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RESEARCH ARTICLE

Antioxidant and antimicrobial activities of branches extracts of five *Juniperus* species from Turkey

Maria Fernanda Taviano[1], Andreana Marino[1], Ada Trovato[1], Valentina Bellinghieri[1], Tommaso Massimo La Barbera[1], Ayşegül Güvenç[2], Muhammed Mesud Hürkul[2], Rita De Pasquale[1], and Natalizia Miceli[1]

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Abstract

Context: Several *Juniperus* species (Cupressaceae) are utilized in folk medicine in the treatment of infections and skin diseases.

Objective: This work was designed to evaluate the antioxidant and antimicrobial potential of methanol and water branches extracts of *Juniperus* species from Turkey: *Juniperus communis* L. var. *communis* (*Jcc*), *Juniperus communis* L. var. *saxatilis* Pall. (*Jcs*), *Juniperus drupacea* Labill. (*Jd*), *Juniperus oxycedrus* L. subsp. *oxycedrus* (*Joo*), *Juniperus oxycedrus* L. subsp. *macrocarpa* (Sibth. & Sm.) Ball. (*Jom*).

Materials and methods: Total phenolics, total flavonoids and condensed tannins were spectrophotometrically determined. The antioxidant properties were examined using different *in vitro* systems. The toxicity was assayed by *Artemia salina* lethality test. The antimicrobial potential against bacteria and yeasts was evaluated using minimum inhibitory concentration and minimum bactericidal concentration (MIC/MBC) measurements. The effect on bacteria biofilms was tested by microtiter plate assay.

Results: Both in the DPPH (1,1-diphenyl-2-picrylhydrazyl) and TBA (thiobarbituric acid) test *Jom* resulted the most active (IC_{50} = 0.034 ± 0.002 mg/mL and 0.287 ± 0.166 µg/mL). *Joo* exhibited the highest reducing power (1.78 ± 0.04 ASE/mL) and Fe^{2+} chelating activity (IC_{50} = 0.537 ± 0.006 mg/mL). A positive correlation between primary antioxidant activity and phenolic content was found. The extracts were potentially non-toxic against *Artemia salina*. They showed the best antimicrobial (MIC = 4.88-30.10 µg/mL) and anti-biofilm activity (60–84%) against *S. aureus*.

Discussion and conclusion: The results give a scientific basis to the traditional utilization of these *Juniperus* species, also demonstrating their potential as sources of natural antioxidant and antimicrobial compounds.

Keywords: *Juniperus*, antioxidant activity, antimicrobial activity

Introduction

Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Kumaran & Karunakaran, 2007). Among the isolated compounds, polyphenols represent the main class of natural antioxidants. Phenolic compounds act as antioxidants in several ways; they are radical scavengers because phenolic groups are excellent nucleophiles and are also able to inhibit lipid peroxidation, acting as breakers of oxidation reaction by binding with free radicals generated through lipid peroxidation; in addition, they could act as chelators of metal ions that induce oxidation (Han & Baik, 2008).

Currently, multiple drug resistance in human pathogenic microorganisms have developed because of the indiscriminate employment of commercial antimicrobial drugs commonly used in the treatment of infectious diseases; for this reason, several studies were carried

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out in order to identify new antimicrobial substances from various sources, like plants, utilized in traditional medicine for several thousand years. Plants are rich in a wide variety of secondary metabolites with antimicrobial properties; polyphenols are well documented to have microbicide activity against a huge number of pathogenic bacteria (Cowan, 1999).

Based on these statements, several researchers are focused on the antioxidant and antimicrobial properties of plant-derived natural products.

The use of traditional medicinal plants for primary health care has steadily increased worldwide in recent years. *Juniperus* genus (Cupressaceae) comprises about 60 monoecious or dioecious evergreen species, trees or shrubs distributed throughout the Northern hemisphere (Welch, 1986). The genus is usually divided into three distinct sections or subgenera: *Juniperus* (syn: *Oxycedrus*, 9 or 10 species), *Caryocedrus* (one species, *J. drupacea* Labill.), and *Sabina* (~50 species) (Adams, 1998). However, concerning this genus, Flora of Turkey reports only two sections, *Juniperus* and *Sabina*, and *J. drupacea* is included in *Juniperus* section (Coode and Cullen, 1965).

Numerous *Juniperus* species are utilized in folk medicine either for humans or domestic animals. *J. communis* L., common juniper, is an aromatic shrub or tree widespread in Europe, Asia and North America. According to the Flora of Turkey, *J. communis* includes two varieties, var. *communis* and var. *saxatilis* Pall. (Farjon, 2000). In folk and official medicine, *J. communis* represents a drug with diuretic, antiseptic, stomachic and carminative properties (Stanić et al., 1998). Decoction of branch with berries is used to relieve kidney infections (Ritch-Krc et al., 1996).

