SUPPLEMENTARY MATERIAL

Antimicrobial activities, toxicity and phenolic composition of *Asphodeline anatolica* E. Tuzlaci leaf extracts from Turkey

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Abstract

The antimicrobial activity of acetone, methanol and aqueous extracts of *Asphodeline anatolica* E. Tuzlaci leaves was evaluated against American Type Culture Collection (ATCC), food and clinical isolates (*Listeria monocytogenes* and *Staphylococcus aureus* including methicillin-resistant strains-MRSA). Biofilm formation, toxicity and characterization of the polyphenolic content were analysed. The acetone extract demonstrated a higher antibacterial bacteriostatic activity against *S. aureus* including MRSA strains, *L. monocytogenes* and *Pseudomonas aeruginosa* than against other extracts. No effect was observed in biofilm formation. The extracts resulted non-toxic against *Artemia salina* Leach. The phytochemical screening of extracts indicated that they mainly contained six polyphenols identified as catechin 3-O-gallate, protocatechuic acid, diosmin, rutin, cirsimaritin and kaempferol glucoside. This study is the first report on antimicrobial activity and phenolic content of *A. anatolica* and contributes to enrich the literature data on the biological properties of this plant. *A. anatolica* leaves have a potential as source of natural antimicrobial compounds.

Keywords: antimicrobials, Biofilm, MRSA, *Listeria monocytogenes*, HPLC analysis, *Artemia salina* test.

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Experimental

Plant material

The leaves of *A. anatolica* were collected from Isparta-Turkey (Sarkikaraagac-Yenisarbademli road, 38°03'07" N, 31°17'51"E, 1144 m) at the end of the flowering season. Taxonomic identification of the plant material was confirmed by senior taxonomist and botanist Dr. Murad Aydin Sanda, from the Department of Biology, Selcuk University. Voucher specimen (number GZ 1001) was deposited in the KNYA Herbarium of Selcuk University, Turkey. The plant material was dried at room temperature. Dried and powdered leaves (10 g) were extracted with acetone or methanol (MeOH) in a Soxhlet apparatus for 6-8 h. The extracts were concentrated under vacuum at 40°C by using a rotary evaporator. To obtain water extract the powdered sample was boiled with 250 ml of distilled water for about 30 min, filtered and lyophilized (-80°C, 48 h). Extracts were stored at 4°C in the dark until use.

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

HPLC-PDA/ESI-MS analyses were performed on a Prominence LC system (Shimadzu, Milan, Italy) equipped with photodiode array (PDA) and mass spectrometry (MS) (LCMS-2020, Shimadzu) detection. The data acquisition was performed by Shimadzu LabSolution software ver. 5.10.153. All solvents were of LC-MS grade and were provided by Sigma-Aldrich (Milan, Italy). For chromatographic separations, an Ascentis Express C18 column (15 cm x 4.6 mm I.D.) packed with 2.7 µm partially porous particles, was employed (Supelco, Bellefonte, PA, USA). The injection volume was 5 µl, and the mobile phase consisted of water/acetic acid (0.075 %) at pH=3 (solvent A) and ACN/acetic acid (0.075 %) (solvent B), respectively in the following linear gradient mode: 0 min, 0% B; 60 min, 40% B; 70 min, 100% B; 71 min, 0% B. The mobile phase flow rate was 1.0 ml/min, and it was split to 0.2 ml/min prior to MS detection. PDA wavelength range was 190-400 nm and the chromatograms were extracted at 280 nm. The extracts (10 mg) were dissolved in acetone and methanol respectively (1 ml), and filtered through 0.45 µm membrane filters (Whatman, Clifton, USA). The quantitative determination of each compound was carried out by means of the external standard method using catechin ($\lambda=278$), gallic acid ($\lambda=270$), rutin ($\lambda=355$), apigenin ($\lambda=336$) and kaempferol ($\lambda=365$) as reference compounds in a concentration range of 1-100 ppm. The results were obtained from the average of three determinations and are expressed as mg/g dried extract $\pm$ percent relative standard deviation (%RSD).

Microbial strains and culture conditions

The following strains were used for the antimicrobial testing and were obtained from the University of Messina's in-house culture collection (Messina, Italy): *Staphylococcus aureus* ATCC 6538P, *S. epidermidis* ATCC 35984, *Pseudomonas aeruginosa* ATCC 9027, *Listeria monocytogenes* ATCC 13932, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, clinical isolates methicillin-resistant *S. aureus* (MRSA, n=6) obtained from specimens of skin infections and surgical infections and food isolates of *L. monocytogenes* belonging to serotypes 1/2a (n=2), 1/2 b (n=2), 1/2 c (n=2) and 4/b (n=2). Cultures for antimicrobial activity tests were grown in Mueller-Hinton Broth (MHB, Oxoid, Basingstoke, United Kingdom) at 37°C (24 h) for bacteria and Sabouraud Dextrose Broth (SDB, Oxoid) at 30°C (48 h) for the yeast.

