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Chemical Composition and Antimicrobial Activity of Essential Oil of *Callistemon Viminalis* from the South Syria

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Abstract : Essential oil from fresh and dried leaves of *Callistemon viminalis* from Syria are obtained by hydro distillation using a Clevenger-type apparatus. The chemical composition of the E. oils was analyzed by GC–MS techniques in order to identify the chemical constituents. The major components obtained are: 1, 8 Cineol (61.25 %), (54.99%); α-pinene (10.94 %), (16.1%); α-terpineol (9.73 %), (8.02%), P-Cymene (5.88%), (5.25%). These E. oils were screened for antibacterial activity using agar disc diffusion technique to determine the diameter of growth inhibition zones. Subsequently, minimal percents concentration of E. oils was determined by micro dilution method. The antimicrobial activity was determined by the disc diffusion and broth micro dilution method against microorganisms obtained from Department of Medical Microbiology and Parasitology, Faculty of Medicine, Damascus University: *Bacellus subitus*, *Staphylococcus aureus, Streptococcus pyogens, Micrococus luteus, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginose, Klebsellia pneumonia, Proteus vulgaris,* and *Vibrio parahaemplyticus*. The E. oils were found active against both Gram-positive and Gram-negative bacteria, and have highest activity against the Gram- negative bacteria *Vibrio parahaemplyticus* and *Pseudomonas aeruginosa*. Minimum inhibitory concentration (MIC) ranged between 0.4 mg/ml and 1.6 mg/ml. The Minimum bactericidal concentration activity (MBC) was ranged from (0.8 mg/ml) to (3.2 mg/ml).
Keywords: *Callistemon viminalis*, GC–MS, Antimicrobial activity, essential oil.

Introduction:

Callistemon is a genus of 34 species an evergreen tree or shrubs belonging to family *Myrtaceae.* Found wildly only on the Australian continent but now all species are endemic to Australia[1-3]. There are also large numbers of hybrids and horticultural varieties based on Australian species. They are found in the more temperate region of Australia, mostly along the east coast and south-west and typically favor moist conditions, so when planted in gardens thrive on regular watering. However some of the species are drought resistant[4]. Species are commonly referred as bottlebrush, because of their flowers are ranged in spikes that can up to 12 cm long, brush like flowers resembling a traditional bottlebrush[5, 6]. *Callistemon* species had a role as traditional medicine. The genus is known in folk medicine for its anticough, antibronchitis, and insecticidal effects. Its essential oils have been used as antimicrobial, antifungal and antioxidant agents [7, 8]. Globally, there has been an unparalleled growth in the plant-derived medicinally useful formulations, drugs and health-care products.

Callistemon plant E. oil is complex mixtures of natural compounds comprised mostly of volatile constituents like lipids, terpenoids, ketones, phenols and oxygenated derivatives with multiple biological

activities such as antimicrobial, insecticidal and antioxidant properties. In recent years, increasing attention has been paid to the exploration of naturally occurring antioxidants and antimicrobials [9-11,29-38].

In the flora of Syria, *Callistemon* species are grown as garden, street trees or ornamental plants due to their decorative flowers. *Callistemon* plant is a handy medium shrub to large tree (5-7 m tall). *C. viminalis* is the most widely cultivated member of the genus *Callistemon*, and the bright red flower spikes are very rich in nectar. The present study was undertaken to assess the composition of E. oil extracted from Syrian *C. viminalis* and to evaluate his antibacterial activity.

Materials and Methods

Plant materials

The Plant material, leaves of *C. viminalis* were obtained from Damascus university campus, Syria in June, 2014 in the early morning. The plant identity was confirmed in Department of Botany, Faculty of Science - Damascus University. Part of collected plant materials (leaves) were dried in shade for about 14 days and made into coarse powder, and another part used fresh.

Isolation of essential oil

Fresh leaves of *C. viminalis* (200 gr) cut into small pieces with knife, were subjected to hydro distillation for (6) hours in a Clevenger-type apparatus, and dried plant parts were ground into small powder by a grinder machine. Then 50 gr of powder of leaves were subjected to hydro distillation for (3) hours in a Clevenger-type apparatus. The Light yellow colored E. oil was separated from aqueous layer, dried over anhydrous sodium sulphate and stored in sealed vial at low temperature (4 ^{0}C) before analysis [12].

