academicJournals

Vol. 9(2), pp. 83-90, 14 January, 2015
DOI: 10.5897/AJMR2014.7135
Article Number: 39A25D950131
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Identification of volatile compounds, antimicrobial properties and antioxidant activity from leaves, cones and stems of *Cupressus sempervirens* from Algeria

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Received 19 September, 2014; Accepted 22 December, 2014

***Cupressus sempervirens L.* (Cupressaceae) leaves, cones and young branches have been used in traditional medicine and aromatherapy. The composition of the isolates obtained by hydrodistillation from the aerial parts (plant material collected at Bainam forest, in Northwest of Alger, Algiers), were analyzed by GC and GC-MS. The leaves isolate (yield 0.22% w/w), was mainly composed of monoterpene hydrocarbons (60.8%), α-pinene (38.4%), δ-3-Carene (13.9%), α-Cedrol (10.6%), α-Terpinyl acetate (3.5%) and E-Totarol (3.0%). The cones isolate (yield 0.34% w/w) was predominantly composed of monoterpene hydrocarbons (33.l8%), with α-pinene (20.3%), δ-3-Carene (6.0%), Tepinene-4-ol (9.0%), α-Terpineol (9.0%), α-Terpinyl acetate (5.9%), α-Cedrol (9.1%), and E-Totarol (4.4%). The major components of stems isolates (yield 0.03% w/w) are rich in diterpenoids (51.9%), namely: α-pinene (5.9%), α-Cedrol (14.4%), Manool (5.6%), E-Totarol (34.7%), Ferrugenol (6.0%). Isolates were also tested against four bacteria (*Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*), and two yeasts (*Saccharomyces cerevisiae* and *Candida albicans*), using the Kirby Bauer disk-diffusion method. All bacteria were susceptible to the *C. sempervirens* volatiles isolates. Antioxidant activity of the isolate was evaluated by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method together with two antioxidant standards, butylated hydroxytoluene (BHA) and tert-butyl-4-hydroxy toluene (BHT). The results show antioxidant effect of all isolates less significant as BHA and BHT.**

Key words: *Cupressus sempervirens*, GC/SM, chemical composition, Antimicrobial activity, Antioxidant activity, using 1, 1-diphenyl-2-picrylhydrazyl (DPPH).

INTRODUCTION

The genus *Cupressus* (Cupressaceae) consists of 12 species spread across North America, the Mediterranean basin, and subtropical Asia at high altitudes. Three species were reported as part of North African flora, for convenience they were called *Cupressus sempervirens aggr;* are often confused, being closely related and similar in external appearance (Greuter et al., 1984). These aggregate species include Algerian endemic

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species *C. dupreziana*, *A. Camus*, and *C. sempervirens L*. (Neffati et al., 1999). *C. sempervirens* is considered to be a medicinal tree, as its dried leaves are used as an emmenagogue and for stomach pain (Castro, 1998), as well as to treat diabetes, and its dried fruit is used to treat inflammation, toothache, and laryngitis as a contraceptive and astringent (Mascolo et al., 1987). In addition, its dried seeds have been used to treat wounds, ulcers, bruises, sores, pimples, pustules, skin eruptions, and erysipelas (Caceres et al., 1987), the branches of this plant are used as antiseptics and antispasmodics (Bellakhder, 1997). The essential oil from the leaves and cones is used externally for headache, colds, cough, and bronchitis. With respect to these medicinal and pharmacological advantages, *C. sempervirens* is widely used as a cosmetic ingredient in perfumery and soap-making, including its essential oil distilled from shoots (Usher, 1974). *C. sempervirens* is very widespread in Algeria and in the entire Mediterranean region. This species has many specific botanical features, including tolerance to drought, air currents, wind-driven dust, sleet, atmospheric gases, a well-developed root system, the ability to flourish in both acidic and alkaline soils (Imededdine, 2013).

