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Chemical Composition and Antimicrobial and Antioxidant Activities of Essential Oils and Various Extracts of *Juniperus phoenicea* L. (Cupressacees)

MONIA ENNAJAR, JALLOUL BOUAJILA, AHMED LEBRIHI, FLORENCE MATHIEU, MANEF ABDERRABA, ALY RAIES, AND MEHREZ ROMDHANE

ABSTRACT: GC-FID and GC-MS analysis of essential oils of *Juniperus phoenicea* resulted in the identification of 30 compounds, representing more than 98% of the total composition. α-pinene (55.7% and 80.7%), δ-3-carene (10.7% and 4.5%), and γ-cadinene (2.9% and 5.1%) were the main components, respectively, in leaves and berries essential oil. Extracts of *J. phoenicea* were obtained by different extraction solvents: methanol, ethanol, ethyl acetate, and dichloromethane and evaluated composition for polyphenols (gallic acid equivalent 52 to 217 g/kg), tannins (catechin equivalent 6.5 to 60.2 g/kg), antocyanins (cyanidin equivalent 84 to 373 mg/kg), and flavonoids (quercetin equivalent 6.4 to 29.3 g/kg). The samples (essential oils and extracts) were subjected to a screening for their antioxidant activity by using DPPH and ABTS assays; antimicrobial activity was tested with 6 bacteria (3 Gram-positive and 3 Gram-negative), 1 yeast, and 2 fungi. The strongest antioxidant activity was obtained by the methanolic extract ($IC_{50} = 6.5 \pm 0.3$ mg/L). Flavonoids are likely to contribute to the antifungal activity against *Saccharomyces cerevisiae*. Correlations were studied between chemical composition and antioxidant and antimicrobial activities.

Keywords: antimicrobial activity, antioxidant activity, essential oil, GC-MS, *Juniperus phoenicea* L. (cupressacees)

Introduction

Phytochemicals are used as natural antimicrobial agents or "biocides" (Smid and Gorris 1999). There is growing interest in correlating phytochemical constituents of plant with its pharmacological activity (Vaidya and Antarkar 1994). The antimicrobial and antioxidant properties of essential oils have been recognized for many years, and their preparations have found applications as naturally occurring antimicrobial and antioxidant agents in the field of pharmacology, pharmaceutical botany, phytopathology, medical, and clinical microbiology, food preservation, and so on. The essential oil and various extracts preparations that possess antimicrobial and/or antioxidant activities have been the subject of many investigations resulting in the screening of a wide variety of plant species, and have revealed structurally unique biologically active compounds. However, less attention was paid to the activity of their main components in essential oils tested. The main advantage of new natural agents is that they do not present the "antibiotic resistance," a phenomenon commonly encountered with the long-term use of antibiotics. The antimicrobial and antioxidant activities of essential oils are assigned to a number of small terpenoids and phenolic compounds (thymol, carvacrol, eugenol), which also in pure form demonstrate high antibacterial and antioxidant activities (Conner 1993). For extracts, different solvent systems have been used for the extraction of polyphenols from plant material (Hernandez and others 2007). Extraction yield is dependent on the solvent and the method of extraction (Hayouni and others 2007).

Juniperus phoenicea L. (Cupressaceae) is a shrub or a small tree from the Mediterranean region (Bonnier and Douin 1990). The species present a very large geographical distribution ranging from Portugal to Saudi Arabia (Meloni and others 2006). It is also native to North Africa as well as the Canary Islands (Adams 2004; Louriero and others 2007). This wide geographical range of the species makes it possible for a vast amount of genetic variability (Meloni and others 2006). In fact, many researchers reported the presence of intraspecific taxa based upon morphological, biochemical (LeBreton and Thivend 1981), and molecular data (LeBreton and Thivend 1981; Adams and others 2002; Adams 2004; Meloni and others 2006; Louriero and others 2007). Recently, on the basis of random amplified polymorphics DNAs fingerprinting, Adams and others (2006) have distinguished between *J. phoenicea* var *phoenicea* and *J. phoenicea* var. *turbinata* and affiliated populations.

The genus Juniperus is considered as an important medicinal plant largely used in traditional medicine. Its leaves are used in the form of decoction to treat diabetes, diarrhea, and rheumatism (Bellakhder 1997). The mixture of leaves and berries of this plant is used as an oral hypoglycaemic agent (Amer and others 1994), whereas the leaves are used against bronco-pulmonary disease and as a diuretic (Bellakhder 1997).

Considering the medicinal interest of the species, several studies related to the chemical characterization of its essential oil were performed. However, these studies were related to only *J. phoenicea*

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grown in north Mediterranean basin (Rezzi and others 2001). On the *J. phoenicea* grown in south Mediterranean basin, a little information is available on its chemical composition (essential oils and extracts) and its infraspecific variation. In the literature, many papers report on the composition of the essential oil from berries and leaves of the *Juniperus* species (Guerra Hernandez and others 1987; Mastelic and others 2000) but few studies have investigated their antimicrobial activity (Stassi and others 1996; Angioni and others 2003). In fact, this study is the first to show the antioxidant activity of leaves and berries *J. phoenicea* essential oils from the south of Mediterranean basin.

