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PHYTOCONSTITUENTS AND BRINE SHRIMPS CYTOTOXIC ACTIVITY OF VOLATILE OIL OF *CALLISTEMON LANCEOLATUS* D.C. LEAVES FROM NEPALESE ORIGIN

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ABSTRACT

The volatile oil of fresh leaves of *Callistemon lanceolatus* D.C. of Nepalese origin was hydro distilled and its chemical constituents were analyzed by gas chromatography-mass spectrometry (GC/MS) technique. A total numbers of thirty eight phytoconstituents were revealed but only thirty five phytoconstituents were identified and representing 95.1% of total oil composition. The major phytoconstituents present in the oil sample were 1, 8-cineole (40.53%), α-pinene (10.87%), α-terpineol (7.69%), linalool (3.36%), 4-carene (3.26%), flavesone (3.24%), geraniol (3.16%), β-pinene (2.84%) and other minor phytoconstituents were a complex mixture of different chemical compounds with very less in the concentration. This volatile oil was found to be moderately cytotoxic against brine shrimps (*Artemia salina*) nauplii due to its LC_{50} value of 146.60μg/ml.

KEYWORDS

Callistemon, Phytoconstituents, Volatile oil, 1, 8-cineole, Cytotoxic and Brine shrimps.

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INTRODUCTON

Callistemon lanceolatus D. C. (*Callistemon citrinus*) is an evergreen, flowering plant belonging to the family Myrtaceae and comprises 34 species of genus. This species are woody, aromatic shrub or tree about 7-10m tall and distributed in wet tropics, Australia, South America and Asia but cultivated all over the world for ornamental purpose mainly due to their decorative crimson or red color flowers[1]. It is commonly named as bottle brush and in Nepal, commonly named as Kagiyo Phool. Its leaves are lanceolate about 3-6mm wide and 40-70mm long in arrangement and very aromatic with prominent vein, mid rib with oil glands. The flowers are borne in spikes of about 40-150 mm long with prominent red stamens with greenish or pale colored, tiny, inconspicuous petals[2,3]. Those bright red flower spikes of this plant are very rich in nectar and attract many birds and honey bees[4]. As Nepal has diverse climatic conditions, this plant can grow effectively in the Hilly and Terai regions below 2,500m altitude.

This plant has been used by tribal communities of India as folk medicine for its anticough, antibronchitis and insecticidal effects and its volatile oil used as antimicrobial and antifungal agents[1], Besides its pharmacological affects, *Callistemon* species are used for forestry, essential oil production, farm tree plantings, degraded land reclamation , ornamental horticulture, weed control and as bioindicators for environmental management[2,5-6]. From the best of our knowledge, the few research works were carried out on the chemical components and bioactivities of this plant therefore the present study was intended to investigate the essential oil compositions of fresh *Callistemon lanceolatus* D. C. leaves from Nepalese origin by gas chromatography-mass spectral analysis and to evaluate its brine shrimps nauplii cytotoxic activity.

MATERIAL AND METHODS

Plant Material

The fresh *Callistemon lanceolatus* D. C. leaves were collected during the month of July 2014 from Kirtipur, Kathamandu district of Nepal which is located in 27^0 40'56" N and 85^0 17'15" E at about 1320 m above sea level. The plant material was taxonomically identified by Prof. Dr. K. K. Shrestha and a voucher specimen has been deposited in the Central Department of Botany, Tribhuvan University, Kirtipur, Nepal. Those collected leaves were washed with cold water to remove the contaminant soil and crushed for volatile oil isolation.

Extraction of Volatile Oil

The volatile oil was isolated by hydro distillation method using Clevenger-type distillation apparatus. An amount of 100 gm crushed fresh leaves were kept into a round bottom flask along with distilled water. The content of the flask was heated in heating mantle at boiling temperature and the process was allowed to continue for 3 hours and followed by standing for one hour at room temperature. The fresh *Callistemon lanceolatus* D.C. leaves volatile oil obtained was kept in anhydrous sodium sulphate to remove water and filtered to store in air tight reagent bottle at 4^0C for further use.