GC-MS Analysis of Essential oil

E. oils composition were analyzed with gas chromatography mass spectrometry (GC/ MS). Gas chromatography analysis was carried out with an Agilent 6890 N gas chromatograph (GC) equipped with Agilent 5973 mass selective detector (MSD),Agilent Auto sampler 7683 and Agilent DB-5MS capillary column (30 m, 0.25 i.d., 0.25 µm film thickness) (Agilent Technologies, Santa Clara, CA, USA). The MS detector was operated in electron impact (EI) mode at 70 eV with interface temperature of 280 ^{0}C; the scan range was 50–550 amu. The injection port temperature was set at 250 ^{0}C. GC was performed in split less mode; carrier gas was helium at a constant flow rate of 1 mL/min. The column temperature was programmed as follows: an initial temperature of 60 ^{0}C increased to 280 ^{0}C at rate of 3^{0}C/min. The injection volume was 1.0 µL.

Identification of components:

Relative percentage amounts were calculated from peaks total area by the apparatus software. The identification of individual compounds was based on comparison of their mass spectra with those obtained from the NIST/NBS, Wiley Libraries spectra. Further confirmation was done from Retention Index data generated from a series of alkane's retention indices (relatives to C7-C20 on the DB-5MS column) (Adams, 2005).

Screening for Antimicrobial Activity

Bacterial Cultures Collection:

C. viminalis E. oils were tested against ten local isolates bacterial strain obtained from the Department of Medical Microbiology and Parasitology, Faculty of Medicine, Damascus University. These microbes were four gram-positive bacteria (*Bacellus subitus*, *Staphylococcus aureus, Streptococcus pyogens, Micrococus luteus)* and six gram-negative bacteria *(Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginose, Klebsellia pneumonia, Proteus vulgaris, Vibrio parahaemplyticus)*. The bacteria cultures were maintained on nutrient agar slant at 37 ^{0}C for 24 hours. The organisms were then sub cultured and preserved at 4 ^{0}C in sterile bottles containing nutrient broth and 15% sterile glycerol.

Agar disc diffusion

The E. oils were tested for antibacterial activity by the agar disc diffusion method [13]. The bacteria cultures were maintained on nutrient agar slant at 37 ^{0}C for 24 hours. Muller Hinton Agar medium (Oxoid) was prepared and sterilized by autoclaving for 20 min. at 121 ^{0}C. The medium was cooled to 45 ^{0}C. About 20 ml of molten nutrient agar medium for each Petri plate was added. Petri plates were allowed to dry and left for 24 hours. After this, the microorganisms were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 5 standard (1.0 x 10^{8}) CFU/ml. 50 mm Petri dishes were inoculated with these microbial suspensions. Sterile Whatman No.1 (6 mm) discs papers were individually placed on the surface of the seeded agar plates and 20 µL of E. oil in dimethylsulfoxide (DMSO) was applied to the filter paper disk. The E. oils were added with variety concentration 25%, 50%, 75%, and 100%. After the plates were kept at room temperature for 30 min to allow the E. oils to diffuse into the agar, they were incubated at 37°C for 24 h. after incubation the diameter of the resulting zones of inhibition (mm) of growth was measured. All tests were performed in triplicates. DMSO served as negative control.

Minimum inhibitory concentration and minimum bactericidal concentration:

The (MICs) of the E. oils were determined using 96-well micro titre dilution method as described previously [14]. Bacterial cultures were incubated in Müeller-Hinton broth overnight at 37 ^{0}C and a 1:1 dilution of each culture in fresh Müeller-Hinton broth was prepared prior to use in the micro dilution assay. Sterile water (100 µl) was pipetted into all wells of the micro titre plate, before transferring 100 µL of E. oil in DMSO. Serial dilutions were made to obtain concentrations 12.8, 6.4, 3.2, 1.6, 0.8, 0.4 and 0.2 mg/mL. 100 µl of bacterial culture of an approximate inoculum size of 1.0 x 10^{8} CFU/mL was added to all well and incubated at 37 ^{0}C for 24 h. The MIC is defined as the lowest concentration of the E.oils at which the microorganism tested does not demonstrate visible growth. The bacteria growth was indicated by the turbidity. To determine MBC, 100 µL broth was taken from each well and inoculated in Mueller-Hinton Agar for 24 h at 37 ^{0}C. The MBC is defined as the lowest concentration of the E. oil at which inoculated bacteria was totally killed. DMSO solution served negative control.

