SUPPLEMENTARY MATERIAL

Comparative study of the phenolic profile, antioxidant and antimicrobial activities of leaf extracts of five *Juniperus* L. (Cupressaceae) taxa growing in Turkey

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Abstract

The purpose of this study was to conduct a comparative evaluation of some biological properties of methanol and water extracts of leaves of five *Juniperus* taxa growing in Turkey: *J. communis* L. var. *communis* (*Jcc*), *J. communis* L. var. *saxatilis* Pall. (*Jcs*), *J. drupacea* Labill. (*Jd*), *J. oxycedrus* L. subsp. *oxycedrus* (*Joo*), *J. oxycedrus* L. subsp. *macrocarpa* (Sibth. & Sm.) Ball. (*Jom*). The antioxidant properties were examined *in vitro*; both in the DPPH and in the reducing power tests, *Joo* methanol extract resulted the most active (IC_{50}=0.09±0.01mg/mL and ASE/mL=2.56±0.06). In the TBA assay, *Jcs* methanol extract exhibited the highest activity (IC_{50}=4.39±0.47μg/mL). The extracts displayed bacteriostatic activity against *Staphylococcus aureus*, and *Jd* methanol extract resulted the most effective (MIC=19.53μg/mL); no effect on the *S. aureus* biofilm formation was observed. The extracts resulted non-toxic in the *Artemia salina* lethality bioassay. Finally, the phenolic profile of the methanol extracts was characterized by HPLC-PDA/ESI-MS.

Keywords*: Juniperus* L.; antioxidant activity; antimicrobial activity; *Artemia salina* Leach; phenolic compounds; HPLC-PDA/ESI-MS.

Content of supplementary data:

Experimental section

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 leaves 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 from different regions of Turkey. The taxonomic identification of the plants was confirmed by Prof. Ayşegül Köroğlu (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 S1. The leaves were dried at room temperature and powdered. The extracts were prepared as described below.

Methanol extracts: powdered leaves (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 “in vacuo” at 40 °C.

Water extracts: powdered leaves (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, referred to 100 g of dried leaves, are given in Table S1.

Antioxidant activity

Free radical scavenging activity

The free radical scavenging activity of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) test (Miceli et al. 2017). An aliquot (0.5 mL) of solution containing different amounts of the extracts 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 versus the DPPH standard solution. The results were obtained from the average of three independent experiments and are expressed as mean 50% inhibitory concentration (IC_{50}) ± SD.

Measurement of Reducing Power

The reducing power of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was evaluated by spectrophotometric detection of Fe^{3+}-Fe^{2+} transformation method (Miceli et al. 2017). Different amounts of the extracts in 1 mL solvent were mixed with 2.5 mL of phosphate buffer (0.2M, pH 6.6) and 2.5 mL of 1% potassium ferrycyanide [$K_3Fe(CN)_6$]. 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 1570 g for 10 min. The resulting supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% fresh ferric chloride ($FeCl_3$), and the absorbance was measured at 700 nm

after 10 min. Ascorbic acid and BHT were used as reference; the results were obtained from the average of three independent experiments and are expressed as ascorbic acid equivalent (ASE/mL) ± SD.

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

The Fe^{2+} chelating activity of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts was estimated by measuring the formation of the Fe^{2+}-ferrozine complex (Miceli et al. 2017). Different concentrations of the extracts in 1 mL solvent were mixed with 0.5 mL of methanol and 0.05 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.1 mL of 5 mM ferrozine. Then, the mixture was shaken vigorously and left standing at r.t. for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as reference standard; the results were obtained from the average of three independent experiments and are expressed as mean IC_{50} ± SD.

Thiobarbituric acid (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 (Taviano et al. 2013). Liposomes were prepared from bovine brain extract in phosphate buffered saline (PBS) (5 mg/mL). The reaction mixture 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 absorbance of the

malondialdehyde (MDA)-TBA complex in the upper layer was determined spectrophotometrically at 532 nm. Propyl gallate was used as reference compound; the results were obtained from the average of three independent experiments and are expressed as mean IC_{50} ± SD.

