

In vitro Antimicrobial and Antioxidant Activities of Methanol and Hexane Extract of *Astragalus* Species Growing in the Eastern Anatolia Region of Turkey

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Abstract: This study was conducted to evaluate the antimicrobial and antioxidant activities of the hexane and methanol extracts of the aboveground parts and roots of some *Astragalus* species grown in the vicinity of Erzurum, located in the eastern Anatolia, Turkey. The methanol extract, which was obtained from the aboveground parts of *Astragalus* species, exhibited mild free radical scavenging activity providing 50% inhibition at the concentrations between 68.8 and 400.4 µg/ml. The hexane/dichloromethane extracts of the aboveground parts of *Astragalus* species did not show any free radical scavenging activity. However, the linoleic acid oxidation inhibition ability of the non-polar extracts seems to be higher than that of the polar methanolic extracts. The methanol root extracts were mild antioxidants but the non-polar root extracts of *A. microcephalus* Willd., *A. macrocephalus* Willd., *A. erinaceus* Fisch. & Mey. ex Fischer, *A. psoraloides* Lam., and *A. argyroides* Becker ex Stapf. were extremely active in DPPH test. IC₅₀ values were 35.2 µg/ml, 21.0 µg/ml, 22.0 µg/ml, 20.3 µg/ml, and 38.0 µg/ml, respectively. In the case of β-carotene-linoleic acid system, the extract obtained from *A. psoraloides* Lam. exhibited strong inhibition among studied *Astragalus* species. Furthermore, the extracts, which were obtained from the tested *Astragalus* species, were evaluated for their possible in vitro antibacterial, anticandidal, and antifungal activities based on disk diffusion assay. The results suggested that none of the extracts has antimicrobial activity on the tested microorganisms.

Key Words: *Astragalus* species, antimicrobial activity, antioxidant activity

Türkiye'nin Doğu Anadolu Bölgesinde Yetişen *Astragalus* Türlerinin Metanol ve Hekzan Ekstrelerinin in vitro Koşullar Altında Antimikrobiyal ve Antioksidan Aktivitesi

Özet: Bu çalışma Türkiye'de Doğu Anadolu'da bulunan Erzurum yöresinde yetişen bazı *Astragalus* türlerinin topraküstü ve kök kısımlarının metanol ve hekzan ekstraktlarının antimikrobiyal ve antioksidan aktivitelerinin araştırılması için yapılmıştır. *Astragalus* türlerinin topraküstü kısımlarından elde edilen metanol ekstraktı 68,8-400,4 µg/ml konsantrasyonları aralığında % 50 inhibisyon ile hafif düzeyde serbest radikal süpürücü etki göstermiştir. *Astragalus* türlerinin topraküstü kısımlarının hekzan/diklorometan ekstraktları serbest radikal süpürücü etki göstermemişlerdir. Nonpolar ekstraktların linoleik asit oksidasyonu inhibisyon özelliklerinin polar metanolik ekstraktlarından yüksek olduğu görülmektedir. Kök kısımlarının metanol ekstraktları hafif antioksidanlar iken, *A. microcephalus* Willd., *A. macrocephalus* Willd., *A. erinaceus* Fisch. & Mey. ex Fischer, *A. psoraloides* Lam. ve *A. argyroides* Becker ex Stapf. türlerinin non-polar ekstraktları DPPH testinde son derece aktif olarak görüldüler. IC₅₀ değerleri sırası ile 35,2 µg/ml, 21,0 µg/ml, 22,0 µg/ml, 20,3 µg/ml ve 38,0 µg/ml olarak tespit edildi. β-karoten-linoleik asit sistemi içinde, çalışılan *Astragalus* türleri arasında en güçlü inhibisyon etkisini *A. psoraloides* Lam. türünden elde edilen ekstrakt gösterdi. Buna ek olarak, disk difüzyon yöntemi ile *Astragalus* türlerinin in vitro antibakteriyel, antikandidal ve antifungal aktiviteleri de çalışılmıştır. Sonuçlar ekstraktların hiçbirinin çalışılan mikroorganizmalar üzerinde antimikrobiyal etkisinin olmadığını göstermiştir.

