

Original Research Article

Total phenolic and flavonoid contents, cytotoxic, immunomodulatory and anti-inflammatory potential of whole plant of *Astragalus creticus* (Fabaceae)

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Abstract

Purpose: To determine total phenolic and flavonoid contents, as well as the cytotoxic, immunomodulatory and anti-inflammatory potentials of the whole plant of *Astragalus creticus* (Fabaceae).

Methods: Folin-Ciocalteu (FCR) method was used for determination of total phenolic and flavonoid contents of the methanol and dichloromethane extracts of *Astragalus creticus*. The cytotoxic potential of the extracts on 3T3 and HeLa cell lines were evaluated using MTT assay. Brine shrimp mortality was determined by lethality bioassay, while inhibitory effects were determined on mouse fibroblast (3T3) and cervical cancer (HeLa) cell lines. In vitro immunomodulatory and in vivo anti-inflammatory effects were assessed using reactive oxygen species (ROS) chemiluminescence and formalin-induced rat paw edema assays, respectively.

Results: Dichloromethane extract had higher contents of phenolics (TPC = 324.75 ± 2.47 mg GAE/g) and flavonoids (TFC = 95.51 ± 0.82 QE/g) than the methanol extract (TPC = 79.82 ± 1.53 mg GAE/g, TFC = 56.11 ± 0.93 QE/g). The dichloromethane extract exhibited high cytotoxic and immunomodulatory potentials, with 76.66 % mortality in brine shrimp lethality bioassay and 83.9 % inhibition (IC₅₀ = 18.0 ± 1.1 µg/mL) in chemiluminescence assay. The extract also resulted in 22 and 13 % inhibition of viability of HeLa and 3T3 cells, respectively, while the methanol extract produced 13 % inhibition of both cell lines. The methanol extract produced very significant anti-inflammatory activity, with a maximum of 49 % inhibition of paw edema at a dose of 160 mg/kg (p < 0.01).

Conclusion: These results suggest that the dichloromethane and methanol extracts of *Astragalus creticus* (Fabaceae) exert cytotoxic, immunomodulatory and anti-inflammatory effects. These findings provide scientific validation for the traditional medicinal use of the *Astragalus* genus.

Keywords: *Astragalus creticus*, Brine shrimp lethality, Flavonoid, Cytotoxic, Immunomodulatory activity

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INTRODUCTION

Fabaceae is popularly known as the pea, legume or bean family which comprises 751

genera and 19000 species [1]. *Astragalus creticus* is an important plant of the *Astragalus* genus which is well known in the field of herbal medicine. Plants of *Astragalus* genus are

traditionally used for treating various types of inflammation, healing of wounds, immunostimulation, and treatment of leukemia. *Astragalus* roots are used for preparation of popular herbal drugs in Traditional Chinese Medicine (TCM). These drugs exert hepatoprotective, immunostimulatory, anti-inflammatory, anti-perspiration, antidiabetic, anti-carcinogenic, and diuretic effects [2]. A list of traditionally-important *Astragalus* plants is presented in Table 1. *Astragalus creticus* is native to the mountainous areas of S. Balkans, Candia, Greece and many parts of Asia. In Pakistan, this species is predominantly found in tribal areas of Southern Punjab and Baluchistan such as Sakhi Sarwar and Fort Manro. The plant, which has the common name of Cretan Milk-vetch, is a rich source of high quality gum tragacanth [3].

The present study was designed as systematic and scientific investigations on the immunomodulatory, cytotoxic and anti-inflammatory potential of *Astragalus creticus* in order to validate its traditional and medicinal uses.

EXPERIMENTAL

Plant collection, identification, and preparation of extracts

The plant was collected near Fort Manro (tribal area of southern Punjab) in March, 2014. It was identified as *Astragalus creticus* by a taxonomist at Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, and

voucher no. (www.the plantlist.org/tpl1.1/record/ild.32000) was deposited at the herbarium of the institute. The whole plant of *Astragalus creticus* was shade-dried for 15 days, ground into coarse powder, and weighed. Then, 1000 g of the dried coarse powder was extracted using maceration in dichloromethane and methanol. The two extracts were concentrated using rotary evaporation under reduced pressure, and were labeled as ACWPD and ACWPM, for dichloromethane extract and methanol extract, respectively.