Several studies on the chemical composition of the essential oil of *C. sempervirens* have been previously reported (Ulukanli et al., 2014). The chemical composition of the essential oil from resin and its Biological activity were studied (Rawat et al., 2010). A detailed study aimed to investigate the chemical composition of fruits (Herzi et al., 2013) have compared the chemical composition and antioxidant activity of essential oil of leaves obtained by hydrodistillation and supercritical extraction. However, almost all of the published studies have examined the chemical composition of the essential oil of the leaves, fruit and stems separately. Therefore, the aim of this work was to study the composition of essential oils from tree organs: leaves, stems and cones, of horizontalys *C. sempervirens* that grow in Algeria and the measure of the antimicrobial and antioxidant activities.

MATERIALS AND METHODS

Plant material and essential oil preparation

Plant material was collected at Bainem forest (May, 2011), in Northwest of Algiers, Algeria. All parts of the plant were dried at room temperature, in the dark, for 15 days. The material was used for hydrodistillation using a Clevenger type apparatus, during 5 h, following the European Pharmacopoeia procedure (Council of Europe, 1997). Condensed volatiles were then recovered from the hydrolyte by extraction with diethyl oxide. Solvent was further eliminated under a gentle stream of nitrogen, rendering extracts with intense odours respectively at yields of 0.22% (w/w); 0.03% (w/w); 0.34% (w/w) for leaves, stems and cones.

Analysis

The volatile isolated were analyzed by gas chromatography (GC) (Hewlett-Packard 6890) equipped with a single injector and two flame ionization detection (FID) systems. For simultaneous sampling, two Supelco fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d. film thickness 0.20 µm), and SupelcoWax-10 (polyethyleneglycol) were used. The oven temperature program was: 70-220°C (3°C.min-1), 220°C (15 min); injector temperature: 250°C; carrier gas: helium; splitting ratio 1:40; detectors temperature: 250°C. GC-MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 µm). GC parameters were as described above; interface temperature: 250°C; MS source temperature: 230°C; MS quadruple temperature: 150°C; ionization energy: 70 eV; ionization current: 60 µA. Compounds were identified by their GC retention indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C8-C23 of n-alkanes (Van den Dool and Kratz, 1963), were compared with those of reference samples included in C.E.F. / Faculty of Pharmacy, University of Coimbra laboratory database. Acquired mass spectra were compared with reference spectra from the laboratory database (Wiley, 2005) and validated literature data (Adams, 2004; Joulain and Koenig, 1998; Cavaleiro et al., 2011). Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

Antimicrobial activity

The essential oils obtained from the aerial parts of C*. sempervirens* were tested against four bacteria (reference strains): *Bacillus subtilis* ATCC 9372, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 4157, and two yeasts: *Saccharomyces cerevisiae* ATCC 601, *Candida albicans* ATCC 24433 strains. The microbial strains were supplied by SAIDAL (pharmaceutical group). The bacteria strains were inoculated into nutrient broth Muler-Hinton (30°C) and incubated for 24 h. Based on modern culture the bacterial suspension was obtained for 18-24 h (48 h for yeasts). 3-5 bacterial colonies were taken far away from each isolate and placed in 6 ml of sterile physiological water. The focus 10^6 CFU/ml for wavelength 450 nm was obtained (Hammer et al., 1999). The paper discs (6 mm in diameter) were separately impregnated with different concentration (20, 100 and 300 µg) of the oil dissolved in Dimethylsulfoxide DMSO (Sigma Aldrich) and placed on the nutrient broth, which had previously been inoculated with the selected test microorganism, respectively. Plates, after 1 h at 4°C, were incubated for bacteria at 37 ◦C for 24 h and for yeasts strains at 30°C for 48 h. The DMSO solvent was used as the negative control. Standard antibiotics (25 µg/disk) Sulfamethoxazol-trimethoprim, Cefixim, Amoxicillin and Lymecyclin were used as positive controls. Antimicrobial activity was assessed by measuring the diameter (DD) of the growth inhibition zone in millimetres (including disc diameter of 6 mm). Tests were carried out in triplicate.