Anahtar Sözcükler: *Astragalus* türleri, antimikrobiyal aktivite, antioksidan aktivite

Introduction

Astragalus L. is the largest genus of flowering plants, containing up to 3000 species (1). This genus is a member of the legume family (Fabaceae) and traditionally classified in the tribe Galageae. The centre of development seems to be in the arid and semi-arid mountainous parts of the Northern Hemisphere. It is mostly diverse in the Irano-Turkish region of southwestern Asia, the Sino-Himalayan Plateau of South Central Asia, the Central Asia region and the Great Basin, and the Colorado Plateau of western North America (2).

It is also the largest genus in Turkey where it is represented by 400 species in 62 sections (3). *Astragali Radix*, the root of *Astragalus membranaceus* (Fisch.) BUNGE, is a crude drug widely used in traditional medicine especially in China. Although *Astragalus* L. is a large family, only a few studies have been reported dealing with the antioxidant ability of this species. Among the many subspecies, *A. membranaceus* is the most investigated species for various biological and medicinal purposes. It was reported that polysaccharides, saponins, and beta glucans, which are the major bioactive compounds obtained from *A. membranaceus*, have various bioactivities including scavenging effects on O_2 and OH. Calycosin and formononetin were found effective among the isoflavones isolated from the roots of *A. membranaceus* (4).

An *Astragalus* saponin isolated from the *A. membranaceus* was reported as an antioxidant agent against superoxide anion and hydroxyl radical (5). This compound was also investigated in vivo for early diabetic nephropathy, which is directly related to the oxidative stress (6).

The antioxidant action of another sub-species, *A. mongholicus*, was also investigated by Yu et al. (7,8). Similar to the study discussed above, formononetin, calycosin, and calycosin-7-O- β -D-glucoside were good free radical scavengers in 1, 1-diphenyl-2-picrylhydrazyl (DPPH) test system (7). However, calycosin and calycosin-7-O- β -D-glucoside were found more active against free radical DPPH than formononetin (8). Additionally, a novel antioxidant chemical astragolose from *A. membranaceus* exhibited significant protective effect in an in vivo study (9).

Antimicrobial properties of *Astragalus* species have not been well investigated. Two new antimicrobial isoflavans, 1-[(3R)-7,8-dimethoxybenzopyran-yl]-hydroxybenzoquinone

(astragaluquinone) and (3S)-7,1'-dihydroxy-8,3'-dimethoxyisoflavan (8-methoxyvestitol), and the known 7-hydroxy-2',3',4'-trimethoxyisoflavan were isolated from roots of *A. alexandrinus* and *A. trigonus* (10).

Antimicrobial properties of *A. membranaceus* species belonging to Chinese traditional medicine were reviewed by Tan and Vanitha (11). In this study *Astragalus* polysaccharides were reported as an antimicrobial agent.

Additionally, the moderate antibacterial activity of *A. sicalus* (12), *A. membranaceus* (13), and *A. melanophrurius* (14) was reported in the literature. In another study, carried out by Pistelli et al. (15), the antimicrobial activity of pure saponins astraverrucins I-VI and crude extracts obtained from aerial parts of *A. verrucocus* were evaluated using disk diffusion method, and antimycotic activity was determined in polar extracts.

In this study, in vitro antimicrobial and antioxidant properties of the methanol and hexane extracts of some *Astragalus* species growing in the eastern part of Turkey were investigated.

Materials and Methods

Plant material

Plant samples of *Astragalus ponticus*, *A. aduncus* Willd., *A. microcephalus* Willd., *A. pinetorum* Boiss., *A. atrocarpus* Champ. & Matthews, *A. lagurus* Willd., *A. macrocephalus* Willd., *A. erinaceus* Fisch. & Mey. ex Fischer, *A. galegiformes* L., *A. trichostigma* Bunge, *A. bicolor* Lam., *A. psoraloides* Lam., *A. argyroides* Becker ex Stapf were collected at flowering stage from different locations in the vicinity of Erzurum, located in the eastern Anatolia, Turkey. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Dr. Meryem Şengül, Department of Biology, Atatürk University, Erzurum-Turkey. The collected plant materials were dried in the shade, and the plant roots were separated from the aboveground parts. The roots and the aboveground parts were separately ground in a grinder with a 2 mm in diameter mesh.

