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Chemical composition, bio-herbicidal and antifungal activities of essential oils isolated from Tunisian common cypress (*Cupressus sempervirens* L.)

Amri Ismail[1]*, Hamrouni Lamia[1, 2], Hanana Mohsen[3], Gargouri Samia[4] and Jamoussi Bassem[5]

[1]Département de Biologie, Faculté des Sciences de Bizerte. Zarzouna, 7021 Bizerte, Tunisie.
[2]Laboratoire d'Ecologie Forestière, Institut National de Recherches en Génie Rural, Eaux et Forêts. BP 10, 2080 Ariana, Tunisie.
[3]Laboratoire de Physiologie Moléculaire des Plantes, Centre de Biotechnologie de Borj-Cédria. BP 901, 2050 Hammam-lif, Tunisie.
[4]Laboratoire de Protection des Végétaux, Institut National de la Recherche Agronomique de Tunisie. Rue Hédi Karray, 2080 Ariana, Tunisie.
[5]Institut Supérieur d'Education et de Formation Continue. Tunis, Tunisie.

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The chemical composition of essential oils obtained by hydrodistillation from leaves, branches and female cones of Tunisian *Cupressus sempervirens* L. was determined by gas chromatography (GC) and CG-mass spectrometry (GC/MS) analysis, 52 compounds were identified; qualitative and quantitative differences between oils were observed. All oils were rich in monoterpene hydrocarbons, and the major constituents were α-pinene (27.5 to 35.8%), α-cedrol (7.7 to 19.3%), δ-3-carene (5.8 to 13.2%) and germacrene D (3.9 to 12.1%). Essential oils of *C. sempervirens* have shown a significant phytotoxic effect against the germination and seedling growth of four weeds: *Sinapis arvensis* L., *Trifolium campestre* Schreb (dicots), *Lolium rigidum* Gaud and *Phalaris canariensis* L. (monocots). Tested oils strongly inhibited the germination and seedling growth of all weeds, in a dose dependent manner. The *in vitro* antifungal activity of the essential oil samples of *C. sempervirens* were evaluated against 10 cultivated crop fungi, and all samples have shown a significant antifungal activity against all tested fungi and it can be suggested to have the potential to be used as a bio-herbicide and alternatives fungicide.

Key words: *Cupressus sempervirens*, essential oils, bio-herbicidal activity, antifungal potential, weeds, allelopathy.

INTRODUCTION

Allelopathy is the science that studies processes in which secondary metabolites from plants and microorganisms are involved, affecting growth and development of biological systems (Qiming et al., 2006). The use of secondary metabolites implicated in allelopathic interactions as sources for news agrochemical models could satisfy the requirements for crop protection and weeds management (Singh et al., 2003). Weeds may be defined as plant with little economic value and possessing the potential to colonize disturbed habitats or those modified by human activities. Fungi can cause disasters on the crops; the metabolites of many fungi

*Corresponding author. E-mail: amri_amri@live.fr.

may have adverse or stimulatory effects on plants, such as suppression of seed germination, malformation, and retardation of seedling growth. Many crop seeds are infected by fungi before harvest or during storage. If conditions are not favourable, the situation is more serious. According to an estimate, in US alone, weeds cause a loss on the crop production in the range of 12% (Pimentel et al., 1991). As per Agrow report, the total value of world's agrochemical market was between US$31 - 35 billion and among the products herbicides accounted for 48% followed by fungicides (22%) (Agrow, 2007). However, the excessive use of synthetic pesticides in the croplands, urban environment, and water bodies to get rid of noxious pests has resulted in an increased risk of pesticide resistance, enhanced pest resurgence, toxicological implications to human health and increased environmental pollution (Gupta et al., 2008; Hong et al., 2009).

In an attempt to reduce the use of synthetic pesticides, extensive investigations into the possible exploitation of plant compounds as natural commercial products, that are safe for humans and the environment were made. Indeed, the search of natural compounds and management methods alternatives to classical pesticides has become an intense and productive research field (Zanie et al., 2008; Dudai et al., 1999).

