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Research Article

Chemical Composition and Antimicrobial Activity of the Essential Oil of *Cupressus sempervirens* L. Leaves in Syria

Mohamad Jawad Khubeiz[1*], Ghaytha Mansour[2] and Bahira Zahraa[1]

[1]*Department of Chemistry, faculty of science, Damascus University, Albarameka, Damascus, Syria.*
[2]*Department of Botany, faculty of science, Damascus University, Albarameka, Damascus, Syria.*

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ABSTRACT

In the present research the essential oil contents and compositions of individual plant from *Cupressus sempervirens* L. *(Cupressaceae),* grown in Damascus / Syria have been investigated. The antimicrobial activity of the essential oil was also discovered. The air-dried leaves were hydro-distilled, and the essential oil analyzed by GC-MS. The essential oil from *C. sempervirens* L. sample was tested for antimicrobial activities using agar disc diffusion technique to determine the diameter of growth inhibition zones. The macro dilution broth susceptibility assay was utilized to determine minimum inhibitory concentrations (MIC), and minimum bactericide concentration (MBC). Fourteen components were identified in the essential oil of *C. sempervirens* L. The main constituents of essential oil leaves were alpha-pinene (36.50%), 3-carene (22.17%), Germacrene D (12.81%), Terpinolen (5.18%), alpha-tepinnyl acetate (4.76), limonene (3.55%) and beta-myrcene (3.16%). The results of the antimicrobial activity tests revealed that the essential oil has rather a strong antimicrobial activity, especially against *Streptococcus pyogenes, Staphylococcus aureus* and *Vibrio parahaemolyticus,* with diameter of inhibition zones ranging from (38.9 ±0.16) to (43.2 ± 0.15) mm, it showed a modest antibacterial effect for *Micrococcus luteus, Klebsiella pneumonia* and *Pseudomonas aeruginosa,* with inhibition zones in the range (8.5± 0.13) to (10.2 ±0.13) mm. Minimum inhibitory concentration (MIC) revealed the lowest activity against *Micrococcus luteus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (3.2mg/mL), while the highest activity was against *Streptococcus pyogenes, Vibrio parahaemolyticus* (1.6 mg/mL) and *Staphylococcus aureus* (0.4 mg/mL). The Minimum bactericidal concentration (MBC) activity was from (1.6 mg/mL) to (4.6 mg/mL).

Keywords: *Cupressus sempervirens* L, Essential oil, Antimicrobial activity, MIC, GC-MS.

INTRODUCTION

There is an increasing interest in using medicinal and aromatic plants all over the world[1]. Bioactive compounds of medicinal plants led them to be used in various industries as botanical drugs, dietary supplements, functional foods, etc[2]. Plants also have been used in ethno pharmacy for various diseases such as hypertension, high cholesterol, eczema and diarrhea for centuries, and today their scientific validation was provided by identification and isolation of bioactive phytochemicals[3,4]. In the last decades, the clinical efficacy of many synthetic antibiotics is being threaded by the emergence of a serious problem which can be defined as multidrug resistant pathogens[5]. Therefore scientists have tried to discover new antimicrobial substances from various sources including plants[6]. Medicinal plants have considerable importance in international trade and their clinical, pharmaceutical, and economic value is still growing[7]. Therefore the use of essential oils is less damaging to the human health[8]. Essential oils of plants are of growing interest both in the industry and scientific research[9,10]. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils[11-13]. Chemically, these volatile products and their oxygenated compounds are derived from terpenes[14,15]. Monoterpenes, sesquiterpenes and diterpenes are natural compounds of essential oils with highly variable structural configurations. Each of these constituents contributes to the beneficial or adverse effects. Many researchers have also focused on plants in order to discover new drugs. *Cupressus* is considered to be a medicinal tree. It is one of the most important trees for multipurpose forestry in the Mediterranean region, since it is well adapted to calcareous, clayish, dry and poor soils[16]. The Mediterranean *Cupressus* (*C. sempervirens* L.) grows over a wide natural range in diverse environments. It has two main varieties: *horizontal* is exhibiting abroad crown with wide angles between branches and stem and *pyramidalis* exhibiting a conical crown form and small angles between branches and stem. The variety *horizontalis* grows naturally and can be found in northern Iran, Syria, Cyprus, Turkey and Greece. *C. sempervirens* L. is only one species native of the genus *Cupressus* in Syria, whereas *C. arizonica* is one of the many species from this genus which has been introduced and cultivated in the country. *Cupressus* leaves and cones have been used as folk remedy in many countries[17]. With