Preparation of Methanol and Hexane extracts

The dried and powdered roots and aboveground parts (500g) were extracted with 1 l methanol and hexane using a Soxhlet extractor (ISOPAD, Heidelberg, Germany) for 72 h at a temperature that is not exceeding the boiling point of the solvent respectively (16). The extract was filtered

using Whatman filter paper (No.1) and then concentrated in vacuo at 40 °C using a Rotary Evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland). The obtained residues were stored in a freezer (Nauire, Plymouth, USA) at -80 °C for further tests.

Antimicrobial activity

Microbial Strains: Methanol and hexane extracts were tested individually against a range of 40 microorganisms including 24 bacteria, 15 fungi, and a yeast species, respectively. The used microorganisms are listed in Tables 1 and 2. Microorganisms were provided by the Department of Clinical Microbiology, Faculty of Medicine, and Plant Diagnostic Laboratory, Faculty of Agriculture, Atatürk University, Erzurum-Turkey. The identities of the

microorganisms used in this study were confirmed by the Microbial Identification System (Sherlock Microbial Identification System Version 4.0, MIDI Inc., Newark, DE, USA) in the Biotechnology Application and Research Center, Atatürk University.

Disk-diffusion assay

The dried plant extracts were dissolved in methanol and hexane to a final concentration of 30 mg/ml and then sterilized by filtration through 0.45 µm Millipore filters (Schleicher & Schuell, MicroScience, Dassel, Germany). Antimicrobial tests were then carried out by the disk diffusion method (17) using 100 µL of suspension containing 10⁸ CFU/ml of bacteria, 10⁶ CFU/ml of yeast, and 10⁴ spore/ml of fungi spread on nutrient agar (NA),

Table 1. Antibacterial activities of *Astragalus* species methanol and hexane extracts against the bacterial strains tested.

Test microorganisms	Plant Extract (MeOH)	Plant Extract (HE)	Antibiotics	
Bacteria	DD ^b	DD ^c	DD ^a	MIC ^d (max)
<i>Acinetobacter baumannii</i> -A8	-	-	18 (OFX)	31.25
<i>Bacillus macerans</i> -A199	-	-	19 (OFX)	15.62
<i>Bacillus megaterium</i> – A59	-	-	9 (SCF)	15.62
<i>Bacillus subtilis</i> -ATCC-6633	-	-	28 (OFX)	62.50
<i>Bacillus subtilis</i> -A57	-	-	28 (OFX)	125
<i>Brucella abortus</i> A77	-	-	12 (SCF)	62.50
<i>Burkholderia cepacia</i> A225	-	-	22 (SCF)	125
<i>Clavibacter michiganense</i> -A227	-	-	25 (SCF)	16.62
<i>Enterobacter cloacae</i> -A135	-	-	20 (NET)	31.25
<i>Enterococcus faecalis</i> -ATCC-29122	-	-	18 (SCF)	31.25
<i>Escherichia coli</i> -A1	-	-	20 (OFX)	62.50
<i>Klebsiella pneumoniae</i> -A137	-	-	12 (OFX)	125
<i>Proteus vulgaris</i> -A161	-	-	12 (OFX)	125
<i>Proteus vulgaris</i> -KUKEM1329	-	-	13 (OFX)	125
<i>Pseudomonas aeruginosa</i> -ATCC9027	-	-	22 (NET)	31.25
<i>Pseudomonas aeruginosa</i> -ATCC27859	-	-	22 (NET)	15.62
<i>Pseudomonas syringae</i> pv. <i>tomato</i> A35	-	-	24 (OFX)	125
<i>Salmonella enteritidis</i> - ATCC-13076	-	-	27 (SCF)	62.50
<i>Staphylococcus aureus</i> - A215	-	-	22 (SCF)	31.25
<i>Staphylococcus aureus</i> -ATCC-29213	-	-	22 (SCF)	62.50
<i>Staphylococcus epidermis</i> -A233	-	-	12 (SCF)	15.62
<i>Streptococcus pyogenes</i> -ATCC-176	-	-	10 (OFX)	62.50
<i>Streptococcus pyogenes</i> -KUKEM-676	-	-	13 (OFX)	31.25
<i>Xanthomonas campestris</i> -A235	-	-	20 (SCF)	31.25

