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Chemical Composition and Antibacterial Activity of Volatile Oil of *Sequoia sempervirens* (Lamb.) Grown in Egypt

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Abstract

The hydrodistilled essential oil of leaves of *Sequoia sempervirens* (Lamb.) belonging to family Cupressaceae was analyzed by GC/MS. Thirty six compounds were identified representing 95.62% of the total oil containing α-phellandrene (29.60%), dl-limonene (15.60%), α-pinene (8.65%) and terpinene-4-ol (3.5%) as major components. The studied essential oil showed a dose-dependent antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl assay (IC_{50}: 32.2 µg/mL). The antimicrobial activity of *S. sempervirens* leaves oil was screened against Gram-positive and Gram-negative bacteria and fungi. The strongest antibacterial effects of oil were on *Bacillus subtilis*, *Trichophyton mentagrophytes* and *Staphylococcus aureus*. *S. sempervirens* oil showed potent cytotoxic activity against the tested carcinoma cell lines; hepatocellular (HEPG2), colon adenocarcinoma (HCT-116) and breast (MCF7) comparing to doxorubicin.

Keywords: *S. sempervirens*; GC/MS; Antioxidant; Antimicrobial; Cytotoxic

Introduction

Cupressaceae family is dioecious or monoecious shrubs or trees containing about 18 genera and 140 species, in which the leaves are in opposite pairs, addressed and scale-like, or needle-like, the cones are usually small, globose too long. The scales of the cones have no spine tips [1]. They distributed in the northern temperate zone, with outlying species in tropical mountains and in temperate America. The family is notable for including the largest, tallest, and stoutest individual trees in the world (Giant Sequoia), and also the second longest lived species in the world (Coast Redwood) [1].

Sequoia is a genus of redwood coniferous trees in the subfamily Sequoioideae of the family Cupressaceae. The only extant species of the genus is *Sequoia sempervirens* in the Northern California coastal forests ecoregion of Northern California and Southwestern Oregon in the United States [2]. The two other genera, Sequoiadendron and Metasequoia, in the subfamily Sequoioideae are closely related to Sequoia [3]. It is a member of the cypress (Cupressaceae) family and like most cypresses is evergreen, as indicated by its species name *sempervirens*, meaning 'always green'. It is one of the tallest trees in the garden [4]. Through evaluation of the state of trees and shrubs of Egypt, it was revealed that some nine indigenous species are threatened with extinction in natural Egyptian Environs of which *Sequoia sempervirens* is one tree in El Orman Garden [5]. Chemical constituents of *S. sempervirens* oil were seldom reported before; in Egypt, no attention has been paid to evaluate the volatile oil, although it has many commercial and medicinal uses even it was possible to propagate *S. sempervirens* tree through tissue culture techniques [6].

The objectives of this work are to demonstrate the antioxidant, antimicrobial and a cytotoxic activity in correlation to phytochemical constituents of oil from *S. sempervirens* (Lamb.) leaves.

Materials and Methods

Plant material

Sequoia sempervirens (D. Don.) leaves were collected during October and May, respectively, from El Orman Garden, Giza, Egypt (2012). The collected plant materials were botanically authenticated by Prof. Dr. Monir Mohamed abd Elghany, The Herbarium, Botany department, Faculty of Science, Cairo University, Egypt, also Voucher specimen of the authenticated plant was deposited at Laboratory of phytochemistry, National Organization for Drug Control and Research. Cairo, Egypt.

Preparation of the essential oil

The fresh leaves of *S. sempervirens* (Lamb.) were subjected to hydro distillation [7]. The essential oil obtained was dried over anhydrous sodium sulphate and kept in a refrigerator for analysis. The percentage of the oil was calculated on fresh weight bases.

Analysis of the essential oil

GC/MS analysis of the essential oil was carried out on an Agilent 6890 equipped with a mass spectrometric detector (MSD), model Agilent 5973, equipped with an HP-5MS column (30 m × 0.25 mm, 0.25 µm); programming from 80 (3 min) to 260°C at 8°C/min, 10 min hold; carrier gas, helium; flow rate, 1.0 ml/min; injection in split mode (60.1); injector and detector temperatures 225°C and 300°C, respectively. The EIMS mode at 70 eV; electron multiplier, 2500 V; ion source temperature, 250°C; mass spectra data were acquired in the scan mode in the m/z range 50 to 700. The essential oil components were identified by comparing their mass fragmentation patterns with those of the available reference [8]. In addition, qualitative analysis was carried out by using internal normalization method (peak area measurement) and compound identification was confirmed by electronic Wiley and NIST mass spectral data base. The retention indices (RI) of the volatile oil components were determined relative to the retention times of series of hydrocarbons. Results are given in Tables 2 and 3.