Therefore, the objectives of this study were to (1) analyze the chemical composition of essential oils by a GC-FID and GC-MS system to determine essential oils chemotype of *J. phoenicea* plants collected from the south eastern region of Tunisia, (2) characterize polyphenols, tannins, flavonoids, and anthocyannins of their extracts, (3) investigate antimicrobial and antioxidant activities of essential oils and various extracts, and (4) the study of possible correlations between chemical compositions and antimicrobial and antioxidant activities.

Materials and Methods

Chemicals

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France): Sigma (sodium acetate [≥99%], potassium chloride [≥99%], NaH_2PO_4 [≥98%], Na_2HPO_4 [≥99%], NaCl [≥98%], gallic acid [≥99%], hydrochloric acid [36.5% to 38%], catechin [≥98%], quercetin [≥98%], cyanidin-3-glucoside [≥95%], nystatin [≥99%], ascorbic acid [≥99%], ampicillin [96 to 100.5%]), Aldrich (aluminium trichloride [99.99%], C5-C24 *n*-alkanes [≥99.0%], 1,1-diphenyl-2-picrylhydrazyl free radical [99%]), Sigma-Aldrich (methanol [≥99%], ethanol [≥99.5%], dichloromethane [≥99.5%], petroleum ether [≥98.5%], anhydrous sodium sulfate [≥99.0%], sodium carbonate [≥99.0%], Folin–Ciocalteu reagent 2 N, acetic acid [≥99.7%], 10% Tween 80, nalidixic acid [≥98%]), and Fluka (ethyl acetate [≥99.7%], potassium persulfate [≥99.0%], vanillin [≥98.0%], H_2SO_4 [≥95%], 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate [≥99.0%]).

Collection of plant material

The leaves and berries of *J. phoenicea* were collected in October 2007 from the southeastern area of Tunisia, precisely in the surroundings of "Matmata" localized in 600 m of altitude, 37° 19′ in the north and 8° 66′ in the east of longitude. Matmata average rainfall was 78 mm for 2007, 6 mm in October 2007, the average temperature was 22.6 °C in October 2007 and humidity was 65% in October 2007 (Pluviometric Directory of Tunisia, 2007–2008, Head Office of the Water Resources, Ministry for Tunisian Agriculture, the Environment and the Hydraulic Resources). Specimens were identified by Dr. Nadia Ben Brahim at the Dept. of Botany, Natl. Inst. of Agronomic Research (INRAT, Tunis) and voucher specimens were deposited at the Herbarium of the Dept. of Botany in the cited institute.

Isolation of essential oils

A portion (100 g) of the dried aerial parts of *J. phoenicea* (leaves and berries) was submitted for 3 h to water distillation, using a clevenger-type apparatus according to the method recommended by the European Pharmacopoeia (1983). Yields are shown in Table 1. The obtained essential oils were dried by anhydrous sodium sulfate and after filtration stored at 4 °C until tested and analyzed.

Preparation of juniper extracts

The air-dried and finely ground leaves and berries (2.5 g) were extracted by stirring with 25 mL of solvent (methanol, ethanol, dichloromethane, or ethyl acetate) at room temperature for 30 min. Extracts were kept for 24 h at 4 °C then filtered through Whattman Millipore Filter paper (GF/A, 110 mm, 8 μm) and evaporated under vacuum. Yields are shown in Table 1.

Gas chromatography and gas chromatography-mass spectrometry

Quantitative and qualitative analysis of essential oils was carried out by gas chromatography-flame ionization detection (GC-FID) and gas chromatography/mass spectrometry (GC-MS). Gas chromatography analyses were carried out on a Varian Star 3400 Cx chromatograph (Les Ulis, France) fitted with a fused silica capillary DB-5MS column (5% phenylmethylpolysyloxane, 30 m × 0.25 mm, film thickness 0.25 μm). Chromatographic conditions were a 60 to 260 °C temperature rise with a gradient of 5 °C/min and 15 min isotherm at 260 °C. A 2nd gradient was applied to 340 °C at 40 °C/min. Total analysis time was 57 min. For analysis, essential oils were dissolved in petroleum ether. One microliter of sample was injected in the split mode ratio of 1 : 10. Helium (purity 99.999%) was used as carrier gas at 1 mL/min. The injector was operated at 200 °C. The mass spectrometer (Varian Saturn GC/MS/MS 4D) was adjusted for an emission current of 10 μA and electron multiplier voltage between 1400 and 1500 V. Trap temperature was 150 °C and that of the transfer line was 170 °C. Mass scanning was from 40 to 650 amu.