^aDD=Diameter of disk diffusion (mm); NET=Netilmicin, (30 µg/disk), used as positive reference standards antibiotic disks (Oxoid).

^bDD= Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 300 µg/disk of methanol extract

^cDD= Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 300 µg/disk of hexane extract

^dMIC= Amphotericin B (µg/ml), used as reference antibiotic in MIC agar dilution (Sigma).

Table 2. Anticandidal and antifungal activities of the methanol and hexane extracts of *Astragalus* species against the yeast and fungi isolates tested.

Test yeast and fungi	Plant Extract (MeOH)	Plant Extract (HE)	Antibiotics	
Yeast	DD ^b	DD ^c	DD ^b	MIC ^d (max)
<i>Candida albicans</i> -A117	-	-	- (NET)	31.25
Fungi	-	-	-	-
<i>Alternaria alternate</i>	-	-	- (NET)	31.25
<i>Aspergillus flavus</i>	-	-	- (NET)	15.62
<i>Aspergillus varicolor</i>	-	-	- (NET)	15.62
<i>Fusarium acuminatum</i>	-	-	- (NET)	62.50
<i>Fusarium oxysporum</i>	-	-	- (NET)	62.50
<i>Fusarium solani</i>	-	-	- (NET)	62.50
<i>Fusarium tabacinum</i>	-	-	- (NET)	62.50
<i>Monilia fructicola</i>	-	-	- (NET)	15.62
<i>Penicillium</i> spp.	-	-	- (NET)	31.25
<i>Rhizopus</i> spp.	-	-	- (NET)	125
<i>Rhizoctonia solani</i>	-	-	- (NET)	31.25
<i>Sclerotinia minor</i>	-	-	- (NET)	125
<i>Sclerotinia sclerotiorum</i>	-	-	- (NET)	62.50
<i>Trichophyton rubrum</i>	-	-	- (NET)	31.25
<i>Trichophyton mentagrophytes</i>	-	-	- (NET)	15.62

^aDD=Diameter of disk diffusion (mm); NET=Netilmicin, (30 µg/disk), used as positive reference standards antibiotic disks (Oxoid).

^bDD= Inhibition zone in diameter (mm) around the disks (6mm) impregnated with 300 µg/disk of methanol extract

^cDD= Inhibition zone in diameter (mm) around the disks (6mm) impregnated with 300 µg/disk of hexane extract

^dMIC= Amphotericin B (µg/ml), used as a reference antibiotic in MIC agar dilution (Sigma).

Sabouraud dextrose agar (SDA), and potato dextrose agar (PDA) medium, consecutively. The disks (6 mm in diameter) impregnated with 10 µl of essential or 10 µl of the methanol solution of the dried plant extracts (300 µg/disk) were placed on the inoculated agar. Negative controls were prepared with same solvents, which were used to dissolve the plant extracts. Ofloxacin (10 µg/disk), sulbactam (30 µg) + cefoperazone (75 µg) (105 µg/disk) and/or netilmicin (30 µg/disk) were used as positive reference standards to determine the sensitivity of one strain/isolate in each tested microbial species. The inoculated plates were incubated at 37 °C for 24 h for clinical bacterial strains, 48 h for the yeast, and 72 h for fungi isolates. Plant-associated microorganisms were incubated at 27 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay was repeated twice.