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Physical characters	*S. sempervirens* oil
Percentage yield (%w/v)	0.68
Color	Pale yellow
Odor	Characteristic, strongly aromatic
RI	1.4256
Sp. gr.	0.96

Table 1: Physical characters of leaf essential oil of *S. sempervirens* (Lamb.).

No.	Compound	%	K.I	RTT*
1.	*α*-pinene	8.65	939	0.49
2.	Sabinene	0.75	975	0.86
3.	b-Myrcene	0.64	985	0.98
4.	*α*-Phellandrene	29.60	1003	1
5.	*p*-Cymene	1.20	1023	1.03
6.	dl-limonene	15.60	1029	1.06
7.	*cis*-*β*-Ocimene	1.35	1040	1.07
8.	*trans*-*β*-Ocimene	0.75	1052	1.09
9.	*m*-Cymene	1.45	1061	1.15
10.	γ-Terpinene	1.82	1062	1.16
11.	Borneol	0.73	1088	1.19
12.	Fenchone	0.85	1091	1.24
13.	Terpinolene	1.32	1143	1.33
14.	Terpinene-4-ol	4.35	1178	1.38
15.	*p*-Cymen-8-ol	0.73	1188	1.39
16.	*α*-Terpineol	0.26	1189	1.43
17.	*cis*-Piperitol	0.65	1200	1.49
18.	*trans*-Piperitol	0.92	1210	1.54
19.	*β*-Citronellol	1.12	1230	1.58
20.	Geraniol	0.34	1253	1.62
21.	Thymol	0.69	1290	1.68
22.	Carvacrol	1.21	1298	1.71
23.	*α*-Terpinyl acetate	3.65	1345	1.76
24.	Thymol acetate	1.45	1350	1.82
25.	Eugenol	0.43	1392	1.84
26.	*β*-Caryophyllene	0.53	1419	1.87
27.	*trans*-*α*-Bergamotene	0.26	1434	1.91
28.	Germacrene D	2.54	1469	1,93
29.	*α*-Humulene	1.08	1454	1.96
30.	*Trans*-*β*-farnesene	0.73	1457	2.21
31.	*E,E*- *α*- Farnesene	0.89	1506	2.45
32.	*β*-Germacrene	4.87	1561	2.58
33.	Spathulenol	0.75	1581	2.63
34.	Citronellyl pentanoate	1.06	1629	2.71
35.	α–Cadinol	0.65	1654	2.79
36.	Phytol	1.53	1940	2.84

KI=Kovat's index; RRT*=Relative retention time; *α*-Phellandrene=1 of RT=10.05.

Table 2: Chemical composition of leaf essential oil of *S. sempervirens* (Lamb.).

Determination of antioxidant activity by the DPPH radical scavenging assay

The free radical scavenging and antioxidant activities of the oil against the stable free radical DPPH were measured. Briefly; 10 µL of different concentrations of essential oils (5-35 µg/ml in DMSO) was added to 190 µL of ethanolic solution of DPPH. After 30 minutes of incubation at room temperature in the dark, the absorbance of the resulting mixture at 517 nm using Spectra max 340 USA (molecular devices). Appropriate blank (DMSO) and standard (Ascorbic acid in distilled water) solutions were prepared and run simultaneously.

Oil constituents	T
% of identified component	95.4
% of unidentified components	4.6
Monoterpene hydrocarbons	63.39
Sesquiterpene hydrocarbons	10.64
Other hydrocarbons	-
Total hydrocarbons	74.03
Oxygenated monoterpenes	17.28
Oxygenated sesquiterpenes	2.13
Other oxygenated constituents	1.96
Total oxygenated compounds	21.37
Total monoterpenes	80.67
Total sesquiterpenes	12.77
Major constituent	*β*- phellandrene

Table 3: The calculated percentage of different classes of components of leaves essential oil of *S. sempervirens* (Lamb.).

Ascorbic acid was used as reference compound. The assay was carried out in triplicate. The percentage inhibition (I %) for each concentration was calculated by using the absorbance (A) values according to the following formula:

$$I\% = [(A_{blank} - A_{oil})/Ablank] \times 100$$

Where: A_{blank} is the absorbance of blank solution and A_{oil} is the absorbance of the oil. The dose-response curve was plotted and IC_{50} value for the oil was calculated [9].