In this regard, greater attention is towards the use of allelopathic plants and their products for pest management in a sustainable manner. Therefore, it is worthwhile to explore the plants as sources of biological active compounds. Species of *Cupressus* genus (Cupressaceae family) are coniferous trees, comprising 12 species which are distributed in the Mediterranean region, North America and subtropical Asia (Bagnoli et al., 2009). Common cypress (*Cupressus sempervirens* L.) is native to the eastern Mediterranean region. This tree is mainly used as an ornamental tree due to its conical crown shape, but it can also be used for timber, as a privacy screen, and protection against wind as well. Moreover, cypress has proved to be very suitable as a pioneer species for reforestation as it can tolerate poor, barren, and superficial soils. For all these reasons, cypress has been introduced in geographic areas that extend far beyond its natural distribution (Bagnoli et al., 2009). Phyto-preparation obtained from the core and young branches of *C. sempervirens* were reported to have antiseptic, aroma therapeutic, astringent, balsamic and anti-inflammatory activities. Cypress is also described to exert antispasmodic, astringent, antiseptic, deodorant, and diuretic effects, to promote venous circulation to the kidneys and bladder area, and finally to improve bladder tone and as a co-adjuvant in therapy of urinary incontinence and enuresis (Rawat et al., 2010). Essential oils and crude extracts of *C. sempervirens* have become a subject for a search of natural antioxidants, antibacterial, insecticidal activities, and inhibition of glucose-6-phosphatase and glycogen phosphorylase (Rawat et al., 2010). There are many reports on the chemical composition of essential oils isolated from various parts of *C. sempervirens*. Most of these reports indicate that monoterpene hydrocarbons like α-pinene and δ-3-carene are the main constituents of these oils (Chanegriha et al., 1993; Chanegriha et al., 1997; Emami et al., 2004, 2006; Sacchetti et al., 2005; Mazari et al., 2010; Milos et al., 2002; Loukis et al., 1991; Chéraif et al., 2005), but to our knowledge, no study has been reported on their herbicidal and antifungal activities and knowing that the chemical composition of essential oils from aromatic plants depends on several factors such as the geographical origin and genetic background of plant from which the oil was obtained, so, the aims of this work were, in a first step, to assay the main constituents of the essential oil obtained from the leaves, cones and branches of *C. sempervirens* growing in Tunisia. In a second step, we assessed their antifungal potential against eight phyto-pathogenic fungi and their herbicidal effects were tested against germination and seedling growth of four common weeds in Tunisia, *Sinapis arvensis* L., *Lolium rigidum* Gaud., *Trifolium campestre* Schreb. and *Phalaris canariensis* L.

MATERIALS AND METHODS

Plant material

The leaves, cones and branches of *C. sempervirens* were collected from the arboretums of the National Institute of Researches on Rural Engineering, Water and Forests in October, 2010 from the region of Makther. Five samples collected from more than five different trees were harvested, mixed for homogenization, and used in three replicates for essential oil extractions. The specimen of the plant was submitted to the herbarium division of the institute and identification was confirmed in the Laboratory of Forest Ecology.

Isolation of the essential oils

The essential oils were extracted by hydrodistillation of fresh plant material (100 g of each sample in 500 ml of distilled water) using a Clevenger-type apparatus for 3 h according to the standard procedure described in the European Pharmacopoeia (2004).

The oils were dried over using anhydrous sodium sulfate (a pinch/10 ml^{-1}) and stored in sealed glass vials at 4°C before analysis. Yield was calculated based on dried weight of the sample (mean of three replications).

Gas chromatography-mass spectrometry

The composition of the oils was investigated by GC and GC/MS. The analytical GC was carried out on an HP5890-series II gas chromatograph (Agilent Technologies California USA) equipped with flame ionization detectors (FID) under the following conditions: the fused silica capillary column, apolar HP-5 and polar HP Innowax (30 m × 0.25 mm ID, film thickness of 0.25 mm). The oven temperature was held at 50°C for 1 min then programmed at rate of 5°C/min^{-1} to 240°C and held isothermal for 4 min. The carrier gas was nitrogen at a flow rate of 1.2 ml/min^{-1}; injector temperature: 250°C, detector: 280°C; the volume injected: 0.1 ml of 1% solution (diluted in hexane). The percentages of the constituents were

calculated by electronic integration of FID peak areas without the use of response factor correction. GC/MS was performed in a Hewlette Packard 5972 MSD System. An HP-5 MS capillary column (30 m × 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to the mass spectrometry. The carrier gas was helium, with a flow rate of 1.2 ml/min^{-1}. Oven temperature was programmed (50°C for 1 min, then 50 to 240°C at 5°C/min^{-1}) and subsequently held isothermal for 4 min. Injector port: 250°C, detector: 280°C, split ratio: 1:50. Volume injected: 0.1 ml of 1% solution (diluted in hexane); mass spectrometer: HP5972 recording at 70 eV; scan time: 1.5 s; mass range: 40 to 300 amu. Software adopted to handle mass spectra and chromatograms was ChemStation. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library). Further confirmation was done from Retention Index data generated from a series of alkanes retention indices (relatives to C9-C28 on the HP-5 column) (Adams, 2007).

Seed germination and seedling growth experiments

Mature seeds of annual seeds of *S. arvensis* L.*, L. rigidum* Gaud, *T. campestre* Schreb and *P. canariensis* L. were collected from parent plants growing in fields in July, 2009. The seeds were sterilized with 15% sodium hypochlorite for 20 min^{-1}. They were then rinsed with distilled water. Empty and undeveloped seeds were discarded by floating in tap water and the remaining seeds were used. Then, the oil was dissolved in tween-water solution (1%; v/v). The final concentrations of the treatments were 0 (control), 1, 2, 3, 4 and 5 µl/ml^{-1}. The emulsions of 8 ml were transferred to Petri dish placed on the bottom two layers of filter paper. Afterward, 20 seeds *S. arvensis*, *P. canariensis, T. campestre* and *L. rigidum* were placed on the filter paper. Petri dishes were closed with an adhesive tape to prevent escaping of volatile compounds and were kept at 25°C on a growth chamber supply with 12 h of fluorescent light (Dudai et al., 1999). The number of germinated seeds and seedling lengths were measured after 10 days and all tests were arranged in a completely randomized design with three replications by treatment.

