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Chemical Composition And Antimicrobial Activity Of The Essential Oils From *Eucalyptus Cinerea, Callistemon Viminalis* And *Calothamnus Quadrifidus* (Myrtaceae)

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ABSTRACT

The composition of the steam distilled oils of the aerial parts of the three entitled plants were separately analyzed by GC and GC/MS techniques. Fifty-one, twenty-nine and thirty-four compounds were identified in *E.cinerea, C.viminalis and C.quadrifidus*, respectively. The majority of these compounds were found to be primarily monoterpenes. Cineole was found to be the major compound in all three oils (82.6%, 78.3% and 80% in *E.cinerea, C.viminalis and C.quadrifidus*, respectively). Results of analysis indicated, however, *E.cinerea* contained no linalool and only traces of myrcene (0.04%); two relatively prominent compounds in both *C.viminalis* (5.16% and 1.68%), and *C.quadrifidus* (0.15% and 3.37%) respectively, β-pinene, though its totally absent in *E.cinerea*, it amounts to (1.30%) in *C.viminalis* and (3.79%) in *C.quadrifidus*. The antimicrobial activity of the three oils revealed similar remarkable results while antifungal activity was found to be moderate in *E.cinerea* and weak in the other two oils. The three oils were found also to inhibit the growth of *M.tuberculosis* inoculated in Lowenstein-Jensen medium.

KEYWORDS

Eucalyptus cinerea;
Callistemon viminalis;
Calothamnus quadrifidus;
Myrtaceae;
Essential Oils;
Cineol;
Antimicrobial.

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INTRODUCTION

Plants belonging to the family Myrtaceae are well known as rich sources of valuable essential oils. Species of the genus *Eucalyptus*, the largest genus among this family, is a very distinguished source of eucalyptus oil, which is well known for its medicinal value. The oil has been used for years in alleviating the symptoms of nasopharyngeal infections, coughs and as a decongestant. It is also used externally in various ointments and liniments, to relief the symptoms of rheumatic pains[1].

Eucalyptus cinerea is among 300 species of this genus, widely known as an indispensable source of eucalyptus oil. The volatile oil obtained from *E.cinerea*, was found to contain cineole, α-terpineol, α-pinene and limonene as major components[2-5]. However, no reports were traced regarding the essential oil from *E.cinerea* cultivated in Egypt.

Callistemon viminalis, known as the weeping bottlebrush, is among 35 species constituting this genus native to Australia. Many reports were traced regarding the chemical composition of the volatile oil from *C.viminalis* and revealed cineole, as the major constituent[6-9]. Only one report was traced to *C.viminalis* cultivated in Egypt[10]. Other *Callistemon* species have been studied primarily for their volatile oils, which have been described to possess the same odour as eucalyptus oils[11]. Various investigators have singled cineole as the major component (ranging from 35-90%) of oils obtained from *C.lanceolatus*, *C.rigidus* and *C.speciosus*[12-19]. The volatile oil of *C.lanceolatus* has been shown to possess strong broad-spectrum antifungal activity; such antifungal activity was stable to autoclaving and long storage[20]. The oil was also reported to exhibit significant inhibition of some plant viruses[21] as well as possess anthelmintic activity[22]. The essential oil of *C.lanceolatus* was also found to possess bee repellant activity[23] and was also reported to possess juvenile hormone activity[24].

This study presents the analysis of the essential oils of three Myrtaceous plants viz., *Eucalyptus cinerea*, *Callistemon viminalis* and *Calothamnus quadrifidus*; though all of which are cultivated in Egypt, yet they are originally native to Australia. The study also aims at a thorough analysis of these essential oils using GC and GC/MS techniques and testing their antimicrobial as well as antifungal activities. A comparative study regarding the chemical composition of the essential oils from *E.cinerea* and *C.viminalis* grown in Egypt was also conducted in relation to their native species. As for *C.quadrifidus*, no references concerning the composition of its oil could be traced in literature; therefore, to the best of our knowledge, it is reported here for the first time to provide full characterization of the oil.

EXPERIMENTAL

Plant material and isolation procedure

The aerial parts of both *C.viminalis* and *C.quadrifidus* were collected from private gardens in Cairo while that of *E.cinerea* was collected from the garden of the Faculty of Agriculture, Cairo University, Egypt. Voucher specimens have been deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt. The three plants were authenticated by Prof. Dr. Abdel Salam El-Nowiahi, Professor of Taxonomy, Faculty of Science, Ain-Shams University, Egypt. The essential oils obtained from the aerial parts (500 g) of each of investigated plant were individually isolated by hydrodistillation in a modified Karlsruhe apparatus[25] for 4 hrs, using n-pentane as solvent.