Antimicrobial screening

A series of bacterial and fungal strains available in stock culture of the Regional Center for Mycology and Biotechnology Antimicrobial Unite (RCMB), Cairo, Egypt, were used for antibiotic sensitivity testing comprising: Gram-positive Bacteria [*Staphylococcus aureus* (RCMB 010015) *Bacillus subtilis* (RCMB010016) *Lactobacillus acidophilus* (RCMB 010023)], Gram-negative Bacteria [*Salmonella typhimurium* (RCMB 010076) *Escherichia coli* (RCMB 010085) *Klebsiella pneumoniae* (RCMB 0010082) *Shigella flexneri* (RCMB 0100876) *Pseudomonas aeruginosa* (RCMB 010043) *Proteous vulgaris* (RCMB 010062)], and four Fungi [*Aspergillus flavus* (RCMB 02554), *Aspergillus niger* (RCMB 05033), *Geotricum candidum* (RCMB 05096), *Trichophyton mentagrophytes* (RCMB 09254)]. The previously prepared essential oil was diluted 1/3 v/v in dimethyl sulphoxide (E-Merck), 30 µl (containing 10 µl of pure oil) were used in the test. Dimethyl sulphoxide (50 µl) was used as a negative control. The agar diffusion method [10] was applied using trypticase soy agar (Difco) medium inoculated with the bacterial or fungal suspension of the test organisms. Discs 5 mm in diameter were impregnated with the oils or the control. Then the discs were placed onto the surface of the culture medium. Discs of ceftriaxon and clotrimazole were used as standard antibacterial and antifungal agent, respectively. The plates were incubated at 35-37°C for 24-48 hours in case of bacteria, 25°C for 48 hours in case of filamentous fungi, while yeasts were incubated at 30°C for 24-48 hours. After incubation, the diameters of inhibition zones were recorded in millimeters and the results were compiled in Table 4. The minimum inhibitory concentrations (MIC) of *S. sempervirens* oil against the tested microorganisms were also determined by micro dilution method [11] results are recorded in Table 5.

Cytotoxic activity

The cytotoxic activity of the oil under investigation was tested against hepatocellular (HEPG2), Colon adenocarcinoma (HCT-116) and breast (MCF7) carcinoma cell lines, obtained from National Cancer Institute,

Tested microorganisms	Essential oil	Standard
Gram-Positive Bacteria		**Ampicillin**
Staphylococcus aureus (RCMB 010015) *Bacillus subtilis* (RCMB010016)	15.6 ± 1.16 17.2 ± 2.24	20.6 ± 1.12 25.2 ± 2.18
Lactobacillus acidophilus (RCMB 010023)	15.1 ± 1.32	26.4 ± 2.34
Gram-negative Bacteria		**Gentamycin**
Salmonella typhimurium (RCMB 010076	13.4 ± 1.39	19.3 ± 1.19
Escherichia coli (RCMB 010085)	12.5 ± 1.16	23.2 ± 1.32
Klebsiella pneumoniae (RCMB 0010082*)*	11.1 ± 2.16	26.1 ± 2.16
Shigella flexneri (RCMB 0100876) *Pseudomonas aeruginosa* (RCMB 010043)	13.4 ± 1.68 14.3 ± 1.19	23.8 ± 1.23 17.7 ± 1.22
Proteous vulgaris (RCMB 010062)	12.8 ± 1.23	25.4 ± 0.97
Fungi		**Amphotericin B**
Aspergillus flavus (RCMB 02554)	NA	24.6 ± 2.10
Aspergillus niger (RCMB 05033)	13.0 ± 1.22	21.8 ± 1.12
Geotricum candidum (RCMB 05096)	12.2 ± 1.13	26.4 ± 1.20
Trichophyton mentagrophytes (RCMB 09254)	16.6 ± 1.17	25.4 ± 2.16

The test was done using the agar disc diffusion technique, Well diameter 6.0 mm, (100 µl was tested), RCMB: Regional Center for Mycology and Biotechnology Antimicrobial unit test organisms. *NA: No activity, data are expressed in the form of mean ± SD. A value of P<0.05 was accepted as significant

Table 4: Antibacterial activity of leaves essential oil of *S. sempervirens* (Lamb.) against the ested microorganisms.