Antioxidant activity

The antioxidant activity was measured using the DPPH assay (Brand-Williams et al., 1995). 1000 µg of essential oils and tested substances in ethanol were added to 3 ml of 0.004% ethanol solution of DPPH. After 30, 60, 90 min at 24 h incubation period at room temperature, the absorbance was read against a blank at 515 nm. Inhibition free radical DPPH in percent (I%) was calculated as: $I\% = \frac{(Ac-Ae)}{Ac} \times 100$; where, Ac was the absorbance of the control reaction (containing all reagents except the test compound), and Ae was the absorbance of the test compound.

Table 1. Volatiles components of the leaves (L), cones (C) and stems (S) of *Cupressus sempervirens*.

RI[a]	RI[b]	Compound	Percent in samples (%)		
			Leaves	Cones	Steams
l924	1030	α-Thujene	0.3	-	0.2
933	1034	α-Pinene	38.4	20.3	5.9
942	1067	α-Fenchene	1.0	0.3	t
966	1127	Sabinene	0.3	-	0.9
970	1118	β-Pinene	1.0	0.9	0.2
982	1163	Myrcene	1.3	1.3	0.5
1007	1156	δ-3-Carene	13.9	6.0	3.0
1011	1185	α-Terpinene	0.2	-	0.3
1013	1274	*P*-Cymene	0.1	-	0.3
1021	1206	Limonene	-	1.5	0.4
1021	1215	β-Phellandrene	2.0	-	t
1047	1248	γ-Terpinene	0.3	0.5	0.2
1048	1456	*Z*-Sabinene hydrate	-	-	0.1
1078	1287	Terpinolene	1.9	2.9	2.2
1084	1539	*E*-Sabinene hydrate	-	-	1.1
1084	1539	Linalool	0.2	-	t
1100	1595	Fenchyl alcool	-	0.1	-
1109	1555	*Z*-p-2-menthen-1-ol	-	0.4	-
1121	1620	*E*-p-2-menthen-1-ol	-	0.2	-
1130	1566	Pinocarvone	-	0.5	-
1145	n.d.	3-Thujene-2-one	0.1	-	-
1147	1664	Borneol	-	2.1	-
1159	1600	Tepinene-4-ol	0.8	9.0	2.1
1170	1692	α-Terpineol	1.0	9.0	t
1226	1598	Carvacrol methyl ether	-	-	0.6
1266	1574	Bornyl acetate (endo)(L)	0.1	1.5	0.3
1332	1692	α-Terpinyl-acetate	3.5	5.9	1.8
1343	1456	α-Cubebene	0.1	0.8	0.9
1368	1488	α-Copaene	-	0.2	-
1394	1567	Longifolene	-	0.1	0.4
1400	n.d.	Iso-allo-longifolene	-	1.9	-
1400	1565	α-Cedrene	0.3	-	-
1406	1591	β-Cedrene	0.2	-	-
1410	1595	Caryophyllene	-	3.4	-
1447	1664	α-Humulene	0.2	-	-
1461	1681	γ-Muurolene	0.2	-	t
1465	1703	Germacrene D	-	2.6	T
1488	1719	*Z*-α-Bisabolene	-	0.5	-
1496	1748	γ-Cadinene	0.2	0.4	-
1506	1748	δ-Cadinene	0.7	0.3	-
1518	1908	α-Calacorene	-	0.4	-
1557	1978	Caryophyllene oxide	0.1	1.2	-
1579	2108	α-Cedrol	10.6	9.1	14.4
1591	2143	Cedrol epi	0.7	-	T

RESULTS AND DISCUSSION

Chemical composition of the essential oils

The composition of the leaves, cones and stems distillates are summarized in Table 1. The detected compounds are listed according to their elution from GC (SPB-1 column) (Figures 1 to 3). The leaves distillation rendered a pale yellow liquid 0.22% (w/w) four times

Table 1. Contd.

