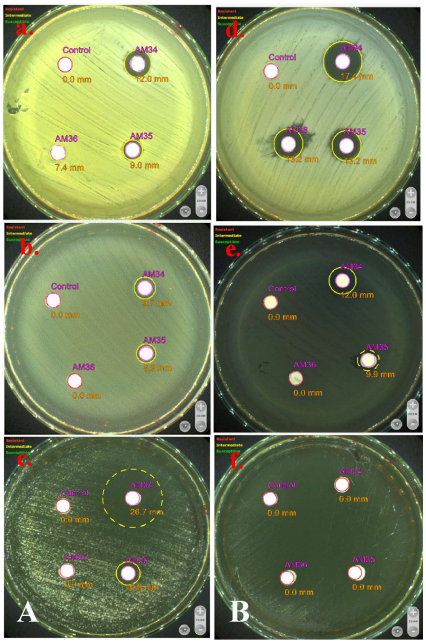


Fig. 3: The representative pictures of the zone of inhibition shown by (A) 50% (B) 100% concentrations of essential oil from *Callistemon citrinus* leaves (marked as AM35) against (a&d) *Staphylococcus aureus* (b&e) *Escherichia coli* and (c&f) *Candida albicans*

Antioxidant activity

The percentage inhibition of DPPH radical and the half of maximum inhibitory concentration (IC_{50}) value was calculated from the calibration curve plotting percentage inhibition against different concentrations of oils (figure 4) to detect the antioxidant activity of oil using commercially available ascorbic acid as a positive control.

The results of the antioxidant activity of *C. citrinus* oil are presented in Table 3. Percentage inhibition of DPPH radical by *C. citrinus* oil showed the inhibition % of 23.08, 27.47, and 42.86 at the concentrations of 100, 200, and 300 µg/mL respectively. The IC_{50} calculated from the calibration curve was found to be 390.73 µg/mL. Similarly, the % inhibition of DPPH radical by control (ascorbic acid) was 51.65, 58.24, and 61.54 % respectively, and the IC_{50} value was calculated to be 55.49 µg/mL.

Table 3: Percentage inhibition of DPPH radicals and IC_{50} value of EO of *C. citrinus* and control (Ascorbic acid)

Components	Concentration (µg/mL)	% Inhibition of DPPH radicals	IC_{50} (µg/mL)
Ascorbic acid	100	51.65	
(Vitamin C)	200	58.24	55.49
	300	61.54	
Callistemon citrinus	100	23.08	
(Bottlebrush) oil	200	27.47	390.73
	300	42.86	

Although the ascorbic acid depicted a greater value of % inhibition and IC_{50} than that of *C. citrinus* oil at a given concentration, the % inhibition of *C. citrinus* exhibited a good antioxidant property. Apart from our study, the extracts from its flower and leaves exhibited excellent scavenging activity as shown in previous studies of Larayetan *et al.,* (2019) and Lagana *et al.,* (2020) [15,29]. The disparities could be in the geographical difference in the availability of the plants.

Furthermore, it is reported that the antioxidant activity present in essential oils is because of the monoterpene (eucalyptol and α-pinene) alcohol, aldehyde, ketone, ethers which are known to contribute to free radical scavenging activity [15, 30-32]. In similar ways, our study also exhibited a high level of eucalyptol and α-pinene which represent for greater applicability of *Callistemon citrinus* oil in natural medicines and healthy food as an antioxidant agent as shown in figure 4.

Conclusion

The findings of this study point out the high availability of some major chemical components such as 1.8-cineole, α-pinene, and α-terpineol, along with other components in a lower amount such as, β-pinene, α-pinene, and linalool, which were already known to exhibit antifungal and bacteriostatic activities.

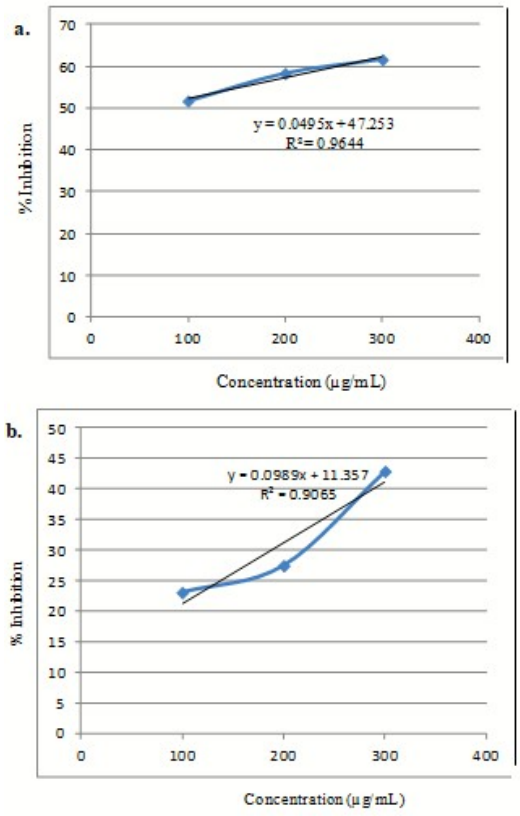


Fig. 4: Calibration curve for (a) Ascorbic acid and (b) *Callistemon citrius*

The presence of these bioactive components revealed by GCMS and physicochemical parameters are the reason behind the antioxidant, antibacterial and antifungal activity

of the essential oil leaf extract. Nevertheless, the presence of minor components could also play a role in biological activity. The antimicrobial components in essential oil justify its conventional and remedial use of the plant to guard against infections caused by bacteria and fungi. These findings suggest that the essential oil from *C. citrinus* could be a potential source of extracting good, cheap, renewable-antimicrobial, and antioxidant compounds. Moreover, the highest percentage yield of essential oil from its leaves and greater application of essential oil for therapeutic and ethnomedicinal purposes recommend for the development of novel natural drugs.

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