GC analyses

An Orion Micromat 412 double column instrument with 25 m fused silica capillaries with CPSil 5 and CPSil 19 (Chrompack) was used. Operating conditions: Linear temperature program from 50 to 230°C, 3°C/min; injector and detector temperatures 200°C and 250°C respectively; split injection, flame ionization detection, carrier gas hydrogen at a flow rate of 0.5 ml/sec.

GC/MS analyses

Electron impact (70eV) GC/MS measurements were carried out on a Hewlett-Packard HP 5890 gas chromatography equipped with a 25 m polydimethylsiloxane (Chrompack CPSil 5 CB) capillary column and coupled to a VG Analytical VG 70-250 S mass spectrometer. Helium is used as a carrier gas at a

flow rate of 0.5 ml/sec. Linear temperature program from 80°C to 270°C, 10°C/min; injector temperature 220°C, transfer line 230°C, ion source 220°C.

Components identification

The identification was performed by computer search of the Wiley/NBS Registry of Mass Spectral Data. Mass Finder Spectral Data, and a user generated library with retention indices and mass spectral data of authentic reference substances.

Antimicrobial testing assay

The following micro-organisms were used: Gram +ve bacteria: *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 19433, *Bacillus subtilis* ATCC 6051 and *Bacillus cereus* ATCC 23260. Gram –ve bacteria: *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 11229. Fungi: *Penicillium cyclopium* ATCC 60762, *Aspergillus aegyptiacus* and *Candida albicans* ATCC 60193. Acid fast bacteria: *Mycobacterium tuberculosis*.

The tween agar diffusion method was used[26]. Sterile nutrient agar was inoculated with the respective micro-organism (200 µl of organism in 20 ml medium) and poured into petri-dishes to give a solid plate. The oil (2 µl) was applied on sterile paper discs (5 mm diameter). The discs were deposited on the surface of inoculated agar plates. Plates were kept for 3hrs in a refrigerator to enable prediffusion of the oil into the agar and then incubated for 24 hrs at 37°C. Ampicillin and tetracycline were used as positive controls. Diameter of inhibition zone around each of the discs (diameter of inhibition zone minus diameter of the disc) was measured and recorded at the end of the incubation time. An average zone of inhibition was calculated for the three replicates. *M.tuberculosis* was inoculated in tween-lowenstein-Jensen medium containing the essential oil in concentration 2µ/1ml. The tubes were then placed upright and incubation continued, at least 6 weeks for clinical specimens[27].

The minimum inhibitory concentration (MIC) were determined using dilution technique[28]. The culture medium used for the bacteria was Müller-Hinton agar, while Sabouraud agar was used for growing the fungi. The incubation conditions used were 24 hrs at 37°C for bacteria and 48 hrs at 28°C for the fungi. The MIC was measured for the oils as well as for standard cineole, which was tested on the same cultures under identical conditions to compare its activity with those of the investigated oils. Initial emulsion of the oils were prepared at 10µl/ml in sterile distilled water with 10% tween 80. Serial dilutions of the stock solutions in broth medium (1000 µl Müller-Hinton broth or on Saboured broth) were prepared in a microtitre plate (96 wells). Then 1 µl of the microbial suspension (in sterile distilled water) was added to each well. For each strain, the growth conditions and sterility of the medium were checked and the plates were incubated as referred above. MICs were determined as the lowest concentration preventing visible growth. Standard antibiotics (Tetracycline and Ampicillin) were used in order to control the sensitivity of the tested bacteria and intraconazole was used in order to control the tested fungi.

RESULTS AND DISCUSSION

Analysis of the essential oils obtained from the three entitled plants by GC and GC/MS techniques, revealed certain differences in oil composition of each plant TABLE 1. Oil of *E.cinerea* recorded the highest number of components (53) followed by *C.quadrifidus* (38) and *C.viminalis* (34). The majority of these compounds were found to be primarily monoterpenes. Cineole was found to be the main component in all three oils (82.6%, 78.3% and 80% in *E.cinerea*, *C.viminalis*, and *C.quadrifidus*, respectively. Results of analysis also indicated *E.cinerea* to contain no linalool and only traces of myrcene; two relatively prominent components in both *C.viminalis* (5.16% and 1.67%) and *C.quadrifidus* (0.15% and 3.37%).