RI[a]	RI[b]	Compound	Percent in samples (%)		
			Leaves	Cones	Steams
1607	2154	γ-Eudesmol	-	0.3	-
1615	2188	α-Muurolol	0.7	-	-
1622	2212	β-Eudesmol	-	0.4	-
1627	2220	α-Cadinol	1.6	-	-
1655	2161	α-Bisabolone oxide	1.6	-	-
1964	2343	Manyol oxide	0.3	-	1.1
1968	2299	Isopimaradiene	0.6	0.2	-
1987	n.d.	Manoyl oxyde 13-epi	-	-	0.4
1988	2331	Cyperone alpha	-	0.2	-
2017	2469	Abietatriene	0.5	-	2.2
2026	n.d.	Manool	-	0.6	5.6
2069	n.d.	Isoabienol	0.7	0.3	-
2232	n.d.	*Z*-Totarol	-	0.8	1.8
2255	n.d.	*E*-Totarol	3.0	4.4	34.7
2273	n.d.	Ferruginol	0.5	0.7	6.0
Monoterpene hydrocarbons			60.8	33.8	13.0
Oxygen containing monoterpenes			2.1	21.4	4.9
Sesquiterpene hydrocarbons			5.8	17.9	3.3
Oxygen containing sesquiterpene			15.3	11.0	14.4
Diterpenoids			5.6	7.3	51.9
Total identified			89.6	91.4	87.5

Compounds listed in order their elution from SPB-1 column: RIa, Retention indice in the SPB-1 column; Rib, Retention indice in the Supelcowax 10 column; n.d, non-determined; t, traces (<0.05%).

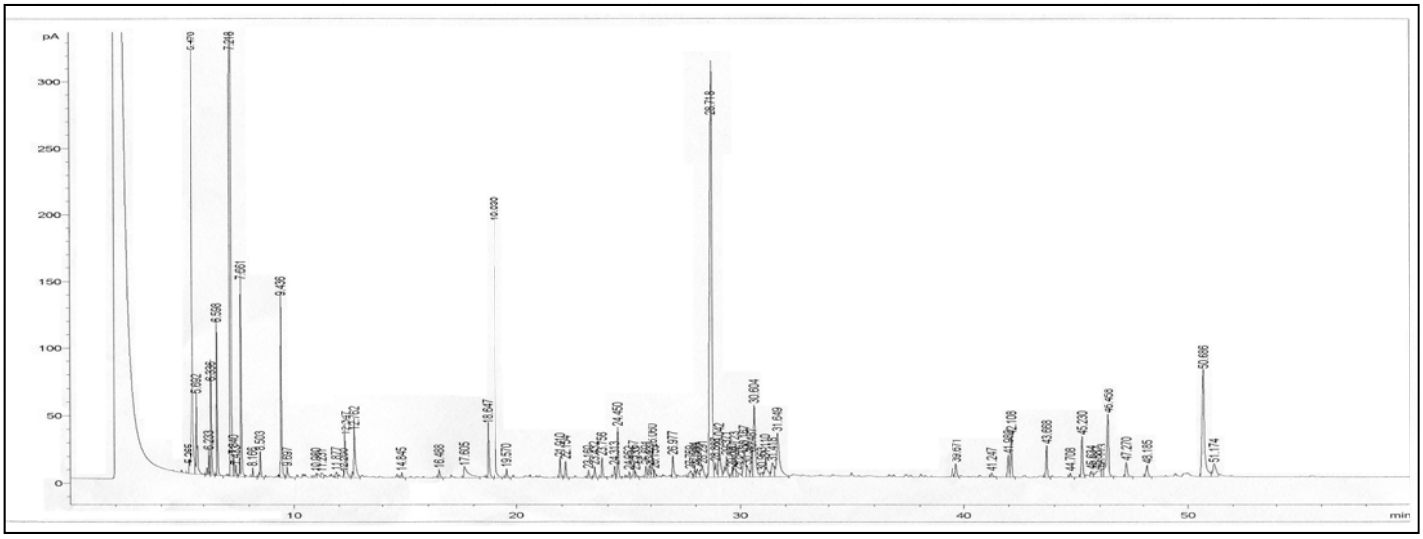

Figure 1. Chromatograms and main compounds of the essential oil of leaves from *C. sempervirens* obtained by SPB-1 column.