Tested microorganisms	MIC (µl/mL)
Staphylococcus aureus (RCMB 010027)	4.2
Staphylococcus epidermidis (RCMB 010024)	6.1
Streptococcus pyogenes (RCMB 010015)	5.3
Neisseria gonorrhoeae (RCMB 010076	7.3
Proteous vulgaris (RCMB 010085)	7.8
Klebsiella pneumoniae (RCMB 0010093*)*	8.4
Shigella flexneri (RCMB 0100542)	7.1
Pseudomonas aeruginosa (RCMB 010043	6.9
Escherichia coli (RCMB 010056)	7.6
Aspergillus fumigatus (RCMB 02564)	ND
Candida albicans (RCMB 05035)	8.2
Geotricum candidum (RCMB 05096)	9.9
Trichophyton mentagrophytes (RCMB 0925)	6.2

ND; Not done, as essential oil (s) has no antimicrobial activity on this microorganism.

Table 5: Minimum inhibitory concentrations (MIC) of essential oil of *S. sempervirens* (Lamb.) leaves against the tested microorganisms.

Kasr El Ainy, Cairo, Egypt, using Sulphorhodamine B assay (SRB) according to the method of [12]. The oil was dissolved in saline solution in a concentration of 100 µg/100 µl, tween 80 was used to help dissolution of insoluble materials. The obtained IC_{50} were compared with that of doxorubicin as reference drug; results are recorded in Table 7.

Statistical analysis

The results were subjected to statistical analysis. Values were reported as Mean ± SD. The data were analysed using Student's t-test to test for differences between treatment and control where a value of P<0.05 was accepted as significant.

Results and Discussion

Physical characters of the essential oil

The color, odor, yield, refractive index (20°C) and the specific gravity (20°C) of the hydro distilled oil were examined. The essential oil prepared from *S. sempervirens* leaves was obtained as pale yellow oil with characteristic, strongly aromatic odor. The percentage yield of the oil was 0.68% v/w (calculated on fresh weight basis), Table 1.

Conc. (µg/ml)	Ascorbic acid	Essential oil
35	80.12 ± 0.55	75 ± 0.47
30	78 ± 0.45	68 ± 0.32
25	76 ± 0.31	54 ± 0.25
20	71 ± 0.26	37 ± 0.41
15	68 ± 0.35	25 ± 0.21
10	28 ± 0.17	19 ± 0.45
5	14 ± 0.08	13 ± 0.15
0	0	0
IC_{50}	16.9	32.2

Data are expressed in the form of mean ± SD. A value of P<0.05 was accepted as significant.

Table 6: *In vitro* DPPH radical scavenging activity of essential oil of *S. sempervirens* (Lamb.) leaves.

***S. sempervirens* oil and drug**	**IC_{50} µl/ml**		
	HEPG2	**HCT-116**	**MCF7**
Essential oil	7.567	8.835	9.782
Doxorubicin	0.6	0.85	0.7

Table 7: Results of cytotoxic activity of of leaf essential oil of *S. sempervirens* (Lamb.).

GC/MS analysis of the essential oils

Thirty-six constituents in the essential oil of *S. sempervirens* leaves were identified corresponding to 95.4% of the total oil. The results revealed that terpenoids (including saturated hydrocarbon, monoterpenoids, sesquiterpenoids and oxygenated ones) in the oil were predominant. The main constituents of the essential oil were α-phellandrene (29.60%), dl-limonene (15.60%), α-pinene (8.65%), β-germacrene (4.87%), respectively. In general the percent of hydrocarbons (74.03%) is higher than the percent of oxygenated compounds 21.37% in the oil sample under investigation, also the percent of monoterpenoid 80.67% compounds is higher than that of sesquiterpenes 12.77%. The percentages of total hydrocarbons, monoterpene and sesquiterpene hydrocarbons as well as the percentages of total oxygenated compounds, oxygenated monoterpenes and sesquiterpenes were calculated and are compiled in Tables 2 and 3. This result agreed with the previous results submitted by Sefidkon et al. [13] who reported that the main components of oil from *S. sempervirens* leaves were b- phellandrene and limonene (13.30%), a- pinene (6.83%), terpinene -4-o1 (6.47%), g-teroubebe (5.44%) and germacrene B (4.17%).

