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Antimicrobial and Antioxidant Activities of Methanol and Hexane Extract of Some Endemic *Astragalus* Species

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The present study is related to evaluate antimicrobial and antioxidant activities of hexane and methanol extracts of four endemic *Astragalus* species grown in the vicinity of Erzurum, located in the eastern Anatolia, Turkey. Methanol extract of *Astragalus* species exhibited better free radical scavenging activity providing 50 % inhibition at the concentration in the range of 21.0-250.3 µg/mL. In the case of β-carotene-linoleic acid system, both extracts obtained from *A. campylosema* and *A. cristianus* were active among studied *Astragalus* species. *A. campylosema* root extracts completely inhibit the oxidation of linoleic acid in the studied test systems. On the other hand, the extracts obtained from *Astragalus* species tested have been evaluated for their possible *in vitro* antibacterial, anticandidal and antifungal activities based on disk diffusion assay. The results suggested that none of the extracts have antimicrobial activity on the microorganisms tested.

Key Words: *Astragalus* species, Antimicrobial activity, Antioxidant activity.

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[2]. It is most diverse in the Irano-Turkish region of South-western Asia, the Sino-Himalayan Plateau of south Central Asia, the Central Asian region and the Great Basin and Colorado Plateau of western North America[2].

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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 has been reported dealing with the antioxidant ability of this species. Among the many subspecies *A. membranaceus* is the most investigated specie for various biological and medicinal purposes. Reported major bioactive compounds were polysaccharides, saponins and β-glucan obtained from *A. membranaceus* known biologically, which various bioactivities including the scavenging effects on O_2^- and OH. Inhibitory effects of isoflavones on lipid peroxidation namely afrormosin, calycosin, farmononetin and odoratin, had been tested in a test system involved lecithin peroxidation. Calycosin and formononetin were found effective among the isoflavones isolated form 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 directly related to oxidative stress[6].

The antioxidant action of another sub-species *A. mongholicus* was also investigated by Yu *et al.*[7,8]. Similar to above discussed study formononetin, calycosin and calycosin-7-O-β-D-glucoside had been found good free radical scavengers in 1,1-diphenyl-2-picrylhydrazyl (DPPH) test system[7]. But calycosin and calycosin-7-O-β-D-glucoside were found more active to free radical DPPH than formononetin[8]. Additionally, a novel antioxidant chemical astragoloside 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 isoflavons, 1-[(3R)-7,8-dimethoxybenzopyranyl]-4-hydroxybenzoquinone (astragaluquinone) and (3S)-7,1'-dihydroxy-8,3'-dimethoxyisoflavan (8-methoxyvestitol) and the known 7-hydroxy-2',3',4'-trimethoxyisoflavan had been isolated from roots of *A. alexandrinus* and *A. trigonus*[10].

Antimicrobial properties of *A. membranaceus* species belonging to Chinese traditional medicine had been reviewed by Tan and Vanitha[11]. In this study astragalus polysaccharides were reported as antimicrobial agent.

Additionally, moderate antibacterial activity of *A. siculus*[12], *A. membranaceus*[13] and *A. melanophrurius*[14] had been reported in the literature. 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 disc diffusion method and antimycotic activity had been determined in polar extracts.

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

EXPERIMENTAL

Plant samples of *A. cristianus, A. campylosema, A. lineatus* and *A. globosus* 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 Sengul, in Department of Biology, Atatürk University, Erzurum, Turkey. Collected plant materials were dried in the shade and the leaves of plant were separated from the stem and ground in a grinder with a 2 mm in diameter mesh.

