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Chemical composition and biological activity of the leaf essential oil of *Callistemon citrinus* from Nepal

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Abstract
The chemical composition of the leaf essential oil of *Callistemon citrinus* from Nepal was determined by gas chromatography – mass spectrometry (GC-MS). Out of a total 47 compounds identified, the major component was found to be 1,8-cineole (52.1%), while α-terpineol (14.7%) and eugenol (14.2%) were other substantial components. The essential oil components from Nepalese *C. citrinus* possessed eugenol in higher quantity than the samples from some other geographical locations. The essential oil was screened for antimicrobial, cytotoxic, larvicidal, nematicidal, and insecticidal activity. *C. citrinus* oil showed insecticidal activity against the fruit fly (*Drosophila melanogaster*) and termites (*Reticulitermes virginicus*).

Keywords: *Callistemon citrinus*, essential oil composition, insecticidal, cluster analysis.

1. Introduction
Callistemon citrinus (Curtis) Skeels (syn. *Callistemon lanceolatus* DC, "crimson bottlebrush") is a flowering plant belonging to the Myrtaceae, and is indigenous to Australia, but is widely found in different parts of the world, including the subtropical and tropical regions of South America and Asia. The plant is commonly known as bottlebrush because the flower-bearing portion of branches resembles cylindrical brush used to clean bottles. *C. citrinus* is the most widely cultivated species among the 34 species of *Callistemon* genus [1]. The plant is a shrub or small tree, which grows up to 7.5 m tall, and possesses crimson flowers and dark red anthers.
C. citrinus has been studied to show several pharmacological affects. The fruit and leaves have exhibited calcium-channel-blocking effects and anti-spasmodic activities [2]. *C. citrinus* has also been used ethnomedicinally to treat conditions like gastrointestinal distress, pain, and infections from bacteria, fungi, virus and parasites [3]. In India, the plant is known as folk medicine for respiratory conditions like cough and bronchitis and is also used as an insecticide, while the essential oil of the plant is used as antimicrobial and antifungal agent [4, 5]. The root of the plant contains phytotoxic leptospermone, which is the structural basis of the synthetic herbicide mesotrione [6].
Besides its medicinal uses, *C. citrinus* is widely gardened throughout the world for its ornamental value [1]. The plant is used in different parts of Nepal for the same purpose. This plant, because of its richness in nectar and pollen, is one of the most widely foraged plants by giant honeybee (*Apis dorsata*), and is a major source of honey in Nepal [7]. In Ghandruk, Nepal, bottlebrush is commonly called 'kalki phool' and is used as fodder besides being used ornamentally [8].
Several groups of scientists have studied the leaf essential oil of *C. citrinus* from different parts of the world and have found 1,8-cineol to be the major compound [9-15]. In the current study we have determined the essential oil components of *C. citrinus* from Nepal and we have screened the oil for several biological activities.

2. Materials and Methods
2.1 Plant Materials
Leaves of *Callistemon citrinus* were randomly collected from one individual tree growing in Biratnagar city (26°28′ N, 87°16′ E, 72 m above sea level), in Morang district of Koshi Zone of Nepal, on May 18, 2011. The plant was identified by Tilak Gautam, and a voucher specimen (1023) has been deposited in the herbarium located in the Botany Department on the Post-Graduate Campus of Tribhuvan University in Biratnagar, Nepal. The fresh leaf

sample (100 g) was crushed and hydrodistilled using a Clevenger type apparatus for 4 h and yielded a clear, pale yellow essential oil (0.5 g), which was stored at 4 °C until analysis.