Antimicrobial activity

The following strains, obtained from the in-house culture collection of the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences (University of Messina, Italy), were used for the antibacterial testing: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404. Bacterial and yeast cultures for antimicrobial activity tests were grown in Mueller-Hinton Broth (MHB, Oxoid, Basingstoke, United Kingdom) at 37°C (24 h) and RPMI-1640 medium supplemented with MOPS (Oxoid) at 30°C (48 h), respectively. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of the extracts were determined according to the standard methods (CLSI 2008; CLSI 2012). Working cultures of bacteria and yeasts were adjusted to the required concentration of 10^5 CFU/mL and 10^3 CFU/mL, respectively. The final concentrations of the extracts adopted were 1.22 to 2500 μg/mL. Ofloxacin (0.2-0.3 μg/mL) and tetracycline (2-8 μg/mL) were used as reference standard drugs to compare the sensitivity of tested bacteria to the extracts; amphoterycin B (0.15 μg/mL) was employed as reference standard drugs for the tested fungi. Growth controls 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 defined as the lowest drug concentration at which there

was complete inhibition of growth. To determine MBC/MFC, bacterial/fungal aliquots were taken from each well and inoculated in Mueller Hinton Agar (MHA) or in Sabouraud Dextrose Agar (SAB), respectively. Plates were incubated for 24 h at 37°C (bacteria) or for 48 h at 35°C (yeasts). MBC or MFC were defined as the lowest drug concentration yielding negative subcultures or only one colony. The results were obtained from the average of three independent experiments and modal results were calculated. Moreover, the effect of leaf extracts on bacterial biofilm formation was evaluated as previously described (Marino et al. 2010).

Overnight culture in TSB with 1% glucose was diluted to standardise suspension of each strain (1×10^5 CFU/mL) and inoculated into sterile flat-bottom polystyrene microtiter plates (Corning Inc., Corning, NY) in the presence of subhinibitory concentration (0.5 MIC) of each *Juniperus* extract or medium (control). Two biofilm controls consisting of TSB medium and TSB-MeOH were included. After incubation for 24 h at 37°C, each well was washed twice with PBS, dried, stained for 1 min with 0.1% safranin and then washed with water. The stained biofilms were resuspended in 200 µL of 30% (v/v) acetic acid and OD_{492nm} was measured using the Microplate Reader. The reduction percentage of biofilm formation in the presence of different *Juniperus* extracts was calculated and the results were derived from three separate experiments and expressed as mean values ± SD.

Artemia salina lethality bioassay

In order to investigate the potential toxicity of *Jcc*, *Jcs*, *Jd*, *Joo*, and *Jom* extracts median lethal concentration (LC_{50}) was determined (Taviano et al. 2018). The extracts, opportunely dissolved and then diluted in artificial seawater, were tested at the final concentrations of 10, 100, 500 and 1000 µg/mL. Ten brine shrimp larvae, were

transferred to each sample vial, and artificial seawater was added to obtain a final volume of 5 mL. After 24 h of incubation (25-28 °C), the surviving larvae were counted. The assay was carried out in triplicate, and LC_{50} values were determined using the probit analysis method. Extracts are considered non-toxic if the LC_{50} is higher than 1000 µg/mL.

Identification of phenolic compounds by HPLC-DAD-ESI-MS analysis

The analyses were carried out using a Shimadzu HPLC system (Milan, Italy) equipped with a CBM-20A controller, LC-20AD pumps, a DGU-20A3 degasser, a SIL-20AC autosampler, a SPD-M20A photo diode array detector (PDA) and a quadrupolar mass analyzer (LCMS-2020, Shimadzu), equipped with an ESI interface, in negative ionization mode. Data acquisition was performed by Shimadzu LabSolution software ver. 5.65.

10 mg of each methanol extracts were dissolved in 1 mL of MeOH. Analyses were carried out on a Ascentis Express C18, 15 cm x 4.6 mm i.d. with particle size of 2.7 µm (Supelco, Bellefonte, PA). The injection volume was 2 µL, mobile phase consisted of water/formic acid (99.9:0.1) (solvent A) and ACN/formic acid (99.9:0.1) (solvent B), the linear gradient profile was as follows: 0 min, 0% B, 5 min, 5% B, 15 min, 10% B, 30 min, 20% B, 60 min, 50% B, 70 min, 100%B, 71 min, 0%B. The flow-rate was 1 mL/min and it was split to 0.2 mL/min prior to MS detection.

PDA conditions. The wavelength range was 200-400 nm and the chromatograms were extracted at 280 nm. Time constant was 25 ms and sample frequency 40 Hz.

MS conditions. The MS acquisition was performed using ESI, in negative mode, under the following conditions: mass spectral range 100-600 m/z; interval: 0.5 sec; nebulizing gas (N2) flow: 1.5 L/min; interface temperature: 350°C Heat block: 300°C, DL

temperature: 300°C; DL voltage -34 V; probe voltage 4.5 kV; Qarray voltage: 1.0 V, RF voltage: 90 V; detection gain 1.0 kV.