Gas Chromatography-Mass Spectral (GC/MS) Analysis

The phytoconstituents of the fresh leaves of *Callistemon lanceolatus* D. C. volatile oils were analyzed by a gas chromatography (Shimadzu GC 2010) having an Rtx-5 MS column (30m X 0.25mm X 0.25μm) using helium as carrier gas. An amount of 10μL of the sample diluted with spectroscopic grade acetone (1:100) was injected into the GC inlet under pressure flow control mode maintaining purge flow 3 mL/min after fixing the split ratio at 100. The initial column oven temperature was set at 90ºC and the injection temperature was 200ºC. The chemical constituents separated were detected and identified by a mass spectrometer (Shimadzu QP 2010 Plus). During the analysis, the ion source and the interface temperature was set at 200ºC and 250ºC respectively. The detector voltage was 0.70 kV, scanning time was from 0.00 to 48.00 min and scan speed was 2000 with m/z range of 40.00 to 400.00. Identification of phytoconstituents were accomplished by comparison of mass spectra with those reported in NIST 05 and FFNSC 1.3 libraries as references.

Brine Shrimp Cytotoxic Activity

The cytotoxic activity of the volatile oil obtained from fresh leaves of *Callistemon lanceolatus* D. C. was evaluated by brine-shrimp bioassay following Mayer *et al*[7]. An amount of 20 mg of the sample volatile oil was dissolved in 2 ml acetone. The solution thus prepared was used as stock solution and from the each stock solution, 500µl (eqv. 1000ppm), 50µl (eqv. 100ppm), 5µl (eqv. 10ppm) were transferred to total nine test tubes, three tubes or each doses level. Then, the solvent was evaporated by standing overnight. After complete evaporation of the solvent, an amount of 5ml artificial sea water and ten mature shrimps were transferred to all test tubes containing samples. Similarly, three control vials (without oil sample) were taken and 10 matured nauplii were introduced in each vial. After 24 hours, the number of survivors was counted with the help of disposable pipettes. LC_{50} values (µg/ml) for the volatile oil was calculated as follows: If 'n' is the number of replicates (here three), 'x' is the log of constituents in µg/ml (here log10, log100 and log1000) for three doses level respectively and 'y' is the average survivors in all replicates, then we have:

$$\alpha = \frac{(\sum y - \beta \sum x)}{n}$$

Where,

$$\beta = \frac{\sum xy - \sum x.\sum y/n}{\sum x^2 - \frac{(\sum x^2)^2}{n}}$$

From Probit regression,

$$Y = \alpha + \beta X$$

$$X = \frac{Y-\alpha}{\beta} \text{and}$$

$$LC_{50} = \text{Antilog } X$$

RESULTS AND DISCUSSION

The volatile oil presents in the fresh leaves of *Callistemon lanceolatus* D. C. obtained by hydro distillation method using Clevenger-type apparatus and its phytoconstituents were determined by gas chromatography-mass spectrometry technique. The percentage yield of volatile oil obtained by hydro distillation was quantified and the result of the observation was found to be 0.5 % (v/w). The obtained volatile oil was colorless, slighty viscous with sweet strong fragrant, having specific gravity and refractive index 0.886 and 1.458 at 27.8^{0}C respectivly. The gas chromatography-mass spectral analysis of the volatile oil of fresh *Callistemon lanceolatus* D. C. leaves revealed the presence of thirty eight phytoconstituents in gas chromatogram out of which thirty five phyto compounds were identified representing 95.01% of the total oil composition shown in Figure No.1. The volatile oil contained a complex mixture of different phytoconstituents, out of these the predominant monoterpenoid was 1, 8-cineole (eucalyptol) (40.53%) with the other major phytoconstituents like α-pinene (10.87%), α-terpineol (7.69%), linalool (3.36%), 4-carene (3.26%), flavesone (3.24%), geraniol (3.16%), and β-pinene (2.84%). The minor phytoconstituents were a complex mixture of chemical compounds with very less in the concentration. The phytoconstituents present in the volatile oil of *Callistemon lanceolatus* D. C. leaves were tabulated below with their gram molecular weight, % peak area, retention time (Rt) and identification method in Table No.1.