2.2 Gas Chromatographic-Mass Spectral Analysis

The leaf essential oil of *Callistemon citrinus* was analyzed by GC-MS using an Agilent 6890 GC with Agilent 5973 mass selective detector (MSD) [operated in the EI mode (electron energy = 70 eV), scan range = 40-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, film thickness of 0.25 µm, length of 30 m, and internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. Injector temperature was 200 °C and detector temperature was 280 °C. The GC oven temperature program was used as follows: 40 °C initial temperature held for 10 min; increased at 3 °C/min to 200 °C; increased at 2 °C/min to 220 °C. A 1% w/v solution of the sample in CH_2Cl_2 was prepared and 1 µL was injected using a split injection technique.

Identification of the oil components was based on their retention indices (RI), determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature [16] and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

2.3 Antimicrobial Screening

The leaf essential oil of *C. citrinus* was screened for antimicrobial activity against Gram-positive bacteria *Staphylococcus aureus* (ATCC No. 29213); Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798), and fungi *Candida albicans* (ATCC No.10231) and *Aspergillus niger* (ATCC No. 16888); minimum inhibitory concentrations (MICs) were determined using the microbroth dilution technique as previously described [17, 18].

2.4 Cytotoxicity Screening

The essential oil was tested for cytotoxicity against human MCF-7 breast adenocarcinoma cell (ATCC No. HTB-22) using the MTT assay for cell viability as previously described [17, 18].

2.5 Nematicidal Assay

A nematicidal assay with *Caenorhabditis elegans* was carried out using a modification of the procedure of Park and co-workers [19]. Briefly, a 1% solution of leaf essential oil in DMSO was used to make dilutions for the sample solutions. The sample solutions were prepared in sterile water beginning with 50 µL of the 1% essential oil solution mixed in 50 µL sterile water. This sample solution was serially diluted (1:1) with sterile water in a 96-well plate. Into each well, 10-30 *C. elegans* (mixtures of juvenile and adult nematodes, male: female: juvenile ~1:1:2) per 50 µL of sample solution was added. Sterile water and serially diluted DMSO were used as controls. The dead and living nematodes were counted after 24 h using a microscope. Dead nematodes were identified by their immobility and straight body, even after transfer to clean water. LC_{50} values were determined using the method of Reed and Muench [20].

2.6 Glassworm Larvicidal Assay

The *C. citrinus* essential oil was screened for larvicidal activity against glassworm (*Chaoborus plumicornis*) [21], which were obtained from a local aquarium shop. For the bioassay, 10 mL of sterile water was placed in five 20-mL vials. Into each vial, 10 larvae were transferred using a soft brush. Three vials were labeled as control with the first one containing 10 µL DMSO, the second containing 100 µL DMSO and the third containing only sterile water. Into the remaining 2 vials were added 10 µL of 1% solution of essential oil in DMSO and 100 µL of 1% essential oil/DMSO solution (*i.e.*, final concentrations of 10 and 100 µg/mL). Surviving larvae were counted after 24 h. The experiments were carried out at 23 ± 2 ºC.

2.7 Fruit Fly Lethality Test

Wild type *Drosophila melanogaster* were obtained from a breeding colony sourced and maintained using a *Drosophila* culture kit (Carolina Biological Supply, Burlington, NC). The *Drosophila* medium (2 mL) was placed into each of five 20-mL glass vials. Of the vials, three vials were labeled as control, the first containing only *Drosophila* medium, the second with 20 µL DMSO, and the third with 10 µL of DMSO. Of the remaining two vials, one contained 20 µL of 1% essential oil solution in DMSO and the second one contained 10 µL of 1% essential oil solution in DMSO. Individual fruit flies were transferred into each vial (10 flies per vial). Each test was done in triplicate. Surviving fruit flies were counted 24 h post initiation of the treatments.

2.8 Termiticidal Activity Screening

Termiticidal activity was determined using worker termites (*Reticulitermes virginicus*) (Item number 143736) purchased from Carolina Biological Supply (Burlington, NC). Assays of activity were done using a six-well culture plate in which each well was fitted with a filter paper disc. The essential oil solution was prepared in 1% aqueous Tween® 80 solutions at 30, 60, and 120 µg/mL. Sample solutions (200 µL) of each concentration were sprayed into three wells. Water and 1% aqueous Tween® 80 solution were used as controls in the remaining wells. In each well, six termites were placed and termiticidal activity was determined 24 h later.