Determination of total phenolic content (TPC)

The test sample (100 μ L) was mixed with 10 μ L of 10 % diluted FolinCiocalteu reagent (FCR), and incubated for 10 min, followed by addition of 90 μ L of 15 % (w/v) aqueous Na_2CO_3 . The tube contents were pre-incubated at 37 $^\circ\text{C}$ for 90 min, prior to measurement of absorbance at 750 nm. Gallic acid was used as positive control. Total phenolic contents (TPCs) were calculated from a gallic acid (GA) calibration curve, and expressed as mg of GA equivalents per gram of dry extract (mg GAE/g) [13]. Each assay was done in triplicate.

Determination of total flavonoid content (TFC)

A calibration curve was drawn using standard quercetin solution (1 mg/mL in methanol). The test sample (100 μ L) was mixed with 25 μ L of 1 % NaNO_2 solution in a test tube, and allowed to stand for 5 min prior to addition of 10 μ L of 10 % AlCl_3 solution and 35 μ L of 4 % NaOH .

Table 1: Traditional uses of various species of *Astragalus* genus

Plant name	Plant part used	Traditional uses	Preparation	Geographical zone(s)	Reference
<i>A. membranaceus</i>	Roots	Anticancer and immunity enhancement	Decoction and tincture	China	[4]
<i>A. pelecinus</i>	Aerial parts	Healing of wounds		Palestine	[5]
<i>A. chrysochlorus</i>	Roots	Healing of wounds and treatment of leukemia.	Aqueous extract	Anatolia (Turkey)	[6]
	Pod	Inflammation of liver, ovaries, spleen and uterus	Decoction	Afghanistan and Pakistan	[7]
<i>A. hamosus</i>	Fruits	Inflammation	Paste	Afghanistan and Pakistan	[8]
	Aerial parts	Inflammation	Decoction	Iran	[9]
<i>A. adsurgens</i>	Roots	Healing of wounds	Decoction	Russia and Tibet	[10]
<i>A. alopecias</i>	Whole plant	Healing of wounds	Powder	Russia and Tibet	[10]
<i>A. trojanus</i>	Roots	Leukemia	Aqueous extract	Anatolia (Turkey)	[11]
<i>A. taipashanensis</i>	Roots	Enhancement of immunity	Teas, infusions and decoction	Taibai mountains (China)	[12]
<i>A. creticus</i>		High quality gum tragacanth			[3]

Following dilution with 30 μ L of methanol, the absorbance of the solution was read at 510nm. The flavonoid contents were calculated and expressed as milligram of quercetin equivalents per gram of dry extract (mg QE/g DE) [13].

Brine-shrimp lethality bioassay

Artificial sea water was prepared by dissolving 3.8 g of sodium chloride (sea salt) in 1000 mL of H₂O, followed by filtration. Then, shrimp eggs were added to the water. When the eggs were hatched, the shrimp larvae were allowed to mature at 22-29 °C in 2 days. Then, graded concentrations of each extract (10, 100, 1000 μ g/mL) was added in triplicate into different vials. Plant extract (20 mg) was dissolved in 2 mL of solvent, and the sample was transferred at volumes of 5, 50, 500 μ L to vials to generate concentrations of 10, 100, 1000 μ g/mL, respectively. Etoposide was used as standard drug. To each vial, 10 shrimps and 5 mL sea water (30 shrimps/dilution) were added, followed by exposure to illuminating light for 24 h. Shrimps that survived were counted and used for Probit analysis (Finney Computer program) [14].

