CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *CALOTROPIS GIGANTEA* LEAF ESSENTIAL OIL

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Keywords: *Calotropis gigantea*, Essential oil; Phytol; Antimicrobial activity.

Essential oil from *Calotropis gigantea* (L) leaves was extracted by hydrodistillation method followed of solvent extraction and characterized by GC-FID and GC-MS techniques. The chemical profiling estimated the presence of 42 components, representing 82.5 % of the total oil composition. Phytol (17.94 %), phenylacetaldehyde (9.16 %), 4-methyl-1-heptanol (4.98 %), benzyl alcohol (4.10 %), 4-vinyl guaiacol (3.87 %), 4-methyl-3-penten-1-ol (3.83%), Gentanol (2.93 %), 2-hexyn-1-ol (2.86 %) and phenethyl alcohol (2.52 %) were found to be the major constituents. Further, biological activities of the extracted oil were studied on the fungal (*Candida albicans*) and bacterial (*Escherichia Coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) pathogens. 100 µl essential oil extracted from leaf shows effective antimicrobial activity against selective bacterial and fungal pathogens. Among bacterial pathogens, leaf oil showed highest antimicrobial activity against *Pseudomonas aeruginosa* followed by *Escherichia coli* and *Staphylococcus aureus*.

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INTRODUCTION

Calotropis is a genus belonging to family Asclepiadaceae, comprised of six species of perennial shrubs distributed in tropical and subtropical Asia, and Africa.[1] Similar to *Calotropis procera*, *Calotropis gigantea* is found in the Indian subcontinent and have great economic importance.[2] The root bark is used to treat dysentery and elephantiasis, while the flowers in small doses give relief in colds, coughs, asthma, and indigestion.[1] The latex and roots are reported as indigenous medicine in birth control.[3] The bark can be used in fiber industries for manufacturing weave carpets, ropes, sewing thread and fishing nets.[4] Leaf extracts are used in agriculture to protect *Oryza sativa* from pathogenic fungus,[5] and in material science to synthesize nanomaterials.[6]

Interestingly, the plant is used as traditional biopesticides in controlling mollusks and mosquitoes.[7,8] Moreover, variety of chemical groups including cardenolides, steroids, flavonoids, terpenoids, cardiac glycosides, resins, fatty acids and non-protein amino acids etc. have also been identified in *C. gigantea*,[9] which leads to various applications in food, flavour and pharma industries to transformed into promising biologically active molecules for chemicals and drugs synthesis. For example, eugenol and guaiacol are important constituents of *Calotropis* essential oil, which is recently being explored as a substitute for fossil-based chemicals for making biobased materials and green chemicals.[10,11] Interestingly, Ashori et al. extensively studied different parts of *C. gigantea* in terms of chemical, morphological, and mechanical properties, and the results show that bark materials have comparatively higher cellulose content than the latex and flower[12].

Consequently, the bark and another woody part of this plant can be utilized and explored as a resource for green chemicals and biofuels as reported in previous literature.[13,14] With diverse chemical functionalities, essential oils (EO) are considered to be promising "green" alternative in the food, pharmaceutical, and agriculture industries[10,11,15] resulted to give effective antimicrobial, nematicidal, antiviral, insecticidal as well as antifungal properties.[16] EO constitutes about 20–60 components from fairly high concentrations (20–70 %) to traces. The products of higher concentrations (e.g. terpenes, terpenoids, aromatics, etc.) are responsible for a biological activities[17] while, minor components of EO shows synergism with major constituents.[15] Variety of plant species and their corresponding parts such as methanol extract of the root bark of *C. gigantea* is reported as potent antifeedant for the desert locust,[18] alcoholic root (100 mg kg^{-1} day^{-1}) and flower extracts are reported correspondingly for pregnancy interceptive and analgesic activities in the rats,[19] while the stem and flower extracts resulted in various chemical and biological activities.[9]

Though, the several kinds of the literature indicated the EO extraction and biological activity of extracts from *Calotropis* sp., very few are available on leaf extracts of EO of the *Calotropis gigantea* in particular. Nevertheless, no study is available on chemical profiling and antimicrobial activities of *C. gigantea*n leaf EO. Thus, in the present study, essential oil extraction of *C. gigantea* leaves and their chemical profiling, are investigated. In order to test biological activities, antimicrobial activities of EO against pathogenic fungal and bacterial species are studied. The chemical compositions of the essential oils were analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometry (GC-MS). This study includes identification of about 42 compounds from the hydrodistilled extract of leaf from *C. gigantea* corresponding to 82.5 % of total oil.