J. oxycedrus L. Is a shrub or small tree growing wild in stony places of the Mediterranean and Near East countries. According to the Flora of Turkey, *J. oxycedrus* L. comprises two subspecies: subsp. *oxycedrus* and subsp. *macrocarpa* (Sibth. & Sm.) Ball. (Farjon, 2000). *J. oxycedrus* is used to prepare empyreumatic oil by destructive distillation of the branches and wood, the so-called oil of Cade, which is widely employed in human and veterinary dermatology (Karaman et al., 2003). Moreover, *J. oxycedrus* is used in folk medicine for the treatment of various ailments, such as hyperglycaemia, obesity, tuberculosis, bronchitis and pneumonia; leaves and fruits are applied externally for parasitic disease (Loizzo et al., 2007; Sezik et al., 1997). Leaves, resin, bark and fruit extracts of *J. oxycedrus* were found to inhibit the growth of numerous microorganisms (Karaman et al., 2003). *J. drupacea* has a limited distribution in the Eastern Mediterranean region. In Turkish folk medicine, *J. drupacea* is utilized as anthelminthic (Yesilada et al., 1993).

This work was designed to make a comparative study of methanol and water extracts of branches of five *Juniperus* species belonging to *Juniperus* section collected from Turkey. Total phenolics, total flavonoids, and condensed tannins were determined. The antioxidant properties of *Juniperus* spp. extracts were examined in different *in vitro* systems and correlated to the phenolic content. In order to have a preliminary value of the toxicity of the extracts, *Artemia salina* lethality test was performed. The antimicrobial potential of *Juniperus* spp. extracts against bacteria and yeasts was evaluated; further, the effect on bacteria biofilms was assessed.

Materials and methods

Chemicals

Tryptone Soya Broth (TSB), Sabouraud Dextrose Agar (SDA), Müeller Hinton Broth (MHB), and Müeller Hinton Agar (MHA) were supplied from Oxoid (Basingstoke, UK). NaOH, HCl, and *n*-butanol were purchased from Merck Chemicals (Milan, Italy). $FeCl_2$ was obtained from Carlo Erba (Milan, Italy). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Plant material and extraction

The branches of the selected plants, *Juniperus communis* L. var. *communis* (*Jcc*), *Juniperus communis* L. var. saxatilis Pall. (*Jcs*), *Juniperus drupacea* Labill. (*Jd*), *Juniperus oxycedrus* L. subsp. *oxycedrus* (*Joo*), *Juniperus oxycedrus* L. subsp. *macrocarpa* (Sibth. & Sm.) Ball. (*Jom*), were collected in 2004 from different regions of Turkey. The taxonomic identification of the plant materials was confirmed by Prof. Ayşegül Güvenç at the Department of Pharmaceutical Botany, Ankara University, Turkey. Voucher specimens are deposited in the Herbarium (AEF) of the Faculty of Pharmacy, Ankara University, Turkey. Collection sites and herbarium numbers of the samples are listed in Table 1.

The branches were dried at room temperature and reduced to moderately coarse powder. The extracts were prepared as described below.

Methanol extracts: Powdered branches (20 g) were extracted twice with methanol (MeOH) (200 mL) at 50°C, under continuous shaking (700 rpm/min), for 8 h. The filtrates of each extract were combined and evaporated to dryness i*n vacuo* at 40°C.

Water extracts: Powdered branches (20 g) were extracted twice with distilled water (200 mL) at 50°C, under continuous shaking (700 rpm/min), for 8 h. The filtrates of each extract were combined and subsequently lyophilized.

The extracts yields (%), referenced to dried branches, are given in Table 1.

Determination of total phenolic content

The total phenolic content of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was measured by the Folin-Ciocalteu reagent (Gao et al., 2000). Each sample solution (100 μL) was mixed with 0.2 mL Folin-Ciocalteu reagent, 2 mL of distilled water, and 1 mL of 15% Na_2CO_3, and the absorbance was measured at 765 nm, after 2-h incubation at room temperature, with a model UV-1601 spectrophotometer (Shimadzu, Milan, Italy). Gallic acid was used as a standard and the total phenolics were expressed as mg gallic

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Table 1. Collection sites and herbarium numbers of the plant samples, and percentage yields of *Juniperus* spp. branches extracts.

Juniperus species	Collection sites	Herbarium numbers	Yields (w/w %)	
			Methanol extracts	Water extracts
Juniperus communis L. *var. communis*	Ağri: Doğubeyazit, Korhan Y.	AEF 23854	6.65	13.76
Juniperus communis L. *var. saxatilis Pall.*	Ankara: Kizilcahamam, işik Dağı	AEF 23801	14.32	18.94
Juniperus drupacea Labill.	Antalya: Antalya-Konya road	AEF 23610	6.40	13.12
Juniperus oxycedrus L. subsp. *oxycedrus*	Ankara: Kizilcahamam, işik Dağı	AEF 23799	11.58	16.20
Juniperus oxycedrus L. subsp. *macrocarpa* (Sibth. & Sm.) Ball.	Izmir: Çeşme, around Çiftlikköy	AEF 23855	9.69	19.23

The yields are referenced to 100 g of dried branches.

acid equivalents (GAE)/g extract (dw) ± standard deviation (SD). The mean value of total phenolic content was obtained from triplicate experiments.