Cineole(82.6%), α-terpineol(4.93%), globulol (1.73%) and α-pinene(0.99%) were found to be the major constituents of the essential oil from *E.cinerea* cultivated in Egypt. Results of analysis of the essential oil from the same species grown in different localities revealed a great resemblance to that of the Moroccon species with a high concentration of cineole(87.80%) and the absence of limonene[2] which is possibly attributed to climatic similarity. The essential oils of *E.cinerea* growing in Argentine, India and Brazil constituted a lower percentage of cineole rang-

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TABLE 1: Essential oil composition of *Eucalyptus cinerea*, *Callistemon viminalis* and *Calothamnus quadrifidus*

Peak #	RI	Compound	*E.cinerea*	*C.viminalis*	*C.quadrifidus*
1	922	α-Thujene	1.10	0.07	0.06
2	929	Tricyclene	0.60	0.07	0.99
3	930	α-Pinene	0.99	0.50	2.34
4	941	α-Fenchene	0.03	-----	tr
5	943	Camphene	0.04	-----	-----
6	963	Sabinene	0.06	0.70	0.73
7	969	β-Pinene	-----	1.30	3.79
8	978	Myrcene	0.04	1.67	3.37
9	994	Δ-2-Carene	-----	-----	0.12
10	1006	α-Phellandrene	0.12	-----	0.06
11	1007	α-Terpinene	-----	0.19	0.22
12	1008	m-Cymene	0.05	-----	-----
13	1010	p-Cymene	1.16	0.40	0.12
14	1020	1,8-Cineole	82.6	78.31	79.95
15	1046	γ-Terpinene	0.35	0.63	0.66
16	1075	2,4 (8)-p-Menthadiene	0.01	0.13	0.17
17	1076	Terpinolene	0.03	0.06	0.06
18	1080	Linalool	-----	5.16	0.15
19	1096	α-Fenchol	0.07	-----	tr
20	1103	β-Phellandrene	-----	-----	0.07
21	1104	α-Campholenal	0.07	-----	-----
22	1121	trans-Pinocarveol	0.38	-----	-----
23	1126	Citronellal	-----	-----	0.06
24	1136	Pinocarvone	0.02	-----	-----
25	1143	δ-Terpineol	0.06	0.12	0.09
26	1147	Borneol	0.07	-----	-----
27	1156	Cymene-9-ol	0.32	-----	-----
28	1159	Terpinene-4-ol	0.35	1.10	1.13
29	1169	α-Terpineol	4.93	4.21	0.99
30	1194	trans-Carveol	0.13	-----	-----
31	1201	Dihydrocarveol	0.36	-----	-----
32	1205	cis-Carveol	0.05	-----	-----
33	1206	Nerol	-----	-----	0.07
34	1210	Neral	0.03	-----	0.17
35	1212	Carvone	0.06	-----	-----
36	1230	Geraniol	-----	0.43	tr
37	1240	Geranial	0.06	-----	0.12
38	1256	Thymol	0.01	-----	-----
39	1266	Carvacrol	0.03	-----	-----
40	1299	Methylgeranate	-----	-----	0.26
41	1358	Geranylacetate	-----	0.18	-----
42	1368	2-Phenylethylacetate	-----	0.08	-----
43	1420	(E)-β -Caryophyllene	0.06	0.08	-----

TABLE 1 Continued

Peak #	RI	Compound	*E.cinerea*	*C.viminalis*	*C.quadrifidus*
44	1442	Aromadendrane	0.17	-----	-----
45	1462	allo-Aromadendrene	0.06	-----	0.04
46	1480	Germacrene D	-----	-----	0.08
47	1495	Bicyclogermacrene	0.12	-----	0.29
48	1558	Viridiflorol	0.21	-----	-----
49	1562	Maaliol	0.10	-----	-----
50	1564	Aromadendrane	-----	-----	0.05
51	1566	Aromadendrane-5-ol	0.17	-----	-----
52	1567	Palustrol	0.05	-----	-----
53	1569	4-Dehydroviridiflorol	-----	-----	0.07
54	1570	Guaia-6,9-dien-4β-ol	tr	-----	-----
55	1572	Spathulenol	-----	0.08	-----
56	1576	Caryophyllenoxide	0.04	tr	-----
57	1580	Globulol	1.73	0.26	0.15
58	1589	Cubebane-11-ol	tr	0.15	-----
59	1596	Rosifoliol	0.20	0.14	-----
60	1600	Ledol	-----	0.07	-----
61	1602	Marsupellone	0.22	-----	-----
62	1617	5-Guaiene-11-ol	0.26	0.19	-----
63	1621	1-epi-Cubenol	-----	0.08	-----
64	1622	Eremoligenol	0.12	-----	------
65	1626	γ-Eudesmol	0.15	-----	-----
66	1627	Isospathulenol	-----	0.08	-----
67	1632	T-Cadinol	0.06	-----	-----
68	1643	β-Eudesmol	0.63	-----	-----
69	1648	α-Eudesmol	0.27	-----	-----

RI, retention indices; tr, trace, <0.02%

ing from 64.65-69% and a significant concentration of limonene; 7.0%, 12.51% and 2.75% respectively[3-5].