MATERIALS AND METHODS

Source of the plant

Fresh leaves of *Calotropis gigantea* were collected during flowering season from the roadsides (April 2014), dry wasteland area adjacent to Muzaffarnagar (29.4723° N, 77.7089° E) having weather (29°C, Wind W at 6 km h^{-1}, 80 % humidity), Uttar Pradesh State, India. Collected plant materials were submitted for identification at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. A voucher specimen is available in the herbarium division of the NISCAIR.[20]

Isolation of essential oil

Leaves were washed properly three times with fresh water and dried in shade till constant weight (difference between two consecutive weight not more than 0.05 mg) in properly ventilated room under fan in ambient conditions (27 °C) to prevent loss of essential oil. Dried leaves were crushed, weighed and subjected to hydro-distillation in 5 batches of 170 g each in 600 mL distilled water in Clevenger type apparatus for 4-5 h. The distillated product was further extracted with n-hexane and passed through anhydrous sodium sulphate to remove residual water. The product was concentrated by evaporating hexane under reduced pressure. Total oil obtained was 0.95 g. The yield of essential oil obtained was found to be 0.11 % (w/w).

Essential oil composition analysis by gas-chromatography (GC)/mass spectrometry (MS)

Essential oil composition of *Calotropis gigantea* was analyzed using Shimadzu GC-2010 equipped with flame ionization detector using Rtx-5MS (30 m x 0.25 mm ID x 0.25 μm) column. Nitrogen was used as carrier gas at 234.6 kPa inlet pressure. Oven temperature was programmed from 50 ºC for 3.0 minutes, 3.0 ºC min^{-1} to 200 ºC for 2.0 minutes, 10 ºC min^{-1} to 280 ºC for 7 minutes. The injector and detector temperature were 250 ºC and 260 ºC respectively. The oil was injected neat with split ratio of 10. Relative amount of individual components are based on GC peak area percentage obtained without FID response factor correction. The retention Indices were obtained from GC by logarithmic interpolation between bracketing n-alkanes. The homogenous series of n-alkanes (C_8-C_{22}, Polyscience, Niles, USA) were used as standard.

GC-MS data were obtained on Shimadzu GCMS-QP2010 plus using same chromatographic conditions and column used for GC-FID i.e. Rtx-5MS (30 m x 0.25 mm ID x 0.25 μm) column and helium as carrier gas. Temperature programming was 50 ºC for 3.0 minutes, 3.0 ºC min^{-1} to 200 ºC for 2.0 minutes, 10 ºC min^{-1} to 280 ºC for 7 minutes.

Identification of constituents

The individual peaks in GC of *Calotropis gigantea* leaf oil were identified by comparison of their retention indices on the SP Rtx-5MS (30 m x 0.25 mm ID x 0.25um) column with literature values[5] and were reported based on its area % response by GC-FID. The essential oil constituents were confirmed by matching of mass spectra of peaks with FFNSC2, NIST08 and Wiley8 library. Identification was done by GC-MS while RI and quantification done by GC-FID.

Antimicrobial activity

Microbial strains used

The antimicrobial activity of essential oil was performed using the following microbial stains procured from the Institute of Microbial Technology, Chandigarh, India. Details of the bacterial and fungal cultures used in the study are given in the Table 1.

Table 1. Lists of microbial stains and their corresponding medium

Organism	Medium	Incubation	
		***T*, °C**	***t*, h**
Escherichia coli ATCC8739	Soyabean-casein digest agar; Soyabean-casein digest broth	37°± 1	24 hours
Pseudomonas aeruginosa ATCC9027	Soyabean-casein digest agar; Soyabean-casein digest broth	37°± 1° C	24 hours
Staphylococcus aureus ATCC6538	Soyabean-casein digest agar; Soyabean-casein digest broth	37°± 1° C	24 hours
Candida albicans ATCC10239	Sabouraud dextrose agar; sabouraud dextrose broth	25°± 1° C	48 hours

Inoculum preparation

Working cultures were prepared from the glycerol culture stocks maintained in the laboratory. A loopful of individual culture from agar slant was inoculated in to the broth medium aseptically and incubated as per the temperature and time combination mentioned in the Table 1. Cultures grown in the broth medium were harvested by centrifugation, washed and resuspended in sterile saline solution (0.85 %) to obtain a culture concentration in the range of 1x 10^5 and 1 x 10^6 cfu mL^{-1}. Culture concentrations were standardised turbidometrically.