Antifungal activity assays

Eight plant pathogenic fungi were obtained from the culture collection of the Tunisian National Institute of Agronomic Research. Cultures of each of the fungi were maintained on potato dextrose agar (PDA) and were stored at 4°C and in 1 ml of glycerol 25% at -20°C. The fungal species used in this study were: *Fusarium culmorum*, *Fusarium oxysporum*, *Fusarium equisiti*, *Fusarium verticillioides*, *Fusarium nygamai*, *Botrytis cinerea, Microdochium nivale* var. *nivale* and *Alternaria* sp. Antifungal activity was studied by using an *in vitro* contact assay which produces hyphal growth inhibition (Cakir et al., 2004). Essential oil was dissolved in 1 ml of Tween 20 (0.1% v/v) and then added into 20 ml PDA at 50°C to obtain a final concentration of 4 µl/ml. A mycelia disk of 5 mm in diameter, cut from the periphery of a 7 day-old culture, was inoculated in the center of each PDA plate (90 mm diameter), and then incubated at 24°C for 7 days. PDA plates treated with Tween 20 (0.1%) without essential oil were used as control. Tests were repeated in triplicate. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control using the following formula: % Inhibition = [(C – T) / C]*100. Where C is an average of three replicates of hyphal extension (mm) of controls, and T is an average of three replicates of hyphal extension (mm) of plates treated with essential oil.

Statistical analysis

Data of germination, seedling growth and fungi inhibition assays were subjected to one-way analysis of variance (ANOVA), using the SPSS 13.0 software package. Differences between means were tested through Student-Newman-Keuls (SNK) and values of $p < 0.05$ were considered significantly different (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

Chemical composition of *Cupressus sempervirens* L. essential oils

The chemical composition of *C. sempervirens* oils, the percentage content of the individual components, the retention indices and percent yields are summarized in Table 1. The oil yields were ranged from 0.1 to 0.65% depending on the part of the plant analyzed. The greatest yields were in cones and leaves (0.65 and 0.43%, respectively) and the oil was lowest in the branches (0.1%). 52 compounds were identified accounting for 93.7, 94.82 and 95.8% of the total oil respectively in leaves, cones and branches. The monoterpene fraction amounted (48.1 to 65.9%), sesquiterpenes accounted for 27.3 to 45.01%, while a low amount of diterpenes (less than 2.6%). In monoterpene fraction, hydrocarbon compounds represent a great amount, accounting for 43.21 and 42.7% respectively in cones and leaves, and 60.4% in branches. The main monoterpene hydrocarbons were α-pinene 27.5% in leaves, 28.91% in cones and 35.8% in branches and δ-3-carene (5.8, 7.2 and 13.2%), respectively in cones, leaves and branches. In sesquiterpene fraction, sesquiterpene hydrocarbons varied from 21.9% in leaves, 18.26% in cones and 14.9% in branches.

The major compounds in this fraction were germacrene D (3.9 to 12.1%), and some other compounds as (*Z*)-caryophyllene, α-humulene and germacrene B. In oxygenated sesquiterpenes fraction (12.4 to 26.75%), α-cedrol was the major compound varying from 7.7% in branches, 18.55% in cones to 19.3% in leaves. So essential oils of Tunisian *C. sempervirens* may be considered as α-pinene, α-cedrol and δ-3-carene chemotype. In previous studies, essential oils of *C. sempervirens* were studied in Iran, Croatia, Italy, Tunisia, Algeria and Greece (Chanegriha et al., 1993; Chanegriha et al., 1997; Emami et al., 2004; Emami et al., 2006; Sacchetti et al., 2005; Mazari et al., 2010; Milos et al., 2002; Loukis et al., 1991; Chéraif et al., 2005). Obtained data of these studies are summarized in Table 2 for each country and each part used for essential oil extraction. According to these studies, generally α-pinene, α-cedrol, δ-3-carene, terpinolene and α-terpenyl acetate were considered the major components on different aerial parts of *C. sempervirens.* Differences found between the main constituents of oils obtained from *C. sempervirens* grown in Tunisia and those from the same species but growing in other countries seem to be related particularly to dry and extraction methods, climate, soils and genetic background of trees.

Table 1. Essential oils composition of leaves, branches and cones of *C. sempervirens* L.