Determination of total flavonoid content

The total flavonoid content of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was measured by using the aluminum chloride colorimetric assay (Chang et al., 2002). An aliquot of appropriately diluted sample solution (0.5 mL) was mixed with 1.5 mL MeOH, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M CH_3COOK and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was spectrophotometrically measured at 415 nm. The amount of 10% $AlCl_3$ was substituted by the same amount of distilled water in blank. Quercetin was used to make the calibration curve and total flavonoids were expressed as mg quercetin equivalents (QE)/g extract (dw) ± SD. The mean value of total flavonoid content was obtained from triplicate experiments.

Determination of condensed tannin content

The condensed tannin content of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was determined according to the method of Julkunen-Titto (1985). An aliquot of each sample solution (50 μL) was mixed 1.5 mL of 4% vanillin in MeOH and then 750 μL of concentrated HCl were added. The well mixed solution was incubated at room temperature in the dark for 20 min; the absorbance of the reaction mixture was spectrophotometrically measured at 500 nm. The amount of 4% vanillin was substituted by the same amount of MeOH in blank. (+)-Catechin was used to make the calibration curve and condensed tannins were expressed as mg catechin equivalents (CE)/g extract (dw) ± SD. The mean value of condensed tannin content was obtained from triplicate experiments.

Antioxidant activity

Free radical scavenging activity

The free radical scavenging activity of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method (Ohnishi et al., 1994). The extracts were tested at different concentrations (0.07, 0.14, 0.21, 0.35, 0.49, and 0.7 mg/mL). An aliquot (0.5 mL) of each sample solution was added to 3 mL of daily prepared methanol DPPH solution (0.1 mM). The optical density change at 517 nm was measured, 20 min after the initial mixing, with a model UV-1601 spectrophotometer (Shimadzu). Butylated hydroxytoluene (BHT) was used as reference standard. The scavenging activity was measured as the decrease in absorbance of the samples vs. DPPH control solution. The radical scavenging activity percentage (%) was calculated by the formula $[(A_o - A_c)/Ao] \times 100$, where A_o is the absorbance of the control and A_c is the absorbance in the presence of the sample or standard. The assays were carried out in triplicate, and the results are expressed as mean 50% inhibitory concentration (IC_{50}) ± SD, calculated by using the Litchfield and Wilcoxon test (1949).

Measurement of reducing power

The reducing power of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was determined according to the method of Oyaizu (1986). Different amounts of each extract (0.07, 0.14, 0.21, 0.35, 0.49, and 0.7 mg/mL) in 1 mL solvent were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. The resulting solution was cooled rapidly, mixed with 2.5 mL of 10% trichloroacetic acid, and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% fresh $FeCl_3$, and the absorbance was measured, after 10 min, at 700 nm; increased absorbance of the reaction mixture indicated increased reducing power. As blank, an equal volume (1 mL) of solvent was mixed with a solution prepared as described above. Ascorbic acid and BHT were used as reference standards. The assays were carried out in triplicate, and the results are expressed as ascorbic acid equivalent (ASE/mL) ± SD (Apáti et al., 2003).

Ferrous ions (Fe^{2+}) chelating activity

The Fe^{2+} chelating activity of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was estimated by the method of Dinis et al. (1994). Briefly, different amounts of each extract (0.07, 0.14, 0.21, 0.35, 0.49, and 0.7 mg/mL) in 0.4 mL solvent were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the total volume was adjusted to 4 mL with MeOH. The mixture was shaken vigorously and left standing at room temperature for 10 min; the absorbance was then measured spectrophotometrically at 562 nm. The

control contains $FeCl_2$ and ferrozine, complex formation molecules.

Ethylenediaminetetraacetic acid (EDTA) was used as reference standard. The percentage of inhibition of the ferrozine-(Fe^{2+}) complex formation was calculated by the formula $[(A_o - A_c)/A_o] \times 100$, where A_o is the absorbance of the control and A_c is the absorbance in the presence of the sample or standard. The assays were carried out in triplicate, and the results are expressed as mean 50% inhibitory concentration (IC_{50}) ± SD (Litchfield & Wilcoxon, 1949).

TBA test

In order to assess the efficacy of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts to protect liposomes from lipid peroxidation thiobarbituric acid (TBA) test was used (Conforti et al., 2002). The extracts were tested at different concentrations (1.625, 3.125, 6.25, 12.5, 25 and 50 μg/mL). Liposomes were prepared from bovine brain extract in phosphate buffered saline (PBS) (5 mg/mL). The reaction mixture (ET) consisted of 0.2 mL of liposomes, 0.1 mL of aqueous $FeCl_3$ (1 mM), 0.1 mL of aqueous ascorbic acid (1 mM), 0.5 mL of PBS, and 0.1 mL of the sample solution. After incubation at 37°C for 20 min, the TBA test was performed by adding 0.1 mL of 2% BHT in ethanol followed by 0.5 mL of 1% w/v TBA in 50 mM NaOH and 0.5 mL of 25% HCl. The tubes were heated at 90°C for 30 min. After cooling, chromogens were extracted with 2.5 mL of *n*-butanol. The mixture was vortexed to ensure complete extraction of the chromogen and then centrifuged at 3500 rpm for 15 min at room temperature.

