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RESEARCH ARTICLE

Antimicrobial and antioxidant properties of methanol extracts of two *Athamanta turbith* subspecies

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Abstract

The methanol extracts of fruits and leaves of *Athamanta turbith* subsp. *hungarica* (Borbás) Tutin (Umbelliferae) and *A. turbith* subsp. *haynaldii* (Borbás & Uechtr.) Tutin were analyzed for their antimicrobial and antioxidant properties. Phenolic compounds (flavonoids and phenolic acids) of the extracts were examined using HPLC. All the extracts were characterized by the presence of caffeic acid derivates, luteolin and its glycosides, with luteolin 7-*O*-glucoside as one of the main compounds. Luteolin 7-*O*-rutinoside was detected only in *A. turbith* subsp. *haynaldii* extracts. Investigation of antimicrobial activity was performed against six bacteria and two fungal strains, using the agar diffusion technique and broth microdilution assay. The extracts of investigated *A. turbith* subspecies exerted similar antimicrobial activity, whereas the best activity was detected against *Candida albicans*. In order to investigate antioxidant properties, ferric ion reducing antioxidant power (FRAP), radical scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical (HO·), and the effect on lipid peroxidation (LP) were examined. All the examined extracts showed moderate antioxidant capacity, whereas the fruit extracts were more active than the extracts of leaves. Also, the extracts of *A. turbith* subsp. *hungarica* exerted higher antioxidant capacity than corresponding *A. turbith* subsp. *haynaldii* extracts. The relationship between estimated activity and chemical composition of the extracts is discussed.

Keywords: *Athamanta turbith subsp. hungarica; Athamanta turbith subsp. haynaldii; antimicrobial activity; antioxidant activity; phenolic compounds*

Introduction

The genus *Athamanta* L. (Umbelliferae) consists of about nine species, which are mainly distributed in southeastern Europe. *Athamanta turbith* (L.) Brot. inhabits limestone rock crevices in southeastern Europe (Italy, Balkan and Carpathians). It is currently considered to contain three subspecies: *A. turbith* subsp. *turbith*, *A. turbith* subsp. *hungarica* (Borbás) Tutin and *A. turbith* subsp. *haynaldii* (Borbás & Uechtr.) Tutin (Tutin, 1968). In the flora of FR Serbia, *A. turbith* subsp. *hungarica* is registered as the species *A. hungarica* Borbás (Obradović, 1986), and *A. turbith* subsp. *haynaldii* as *A. haynaldii* Borbás & Uechtr. (Nikolić, 1973).

A. turbith subsp. *hungarica* is distributed in gorges of the southern Carpathians and northeastern Serbia. *A. turbith* subsp. *haynaldii* is an endemic Dinaric alpine plant. These two plants have very similar habitus. Morphologically, the only difference that can be noticed is the stylopodium shape: each half of *A. turbith* subsp. *hungarica* stylopodium is 1.5–2 times as long as it is wide, while each half of *A. turbith* subsp. *haynaldii* stylopodium is about as long as it is wide (Tutin, 1968).

In traditional medicine, *Athamanta cretensis* and *Athamanta macedonica* are used in the therapy of sclerosis, *Athamanta oreoselinum* as an antiseptic and diuretic (Duke, 2004), while *Athamanta sicula* is used to expel renal calculi (Lentini, 2000).

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It has been recognized that naturally occurring substances in plants have antimicrobial activity. There is a growing interest in research of antimicrobial properties of plant extracts, since resistance to antibiotics has become an important and pressing global problem. Over the past decade, great interest has also been devoted to the antioxidant activity of phenolic compounds from plant extracts, which is due to their ability to reduce free radical formation and to scavenge free radicals. In this study, *in vitro* antimicrobial and antioxidant activity of methanol extracts of mature fruits and leaves of *A. turbith* subsp. *hungarica* and *A. turbith* subsp. *haynaldii* were investigated.

