

Original Research Article

Protective effects of two *Astragalus* species on ulcerative colitis in rats

Gamal A Soliman^{1,2}, Gamal A Gabr^{1,3}, Fahad I Al-Saikhan⁴, Mohd Nazam Ansari^{1*}, Tajdar H Khan¹, Majid A Ganaie¹, Saeedan S Abdulaziz¹ and Özgen Alankuş-Çalışkan⁵

¹Department of Pharmacology, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, Kingdom of Saudi Arabia, ²Department of Pharmacology, College of Veterinary Medicine, Cairo University, ³Agricultural Genetic Engineering Research Institute, Agric. Res. Center, Giza, Egypt, ⁴Department of Clinical Pharmacy, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, Kingdom of Saudi Arabia, ⁵Department of Chemistry, Faculty of Science, Ege University, Bornova, Izmir, 35100, Turkey

*For correspondence: **Email:** m.ansari@psau.edu.sa; **Tel:** +966-11-5886037

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Abstract

Purpose: To investigate the anti-inflammatory activities of root extracts of *Astragalus gummifera* and *Astragalus kurdicus*, as well as their protective effects against acetic acid-induced ulcerative colitis (UC) in rats.

Methods: The roots of both species were shade-dried, pulverized to fine powder and extracted with 80 % ethanol. The extracts were lyophilized using freeze-dryer, and their anti-inflammatory effects were evaluated in rats by carrageenan-induced paw edema. In UC study, the extracts, at doses of 200 and 400 mg/kg, were given orally to rats for 5 days, with the last dose given 2 h prior to colitis induction. Histopathological studies were carried out on the colon tissues. Dexamethasone (DEX) was used as standard anti-inflammatory drug.

Results: *A. gummifera* (400 mg/kg) and *A. kurdicus* (200 and 400 mg/kg) significantly reduced carrageenan-induced edema, offered protection against UC and arrested UC-induced increases in myeloperoxidase (MPO) activity. Histological examination of colon sections also revealed evidence of extract-induced protection from UC.

Conclusion: These results suggest that *A. gummifera* and *A. kurdicus* root extracts have promising potential as sources of anti-inflammatory agents which may be useful in the treatment of UC.

Keywords: Colon ulcer, *Astragalus gummifera*, *Astragalus kurdicus* Inflammation, Myeloperoxidase, Ulcer index

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INTRODUCTION

UC is a chronic gastro-intestinal disease characterized by inflammatory changes and mucosal tissue injury. The pathogenesis of UC is still not clear, but some causative factors have been suggested. These include environmental, genetic and microbial and immuno-regulatory factors [1]. The principal drugs used for

management of UC are corticosteroids, 5-aminosalicylate, azathioprine, methotrexate, cyclosporine, vancomycin, ciprofloxacin and metronidazole [2]. Although these medications have good treatment outcomes, they produce adverse effects which have reduced their clinical applications. Despite lots of research efforts on this disease, no specific treatments have been developed so far [3]. It is known that plant

products have less toxic effects than synthetic ones and are good sources of novel therapeutic agents [4]. Thus in recent years, attention has considerably shifted to use herbal medicine in treating UC.

Astragalus which belongs to the family Fabaceae, consists of 380 species and is the largest genus of flowering plants in the flora of Turkey [5]. Different species of *Astragalus* genus are reported to possess numerous pharmacological activities such as immune-stimulatory effects [6], anti-bacterial and antiviral properties; ability to promote nucleic acid synthesis in the liver, hepato-protective activities, anti-inflammatory activities, and cardiovascular effects such as hypotensive and vasodilatory properties [7,8].

Astragalus has also been used in the treatment of diabetes mellitus, nephritis, leukemia and uterine cancer [9]. It is usually used as topical therapy for relief of rheumatoid arthritis, toothache, and neck pain, or orally for treating stomach pain [10]. The active constituents of *Astragalus* plants include flavonoids, saponins, polysaccharides, anthraquinones, alkaloids, β -sitosterol and amino acids. Flavonoids have marked antioxidant properties and show protective activities against pathological changes in liver, lung, kidney and heart [11,12]. *Astragalus* polysaccharides have been shown to induce endogenous interferon production in animals and humans, and to potentiate the actions of interferon in viral infections [13].