The absorbance of the malondialdehyde (MDA)-TBA complex in the upper layer was determined spectrophotometrically at 532 nm. The percentage of lipid peroxidation inhibition was assessed by using the following formula: $[(FRM - B) - (ET - B - EA)/(FRM - B)] \times 100$, where FRM is the absorbance of the control reaction and ET is absorbance in the presence of the sample. The absorbance of liposomes alone (B) and extract alone (EA) was also taken into account. Propyl gallate was used as reference compound. The assays were carried out in triplicate, and the results are expressed as mean 50% inhibitory concentration (IC_{50}) ± SD (Litchfield & Wilcoxon, 1949).

Artemia salina lethality test

Medium lethal concentration (LC_{50}) determination was carried out according to the method of Meyer et al. (1982). For the determination different amounts of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts, opportunely dissolved and then diluted in artificial seawater, were transferred to vials to obtain 10, 100, 500 and 1000 μg/mL final concentrations. Ten brine shrimp larvae (*Artemia salina* Leach), taken 48 h after initiation of hatching in artificial seawater, were transferred to each sample vial, and artificial seawater was added to obtain a final volume of 5 mL. After 24 h of incubation at 25–28°C, the vials were observed using a magnifying glass, and surviving larvae were counted. The assay was carried out in triplicate. LC_{50} was determined using the probit analysis method described by Finney (1971). *Juniperus* spp. extracts dilutions that did not show cytotoxicity at 1000 μg/mL were considered non-toxic.

Antimicrobial activity

The following strains were used as indicators for antimicrobial testing and were obtained from the in-house culture collection of Pharmaco-Biological Department (University of Messina, Italy): *Staphylococcus aureus* strain ATCC 6538P, *Staphylococcus epidermidis* G1, *Enterococcus hirae* V3, *Bacillus subtilis* P3, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9021, *Proteus mirabilis* G4 were grown at 37°C in TSB; *Candida albicans* ATCC10231 and *Candida parapsilosis* P7 were grown at 35°C on SDA.

The minimum inhibitory concentrations (MICs) of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts were determined in MHB using a broth dilution micromethod in polystyrene microtitre plates according to the National Committee for Clinical Laboratory Standards (NCCLS, 2008). The microplates were maintained under shaking conditions to prevent sedimentation and to increase aeration. The final concentrations of the extracts adopted to evaluate the antibacterial activity were 1.22–2500 μg/mL. Ofloxacin (range from 0.2 to 0.3 μg/mL) and tetracycline (range from 2 to 8 μg/mL) were used as positive reference standard drugs. Two growth controls consisting of MHB medium and MHBMeOH were included. Microbial growth was determined by visual readings and spectrophotometric values (550 nm) using the Microplate Reader, Model 550 (BIO-RAD Laboratories Milano, Italy). The MIC was considered as the lowest concentration of each extract at which there was no microbial growth after 24 h of incubation.

As an indicator of bacterial growth, 20 μL of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) dissolved in water was added to the wells and incubated at 37°C for 30 min. Where bacterial growth was inhibited, the solution in the well remained clear after incubation with TTC. The minimum bactericidal concentrations (MBCs) were determined by seeding 20 μL from all clear MIC wells onto MHA plates (NCCLS, 2008). The MBC was defined as the lowest extract concentration that killed 99.9% of the final inoculums after 24 h of incubation. The data from at least three replicates were evaluated and modal results were calculated.

The MICs of *Juniperus* spp. extracts on yeasts were determined in RPMI1640 buffered to a pH of 7.0 with 3-(*N*-morpholino) propanesulfonic acid (MOPS) according to National Committee for Clinical Laboratory Standards (NCCLS, 2002). The microplates were maintained under shaking conditions to prevent sedimentation and to increase aeration. The final concentrations of the extracts adopted to evaluate the activity were 1.22–2500 μg/mL. Amphotericin B (0.15 μg/mL) was used as positive reference standard drug. Two growth controls consisting of RPMI 1640 medium and RPMI1640MeOH were included.

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Microbial growth was determined by visual readings and spectrophotometric values (550 nm) using the microplate reader (BIO-RAD). The MIC was considered as the lowest concentration of each extract at which there was no microbial growth after 24–48 h of incubation.

The minimum fungicidal concentrations (MFCs) were determined by seeding 20 μL from all clear MIC wells onto SDA plates (NCCLS, 2002). The MFC was defined as the lowest extract concentration that killed 99.9% of the final inoculums after 24–48 h of incubation. The data from at least three replicates were evaluated and modal results were calculated.