Materials and methods

Chemicals

1,1-Diphenyl 2-picryl hydrazyl (DPPH), Folin-Ciocalteu reagent, 2,4,6-tripyridyl-*s*-triazine (TPTZ), apigenin and caffeic acid were obtained from Sigma Chemical Co. (St. Louis, MO); thiobarbituric acid (TBA) from Merck (Darmstadt, Germany); trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and L-ascorbic acid from Lachema (Neratovice, Czech Republic); 2-deoxyribose from Acros Organics (New Jersey, USA); rutin, quercetin, umbelliferone and kaempferol from Fluka AG (Buchs, Switzerland); cynarine from Roth (Karlsruhe, Germany); all other reagents used were of analytical grade. Luteolin, luteolin 7-*O*-glucoside and luteolin 7-*O*-rutinoside were isolated from *Hieracium gymnocephalum* Griseb. ex Pant. (Petrović et al., 1999). Scopoletin and 3,5-di-*O*-caffeoylquinic acid were isolated from *Anthemis triumfetti* (L.) DC. (Pavlović et al., 2006). Ampicillin, amikacin and nystatin were obtained from Galenika (Belgrade, Serbia); Müeller-Hinton agar and Sabouraud agar from Institute of Immunology and Virology, Torlak (Belgrade, Serbia).

Plant material

The plants were collected in July 2004 from two localities in Serbia: Đerdap gorge (Iron Gate) (*A. turbith* subsp. *hungarica*) and Ovčar-Kablar gorge (*A. turbith* subsp. *haynaldii*), and were identified by Marjan Niketić. Voucher specimens (ko2004071 and ko2004072, respectively) were deposited at the Herbarium of the Natural History Museum in Belgrade (BEO).

Extraction

Air-dried, powdered plant material (mature fruits and leaves, separately) was macerated with chloroform for three days, and after filtration, for two more days. The extracts were filtered and the residues extracted with methanol, under the same conditions. The methanol extracts were evaporated to dryness under vacuum and were used for further analyses. Yields of methanol extracts of fruits and leaves of *A. turbith* subsp. *hungarica* were 6% and 7.2%, and of *A. turbith* subsp. *haynaldii* 6.5% and 10.9%, respectively.

High Performance Liquid Chromatography (HPLC) analysis

Phenolic compounds of the extracts were assayed by HPLC on an Agilent 1100 series system consisting of a G1312A binary pump, a G1328B injector (20 μL sample loop) and G1315B DAD detector, equipped with ZORBAX Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm). A gradient elution was performed with solvent A (H_3PO_4 in H_2O, pH = 2.75) and solvent B (solvent A: acetonitrile = 10:90; solvent B: H_3PO_4 in H_2O, pH = 2.75: acetonitrile = 10:90) as follows: in 0 min 10% B; in 5 min 25% B; in 15 min 25% B; in 20 min 30% B; in 25 min 50% B, in 30 min 70% B and in 35 min 10% B. The column temperature was 25°C, flow rate 0.8 mL/min. Luteolin, luteolin 7-*O*-glucoside, luteolin 7-*O*-rutinoside, apigenin, quercetin, kaempferol, caffeic acid, 3,5-dicaffeoylquinic acid, cynarine, scopoletin and umbelliferone were used as standards (1 mg/mL in acetonitrile:H_2O = 1:1). Dry methanol extracts were dissolved in methanol in concentration of 1.5%. Chromatograms of extracts and standards were recorded under the same conditions. Detection was performed at 350 nm. Identification of compounds was carried out by comparing their spectra and their retention times with those of standards when available. UV spectra were also compared with literature data (Mabry et al., 1970).

Antimicrobial activity

Microbial strains

Methanol extracts were tested against six bacteria and two fungal strains, listed in Table 1. Microorganisms were provided by the Institute for Immunology and Virology, Torlak, Belgrade.

Agar diffusion assay

Antimicrobial activity was assayed using the agar diffusion method (Acar & Goldstein, 1996), in comparison to ampicillin, amikacin and nystatin.

