

Preliminary Phytochemical Screening and In-vitro Evaluation of Antioxidant and Antimicrobial Activities for *Astragalus pelecinus* from Palestine

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Received 11 Aug 2016,
Revised 18 Jan 2017,
Accepted 20 Jan 2017

Keywords

- ✓ *Astragalus pelecinus*;
- ✓ Phytochemical screening;
- ✓ Antioxidants;
- ✓ Antimicrobial

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ABSTRACT

Astragalus pelecinus aerial parts have been utilized in the Palestinian traditional medicine for healing of the infected wounds. This study aimed to screen the phytochemical compounds in the acetone, ethanol, methanol and aqueous extracts, evaluate the antimicrobial and antioxidant activities of the plant aerial parts. The antioxidant property of extract was evaluated using 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity method. Detection of antimicrobial activity and determination of minimal inhibitory concentration of aqueous and methanolic extracts of *A. pelecinus* were performed using broth micro-dilution method. The antimicrobial activity was examined against American Type Culture Collection *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, as well as against *Staphylococcus aureus* (MRSA) and a clinical isolate of *Candida albicans*. The results showed that *A. pelecinus* methanol, acetone, aqueous, and ethanolic extracts contain proteins, carbohydrates, phenolic tannins, glycosides, flavonoids, terpenoids and alkaloids. Revealed that, this plant had antioxidant activity ($IC_{50} = 70.78 \pm 1.12 \mu\text{g/ml}$) and its aqueous extract possessed antimicrobial activity against *Staphylococcus aureus*, MRSA, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*, the MIC values were 33, 33, 33, 66 and 33 mg/ml, respectively. On the other hand, methanol extract exhibited antimicrobial effect against *Staphylococcus aureus*, MRSA and *Escherichia coli*, where the MIC value was 51.5 mg/ml in all cases. The present study showed that *Astragalus pelecinus* plant had biological active molecules which explained its traditional use in healing of infected wounds. *A. pelecinus* can provide natural source of antimicrobial and antioxidant medications and may be useful in preventing various dangerous diseases including diabetes mellitus, cardiac diseases and cancer.

1. Introduction

The use of herbs for manufacturing of medicaments has historical roots and since prehistoric times, they played an important role in treatment and prevention of various diseases and in controlling different human pathogens. Throughout history, many of herbal remedies were described, but few of them are accepted in the modernistic medicine. In the 20th century, the discovery of effective and safe new drugs had come to depend on the application of scientific principles, to understand why some treatments are effective and others are not [1].

It is rational to suppose that many medications can be found in the nature especially in the plant kingdom if the search is carried out in a systematic organized manner since there are at least 250,000 species of higher plants in the world. Twenty percent of plants found on earth have been considered as medicinal plants containing biologically and pharmacologically active compounds [2,3].

The first and most important step in manufacturing of new phyto-genic medication should be started with gathering information on the folk uses of these plants from various nations' cultures. Ethnopharmacology can provide the basic information as a 'pre-screen' to select plants for experimental biological and pharmacological experiments [4]. The plants biosynthesized a huge number of phyto-chemicals called secondary metabolites which are divided into different classes based on their pharmacological function like bacteriostatic, chemotherapeutic, bactericidal, antifungal agents and others [5]. There is pressing urgent need to search new antimicrobial and antioxidant medications with diverse mechanisms of action and various chemical structures

for re-emerging infectious and chronic diseases, especially in fighting the resistant of pathogenic microorganisms, cancer, diabetes mellitus, cardiovascular diseases and others [6,7]. The investigation of drugs from medicinal plants is slow; however, their use is becoming popular due of their safety issue [8].

The *Astragalus* plant genus is the largest class of flowering herbs belonging to the Leguminosae family, with about 3000 species. Most species are herbaceous annual none climbing plants. The bulk of diversity in *Astragalus* is centered in central and southwestern regions of Asia. In Palestine, 47 *Astragalus* species were found and widely distributed mainly in warm arid, semiarid and cool regions of the northern areas [9-14]. The leaves of *Astragalus pelecinus* are sticky, linear, glandulous, short-stalked, indented at the top, compound impair-pinnate, oblong to round alternate with 8-12 pairs of leaflets, while the flowers are from 4 to 6 mm long, symmetrically bilateral with purple or blue color as well as the fruits form is pods (legumes) from 5 to 10 mm across and from 10 to 40 mm long, curling on a face and flat on the other with two toothed margins [15]. To the best of author's knowledge, there were no previous studies about the phytoconstituents, biological and pharmacological potentials of *A. pelecinus* aerial parts and our study will be the first one.