Methanol used in preparing the methanol extract.was obtained from Sigma (St. Louis, MO). Hexane used in preparing the hexane extract was obtained from Sigma (St. Louis, MO). Nutrient agar (NA), sabouraud dextrose agar, potato dextrose agar (PDA) used for Disk-diffusion assay were obtained from Fluka. Ofloxacin, sulbactam cefoperazone, netilmicin were obtained from Oxoid. Dimethyl sulfoxide for micro-well dilution assay was obtained from Sigma (St. Louis, MO). Chemicals used in antioxidant activity and total phenolics assays were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). All solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Preparation of the extracts: The dried and powdered leaves (500 g) were extracted with 1 L of methanol and hexane using a Soxhlet extractor (ISOPAD, Heidelberg, Germany) for 72 h at a temperature 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 ºC using a Rotary evaporator (BÜCHI Labortechnic AG, Flawil, Switzerland). The residues obtained were stored in a freezer (Nauire, Plymouth, USA) at -80 ºC until 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 microorganisms used 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 at Atatürk University, Erzurum, Turkey. The identity of the microorganisms used in this study was confirmed by the Microbial Identification System (Sherlock Microbial Identification System Version 4.0, MIDI Inc., New York, USA) in the Biotechnology Application and Research Center at Atatürk University.

TABLE-1
ANTIMICROBIAL ACTIVITIES OF *Astragalus* SPECIES METHANOL AND HEXANE EXTRACTS AGAINST THE BACTERIAL STRAINS TESTED

Test microorganisms	Plant extract (MeOH)	Plant extract (HE)	Antibiotics	
	DD[b]	DD[c]	DD[a]	MIC[d] max
Acinetobacter baumanii-A8	-	-	18 (OFX)	31.25
Bacillus macerans-A199	-	-	19 (OFX)	15.62
Bacillus megaterium – A59	-	-	9 (SCF)	15.62
Bacillus subtilis-ATCC-6633	-	-	28 (OFX)	62.50
Bacillus subtilis-A57	-	-	28 (OFX)	125.00
Brucella abortus A77	-	-	12 (SCF)	62.50
Burkholdria cepacia A225	-	-	22 (SCF)	125.00
Clavibacter michiganense-A227	-	-	25 (SCF)	16.62
Enterobacter cloacae-A135	-	-	20 (NET	31.25
Enterococcus faecalis-ATCC-29122	-	-	18 (SCF)	31.25
Escherichia coli-A1	-	-	20 (OFX)	62.50
Klebsiella pneumoniae-A137	-	-	12 (OFX)	125.00
Proteus vulgaris-A161	-	-	12 (OFX)	125.00
Proteus vulgaris-KUKEM1329	-	-	13 (OFX)	125.00
Pseudomonas aeruginosa-ATCC9027	-	-	22 (NET)	31.25
*Pseudomonas aeruginosa*ATCC27859	-	-	22 (NET)	15.62
Pseudomonas syringae pv.*tomato* A35	-	-	24 (OFX)	125.00
Salmonella enteritidis- ATCC-13076	-	-	27 (SCF)	62.50
Staphylococcus aureus- A215	-	-	22 (SCF)	31.25
Staphylococcus aureus-ATCC-29213	-	-	22 (SCF)	62.50
Staphylococcus epidermis-A233	-	-	12 (SCF)	15.62
Streptococcus pyogenes-ATCC-176	-	-	10 (OFX)	62.50
Streptococcus pyogenes-KUKEM-676	-	-	13 (OFX)	31.25
Xanthomonas campestris-A235	-	-	20 (SCF)	31.25

[a]DD = Diameter of disk diffusion (mm); NET = Netilmicin, (30 µg/disk) were used as positive reference standards antibiotic disks (Oxoid).

[b]DD = Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 300 µg/disk of methanol extract

[c]DD = Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 300 µg/disk of hexane extract

[d]MIC = Amphotericin B (µg/mL) was 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	
	DD[b]	DD[c]	DD[a]	MIC[d] max
Candida albicans-A117	-	-	- (NET)	31.25
Fungi	-	-		
Alternaria alternate	-	-	- (NET)	31.25
Aspergillus flavus	-	-	- (NET)	15.62
Aspergillus variecolor	-	-	- (NET)	15.62
Fusarium acuminatum	-	-	- (NET)	62.50
Fusarium oxysporum	-	-	- (NET)	62.50
Fusarium solani	-	-	- (NET)	62.50
Fusarium tabacinum	-	-	- (NET)	62.50
Moniliania fructicola	-	-	- (NET)	15.62
Penicillum spp.	-	-	- (NET)	31.25
Rhizopus spp.	-	-	- (NET)	125.00
Rhizoctonia solani	-	-	- (NET)	31.25
Sclorotinia minor	-	-	- (NET)	125.00
Sclorotinia sclerotiorum	-	-	- (NET)	62.50
Trichophyton rubrum	-	-	- (NET)	31.25
Trichophyton mentagrophytes	-	-	- (NET)	15.62