S/No.	Compounds	RI	Leaves	Cones	Branches	M. I.
1	Tricyclene	926	0.1	-	0.1	RI, MS
2	α-thujene	931	0.1	0.1	-	RI, MS
3	α-pinene	939	27.5	28.91	35.8	RI, MS, Co-Inj
4	α-fenchene	950	0.6	0.2	0.7	RI, MS
5	Sabinene	968	0.2	0.6	1.3	RI, MS, Co-Inj
6	β-pinene	976	0.8	0.9	2.5	RI, MS
7	β-myrcene	991	1	1.5	1.9	RI, MS
8	α-phellandrene	1007	1.4	1.8	-	RI, MS
9	δ-3-carene	1011	7.2	5.8	13.2	RI, MS, Co-Inj
10	1.8.cineole	1021	1	0.6	-	RI, MS
11	*p*-cymene	1026	0.2	1.7	1.1	RI, MS
12	Limonene	1031	2.2	0.6	1.9	RI, MS, Co-Inj
13	β-phellandrene	1032	0.1	0.2	-	RI, MS
14	α-terpinolene	1088	1.3	0.9	1.9	RI, MS
15	linalool	1098	0.1	0.3	-	RI, MS
16	α-campholenal	1126	0.2	0.2	0.9	RI, MS
17	Camphre	1142	0.1	-	0.1	RI, MS
18	Borneol	1149	0.2	0.3	-	RI, MS
19	δ-terpineol	1163	0.1	0.7	1.7	RI, MS
20	Myrtenal	1168	0.1	-	-	RI, MS
21	Myrtenol	1176	0.2	-	0.1	RI, MS
22	Terpen-4-ol	1179	1.8	1.9	1.5	RI, MS
23	α-terpineol	1196	1.1	0.8	-	RI, MS
24	*iso*-bornyl acetate	1279	0.3	0.4	0.7	RI, MS
25	α-terpenyl acetate	1337	0.2	0.4	0.5	RI, MS
26	longifolene	1398	0.6	1.2	0.6	RI, MS
27	(*Z*)-caryophyllene	1420	2.2	1.9	1.1	RI, MS, Co-Inj
28	α-cedrene	1432	0.6	1.8	1.3	RI, MS
29	α-humulene	1448	2.1	2.4	1.9	RI, MS
30	Ermacrene D	1478	12.1	6.36	3.9	RI, MS, Co-Inj
31	β-selinene	1486	0.6	1	1.8	RI, MS
32	α-murrolene	1499	0.5	0.1	0.5	RI, MS
33	*epi*-zonarene	1501	0.2	0.3	0.6	RI, MS
34	β-bisabolene	1508	0.5	1.1	0.4	RI, MS
35	Cubebol	1510	0.1	0.6	0.3	RI, MS
36	*Cis*-calmanene	1521	0.2	-	-	RI, MS
37	δ-cadinene	1524	0.2	0.4	0.6	RI, MS
38	α-copan-11-ol	1540	0.3	0.3	0.1	RI, MS
39	α -calacorene	1542	0.2	0.2	0.1	RI, MS
40	Elemol	1551	0.1	1.4	-	RI, MS
41	Germacrene B	1552	1.5	0.9	1.2	RI, MS
42	β-calacorene	1560	0.6	0.8	1	RI, MS
43	Caryophyllene oxide	1576	0.3	0.6	1.1	RI, MS
44	α-cedrol	1592	19.3	18.55	7.7	RI, MS
45	*T*-cadinol	1616	0.5	1.1	1.3	RI, MS
46	*T*-murrolol	1627	0.6	1.7	0.1	RI, MS
47	Manoyl oxide	1993	0.9	2.3	1.7	RI, MS
48	Abietatriene	2044	0.4	0.1	0.8	RI, MS
49	Abietadiene	2080	0.4	0.3	0.5	RI, MS
50	Nezukol	2080	0.3	0.2	0.6	RI, MS
51	Sempervirol	2283	0.1	0.4	0.4	RI, MS

Table 1. Contd.

52	*(Z)*- tartarol	2313	0.2	-	0.3	RI, MS
	Yield % (w/w):		0.43	0.65	0.1	
	Total identified compounds		93.7	94.82	95.8	
	Monoterpene hydrocarbons		42.7	43.21	60.4	
	Oxygenated monoterpenes		5.4	5.6	5.5	
	Sesquiterpene hydrocarbons		21.9	18.26	14.9	
	Oxygenated sesquiterpenes		22.3	26.75	12.4	
	Diterpene hydrocarbons		0.8	0.4	1.3	
	Oxygenated diterpenes		0.6	0.6	1.3	

RI, Retention index on apolar HP-5 MS column; MS, mass spectrometry; percentage calculated by GC-FID on apolar HP-5 MS column; MI, methods of identification; Co-inj, co-injection; -, not detected.

Table 2. Major constituents of essential oils of *C. sempervirens* from different origins previously reported.

Country	Used part	Major compounds	References
Iran	Leaves	α-pinene (30%), Δ-3-carene (24%), terpinolene (6.6%), α-terpenyl acetate (6.6%).	Emami et al. (2004)
	Cones	α-pinene (39%), Δ-3-carene (24%), α-terpenyl acetate (5.6%).	
Iran	Leaves	α-pinene (21.4%), Δ-3-carene (16%), germacrene D (13%).	Emami et al. (2006)
	Cones	α-pinene (46%), Δ-3-carene (27%), α-terpinolene (6.4%).	
Italy	Leaves	α-pinene (19.3%), sabinene (39.6%), limonene (7.31%), zingibirene (6.9%), δ-terpinene (6.14%), δ-cadinene (5.45%).	Sacchetti et al. (2005)
Greece	Cones	α-pinene (39.54%) and γ-terpinene (11.56%).	Loukis et al. (1991)
Croatia	Leaves	α-pinene (28.4 - 79.2%), γ-3-carene (9.1 - 32.6%), α-cedrol (1.2 - 12.9%), limonene (1.4 - 8.7%)	Milos et al. (2002)
Algeria	Leaves	α-pinene (47.00 - 52.76%), δ-3-carene (19.35 - 21.13%), α-terpinyl acetate (4.10 - 6.47%), cedrol (2.03 - 3.92%), myrcene (3.11 - 3.48%) and limonene (2.28 - 3.31%).	Chanegriha et al. (1993)
Algeria	Leaves	α-pinene (2.8 - 44.9%), δ-3-carene (31 - 10.6%) and α-terpinyl acetate (5.5 - 12.0%)	Chanegriha et al. (1997)
Algeria	Leaves	α-pinene (60.5%), cedrol (8.3%),	Mazari et al. (2010)
Tunisia	Branches	α-pinene (20%), δ-3-carene (22.9%), α-terpinolene (9.4%), α-terpinyl acetate (7.5%), limonene (5.1%)	Chéraif et al. (2005)