Cytotoxicity assay

Cell viability was determined by checking the ability of the cells to oxidize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) and generate formazan crystals. The 3T3 and HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) and minimum essential medium eagle (MEME), respectively. The culture media were supplemented with penicillin (100 units/mL), 5 % FBS, and streptomycin (100 μ g/mL) in a 75-cm² culture flask, and placed in a 5 % carbon dioxide incubator at 37 °C. The cells were counted with a hemocytometer, and diluted in appropriate specific medium. Each cell was cultured at a density of 5 x 10⁴ cells/mL in 96-well plates at a volume of 100 μ L per well. After overnight incubation, the medium in each well was replaced with 200 μ L fresh medium containing a different concentration of sample extract, with extract concentrations ranging from 1 to 30 μ M). Then, the wells were incubated for 48 h, after which 200 μ L MTT (0.5 mg/mL) was added to each well, followed by further incubation for 4 h. Thereafter, the medium in each well was discarded and replaced with 100 μ L of DMSO, in order to solubilize the resultant formazan crystals. The absorbance of the formazan solution in each well was measured at 540 nm in a Spectra Max plus microplate reader, and the inhibition of cell viability (H) was calculated as shown in Eq 1.

$$H (\%) = \{100 - (As - An)/(Ac - An)\} \times 100$$

where As, An and Ac are the absorbance of sample, negative and positive control samples, respectively.

Soft-Max Proof software (US) was used for calculation [15].

Evaluation of immunomodulatory effect

Diluted whole blood (1:5 in sterile PBS, pH 7.4, 25 μ L) and PMNs (1x10⁶) suspended in HBSS⁺⁺ were incubated with 25 μ L of various concentrations of each extract (1, 10 and 100 μ g/mL) in triplicate in 96-well plates. The control wells contained only cells and HBSS⁺⁺. Then 96-well plates were incubated at 37 °C for 15 min in a luminometer thermostat chamber. Thereafter, 25 μ L of SOZ and 25 μ L of ROS probe were introduced to each well. The ROS levels were determined in the luminometer in terms of RLU [16].

Determination of anti-inflammatory activity

White albino mice weighing 25-30 g were kept safely in steel cages and provided free access to standard chow feed and water in a well-ventilated room at 25±2 °C at 12-h light/12-h dark cycle. The animal experiments were carried out in strict compliance with the policies and protocols recommended by Institutional Animal Ethical Committee of Faculty of Pharmacy, Bahauddin Zakriya University, Multan. The committee also granted approval for the various experiments (approval ref no. 13/PEC/2019). International guidelines for animal studies were followed.

Establishment of formalin-induced rat paw edema

Albino mice were randomly assigned to 4 groups (n = 5). Group 1 mice received normal saline only, at a dose of 0.1mL/kg. Group 2 was treated intraperitoneally with indomethacin at a dose of 10 mg/kg, while ACWPM was administered to the 3rd and 4th experimental groups at doses 80 and 160mg/kg, respectively. Subcutaneous formalin injection (10 % w/v; 0.5 mL) was administered to groups 2, 3 and 4 after 30 min of their respective treatments as indicated above. Then, at 0, 1, 2 and 3 h after the administration of formalin, paw size was measured by wrapping a cotton thread around each rat paw and measuring the length of the thread using a metric ruler [17].

Statistical analysis

All results are expressed as mean \pm SEM (n = 5). Student's *t*-test was used for statistical analysis of differences between two groups, while one-way analysis of variance (ANOVA) and Dunnett's test for multiple comparisons were used to analyze differences in anti-inflammatory data among groups. All statistical analyses were done with SPSS version 24 software. Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Total phenolic (TPC) and total flavonoid contents (TFC)

Total phenolic and flavonoid contents were calculated in dichloromethane and methanol extracts of whole plant of *Astragalus creticus*. The results illustrate that dichloromethane extract (ACWPD) contained higher phenolic; 324.75 \pm 2.47 and flavonoid; 95.51 \pm 0.82 contents as compared to methanol extract (ACWPM) of *Astragalus creticus*. The flavonoid/phenolic ratio (F/P) of ACWPD and ACWPM extracts was indicated at 0.29 and 0.70, respectively (Table 2).

