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Comparative chemical and biological investigations of three Saudi *Astragalus* species

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ABSTRACT

Aim: The chemical profile and biological activities of alcoholic extracts of the aerial parts of three Saudi *Astragalus* species have been comparatively investigated in this research.
Materials and Methods: Three Saudi *Astragalus* species (*A. spinosus* Vahl, *A. armatus* Willd, and *A. sieberi* DC.) were collected from the wild area of Rafhaa city, Northern border region in Saudi Arabia. Phytochemical screening was carried out using the general standard procedure, total flavonoid content (TFC) and total polyphenolic content (TPC) were determined by $AlCl_3$ colorimetric method and Folin–Ciocalteu reagent method, respectively. Flavonoid markers (kaempferol, apigenin, rutin, luteolin, and quercetin) and phenolic compounds (gallic, caffeic, coumaric, ferulic, cinnamic, syringic, and chlorogenic acids) were quantitatively traced for the first time in these Saudi *Astragalus* species using high performance liquid chromatography (HPLC) method. The antibacterial and antifungal studies were carried out by well diffusion method. Cytotoxic activities studies were carried out against Hep G-2, HCT-116, and A-549 cancer cell lines using 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay procedure. Antioxidant activities were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Immunostimulant activity was examined using lymphocyte proliferation method.
Results: The chemical screening confirmed the presence of triterpenes, flavonoids, sterols, glycosides, saponins, and polyphenolic compounds and absence of anthraquinones in all species, while *A. spinosus* shows the highest percentages of TFC and TPC. Ethyl acetate fractions of *A. spinosus* and *A. sieberi* showed potent cytotoxic activities, expressed as 50% inhibitory concentration (IC_{50}) = 50.2, 22.6, and 29.1 µg/ml for *A. spinosus* and 39.8, 28.8, and 47.2 µg/ml for *A. sieberi* against tumor cell lines, HepG-2, HCT-116, and A-549, respectively. *Astragalus spinosus* showed a DPPH radical scavenging effect (IC_{50}) = 69 µg/ml, compared with other two species (IC_{50}) = 161 and 313 µg/ml for *A. armatus* and *A. sieberi*, respectively. The *Astragalus* samples showed mild antimicrobial activities and immunomodulating activities.
Conclusion: The present research shows the quality control testing, for the first time, of three Saudi *Astragalus* species and *Astragalus*-containing recipes. The present work provides valuable information for new drug or food supplement research and development.

1. INTRODUCTION

The plants of medicinal values still play a significant role in maintaining health status of the mankind and communities. These plants contain natural substances that exert specific physiological effects on the human body [1]. The modern medicines are usually natural, natural mimics, or modified natural product structures. Natural products and their new structures are still the most important source for drug discovery and drug design in the modern medicine. For example, the period approximately between the 1940s and 2014, 131 out of 175 (i.e., 75%) approved small molecules are natural or natural mimics, with 85 (49%) actually being either natural products or natural product derivatives [2]. Furthermore, The World Health Organization supports the use of traditional medicine, provided that it is proven effective and safe. In developing and developed countries, a large number of people live in extreme poverty and some suffer and probably die due to

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lack of safe water and medicine, they have no choice other than primary health care [3]. It is always published that 80% of people in Asia and Africa (approximately 80% of the world's mankind) use traditional medicines [4]. Therefore, it is necessary to look inward to search for herbal medicines in order to validate the ethnomedicinal use and subsequently to isolate and characterize compounds that will be considered as new drug leads or new drugs in the near future [3]. Genus *Astragalus* is one of the largest genera (about 3,000 *Astragalus* species) of Fabaceae worldwide [5–6]. *Astragalus* species are used in folk medicine for treatment of several human ailments in rural areas such as cough, hypertension, bronchitis, stomach ulcer, gynecological disorders, diabetes, and scorpion bites [7]. Some *Astragalus* species are considered as valuable sources for the economically important natural products, e.g., gum tragacanth which is obtained from *A. gummifer* and the extract of dried roots of *A. membranceus* grown in East Asia which are applied well in traditional Chinese Medicines for treatment of many diseases, e.g., impaired immunity, inflammation, nephropathy, high blood pressure, diabetes mellitus, liver cirrhosis, leukemia, and uterine cancer [8–14]. In North Africa, some species are applied for cough, asthma, arthritis, and scorpions' stings [15]. In academia, several *Astragalus* species have been investigated using many biological screening modules, for example, anti-inflammatory, analgesic, antiviral, free radical scavenging, anticancer, immunostimulant, and cardiotonic activities [16–20]. After the scientific scrutiny, it was found that there are no available data about the chemical and/or biological activities of the Saudi *Astragalus* species (*A. spinosus, A. armatus,* and *A. sieberi*). The present study deals comparatively with the biological activities and chemical profiling of the *Astragalus* species of northern border region of KSA. Targeted biological evaluation comprises cytotoxicity, antimicrobial, immunostimulant, and antioxidant activities. The chemical study in the present work includes phytochemical screening, determination of total polyphenolic contents (TPCs), and total flavonoid contents (TFC) in addition to HPLC tracing of flavonoid and phenolic acid markers. The outcome of this study is expected to provide new valuable information regarding the phytochemical profile and biological activities of these *Astragalus* species which help in the identification of these *Astragalus* species and support new drug and food research and development.