Müller-Hinton agar and Sabouraud agar were used to test sensitivity of bacteria and yeasts, respectively. The diffusion technique was carried by pouring agar into Petri dishes to form 4 mm thick layers and adding dense inocula of the tested microorganisms (10^6 microorganisms/mL) in order to obtain semiconfluent growth. Dry methanol extracts were dissolved in methanol in the concentration of 500 and 250 μg/mL. One drop of each extract solution was poured on the agar prepared as required. Incubation lasted 18 h at 37°C for bacteria

Table 1. HPLC analysis of *Athamanta turbith* subsp. *hungarica* and *A. turbith* subsp. *haynaldii* methanol extracts.

RT (min)	Phenolic compound	*A. turbith* subsp. *hungarica* Fruits	*A. turbith* subsp. *hungarica* Leaves	*A. turbith* subsp. *haynaldii* Fruits	*A. turbith* subsp. *haynaldii* Leaves
7.9	Chlorogenic acid	tr	tr	—	—
9.8	Luteolin glycoside	—	—	+	+
10.2	Luteolin 7-*O*-rutinoside	—	—	+	++
10.7	Luteolin glycoside	++	++	tr	tr
11.4	Luteolin 7-O-glucoside	++	+++	+	+
13.1	Caffeic acid derivate	—	—	+	—
13.4	Caffeic acid derivate	++	tr	—	—
14.0	Luteolin glycoside	—	—	+	+
14.6	3,5-Dicaffeoylquinic acid	++	+	+	tr
15.7	Luteolin glycoside	+	+	tr	tr
16.3	Caffeic acid derivate	++	+	+	tr
17.4	Luteolin glycoside	—	—	tr	+
19.3	Caffeic acid derivate	tr	—	tr	—
26.8	Luteolin	++	+	++	tr

Note: +++, major; ++, strong; +, present; tr, trace; -, not detected. Estimated from the areas of the HPLC peaks.

and 48 h at 26°C for *Candida albicans*. Reading of results was carried out by measuring diameters of zones of inhibitions in mm (in quadruplicate). Each assay in this experiment was repeated twice.

Broth microdilution assay

For the determination of minimal inhibitory concentration (MIC), a broth microdilution assay was used, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2001). Tests for antibacterial activity were performed in Müller-Hinton broth, and in Sabouraud dextrose broth for antifungal activity. Test strains were suspended in broth to give a final density of 5×10^5 cfu/mL. Dilutions ranging from 500 to 62.5 μg/mL of the methanol extracts were prepared in 96-well plates. As a positive control of growth, the wells containing only microorganisms in the broth were used. The contents of each well were mixed and incubated for 18 h at 37°C for bacteria and 48 h at 26°C for yeast. Bacterial growth was indicated by the presence of a pellet on the well bottom. The extracts were screened twice against each microorganism. The MIC of ampicillin, amikacin and nystatin were determined in parallel experiments.

Antioxidant activity

In order to investigate the antioxidant properties of the examined extracts, ferric ion reducing antioxidant power (FRAP), radical scavenging capacity (RSC) against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical ($HO^{\cdot}$), and the effect on lipid peroxidation (LP) were examined. All the tests were carried out in triplicate.

FRAP assay

FRAP reagent was freshly prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL 2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ) solution (10 mM TPTZ in 40 mmol/L HCl) and 2.5 mL $FeCl_3$ (20 mM) water solution. Each sample (150 μL) (0.5 mg/mL) dissolved in methanol was added in 4.5 mL of freshly prepared FRAP reagent and stirred, and after 5 min, absorbance was measured at 593 nm, using FRAP working solution as blank (Szőllősi & Szőllősi Varga, 2002). A calibration curve of ferrous sulfate (100-1000 μmol/L) was used, and results were expressed in μmol Fe^{2+}/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid, quercetin, and rutin.

DPPH radical assay

For measuring RSC against DPPH radical, 1 mL of 0.5 mM methanol solution of DPPH was added to 4 mL of methanol solution of the investigated extract, in four different concentrations. Absorbance at 517 nm was determined after 30 min, using methanol as blank (Cuendet et al., 1997). Scavenging of DPPH radical was calculated using the equation: $S(\%) = 100 \times (A_0 - A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the tested sample. Results were compared with the activity of L-ascorbic acid, quercetin, and rutin.