Herbicidal activity of essential oils from *Cupressus sempervirens*

Phytotoxic effects of essential oils obtained from aerial parts of *C. sempervirens* were tested on germination and seedling growth of *S. arvensis*, *T. campestre, L. rigidum* and *P. canariensis* which are very invasive weeds in cultivated areas. Providing statistical analysis, phytotoxic effects of tested oils were significantly influenced by doses, tested weeds and the sample oils.

The results (Tables 3, 4 and 5) show that all oils completely inhibited the emergence of these four weeds

Table 3. Inhibitory effects of essential oils of *C. sempervirens* on weeds germination.

Weed	Doses (µl/ml^{-1})	Germination %		
		Leaves	Cones	Branches
S. arvensis	0	95 ± 5^{a}	95 ± 5^{a}	95 ± 5^{a}
	1.25	60 ± 5^{b}	61.66 ± 5.77^{b}	58.33 ± 2.88^{b}
	2.5	23.33 ± 7.63^{c}	30 ± 5^{c}	50 ± 5^{b}
	3.75	0.0 ± 0.0^{d}	8.33 ± 2.88^{d}	38.33 ± 5.77^{c}
	5	0.0 ± 0.0^{d}	0 ± 0^{e}	15 ± 0^{d}
T. campestre	0	88.33 ± 2.88^{a}	88.33 ± 2.88^{a}	88.33 ± 2.88^{a}
	1.25	73.33 ± 10.4^{b}	66.66 ± 2.88^{b}	65 ± 8.66^{b}
	2.5	40 ± 13.22^{c}	46.66 ± 2.88^{c}	48.33 ± 2.88^{c}
	3.75	13.33 ± 5.77^{d}	13.33 ± 7.63^{d}	31.66 ± 2.88^{d}
	5	0 ± 0^{d}	0 ± 0^{e}	5 ± 5^{e}
L. rigidum	0	81.66 ± 7.63^{a}	81.66 ± 7.63^{a}	81.66 ± 7.63^{a}
	1.25	73.33 ± 7.63^{a}	71.66 ± 2.88^{a}	61.66 ± 2.88^{b}
	2.5	45 ± 8.66^{b}	51.66 ± 2.88^{b}	35 ± 5^{c}
	3.75	16.66 ± 2.88^{c}	18.33 ± 10.4^{c}	26.88 ± 2.88^{c}
	5	0 ± 0 d	0 ± 0^{d}	26.66 ± 2.88^{c}
P. canariensis	0	81.66 ± 2.88^{a}	81.66 ± 2.88^{a}	81.66 ± 2.88^{a}
	1.25	53.33 ± 5.77^{b}	63.33 ± 10.4^{b}	58.33 ± 7.63^{b}
	2.5	31.66 ± 2.88^{c}	36.66 ± 5.77^{c}	43.33 ± 12.58^{c}
	3.75	11.66 ± 2.88^{d}	18.33 ± 5.77^{d}	38.33 ± 5.7^{c}
	5	0 ± 0^{e}	0 ± 0^{e}	15 ± 0^{d}

Means in the same column by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$). (Mean of three replicates).

relative to the control. In general, a dose-response relationship was observed and the emergence declined with the increase amount of cypress oils. At the doses of 1.25, 2.5 and 3.75 µl/ml^{-1}, weeds germination was partially reduced by all oils, and totally inhibited at 5 µl/ml^{-1}, while the germination of *S. arvensis* was totally inhibited by leaves oil at the dose 3.75 µl/ml^{-1}. When germination was partially inhibited, not only emergence, even the seedling growth measured as roots and shoots lengths were significantly reduced, the reduction was greater with increasing amount of cypress oil. In the literature, herbicidal effects of essential oils from Lamiaceae, Anacardiaceae, Verbenaceae, Rutaceae, Asteraceae, Cupressaceae, Myrtaceae and other family against weeds have been previously reported (Barney et al., 2005; Ens et al., 2009; Angelini et al., 2003; Amri et al., 2012a, b, c; Batish et al., 2008; De Feo et al., 2002; Verdeguer et al., 2009); on the other hand, nothing was reported on the phytotoxic effects of *C. sempervirens*. In recent reports, we have demonstrated the herbicidal effects of essential oils obtained from Cupressaceae family that *Juniperus oxycedrus* and *Juniperus phoniceae*, the chemical analysis of these oils indicate their richness in monoterpenes hydrocarbons like α-pinene (Amri et al., 2011a, 2012a), which is consistent with obtained results in this study. Based on previous reports, we can conclude that phytotoxic effects of essential oils were attributed to individual components, while synergism and antagonism does play an important role on the biological activity. Previous studies have reported that essential oils and individual monoterpenes, such as α-pinene, limonene, terpinen-4-ol, camphor, 1,8-cineole, thymol and carvacrol strongly inhibit seed germination and seedling growth of some agricultural crops and weeds (Ens et al., 2009; De Feo et al., 2002; Singh et al., 2006; Scrivanti et al., 2003; Tworkoski et al., 2002; Wang et al., 2009; Kil et al., 2000; De Martino et al., 2010; Bulut et al., 2006). Looking at the chemical composition of the oil of *C. sempervirens*, more than 14 compounds are known to have herbicidal activity; α-pinene, β-pinene, β-myrcene, limonene, δ-3-carene and *p*-cymene are six hydrocarbonated monoterpenes that are present in our oil, indeed, these compounds have been reported to have herbicidal activities (Vokou et al., 2003; De Martino et al., 2010). Linalool, terpen-4-ol, myrtenal, α-terpineol borneol, 1.8-cineoole and bornyl acetate are 7 oxygenated monoterpenes; these compounds are present in the oil of *C. sempervirens* with