2. EXPERIMENTAL

2.1. Plant Material

The aerial parts of *A. spinosus, A. armatus,* and *A. sieberi* (Fig. 1) were collected from wild area of Rafhaa city, Northern border region, Saudi Arabia in May 2017. Voucher specimens are kept in the herbarium of Natural product chemistry Dept., College of Pharmacy, Northern Border University, Rafhaa, Saudi Arabia. The plants were air dried in shade then powdered. Each plant powder was extracted separately with 70% methanol till exhaustion; the extracts were dried under vacuum using rotatory evaporator at 45°C and kept in refrigerator.

2.2. Phytochemical Screening

Phytochemical screening of the aqueous-methanolic extracts of *Astragalus* samples were carried out to test for the presence of major phytoconstituents. The general phytochemical tests were carried out using the standard procedures as described by Mir et al. [3] and Dyayiya et al. [21].

Figure 1: Picture of *Astragalus* species.

2.3. Determination of Total Flavonoid Content and Total Polyphenolic Content

Determination of TFCs by $AlCl_3$ colorimetric method [22] and determination of TPCs using Folin–Ciocalteu reagent method [22] for the *Astragalus* samples were determined using UV/Vis spectrophotometer (6105, Jenway Ltd., England).

2.4. HPLC Tracing of Flavonoid and Phenolic Acid Markers

Five standard flavonoid markers (kaempferol, luteolin, rutin, apigenin, and quercetin) and seven phenolic acids (gallic, chlorogenic, caffeic, coumaric, ferulic, cinnamic, and syringic acids) were used as reference compounds in this experiment. The presence of these compounds was traced in *Astragalus* samples and their concentrations in the samples were tentatively determined using HPLC analytical methods by comparison of the areas under the peaks with those of serial dilutions of reference compounds. For HPLC analyses, the following units and conditions were applied; Detector: UV/vis Detector (GBC); Pump: LC 1110 Pump (GBC); Software: Win Chrome Chromatography Version 1.3; Column: KROMASIL 150 * 4.6 mm; Flow Rate: 0.8 ml/minute (for flavonoids) and 1.0 ml/minute (for phenolic acids); Detection: UV 356 nm (for flavonoids) and UV 280 nm (for phenolic acids); Eluent: [Acetonitrile:water:formic acid, (85:14:1)] (for flavonoids) and Methanol:water:tetrahydrofuran:acetic acid, (23:75:1:1) (for phenolic acids).

2.5. Antioxidant Activity

The antioxidant activity of *Astragalus* samples were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay [23] in triplicate and average values were considered, where freshly prepared (0.004% w/v) methanol solution of DPPH radical was prepared and stored at 10°C in the dark. A methanol solution of the test sample was prepared. A 40 µl aliquot of the methanol solution was added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously with data being recorded at 1-minute intervals until the absorbance was stabilized (16 minutes). The absorbance of DPPH radical without antioxidant (control) and the reference compound (Ascorbic acid) was also measured. All determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = {(AC-AT)/(AC)}×100 ; Where: AC = Absorbance of the control at $t = 0$ minute, AT = absorbance of the test sample + DPPH at $t = 16$ minutes.