Statistical Analysis:

All determinations in this work were carried out in triplicates. SPSS Statistics 19.0 Software was used to evaluate one-way analysis of variance (ANOVA) at $p \leq 0.05$. Canonical Discriminate Analysis was also used to establish differences between samples, and to evaluate the importance of different variables on discrimination.

Results and discussion

The E. oil obtained by hydro distillation from both fresh and dried leaves of *C. viminalis* was light yellow in color. The yield was 0.28 % v/w and 0.63% v/w respectively. The analysis of the volatile constituents was carried out GC-MS systems. (Table 1) shows the main identified compounds, their percentage of composition and their retention indices values (RI) listed in order of elution from the DB-5MS capillary column. Thirty constituents were identified in the E. oils representing 97.78% (fresh) and 96.04% (dried) respectively of all the components found in the E. oil samples. The relative amounts of individual components of the E. oil were, expressed as percentages of the peak area relative to the total peak area (fig 1.1, 1.2). The yield of *C. viminalis* E. oil extracted from the fresh leaves was considerably smaller than that obtained from dried leaves. The volatile constituents often represent a mixture of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives. The E. oil is characterized by the dominance of 1,8 Cineol (61.25 %), (54.99%), α -pinene (10.94 %), (16.1%), α - terpineol (9.73 %), (8.02%), O-Cymene (5.88%), (5.25%), Terpinen-4-ol (1.41%), (1.25%), trans-Pinocarveol (1.05%), (0.66%), β -Pinene (0.86 %), (1.36%) and α -phellandrene (0.72%), (1.20%) respectively. Of this total are reported: non-oxygenated monoterpenes (19.49% and 25.65%), oxygenated monoterpenes (76.14% and 67.11%), non-oxygenated sesquiterpenes (1.19% and 1.30%), and oxygenated sesquiterpenes (0.99% and 1.98%) respectively. The main difference between the rates of extraction components of the E. oils of fresh and dry leaves *C. Viminalis* was the percentage of 1,8 Cineole ratio decreased from 66% to 55% and α -pinene increased from 10% to 16%. When, the change in the rest of the ingredients was slight.

Table 1 Chemical Compositions of E. oils from Fresh and Dried *C. viminalis* Leaves

Dried Area %	**Fresh** Area %	**Compositions**	**R.T**	**No.**
25.65	**19.46**	**Monoterpene Hydrocarbons**		
0.54	0.18	α-Thujene	924	1
16.10	10.94	α -Pinene	932	2
1.36	0.86	β -Pinene	974	3
0.18	0.07	β -Myrcene,	988	4
1.20	0.72	á -phellandrene	1002	5
0.35	0.47	3-Carene	1008	6
5.25	5.88	*p*-cymene	1020	7
0.49	0.20	γ -Terpinene,	1054	9
0.18	0.14	α-Terpinolene	1086	10
67.11	**76.14**	**Oxygenated Monoterpenes**		
54.99	61.25	1,8-cineole	1026	8
0.69	0.56	Linalool	1095	11
0.66	1.05	trans-Pinocarveol	1135	12
0.13	0.20	Pinocarvone,	1160	13
0.23	0.41	borneol	1165	14
1.25	1.41	Terpinen-4-ol	1174	15
0.18	0.31	Cryptone,	1183	16
8.02	9.73	α -Terpineol	1189	17
0.22	0.32	trans-Carveol,	1215	18
0.15	0.23	m-Cumenol	1224	19
0.17	0.09	Trans-Geraniol	1249	20
0.42	0.58	1,5-Menthadien-7-ol	1286	21
1.30	**1.19**	**Sesquiterpene hydrocarbons**		
0.67	0.58	alloaromadendrene	1459	22
0.15	0.23	Viridiflorene	1496	23
0.11	0.11	γ -Cadinene	1513	24
0.37	0.27	Cadala-1(10),3,8-triene	1548	26
1. 29	**0.64**	**Oxygenated Sesquiterpenes**		
0.66	0.27	spathulenol	1566	25
0.63	0.37	Globulol	1579	28
94.06	**97.46**		**Total**	