inferior than the Cameroonian 1% (w/w) (Tapondjou et al., 2005). In this isolate, 37 components (89.6%) were identified of which α-Pinene, (38.4%) followed by δ-3 Carene (13.9%), α-Cedrol (10.6%), α-Terpinyl acetate (3.5%), E-Tatarol (3.0%) and β-Phellandrene (2.0%), were the most abundant. Monoterpene hydrocarbons contribute to (60.8%), for the global composition. The major constituents were com-pletely different from those of previous report on the chemistry of these oils (Hosni et al., 2014).

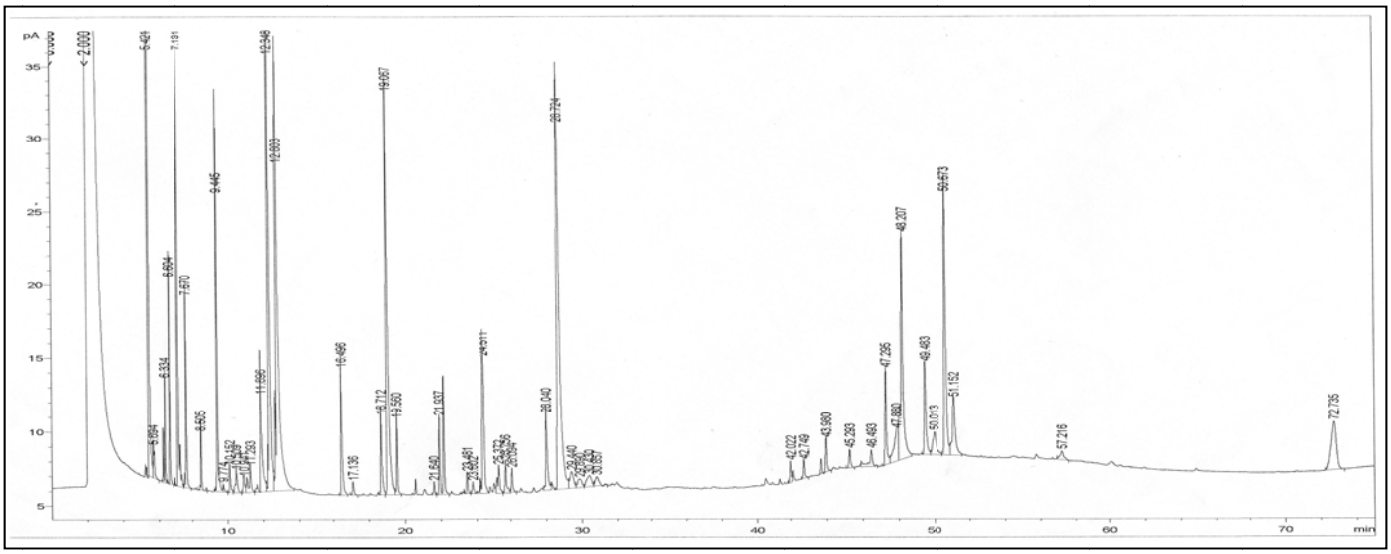

Figure 2. Chromatograms and main compounds of the essential oil of cones from *C. sempervirens* obtained by SPB-1 column.