2.6. Cytotoxic Activity

The total extract of each plant sample was diluted with distilled water, defatted with n-hexane, and fractionated into two fractions; FA (ethyl acetate fraction) and FB (rest of the total extract). The obtained fractions of each sample were tested for cytotoxic activity using three mammalian cell lines: HepG-2 cells (human hepatocellular cancer cell line), HCT-116 (colon carcinoma), and A-549 cells (human lung carcinoma) which were obtained from VACSERA Tissue Culture Unit. The procedure for cytotoxicity evaluation was applied using viability assay [24].

2.7. Antimicrobial Activity

Antimicrobial activities of different FA and FB fractions of each *Astragalus* sample were carried out using well diffusion method [24] at antimicrobial activity unit in the Regional Center for Mycology and Biotechnology, Faculty of Science, Al-Azhar University, Cairo, Egypt, using two fungal strains *Aspergillus fumigatus* (RCMB 002008) and *Candida albicans* RCMB 005003 (1) ATCC 10231, two Gram-positive bacteria *Staphylococcus aureus* (RCMB 010010) and *Bacillus subtilis* RCMB 015 (1) NRRL B-543, and two Gram-negative bacteria *Proteus vulgaris* RCMB 004 (1) ATCC 13315 and *Escherichia coli* (RCMB 010052) ATCC 25955; the diffusion agar technique was applied, Well diameter: 6.0 mm (100 µl was tested), where Ketoconazole (100 µg/ml), Gentamycin (4 µg/ml) were used as positive control for antifungal and antibacterial activities, respectively, the samples were tested at 10 mg/ml concentration.

2.8. Immunostimulant Activity

2.8.1. Isolation of lymphocytes from rat blood

Blood was withdrawn from orbital plexus of Wistar albino rats and lymphocytes were isolated using Ficoll histopaque. The lymphocytes were washed twice with phosphate buffered saline (PBS) and resuspended in complete RPMI 1640 medium (RPMI 1640 with 10% fetal calf serum and 1% antibiotic-antimycotic solution). The cell number was adjusted to 1×10^6 cells/ml by counting in hemocytometer and cell viability was tested by the trypan blue dye exclusion technique.

2.8.2. Lymphocyte proliferation assay

Effect of the tested samples on lymphocyte proliferation was carried out using MTT assay. The number of lymphocytes was adjusted to 10^6 cells/ml in RPMI-1640 and 100 µl cell suspensions were seeded into a 96-well tissue culture plate (Nunc, Denmark). Each control ($n = 4$) and treated ($n = 4$) cell suspension was repeated in triplicate. The final volume of the wells was made up to 200 µl with the tested compound at a concentration of 100 µg/ml of each *Astragalus* sample and 10 mg of lipopolysaccharide (LPS) sample. The plate was incubated at 28°C for 48 hours in a 5% CO_2 atmosphere. The colorimetric MTT method [25] was used to determine the proliferation of lymphocytes. After 48 hours culture, 20 µl of MTT (5 mg/ml PBS) was added to each well of the leukocyte culture and incubated at 28°C for 4 hours. The formazan crystals were dissolved by adding 150 µl of dimethyl sulphoxide (DMSO) (Sigma, USA) to each well, followed by 25 µl glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The contents of the wells were mixed thoroughly with a micropipette and incubated at room temperature for 10 minutes. Formazan development was read at 595 nm using a microplate reader (SunRise, Tecan, USA). One set was treated with mitogen LPS (10 µg/ml in PBS). The proliferation stimulation index (SI) was calculated according to the following equation: [SI = O.D. (optical density) of experimental/ O.D. of control]. The results are expressed as means ± standard error [26].

2.8.3. Chemicals

LPS and MTT were purchased from Sigma Aldrich chemical company, St. Louis, USA.

3. RESULTS AND DISCUSSIONS

3.1. Percentage of Extractive Matter

Fifty-gram dry plant powder of each *Astragalus* sp. was extracted with 70% aqueous methanol. The plant extract of each sample was concentrated under vacuum at 45°C till dryness. The percentage of dry extracts of *A. spinosus, A. armatus*, and *A. sieberi* were 11.1%, 12.0%, and 14.5%, respectively.

3.2. Phytochemical Screening

The comparative phytochemical screening confirmed the great similarity of all species (Table 1), all *Astragalus* samples showed positive results for the presence of carbohydrates and/or glycosides, sterols and/or triterpenes, flavonoids, tannins and/or phenolic compounds, and saponins in all sample. Also, it shows the presence of traces of alkaloids and/or nitrogenous compounds in *A. sieberi* and *A. armatus* only; while anthraquinones were not detected in all samples.

