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Chemical Composition and Biological Potential of Essential Oil from Tunisian *Cupressus sempervirens* L.

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Abstract: The essential oil was obtained by hydro-distillation of the aerial part of *Cupressus sempervirens* L. cultivated in Sfax gardens, Tunisia. The obtained oils were analyzed by gas chromatography-mass spectrometry (GC-MS) and 24 compounds were identified. The antioxidant activity of *Cupressus sempervirens* L. essential oil was evaluated by measuring the radicals-scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and by using the 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay. Antimicrobial activity were obtained on ten food-spoilage yeasts *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Halomonas elongate, Salmonella typhimurium, Enterococcus hirae, Aspergillus niger, Candida albicans and Trichoderma reesei*. The results suggest that the essential oil of *C. sempervirens* might be used as a readily accessible source of antimicrobial agent and as natural antioxidant.

Keywords: Antimicrobial activity, Antioxidant activity, *Cupressus sempervirens* L., Essential oil

1. Introduction

The use of essential oils as functional ingredients in foods, drinks, toiletries and cosmetics is gaining momentum, both for the growing interest of consumers in ingredients from natural sources and also because of increasing concern about potentially harmful synthetic additives (Reische *et al*., 1998). Actually, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey *et al*., 2001; Yangui *et al*., 2009). The genus *Cupressus* (cupressaceae), comprising twelve species, is distributed in North America, the Mediterranean region and subtropical Asia at high altitudes (Rawat *et al*., 2010). In Tunisia only one species of the genus *Cupressus*, *Cupressus sempervirens* L. (Cuénod, 1954) was native. The antimicrobial properties of essential oils have been recognised for many years (Yan *et al*., 2009; Gonzalez and Marioli 2010). Indeed, phytopreparation obtained from the core and young branches of *C. sempervirens* were reported to have antiseptic, aromatherapeutic, astringent, balsamic and anti-inflammatory activities (Milos *et al*., 2002).

In the present paper, we report the results of a study aimed to define functional antioxidant, antiradical and antimicrobial properties of cypress oil with some peculiarities related to chemical composition.

2. Materials and Methods

2.1. Plant materials

The aerial parts of *Cupressus sempervirens* L. were collected from the random gardens in Sfax (Tunisia) on April 2010. *C. sempervirens* is largely used as windbreaks and ornamentals throughout northern and center of Tunisia, and used as traditional medicines to treat cough, influenza, and rheumatism by local people. Identification of the species was carried out by Professor Mekki Boukhris from Botanic Ecology of Faculty of Sciences of the University of Sfax, Tunisia. A voucher specimen was deposited at the herbarium of this laboratory under the code LBPE.11 C.

2.2. Essential oils extraction

The essential oils were extracted by hydrodistillation of fresh aerial part of *C. sempervirens*. Material (1000 g of sample in 4L of distilled water) was subjected to hydro -distillation during approximately 4 h in a Clevenger-type apparatus. The oils were dried over anhydrous sodium sulphate and stored in sealed glass vials at 4-6°C prior to analysis.

2.3. GC/MS analysis

The analysis of the essential oils was performed on a GC-MS HP model 5975B inert MSD (Agilent Technologies, J &W Scientific Products, Palo Alto, CA, USA), equipped with an Agilent Technologies capillary DB-5MS column (30 m length; 0.25 mm i.d.; 0.25 mm film thickness), and coupled to

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a mass selective detector (MSD5975B, ionization voltage 70 eV; all Agilent, Santa Clara, CA). The carrier gas was He and was used at 1 $mL.min^{-1}$ flow rate. The oven temperature program was as follows: 1 min at 100°C ramped from 100 to 260°C at $4°C.min^{-1}$ and 10 min at 260°C. The chromatograph was equipped with a split/splitless injector used in the split mode. The split ratio was 1:100. Identification of components was assigned by matching their mass spectra with Wiley and NIST library data, standards of the main components and comparing their Kovats retention indices with reference libraries (Bouaziz *et al*., 2009) and from the literature. The component concentration was obtained by semi-quantification by peak area integration from GC peaks and by applying the correction factors.

2.4. Antioxidant Potential of Essential oil

2.4.1. DPPH assay

DPPH assay was carried out as described by Bouaziz *et al* (2010). Various concentrations of essential oil in ethanol (50 µL) were mixed with methanolic solution containing DPPH radicals (6 µM). The mixtures were shaken vigorously and left to stand for 30 min in the dark. Decrease in colorization was measured spectrophotometrically at 517 nm using a UV-1800PC, Japan spectrophotometer. The radical scavenging activity (RSA) was calculated using the equation:

$$\% RSA = [(A_{DPPH} - A_E) / A_{DPPH}] \times 100$$

Where A_E was the absorbance of solution containing antioxidant extract and A_{DPPH} was the absorbance of DPPH solution. The extract concentration providing 50% inhibition (IC_{50} $\mu g.mL^{-1}$) was calculated from the graph of RSA percentage against extract concentration. 2, 6-di-tert-butyl-4 -hydroxy-boxylic acid (BHT) was used as reference compound.