The effect of branches extracts on biofilm formation was evaluated as described by Cramton et al. (1999) with some modifications. Overnight culture in 10 mL 1% glucose (TSBG) glucose was diluted to standardise suspension of each strain 1×10^6 CFU mL. Aliquots of 100 μL were dispensed into each well of sterile flat-bottom 96-well polystyrene microtiter plates (Corning Inc., Corning, NY) in the presence of 100 μL subinhibitory concentration (0.5 MIC) of each *Juniperus* extract or 100 μL medium (control). The final volume in each well was 200 μL. The microtiter plates were incubated for 24 h at 37°C under shaking conditions to prevent extract sedimentations and to increase aeration. Two biofilm controls consisting of TSBG medium and TSBGMeOH were included. The medium was then aspirated and the wells, rinsed twice with PBS, were fixed by drying for 1 h. Once the wells were fully dry, 200 μL of 0.1% safranin were added to the wells for 2 min. The contents of the wells were then aspirated and after rinsing with water, 200 μL of acetic acid 30% (v/v) were added to the wells for spectrophotometric analysis ($OD_{492\,nm}$).

The results were derived from three separate experiments. The $OD_{492\,nm}$ value obtained without extracts was used as the control. The reduction percentage of biofilm formation in the presence of different *Juniperus* spp. extracts was calculated employing the ratio between the values of $OD_{492\,nm}$ with and without extracts, adopting the following formula: [100 – ($OD_{492\,nm}$ with extract/$OD_{492\,nm}$ without extract) × 100]. Data were expressed as mean values ± SD.

Results and discussion

Total phenolic content

Phenolic compounds are called high level antioxidants because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide free radicals and hydroxyl radicals (Jayabalan et al., 2008). The antioxidant activities of plant extracts are often explained with their total phenolic content with good correlation; therefore, it is worthwhile to determine their total amount in the extracts utilized for the study.

The estimation of phenolic content of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* branches extracts was done using Folin-Ciocalteu phenol reagent; the total phenolics were found to be higher in methanol extracts respect to water extracts. Among methanol extracts, total phenolic content varied from 170.43 ± 2.13 mg GAE/g extract (*Jcc*) to 253.29 ± 3.16 mg GAE/g extract (*Joo*), and it decreased in the order: *Joo* > *Jom* > *Jcs* > *Jd* > *Jcc*. Among water extracts, the amount of total phenolics varied from 98.74 ± 0.49 mg GAE/g extract (*Jd*) to 212.88 ± 2.95 mg GAE/g extract (*Jom*), and it decreased in the order: *Jom* > *Jcs* > *Joo* > *Jcc* > *Jd* (Table 2).

Total flavonoid content

Total flavonoid content was found to be higher in water extracts respect to methanol extracts. Among methanol extracts, the flavonoid content ranged from 6.60 ± 0.08 mg QE/g extract (*Jom*) to 14.44 ± 1.05 mg QE/g extract (*Jcc*), and it decreased in the order: *Jcc* > *Jcs* > *Joo* > *Jd* > *Jom*. Among water extracts, total flavonoids varied from 10.50 ± 0.43 mg QE/g extract (*Jcs*), to 26.70 ± 0.50 mg QE/g extract (*Jcc*); the total flavonoid content decreased in the order: *Jcc* > *Jd* > *Joo* > *Jom* > *Jcs* (Table 2).

Condensed tannin content

Vanillin-concentrated HCl test is a quite reproducible and sensitive assay for the estimation of flavanols, including catechins and proanthocyanidins (Julkunen-Titto, 1985). The results revealed that, among phenolic compounds, *Juniperus* spp. branches extracts contained remarkable levels of condensed tannins, which were found to be higher in methanol extracts respect to water extracts. Among methanol extracts, the tannin content ranged from 125.34 ± 3.35 mg CE/g extract (*Joo*) to 188.44 ± 12.21 mg CE/g extract (*Jcs*), and it decreased in the order: *Jcs* > *Jom* > *Jcc* > *Jd* > *Joo*. Among water extracts, the amount of condensed tannins varied from 51.22 ± 1.14 mg CE/g extract (*Jd*) to 155.03 ± 7.88 mg CE/g extract (*Jom*), and it decreased in the order: *Jom* > *Jcs* > *Jcc* > *Joo* > *Jd* (Table 2).

Table 2. Quantitative determination of total phenolics (calculated as gallic acid), total Flavonoids (calculated as quercetin), and Condensed Tannins (calculated as catechin), of *Juniperus* spp. branches extracts.

	Total Phenolics mg GAE/g extract (dw)		Total Flavonoids mg QE/g extract (dw)		Condensed Tannins mg CE/g extract (dw)	
Juniperus species	Methanol extracts	Water extracts	Methanol extracts	Water extracts	Methanol extracts	Water extracts
Jcc	170.43 ± 2.13	149.88 ± 2.67	14.44 ± 1.05	26.70 ± 0.50	138.54 ± 4.40	103.10 ± 3.37
Jcs	196.65 ± 3.41	156.00 ± 1.50	10.63 ± 0.77	10.50 ± 0.43	188.44 ± 12.21	107.37 ± 0.11
Jd	184.23 ± 4.33	98.74 ± 0.49	9.76 ± 0.44	15.70 ± 0.04	128.23 ± 1.94	51.22 ± 1.14
Joo	253.29 ± 3.16	151.06 ± 2.00	9.79 ± 0.39	12.18 ± 0.37	125.34 ± 3.35	85.28 ± 1.09
Jom	227.06 ± 4.66	212.88 ± 2.95	6.60 ± 0.08	11.41 ± 0.27	163.60 ± 8.16	155.03 ± 7.88

Values are expressed as the mean ± SD ($n = 3$).