Table 1: Phytochemical screening results of *Astragalus* samples.

Plant species	*A. Sieberi*	*A. spinosus*	*A. armatus*
Carbohydrates and/or glycosides	++	++	++
Sterols and/or triterpenes	++	++	++
Flavonoids	++	++	++
Anthraquinones	−	−	−
Saponins	++	++	++
Alkaloids/nitrogenous bases	+	−	+
Tannins/phenolic compounds	++	++	++

Key: (−) not detectable, (+) trace amount, (++) abundant.

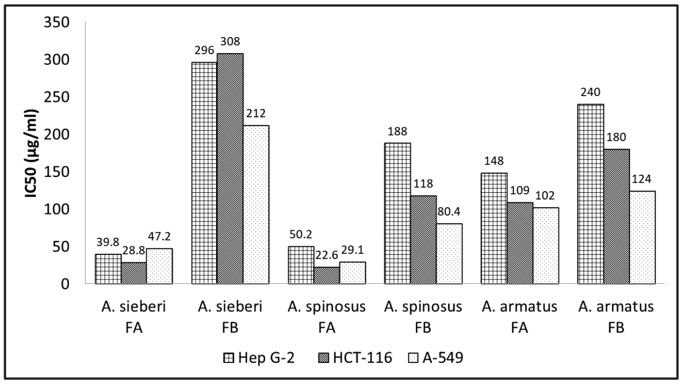

Figure 2: Cytotoxic activity (IC_{50}) of *Astragalus* samples versus three cancer cell lines.

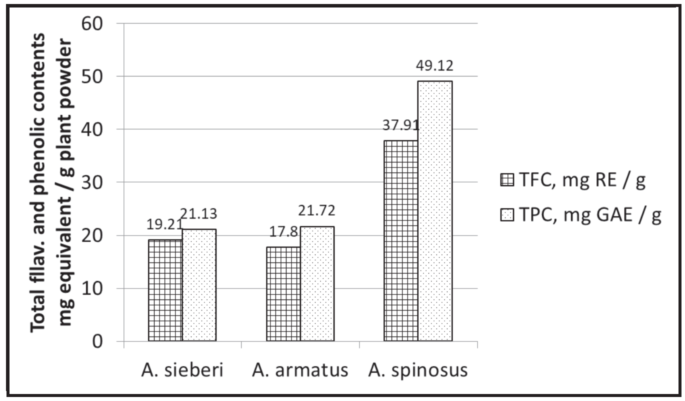

Figure 3: Results of TFC and TPC determination of *Astragalus* samples. TFC = total flavonoid content; TPC = total polyphenolic content; RE = Rutin equivalent; GAE = gallic acid equivalent.

3.3. Cytotoxic Activity

The activity of the fractions, FA and FB, of each *Astragalus* sample were tested *versus* three human cell lines HepG-2, HCT-116, and A-549 cells (Fig. 2). Cytotoxic activity study showed promising results, especially for ethyl acetate fractions of *A. spinosus* and *A. sieberi* where the IC_{50} were 50.2, 22.6, and 29.1 µg/ml for ethyl acetate fraction of *A. spinosus*, and 39.8, 28.8, and 47.2 µg/ml for ethyl acetate fraction *A. sieberi versus* the cancer cell lines, HepG-2, HCT-116, and A-549, respectively.

3.4. Antimicrobial Activity

The antimicrobial activities are expressed as zone of inhibition in mm beyond well diameter (6 mm). The experiment showed the poor antimicrobial effect of all sample extracts; however, mild effects (inhibition zones 10, 11, and 8 mm) were recorded for *A. Sieberi* FA, *A. spinosus* FA, *A. armatus* FA, respectively against *Escherichia coli* (RCMB 010052). While *A. Sieberi* FB, *A. Spinosus* FB showed inhibition zones 9 and 8 mm, respectively, against *Proteus vulgaris* RCMB 004 (1) ATCC 13315 strain.

3.5. Antioxidant Assay

The result of antioxidant activity assay regarding the concentrations responsible for 50% DPPH scavenging activities (IC_{50}) confirmed the potent effect of *A. spinosus* (IC_{50}) = 69 µg/ml, compared with the other two species (IC_{50}) = 161, and 313 µg/ml for *A. armatus* and *A. sieberi* total extract, respectively, while IC_{50} of the reference material (ascorbic acid) was 14.2 µg/ml.