Compounds were identified by comparison of their KI (Kovats indices) relative to C5-C24 *n*-alkanes obtained on a nonpolar DB-5MS column, with those provided in the literature, by comparison of their mass spectra with those recorded in NIST 08 (Natl. Inst. of Standards and Technology) and reported in published articles and by co-injection of available reference compounds. The samples were analyzed in duplicate. The percentage composition of essential oils was computed by the normalization method from the GC peak areas. Results were calculated as mean values of 2 injections from each essential oil, without using correction factors. All determinations were performed in duplicate and averaged.

Total amount of phenolic compounds

The total phenolics of each extract were determined by the Folin–Ciocalteu method (Folin and Ciocalteu 1927). Preparation of Folin and Ciocalteu reagent 2 N (Fluka): dissolve 10 g sodium tungstate and 2.5 g sodium molybdate in 70 mL water. Add

Table 1 — Extraction yields (percent) of essential oils and various extracts of *J. phoenicea*.

	Water distillation (essential oil)	Methanol	Ethanol	Ethyl acetate	Dichloromethane
Leaves	0.90	26.2	35.4	18.0	1.1
Berries	3.95	16.4	35.8	6.4	0.8

The values are mean with standard deviations (SD) that did not exceed 5% for triplicate determinations.

5 mL 85% phosphoric acid and 10 mL concentrated hydrochloric acid. Reflux for 10 h. Add 15 g lithium sulfate, 5 mL water, and 1 drop bromine. Reflux for 15 min. Cool to room temperature and bring to 100 mL with water. Hexavalent phosphomolybdic/phosphotungstic acid complexes with the following structures are formed in solution.

The diluted aqueous solution of each extract (0.5 mL) was mixed with Folin–Ciocalteu reagent (0.2 N, 2.5 mL). This mixture was allowed to stand at room temperature for 5 min and then sodium carbonate solution (75 g/L in water, 2 mL) was added. After 1 h of incubation, the absorbances were measured at 765 nm against water blank using a Helios spectrophotometer (Unicam, Cambridge, U.K.). A standard calibration curve was plotted using gallic acid (0 to 300 mg/L). The results were expressed as gram of gallic acid equivalents (GAE)/kg of dry mass.

Condensed tannin content

Catechins and proanthocyanidins reactive to vanillin were analyzed by the vanillin method (Broadhurst and Jones 1978; Naczk and others 2001). One milliliter (1 mL) of each extract solution was placed in a test tube and 2 mL of vanillin (1% in 7 M H_2SO_4) in an ice bath and then incubated at 25 °C. After 15 min, the absorbance of the solution was read at 500 nm. Concentrations were calculated as gram catechin equivalents (CE)/kg dry mass from a calibration curve.

Total flavonoids determination

The total flavonoids were estimated according to the Dowd method as adapted by Arvouet-Grand and others (1994). A diluted methanolic solution (4 mL) of each extract was mixed with a solution (4 mL) of aluminum trichloride ($AlCl_3$) in methanol (2%). The absorbance was read at 415 nm after 15 min against a blank sample consisting of a methanol (4 mL) and extract (4 mL) without $AlCl_3$. Quercetin was used as reference compound to produce the standard curve, and the results were expressed as gram of quercetin equivalents (QE)/kg of dry mass.

Determination of total anthocyanin content

Total anthocyanin content was measured with the pH differential absorbance method, as described by Cheng and Breen (1991). Briefly, absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetic acid-sodium acetate, 1 M). The wavelength reading was performed after 15 min of incubation. Anthocyanin content was calculated using a molar extinction coefficient (ε) of 29600 (cyanidin-3-glucoside) and absorbance of $A = [(A_{510} - A_{700})_{\mathrm{pH1.0}} - (A_{510} - A_{700})_{\mathrm{pH4.5}}]$. Results were expressed as milligram cyanidin-3-glucoside equivalent (C3GE)/kg of dry mass.

Microbial strains

The different essential oils and extracts were individually tested against a panel of microorganisms. Six bacteria including 3 Gram-positive (*Bacillus subtillis* ATCC 6633, *Staphylococcus aureus* CIP7625, *Listeria monocytogenes* Scott A 724) and 3 Gram-negative (*Pseudomonas aeruginosa* CIPA22, *Escherchia coli* ATCC10536, *Klebsiella pneumoniae* CIP8291), 1 yeast (*Saccharomyces cerevisiae* ATCC 4226 A), and 2 fungi (*Mucor ramamnianus* ATCC 9314, *Aspergillus westerdijkiae* [*ex: ochraceus*]) were used.

Well-diffusion assay

Agar well-diffusion method (Kalemba and Kunicka 2003; Oke and others 2009) was employed for the determination for antimicrobial activity of essential oils and extracts. Briefly, a suspension of the tested microorganism (0.1 mL of 10^8 cells/mL) was spread on nutrient agar. Essential oils or extracts were serially diluted, using 10% Tween 80 in distilled sterile water. The wells with a diameter of 10 mm, were injected with 10 μL (9 μL of the essential oil + 1 μL of 10% Tween 80) of essential oils and with 100 μL (3 mg/mL) of extracts (300 μg/well) and placed on the inoculated plates. Ampicillin and nalidixic acid (30 μg/well) were used as positive reference standards to determine, respectively, the sensitivity of Gram-positive and Gram-negative bacterial. Nystatin (30 μg/well) was used as positive reference standard to determine the sensitivity of yeast and fungi species.