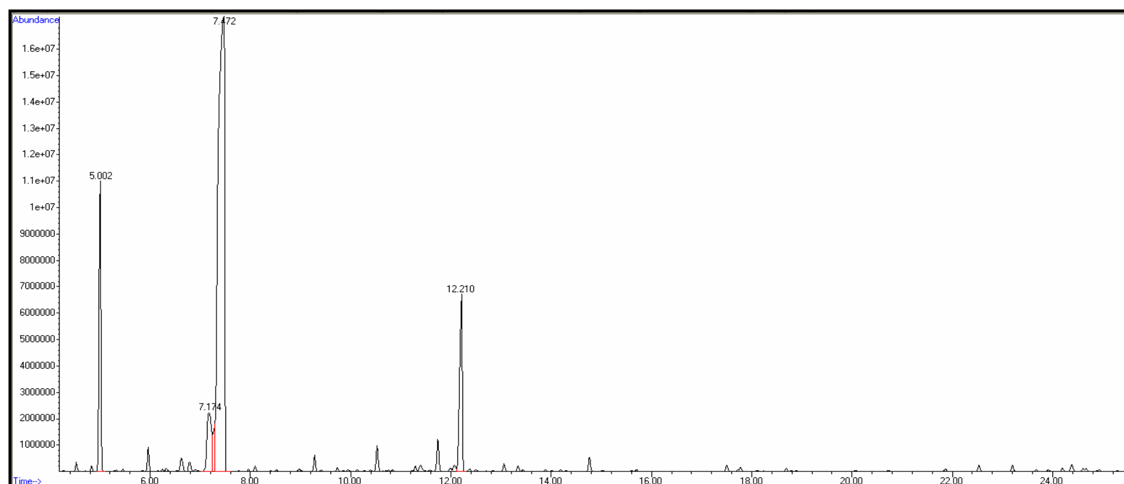

Fig 1.1 GC/MS Chromatogram of Fresh *C. viminalis* Leaves E. oil.

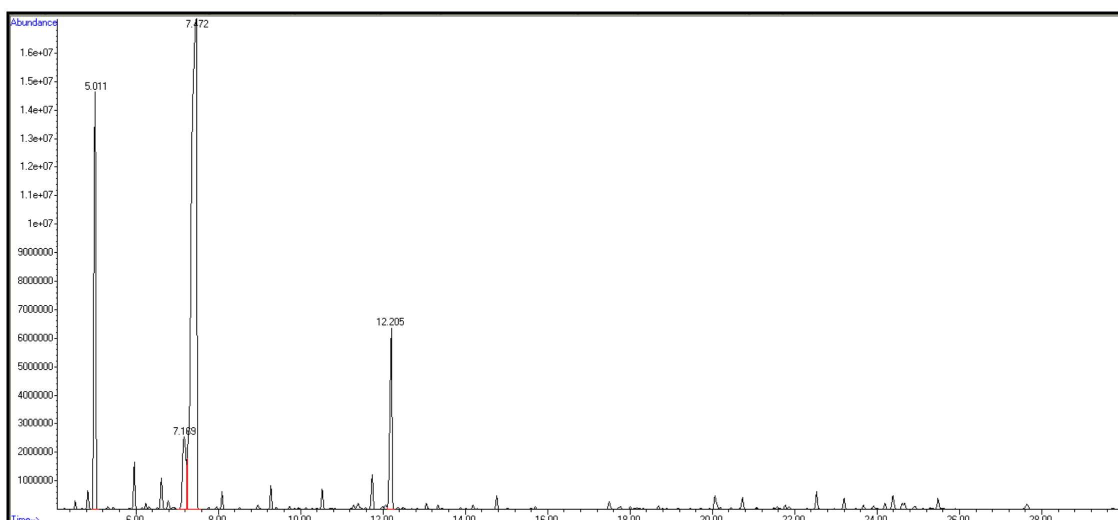

Fig 1.2 GC/MS Chromatogram of Dried *C. viminalis* Leaves E. oil.