Antimicrobial testing

The methanol, acetone and water extracts of *A. anatolica* leaves were dissolved in dimethyl sulfoxide (DMSO), incorporated into nutrient broth medium to obtain the final concentration of 2000 µg/ml and then serially diluted to 1.95 µg/ml. The Minimum Inhibitory Concentration (MICs), the Minimum Bactericidal Concentration (MBC) and the Minimum Fungicidal Concentration (MFC) of the extracts were determined according to the standard methods (CLSI 2011). As an indicator of microbial vitality, 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 (Marino et al. 2010). Ofloxacin (range from 0.2 to 0.3 µg/ml), tetracycline (range from 2 to 8 µg/ml) and amphotericin B (range from 0.2 to 0.3 µg/ml) were used as positive reference standard drugs. The biofilm formation of *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *L. monocytogenes* was performed according to the method previously reported (Marino et al. 2010). All experiments were performed in triplicate on three independent days and modal results were calculated.

Artemia salina lethality test

The *Artemia salina* Leach (brine shrimp) lethality bioassay was employed to predict the potential toxicity of acetone, methanol and water extracts of *A. anatolica* leaves. Medium lethal concentration (LC₅₀) determination was carried according to the method previously reported (Taviano et al. 2011). The assay was carried out in triplicate, and LC₅₀ was determined using the probit analysis method. The extracts dilutions that did not show cytotoxicity at 1000 µg/mL were considered non-toxic.

Table S1. HPLC-PDA/ESI-MS identification and quantification of polyphenolic compounds contained in acetone and methanol extracts obtained from *A. anatolica* leaves. Data are expressed as mean of three experiments $\pm$ relative standard deviation (RSD).

Peak	t_R (min)	Compounds (expressed as)	[M-H] ⁻	UV/Vis		mg/g extract $\pm$ % RSD	
				Max	Extracted	DMSO	MeOH
1	14.6	Catechin 3-O-gallate	441, 289	240, 278	278	3.69 $\pm$ 3.54	17.89 $\pm$ 2.52
2	22.08	Protocatechuic acid	153	225, 270	270	4.52 $\pm$ 2.81	5.66 $\pm$ 3.1
3	22.59	Diosmin	607, 299	255, 355	355	19.55 $\pm$ 1.56	19.85 $\pm$ 1.52
4	22.98	Rutin	609, 301	255, 355	355	9.77 $\pm$ 2.65	43.92 $\pm$ 3.26
5	27.21	Cirsimaritin	313, 293	276, 336	336	24.43 $\pm$ 0.36	16.53 $\pm$ 2.59
6	33.03	Kaempferol glucoside	447, 285	266, 348	348	8.93 $\pm$ 1.31	4.06 $\pm$ 1.56

Table S2. MICs of *A. anatolica* leaf extracts. Values represent the mean of three determination.

Strains	Acetone	Methanol	Water
	MIC (μg/ml)		
Gram-positive bacteria			
<i>S. aureus</i> ATCC 6538P	31.25	125	> 2000
<i>S. aureus</i> 815	62.50	500	> 2000
<i>S. aureus</i> 14*	31.25	250	> 2000
<i>S. aureus</i> 526*	62.50	500	> 2000
<i>S. aureus</i> 543*	62.50	250	> 2000
<i>S. aureus</i> 550*	62.50	250	> 2000
<i>S. aureus</i> 588*	31.25	250	> 2000
<i>S. epidermidis</i> ATCC 35984	1000	250	> 2000
<i>L. monocytogenes</i> ATCC 13932	125	1000	> 2000
<i>L. monocytogenes</i> A149 (1/2b)**	125	1000	> 2000
<i>L. monocytogenes</i> A216 (1/2a)**	125	1000	> 2000
<i>L. monocytogenes</i> A222 (4b)**	125	1000	> 2000
<i>L. monocytogenes</i> A241 (1/2a)**	125	1000	> 2000
<i>L. monocytogenes</i> A282 (1/2b)**	125	1000	> 2000
<i>L. monocytogenes</i> G152 (1/2c)**	125	1000	> 2000
<i>L. monocytogenes</i> G193 (1/2c)**	125	1000	> 2000
<i>L. monocytogenes</i> G197 (4b)*	125	1000	> 2000
<i>B. subtilis</i> ATCC 6633	> 2000	> 2000	> 2000
Gram-negative bacteria			
<i>P. aeruginosa</i> ATCC 9027	500	> 2000	> 2000
Yeasts			
<i>C. albicans</i> ATCC 10231	> 2000	> 2000	> 2000

* clinical isolate methicillin-resistant *S. aureus*

** food isolate

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