2-Deoxyribose assay

RSC against $HO^{\cdot}$ radical was determined in non-site-specific deoxyribose assay. Methanol solution (20 μL) of the examined extract (five different concentrations) was mixed with 100 μL of 0.004 M $FeCl_3$, 100 μL 0.004 M ethylenediaminetetraacetic acid (EDTA), 200 μL of 2-deoxyribose (0.05 M), 20 μL of 1.5% H_2O_2 and 100 μL of L-ascorbic acid (0.004 M) in phosphate buffer, pH = 7.4 (4 mL final solution). The mixtures were then incubated at 37°C for 60 min. When adding 1 mL of 1% (w/v) solution of thiobarbituric acid (TBA) in 0.05 M NaOH and 1 mL of 2.8% (w/v) solution of trichloroacetic acid (TCA), the mixtures were heated at 100°C for 15 min, and then cooled on ice. Absorbance of samples was measured at 532 nm (Lee et al., 2003). Inhibition of 2-deoxyribose degradation was calculated the same way as described in DPPH radical assay. Quercetin and rutin were used as standards.

TBA assay

For measuring the inhibitory effect on Fe^{2+}/ascorbate induced lipid peroxidation (LP) in liposomes, the TBA test was used. Liposomes were prepared from the commercial preparation "Lipotech 10", containing 10% lecithin, diluted with distilled water in ultrasonic bath for 30 min. Liposomes (0.03 mg lecithin/mL) were incubated at 37°C for 60 min with 20 μL of $FeSO_4$ (0.075 M), 10 μL of L-ascorbic acid (0.1 M) and 10 μL methanol solution of

the examined extract (five different concentrations) in phosphate buffer, pH = 7.4 (4 mL final solution). LP was terminated by adding 0.2 mL 0.1 M EDTA and 1.5 mL of TBA reagent (3 g TBA, 120 g TCA and 10.4 mL $HClO_4$ in 800 mL of distilled water). Mixtures were heated for 15 min at 100°C. After cooling on ice, samples were centrifuged for 10 min (3000 rpm) and absorbance of supernatants was measured at 532 nm (Afanas'ev et al., 1989). Inhibition of LP was calculated the same way as described in DPPH radical assay. Results were compared with the activity of quercetin.

Statistical analysis

Results were expressed as means ± standard deviations (SD). Statistical analysis was performed by ANOVA. Differences were accepted as statistically significant when $p < 0.05$.

Results and discussion

HPLC analysis

The extracts of both *A. turbith* subspecies are characterized by the presence of luteolin, its glycosides and caffeic acid derivates (Table 1). Luteolin 7-*O*-glucoside and 3,5-dicaffeoylquinic acid were detected in all four investigated extracts. Between the extracts of fruits and leaves of each subspecies there are only quantitative differences. In the extracts of fruits, free luteolin is the dominant compound, while in the extracts of leaves, luteolin glycosides are present in larger quantity. However, between investigated subspecies there are qualitative differences: in the *A. turbith* subsp. *haynaldii* extracts more luteolin glycosides have been detected, with luteolin 7-*O*-rutinoside being one of the major compounds, while in *A. turbith* subsp. *hungarica* extracts this component has not been detected. Similar flavonoid patterns of the investigated plants support their taxonomic classification in the same species. Also, concerning estimated differences, flavonoids could be regarded as good chemotaxonomic markers for the investigated *A. turbith* subspecies.

Antimicrobial activity

The extracts of investigated *A. turbith* subspecies exerted similar antimicrobial activity. All the extracts showed strong activity against *Candida albicans*. As for antibacterial activity, at the applied concentrations, the extracts exerted moderate activity against *Staphylococcus aureus*, slightly weaker against *Enterococcus faecalis* and *Escherichia coli*, and weak against *Pseudomonas*

Table 2. Antimicrobial activity of *Athamanta turbith* subsp. *hungarica* and *A. turbith* subsp. *haynaldii* methanol extracts tested by agar diffusion assay.