These plates, after staying at 4 °C for 2 h to allow dispersal, were incubated at 37 °C (bacteria) and at 30 °C (yeast and fungi) during 48 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. The diameter of the inhibition zones was measured in millimeters. Tests were carried out in triplicate.

Free radical scavenging activity DPPH test

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Blois (1958) with some modifications; 1.5 mL of various dilutions of the test materials (pure antioxidants or plant extracts) were mixed with 1.5 mL of a 0.2 mM methanolic DPPH solution. After an incubation period of 30 min at 25 °C, the absorbance at 520 nm, the wavelength of maximum absorbance of DPPH, were recorded as $A_{(\mathrm{sample})}$. A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as $A_{(\mathrm{blank})}$. The free radical-scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\%\,\mathrm{inhibition} = 100\,(A_{(\mathrm{blank})} - A_{(\mathrm{sample})})/A_{(\mathrm{blank})}$$

Antioxidant activity of essential oils or extracts was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Ascorbic acid was used as a standard. All measurements were performed in triplicate.

ABTS radical-scavenging assay

The radical scavenging capacity of the samples for the 2,2-AzinoBis-3-ethylbenzoThiazoline-6-Sulphonate radical cation (ABTS) was determined as described by Re and others (1999). ABTS was generated by mixing a 7 mM of ABTS at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, and 154 mM NaCl) with 2.5 mM potassium persulfate (final concentration) followed by storage in the dark at room temperature for 16 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using spectrophotometer. For each sample, diluted methanol solution of the sample (100 μL) was allowed to react with fresh ABTS solution (900 μL), and then the absorbance was measured 6 min after initial mixing. Ascorbic acid was used as a standard and the capacity of free radical scavenging was expressed by IC_{50} (mg/L) values calculated denote the concentration required to scavenge 50% of ABTS radicals. The capacity of free radical scavenging IC_{50} was determined using the same previously used equation for the DPPH method. All measurements were performed in triplicate.

Statistical analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P < 0.05$. Correlation coefficients (r) to determine the relationship between

chemical composition and antioxidant or biological activity were calculated using MS Excel software (CORREL statistical function).

Results and Discussion

Chemical composition

Chemical composition of essential oils. Essential oils presented a white-yellow color. By hydrodistillation of leaves and berries of *J. phoenicea*, yields (relative to dry weight material) of 0.90% (w/w) and 3.95% (w/w), respectively, were obtained. El-Sawi and others (2007) obtained a berry's yield superior to that of leaf (0.36% and 1.96%, respectively), during its study of Egyptian juniper. Tunisian *J. phoenicea* has a yield at least twice higher than the Egyptian. Barrero and others (2006) have obtained yield 0.70% of Morocco *J. phoenicea* leaves.

The results of analyses of essential oils by GC-FID and GC-MS are given in Table 2, where the compounds were listed according to their KI. Twenty-nine constituents, representing 97.9% of the total components in the essential oil from the leaves of *J. phoenicea*, were identified. Monoterpenes (75.9%) represent the main fraction of the essential oil; α-pinene (55.7%) and δ-3-carene (10.7%) were the main components of this fraction. Also, α-terpineol (2.2%) and β-myrcene (2.1%) were present in reasonable amounts. A similar result was obtained by Barrero and others (2006), in his study of Marcocain *J. phoenicea* when the fresh leaves were treated by steam distillation and found that the largest group of constituents in the essential oil was the monoterpenes (71.1%), the major components were α-pinene (45.5%) and δ-3-carene (13.0%). It is clear that the Tunisian juniper was the highest content in different groups. The sesquiterpene compounds constituted 20% of the total essential oil (Table 2). γ-cadinene (2.9%), germacrene B (2.0%), β-caryophyllene (1.9%), and α-cubebene (1.5%) were the main components of the sesquiterpenes, which accounted for 20% of the essential oil. Also, in Morocain leaves essential oil, β-caryophyllene (3.4%) was the major component followed by γ-elemene (2.7%), which in the Tunisian leaves oil did not exceed 1.3%.

The chemical composition of the essential oil from *J. phoenicea* berries is also shown in Table 2. Nine compounds were identified, which present 98.8% of the total essential oil composition. The monoterpenes represent the major fraction, in which α-pinene (80.7%) was the main component. γ-cadinene (5.1%) represented the major compound of sesquiterpenes. There was a large difference between *J. phoenicea* leaves and berries essential oils. The 2 types of essential oils revealed a wide variation in chemical composition, their contents and the number of identified compounds.