The results indicated that identified compounds were nine monoterpenes, two cyclic ether monoterpenoids, eight monoterpene alcohols, one acyclic ether, three acyclic ester, one phenyl propanoid, three triketones, three sesquiterpenes, four sesquiterpene alcohols and one phenol derivative. The present study revealed that the percentage compositions and phytoconstituents are different from previously published works on gas chromatography-mass spectral analysis of the volatile oil of *Callistemon lanceolatus* D. C. leaves collected from different geographical locations. The volatile oil of *Callistemon lanceolatus* D.C. leaves from Biratnagar district, Nepal was identified fourtysix compounds, out of these compounds 1, 8-cineole (52.1%), and α-terpineol (14.7%), eugenol (14.2%) as major phytoconstituents[8] but comparision with our sample, there was less number of phytoconstituents with more concentration of α-pinene (10.87%), less concentration of 1, 8-cineole (40.53%) α-terpineol (7.69%), and also very less concentration of eugenol (0.35%). The volatile oil of this plant from Pakistan origin[9] was identified sixteen compounds only, out of these compounds 1, 8-cineole (68.0%), α-pinene (2.9%) and α-terpineol (10.6%) were found to be major phytoconstituents. Similarly Australian[10], Brazil[11], India[12,13], Reunion Island[14] and South Africa[15] origin species of volatile oil of *Callistemon* leaves were found to be rich in 1, 8-cineole (45-80%) with α-pinene, limonene and α-terpineol. The percentage of linalool and geraniol were found to be more than 3% each in the volatile oil of our sample studied while it was reported to be less than 1% each in the volatile oil sample of the same species from South Africa[15]. This findings are expected as the plant's response to the different climatic and environmental conditions lead to the huge variance in their secondary metabolites and their activities. This volatile oil showed a very good medicinal potential due to presence of high concentration of 1, 8-cineole[16] which is a great evolution for medicinal essential oil classification.

The volatile oil from *Callistemon lanceolatus* D. C. leaves was found to be moderately cytotoxic against brine shrimps nauplii as shown by its LC_{50} value of 140.60 µg/ml, much below the accepted maximum potential value of 1000µg/ml.

This moderately cytotoxic effect due to synergistic effect of the volatile oil containing different types of phytoconstituents in high and low concentration.

Table No.1: Phytoconstituents of volatile oil of *Callistemon lanceolatus* D. C. leaves

S.No	Name of Phytoconstituents	Gram Molecular weight	Retention Time (Rt)	Peak Area %	Identification Method
1	Isoamyl acetate	130	2.591	0.69	Rt, MS
2	Propanoic acid, 2-methyl-, 2-methylpropyl ester	144	2.860	0.90	Rt, MS
3	α-thujene	136	3.010	0.63	Rt, MS
4	α-pinene	136	3.111	10.87	Rt, MS
5	Sabinene	136	3.374	0.52	Rt, MS
6	β-pinene	136	3.424	2.84	Rt, MS
7	4-carene	136	3.614	3.26	Rt, MS
8	1,8-cineol (Eucalyptol)	154	3.964	40.53	Rt, MS
9	γ-terpinene	136	4.054	0.79	Rt, MS
10	Terpinolene	136	4.268	0.39	Rt, MS
11	Linalool	154	4.315	3.36	Rt, MS
12	3,4-Dimethyl-1-penten-3-ol	112	4.627	0.46	Rt, MS
13	Trans-pinocarveol	152	4.698	0.48	Rt, MS
14	δ-terpineol	154	4.877	0.56	Rt, MS
15	1-Terpinen-4-ol	154	4.966	1.81	Rt, MS
16	α-terpineol	154	5.081	7.69	Rt, MS
17	Nerol	154	5.276	0.32	Rt, MS
18	Geraniol	154	5.462	3.16	Rt, MS
19	n-Amyl ether	158	5.552	0.76	Rt, MS
20	3-Allyl-6-methoxyphenol	164	6.231	1.19	Rt, MS
21	Exo-2-hydroxycineole acetate Geranyl acetate	154	6.267	0.41	Rt, MS

22	E-caryophyllene	196	6.328	1.64	Rt, MS
23	Eugenol	204	6.732	0.35	Rt, MS
24	Neryl isobutyrate	164	6.852	0.35	Rt, MS
25	Bicyclogermacrene	224	7.163	0.36	Rt, MS
26	Cinerolon	204	7.218	0.47	Rt, MS
27	Flavesone	166	7.310	0.89	Rt, MS
28	Spathulenol	252	7.443	3.24	Rt, MS
29	Globulol	220	7.741	1.15	Rt, MS
30	Viridiflorol	222	7.787	1.22	Rt, MS
31	iso-Leptospermone	220	7.842	0.88	Rt, MS
32	Leptospermone	266	7.900	0.66	Rt, MS
33	α-elemol	266	7.955	1.15	Rt, MS
34	1(2H)-Naphthalenone, octahydro-	222	8.007	0.58	Rt, MS
35	4a,8a-dimethyl-7-(1-methylethyl)-[4Ar-(4a.α.7β.8aα)]	222	8.326	0.43	Rt, MS