	A. turbith subsp. *hungarica*				*A. turbith* subsp. *haynaldii*						
	Fruits		Leaves		Fruits		Leaves		Ampicillin	Amikacin	Nystatin
Microorganism	500 µg/mL	250 µg/mL	500 µg/mL	250 µg/mL	500 µg/mL	250 µg/mL	500 µg/mL	250 µg/mL	10 µg/disk	30 µg/disk	100 units/disk
Staphylococcus aureus ATCC 25923	16.00 ± 1.00	15.00 ± 1.00	8.67 ± 0.58	5.67 ± 0.58	16.00 ± 1.00	13.33 ± 0.58	15.33 ± 0.58	—	27.0 ± 0.5	26.0 ± 0.0	nt
Enterococcus faecalis ATCC 29212	10.33 ± 1.15	9.00 ± 1.00	—	—	13.00 ± 0.00	—	—	—	26.0 ± 0.0	nt	nt
Escherichia coli ATCC 25922	9.67 ± 0.58	8.33 ± 0.58	6.00 ± 0.00	—	14.67 ± 0.58	11.33 ± 0.58	14.33 ± 0.58	4.00 ± 0.00	18.0 ± 0.0	24.0 ± 0.0	nt
Pseudomonas aeruginosa ATCC 27853	11.00 ± 0.00	—	—	—	12.67 ± 0.58	—	—	—	nt	26.0 ± 0.5	nt
Klebsiella pneumoniae ATCC 29655	10.67 ± 0.58	—	—	—	8.33 ± 0.58	—	—	—	17.0 ± 0.0	nt	nt
Klebsiella pneumoniae NCIMB 9111	12.00 ± 1.00	—	—	—	10.67 ± 0.58	—	—	—	nt	nt	nt
Candida albicans ATCC 24434	18.00 ± 0.00	17.00 ± 0.00	14.67 ± 0.58	13.67 ± 0.58	20.00 ± 1.00	17.00 ± 1.00	13.67 ± 0.58	12.00 ± 0.00	nt	nt	20.0 ± 0.8
Candida albicans ATCC 10259	20.00 ± 1.00	18.00 ± 1.00	15.00 ± 0.00	11.67 ± 0.58	19.00 ± 1.00	14.00 ± 0.00	17.67 ± 0.58	11.33 ± 0.58	nt	nt	20.0 ± 0.8

Results are presented as diameters of zones of inhibition (mm) and values are the average of four determinations (± SD).
nt, not tested.

Table 3. Antimicrobial activity of *Athamanta turbith* subsp. *hungarica* and *A. turbith* subsp. *haynaldii* methanol extracts tested by broth microdilution assay.

	MIC[a] (μg/mL)						
	A. turbith subsp. *hungarica*		*A. turbith* subsp. *haynaldii*				
Microorganism	Fruits	Leaves	Fruits	Leaves	Ampicillin	Amikacin	Nystatin
Staphylococcus aureus ATCC 25923	125	125	250	500	1	2	nt[b]
Enterococcus faecalis ATCC 29212	250	>500	500	>500	2	nt	nt
Escherichia coli ATCC 25922	250	500	125	250	8	4	nt
Pseudomonas aeruginosa ATCC 27853	>500	>500	>500	>500	nt	2	nt
Klebsiella pneumoniae ATCC 2655	500	>500	>500	>500	nt	nt	nt
Klebsiella pneumoniae NCIMB 9111	500	>500	>500	>500	nt	nt	nt
Candida albicans ATCC 24433	125	125	250	250	nt	nt	3
Candida albicans ATCC 10259	125	125	250	250	nt	nt	3

[a]MIC, minimal inhibitory concentration.
[b]nt, not tested.

Table 4. Antioxidant properties of *Athamanta turbith* subsp. *hungarica* and *A. turbith* subsp. *haynaldii* methanol extracts.