Table 2: TPC and TFC of the two *Astragalus creticus* extracts

Extract	TPC (mg GAE/g of DE)	TFC (mg QE/g of DE)	Flavonoid: phenolic (F:P) ratio
ACWPD	324.75 \pm 2.47	95.51 \pm 0.82	0.29
ACWPM	79.82 \pm 1.53	56.11 \pm 0.93	0.70

Values are expressed as mean \pm SEM

Cytotoxic activity

Brine-shrimp lethality bioassay

The results showed that ACWPD produced better cytotoxic effect, with 6.66, 10 and 76.66 % mortality values, than ACWPM which produced mortality values of 10, 16.66 and 20.00 % against brine shrimp larvae at concentrations of 10, 100 and 1000 μ g/mL, respectively. The standard drug (etoposide) resulted in 46.66 % mortality at a concentration of 7.46 μ g/mL (Table 3).

Cytotoxicity against 3T3 cell line

Results from MTT assay on 3T3 cells revealed that ACWPD and ACWPM produced 13 % inhibition, relative to the standard drug (cycloheximide) which produced 70 % inhibition at IC₅₀ of 0.8 \pm 0.2 μ g/mL (Table 4).

Cytotoxicity against HeLa cell line

The anticancer effects of the extracts were further tested against HeLa cell lines using MTT assay. Both samples (ACWPD and ACWPM) produced moderate inhibitory effects: 22 and 13 % respectively, relative to the standard drug cycloheximide which produced 70 % inhibition at IC₅₀ value of 1.2 \pm 0.2 μ g/mL (Table 4).

Immunomodulatory effect

The immunomodulatory effects of ACWPD and ACWPM were determined using oxidative burst assay. The results showed that ACWPD exhibited high level of immunomodulatory effect, with 83.9 % inhibition, and IC₅₀ value of 18.0 \pm 1.1 μ g/mL. In contrast, ACWPM exerted

Table 3: Cytotoxic effect of *Astragalus creticus* extracts in brine shrimp lethality assay

Extract	Concentration (μ g/ml)	No. of shrimps taken	No. of shrimps that survived	Mortality (%)
ACWPD	10	30	28	06.7
	100	30	27	10.0
	1000	30	07	76.7
ACWPM	10	30	27	10.0
	100	30	25	16.7
	1000	30	24	20.0
Etoposide (standard drug)	7.46	30	16	46.7

Table 4: Cytotoxic effect of *Astragalus creticus* extracts against HeLa and 3T3 cell lines assay

Extract	Concentration (μ g/mL)	Inhibition (%)	
		HeLa cell line (%)	3T3 cell line (%)
ACWPD	30	22	13
ACWPM	30	13	13
Cycloheximide (standard drug)	30	70	70

Table 5: Immunomodulatory effect of *Astragalus creticus* extracts

Extract	Concentration (µg/mL)	Inhibition (%)	IC ₅₀ (µg/mL)
ACWPD	25	83.9	18.0±1.1
ACWPM	25	38.6	
Ibuprofen (standard drug)	25	73.2	11.2±1.9

Table 6: Anti-inflammatory effects of *Astragalus creticus* extracts

Group	Dose (mg/kg)	Change in mean paw edema (cm)				Inhibition (%)		
		Normal	1 h	2 h	3 h	1 h	2 h	3 h
Control	-	1.04±0.17	3.33±0.75	4.45±0.31	5.07±0.28	-	-	-
Indomethacin	10	0.99±0.08	1.63±0.42**	1.83±0.14**	1.97±0.12**	51%	59%	61%
ACWPM	80	0.96±0.13	2.43±0.46*	2.45±0.13**	2.74±0.13**	26%	46%	45%
ACWPM	160	1.01±0.05	2.43±0.52	2.43±0.21*	2.56±0.17**	27%	46%	49%

Values shown are mean ± SEM (n = 5). **P* < 0.05; ***p* < 0.01

moderate activity, with 38.6 % inhibition, when compared with Ibuprofen (73.2 % inhibition, with IC₅₀ of 11.2±1.9µg/mL, Table 5).

Anti-inflammatory activity

The values of % inhibition of inflammation for ACWPM and indomethacin (standard drug) were determined at various time intervals i.e., 1, 2 and 3 h. The results showed that ACWPM exerted significant anti-inflammatory potential when tested at concentrations of 80 and 160 mg/kg. The extract ACWPM produced a maximum of 46 % inhibition at a dose of 80 mg/kg after 2 h. At a dose of 160 mg/kg, ACWPM administration resulted in 49 % inhibition of paw edema after 3 h. The changes in mean paw edema and the corresponding % inhibitions are presented in Table 6.