In addition to cineole (78.3%), other major constituents of the essential oil from *C.viminalis* are included such as linalool (5.16%), α-terpineol (4.21%), myrcene (1.67%), α-pinene (1.3%), and β-pinene (0.50%). Similar major components and concentrations were recorded for the same species growing in Northern Australia[7,8] and India[9]. Interestingly, a high concentration of linalool was found to be a unique characteristic of the Egyptian oil as observed from the analysis presented (5.16%) and the recent report mentioned earlier (13.3%)[10].

In case of *C.quadrifidus*, this is considered as the first report regarding GC/MS analysis of its essential oil. Though the results of analysis revealed relatively similar major components to that of *C.viminalis*; yet *C.quadrifidus* oil is considered as a new additional rich source of cineole (80%), besides other constituents viz., α-pinene (2.34%), β-pinene (3.79%), myrcene (3.37%), linalool (0.15%), and α-terpineol (0.99%).

The antimicrobial study for the three oils along with authentic cineole, common major component of the three oils, revealed very close remarkable antimicrobial activity; this is expected due to the close resemblance of their oil composition. The antifungal activity was found to be moderate in *C.cinerea* and weak in the other two oils. The three oils were also found to inhibit the growth of *Mycobacterium tuberculosis* inoculated in lowenstein-Jensen medium. Results of antimicrobial activity and minimum inhibitory concentrations (MICs) of the three oils as well as their major component cineole are compiled in TABLES 2 and 3.

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TABLE 2: Antimicrobial activity of essential oils of *E.cinerea*, *C.viminalis* and *C.quadrifidus* and their main component (cineole)

Microorganism	Inhibition zone diameter (cm)					
	*E.Cinerea**	*C.viminalis**	*C.quadrifidus**	Cineole*	Tetracycline**	Ampicillin**
Gram +ve						
Staphylococcus aureus	2.2	1.8	1.8	2.4	2.6	2.1
Enterococcus faecalis	1.6	1.6	1.4	1.6	1.8	2.2
Bacillus subtilis	0.9	0.7	0.9	1.6	2.1	1.7
Bacillus cereus	---	---	---	----	2.5	2.1
Gram –ve						
Pseudomonas aeruginosa	1.0	0.8	0.7	0.9	1.1	1.5
Escherichia coli	1.5	1.2	1.2	1.4	0.9	1.6
Fungi					Intraconazole**	
Candida albicans	0.6	0.5	0.3	----	2.7	
Penicillium cyclopium	---	---	---	----	1.9	
Aspergillus aegyptiacus	---	---	---	----	0.9	
Acid fast bacteria						
Mycobacterium tuberculosis	Growth inhibition					

***Concentration = 2 µl in each disc; **Concentration = 0.1 mg in each disc**

Inhibition zone: 0.8 cm or greater, remarkable activity; 0.6-0.7 cm moderate activity; 0.4-0.5 cm, weak activity; 0.2-0.3 cm very weak, activity

TABLE 3: Minimum inhibitory concentration (MIC mg/ml) of essential oils of *E.cinerea*, *C.viminalis* and *C.quadrifidus* and their main component (cineole)

Microorganism	Inhibition zone diameter (cm)					
	E.Cinerea	*C.viminalis*	*C.quadrifidus*	Cineole	Tetracycline	Ampicillin
Gram +ve						
Staphylococcus aureus	0.32	0.45	0.40	0.25	1×10^{-2}	3×10^{-3}
Enterococcus faecalis	0.42	0.52	0.45	0.29	1×10^{-1}	$2.\times10^{-3}$
Bacillus subtilis	0.65	0.59	0.55	0.32	1×10^{-2}	1×10^{-2}
Bacillus cereus	---	---	---	----	1×10^{-3}	2×10^{-3}
Gram –ve						
Pseudomonas aeruginosa	0.59	0.60	0.65	0.52	0.5×10^{-3}	0.3×10^{-3}
Escherichia coli	0.45	0.42	0.50	0.40	0.4×10^{-3}	0.2×10^{-3}
Fungi					Intraconazole	
Candida albicans	0.62	0.77	0.75	----	1×10^{-3}	
Penicillium cyclopium	---	---	---	----	1×10^{-1}	
Aspergillus aegyptiacus	---	---	---	----	0.5×10^{-1}	
Acid fast bacteria						
Mycobacterium tuberculosis	Growth inhibition 2µl/ml					

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