Antimicrobial activity

Antibacterial activity by well diffusion method:

The essential oil obtained from hydro-distillation of shade dried leaf was screened for their antibacterial activity in vitro by well diffusion method. Lawn culture was used using the test organism on Soyabean-casein digest agar in triple section Petri plate. The inoculated plates were kept aside for

few minutes, and using well cutter, wells were made in center of each section under aseptic condition. A fixed volume 100 µl of *Calotropis gigantea* leaf essential oil was then introduced into the well. The plate with bacteria was incubated at 37 ˚C for 24 h. The activity of essential oil was determined by measuring the diameter of zone of inhibition.

Antifungal activity by well diffusion method:

The essential oil obtained from hydrodistillation of shade dried leaves was also screened for antifungal activity in vitro using well diffusion method. Lawn culture was used using the test organism on Sabouraud dextrose agar in triple section plate. The inoculated plates were kept aside for few minutes, and using well cutter, wells were made in center of each section under aseptic condition. A fixed volume 100 µl of *C. gigantea* leaf essential oil was then introduced into the well. The plate of fungi was incubated at room temperature for 48 h. The activity of essential oil was determined by measuring diameter of zone of inhibition.

RESULTS AND DISCUSSION

Chemical investigation of essential oil of traditionally useful *Calotropis gigantea* which is reported for the first time in the literature and total ion chromatogram (TIC) of this oil showed 42 compounds corresponding to 82.5 % total area percentage. The yield of essential oil obtained by hydro distillation from *C. gigantea* was 0.11 % (w/w) with respect to total dry mass of the leaves. In order to explore useful products of C. *gigantea*, significant contributions have been made to identify phytochemical constituents and important activities[2,9,19] studied in different parts of the plant except for the leaves. In addition to the beneficial effect of latex,[3,7,8] root, and flowers,[1,2,9] Kumar et al. recently studied aqueous leaf extract of C. *gigantea* for antibacterial activity and found effective against *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Micrococcus luteus* and *Klebsiella pneumonia* species.[22] Motivated from this study, the extraction and identification of chemical composition of C. *gigantea* leaf essential oil was performed and analyzed on GC-FID and GC-MS for further structural confirmation under similar chromatographic conditions as mentioned in the material method section. The chemical profiling of the EO reveals the dominance of oxygenated diterpenes (phytol), aromatic alcohol (benzyl alcohol) and linear chain alcohol (4-methyl-1-heptanol) as shown in the Table-2 (Supplementary Information). Among major constituents of leaf essential oil, the main peaks were dominated with phytol (17.94 %), phenylacetaldehyde (9.16 %), 4-methyl-1-heptanol (4.98 %), benzyl alcohol (4.10 %), 4-vinyl guaiacol (3.87 %), 4-methyl-3-penten-1-ol (3.83 %), gentanol (2.93 %), 2-hexyn-1-ol (2.86 %) and phenethyl alcohol (2.52 %).

The result of *C. gigantea* leaf is in good agreement with the result of Schmelzer et al. on another species (*Gongronema lantifilium*) of the same family *Asclepiadaceae* where phytol was measured to be 15.5% along with 19.5 % linalool. The compositional changes may be due to the geographical areas, a method of extraction as well as species of the plant. For example, the essential oil extracted from *Calotropis* procera from Nigeria is reported to be 33.6 % phytol while the same species from Iran shows about 5 % more phytol content (38.2%).[23,24]

Phytol, phenylacetaldehyde and 4-vinylguaiacol are an important fragrant ingredient used in many house-hold products including cosmetics, fine fragrances, shampoos, toilet soaps and detergents.[25,26,27] In addition to that, the derivatives of phytol show crucial pharmacological effects in humans and other animals,[25] while 4-vinylguaiacol can act as a green source to produce acetovanilline and ethyl guaiacol (used in perfumery) as well as biodegradable polystyrene.[27]