3.6. Determination of Total Flavonoid and Total Polyphenolic Contents

Although the comparative phytochemical screening confirmed the great similarity of all three species, total flavonoid, and total phenolic estimations shows a quite differences, where *A. spinosus* contain the highest percentages of both TFC and TPC; while the least percentage were detected in *A. armatus*. TFCs were calculated as 19.21, 17.8, and 37.91 mg rutin equivalent/g plant powder for *A. sieberi, A. armatus*, and *A. spinosus*, respectively; while TPCs were calculated as 21.13, 21.72, and 49.12 mg gallic acid equivalent/g plant powder for *A. sieberi, A. armatus*, and *A. spinosus,* respectively (Fig. 3). These findings can probably explain the great efficacy of *A. spinosus* extract compared with other species, especially *A. armatus.*

3.7. HPLC Tracing of Flavonoid and Phenolic Acid Markers

HPLC is a powerful and famous technology and has many beneficial applications in natural product research, including detection, isolation, purification, structure elucidation, qualitative, and quantitative estimation of natural products [27,28]. Tracing of flavonoid markers by HPLC shows the highest percentages of quercetin, luteolin, and apigenin in *A. spinosus* compared with other species. However, *A. armatus* contains the highest amount of kaempferol and rutin (Table 2). Tracing of phenolic acid markers by HPLC also shows the highest percentages of coumaric, cinnamic, and gallic acids in *A. spinosus*, while *A. sieberi* showed high percentages of gallic, chlorogenic, ferulic, and syringic acids, whereas *A. sieberi* showed high percentages of chlorogenic, caffeic, ferulic, and syringic acids (Table 3).

3.8. Immunostimulant Activity

The immunostimulant assay was done in the present study in order to compare the obtained results with those reported for root extract of some *Astragalus* species (e.g., *A. membranaceous* and *A. mongholicus*). The proliferation SI for each mitogen (i.e., different *Astragalus* extracts and LPS) was obtained from the equation: (SI = O.D. of experimental/O.D. of control). The proliferation stimulation indices obtained from immunostimulant assay were 2.63 ± 0.46; 1.25 ± 0.13; 0.98 ± 0.08 for *A. spinosus, A. sieberi, A. armatus*, respectively. This result confirmed the superiority of *A.*

Table 2: HPLC tracing of standard flavonoid markers.

Flavonoids	*A. spinosus* (µg/g)	*A. sieberi* (µg/g)	*A. armatus* (µg/g)
Kaempferol	22.6	21.1	35.0
Luteolin	27.9	19.3	14.5
Rutin	18.4	17.7	35.0
Apigenin	31.0	33.5	17.2
Quercetin	39.1	14.3	23.3

Table 3: HPLC tracing of standard phenolic acid markers.

Phenolic acids	*A. spinosus* (µg/g)	*A. sieberi* (µg/g)	*A. armatus* (µg/g)
Gallic	3.43	3.61	2.36
Chlorogenic	1.86	3.91	3.24
Caffeic	2.08	1.13	2.95
Coumaric	3.00	1.97	1.85
Ferulic	2.45	3.33	2.91
Cinnamic	3.49	1.00	2.08
Syringic	1.91	3.10	2.63

spinosus compared with other two species, however, all samples showed weak immunostimulant effect compared with the reference mitogen, LPS, SI = 3.84 ± 0.51, therefore immunostimulant effect of the aerial parts of the test samples can be considered mild or no effect compared with the reported promising effects of *A. membranaceous* and *A. mongholicus* root extracts. However, the immunostimulant effects of the root extracts of these Saudi *Astragalus* species are not tested yet.

4. CONCLUSION

This comparative study provided valuable information about the promising biological activities, phytochemical profiles, and helps in chemical identification and characterization of three Saudi *Astragalus* species. However, the research will continue to isolation and identification of the bioactive natural products, especially of the ethyl acetate fractions of these species. The future research studies have also extend to include the chemical and biological evaluation of the root extracts of these Saudi species, especially the immunomodulation activities with the aim of isolation and identification of individual secondary metabolites which may be considered as effective drug leads in the near future.

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