2. Materials and methods

2.1. Reagents

The following reagents were used in evaluation of antioxidant activity: Methanol, n- hexane (Lobachemie, India), Trolox ((s)-(-)-6 hydroxy-2, 5, 7, 8-tetramethylchroman-2 carboxylic acid) (Sigma-Aldrich, Denmark), (DPPH) 2, 2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Germany).

Phytochemical and antimicrobial screening tests included the following reagents: Millon's reagent, Ninhydrin solution (Alfa Agar, England), Benedict's reagent, Molish's reagent, H₂SO₄, iodine solution (Alfa aesar, England), NaOH, chloroform HCl (Sigma Aldrich, Germany) magnesium ribbon, acetic acid, FeCl₃ (Riedeldehan, Germany), Mueller-Hinton broth (Himedia, India) and Dimethyl sulfoxide (DMSO) (Riedeldehan, Germany).

2.2. Instrumentation

Shaker device (Memmert Shaking Incubator, Germany), rotary evaporator (Heidolph OB2000, Germany), spectrophotometer (Jenway 7135, England), freeze dryer (Mill rock technology, model BT85, Danfoss, China), grinder (Moulinex, Uno, China), balance (Radw ag, AS 220/c/2, Poland), filter paper (Machrery-Nagel, MN 617 and Whatman no.1, USA), micropipettes (Finnpipette, Finland), incubator (Nuve, Turkey), syringe filter 0.45 μ m pore size (Microlab, China) and micro broth plate (Greiner bio-one, North America).

2.3. Collection and preparing plant materials

The aerial parts of *Astragalus pelecinus* were collected in June, 2014 during the flowering time from Jerusalem mountains of Palestine. Botanical identification was carried out at the Pharmacognosy and Herbal Products Laboratory at An-Najah National University by the Pharmacognosist Dr. Nidal Jaradat by using herbarium and photos from books [16] and the voucher specimen code was (Pharm-PCT-314).

The aerial *A. pelecinus* parts were washed, and then dried in the shade at room temperature until all the plants parts became well dried. After drying process, the plants materials were ground well into fine powder using mechanical blender and transferred into special containers with proper name labeling for future use.

2.4. Preparation of plant extracts for phytochemical analysis

The phytochemical extraction was performed using organic solvent extraction as well as aqueous extraction. The organic extraction was performed by Soxhlet extraction method. This extraction was conducted by taking 20 g of dried *A. pelecinus* powder and was uniformly packed into a thimble and extracted with 250 ml of different solvents separately (ethanol, acetone and methanol). After that the extract was heated on hot water bath at 30-40°C till all the solvent evaporated. The dried plant crude extract was kept in refrigerator at 2-8 °C for their future use. The aqueous extraction was done by taking five grams of the powdered plant and was placed in a beaker with 200 ml of distilled water. The mixture was heated on a hot plate at 30-40 °C and mixed with continuous stirring for 20 minutes. The mixture was filtered using whatman filter paper and the filtrate was used for the phytochemical analysis.

2.5. Preparation of plant extracts for biological tests

A total of 25 g of the powder of *A. pelecinus* plant was weighed and exhaustively extracted by adding 50 ml of n-hexane and 125 ml of 50% ethanol in triply distilled water. The mixture was placed in the shaker for 72 hours

at 25 °C with continuous shaking at (200 rpm) then filtered by using suction flask and Buchner funnel filtration. The resulting liquid filtrate was separated by separatory funnel into 2 phases. The lower phase, which is the aqueous phase representing the first aqueous extract and the upper phase, which is the organic phase representing the organic extract. The remaining solid filtrate was extracted again by adding 125 ml of 50% ethanol in triple distilled water and was placed in the shaking incubator for 72 hours at 25°C with continuous shaking at (200 rpm) as before. After that it was filtered to obtain the second aqueous extract. Both first and second aqueous extracts were pooled together and placed in the rotary evaporator for 1 hour at 40 °C to evaporate any leftover organic solvents. Then they were dried completely for 24 hours and stored in refrigerator at 4 °C.

2.6. Preparation of plant extracts for antioxidant evaluation

About 10 g of the grounded plant were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours at room temperature and stored in refrigerator for 4 days. The extracts were then filtered using filter papers. The extract was then concentrated under vacuum using a rotary evaporator. The crude extract was stored at 4°C for further use.