Although leaves essential oil is richer in compounds than berries but it has a lower content in α-pinene. A similar result was found by Angioni and others (2003) when α-pinene of berries essential oil was at 87.5% but in leaves essential oil the percentage was 48.9%. In contrast with Afifi and others (1992), α-pinene was the major constituent of both essential oils recording a little difference by 39.3% for berries and 38.22% for leaves. β-pinene was present in not negligible amounts in both essential oils (2.45% and 0.48% in berries and leaves essential oils, respectively) compared with our study (0% and 1.1% in berries and leaves essential oils, respectively). The results were in agreement with those reported on the analysis of other

Table 2—Chemical composition (percent) of *J. phoenicea* essential oils.

Nr	KI	M (g/mol)	Compounds	Leaves	Berries
1	936	136	α-pinene	55.7	80.7
2	974	136	β-pinene	1.1	ND
3	985	136	β-myrcene	2.1	1.8
4	1011	136	δ-3-carene	10.7	4.5
5	1028	136	limonene	0.7	ND
6	1030	136	α-phellandrene	0.8	ND
7	1057	136	γ-terpinene	0.7	1.3
8	1086	136	α-terpinolene	0.7	ND
9	1185	154	α-terpineol	2.2	ND
10	1237	148	cuminaldehyde	0.6	ND
11	1272	196	isopulegyl acetate	0.6	ND
12	1345	204	α-cubebene	1.5	ND
13	1415	204	β-caryophyllene	1.9	2.6
14	1428	204	γ-elemene	1.3	ND
15	1432	204	β-gurjunene	0.7	ND
16	1450	204	α-himachalene	0.8	0.8
17	1454	204	α-humulene	0.5	ND
18	1460	204	*allo*-aromadendrene	0.8	ND
19	1480	204	γ-muurolene	0.8	ND
20	1482	204	β-selinene	2.0	ND
21	1488	204	α-amorphene	0.7	ND
22	1502	204	α-chamigrene	0.7	ND
23	1513	204	γ-cadinene	2.9	5.1
24	1514	222	cubenol	1.5	ND
25	1524	204	δ-cadinene	1.9	ND
26	1540	222	α-calacorene	0.8	ND
27	1554	204	germacrene B	2.0	1.5
28	1960	272	13-isopimaradiene	ND	0.5
29	2328	286	*trans*-ferruginol	0.6	ND
30	nd	136	heptane 2,4-dimethyl[a]	0.6	ND
			Total	97.9	98.8
			Monoterpene hydrocarbons	72.5	88.3
			Monoterpenes oxygenated	3.4	0
			Sesquiterpenes hydrocarbons	18.5	10.0
			Sesquiterpenes oxygenated	1.5	0
			Others	2.0	0.5

[a]Tentatively identified supported by good match of MS. nd = not determined. ND = not detected.

juniper oils (Adams and others 1996) in which pinene derivatives are more common.

Data reported by Adams and others (1996) on the chemical composition of the essential oil of Spanish *J. phoenicea* ssp. *turbinata* leaves showed that it was dominated by α-pinene (28.3%), β-phellandrene (25.3%), β-myrcene (7.2%), and α-phellandrene (4.1%); while limonene and δ-3-carene were absent. In our study, we note the absence of β-phellandrene and presence of a high content of δ-3-carene (10.7%) as the 2nd major component; nevertheless, β-myrcene and α-phellandrene were in a lower value (2.1% and 0.8%, respectively). This important variation in chemical composition could be due to geographical and bioclimatic factors of the region.

Rezzi and others (2001) reported the composition of leaves essential oil of *J. phoenicea* from Corsica. They found 2 different main compositions, identified as Cluster I and II, with the former rich in α-pinene (70%) and the latter rich in α-pinene (33.0%), β-phellandrene (21.1%), and α-terpinyl acetate (8.2%). In the essential oil from *J. phoenicea* leaves from Portugal, Cavaleiro and others (2001) have recognized 3 clusters based on the α-pinene/β-phellandrene ratio. We observed absence of β-phellandrene and α-terpinyl acetate in our study but contrary to this, δ-3-carene was the 2nd major compound (10.7%) in this work and it was low in study of Cavaleiro and others (2001). The variability in essential oil composition was present even in *J. phoenicea* and these variations, sufficient to allow the distinction of different specimens, were the result of an adaptative process to particular ecologic and geographic conditions.

Chemical composition of extracts. According to similarity and miscibility, 4 solvents having different polarities, methanol, ethanol, ethyl acetate, and dichloromethane, were used to extract the different secondary metabolites of *J. phoenicea*. Yields of different extracts obtained from leaves and berries are presented in Table 1. The highest yield, for leaves and berries, was achieved by the ethanol (35.4% and 35.8%, respectively), followed by methanol (26.2% and 16.4%, respectively) and ethyl acetate (18% and 6.4%, respectively), and the lowest was dichloromethane (1.1% and 0.8%, respectively). Variation in the yields of various extracts was attributed to polarities of different compounds in the leaves and berries, such differences have been reported in the literature concerning berries (Jayaprakasha and others 2001).