Table 4. Inhibitory effects of essential oils of *C. sempervirens* on roots growth of weeds.

Weed	Doses (µl/ml^{-1})	Germination %		
		Leaves	Cones	Branches
S. arvensis	0	13.13 ± 0.66^{a}	13.13 ± 0.66^{a}	13.13 ± 0.66^{a}
	1.25	8.2 ± 1.1^{b}	9.93 ± 1.8^{b}	8.66 ± 1.7^{b}
	2.5	2.93 ± 0.6^{c}	5.73 ± 0.64^{c}	6.03 ± 0.89^{c}
	3.75	0 ± 0^{d}	2.23 ± 0.25^{d}	4.13 ± 0.41^{d}
	5	0 ± 0^{d}	0 ± 0^{e}	1.8 ± 0.72^{e}
T. campestre	0	10.55 ± 1^{a}	10.55 ± ^{a}	10.55 ± 1^{a}
	1.25	9.4 ± 1.44^{a}	8.53 ± 1.16^{b}	8.46 ± 0.47^{b}
	2.5	5.33 ± 0.7^{b}	6.16 ± 0.15^{c}	5.1 ± 0.26^{c}
	3.75	2.5 ± 0.5^{c}	2.33 ± 0.65^{d}	5.1 ± 0.36^{c}
	5	0 ± 0^{d}	0 ± 0^{e}	1.5 ± 0.45^{d}
L. rigidum	0	13.56 ± 0.6^{a}	13.56 ± 0.6^{a}	13.56 ± 0.6^{a}
	1.25	9.96 ± 1.19^{b}	7.4 ± 1.05^{b}	8.03 ± 0.55^{b}
	2.5	5.73 ± 0.58^{c}	5.3 ± 0.75^{c}	5.43 ± 1.4^{c}
	3.75	3.9 ± 0.85^{d}	2.43 ± 0.45^{d}	3.7 ± 0.62^{d}
	5	0 ± 0^{e}	0 ± 0^{e}	0.93 ± 0.11^{e}
P. canariensis	0	13.03 ± 0.47^{a}	13.03 ± 0.47^{a}	13.03 ± 0.47^{a}
	1.25	8.6 ± 1.65^{b}	7.96 ± 2.21^{b}	9.96 ± 1.26^{b}
	2.5	4.83 ± 0.76^{c}	4.7 ± 0.7^{c}	5.4 ± 0.55^{c}
	3.75	1.63 ± 0.41^{d}	1.56±0.45^{d}	3.53 ± 0.47^{d}
	5	0 ± 0^{e}	0 ± 0^{d}	2.03 ± 0.55^{e}

Means in the same column by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$). (Mean of three replicates).

different percentages and they are known for their potential herbicide (Vokou et al., 2003). In addition, in our study, the oil was rich in sesquiterpenes that (*Z*)-caryophyllene which are known for their phytotoxic effects (Kil et al., 2000; De Feo et al., 2002; Singh et al., 2006; wang et al., 2009). The exact mechanism by which germination and seedling growth are affected by *C. sempervirens* volatile oil is unknown and not prospected in our study. However, such inhibitory effects could be caused by allelochemicals interfering with physiological and biochemical processes in target species (Singh et al., 2006; Scrivanti et al., 2003; Kaur et al., 2010). Indeed, it has been reported that the inhibition of germination may be the consequence of the inhibition of water uptake, increased abscisic acid content, decreased indole-3-acetic acid and zeatin riboside contents and disruption of the activity of metabolic enzymes that are involved in glycolysis and oxidative pentose phosphate pathway (Yang et al., 2008; Muscolo et al., 2001). On the other hand, previous studies showed that essential oils have phytotoxic effects that may cause anatomical and physiological changes in plant seedlings, leading to accumulation of lipid globules in the cytoplasm, reduction in some organelles such as mitochondria, possibly due to inhibition of DNA synthesis or disruption of membranes surrounding mitochondria and nuclei (Koitabashi et al., 1997). Muscolo et al. (2001) reported that the inhibition of seed germination in *Pinus laricio* was attributed to a disruption of the activity of metabolic enzymes that are involved in glycolysis and the oxidative pentose phosphate pathway. Another suggested mechanism for the inhibition of seed germination and radicle elongation is the disruption of dark or mitochondrial respiration. At this point, it has been shown that some volatile constituents such as α-pinene strongly affected the respiratory activity by interfering with the electron flow in the cytochrome pathway, resulting in decreased adenosine triphosphate (ATP) production and hence, alteration of other cell processes which are energy-demanding (Abrahim et al., 2001). In contrast, due to the difficulties to measure the allelochemicals effects on mitochondrial respiration in intact plants because many of these effects are masked by photorespiration, it has been hypothesized that the ability of monoterpenes to act as allelochemicals on intact seeds was probably directly related to their ability to permeate intracellular compartments (Abrahim et al., 2001; Zunino et al., 2004; Xu et al., 2006). Concerning the negative effects of

Table 5. Inhibitory effects of essential oils of *C. sempervirens* on shoots growth of weeds.