Antimicrobial activity

In the present study, the antibacterial activity of *S. sempervirens* leaves essential oil was tested by the disc diffusion method against nine bacterial species. Results showed that the leaves essential oil, at concentration of 20 µL/disc, gave inhibition activities (more than 6 mm diameter hollow zones) against all tested Gram-positive species and all tested Gram-negative bacteria species, they were all sensitive to the essential oil comparing to Ampicillin and Gentamycin, respectively. Antifungal activity of the essential oil of leaves of *S. sempervirens* was tested against four fungal species. All tested fungal species were susceptible to the discs impregnated with the tested essential oil with the exception of *Aspergillus flavus* which was totally resistant to leaves essential oil (Table 4). Although the essential oil of *S. sempervirens* had better antibacterial activity against Gram-positive species, it showed broad-spectrum antibacterial and antifungal action as well. Accordingly, the essential oil of the leaves should be further investigated to evaluate its efficiency and safety in clinical practice. The MIC of leaves

essential oil against the tested microorganism is presented in Table 5. The results revealed variability in the inhibitory concentrations of the essential oil against each microorganism, in the range (concentrations) from 4.2 to 9.9 µl/mL. The lowest variation was observed for essential oil on *Staphylococcus aureus* followed by *Streptococcus pyogenes* and *Staphylococcus epidermidis*. Previously, the volatile oil of *S. sempervirens* showed antifungul activity against *Pleuroplaconema sp*. [14].

Earlier papers on the antibacterial activities of α-phellandrene, limonene, α- pinene, and β-caryophyllene have shown that they have varying degrees of growth inhibitory effects against some bacteria [15-17]. The current study shows that the antimicrobial activity of the oils from *S. sempervirens* leaves could, in part, be associated with its major components (α-Phellandrene,dl-limonene and α-pinene).

Antioxidant activity

Antioxidant potential of essential oil of *S. sempervirens* leaves was determined as its DPPH radical scavenging ability, Table 6. The essential oil exhibited higher free radical scavenging activity (75 ± 0.47%) at a conc. of 35 µg/ml with IC_{50} value equal to 32.2 µg/ml. Studies by Bajpai et al. [18]. evaluated the antioxidant activity of the essential oil obtained from of *Metasequioa glyptostroboides* Miki leaves, a species of the same family verified the free radical scavenging activity of the oil was found to be 11.32 µg/ml. The antioxidant activity of essential oil can be attributed to the synergistic activities of multiform unsaturated compounds such as α-phellandrene, limonene and α-pinene which reported to have antioxidant activities [19,20].

Anticancer activity

The essential oil of *S. sempervirens* leaves showed antitumor activities against all tested cell lines (IC_{50} Values <10 µg/ml). The IC_{50} values were 7.567, 8.835 and 9.782 µg /ml for HEPG2, HCT-116 and MCF7 respectively. The cytotoxic activity of the essential oil of *S. sempervirens* leaves could be attributed to its hydrocarbon contents [21-23]. Other studies has verified that α-Phellandrene, dl-limonene, α-pinene, have been described as cytotoxic compounds against different cell lines [24-27]. Regarding that bioactive cytotoxic compounds have been found in the essential oil from *S. sempervirens* leaves, it is expectable to observe cytotoxic activity against the examined cell lines In addition, the components with lower concentrations, such as α- Farnesene, β-Germacrene α- humulene and β-caryophyllene, may also be contributing to the cytotoxic activity of the oil. Therefore, the synergistic effects of the major and minor components of the essential oils should be taken into consideration to account for the oil biological activity [28-30].

Conclusion

The antimicrobial, antioxidant and cytotoxic activities of the essential oils from many plants are of great interest to both the academe and the pharmaceutical, cosmetic, and food industries because of their possible use as natural additives to replace synthetic compounds. For the first time, we reported that the essential oil of *S. sempervirens* leaves exhibits antioxidant, cytotoxic activities and successfully inhibits the growth of different microorganisms. The results obtained in this study show that the essential oil of *S. sempervirens* leaves may be a new potential source of natural antimicrobial, antioxidant and cytotoxic agents so they could be used in pharmaceutical formulations. However, further studies need to be conducted to understand the mechanism of the activity and obtain more information on the safety and toxicity of the oil. In general, we recommend that a further study under the *in vivo* conditions to further elaborate the antimicrobial, antioxidant and cytotoxic principles of *S. sempervirens* essential oil for various useful applications. The investigated essential oil may be used for the preservation of processed foods as well as pharmaceutical and natural therapies for the treatment of infectious diseases in humans and plants.

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Declaration of Interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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