The phytochemical analysis of leaf EO was investigated using nonpolar solvent, n-hexane. Nonpolar solvents are mostly used for defatting the dried plant material.[29] During the chromatographic analysis of EO, the compound eluted in order of their molecular weight, boiling point, and chemical structures. The molecule with low boiling point eluted first followed by the increasing boiling point/molecular weight of the molecule. As a result, phytol with higher molecular weight (296.53 g mol^{-1}) eluted at the end (Table 2, Fig 1). Linear chain and the aromatic compound have a chain in their structure. Gentanol (116.2 g mol^{-1}), for example, is a linear chain alcohol with seven carbon chain eluted before benzyl alcohol (108.14 g mol^{-1}). This could be due to its interaction with the stationary phase column. Similarly, 4-methyl-3-penten-1-ol (100.16 g mol^{-1}) eluted before 2-hexyn-1-ol (98.15 g mol^{-1}) since it has single covalent bond whereas 2-hexyn-1-ol has a triple bond.

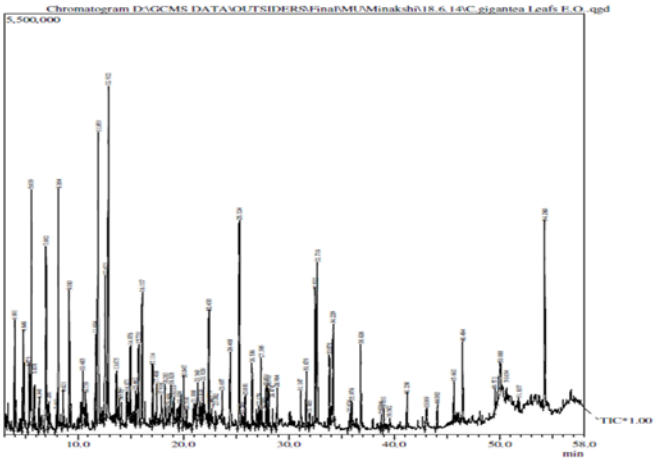
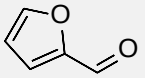
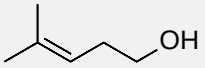
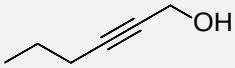
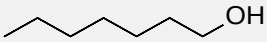
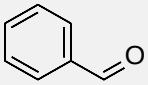
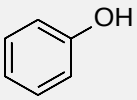
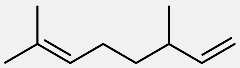
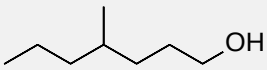
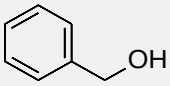
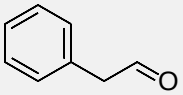
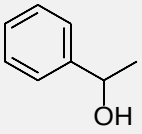
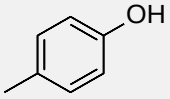
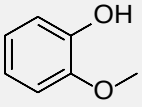


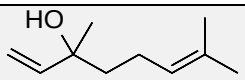
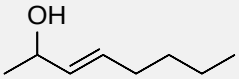
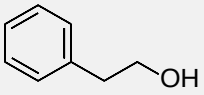
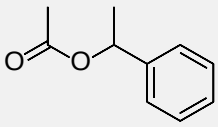
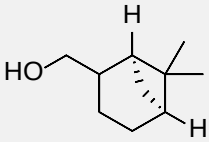
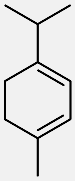
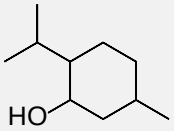
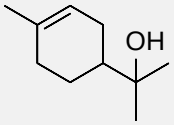
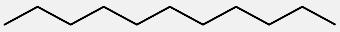
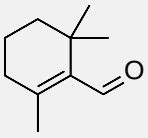
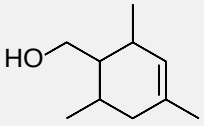
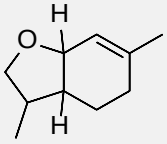
Figure 1. GC/MS spectra of essential oil from leaf of *Calotropis gigantea*