The amounts of the total polyphenols, tannins, flavonoids, and antocyanins in the extracts fractions of leaves and berries of *J. phoenicea* are shown in Table 3. The amount of total phenolics, in leaves and berries, varied in the different extracts and ranged from 52 ± 1 to 217 ± 2 g GAE/kg of dry material. The leaves of *J. phoenicea* were richer in polyphenols than berries. The highest amount of polyphenols was in methanolic leaves extract followed by ethanol extract. Also, Hayouni and others (2007) approved that the highest yield of berries *J. phoenicea* was 36.2% and the important amount of polyphenolics was 202 ± 0.43 g GAE/kg of dry material. In addition, for tannins and flavonoids, the ethanolic and methanolic leaves extracts are richer than berries extracts. While for antocyanins, berries are richer than the leaves and the highest amount was obtained by ethyl acetate extract (373 ± 13 mg C3GE/kg of dry material). Among these compounds, anthocyanins was at the lowest rate 84 to 373 mg C3GE/kg of dry mass compared with the amounts of polyphenols, flavonoids, and tanins, which were expressed in g/kg of dry mass. To the best of our knowledge, this is the 1st study reporting the amount of flavonoids, tanins, and anthocyanins of *J. phoenicea* leaves and berries extracts. Hence, it could be used as an important descriptor to characterize the extracts of *J. phoenicea* and consequently this endemic species itself.

Antimicrobial activity

The *in vitro* antimicrobial potential of *J. phoenicea* essential oils and extracts against microorganisms was quantitatively assessed by the presence or absence of inhibition zones (Table 4).

Antimicrobial activity of essential oils. As can be seen in Table 4, essential oils of leaves and berries showed reasonable *in vitro* antimicrobial activity compared with references against all the tested microorganisms including Gram-positive, Gram-negative, fungi, and yeast with diameter zones of inhibition 11 to 18 mm. The leaves essential oil has an antimicrobial activity greater than that of berries (except *M. ramamnianus*) that may be due to the wealth of leaves essential oil by oxygenated compounds that are totally absent in the berries essential oil. In addition, the amount of sesquiterpenes in the leaves essential oil was higher than in berries essential oil.

Essential oils of many *Juniperus* species were known to exhibit antimicrobial activity against several microorganisms (Stassi and others 1996; Cavaleiro and others 2001; Angioni and others 2003). The antibacterial activity of the essential oil of *J. phoenicea* buds could, in part, be associated with major constituents such as α-pinene, δ-3-carene, α-terpineol, β-myrcene, limonene, γ-terpinene, and α-amorphene. These components have been reported to display antibacterial effects (Cosentino and others 1999; Alessandra and others 2005). Terpenes were active against bacteria (Cosentino and others 1999).

As described previously by other researchers, essential oils containing terpenoids are more active against Gram-positive bacteria than against Gram-negative ones (Djoukeng and others 2005). In addition, the components in lower amount may also contribute to antibacterial activity of the essential oil, involving probably some type of synergism with other active compounds. Stassi and others (1996) have tested the antimicrobial activity of essential oils of 4 *Juniperus* spices against Gram-positive and Gram-negative bacteria. They approved that essential oils of the berries generally appear more active than essential oils of the leaves. Essential oils of the berries inhibit the growth of *E. coli*, *P. aeruginosa*, and *S. aureus*, while essential oils of the leaves do not. The same results were observed with our essential oils but not with *E. coli*. Essential oils of

Table 3 — Chemical composition of *J. phoenicea* extracts.

	Samples	Polyphenols (GAE)[a]	Tannins (CE)[a]	Flavonoids (QE)[a]	Anthocyanins (C3GE)[b]
Leaves	Ethyl acetate	116 ± 2	18.4 ± 0.7	24.6 ± 0.7	218 ± 10
	Dichloromethane	56 ± 1	9.3 ± 0.4	16.7 ± 0.7	219 ± 11
	Ethanol	169 ± 2	60.2 ± 2.8	29.3 ± 1.3	249 ± 12
	Methanol	217 ± 2	57.2 ± 2.8	20.9 ± 1.0	84 ± 4
Berries	Ethyl acetate	79 ± 1	13.3 ± 0.5	15.0 ± 0.7	373 ± 13
	Dichloromethane	93 ± 1	20.3 ± 0.6	6.5 ± 0.2	275 ± 11
	Ethanol	96 ± 1	6.5 ± 0.2	6.4 ± 0.3	186 ± 8
	Methanol	52 ± 1	14.7 ± 0.4	8.6 ± 0.4	278 ± 11

[a]g/kg dry mass; [b]mg/kg dry mass. Standard deviations (SD) did not exceed 5%.

Table 4 – Antimicrobial activity (diameter of the inhibition zones in mm) of essential oils and extracts of *J. phoenicea*.