Weed	Doses (µl/ml^{-1})	Germination %		
		Leaves	Cones	Branches
S. arvensis	0	12.93 ± 1.77^{a}	12.93 ± 1.77^{a}	12.93 ± 1.77^{a}
	1.25	7.56 ± 0.6^{b}	6.96 ± 0.4^{b}	6.7 ± 0.81^{b}
	2.5	4.56 ± 0.6^{c}	4.8 ± 0.76^{c}	4.16 ± 0.76^{c}
	3.75	0 ± 0^{d}	2.7 ± 0.49^{d}	3.1 ± 0.52^{c}
	5	0 ± 0^{d}	0 ± 0^{e}	1.56 ± 0.4^{d}
T. campestre	0	9.23 ± 0.75^{a}	9.23 ± 0.75^{a}	9.23 ± 0.75^{a}
	1.25	9 ± 1.32^{a}	6.9 ± 0.55^{b}	8.56 ± 0.66^{ab}
	2.5	6.3 ± 0.9^{b}	5.96 ± 0.45^{b}	7.23 ± 1.12^{b}
	3.75	3.93 ± 0.4^{c}	3.46 ± 0.85^{c}	4 ± 0.86^{c}
	5	0 ± 0^{d}	0 ± 0^{d}	3.53 ± 0.89^{c}
L. rigidum	0	12.83±1.6^{a}	12.83 ± 1.6^{a}	12.83 ± 1.6^{a}
	1.25	7.96±0.55^{b}	6.6 ± 0.36^{b}	8.83 ± 0.58^{b}
	2.5	6.2±0.91^{c}	4.8 ± 0.2^{c}	7.16 ± 1.25^{b}
	3.75	4.6±0.45^{c}	3.83 ± 0.2^{c}	4.36 ± 1.19^{c}
	5	0±0^{d}	0 ± 0^{d}	3.1 ± 0.36^{c}
P. canariensis	0	15.5±0.86^{a}	15.5 ± 0.86 ^{a}	15.5 ± 0.86^{a}
	1.25	9.43±1.1^{b}	7.5 ± 1.37^{b}	9.1 ± 1.34^{b}
	2.5	6.2±1.31^{c}	6.7 ± 0.26 ^{b}	5.1 ± 0.52^{c}
	3.75	4.33±0.8^{d}	4.6 ± 0.65^{c}	3.13 ± 1.2^{d}
	5	0±0^{e}	0 ± 0^{d}	2.16 ± 0.58^{d}

Means in the same column by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$). (Mean of three replicates).

volatile oils on seedling growth, Nishida et al. (2005) and Singh et al. (2009) have reported that the exposure to α-pinene, β-pinene, 1,8-cineole and camphor inhibited root growth of *Brassica campestris* by inhibiting cell proliferation in root apical meristems, and decreased the mitotic index. Beside these manifestations, the latter authors also found that α-pinene disrupts membrane permeability resulting in solute leakage and bio-energetic failure which induce a cell death by apoptosis and necrosis (Singh et al., 2003; Kaur et al., 2010). The data obtained by Abrahim et al. (2003) indicate that α-pinene affects energy metabolism of isolated mitochondria from maize coleoptiles and primary roots by two mechanisms: uncoupling of oxidative phosphorylation and inhibition on the electron transfer chain which result the uncoupling of mitochondrial energy metabolism and inhibition of the mitochondrial ATP production. In the same report it demonstrates that the actions of α-pinene on isolated mitochondria are consequences of unspecific disturbances in the inner mitochondrial membrane. According to Weir et al. (2004), the decrease in membrane permeability was attributable to the accumulation of reactive oxygen species (ROS). The latter components such as singlet oxygen ($^{1}O_2$) and superoxide (O^{2-}), hydroxyl (OH) as well as hydroperoxyl (HO^2) radicals can affect membrane permeability, cause damage to DNA and proteins, and generate lipid peroxide signaling molecules. Moreover, it has been shown that the increased ROS generation following the exposure of *Cassia occidentalis* roots to α-pinene, was concomitant to enhanced activity of anti-oxidant enzymes mainly superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase, peroxidase and catalase (Singh et al., 2006). Despite the absence of comprehensive and systemic investigations in functional mechanism of allelopathy of cypress volatile oils, we can conclude that the strong inhibitory effects on seed germination and radicle elongation in weeds are attributable to one or more of the above-mentioned mechanisms. Deep physiological and biochemical investigations should be performed.

Antifungal activity of essential oil

Essential oils isolated from leaves, cones and branches of *C. sempervirens* L. were tested for their antifungal activity against eight plant pathogenic fungal species.

Table 6. Antifungal activity of essential oil extracted from aerial parts of *C. sempervirens* L.