The objective of the present investigation was to evaluate the anti-inflammatory activities of *A. gummifera* and *A. kurdicus* root extracts and their potential to protect against UC in an experimental model of acetic acid-induced UC in rats.

EXPERIMENTAL

Chemicals

Carrageenan and DEX were procured from Sigma–Aldrich (St Louis, MO, USA). Ethanol was purchased from Scharlab, S.L. Spain. Other chemicals were of analytical grade.

Plant material

Astragalus gummifera Labill was collected along Elazig-Hazargolu Road, East Anatolia, Turkey, in June 2010. The plant was authenticated by Prof Zeki Aytac, of Gazi University, Faculty of Science and Art, Department of Biology, Ankara, Turkey. A voucher specimen (no. HUEF 00-23) was

deposited in the Herbarium of the Pharmaceutical Botany Department, Pharmacy College, Hacettepe University, Ankara, Turkey. *Astragalus kurdicus* Boiss. var. *kurdicus* was collected from Van: Gevaş, Alacabük mountain (southern slopes, 2850m altitude), East Anatolia, Turkey in June 2010. It was identified by Associate Professor Dr Fevzi Özgökçe, Yüzüncü Yıl University, Science and Art College, Department of Biology, Van, Turkey. A voucher specimen (no: VANF 13805) was deposited in the Herbarium of Yüzüncü Yıl University, Van, Turkey.

Preparation of plant extract

Roots collected from the plants were shade-dried and then separately pulverized to fine powders. The powdered roots (900 g) were extracted with 80 % ethanol (2 × 3 L) under reflux. The ethanol extract of each plant was subjected to rotary evaporation (70 ± 2 °C) and lyophilized using freeze drier. The extracts were stored in the refrigerator. Required concentrations were prepared immediately before use.

Animals

Wistar albino rats weighting 180 - 200 g were used. They were obtained from Laboratory Animal Care Unit, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Saudi Arabia. The animals were kept under standard conditions of temperature (22 ± 1 °C), relative humidity (55 ± 5 %), and 12 h / 12 h light / dark cycle, and fed with standard pellet diet with water provided ad libitum. All experimental procedures followed the guiding principles of the Institute for Laboratory Animal Research (2011) in Guide for the Care and Use of Laboratory Animals [14] and were approved by the Ethical Review Committee (Protocol no. PHARM-3-3-2015), Pharmacy College, Prince Sattam Bin Abdulaziz University, Al-Kharj, KSA.

Acute toxicity studies

Acute toxicity test was carried out in rats according to OECD - 423 Guidelines [15]. Two groups of Wistar albino rats (n = 6) were fasted overnight, and then treated with *A. gummifera* and *A. kurdicus* extracts, respectively at an oral dose of 2000 mg/kg. Control animals received vehicle (3 % v/v Tween 80 in distilled water). Each animal was observed for symptoms of toxicity and/or death every 15 min in the first 4 h after treatment, then every 30 min for successive 6 h intervals, and then daily for the next 48 h. Since there were no deaths at this dose; the dose of the tested extracts was

increased to 4 g/kg and the rats were monitored for another 48 h.

Justification of dose selection

A. gummifera and *A. kurdicus* extracts were safe at 4.0 g/kg and thus 1/20th and 1/10th (200 and 400 mg/kg, respectively) were chosen for the present investigation.

Anti-inflammatory activity

Anti-inflammatory effect was evaluated in rats using carrageenan-induced paw edema [16]. Six groups of animals (n = 6) were fasted overnight prior to the study, with free access to water. The 1st and 2nd groups (normal control and reference, respectively) were treated orally with vehicle (5 mL/kg) and DEX (0.2 mg/kg), respectively. *A. gummifera* extract (at 200 and 400 mg/kg) was administered orally to the 3rd and 4th groups, respectively; while *A. kurdicus* extract was administered orally to the 5th and 6th groups at doses of 200 and 400 mg/kg, respectively. After 30 min, inflammation was induced by sub-plantar injection of 1 % carrageenan (0.1 mL) in the left hind paw of all animals. The paw volumes up to the tibiotarsal joint were measured in mL using a plethysmometer (Apelex, France), immediately before and at 1, 2 and 3 h after carrageenan administration. Mean increases in injected paw volume were measured, and the percentage inhibition of paw edema was calculated using the equation below:

$$\text{Inhibition (\%)} = \{1 - (V_t/V_c)\}100 \dots \dots \dots (1),$$

where V_t and V_c are the mean changes in paw volume of treated and control rats, respectively.