2.9 Hierarchical Cluster Analysis

To develop a hierarchical cluster analysis, essential oil composition of *C. citrinus* leaf from seven other geographical locations locations, obtained from published literature [9-15], were treated as operational taxonomic units (OTUs). The chemical relationship among the essential oil samples were determined by the agglomerative hierarchical cluster (AHC) analysis using the XLSTAT software, version 2014.4.09. Pearson's correlation was selected as a measure of similarity, and the unweighted, pair-group method with arithmetic average (UPGMA) was used for cluster definition and to develop a dendrogram for the *C. citrinus* selections.

3. Results and Discussion

The chemical composition of *C. citrinus* was determined by GC-MS and the percentage composition and retention indices are summarized in Table 1. A total of 47 compounds were identified, of which 35 compounds accounted for 99.7% of total oil composition. The most abundant compound was 1,8-

cineole with 52.1 % of total composition. α-Terpineol (14.7%) and eugenol (14.2%) represented two additional important compounds. Besides these compounds, α-pinene (2.9%) and (*E*)-caryophyllene (2.1%) were also present, and all other compounds were in concentration of less that 2%. Oxygenated monoterpene formed the major class of compound present with over 90.6% of total composition.

Table 1: Essential oil composition of *Callistemon citrinus* leaves from Nepal.

RI	Compounds	%	RI	Compounds	%
793	2,4-Dimethyl-3-pentanone	tr	1386	Geranyl acetate	0.2
808	2-Hexanol	0.1	1421	(*E*)-Caryophyllene	2.1
936	α-Pinene	2.9	1439	Aromadendrene	tr
979	β-Pinene	0.2	1448	(*E*)-Cinnamyl acetate	1.7
1046	1,8-cineole	52.1	1454	α-Humulene	0.5
1061	γ-Terpinene	0.1	1481	Germacrene D	tr
1089	Terpinolene	0.1	1486	β-Selinene	tr
1103	Linalool	1.5	1495	Viridiflorene	0.1
1113	*endo*-Fenchol	tr	1516	Geranyl isobutanoate	0.1
1121	*cis*-*p*-Menth-2-en-1-ol	0.1	1523	Dihydroxydurene	1.6
1138	*trans*-Pinocarveol	0.5	1529	Eugenol acetate	0.1
1167	δ-Terpineol	0.3	1538	β-Thujaplicinol	0.2
1177	Terpinen-4-ol	0.8	1547	Flavesone	1.3
1199	α-Terpineol	14.7	1579	Spathulenol	0.5
1200	Myrtenol	tr	1585	Caryophyllene oxide	0.5
1202	*cis*-Piperitol	tr	1592	Viridiflorol	tr
1219	*trans*-Carveol	0.1	1604	Methyl eugenol	0.1
1227	*cis*-*p*-Mentha-1(7),8-dien-2-ol	0.1	1609	Humulene epoxide II	tr
1258	Geraniol	1.9	1620	Leptospermone	0.2
1269	(*E*)-Cinnamaldehyde	tr	1637	*iso*-Spathulenol	0.1
1366	Eugenol	14.2	1652	Selin-11-en-4α-ol	0.1
1369	Neryl acetate	0.3	1717	(2*E*,6*Z*)-Farnesol	tr
1372	Hydrocinnamyl acetate	0.1	2110	Phytol	tr
1376	α-Copaene	0.3		Total Identified	99.7

The chemical composition of the leaf essential oil from Nepal was similar to those from India [12, 13], South Africa [14], Réunion Island [10], Brazil [15], Pakistan [9], and Australia [11], with 1,8-cineole being the most abundant compound. However, the presence of eugenol from the leaves in Nepal in such large quantity (14.2%) is considerably different from the oil from the other geographical locations where eugenol is present in trace amount, or is absent. The cluster analysis (Fig. 1) reflects the similarities in the essential oils. The only "outlier" would be the oil from Pakistan with 1,8-cineole < 50% and α-terpineol > 30% the difference in the composition may be due to the geographical/environmental differences in location of plant growth.