2.7. Antioxidant activity

A stock solution of a concentration of 1 mg/ml in methanol was firstly prepared for the plant extract and Trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/ml) were prepared by serial dilution with methanol from the stock solution.

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ratio of 1:1:1. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in dark for 30 minutes at room temperature before the absorbance readings were recorded at 517 nm. The percentage of antioxidant activity of the plants and the Trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = $(A-B)/A \times 100\%$

Where: A = optical density of the blank, B = optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC₅₀) for the plant samples and the standard deviation were calculated using BioDataFit edition 1.02 (data fit for biologist). The antioxidant activity was reported as percentage of inhibition. The inhibition of *A. pelecinus* plant and Trolox standard at different concentration were plotted and tabulated and the IC₅₀ for each of them was calculated using the BioDataFit fitting program.

2.8. Qualitative phytochemical analysis

The crude ethanolic, methanolic, acetone and aqueous extracts were tested for the presence of phytochemical compounds by using standard identification methods described by Trease and Evans, 1983 and Harborne, 1998 [17,18].

2.9. Antimicrobial tests

Antibacterial activities of aqueous and organic extracts of *A. pelecinus* were examined against 4 reference bacterial strains obtained from the American Type Culture Collection (ATCC), which were *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MRSA Positive), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Furthermore, antifungal activities of aqueous and organic extracts of *Astragalus pelecinus* were examined against a clinical isolates of *Candida albicans*, which was obtained from Talkarim hospital in Palestine. Identification of the isolate was confirmed by colony morphology, microscopic examination, germ tube and chlamyospore production tests.

The detection of *A. pelecinus* antibacterial and antifungal activity of the methanolic and aqueous extracts of *A. pelecinus* were carried out using broth microdilution method. The applied method was similar to that of CLSI [19,20]. Aqueous and organic extract were dissolved in 5% Dimethyl sulfoxide (DMSO) achieving a concentration of 132 mg/ml and 103 mg/ml, respectively. Syringe filters with 0.45 µm pore size were used to sterilize the resulting solutions. Plant extract solutions were serially diluted (2-fold) 11 times with Mueller-Hinton broth. Well number 11 was considered negative control of microorganism growth, while well number 12 contained Mueller-Hinton broth only and was used as positive control of microbial growth. The final bacterial concentration in each well (except negative control) was adjusted to 5×10^5

CFU/ml (CFU: colony-forming unit). On the other hand, yeast concentration in each well was adjusted to 0.5×10^3 to 2.5×10^3 CFU/ml.

In antibacterial activity test, the achieved 10 concentrations of aqueous and organic plant extract were from 0.129 to 66 mg/ml and 0.1 to 51.5 mg/ml, respectively. While the concentration in antifungal test was 0.065 to 33 mg/ml and 0.05 to 25.75 mg/ml in of aqueous and organic plant extract, respectively. After inoculation of microorganisms, the plates were covered and incubated at 35 °C. The incubation period was 18 hours for bacterial isolates and 48 hours for *Candida albicans*. Each microorganism isolate was examined in duplicate. The lowest concentration of plant extract that did not allow any visible microorganism growth in the test broth was considered minimal inhibitory concentration (MIC).

3. Results and Discussion

3.1 Phytochemical screening

The phytochemical characteristics of *A. pelecinus* aerial parts were summarized in Table 1. It could be seen that proteins, phenols, tannins, flavonoids, glycosides and alkaloids were present which point out towards the richness of plant in secondary metabolites. On the other hand saponin and steroids were absent.

Table 1: Phytochemical constituents of *A. pelecinus* plant

Plant extracts	Proteins	Carbohydrate	Phenols/Tannins	Flavonoids	Saponin	Glycosides	Steroids	Terpenoids	Alkaloids
<i>A. pelecinus</i> methanolic extract	+	+	+	+	-	+	-	+	+
<i>A. pelecinus</i> aqueous extract	+	+	+	+	-	+	-	+	+
<i>A. pelecinus</i> acetone extract	+	+	+	+	-	+	-	+	+
<i>A. pelecinus</i> ethanolic extract	+	+	+	+	-	+	-	+	+

3.2. Antioxidant capacity

The free radical scavenging activity of the methanolic extract of *A. pelecinus* has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100 µg/ml. The zero inhibition was considered for the solution, which contained only DPPH without any plant extract. The results revealed a mild antioxidant activity with IC_{50} 70.78 ± 1.12 µg/ml which is comparable to Trolox standard. The detailed results are shown in Table 2.