Antioxidant activity

Antioxidant activity occurs by different mechanisms, such as prevention of chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts (Bounatirou et al., 2007). It is thus important to evaluate the effectiveness of antioxidants by using several analytical methods and different substrates.

It is known that the antioxidant activity can be primary and secondary: primary antioxidants scavenge radicals to inhibit chain initiation and break chain propagation; secondary antioxidants suppress the formation of radicals and protect against oxidative damage (Miceli et al., 2009). In this study, we evaluated primary antioxidant properties by the DPPH test, which measures the ability of the extract to donate hydrogen to the radical, and reducing power assay, which measures the ability of the extract to donate electron to Fe (III); secondary antioxidant ability was assayed by Ferrous ions chelating activity. Moreover, TBA test was carried out in order to evaluate the anti-lipid peroxidation activity of the extracts.

Antioxidant properties, particularly radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological systems. In this study, the free radical scavenging activity of *Juniperus* spp. branches extracts was determined using the DPPH test. The method has been used extensively to predict antioxidant activities of single compounds, as well as of different plant extracts because of the relatively short time required for the analysis; in addition, it is reproducible and strongly correlated with phenolic compounds (Maisuthisakul et al., 2008).

The results show that the extracts have a noticeable effect on scavenging free radicals; moreover, methanol extracts of all *Juniperus* species showed stronger DPPH scavenging activity than water extracts, with the exception of *Jom*. Among methanol extracts, *Joo* exhibited the highest scavenging effect ($IC_{50} = 0.046 \pm 0.004$ mg/mL); the activity of methanol extracts was stronger than that of BHT, except for *Jcc*, and it decreased in the order: *Joo* > *Jom* > *Jcs* > *Jd* > BHT > *Jcc*. Among water extracts, the highest activity was observed in *Jom* ($IC_{50} = 0.034 \pm 0.003$ mg/mL); in addition, the scavenging effect of water extracts and BHT on DPPH radical decreased in the order: *Jom* > *Jcs* > BHT > *Joo* > *Jcc* > *Jd* (Table 3). Finally, a positive correlation was found between total phenolic content and DPPH radical scavenging activity both in methanol ($r^2 = 0.8303$) and water extracts ($r^2 = 0.7154$).

Different studies have indicated that the electron donation capacity, reflecting the reducing power, of bioactive compounds is associated with antioxidant activity (Siddhuraju et al., 2002). The reductive capabilities of *Juniperus* spp. extracts were evaluated by reducing power assay. All methanol extracts exhibited stronger reducing power than water extracts; so, it could be assumed that methanol extracts contain higher amounts of reductants, which could react with free radicals to convert them to more stable products and block radical chain reaction. Moreover, like the radical scavenging activity, the reducing power of *Joo* was the highest among methanol extracts (1.781 ± 0.040 ASE/mL), and the reducing power of *Jom* was the highest among water extracts (3.486 ± 0.010 ASE/mL). The reducing power of methanol extracts and standard decreased in the following order: BHT > *Joo* > *Jom* > *Jcs* > *Jd* > *Jcc*. The reducing power of water extracts and BHT decreased in the order: BHT > *Jom* > *Jcs* > *Jcc* > *Joo* > *Jd* (Table 3). A strong positive correlation was found between phenolic content and reducing power both in methanol ($r^2 = 0.8004$) and water extracts ($r^2 = 0.9244$).

Among the transition metals, iron is known as the most powerful lipid oxidation pro-oxidant due to its high reactivity (Gülçin, 2006). Minimizing ferrous ions may afford protection against oxidative damage within living organism by inhibiting production of ROS and lipid peroxidation. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidized form of the ions (Kumaran & Karunakaran, 2007). Hence, the ability of substances to chelate iron can be a valuable method of antioxidant capability. The results of the Ferrous ions (Fe^{2+}) chelating activity show that both methanol and water extracts interfered with the complex formation, suggesting that they have chelating activity and are able to capture ferrous ions before ferrozine. In addition, water extracts of all *Juniperus* species showed a more marked capacity for iron binding than methanol ones.

Table 3. Free radical scavenging activity (DPPH test), reducing power, ferrous ions (Fe^{2+}) chelating activity, and anti-lipid peroxidation activity (TBA assay) of *Juniperus* spp. branches extracts.