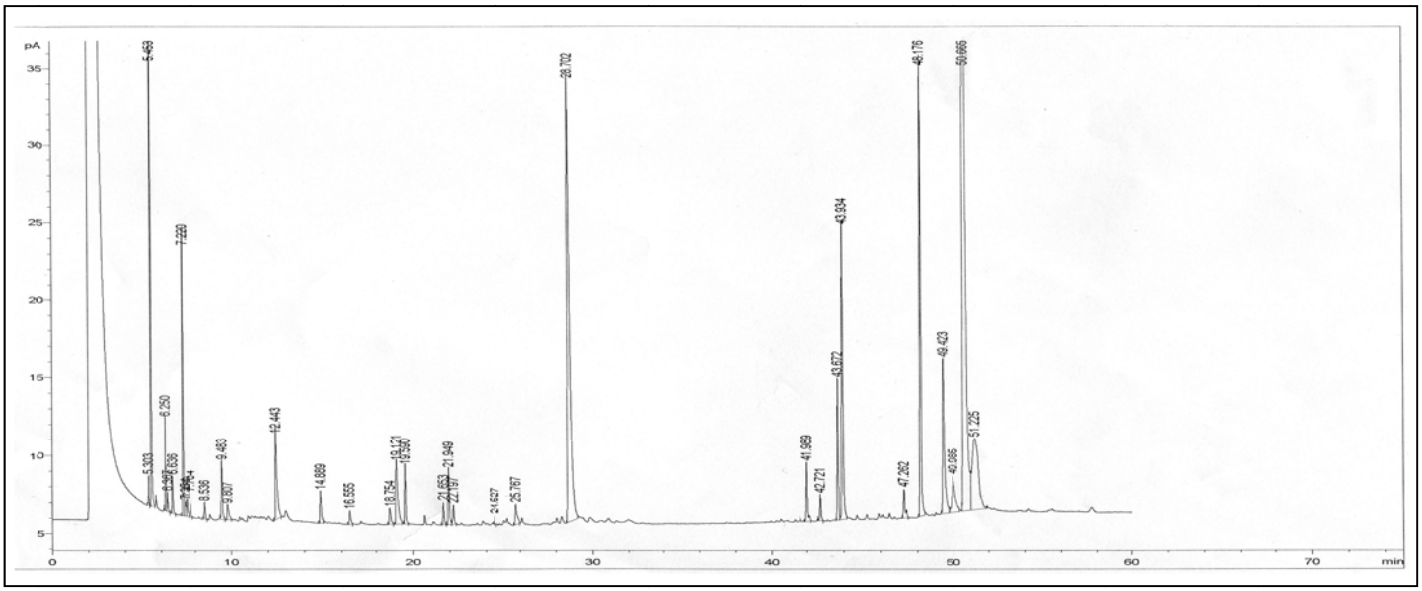

Figure 3. Chromatograms and main compounds of the essential oil of stems from *C. cempervirens* obtained SPB-1 column.

The chemical composition of isolated essential oils from leaves are in accordance with those previously reported (Amri et al., 2013). Thirty-eight (38) components were identified (91.4%) in the cones isolate. The most abundant constituents were the monoterpenes hydrocarbons (33.8%): α-Pinene (20.3%) and δ-3 Carene (6.0%), the monoterpenes oxygenes (21.4%): α-Terpinyl acetate (5.9%) and the oxygenateds sesquitepenes (11%): α-Cadinol (9.1%). The stems isolate was chiefly composed of deterpinoids (51.9%) with E-Tatarol (34.7%) and α-Cedrol (14.4%) as major components. The amount of α-Pinene and δ-3 Carene were lower in cones and stems than leaves isolates, decreasing from 60.8 to 33.8, 13.0 and from 13.9 to 6.0, 3.0%, respectively.