The main E. oil compositions of *C. viminalis* leaves from different countries are 1,8-cineole, α-pinene and α – terpineol. On the other hand, the abundance of 1, 8-cineole in the essential oil of *C. viminalis* makes it similar to those obtained in the previous studies such as Brazil (65.0%, 12.0% and 13.0%) [15], India (61.7%, 24.2% and 2.3%) [16], Cameroon (58.49%, 0.93% and 7.38) [17], South Africa (83.2%, 6.4% and 4.9%) [18], Australia S1 (64.2% 0.7% and 1.2%), S2 (48.7%, 18% and 11.8%) [19], Egypt (66.36%, 20.43% and 6.65%) [20], (47.49%, 3.29% and 7.56) [21], (78.31%, 0.50% and 4.21%) [22], Malaysia (61.51%, 21.53% and 12.47%) [23] respectively. So, our results showed that *C. viminalis* can be considered as a good source for 1, 8-cineole (61.25%) and this finding is in agreement with those reported before in other parts of the world. There are differences in the yield and profile of the E. oil constituents, which could be attributed to many environmental factors viz. latitude, geographical distribution etc.[24]

The in vitro antimicrobial activity of *C. viminalis* E. oils against the microorganisms and its potentials activity were qualitatively and quantitatively assessed by microdilution methodology. The inhibition zones and disc diameters of the E. oil against the tested microorganisms are shown in (Table 2) and (Table 3). The data obtained from this method indicated that the E. oils no displayed a variable degree of antimicrobial activity on different tested strains. The results obtained from this method for the E. oils revealed that were highest activity against the gram negative bacteria *Vibrio parahaemplyticus* and *Pseudomonas aeruginosa*. And it showed moderate activity against the *Klebsiella pneumonia, Proteus vulgaris* and *Escherichia coli* (gram negative) and *Bacellus subitus, Micrococus luteus and Staphylococcus aureus* (gram positive). Whereas it showed no activity against the *Streptococcus pyogens* (gram positive) and *Salmonella Typhimurium* (gram negative). The antibacterial activity showed by the E. oil of *C. viminalis* could be attributed to the presence of some major components such as 1.8-cineole, α-pinene and α-terpineol, along with other components in lower amount such as, β-pinene and linalool, which were already known to exhibit antimicrobial activities [25-27].

Table 2 Antibacterial activity of the E. oil (diameter of IZ in mm) of fresh *C. Viminalis* leaves.

Different concentrations (percentages) of essential oil				Test Organisms
100%	75%	50%	25%	
				gram-positive bacteria
14.12 ± 0.12	12.14 ± 0.15	11.12 ± 0.15	8.90 ± 0.17	*Bacellus subitus*
14.11± 1.14	13.33 ± 0.17	12.11± 0.17	8.12 ± 0.11	*Micrococus luteus*
NA	NA	NA	NA	*Streptococcus pyogens*
14.16 ± 1.13	13.34 ± 1.14	13.21 ± 0.11	12.11 ±0.15	*Staphylococcus aureus*
				gram-negative bacteria
13.31 ±0.13	12.51 ±0.14	12.11 ±0.15	11 ± 0.13	*Klebsiella pneumoniae*
19.04 ± 0.17	18.16 ± 0.13	12.14± 0.10	9.17 ± 0.14	*Vibrio parahaemplyticus*
13.21 ± 0.12	12.13 ± 0.14	10.21 ±0.11	9.11 ±0.14	*Proteus vulgaris*
NA	NA	NA	NA	*Salmonella Typhimurium*
12.12 ± 0.23	11.14 ± 0.11	10.22 ± 0.15	9.20 ± 0.11	*Escherichia coli*
17.22 ± 0.13	16.21 ± 0.14	14.31 ±0.12	12.41 ±0.13	*Pseudomonas aeruginosa*

Data are mean ± SEM of three independent experiments. NA: Not activation.

Table 3 Antibacterial activity of the E. oil (diameter of IZ in mm) of dried *C. Viminalis* leaves.