[a]DD = Diameter of disk diffusion (mm); NET = Netilmicin, (30 µg/disk) were used as positive reference standards antibiotic disks (Oxoid).
[b]DD = Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 300 µg/disk of methanol extract.
[c]DD = Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 300 µg/disk of hexane extract.
[d]MIC = Amphotericin B (µg/mL) was used as reference antibiotic in MIC agar dilution (Sigma).

Disk-diffusion assay: The dried plant extracts were dissolved in methanol and hexane to a final concentration of 30 mg/mL and sterilized by filtration through 0.45 µm Millipore filters, respectively (Schleicher & Schuell, Microscience, Dassel, Germany). Antimicrobial tests were then carried out by the disk diffusion method[17] using 100 µL of suspension containing 10^8 CFU (Colony Forming Units)/mL of bacteria, 10^6 CFU/mL of yeast and 10^4 spore/mL of fungi spread on nutrient agar (NA), sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) medium, respectively. 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/disc) were placed on the inoculated agar. Negative controls were prepared with the same solvents 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 microbial species tested. The inoculated plates were incubated at 37 ºC 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 ºC. 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, Germany) was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. Following reduction its deep violet colour in methanol bleaches to yellow that means a significant absorption decrease at 517 nm[18]. 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 0.5 h incubation period at room temperature the absorbance was read against a blank at 517 nm (ATI-Unicam UV-2 UV-Vis spectrophotometer, Cambridge, UK). Inhibition free radical DPPH in per cent (I %) was calculated as follows:

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

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 (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate and butylated hydroxy-toluene (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 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 (0.5 h at 100 mL/min) was added with 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 of the mixtures were measured at 490 nm. 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 averaged ± 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 endemic *Astragalus* species. The results of hexane and methanol extracts of the roots and ground above parts (stalks) of *Astragalus* species are given in Table-1. Butylated hydroxytoluene (BHT) was used as positive control for both test method.

Methanol extract of *Astragalus* species exhibited better free radical scavenging activity providing 50 % inhibition at the concentration in the range of 21.0-250.3 μg/mL. Although activities were lower than BHT, studied species could be considered as effective radical scavenger. Free radical scavenging action of hexane/dichloromethane extracts of all *Astragalus* species were observed except *A. cristianus*. Hexane/dichloromethane extracts were not soluble in methanol over 10 mg/mL concentration. Therefore, these extracts were found not active for lower concentration. But both extracts obtained from the roots and stalks of *A. cristianus* were extremely active in DPPH test. IC_{50} values were 21.0 and 20.3 μg/mL, respectively.

In the case of β-carotene-linoleic acid system, extracts obtained from *A. campylosema* and *A. cristianus* were also very active among studied *Astragalus* species. *A. campylosema* root extracts (both methanol and hexane/dichloromethane) completely inhibit the oxidation of linoleic acid in the studied test systems. Methanol or hexane extraction is used for flavonoid, flavone or saponin containing plants. It is known that *Astagalus* species contain antioxidant isoflavones[4] and saponins[5]. The present results shows that methanolic extracts from stem and roots exhibit excellent antioxidant ability. Additionally, a very good inhibition effect was also observed in the non polar extracts. Since these activities observed in only crude extracts, isolation and identification of the bioactive components should be considered in details. The present results show that methanolic extracts from above ground part and roots exhibit excellent antioxidant ability. Additionally, a very good inhibition effect was also observed in the non polar extracts. Since these activities observed in only crude extracts, isolation and identification of the bioactive components should be considered in

details. Antimicrobial test results showed that none of the extracts have inhibitory activity against any of the microorganisms tested. Therefore, the data of the present study indicate that the extracts of four endemic Astragallus species examined possess antioxidant but not antimicrobial compounds.

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