Antioxidant activity

DPPH assay: *T* Radical scavenging activity of plant extracts against stable free radical 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH, Sigma-Aldrich Chemie, Steinheim, Germany) was determined

spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. Following the reduction, its deep violet color in methanol bleaches to yellow that shows a significant absorption decrease at 517 nm (18). Then 50 µl of various concentrations of the extracts dissolved in methanol was added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm (ATI-Unicam UV-2 UV-Vis spectrophotometer, Cambridge, UK). Free radical DPPH inhibition in percent (I%) was calculated as follows:

$$I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC₅₀) was calculated from the graph plotted as inhibition percentage against extract concentration. Tests were carried out in triplicate, and butylated hydroxytoluene (BHT) was used as positive control.

β-Carotene-linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides, which are arising from linoleic acid oxidation.

A stock solution of β-carotene/linoleic acid (Sigma-Aldrich) was prepared as follows: First, 0.5 mg of β-carotene was dissolved in 1 ml of chloroform (HPLC grade), then 25 µl of linoleic acid and 200 mg of Tween 40 (Merck) were added. The chloroform was subsequently evaporated using a vacuum evaporator (Büchi, Flawil, Switzerland). Then, 100 ml of distilled water saturated with oxygen (30 min at 100 ml/min) was added by vigorous shaking. Aliquots (2.5 ml) of this reaction mixture were transferred to test tubes, and 350 µl portions of the extracts (2 g/l in ethanol) were added before incubating for 48 h at room temperature. The same procedure was repeated with BHT at the same concentration and a blank containing only 350 µl of ethanol. After the incubation period, the absorbance values of the mixtures were measured at 490 nm. The antioxidant capacities of the samples were compared with those of BHT and the blank.

Statistical analysis

SPSS for Windows version 11.0 was used for statistical analysis. Results were expressed as average ± standard deviation (SD).

Results and Discussion

Two in vitro test methods, DPPH test and β-carotene/linoleic acid oxidation test, were used to evaluate the antioxidant properties of *Astragalus* species. The results of the hexane and the methanol extracts of the roots and the aboveground parts (stem) of *Astragalus* species were given in Tables 3 and 4. 4-Buthylatedhydroxytoluene (BHT) was used as positive control for both test methods.

Antioxidant activity of the extracts obtained from the aboveground part of *Astragalus* species: The methanol extract of *Astragalus* species exhibited mild free radical scavenging activity providing 50% inhibition at the concentrations between 68.8 and 400.4 µg/ml. Hexane/dichloromethane extracts of all *Astragalus* species did not show free radical scavenging activity. Hexane/dichloromethane extracts were not soluble in methanol over 10 mg/ml concentration. Therefore, these extracts were found not active for lower concentration. However, the linoleic acid oxidation inhibition ability of the non-polar extracts seems to be higher than the polar methanolic extracts. Especially, *A. pinetorum*, *A. macrocephalus*, and *A. argyroides* are strongly inhibiting the oxidation.

Antioxidant activity of the extracts obtained from roots: The methanolic root extracts were mild antioxidants, but the non-polar extracts of *A. microcephalus*, *A.*

Table 3. IC₅₀ and relative antioxidant activity (RAA%) of the aboveground part of the extracts of the studied *Astragalus* species.

Species	Stalk			
	Methanol		Hexane/Dichloromethane	
	DPPH IC ₅₀ (µg/ml)±0.5	β-Carotene RAA%±1	DPPH IC ₅₀ (µg/ml)±0.5	β-Carotene RAA%±1
<i>A. ponticus</i>	141.1	53.2	N.A.	57.0
<i>A. aduncus</i> Willd.	N.A.	61.4	N.A.	82.5
<i>A. microcephalus</i> Willd.	200.0	45.4	N.A.	61.8
<i>A. pinetorum</i> Boiss.	400.4	61.4	N.A.	100.0
<i>A. atrocarpus</i> Champ. & Matthews	297.0	42.0	N.A.	85.3
<i>A. lagurus</i> Willd.	366.3	40.7	N.A.	46.0
<i>A. macrocephalus</i> Willd.	217.8	52.3	N.A.	93.1
<i>A. erinaceus</i> Fisch. & Mey. ex Fischer	170.4	83.3	N.A.	56.5
<i>A. galegiformes</i> L.	280.0	39.7	N.A.	71.7
<i>A. trichostigma</i> Bunge	221.0	43.8	N.A.	37.9
<i>A. bicolor</i> Lam.	190.1	42.2	N.A.	72.7
<i>A. psoraloides</i> Lam.	N.D.	N.D.	N.D.	N.D.
<i>A. argyroides</i> Becker ex Stapf	68.8	26.4	N.A.	94.0