	Samples	Gram-positive bacteria			Gram-negative bacteria			Fungi and yeast		
		B. subtillis	*L. monocytogenes*	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*	*E. coli*	*S. cerevisiae*	*M. ramamnianus*	*A. westerdijkiae*
Essential oils	Leaves	18	–	15	14	–	–	11	16	13
	Berries	16	–	16	13	–	–	11	17	–
Leaves extracts	Methanol	34	–	15	17	–	–	14	16	–
	Ethyl acetate	24	–	13	15	–	–	13	12	–
	Ethanol	30	–	15	16	–	–	15	20	–
	Dichloromethane	20	–	11		–	–	11	12	–
Berries extracts	Methanol	34	–	17	20	–	–	12	20	–
	Ethyl acetate	34	–	19	23	–	–	11	12	–
	Ethanol	28	–	18	17	–	–	11	22	–
	Dichloromethane	17	–	16	11	–	–	–	–	–
References standard	Ampicillin	54	31	20						
	Nalidixic acid				19	28	30			
	Nystatin							29	31	24

– = absence of inhibition zone detected.

both the leaves and the berries inhibit the growth of *B. subtulis* at the same concentration. Furthermore, in our study, the most sensitive microorganism for the 2 essential oils was *B. subtilus*. Nevertheless, for the fungi, it turned out that the berries essential oil was inactive against *A. westerdijkiae* while leaves essential oil was active.

The activity of the essential oil varies with its concentration and the bacteria. These differences in the susceptibility of the test organisms to the essential oil could be attributed to variation in the rate of the essential oil constituents penetration through the cell wall and cell membrane structures. The ability of the essential oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control are the most likely reasons for its lethal action.

Antimicrobial activity of extracts. The antibacterial activity of the different extracts of *J. phoenicea* (methanol, ethanol, ethyl acetate, and dichloromethane) was determined against 9 microbial strains, considered as among the common foodborne microorganisms. The inhibition zone, measured in millimeters, including the diameter of the well, was used as the criterion for measuring the antimicrobial activity of *J. phoenicea* extracts. Table 4 shows that the tested extracts could be classified according to their activity. The diameter of inhibition zones was obtained in the range of 11 to 34 mm. Methanol extracts of leaves and berries showed the highest activity. For Gram-positive, the most sensitive microorganism was the *B. subtilis* followed by *S. aureus* while *L. monocytogenes* was proved resistant against all samples. On the other hand, the most sensitive bacteria in Gram-negative was *P. aeruginosa* with a 23 mm inhibition obtained with ethyl acetate extract berries. *K. pneumoniae* and *E. coli* are completely resistant against the different samples. Our results are in agreement with the those of Hayouni and others (2007) when *E. coli* and *L. monocytogenes* were not inhibited by acetone extracts of *J. phoenicea* fruits. In the case of fungi, the highest activity of ethanol extract berries was against *M. ramamnianus* (22 mm), while all extracts were inactive against *A. westerdijkiae*. Finally, dichloromethane extracts had the lowest activity compared to all extracts (Table 4); this activity was due to compounds that presented a low polarity of secondary metabolites of *J. phoenicea*.

These data indicate that Gram-positive bacteria are the most sensitive tested strain to the different extracts. The tendency of polyphenols, flavonoids, tannins, and anthocyanins could be explained by that, the structures of cell envelope, including cytoplasmic membrane and cell-wall component, are different between Gram-positive and Gram-negative bacteria. Gram-negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering. Without outer membrane, the cell wall of Gram-positive bacteria can be permeated more easily and can disturb the cytoplasmic membrane, disrupt the proton motive force, electron flow, active transport, and coagulation of cell contents (Burt 2004). Therefore, the structural difference of bacteria plays an important role in their susceptibility.

Furthermore, the content of flavonoids in extracts correlates with their antifungal activity, confirming that flavonoids are likely to contribute to the antifungal activity against *S. cerevisiae* ($r = 0.67$). Similar results were reported for different plants by various studies (Rauha and others 2000; Sohn and others 2004) when they proved that there is a relationship between the content of flavonoids and antifungal activity; flavonoids cloud creased the activity of some extracts of Finnish and Korean plants against *S. cerevisiae*.

Although the concentrations of essential oils and extracts were generally about 10 times higher than those of the standard

Table 5 — Free radical scavenging capacities of essential oils and extracts of *J. phoenicea*.