	A. turbith subsp. *hungarica*		*A. turbith* subsp. *haynaldii*				
Assay	Fruits	Leaves	Fruits	Leaves	L-Ascorbic acid	Quercetin	Rutin
Flavonoid content (%)[a]	0.06 ± 0.00	0.16 ± 0.01	0.12 ± 0.01	0.32 ± 0.02			
FRAP values[b]	1.98 ± 0.04	1.60 ± 0.07	0.93 ± 0.04	0.70 ± 0.04	7.41 ± 0.09	7.69 ± 0.04	5.92 ± 0.01
DPPH radical (SC_{50} (μg/mL))	40.25 ± 0.25	45.00 ± 0.50	55.40 ± 0.28	76.67 ± 2.08	4.09 ± 0.08	2.75 ± 0.37	5.75 ± 0.18
Hydroxyl radical (SC_{50} (μg/mL))	47.33 ± 2.52	71.67 ± 2.08	59.20 ± 2.99	111.33 ± 4.16	nt	3.10 ± 0.24	1.45 ± 0.18
Inhibition of LP (I_{max} (%))	47.09 ± 1.10	43.76 ± 1.06	38.28 ± 0.52	34.50 ± 1.09	nt	75.70 ± 0.98	nt

The values are the average of three determinations (± SD).
[a]Determined according to the procedure of DAB 10 (for *Crataegi folium et flores*) (German Pharmacopoeia, 1996).
[b]Expressed as μmol Fe^{2+}/mg dry weigh extract.
SC_{50}, concentration of the sample required to scavenge 50% radical; I_{max}, maximum of inhibition achieved; nt, not tested.

aeruginosa and *Klebsiella pneumoniae* (Tables 2 and 3). In previous investigations on antibacterial properties of luteolin, the best activity was also detected against *S. aureus*, while its activity against *E. faecalis*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* was weaker (Tshikalange et al., 2005; Sato et al., 2000). Zhu et al. (2004) reported that 3,5-dicaffeoylquinic acid and luteolin 7-*O*-rutinoside were active against *S. aureus*, *E. coli*, and *C. albicans*, but had no effect against *P. aeruginosa*.

Antioxidant activity

Results obtained from antioxidant capacity assays are given in Table 4. In the FRAP assay, the extracts of *A. turbith* subsp. *hungarica* exerted higher activity than *A. turbith* subsp. *haynaldii* extracts. Investigated extracts exhibited dose-dependent scavenging of DPPH radical. SC_{50} values for *A. turbith* subsp. *hungarica* extracts suggest high antiradical activity (<50 μg/mL), while activity of *A. turbith* subsp. *haynaldii* extracts could be characterized as moderate (SC_{50} values were slightly above 50 μg/mL) (Cho et al., 2003). The extracts of *A. turbith* subsp. *hungarica* also exhibited higher $HO^{\cdot}$ radical scavenging capacity in 2-deoxyribose assay. In the TBA assay, none of the extracts reached 50% of inhibition.

Antioxidant properties of luteolin and its glycosides were previously confirmed (Heim et al., 2002; Wu et al., 2006). It has been found that presence of the 3′,4′-dihydroxyl phenyl substitution pattern in B-ring, as well as presence of 2,3-double bond in conjugation with a 4-oxo group in C-ring is very important for antioxidant activities of luteolin. Luteolin was also shown to be a significantly stronger antioxidant than its glycosides (Igile et al., 1994; Cao et al., 1997; Arora et al., 1998; Heim et al., 2002; Wu et al., 2006). Higher content of luteolin in the extracts of fruits could explain their higher antioxidant activity in comparison to the extracts of leaves.

Although flavonoid content in *A. turbith* subsp. *hungarica* extracts was about two times lower, their higher antioxidant capacity in comparison to *A. turbith* subsp. *haynaldii* extracts could be explained by the presence of other phenolic compounds such as caffeic acid derivates. In general, caffeic acid derivates are considered as effective antioxidants because the catechol structure donates the phenolic hydrogens or electron to the acceptors such as lipid peroxyl groups or reactive oxygen species (Chakraborty & Mitra, 2007). Caffeic acid was shown to have the same antioxidant capacity as L-ascorbic acid (Kim & Lee, 2004).

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