**Author for Correspondence*

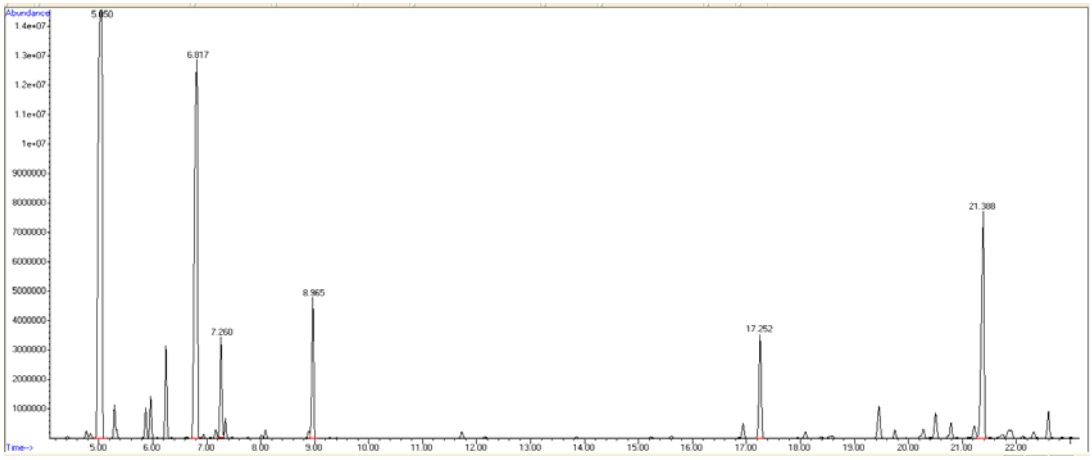

Figure 1: GC/MS Chromatogram of dried *C.sempervirens* L .leaves essential oil.

Table 1: Chemical compositions of dried *C. sempervirens* L. leaves essential oil.

S. No.	R.I	R.I[A]	Compositions	Area %
			Monoterpene Hydrocarbons	74.23
1.	939	932	Alpha-Pinene	36.50
2.	949	946	Camphene	1.13
3.	976	974	Beta-Pinene	1.04
4.	980	982	Cis-Pinane	1.50
5.	993	988	Beta-Myrcene	3.16
6.	1014	1008	3-Carene	22.17
7.	1030	1024	Limonene	3.55
8.	1090	1086	Terpinolen	5.18
			Oxygenated Monoterpenes	4.76
9.	1351	1346	Alpha-Terpinyl acetate	4.76
			Sesquiterpene hydrocarbons	19.33
10.	1421	1417	Alpha -Caryophyllene	1.61
	1431	1430	β-Copaene	0.72
	1449	1449	Alpha -Himachalene	0.45
11.	1455	1455	Alpha-Humulene	1.41
12.	1465	1464	9-epi-Caryophyllene -E	0.84
13.	1484	1484	Germacrene D	12.81
	1497	1498	Alpha -Selinene	0.68
14.	1525	1522	delta-Cadinene	1.31
			Total	98.82

RI: retention index (Kovalts) relative to n-alkanes (C8–C20) on a non-polar DB-5 column. RI[A]: retention index in the literature on a non-polar DB-5 column (Adams, 2007).

respect to these medicinal and pharmacological advantages, *C. sempervirens* L. widely used as a cosmetic in gradient in perfumery and soap-making, including its essential oil from it[18]. The aim of this work is to assay the main constituent of the essential oil obtained from leaves of *C. sempervirens* L. Which is grown in Syria, to compare their chemical composition with those extracted from the same species from different geographical origins, and to carry out a comparative evaluation of their antibacterial activity.

EXPERIMENTAL

Plant Material

Fresh leaves of *C. Sempervirens* L. growing in the campus of Damascus University faculty of science, was randomly collected in May 2014, and authenticated by the taxonomist of Department of Botany. The dirt was removed with tap water. Collected leaves were dried in the shade for about 14 days. The dried leaves of plant parts were ground into small powder by a grinder machine.