	DPPH test IC_{50} (mg/mL)		Reducing power assay (ASE/mL)		Fe^{2+} chelating activity IC_{50} (mg/mL)		TBA test IC_{50} (μg/mL)	
Juniperus species	Methanol extracts	Water extracts	Methanol extracts	Water extracts	Methanol extracts	Water extracts	Methanol extracts	Water extracts
Jcc	0.164 ± 0.005	0.186 ± 0.004	3.564 ± 0.142	4.907 ± 0.228	33.109 ± 0.401	3.530 ± 0.254	3.476 ± 1.188	12.181 ± 3.118
Jcs	0.079 ± 0.004	0.132 ± 0.004	3.421 ± 0.094	4.773 ± 0.039	30.548 ± 0.480	3.467 ± 0.003	3.201 ± 0.156	12.060 ± 2.131
Jd	0.120 ± 0.002	0.898 ± 0.052	3.524 ± 0.102	6.789 ± 0.238	4.075 ± 1.322	1.004 ± 0.001	4.356 ± 2.159	38.886 ± 1.871
Joo	0.046 ± 0.004	0.136 ± 0.002	1.781 ± 0.040	5.702 ± 0.056	6.829 ± 1.159	0.537 ± 0.006	0.349 ± 1.210	6.759 ± 1.238
Jom	0.054 ± 0.003	0.034 ± 0.003	3.158 ± 0.098	3.486 ± 0.010	1.408 ± 0.240	0.715 ± 0.007	0.287 ± 0.166	6.302 ± 0.586
Standard	BHT (0.134 ± 0.001)		BHT (1.754 ± 0.049)		EDTA (0.009 ± 0.001)		Propyl gallate (0.179 ± 0.402)	

Values are expressed as the mean ± SD ($n = 3$).

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Among methanol extracts *Jom* exhibited the highest activity (IC_{50} = 1.408 ± 0.240 mg/mL); the metal chelating effect of methanol extracts and EDTA decreased in the order: EDTA >*Jom* > *Jd* > *Joo* > *Jcs* > *Jcc*. Among water extracts *Joo* resulted the most active one (IC_{50} = 0.537 ± 0.006 mg/mL); the activity of water extracts and standard decreased in the order: EDTA > *Joo* > *Jom* > *Jd* > *Jcs* > *Jcc* (Table 3). No correlation between metal chelating activity and total phenolic content was found.

Lipid peroxidation can inactivate cellular components that play a major role in oxidative stress in biological systems. Further, several toxic byproducts of the peroxidation can damage other biomolecules including DNA away from the site of their generation. Therefore, compounds possessing anti-lipid peroxidation activity are really important for health benefits and food preservation (Rathee et al., 2006). The results of the determination of anti-lipid peroxidation activity show that methanol extracts of all *Juniperus* species have a more marked effect than water extracts. Among methanol extracts *Jom* exhibited the highest anti-lipid peroxidation ability (IC_{50} = 0.287 ± 0.166 μg/mL); the activity of methanol extracts and standard decreased in the order: propyl gallate > *Jom* > *Joo* > *Jcs* > *Jcc* > *Jd*. Among water extracts, the highest effect was observed in *Jom*, too (IC_{50} = 6.302 ± 0.586 μg/mL); the activity of water extracts and standard decreased in the order: propyl gallate > *Jom* > *Joo* > *Jcs* > *Jcc* > *Jd* (Table 3). In addition, a positive correlation was found between total phenolic content and anti-lipid peroxidation activity both in methanol (r^2 = 0.8208) and water extracts (r^2 = 0.6786).

On the basis of the results obtained, it is evident that *Juniperus* spp. branches extracts can act both as primary and secondary antioxidants.

In this study, a positive correlation between primary antioxidant activity and total phenolic content was found, as indicated by the coefficient of determination (r^2). In addition, it was observed that methanol extracts, containing higher amounts of total phenolics, exhibited stronger primary antioxidant properties than water extracts. The chemical composition of *Juniperus* extracts indicates the presence of flavonoids and condensed tannins. Extensive investigations on antiradical and antioxidant activities of small phenolics, including flavonoids and phenolic acids, have been reported; moreover high molecular weight phenolics (tannins) have shown ability to quench free radical and their effectiveness depends on the molecular weight, the number of aromatic rings, and nature of hydroxyl groups substitution and the specific functional groups (Siddhuraju & Manian, 2007). Hence, it can be hypothesized that primary antioxidant activity is related to the synergic effect of these phenolic compounds.

With regard to secondary antioxidant properties, our results show that *Juniperus* spp. extracts may offer protection against oxidative damage through their chelating properties. However, it should be pointed out that there was not correlation between metal chelating activity and total phenolic content. Besides, water extracts, with lower phenolic content, displayed greater activity than methanol ones. It is known that the extracts are very complex mixtures of many different compounds and that non-phenolic antioxidants can contribute to their antioxidant activity; therefore, it can be speculated that the observed activity could depend on other phytochemicals that are extracted together with phenolics.

The results of the determination of anti-lipid peroxidation activity are positively correlated to the phenolic content of the extracts. In this test methanol extracts, containing higher levels of phenolic compounds, resulted more active than water extracts. Most lipid oxidation inhibition assays measure a combination of transition metal (usually iron) chelation and radical scavenging properties (Jayabalan et al., 2008). On the basis of the obtained results, it can be hypothesized that the anti-lipid peroxidation activity of *Juniperus* spp. extracts is related mainly to the radical scavenging properties of phenolic compounds.

Artemia salina lethality test

The brine shrimp larva (*Artemia salina*) is an invertebrate used in the alternative lethality bioassay, that is considered a useful tool for preliminary assessment of toxicity; it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, and pesticides. This bioassay has the advantage of being rapid, inexpensive, and simple (Krishnaraju et al., 2005).

