SUPPLEMENTARY MATERIAL

Phenolic Content, Antioxidant Capacity, and Antimicrobial Activity of Leaf Extracts from *Pistacia atlantica.*

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Abstract

The aim of this study was to investigate the secondary metabolites related to *Pistacia atlantica* Desf as well as their biological activities. Results show that using ethanol as a solvent exhibited the highest content of total phenolics and flavonoids (68.23 mg GAE. g^{-1} of DW and 44 mg RE. g^{-1} of DW), followed by aqueous one (20.07 mg GAE. g^{-1} of DW and 15 mg RE. g^{-1} of DW). Good correlation (Data not shown) was obtained between the DPPH radical-scavenging activities and total phenolics contents. Many similarities were observed between the results of the DPPH (IC_{50} = 32 and 200 µg. ml^{-1}) and ABTS (IC_{50} = 42 and 300 µg. ml^{-1}) assays. All tested extracts contained phenolic compounds exhibited an antimicrobial effect against Gram-positive and Gram-negative bacteria. Further researches on identification and purification of phenolic compounds are required.

Keywords*:* *Pistacia atlantica* Desf, phenolic compounds, DPPH, medicinal plants.

Experimental section

1. Plant material

Plant material used in the present research includes *Pistacia atlantica* Desf. leaves. Fully expanded, green, healthy leaves with petioles were collected from El Feija, Jendouba (North-East of Tunisia) on November 2014. The sample size was 350 g. The plant material was air dried in the shade at room temperature. The leaf petioles were carefully manually separated and dry leaves were pulverized (3 × 1 min in a high speed grinder) into powder.

Concerning the botanical identification of *Pistacia atlantica*, it was carried out by Professor Zouheir Nasr. Voucher specimens PA2014 is deposited at the Laboratory of Management and Valorization of Forest Resources.

2. Extractions of Polyphenols

The polyphenolic compounds were extracted from the homogenized dry plant material (10 g) using 10 mL of two solvents: ethanol and water at room temperature, contact time was 24 h (Rigane et al. 2013). Each extract was filtered with Whatman No. 1 filter paper and the residual tissue was washed with 2 × 10 mL of each solvent. Each filtrate was combined in total volume, which was dried under a vacuum using a rotary evaporator, at 50 °C. The dry residues were redissolved with the appropriate solvents reaching a volume of 10 mL. Extractions were done in three repetitions and extracts (3 × 10 mL) were combined in total leaf extract (LE). Thus, obtained LE was centrifuged at 5000 rpm for 10 min and used for further analysis. Tow extracts were obtained for each solvent's extract.

3. Determination of Total Phenolic Content (TPC)

The TPC was determined by a Folin - Ciocalteu assay with slight modifications (Rigane et al. 2013) using gallic acid (GA) as the standard. The absorbance was measured at 765 nm against a reagent blank. The TPC was expressed as milligrams of gallic acid equivalents (mg of GAE.g^{-1} of Dry weight) through the calibration curve of gallic acid. The linearity range of the calibration curve was 20 – 100 µg.mL^{-1} (r^2 =0.9892).

4. Determination of total flavonoids content (TFC)

Total flavonoids content was determined using the aluminium trichloride method as described by Papova et al. (1996). Briefly, 1 ml of each extract solution was added to a test tube with 1 ml of $AlCl_3$ (2 % in ethanol). The mixture was shaken vigorously and incubated for 30 min at room temperature in the dark. The absorbance of the mixture was measured at 430 nm (UV-vis spectrophotometer, Shimadzu UV-160A PC, Shimadzu Corporation, Kyoto, Japan) immediately, and the total flavonoids content was expressed as mg rutin equivalent (RE) .g^{-1} Dry weight. The linearity range of the calibration curve was 5 - 25 µg mL^{-1} (r^2= 0.99).

5. Antioxidant activity evaluation

To assess the antioxidant potential of bioactive compounds, the application of at least two different assays varying in their mechanisms of antioxidant action has been recommended (Rigane et al., 2013). The antioxidant capacity of the studied samples was determined applying the DPPH and ABTS assays.

5.1. DPPH Assay. The method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant. DPPH solutions show a strong absorption band at 517 nm with a deep violet colour. The absorption vanishes and the resulting decolouration is stochiometric with respect to degree of reduction. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant.

The DPPH radical scavenging capacity was measured according to Ghazghazi et al (2014) with modifications. Various concentrations from ethanolic or aqueous extracts of *P. atlantica* leaves (10- 450 µg.mL^{-1}) (1 mL) was added to 250 µL of ethanolic solution of DPPH. The mixture was stirred vigorously and then incubated at room temperature for 30 min in the dark. Each experiment was analyzed in triplicate.

5.2. Measurement of radical scavenging activity by ABTS. The radical scavenging activity of the two extracts of *Pistacia atlantica* growing in Tunisia was also measured using the method of Re et al. (1999) with some modifications. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 24 h before use. Afterwards, the ABTS^{+} solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. After that, various concentrations from ethanolic or aqueous extracts of *P. atlantica* leaves (10- 450 µg.mL^{-1}) (10 mL) was added to 990 ml of diluted ABTS^{+} solution, the absorbance reading was taken at 30°C exactly 6 mn after initial mixing, at 734 nm. Tests were carried out in triplicate.

6. Antimicrobial activity

6.1. Microbial strains. The different extracts of *P. atlantica* were tested for their antibacterial activities against seven bacteria reference pathogenic (*Escherichia coli* ATCC 8739, *Salmonella typhimurium* NCTC 6017, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC27853, *Aeromonas hydrophila* EI, *Listeriamonocytogenes* ATCC 7644, and *Bacillus cereus* ATCC 1247).

6.2. Disk diffusion assay. Antibacterial activity was evaluated using the method described by Choi et al. (2006). The principle is to use Whatman paper discs of 6mm in diameter. The discs were impregnated with essential oil diluted in hexane. A disc soaked in hexane was used as negative control. These discs are then deposited on the surface of a middle swab with a bacterial suspension to an optical density of 0.5 Mc Farlend standards. Bacterial strains were grown in Muller-Hinton agar. We used the bacterial strains for the culture medium. At the end of the incubation, 24 hours at 37°C, the diameters of the zone of inhibition were measured. Tests were carried out in triplicate.

6.3. Determination of MIC and MBC

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined using a broth dilution method as described by Ghazghazi et al. (2014). All antibacterial and tests were performed Muller-Hinton agar. Overnight broth cultures were diluted in peptone water (0.1% (v/v)) to obtain working culture (10^5 CFU/mL). Serial dilutions, ranging from 0.07 to 50 mg/mL of the tested extracts, were used, including one growth control (Muller-Hinton agar) and one sterility control (appropriate medium + tested extract). Tubes were incubated for 24 h at 37°C and the MICs and MBCs were determined. Microbial growth was indicated by the presence of turbidity and a ‘pellet’ on the tube bottom. MICs were determined

presumptively as the first tube, in ascending order, which did not produce a tube bottom. To confirm MICs and to establish MBCs, 10 μL of broth was removed from each well and inoculated on trypto-caseine soja agar. After incubation, the number of surviving organisms was determined. The MIC was the lowest concentration which resulted in a significant decrease in inoculum viability (> 90%), while the MBC was the concentration where 99.9% or more of the initial inoculum was killed. MIC and MBC values were selected.

7. Statistical Analysis

Results were evaluated using ANOVA and means comparison by Tukey's test at 5% probability using SAS version 9.1 for Windows (SAS Institute, Cary, NC, USA).

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