Induction of UC in rats

After 16 h fast, the rats were lightly anesthetized with pentobarbital sodium (30 mg/kg, i.p.), and an 8 cm polyethylene catheter was introduced into the rectum of each rat. UC was induced in the rats (except the normal control group) by infusing 1 mL of 4 % acetic acid (in 0.9 % NaCl) through the polyethylene catheter. Thereafter, the rats were maintained in a head-down position for 30 s to limit ejection of the solution. Two days after colitis induction, the animals were euthanized and the distal 8 cm of the colon was resected for estimation of different parameters.

Experimental design

Seven groups of rats (n = 6) were used. Groups 1 and 2 (normal control and colitis control,

respectively) were treated with vehicle (5 mL/kg). Group 3 rats (reference) received DEX at 0.2 mg/kg. Rats in groups 4 and 5 were treated with extract of *A. gummifera* at 200 and 400 mg/kg, respectively, while rats in Groups 6 and 7 were treated with *A. kurdicus* extract at 200 and 400 mg/kg, respectively. All treatments were given orally via an orogastric cannula, once a day for 5 consecutive days, and the last dose was administered 2 h before colitis induction.

Macroscopic assessment of ulcerative colitis

The rats were sacrificed under anesthesia 24 h after colitis induction [17]. Each rat abdomen was opened to expose the colon. Distal colon, 8 cm in length and 2 cm proximal to the anus, was excised, flushed out with cold saline solution, opened longitudinally and imaged. The colon samples were weighted, and wet weight/length ratios were calculated (g/cm). Macroscopic damage of the colon was assessed by the scoring method: 0 = normal tissue; 1 = redness without ulcers or swelling; 2 = redness and swelling; 3 = one or two ulcers; 4 = more than two ulcers or one large ulcer; 5 = mild necrosis; 6 = severe necrosis.

Furthermore, ulcer area was estimated for each colon sample with the aid of a 1-mm² grid. Ulcer index (U) was computed as the sum of ulcer area (Ua, cm²) and macroscopic score (Ms)[18].

Assessment of colonic MPO activity

The activity of MPO (enzyme marker of neutrophil infiltration) was assayed in colon tissues [19]. Specimens from the distal colon (without ulceration) were kept at -80 °C before analysis. Colon samples were weighed then homogenized (50 mg tissue/ml) in a solution containing hexadecyl trimethyl-ammonium bromide (0.5 %) dissolved in 10 mM sodium phosphate buffer (pH 7.4) in an ice bath using homogenizer (IKA Werke GmbH, Germany). Colon homogenates were centrifuged at 20,000 rpm and 4 °C for 30 min. An aliquot of the supernatant was allowed to react with tetramethyl benzidine (1.6 mM) and 0.1 mM hydrogen peroxide solution. Change in absorbance was estimated spectrophotometrically at 650 nm. MPO activity was determined as the quantity of enzyme degrading 1 μmole of peroxide/min at 37 °C and was expressed in units/mg wet tissue.

Microscopic assessment of UC

Samples from colon were preserved in 10 % formaldehyde solution for 1 week. They were

dehydrated in graded ethyl alcohol and embedded in paraffin. Sections of 7 μm were deparaffinized with xylene and stained with haematoxylin and eosin (H&E), and then examined microscopically.

Statistics

Data are expressed as mean \pm standard error of mean (SEM). Statistical differences between treatment groups were analyzed according to one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS program (version 8) software package (SPSS Inc, USA).

RESULTS

Acute toxicity

A. gummifera and *A. kurdicus* extracts were well tolerated by the rats. In the 48 h period of acute

toxicity evaluation, rats given single dose of 400 mg/kg of *A. gummifera* or *A. kurdicus* extracts, showed no mortality and none of them showed any symptoms of toxicity.

Anti-inflammatory activity

The control group of rats showed paw volumes of 2.44 ± 0.19 , 2.46 ± 0.20 and 2.49 ± 0.22 mL at 1, 2 and 3 h, respectively, following carrageenan injection (Table 1). DEX significantly reduced carrageenan-induced paw edema. The mean reduction in paw volume of rats treated with DEX was 44.97 % (Fig. 1). *A. gummifera* also reduced carrageenan-induced edema, but this effect was significant at 400 mg/kg dose only. Treatment with 200 and 400 mg/kg of *A. kurdicus* elicited marked decreases in carrageenan-induced paw edema, at the first (28.68 and 32.37 %, respectively), second (23.17 and 29.67 %, respectively) and third (22.48 and 31.72 %) hours, respectively.