The results of the assay show that all *Juniperus* spp. branches extracts did not display any toxicity against brine shrimps (LC_{50} > 1000 μg/mL), except for *Jcs* methanol extract, which showed a weak toxicity (LC_{50} = 658.6 μg/mL).

Antimicrobial activity

The branches extracts of *Juniperus* spp. were tested at various concentrations (1.22–2500.00 μg/mL) and the MIC values ≤ 250.00 μg/mL are reported in Table 4A. *Juniperus* spp. extracts showed moderate to good inhibitory activity against Gram-positive bacteria only. The most sensitive species was *S. aureus* followed by *S. epidermidis* and *E. hirae*, whereas *B. subtilis* was the less sensitive to extracts. The inhibitory effect of extracts against all the tested strains was more bacteriostatic than bactericidal within the concentrations range tested.

Particularly, *Jcc* showed bactericidal activity against *S. epidermidis* (with MBC 78.12 μg/mL), *Jcc* and *Jcs* vs. *S. aureus* (with MBC 1250.00 μg/mL), *Jd* (with MBC 78.12 μg/mL) and *Joo* and *Jom* (with MBC 312.50 μg/mL) against *E. hirae*, *Jom* vs. *B. subtilis* (with MBC 2500.00 μg/mL). MIC and MBC concentrations (78.00 μg/mL) of *Jcc* and *Jd* methanol extracts showed equivalent effect against *S. epidermidis* and *E. hirae* respectively. Among the extracts, *Jd* (with MIC 9.76 and MBC 625.00 μg/mL) and *Jom* (with MIC 4.88 and MBC 625.00 μg/mL) methanol and water extracts, respectively, were the most

Table 4. MIC values (A) and percentage of biofilm reduction (B) of *Juniperus* spp. branches extracts.

	Methanol extracts					Water extracts					
Microorganisms	*Jcc*	*Jcs*	*Jd*	*Joo*	*Jom*	*Jcc*	*Jcs*	*Jd*	*Joo*	*Jom*	Sd
A[a]	MIC (μg/mL)										
Gram-positive											
**S. aureus* ATCC 6538P	19.53	19.53	9.76	19.53	19.53	19.53	19.53	39.10	19.53	4.88	0.3[†]
S. epidermidis G1	78.12	156.25	156.25	156.25	156.25	78.12	78.12	156.25	156.25	78.12	0.3[†]
E. hirae V3	156.25	156.25	78.12	156.25	156.25	>250.00	>250.00	>250.00	>250.00	>250.00	0.2[†]
B[b]	Biofilm reduction %										
**S. aureus* ATCC 6538P	82	81	78	84	81	68	66	70	78	60	

[a]MIC values ≤250.00 μg/mL are reported.
[b]Strains resistant to anti-biofilm effect of the extracts are not shown.
Standard drug (Sd): [†]ofloxacin.

effective against *S. aureus*. The MIC values of extracts against *B. subtilis* were >250.00 μg/mL. No antimicrobial activity vs. Gram-negative strains and yeasts was observed. The results of negative controls containing methanol (maximum 1% v/v) indicated the complete absence of inhibition of all the strains tested (data not shown).

In Table 4B the percentage of biofilm reduction is reported. Subinhibitory extract concentrations (0.5 MIC) demonstrated a good inhibiting activity on *S. aureus* biofilm formation (with reduction of 60–84%). Particularly, the mean biofilms quantified by this method for *S. aureus* treated with methanol extracts was 0.15 ± 0.03 with a range of 0.11 ± 0.02 to 0.20 ± 0.06, whereas for the strain treated with water extracts the mean biofilms was 0.09 ± 0.01 with a range of 0.08 ± 0.01 to 0.11 ± 0.02. The *S. aureus* control value was 0.55 ± 0.03. No inhibition of biofilm formation has been recorded vs. other strains.

These results highlight the best activity of the extracts both on growth and biofilm formation of *S. aureus* compared to other tested microorganisms. As demonstrated in our results *Juniperus* spp. branches extracts contain phenolic compounds including flavonoids and condensed tannins. The major compound group believed to be responsible for the antimicrobial activities of most plant extracts are the phenolics (Cowan, 1999). Investigation into the mechanism of their activity has shown that these compounds have multiple cellular targets, rather than one specific site of action (Cushnie & Lamb, 2005). The phenolic compounds potentially disturb the function of bacterial cell membranes which causes retardation of growth and multiplication of bacteria. Phenolic compounds are involved in adhesion binding, protein and cell wall binding, enzyme inactivation, intercalation into the cell wall and/or DNA during inactivation of pathogens (Davidson, 2001).

Conclusion

The obtained results give support to the ethnopharmacological use of these Turkish *Juniperus* species under *Juniperus* section in the treatment of infections and skin diseases, as well as demonstrating the potential of these plants as sources of natural antioxidant and antimicrobial compounds. Bioassay-guided fractionation procedures are necessary to characterize and isolate the active constituents.

Declaration of interest

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