Different concentrations (percentages) of essential oil				Test Organisms
100%	75%	50%	25%	
				gram-positive bacteria
14.13 ± 0.12	12.14 ± 0.11	11.32 ± 0.15	8.90 ± 0.12	*Bacellus subitus*
14.21 ± 1.12	13.23 ± 0.12	12.33 ± 0.13	8.32 ± 0.31	*Micrococus luteus*
NA	NA	NA	NA	*Streptococcus pyogens*
14.32 ± 1.23	13.44 ± 1.11	13.31 ± 0.41	12.21 ± 0.12	*Staphylococcus aureus*
				gram-negative bacteria
13.23 ± 0.23	12.11 ± 0.21	12.33 ± 0.14	11.3 ± 0.12	*Klebsiella pneumoniae*
18.44 ± 0.12	18.46 ± 0.17	12.24 ± 0.13	9.16 ± 0.14	*Vibrio arahaemplyticus*
13.34 ± 0.22	12.22 ± 0.12	10.31 ± 0.11	9.33 ± 0.14	*Proteus vulgaris*
NA	NA	NA	NA	*Salmonella yphimurium*
12.42 ± 0.12	11.34 ± 0.12	10.42 ± 0.13	9.40 ± 0.13	*Escherichia coli*
17.32 ± 0.11	16.41 ±0.24	14.44 ±0.33	12.31 ± 0.12	*Pseudomonas eruginosa*

Data are mean ± SEM of three independent experiments. NA: Not activation.

The results of the antimicrobial broth micro-dilution assay are summarised in Table 4. Results for both dry and fresh cases were the same. MIC ranged between 0.4 mg/ml and 1.6 mg/ml, with the lowest activity for *Bacillus subtilis* (1.6 mg/ml), while the highest activity was observed against *Vibrio parahaemplyticus*, and *Pseudomonas aeruginosa* (0.4 mg/ml). The lowest MBC was 0.8 mg/ml for *Staphylococcus aureus* while *Micrococus luteus* and *Klebsiella pneumoniae* had the highest MBC of 3.2 mg/ml.

Our research results revealed that medicinal plants C. *viminalis* can play an important role in fighting the bacterial resistance. The applications of natural antimicrobial agents are likely to grow steadily in the future because of greater consumer demands for minimally processed foods and those containing naturally derived preservation ingredients [28].

Table 4 (MIC) and (MBC) of the fresh and dried *C. Viminalis* leaves E. oil (Values in mg/ml).

MBC	MIC	Microorganism
		gram-positive bacteria
1.6	1.6	*Bacillus subtilis*
3.2	0.8	*Micrococus luteus*
NA	NA	*Streptococcus pyogens*
1.6	0.8	*Staphylococcus aureus*
		gram-negative bacteria
3.2	0.8	*Klebsiella pneumoniae*
1.6	0.4	*Vibrio parahaemplyticus*
1.6	0.8	*Proteus vulgaris*
NA	NA	*Salmonella Typhimurium*
1.6	0.8	*Escherichia coli*
0.8	0.4	*Pseudomonas aeruginosa*

NA: Not activation.

Conclusions

This paper describes a study of the chemical composition and antibacterial properties of the E. oil obtained from the leaves of C. *viminalis* in Syria. The study proves that C. *viminalis* is very rich in E. oil, with especially high amount of 1,8-cineole, and could be regarded as a potential source for food, pharmaceutical and cosmetic industries. In addition, the antibacterial assays demonstrated moderate activity against *Vibrio parahaemplyticus* and *Pseudomonas aeruginosa*. It showed moderate activity against *Klebsiella pneumonia, Proteus vulgaris, Escherichia coli* and *Bacellus subitus, Micrococus luteus and Staphylococcus aureus*. As a whole the results presented here support the potential use of the E. oil from C. *viminalis*, but at the same time indicate the need for additional investigations to valorize its application as health-beneficial phytochemical and new remedy.

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Conflict of interest

We do not have any conflict of interest. We have read the final version of the manuscript and are responsible for what is said in it. No one who has contributed significantly to the work has been denied authorship and those who helped have been duly acknowledged.

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