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, *Sequoiodendron* 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 Environments 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.
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 spectroscopy) was measured. Briefly; 10 μL of a series 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 spectroscopy) was measured. Briefly; 10 μL of a series 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 spectroscopy) was measured.
The test was done using the agar disc diffusion technique, Well diameter 6.0 mm, against the tested microorganisms.

Table 5: Minimum inhibitory concentrations (MIC) of essential oil of S. sempervirens (Lamb.) leaves. A value of P<0.05 was accepted as significant.

| Tested microorganisms | Essential oil | Standard |
|------------------------|---------------|----------|
| Gram-Positive Bacteria |              |          |
| Staphylococcus aureus (RCMB 010015) Bacillus subtilis (RCMB010016) Lactobacillus acidophilus (RCMB 010023) | 15.6 ± 1.16 17.2 ± 2.24 15.1 ± 1.32 | Amoxicillin 20.6 ± 1.12 25.2 ± 2.18 26.4 ± 2.34 |
| Gram-negative Bacteria |              |          |
| Salmonella typhimurium (RCMB 010076) Escherichia coli (RCMB 0100885) Klebsiella pneumoniae (RCMB 0010882) Shigella flexneri (RCMB 0010876) Pseudomonas aeruginosa (RCMB 010043) Proteus vulgaris (RCMB 010062) | 13.4 ± 1.39 12.5 ± 1.16 11.1 ± 2.16 13.4 ± 1.68 14.3 ± 1.19 12.8 ± 1.23 | Gentamycin 19.3 ± 1.3 23.2 ± 1.32 26.1 ± 2.16 23.8 ± 1.23 17.7 ± 1.22 25.4 ± 0.97 |
| Fungi |              |          |
| Aspergillus flavus (RCMB 02554) Aspergillus niger (RCMB 05033) Geotricum candidum (RCMB 05096) Trichophyton mentagrophytes (RCMB 09254) | NA 13.0 ± 1.22 12.2 ± 1.13 16.6 ± 1.17 | Ampicillin B 24.6 ± 2.10 21.8 ± 1.12 26.4 ± 1.20 25.4 ± 2.16 |

The test was done using the agar disc diffusion technique, Well diameter 6.0 mm, against the tested microorganisms.

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

| Tested microorganisms | MIC (μL/mL) |
|------------------------|------------|
| Staphylococcus aureus (RCMB 01027) | 4.2 |
| Escherichia coli (RCMB 010024) | 6.1 |
| Streptococcus pyogenes (RCMB 010015) | 5.3 |
| Neisseria gonorhoeae (RCMB 010076) | 7.3 |
| Proteus vulgaris (RCMB 010085) | 7.8 |
| Klebsiella pneumoniae (RCMB 001093) | 8.4 |
| Shigella flexneri (RCMB 0100842) | 7.1 |
| Pseudomonas aeruginosa (RCMB 010043) | 6.9 |
| Escherichia coli (RCMB 010056) | 7.6 |
| Aspergillus fumigatus (RCMB 25564) | ND |
| Candida albicans (RCMB 05035) | 8.2 |
| Geotricum candidum (RCMB 05096) | 9.9 |
| Trichophyton mentagrophytes (RCMB 09254) | 6.2 |

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

Kas 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, then 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.

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 21.57% in the oil sample under investigation, also the percent of monoterpenoids 80.67% compounds is higher than that of sesquiterpenes 12.77%. The percentages of total hydrocarbons, monoterpenes and sesquiterpenes 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%), α- pinene (6.83%), β-germacrene (4.87%), respectively.

Antimicrobial activity

In the present study, the antibacterial activity of S. sempervirens leaves 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 antifungal activity against Pleurophacomena 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 be, in part, be associated with its major components (α-Phellandrene,dll-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 IC50 value equal to 32.2 μg/ml. Studies by Bajpai et al. [18], evaluated the antioxidant activity of the essential oil obtained from of Metasequoia 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 multifrom 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 (IC50 Values <10 μg/ml). The IC50 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.

Acknowledgements

The authors thank the Regional Center for Mycology and Biotechnology Antimicrobial Unite (RCMB), Cairo, Egypt for supplying microbial stains for performing the antimicrobial study.

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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