Antioxidant activity

The results obtained during the test measurement of the percentage of inhibition of DPPH radical are shown in Figure 4. It seems that the percentage inhibition of free radicals increased with increasing time for the essential oil of *C. sempervirens*. The inhibition percentage of free radicals for the essential oil is lower than that of BHA and BHT. These results show that the essential oil of *C.*

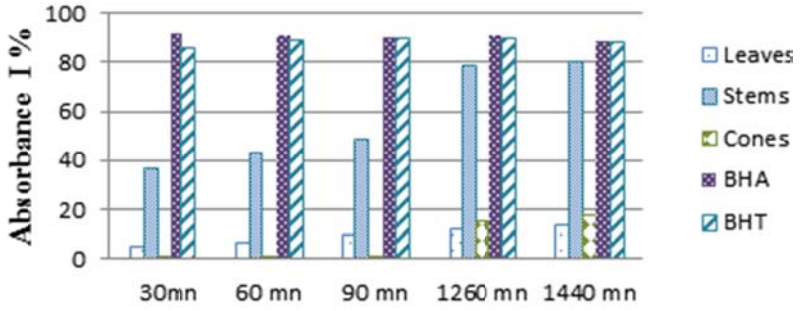

Figure 4. The reducing power of various times of: BHA, BHT, leaves, cones, stems.

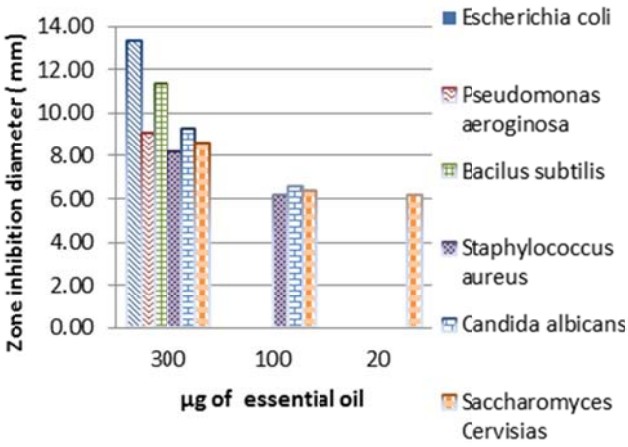

Figure 5. Antimicrobial activities of the essential oil of leaves.

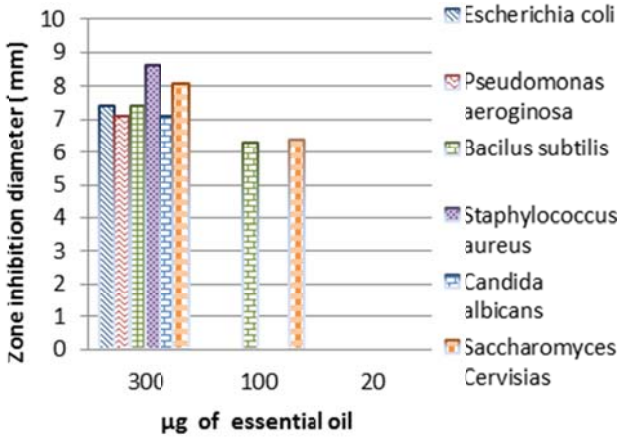

Figure 6. Antimicrobial activities of the essential oil of cones

sempervirens has antioxidant activity in concentration of 1000 µg but it is less effective than BHA and BHT for leaves and cones. As shown in Figure 1, the inhibition activity of the stems essential oils was same to the activity of BHA and BHT. This may be due to the presence of diterpinoids (51.9%) specifically to E- Tatarol which is a major component of the essential oil studied

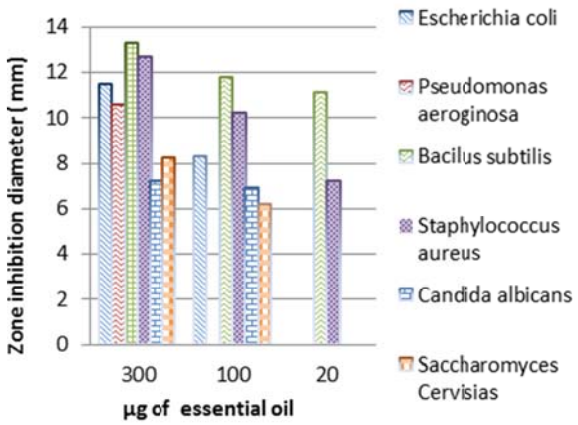

Figure 7. Antimicrobial activities of the essential oil of stems

(34.7%) and has a strong antioxidant activity (Haraguchi et al., 1997). Moreover, the presence of carvacrol even at low concentrations in the stems essential oil (0.6%) may explain the scavenging activity of DPPH radical.