Table 2: Inhibition activity for Trolox standard and *A. pelecinus* plant.

Concentration	% of inhibition by Trolox, \pm SD	% of inhibition by <i>A. pelecinus</i> , \pm SD
1	33.83 \pm 1.62	7.06 \pm 1.17
2	36.43 \pm 1.76	7.80 \pm 1.14
3	41.26 \pm 1.33	8.55 \pm 1.03
5	54.65 \pm 1.21	10.03 \pm 1.68
7	69.52 \pm 1.54	11.52 \pm 1.22
10	93.68 \pm 1.17	13.38 \pm 1.08
20	95.17 \pm 1.32	14.87 \pm 1.25
30	95.54 \pm 1.88	22.30 \pm 1.00
40	95.54 \pm 1.93	26.77 \pm 1.36
50	95.54 \pm 1.15	33.46 \pm 1.84
80	95.54 \pm 1.77	53.53 \pm 1.42
100	95.54 \pm 1.46	58.00 \pm 1.33
IC_{50}	3.26 \pm 0.22	70.78 \pm 1.12

The multiple drug resistance not only increases mortality and morbidity but also increased expenditure on patient implementation and management of infection control measures [21]. The current study, DPPH method, which relies on the reduction of 2, 2-diphenylpicrylhydrazyl (DPPH) radical, was used. This method is simple, fast and inexpensive for measuring the antioxidant capacity. Furthermore, it is not specific to any particular antioxidant component and could be applied to either solid or liquid samples. The DPPH with free radical has a purple color and a strong absorption maximum at 517 nm. When the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant it will be reduced to DPPH-H, the color becomes yellow and the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 [22]. The antioxidant activity is usually compared with a reference standard and a common example is Trolox. Trolox is a (Hoffman-La Roche) trade name for (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); a water soluble vitamin E analogue used in this research as an antioxidant standard [23].

3.3. Antimicrobial activity

The aqueous extract of *A. pelecinus* exhibited antimicrobial activity against all examined microorganisms. It inhibited the growth of *S. aureus*, MRSA and *E. coli* at the same level (MIC = 33 mg/ml). Higher concentration of aqueous extract (MIC = 66 mg/ml) was required to inhibit the growth of *P. aeruginosa*. Growth of *C. albicans* was also inhibited by aqueous extract (MIC= 33 mg/ml). Methanol extract of *A. pelecinus* failed to show antimicrobial activity against *P. aeruginosa* and *C. albicans*. Furthermore, lower level of inhibition by its methanolic extract (MIC= 51.5 mg/ml) was detected against *S. aureus*, MRSA and *E. coli* as shown in Table 3.

Table 3: Antimicrobial activities of aqueous and methanolic extracts of *A. pelecinus*

Microorganism	MIC value (mg/ml)	
	Aqueous extract	Methanol extract
<i>Escherichia coli</i> (ATCC 25922)	33	51.5
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	66	No inhibition
<i>Staphylococcus aureus</i> (ATCC 25923)	33	51.5
<i>Staphylococcus aureus</i> (MRSA Positive)	33	51.5
<i>Candida albicans</i>	33	No inhibition

In comparing with other species of Astragalus plant which is *A. membranaceus* considered the most common Astragalus species used in the Traditional Chinese Medicine specifically, its constituents of the dried roots provided significant protection against brain, heart, intestine, kidney, lung and liver injury in various models of oxidative stress related diseases [24]. Meanwhile, *A. membranaceus* had many studies which were performed about its anticancer [25], anti-diabetic [26] and cardio-protective effects [27].

To the best of our knowledge, there were no previous studies about the antioxidant and antimicrobial activities on *A. pelecinus* and this study is the first one. It is worth to notify that, it is important in the future to study its effects against cancer, diabetes and cardiac diseases as well as its neighbor species had these effects.

Conclusions

In this study, the phytochemical, antimicrobial and antioxidant properties of the extracts of *A. pelecinus* were evaluated. Its aqueous extract showed antibacterial and antifungal activities, while its methanol extract possessed antibacterial activity not to all the examined strains. The demonstration of broad spectrum of antimicrobial activity by *A. pelecinus* may help in discovering new chemical classes of antibiotics that could serve as selective agents for infectious disease chemotherapy, and to be one of most important arms in fighting bacterial infections, and to play important role in improving the life quality of humans.

Acknowledgments- This study was not funded. Sincere thanks to all participants in this study are also acknowledged.

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(2017) ; <http://www.jmaterenviromsci.com>