2.4.2. ABTS assay

The free-radical scavenging capacity was measured using the ABTS decoloration method (Re *et al*., 1999) with some modifications. Briefly, ABTS was dissolved in water to get a 7 mM concentration. ABTS radical ($ABTS^{\cdot+}$) was produced by reacting this stock solution with a 2.45 mM $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 730 nm. After the addition of 100 µL of sample, ethanol as a blank, or Trolox standard to 2.9 mL of diluted $ABTS^{\cdot+}$ solution, absorbance readings were taken after 6 min. ethanolic solutions of known concentrations of Trolox were used for calibration. This standard curve was linear between 0.2 mM and 0.8 mM of Trolox ($y = 0.6792\ x$, $r^2 = 0.9965$). The Results were expressed in TEAC (mM).

2.5. Antimicrobial activity

2.5.1. Test organisms and culture media

The most of the test organisms used were from the international culture collection strains of Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, *Enterococcus hirae* ATCC 10541, *Bacillus subtilus* ATCC 6633, Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 10536, *Salmonella typhimurium* TA 97, the yeast mould *Candida albicans* ATCC 10231, and filamentous fungi *Aspergillus niger* ATCC 16404, *Trichoderma reesei* Rut C30.

The bacteria were cultivated in Tryptic Soy Broth (TSB) or Agar (TSA) (Sigma) at the appropriated temperature (30°C or 37°C) of the strain. Fungi and yeasts were cultured on Malt Extract Broth (MEB) or Agar (MEA) (Fluka) at 28°C. Inocula were prepared by adjusting the turbidity of each bacterial and yeast cultures to reach an optical comparison to that of a 0.5 McFarland standard, corresponding to approximately 1-5 10^8 $cfu.mL^{-1}$. The concentration of spore suspensions was determined using a hematocytometer (Thoma cell) and adjusted to 1-5 10^8 spores mL^{-1}.

2.5.1. Minimal Inhibitory Concentration (MIC), Minimal Cidal Concentration (MCC)

This test was used to determine MIC and MCC of the essential oil of *C. sempervirens* against the test organisms as recommended by the National Committee for Clinical Laboratory Standard (NCCLS, 2002). This test was performed in a sterile 96-well microplate. The inhibitory activity of the essential oil was properly prepared and transferred to each microplate well in order to obtain a twofold serial dilution of the original sample. To obtain stable diffusion, a stock solution of the essential oil was prepared in 0.1% ethanol. The inocula (100 µL) containing 104 cfu of bacteria were added to each well. Control wells contained bacteria or fungi only, adequate medium only and chloramphenicol or gentamycin antibiotics (for bacteria and fungi respectively) (10 $\mu g.L^{-1}$). Thereafter, 30 µL of 0.02% resazurin and 12.5 µL of 20% Tween 80 were added. Plates were aerobically incubated at 30°C for 16-20 h. After incubation, the wells were observed for a color change from blue to pink. MIC was defined as the lowest concentration at which no growth was observed (blue colored) after incubation. Ethanol at 0.1% had no inhibition effect.

To determine MCC values, 10 µL of each culture medium with no visible growth were removed of each well and inoculated in TSA or MEB plates. After aerobic incubation at 30°C during 16-20 h and the number of surviving organisms was determined.

3. Results and Discussion

3.1. Chemical composition

In this study, a total of 24 compounds were identified, which accounted for 92.95% of the essential oil (**Table 1**). The major components of different aerial parts of cypress were α-pinene (37.14%), δ-3-carene (19.67%), limonene (5.43%) and α-terpinolene (4.69%). The monoterpenic hydrocarbons were found to be high in this oil. The dominant compounds in cypress oil were monoterpenes hydrocarbons (84.38%), followed by oxygenated monoterpenes (3.81%), sesquiterpenes hydrocarbons (3.07%) and oxygenated sequiterpenes (1.69%) (Table 1).

3.2. Antioxidant Potential of essential oil

To assess the antioxidant potential of essential oil, the application of at least two different assays varying in their mechanisms of antioxidant action has been recommended.

Table 1. Chemical composition of essential oil of *Cupressus sempervirens* L.

N°	*TR (min)*	*Compounds*	*KI*[a]	*%*
1	8.52	Tricyclene	915	0.35
2	8.71	α-thujene	922	1.66
3	9.01	α-pinene	930	37.14
4	9.28	α-fenchene	945	1.31
5	9.33	Camphene	947	0.49
6	10.18	β- pinene	980	2.11
7	10.65	β-myrcene	998	3.63
8	11.27	δ-3-carene	1012	19.67
9	11.41	1,3-p-menthadiene	1015	0.71
10	11.65	o-cymene	1021	1.22
11	11.78	β-limonene	1024	4.42
12	11.86	1,8-cineol	1026	1.18
13	12.69	ν-terpinene	1044	1.20
14	13.60	α-terpinolene	1064	4.69
15	15.27	Camphor	1104	0.37
16	16.25	4-Terpineol	1161	2.26
17	19.31	Bronyl acetate	1289	0.25
18	19.72	Carvacrol	1304	0.35
19	21.04	R(+)-limonene	1355	5.43
20	22.74	trans-Caryophyllene	1421	0.49
21	23.74	α-humulene	1459	0.36
22	24.42	Germacrene-D	1485	1.36
23	25.42	δ-cadinene	1527	0.61
24	27.33	α-cedrol	1610	1.69
		Total		92.95
		Monoterpenes		84.38
		Oxygenated monoterpenes		3.81
		Sesquiterpenes		3.07
		Oxygenated sesquiterpenes		1.69

[a] KI, retention index (Kovalts) relative to n-alkanes (C9–C26) on a polar HP-5 column.