Quantitative determination was carried using calibration curves of three standards, namely gallic acid, rutin and amentoflavone. Standard calibration curves were prepared in a concentration range 0.1-50 mg/L with five different concentration levels. Triplicate injections were made for each level, and a linear regression was generated. The calibration curves with the external standards were obtained using concentration (mg/L) with respect to the area obtained from the integration of the PDA peaks at a wavelength of 270 nm and 350 nm. The amount of the compound was finally expressed in mg/g of extract.

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Table S1. Collection sites and herbarium numbers of the plant samples, and percentage yields of the extracts of *Juniperus* leaves.

***Juniperus* taxa**	**Collection sites**	**Herbarium numbers**	**Yields (w/w%)**	
			Methanol extracts	Water extracts
Jcc	Ağrı: Doğubeyazıt, Korhan Y.	AEF 23854	17.53	45.77
Jcs	Ankara: Kızılcahamam, Işık Dağı	AEF 23801	6.05	38.48
Jd	Antalya: Antalya-Konya road	AEF 23610	21.08	32.13
Joo	Ankara: Kızılcahamam, Işık Dağı	AEF 23799	22.61	46.25
Jom	İzmir: Çeşme, around Çiftlikköy	AEF 23855	21.43	42.71

The yields are referred to 100 g of dried leaves.

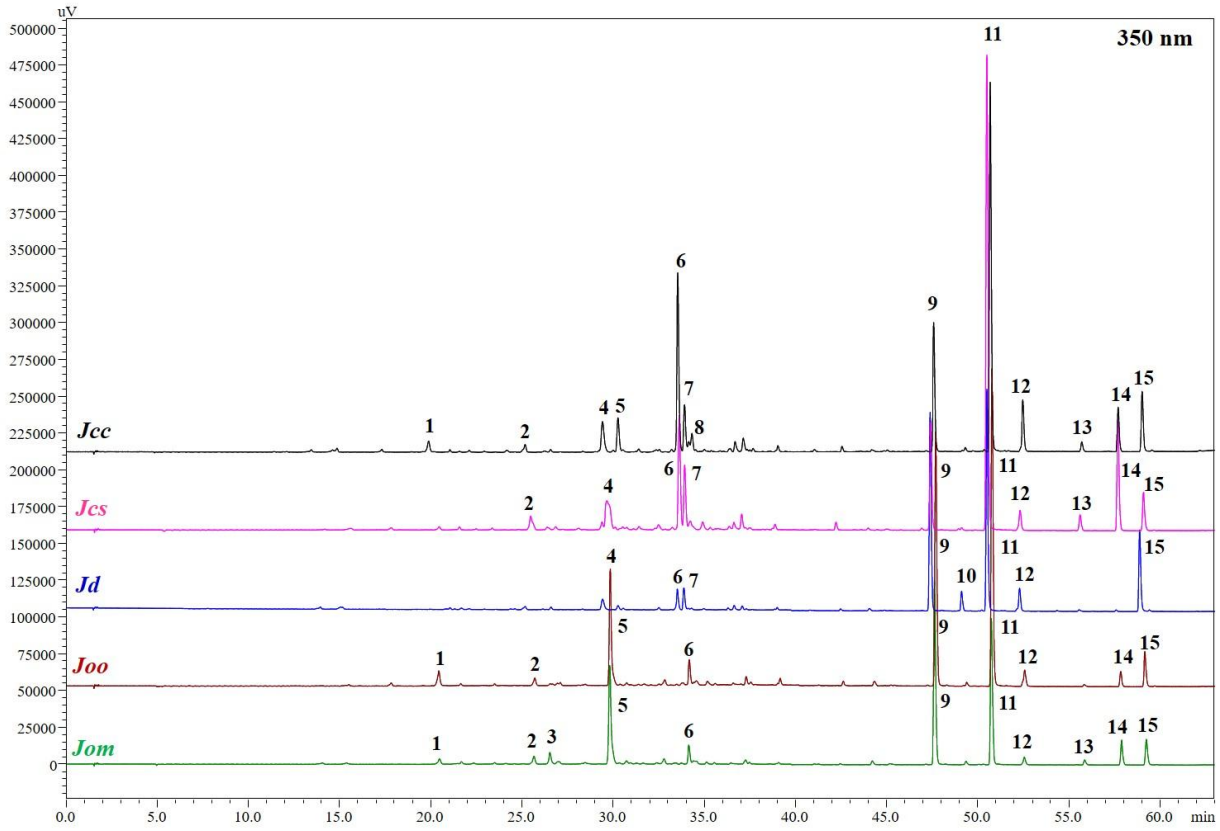

Figure S1. HPLC-PDA/ESI-MS (negative ionization mode) phenolic fingerprint of methanol extracts of *Juniperus* leaves. Column: C_{18}, 15 cm x 4.6 mm, 2.7 µm particles (Ascentis® Express). For peak identification, see Table 2.