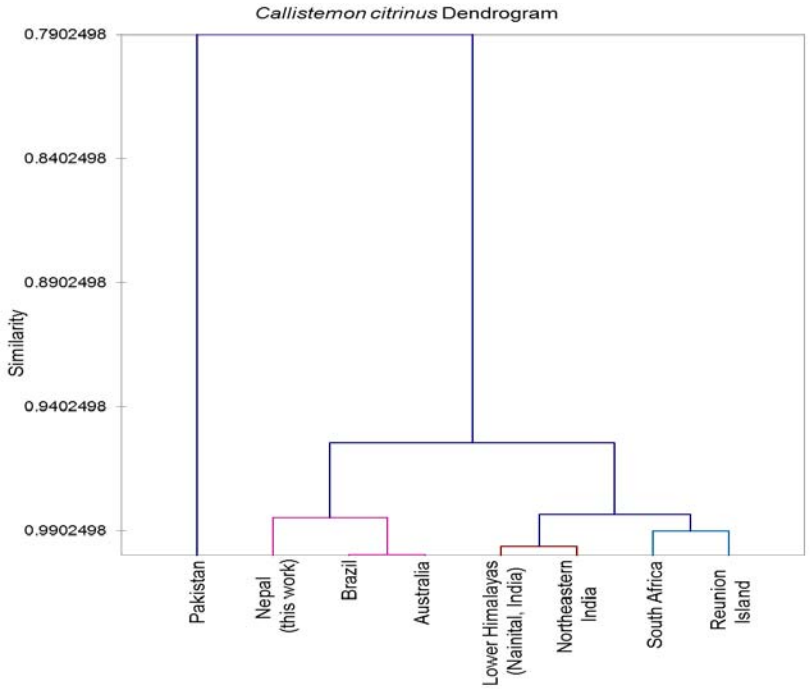

Fig 1: Dendrogram obtained from agglomerative hierarchical cluster of eight *Callistemon citrinus* essential oil compositions.

C. citrinus leaf oil was found to be ineffective against the microbes it was tested against, with MIC of 625 µg/mL against *A. Niger*, 1250 µg/mL against *P. aeruginosa*, and 2500 µg/mL against *E. coli, S. aureus* and *C. albicans*. *C. citrinus* essential oils from South Africa [14] and Brazil [15] were only marginally antibacterial. The oil was also ineffective against MCF-7 cells as a cytotoxic agent with only 17.5% kill at the oil concentration of 100 µg/mL. The oil was also inactive against *C. elegans* nematodes with LC_{50} of greater than 2500 µg/mL. However, the essential oil was very active against fruit fly with LC_{50} of 57.4 µg/mL, and against worker termites with LC_{50} of 38 µg/mL. By comparison, *Aegle marmelos* essential oil had LC_{50} of 238 and 500 µg/mL against *D. melanogaster* and *R. virginicus* [21], respectively, while LC_{50} for *Cannabis sativa* oil was 500 and 354 µg/mL against these insects [22]. The insecticidal activity of *C. citrinus* leaf oil can be attributed to the major components 1,8-cineole [23-25], α-terpineol [26], and eugenol [27].

4. Conclusions

Like the leaf essential oils of *C. citrinus* from other parts of the world, the sample from Nepal also has 1,8-cineole as its major component, with varying quantities of other substantial constituents like α-pinene, α-terpineol, eugenol and β-pinene. Although *C. citrinus* leaf oil was not antimicrobial, larvicidal, or nematicidal, the insecticidal activity of the oil is consistent with the traditional use of this plant as an insecticide.

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6. References

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