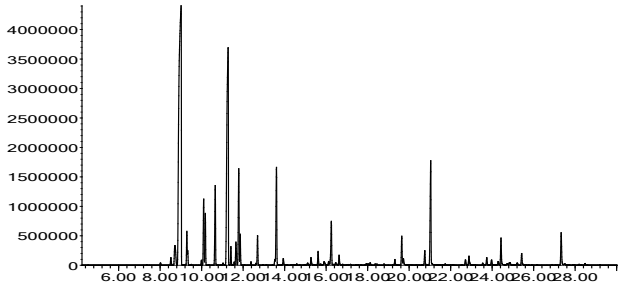

Fig. 1. GC Chromatogram of *Cupressus sempervirens* L.

Table 2. Antioxydant potential of *Cupressus sempervirens* L. essential oil.

Compounds	DPPH (µg/mL)	$ABTS^{\cdot+}$ (TEAC, mM Trolox)
Cypress oil	7.70±0.70	2.14±0.51
BHT	8.13±1.07	2.41±0.10

The antioxidant capacity of *C. sempervirens* essential oil was determined applying the DPPH and ABTS assays.

On the other hand, essential oil showed high antioxidant activity (7.7 $\mu g.mL^{-1}$ and 2.14 mM Trolox for DPPH and ABTS assays, respectively) when compared to BHT. The antioxidant activity of essential oil could be assigned to the synergistic effects of two or more compounds present in the oil. In this context, Lu and Foo (2001) reported that most natural antioxidative compounds often work synergistically with each other to produce a broad spectrum of antioxidative properties that create an effective defense system against free radicals.

3.3. Antimicrobial activity of essential oil

The antimicrobial activity of the essential oil of *C. sempervirens* was examined by broth microdilution susceptibility assay. The results presented in table 3, reveal that the oil of *C. sempervirens* inhibited the growth of susceptible bacteria, filamentous fungi and yeasts. The MIC and MCC values indicate that *C. sempervirens* essential oil was highly effective. In addition, MIC/MCC ratio confirmed a bactericidal and fungicidal activity of the essential oil.

The antimicrobial activity of the *C. sempervirens* essential oils was more pronounced against Gram-positive than Gram-negative bacteria. This is a general observation derived from studies with essential oils from many other species (Burt, 2004; Delamare *et al*., 2007). Generally, the higher resistance among Gram-negative bacteria could be ascribed to the presence of their outer phospholipidic membrane, almost impermeable to lipophilic compounds (Rattanapitigorn *et al*., 2006). The absence of this barrier in Gram-positive bacteria allows the direct contact of the essential oils hydrophobic constituents with the phospholipids bilayer of the cell membrane, where they bring about their effect, causing either an increase of ion permeability and leakage of vital

Table 3. Antimicrobial activity of essential oil of *Cupressus sempervirens* L.

	MIC ($\mu g\,mL^{-1}$)	MCC ($\mu g\,mL^{-1}$)	MCC/MIC
Pseudomonas aeruginosa	0.62-0.31	0.62-0.31	1
Escherichia coli	0.62-0.31	0.62-0.31	1
Staphylococcus aureus	0.15-0.07	0.15-0.07	1
Bacillus subtilis	0.62-0.31	0.62-0.31	1
Halomonas elongate	0.62-0.31	0.62-0.31	1
Salmonella typhimurium	0.62-0.31	0.62-0.31	1
Enterococcus hirae	0.15-0.07	0.15-0.07	1
Aspergillus niger	2.5-1.25	2.5-1.25	1
Candida albicans	0.15-0.07	0.15-0.07	1
Trichoderma reesei	0.62-0.31	0.62-0.31	1

MIC: Minimal Inhibitory Concentration, MCC: Minimal Cidal Concentration

intracellular constituents, or impairment of the bacteria enzyme (Dorman and Deans, 2002; Burt, 2004). The essential oil exhibited a broad antifungal spectrum, with higher activity (2.5-1.25 $\mu L.mL^{-1}$) against yeasts strains (0.07-0.15 $\mu L.mL^{-1}$) (**Table 3**). However, Pinto *et al*. (2007) reported that filamentous fungi are more susceptible than yeasts to the essential oils of *C. sempervirens*.

4. Conclusion

Essential oil extracted from aerial part of *C. sempervirens*. growing in Tunisia, comparing with the same species cultivated in other countries, was found to be rich in α-pinene, δ-3-carene, limonene and α-terpinolene. These differences could be due to climatic conditions. Composition of the Tunisian essential oil showed remarkable antioxidant and antimicrobial activities.

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