	Samples	DPPH IC_{50} (mg/L)	ABTS IC_{50} (mg/L)
Essential oils	Leaves	5364 ± 121	189 ± 5
	Berries	14716 ± 411	87 ± 3
Leaves extracts	Ethyl acetate	273 ± 4	30 ± 1
	Dichloromethane	1052 ± 12	16.0 ± 0.5
	Ethanol	49.1 ± 0.6	9.1 ± 0.3
	Methanol	8.5 ± 0.3	6.5 ± 0.3
Berries extracts	Ethyl acetate	642 ± 7	35 ± 1
	Dichloromethane	2120 ± 24	131 ± 4
	Ethanol	54 ± 1	100 ± 4
	Methanol	664 ± 7	81 ± 4
Reference standard	Vitamin C	4.4 ± 0.2	1.9 ± 0.1

Standard deviations (SD) did not exceed 5%.

antibiotics, they showed marked antibacterial and antifungal activities. This difference can be explained of the fact that the active components in essential oils and extracts comprise only an active fraction. Therefore, the concentration of the active components could be much lower than the standard antibiotics used. It is important to note that, if the active components were isolated and purified, they would probably show higher antimicrobial activity.

Antioxidant activity

Antioxidant activity of essential oils and subfractions of the various extracts of *J. phoenicea* leaves and berries has been determined by 2 different test systems, namely, DPPH and ABTS assays. All of the data are presented in Table 5.

DPPH assay. In the present study, radical-scavenging activity by DPPH was exhibited by essential oils of leaves and berries (IC_{50} value 5364 ± 121 and 14716 ± 411 mg/L, respectively). The low reactivity was explained by the high concentration of terpenes hydrocarbons of essential oils (91.0% for leaves and 98.3% for berries).

For extracts, antioxidant activity of leaves methanol extract was superior to all samples tested with an IC_{50} value of 8.5 ± 0.3 mg/L, followed by leaves ethanolic extract (49.1 ± 0.6 mg/L). On the other hand, the leaves extracts prepared with ethyl acetate or dichloromethane have exhibited moderate radical-scavenging activity (273 ± 4 mg/L and 1052 ± 12 mg/L, respectively). For the berries, the major activity was detected in ethanol extract (54 ± 1 mg/L) followed by ethyl acetate and methanolic (642 ± 7 and 664 ± 7 mg/L, respectively). We can deduce that the antioxidant activity of *J. phoenicea* leaves (the essential oil and extracts) was better than the essential oil and extracts of berries. Methanol leaves extract showed a comparable activity to vitamin C (4.4 ± 0.2 mg/L). As can be seen in Table 5 that extracts prepared by different solvents exhibited varying degrees of antioxidant activity; this change in solvent polarity alters its ability to dissolve a group of antioxidant compounds and influences activity estimation (Hayouni and others 2007).

ABTS assay. According ABTS assay, the essential oil of berries has a more important activity than leaves (IC_{50} = 87 ± 3 and 189 ± 5 mg/L, respectively). This result was opposite with the DPPH assay. In fact, this important activity compared to other samples was due to the specificity of ABTS radical cation.

For extracts, methanolic leaves presented a good antioxidant activity value, proving its capacity to scavenge the ABTS. However, compared with values of DPPH activity, those of ABTS assay exhibited more antioxidant activity whatever the extracting solvent.

We observed that the content of phenolics in the extracts correlates with their antioxidant activity, the correlation coefficients (r) between data of DPPH and ABTS assays and total phenolic compounds are 0.70 and 0.71, respectively. This confirms that phenolic compounds contribute to the radical scavenging activity of *J. phoenicea* extracts. In general, these properties depend on the ability to donate hydrogen or electron to a free radical. In *Juniperus*, a similar correlation was reported by Djeridane and others (2006) who found $r = 0.79$ between antioxidant activity and total phenolics but in 70% (v/v) aqueous ethanol extract of *J. oxycedrus*.

The highest correlation was detected between tannins against ABTS assay with $r = 0.76$. Falleh and others (2008) found the same behavior between tannins and DPPH assay ($r = 0.96$) in their study of phenolic compounds of *Cynara cardunculus* L. In the best of our knowledge, our study is the 1st research inducing the relationship between tannins and ABTS antioxidant assay. The high correlation explains the important antioxidant power of tannins of Tunisian *J. phoenicea* extracts.

The results indicated that methanol extract presented higher total phenolic quantity (217 ± 2 g GAE/kg of dry material) between the different extracts. Phenolic compounds, biologically active components, are the main agents that can donate hydrogen to free radicals. Essential oils had a weak antioxidant activity. The results indicated that methanolic extracts were effective antioxidants in different *in vitro* assays including DPPH radical scavenging and ABTS activity. Besides, ethanolic leaves extract also exhibited strong scavenging effect on DPPH free radical.

Conclusions

The structure/antioxidant activity relationship evaluation of these compounds suggested that the number of hydroxyl groups was the most important factor in determining the antioxidant activity of the phenolic compounds. Therefore, from the above-mentioned results, we can conclude that essential oils and methanolic extracts derived from *J. phoenicea* leaves and berries could be considered as potential alternatives for synthetic bactericides and natural antioxidants for use in the food industry along with their possible applications in the pharmaceutical industry for the prevention or treatment caused by microorganisms and free radicals.

Further studies are necessary to assess the methanolic extraction of leaves of the compounds responsible for the antioxidant activity which could have far more capacity effective than vitamin C and might be used as a new antioxidant and alternative to synthetic antioxidants.

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