N.A.: Not Active

N.D.: Not Determined

BHT: IC₅₀ = 19.8 ± 0.5 µg/ml and RAA% = 100 ± 1

Table 4. IC₅₀ and relative antioxidant activity (RAA%) of the root extracts of the studied *Astragalus* species.

Species	Root			
	Methanol		Hexane/Dichloromethane	
	DPPH IC ₅₀ (µg/ml)±0.5	β–Carotene RAA%±1	DPPH IC ₅₀ (µg/ml)±0.5	β–Carotene RAA%±1
<i>A. ponticus</i>	207.9	49.4	N.A	69.8
<i>A. aduncus</i> Willd.	N.D.	N.D.	N.D.	N.D.
<i>A. microcephalus</i> Willd.	227.7	51.4	35.2	58.1
<i>A. pinetorum</i> Boiss.	N.D.	N.D.	N.D.	N.D.
<i>A. atrocarpus</i> Champ. & Matthews	N.D.	N.D.	N.D.	N.D.
<i>A. lagurus</i> Willd.	N.D.	N.D.	N.D.	N.D.
<i>A. macrocephalus</i> Willd.	170.5	58.6	21.0	64.4
<i>A. erinaceus</i> Fisch. & Mey. ex Fischer	328.5	38.2	22.0	61.2
<i>A. galegiformes</i> L.	N.D.	N.D.	N.D.	N.D.
<i>A. trichostigma</i> Bunge	N.D.	N.D.	N.D.	N.D.
<i>A. bicolor</i> Lam.	N.A.	23.8	N.A.	56.3
<i>A. psoraloides</i> Lam.	115.1	88.1	20.3	100.0
<i>A. argyroides</i> Becker ex Stapf	126.7	45.3	38.0	20.0

N.A.: Not Active

N.D.: Not Determined

BHT: IC₅₀ = 19.8 ± 0.5 µg/ml and RAA% = 100 ± 1

macrocephalus, *A. erinoceus*, *A. psoraloides*, and *A. argyroides* were extremely active in DPPH test. IC₅₀ values were 35.2 µg/ml, 21.0 µg/ml, 22.0 µg/ml 20.3 µg/ml, and 38.0 µg/ml, respectively.

In the case of β–carotene-linoleic acid system, the extract obtained from *A. psoraloides* exhibited strong inhibition among studied *Astragalus* species.

Methanol or hexane extraction is used for flavonoid, flavone, or saponin containing plants. It is known that *Astragalus* species contain antioxidant isoflavones (4) and saponins (5). Our results showed that methanolic extracts from the aboveground part and roots exhibit excellent antioxidant ability. Additionally, a very good inhibition effect is also observed in the non-polar extracts. Since these activities observed in only crude extracts, the isolation and identification of the bioactive components should be considered in details.

Although it is known from the previous study, the extracts of *A. siculus*, *A. gummifer*, *A. membranaceus*, *A. melanophrurius*, and *A. verrucosus* exhibit moderate

antibacterial effect against Gram-positive and Gram-negative tested strains (15), in our study antimicrobial test results showed that none of the extracts tested has inhibitory activity against any of the microorganisms tested.

As a result, the data of the present study indicate that the extracts of 4 examined endemic *Astragalus* species possess antioxidants but not antimicrobial compounds.

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