Fungi	Inhibition of fungi growth %.		
	Leaves	**Cones**	**Branches**
F. nygamai	60.91 ± 4.02^{bcB}	78.56 ± 3.81^{aA}	54.39 ± 4.02^{aB}
Alternaria sp	75.21 ± 6.1^{aA}	75.43 ± 5.37^{aA}	51.9 ± 7.33^{aB}
M. nivale	71.11 ± 6.81^{bcA}	78.33 ± 6.17^{aA}	58.49 ± 3.8^{aB}
F. culmorum	72.06 ± 3.78^{bcA}	71.11 ± 10.87^{abA}	53.73 ± 9.02^{aA}
B. cinerea	70.46 ± 2.99^{bcB}	82.46 ± 2.03^{aA}	56.76 ± 6.58^{aC}
F. equisiti	71.63 ± 4.53^{bcA}	58.49 ± 6.2^{bcA}	53.45 ± 12.63^{aA}
F. oxysporum	58.51 ± 2.79^{bcB}	69.35 ± 5.01^{abA}	63.51 ± 3.83^{aAB}
F. verticilloides	66.73 ± 8.5^{bcB}	79.16 ± 2.18^{aA}	52.81 ± 5.63^{aC}

Small letters compare means in the lines and capital letters in the columns. Means in the same column by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$). (Mean of three replicates). Means in the same line by the same letter are not significantly different of the test Student-Newman-Keuls ($p \leq 0.05$).

According to obtained results in Table 6, essential oils of *C. sempervirens* showed significant inhibition of fungal growth, this study also indicated that the antifungal activity is variable depending on the dose, fungal strain and tested oils. According to statistical analysis, the highest inhibitions were obtained with cones and leaves, while weak activities were obtained with branches oils. Different degrees of sensitivity were recorded as *Alternaria* sp was the most sensitive to the oil of leaves, whereas, *Alternaria* sp, *F. verticilloides*, *F. nygamai* and *M. nivale* were the most sensitive to cones oil, however, all fungi showed the same sensitivity behavior to branches oil. Essential oils of *C. sempervirens* showed a significant inhibition of the growth of all fungi, in general, there was a correlation between the antifungal activity and percentage of some major components. As mentioned above, cypress oils were characterized by relatively high content of monoterpenes hydrocarbons (40.2 to 60%) as α-pinene, δ-3-carene and oxygenated sesquiterpenes like α-cedrol which could be responsible for the antifungal activity observed in this study. Indeed, several authors have attributed the antifungal capacity of essential oils to the presence of these components (Amri et al., 2011a, b, 2012a, b; Sokovic et al., 2006). Besides, Sokovic and Van Griensven (2006) showed that limonene and α-pinene have a strong antifungal activity against *Verticillium fungicola* and *Trichoderma harzianum* (Sokovic et al., 2006). Moreover, Chang et al. (2008) showed the fungicide activity of limonene, α- and β-pinene against *Fusarium solani* and *Colletotrichum gloeosporioides*. Thus, the antifungal activity of the oil in this study is not attributed only to the high proportions of the monoterpenes, however, other major or trace components in the oil could give rise to its antifungal activity. Indeed, there are synergistic and antagonistic interactions between oil components. The mode of action of essential oils was investigated by many authors who suggested that the antimicrobial activity is produced by interactions provoked by terpenes in the enzymatic systems related with energy production and in the synthesis of structural components of the microbial cells (Omidbeygi et al., 2007). Other reports suggested that the components of the essential oils cross the cell membrane, interacting with the enzymes and proteins of the membrane such as the H^+/ATPase pumping membrane, so producing a flux of protons toward the cell, exterior which induces changes in the cells and ultimately their death. Besides, several authors (Cristani et al., 2007; Lucini et al., 2006; Tatsadjieu et al., 2009) reported that the antimicrobial activity is related to ability of terpenes to affect not only permeability but also other functions of cell membranes, these compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. In addition, Daferera et al. (2000) reported that the fungitoxic activity of essential oils may have been due to formation of hydrogen bonds between the hydroxyl group of oil phenols and active sites of target enzymes. These components would increase the concentration of lipidic peroxides such as hydroxyl, alkoxyl, and alkoperoxyl radicals and so bring about cell death (Daferera et al., 2000). Other reports showed that the essential oils would act on the hyphae of the mycelium, provoking exit of components from the cytoplasm, the loss of rigidity and integrity of the hyphae cell wall, resulting in its collapse and death of the mycelium (Daferera et al., 2000; Sharma et al., 2006). Even though the inhibitory effect of the essential oils was lower than those obtained by the chemical fungicide, however, essential oils could reduce significantly the growth of all fungi tested.

Conclusion

Our study could give the solution, which in its first part had focused on the correlation between the chemical composition and the effectiveness as antifungal and herbicidal agents of three essential oils extracted from common Tunisian cypress (leaves, cones and branches of *C. sempervirens*). Results of essential oils bioactivities

showed that *C. sempervirens* exhibited stronger phytotoxic and antifungal effects. Based on our preliminary results, the essential oils of *C. sempervirens* could be suggested as alternative herbicides and fungicides. However, further studies are required to determine the cost, applicability, safety and phytotoxicity against the cultured plants of these agents as potential bio-pesticide.

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