DISCUSSION

Phenolics are widely distributed in the plant kingdom. Studies have shown that phenolic compounds are beneficial for the prevention and treatment of bacterial and viral infections, as well as cancer and inflammatory diseases [18]. Thus, the quantification of phenolic compounds in plants is of paramount medicinal importance. Cancer is a malignant disease associated with millions of deaths worldwide. In 2015 alone, there were at least 17.5 million new cancer cases, and 8.7 million deaths worldwide [19]. In spite of all improvements in cancer therapy, survival from cancer is still low, and many cancer patients suffer from relapse as well as adverse effects of anti-cancer drugs. Moreover, the development of resistance to existing chemotherapeutic agents is a vital problem in cancer treatment. Therefore, there is need to evolve novel chemotherapeutic strategies for cancer [20].

The brine-shrimp lethality bioassay gives frontline cytotoxicity information which can be backed up with more sophisticated and specific bioassays. It has been reported that the brine shrimp lethality bioassay has a good correlation with the cytotoxicity of plant bioactive substances against some human solid tumors [14]. Indeed, several natural pesticidal and antitumor agents have been identified through screening using this assay. The 3T3 cells are mouse embryo fibroblasts colonial cells which are responsible for oncogenic activity of spontaneous human tumor-transfected DNA. Despite their limited susceptibility to transformation after transfection with DNA from neoplastic tissue, 3T3 cells play a vital role in the development of the molecular genetics of cellular oncogenes [21].

It has been revealed that the anticancer activities of plant extracts on HeLa cells are due to induction of apoptosis via the intrinsic and extrinsic pathways. This hypothesis is supported by previous studies which demonstrated the antiproliferative effects of extracts of *Emilia sonchifolia*, *Solanum lyratum*, *Vatica diospyroides*, *Cotinus coggygri*, *Rosa damascene*, *Colchicum sanguicolle* and *Centaurea antiochia* [22]. The choice of HeLa cells is based on the fact that cervical cancer is the fourth most widely diagnosed cancer in women worldwide. Thus, new therapies are required for cervical cancer.

Reactive oxygen species (ROS) are generated via oxidative burst which has been implicated as the key underlying mechanism of tumoricidal and antimicrobial effects. Oxidative stress is closely linked to carcinogenesis because ROS function in various epigenetic factors involved in the induction, promotion, and modulation of cervical and breast cancers [24]. Recent findings indicate

improved outcomes in the use of immune-modulatory drugs for treatment of cancer patients. Thus, ongoing investigations on the use of various treatment combinations for enhancing immunity against cancer are of prime importance in combating this disease [25].

Formalin-induced paw edema is a widely used experimental model for studying acute inflammatory response. Plant extracts that show anti-inflammatory potential are likely to inhibit the release of inflammatory mediators, and suppress accumulation of macrophages and neutrophils within 1-5 h following both phases of edema [26]. In this study, the results obtained with *Astragalus creticus* are consistent with the folkloric uses and experimentally-tested anti-inflammatory potential of various *Astragalus* species such as *A. fasciculifolius*, *A. membranaceus*, *A. sinicus*, *A. gummifer*, and *A. hamosus* [27]. Phenolic compounds derived from ginger (gingerol and 6-shogaol) exhibit significant anticancer potential against breast, brain and lung cancers. The present study showed that *Astragalus creticus* extracts were rich in phenolic and flavonoid contents which were responsible for the observed cytotoxic effects against brine shrimp lethality bioassay, 3T3 and HeLa cell lines [28].

CONCLUSION

The results of the present study demonstrate the potential cytotoxic, immunomodulatory and anti-inflammatory activities of *Astragalus creticus*. Phenolics and flavonoids, being the major phyto-constituents, may be responsible for these activities. Further research is required, using other models, to elucidate the possible mechanism of action and to identify bioactive components that are responsible for the observed activities.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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