Antimicrobial activity

The antibacterial activity of the essential oils was evaluated against four microorganisms and two yeast, using disc diffusion methods. The disc diameters (DD) of essential oils inhibition zone for the tested microorganisms are shown in Figures 5 to 7. Results show that all the oils inhibited the growth of microbial strains. A zone diameter of inhibition from 6.2 to 13.3 mm was observed, depended on susceptibility of the tested microbial. The oil of leaves, cones and stems have a high antimicrobial activity against the bacterial strains and yeasts at a concentration of 300 µg, however, at this concentration the oil extracted from leaves did not inhibit *P. aeruginosa*. The leaves showed better inhibitory effect on *E. coli* and *S. Cerevisiae* when compared to the cones and stems. The presence of α-Pinene (38.4%) and δ-3 Carene (13.9%) in leaves isolate are attributed the antimicrobial (Guy et al., 2001; Jiang et al., 2011; Ojeda-

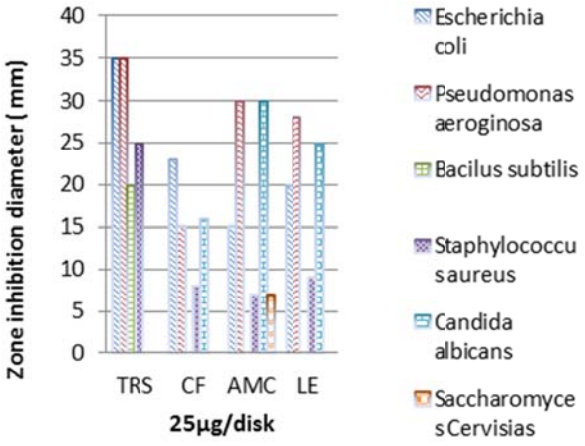

Figure 8. Antimicrobial activities of standards antibiotics. TRS, Trimethoprim-sulfamethoxazol; CF, cefixim; AMC, Amoxicillin; LE, Lymecyclin.

Sana et al., 2013; Hmamouchi et al., 2001). The presence of Tepinene-4-ol in the stems and cones with respectively 2.1 and 9.0% give excellent inhibition against S*. aureus* and *P. aeruginosa* (Jirovetz et al., 2005).

Stems have a good inhibitory activity against *B. subtilis* compared to cones and leaves; diterpinoids (51%) are effective against bacteria (Kotan et al., 2007). The antimicrobial activity of the essential oils extracted from different parts of C*. sempervirens* are, in part, associated with their major constituents such as α- Pinene, β-Phellandrene, α-Terpinyl acetate and Cedrol. These components have been reported to display antimicrobial effects (Cosentino et al., 1999; Alessandra et al., 2005; Yang et al., 2007; Demirci et al., 2007).

The essential oils containing terpenes are also reported to possess antimicrobial activity (Dorman and Deans, 2000), which are consistent with our present study. *Saccharomyces cerevisiae* exhibited high resistance to any standard antibiotic; however its activity was inhibited by the essential oils extracted from different parts of the plant (Figure 8).

Conclusion

Chromatographic analyses have identified 37 and 38 components with a codominance of α-pinene (38.4 and 20.3%) for leaves and cones, respectively, and 34 components with E-Tatarol (34.7%) as the major component for the stems. The results obtained in this work show that the C*. sempervirens* essential oils possess antimicrobial properties, which can be used as natural antimicrobial agents for human and infectious diseases and in food preservation. The stems essential oil exhibited a better free radicals inhibition compared with leaves and cones and was same microbiological activity that BHA and BHT.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

Thanks are due to Fundação para a Ciência e Tecnologia (FCT), POCI 2010/FEDER for the financial support.

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