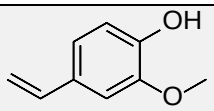
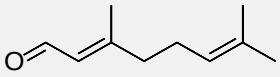
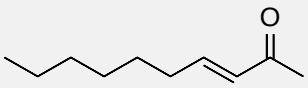
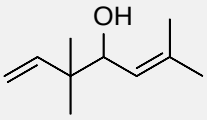
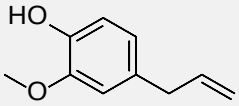
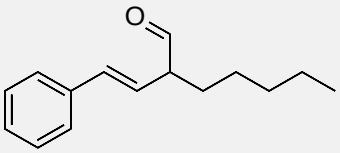
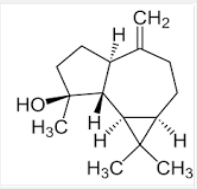
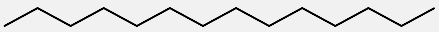
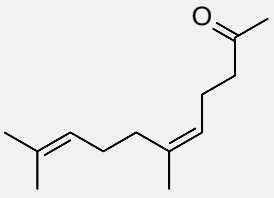
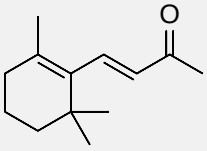
Moreover, a number of other odor-active compounds such as ±linalool (0.49 %), hexanol (0.48 %), α-citral (0.06 %), eugenol (0.30 %) etc. are identified in this study. These minor products are used extensively in chemical industries as perfumery or precursors for making value-added products along with some pharmaceutical purposes.[28,11,29] α-Citral, (±)-linalool and eugenol can also show biological activities against pathogenic fungus. In addition, compounds containing strong antimicrobial activity like benzyl alcohol (4.10%), (+)-β-citronellene (1.39 %), p-cresol (1.07 %), and guaiacol (0.86 %) have also been identified in the leaf

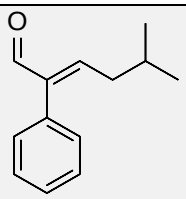
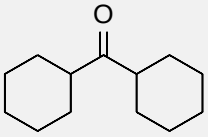
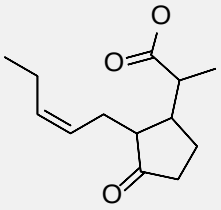
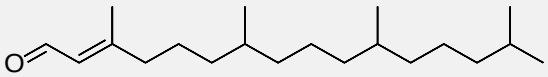
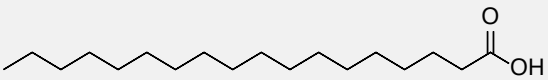
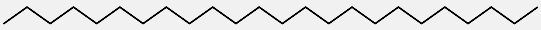
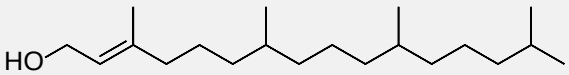
essential oil. These products can also show repellent properties against some insects, and thereby being used extensively as an important ingredient in insecticide formulation.

Table 2. Major identified products of Essential oil from *Calotropis gigantea* Leaf EO as measured by GC-FID[a]

Peak#	R.Time	Area	Area%	RI	Structure and Name	M/Z
1	4.848	431538	2.4992	1026	 2-Furancarboxaldehyde	96
2	5.619	661265	3.8296	1058	 4-Methyl-3-penten-1-ol	100
3	7.002	494423	2.8634	1112	 2-Hexyn-1-ol	98
4	8.164	506301	2.9322	1148	 Gentanol	116
5	9.183	324934	1.8818	1180	 Benzaldehyde	106
6	10.483	273777	1.5855	1214	 Phenol	94
7	11.694	241298	1.3974	1225	 β-Citronellene	138
8	11.953	859671	4.9787	1251	 4-Methyl-1-Heptanol	130
9	12.625	708290	4.102	1258	 Benzyl alcohol	108
10	12.922	1582376	9.1641	1266	 Phenyl acetaldehyde	120
11	13.675	114682	0.6642	1276	 alpha-Methylbenzyl alcohol	122
12	14.671	185932	1.0768	1304	 p-Cresol	108
13	14.976	148515	0.8601	1310	 Guaiacol	124

14	15.462	85003	0.4923	1316	 Linalool	154
15	15.77	215671	1.249	1329	 Oct-3-en-2-ol	128
16	16.117	435391	2.5215	1337	 Phenethyl alcohol	122
17	17.114	109729	0.6355	1350	 Gardenol	164
18	17.918	125137	0.7247	1367	 cis-Myrtanol	154
19	18.283	144493	0.8368	1392	 α-Terpinene	136
20	18.828	49071	0.2842	1413	 Menthol	156
21	19.658	110346	0.6391	1431	 Alpha-Terpineol	154
22	20.047	285531	1.6536	1436	 n-Undecane	156
23	21.008	95278	0.5518	1457	 β-Cyclocitral	152
24	21.779	43108	0.2497	1492	 Isocyclogeraniol	154
25	23.697	79634	0.4612	1529	 Dill ether	152