Essential Oil Extraction

Dried *C. sempervirens* L. leaves (100 g) was subjected to hydro-distillation in a Clevenger-type apparatus for (3) hrs. in accordance with the standard procedure described in the European Pharmacopoeia[19]. The sample was added to distilled water (1000 m l) in a 2 L round - bottomed flask and heated to boiling, after which the essential oil was evaporated together with water vapor, and finally collected in a separating funnel. The upper phase that contained the essential oil was separated from the lower one and the essential oil was dried over anhydrous sodium sulfate and preserved in a sealed sample tube and stored

Table 2: Antibacterial activity of dried *C. sempervirens* L .leaves essential oil.

Test Organisms	Different concentrations of essential oil				Amoxicillin
	25%	50%	75%	100%	30mg/mL
gram-positive bacteria					
Bacillus subtilis	NA	NA	NA	NA	40 ± 0.11
Micrococcus luteus	7.40 ± 0.16	7. 70 ± 0.14	8.10 ± 0.17	8.50 ± 0.13	17 ± 0.21
Streptococcus pyogenes	13.50 ± 0.13	15.60 ± 0.16	18.40 ± 0.13	38.90 ± 0.16	NA
Staphylococcus aureus	14.40 ± 0.12	19. 50 ± 0.11	27.40 ± 1.15	42.30 ± 0.18	45± 0.13
gram-negative bacteria					
Klebsiella pneumoniae	7.60 ± 0.12	8.10 ± 0.17	9.10 ± 0.15	10.20 ± 0.13	NA
Vibrio parahaemolyticus	19.20 ± 0.13	25. 20 ± 0.17	39.18 ± 0.19	43.20 ± 0.15	30± 0.32
Proteus vulgaris	NA	NA	NA	NA	45± 0.12
Salmonella Typhimurium	NA	NA	NA	NA	16± 0.31
Escherichia coli	NA	NA	NA	NA	NA
Pseudomonas aeruginosa	7. 40 ± 0.11	8. 20 ± 0.10	8.70 ± 0.14	9.40 ± 0.16	NA

Data are mean ± SD of three independent experiments. NA: Not active

Table 3: MIC and MBC (mg/mL) of dried *C. sempervirens* L. leaves essential oil.

Microorganism	MIC (mg/mL)	MBC (mg/mL)
Bacillus subtilis	NA	NA
Micrococcus luteus	3.2	6.4
Streptococcus pyogenes	1.6	1.6
Staphylococcus aureus	0.4	1.6
Klebsiella pneumoniae	3.2	6.4
Vibrio parahaemolyticus	1.6	3.2
Proteus vulgaris	NA	NA
Salmonella Typhimurium	NA	NA
Escherichia coli	NA	NA
Pseudomonas aeruginosa	3.2	6.4

NA: Not active

in the fridge at 4ºC until analysis[20].

Analysis of the Essential Oil (GC/MS)

Essential oil composition was studied 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 70eV with interface temperature of 280ºC; the scan range was 50–550amu. The injection port temperature was set at 250 °C. But 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 3ºC/min. The injection volume was 1.0 µL.

Identification of Components

Relative percentage amounts were calculated from peak area by apparatus software. The identification of individual compounds was based on comparison of their mass spectra with those obtained from the Wiley Libraries spectra, stored in the GC-MS database**.** Further confirmation was done from Retention Index data generated from a series of alkane's retention indices (relatives to C8-C20 on the DB-5MS column) and (Adams, 2007).

Screening for Antimicrobial Activity

The essential oil from *C. sempervirens* L. sample was tested for antimicrobial activities using agar disc diffusion technique to determine the diameter of growth inhibition zones while broth macro-dilution method was used to determine the MIC and MBC. The biological assays were carried out on the essential oil against ten local human bacteria isolates. These microbes were four gram-positive bacteria (*Bacillus subitus, Staphylococcus aureus, Streptococcus pyogenes, Micrococcus luteus*) and six gram-negative bacteria (*Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginose, Klebsellia pneumonia, Proteus vulgaris, Vibrio parahaemolyticus*) obtained from the Department of Medical Microbiology and Parasitology, Faculty of Medicine, Damascus university. The bacteria cultures were maintained on nutrient agar slant at 37ºC for 24 hrs. The organisms were then sub cultured and preserved at 4ºC in sterile bottles containing both nutrient broth and 15% sterile glycerol. The essential oil of the dried leaves was tested for antimicrobial activity against the tested organism using the agar disc diffusion method[21]. Each of the organisms was transferred into a separate test- tube containing nutrient broth to reactivate them by culturing overnight at 37ºC. A suspension of the tested bacteria (0.1 ml of 10^6 CFU/mL) was spread on the solid media plates. Sterile apery discs (5 mm diameter) prepared from Whatman Number 1. Filter paper discs were loaded with 20 µL of the essential oil and placed on the inoculated plates and, after staying at 4ºC for 2 hrs, the plates were incubated at 37ºC for 24 hrs. Antimicrobial activities studied with four concentrations 100%, 75%, 50% and 25% of the essential oil, which was dissolved in dimethyl sulphoxide (DMSO). The zones of inhibition were measured at the end of the incubation period. Antimicrobial activities of the essential oil leaves were expressed by mm in diameter. The result recorded for each bioassay was the average of triplicate test. Negative control was prepared using DMSO solvent. And