Table No.2: Calculation of LC_{50} value of volatile oil from *Callistemon lanceolatus* D. C. Leaves

S.No	Sample	Z (concentration in µg/ml)	x = log Z	y	xy	x^2	β	α	X	LC_{50} = Antilog X
1	Essential Oil from *C. citrinus* leaves	10	1	9	9	1	-4.5	14.66	2.148	140.60 µg/ml
		100	2	8	16	4				
		1000	3	0	0	9				

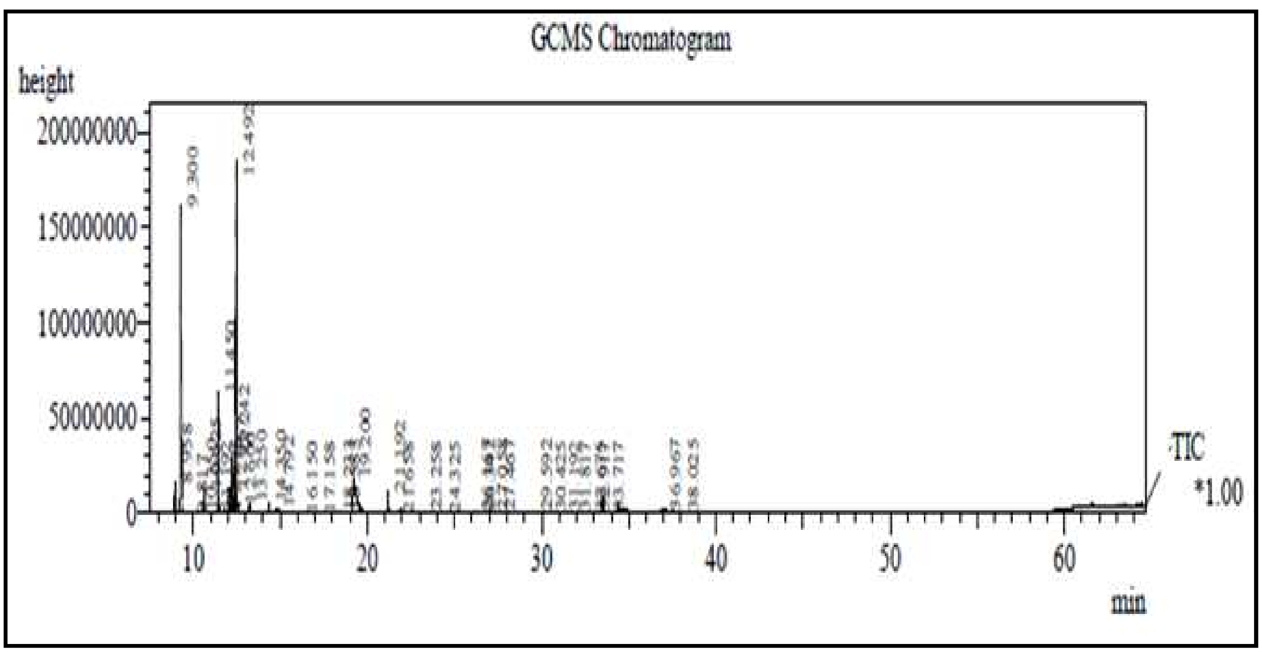

Figure No.1: GC/MS chromatogram of volatile oil of *Callistemon lanceolatus* D. C. leaves

CONCLUSION

In the present study, we have discussed the chemical compositions of the less studied volatile oil of the fresh *Callistemon lanceolatus* D.C. leaves. 1, 8-Cineole (40.53%), α-pinene (10.87%), α-terpineol (7.69%), linalool (3.36%), 4-carene (3.26%), flavesone (3.24%), geraniol (3.16%), β-pinene (2.84%) were found to be the major constituents of volatile oil from Kathmandu district, Nepal and the other minor phytoconstituents were a complex mixture of chemical compounds with very less in the concentration. This volatile oil was found to be moderatly cytotoxic against brine shrimps (*Artemia salina*) nauplii due to its LC_{50} value of 146.60µg/ml. Due to this cytotoxic activity, pharmacological importance of volatile oils of *Callistemon lanceolatus* D. C. leaves is gradually increasing more in the world and further support the ethno botanical approach to screening plants as potential source of bioactive phytoconstituents.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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