26	25.324	667795	3.8674	1546	 4-Vinylguaiacol	150
27	25.881	10643	0.0616	1583	 α-Citral	152
28	26.536	83596	0.4841	1606	 Heptylidene acetone	154
29	26.957	199462	1.1552	1613	 Artemisia alcohol	154
30	27.179	52433	0.3037	1621	 Eugenol	164
31	27.873	166014	0.9614	1676	 Z-Amyl cinnamaldehyde	216
32	28.474	67332	0.3899	1692	 Spathulenol	220
33	28.904	52044	0.3014	1704	 n- Tetradecane	198
34	31.147	90802	0.5259	1768	 Nerylacetone	194
35	32.522	316913	1.8354	1808	 β-Ionone	192

36	32.718	426348	2.4691	1814	 Hex-2-enal (5-methyl-, 2-phenyl-)	188
37	33.87	167676	0.9711	1848	 Cyclohexyl ketone	194
38	38.794	66462	0.3849	1939	 Methyl-Jasmonate	224
39	45.662	51359	0.2974	2228	 Phytone	268
40	50.088	116506	0.6747	2362	 Stearic acid	284
41	50.654	122057	0.7069	2378	 n-Tetracosane	338
42	54.28	3097846	17.9408	2659	 Phytol	296

where RT = Retention time, RI = Retention index, [a] List of products as confirmed by GC-MS

Since the plant is reported effectively against fungal and bacterial infections such as leprosy, tuberculosis and lupus, hence there is a need to investigate products that can serve as an effective antimicrobial and anti-biotic agent. In order to test these properties in leaf extracts of C. *gigantea* EO, a fixed volume 100 µl of leaf EO was used by well plate method against *Candida albicans*, a fungal pathogen as well as bacterial pathogens such as *Pseudomonas aeruginosa*, *Escherichia Coli*, and *Staphylococcus aureus* respectively. The activities of EO was determined by measuring zone of inhibition (mm). The results show that extracted EO gave promising biological activities and thereby exhibit strong inhibitory effect (5mm) against fungal pathogen, *Candida albicans* as presented in Table 3.

Table 3. Antifungal activity of essential oil from leaves of *Calotropis gigantea*

Microbial Strains	Fungus/Bacteria	ZOI (mm)
Candida albicans	fungus	5
Staphylococcus aureus	bacterium	4
Escheriachia coli	bacterium	5
Pseudomonas areuginosa	bacterium	7

ZOI, zone of inhibition

This is probably due to high benzyl alcohol (+)-β-citronellene, p-cresol, and guaiacol content. Similarly, the oil shows good activity against bacteria *Pseudomonas aeruginosa* followed by *Escherichia Coli* and *Staphylococcus aureus* which cover 7 mm, 5 mm and 4 mm zone of inhibition (ZOI) respectively.

CONCLUSION

This study reports EO extraction from *C. gigantea* leaf, their compositional analysis and tests for biological activity for the first time to the best of our knowledge, and a high phytol content 17.94 % was obtained in the oil. In addition, phenylacetaldehyde (9.16 %), 4-methyl-1-heptanol (4.98 %), benzyl alcohol (4.10 %), 4-vinylguaiacol (3.87 %), 4-methyl-3-penten-1-ol (3.83 %), gentanol (2.93 %), 2-hexyn-1-ol (2.86 %) and phenethyl alcohol (2.52 %). The biological activities of EO were tested against *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* and showed promising results. Due to the presence of diverse functionality, the EO or isolated molecules can be applied as a natural preservative, fuel additives, and drugs including pesticides and bulk chemicals.

eugenol and guaiacol are important building block chemicals in the range of traditionally produced molecules from fossil resources. The production/isolation of these chemicals from *Calotropis gigantea* could help in exploring biobased chemicals production to fulfill future energy and chemical demands.

ACKNOWLEDGMENTS

Authors acknowledge Dr. Imteyaz Alam of Department of Chemical Engineering, Institute of Technology (IIT) Delhi, Dr. Ovais Rizvi and Dr. Pooja Sharma of Department of Chemistry, Jamia Hamdard for their continuous support and scientific discussions.

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Received: 15.11.2018.
Accepted: 13.12.2018.