Table 4: Main constituents of dried *C. sempervirens* L. leaves essential oil from different origins.

No.	α-pinene	β-myrcene	3-carene	α-terpinylacetate	α-terpinolene	Germacrene D	α-cedrol
Our study	36.50	3.16	22.17	4.76	5.18	12.81	-
24	30.00	4.10	24.00	6.60	6.60	4.00	-
25	21.40	5.00	16.00	5.60	5.60	13.00	3.30
26	60.50	3.90	0.20	-	2.00	2.30	8.30
27	28.40-79.20	-	9.10-32.60	0.80-4.50	0.60-7.00	-	1.20-12.90
28	54.1	4.6	10.8	5.5	4.1	2.4	4.9
29	48.60	4.10	22.10	-	4.50	1.60	3.50
30	37.10	3.60	19.60	-	4.70	1.30	1.69
31	27.5	1.0	13.2	0.2	7.2	12.1	19.3
32	6.9	-	17.85	-	9.17	2.75	21.29

Amoxicillin was used as a positive control.

Determination of the MIC and MBC

The MIC values of essential oil were tested by two-fold serial dilution method[22]. The test samples of essential oil were first dissolved in 5% DMSO, and incorporated into nutrient broth medium in a tube to obtain a concentration of 12.8 mg/mL, and serially diluted to achieve 6.4, 3.2,1.6 and 0.8 mg/mL, respectively. Ten microliters of standardized suspension of each test organism (10^6 CFU/mL) was transferred to the tubes, and incubated at 37°C for 24 hrs. The MIC was determined as the lowest concentration (mg/mL) of the essential oil where no visible growths of test organisms occur. The microorganism growth was indicated by turbidity. MBC is usually an extension from the MICs, where the organisms quantitatively indicate the minimum concentration when no viable organism appears in the culture[23].

Statistical Analysis

All determinations in this article were carried out in triplicates and 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 yield of *C. sempervirens* L. leaves essential oil from the hydro distillation of Sample was 1.28% v/w. Table 1 shows the identified constituents of this essential oil percentage and fig 1 shows their GC/MS Chromatogram. A total of fourteen compounds amounting to 98.82% in the *C. sempervirens* L. leaves essential oil were identified. Among these 74.23% were monoterpene hydrocarbons, 4.76% were oxygenated monoterpenes, and it also contained 19.33% sesquiterpene hydrocarbons and no oxygenated sesquiterpenes found. The major constituents in the *C. sempervirens* L. leaf essential oil were alpha-pinene 36.50%, 3-carene 22.17%, Germacrene D 12.81%, Terpinolen 5.18%, alpha-terpinnyl acetate 4.76, limonene 3.55% and beta-myrcene 3.16%. The in vitro antibacterial activities of essential oil of *C. sempervirens* L. leaves against the bacteria used were qualitatively assessed by the presence or absence of inhibition zones. A total of ten bacterial strains, including four Gram-positive and six Gram-negative bacteria were tested. The essential oil exhibited antibacterial activity against three Gram-positive and three Gram-negative bacteria. They exhibited a potent inhibitory effect against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*, with diameter of inhibition zones ranging from 38.9 ± 0.16 to 43.2 ± 0.15 mm, as shown in Table 2.

On the other hand, essential oil exhibited the modest antibacterial effect for *Micrococcus luteus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, with inhibition zones in the range 8.5± 0.13 to 10.2 ± 0.13 mm, whereas it showed no activity against the *Bacillus subtilis* (gram positive) and *Proteus vulgaris, Salmonella Typhimurium* and *Escherichia coli* (gram negative). The antimicrobial activity of the *C. sempervirens* L. essential oil was more pronounced against Gram-positive than Gram-negative bacteria. The results of the tests for MIC and MBC revealed that the MIC and MBC values for *Micrococcus luteus* 3.2, 6.4 mg/ mL, *Klebsiella pneumonia* 3.2, 6.4 mg/ mL and *Pseudomonas aeruginosa* 3.2, 6.4 mg/ mL respectively, were bigger than that of *Streptococcus pyogenes* 1.6, 1.6 mg/ mL and *Vibrio parahaemolyticus* 1.6, 3.2 mg/ mL respectively, while no active MIC and MBC for *Bacillus subtilis*, *Proteus vulgaris, Salmonella Typhimurium* and *Escherichia coli* as shown in Table 3. It is difficult to compare between different studies that concerning essential oil composition of *C. sempervirens* L. due to the difference in compounds. In previous studies, essential oil of *C. sempervirens* L. was studied in Iran[24,25], Algeria[26], Croatia[27], Greece[28], Saudi Arabia[29], Tunisia[30,31] and Egypt[32]. The major components were α-cedrol, 3-carene, terpinolene, Germacrene D and α-terpenyl acetate. According to these studies, α-pinene is the first major component of leaves essential oil, these findings are in general agreement with our reports, but α-pinene is the fourth major component in the previous research[33]. 3-carene is the second major component in the reported investigation for researches[24-30], whereas it was in third major component in (31), and the second in (32). In addition, Germacrene D is the third component in the previous research (29), and the fourth in (31). Ultimately, α-cedrol is the first major component in (32), but in (26, 31) the second major component, when it is third in (27) and fourth in (28, 29). In comparison with our results, the major components are α-pinene, 3-carene, Germacrene D, α-terpinyl acetate, α-terpinolene respectively, while no α-cedrol included. The study of essential oil of *C. sempervirens* L. grows

in Mediterranean countries, as Syria (this study), Algeria[26], Greece[28], Tunisia[30,31], and Egypt[32]. It showed dissimilarity in components of essential oil, although they have the same nature of the climate. Finally, the differences between the main constituents and their percentages of dried *C. sempervirens* L. leaves essential oil. growing in other countries seem to be related particularly to dry and extraction methods, soils and genetic background of trees. Obtained data of some of these studies are summarized in Table 4. The antimicrobial effects of essential oil showed a higher resistance among Gram-negative bacteria Table 2 It has been noticed that the *P. aeruginosa* has less sensitivity to the tested *Cupressus* essential leaves oil. The essential oil had no antimicrobial activity against *C. albicans*, *E. coli* and *S. aureus*. This is in general agreement with the Egyptian report indicating that the essential oil was active against *P. aeroginosa* and *S. aureus,* while it was inactive against *B. subtilis* and *E. coil*[33,34]. The antimicrobial activity of the essential oil of *C. sempervirens* L. leaves could, in part, be associated with theirs major constituents such as α-pinene and cedrol. These components have been reported to display antimicrobial effects[35,36]. The essential oil containing terpenes are also reported to possess antimicrobial activity[37], which are consistent with our present study. The strains which presented the biggest inhibition zones are not always the most sensitive (value of MIC was lower) because the diameter of IZ does not reflect the antibacterial activity of a compound[38].

CONCLUSIONS

The results of this work prove that the main constituents essential oil of leaves were alpha-pinene 36.50%, 3-carene 22.17%, Germacrene D 12.81%. The *C. sempervirens* L**.** leaves essential oil is rich in aromatic compounds, possesses antimicrobial properties, which can be used as a natural antimicrobial agent for human infectious diseases. Ten bacterial strains, including four Gram-positive and six Gram-negative bacteria were tested. The essential oil exhibited antibacterial activity against three Gram-positive and three Gram-negative bacteria, with diameter of inhibition zones ranging from 38.9 ± 0.16 to 43.2 ± 0.15 mm. The development of natural antimicrobial agents will help to decrease negative effects of synthetic chemicals and drugs. The quality of natural products depend considerably on their geographic origin, thus the leaves essential oil components of the Syrian *C. sempervirens* L**.** must be known for each region to confirm the quality of essential oil which is important to determine the possibility of utilizing it in food preservation, cosmetics and pharmaceutical industries.

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