Table 1: Anti-inflammatory effect of DEX and ethanol extracts of *A. gummifera* and *A. kurdicus* in carrageenan-induced rat paw edema.

Group	Dose (mg/kg)	Paw volume (mL)			
		0 h	1 h	2 h	3 h
Control	0.0	1.97 \pm 0.10	2.44 \pm 0.19	2.46 \pm 0.20	2.49 \pm 0.22
DEX	0.2	1.91 \pm 0.11	1.28 \pm 0.12*	1.39 \pm 0.11*	1.37 \pm 0.10*
<i>A. gummifera</i>	200	1.99 \pm 0.09	1.89 \pm 0.17	1.99 \pm 0.16	2.06 \pm 0.19
	400	1.93 \pm 0.08	1.67 \pm 0.14*	1.79 \pm 0.14*	1.81 \pm 0.14*
<i>A. kurdicus</i>	200	1.98 \pm 0.09	1.74 \pm 0.16*	1.89 \pm 0.15*	1.93 \pm 0.12*
	400	1.92 \pm 0.07	1.65 \pm 0.13*	1.73 \pm 0.13*	1.70 \pm 0.11*

Values represent the mean \pm SEM (n = 6); *significantly different from the values of control rats at $p < 0.05$

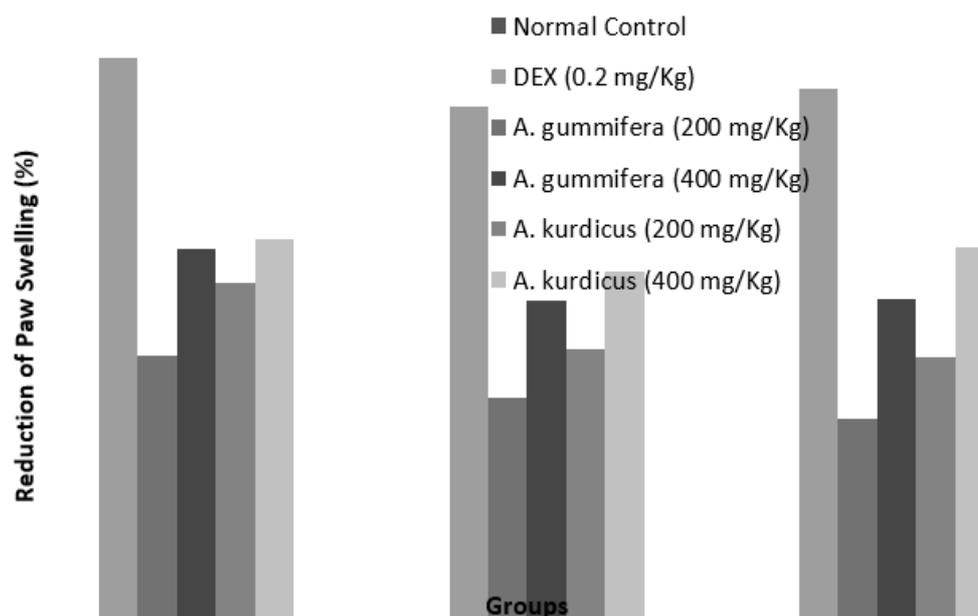


Figure 1: Anti-inflammatory activities of DEX and *A. gummifera* and *A. kurdicus* extracts against carrageenan-induced paw edema in rats

Macroscopic features of UC

In this investigation, no abnormal alterations were observed in untreated, normal rats, indicating that handling process did not interfere with the experimental outcomes. After intra-colonic administration of acetic acid, the rats with induced colitis showed prostration, piloerection and hypomotility (data not shown). Incidence of diarrhea, as well as colon weight/length lesion score, ulcer area and ulcer index were used as parameters for assessing the protective efficacies of *A. gummifera* and *A. kurdicus* extracts against UC in the rats. In the normal group, diarrhea was absent, while majority of the colitis rats showed marked signs of diarrhea (83.33 %). DEX, *A. gummifera* (400 mg/kg) and *A. kurdicus* (200 and 400 mg/kg) decreased incidence of diarrhea to 33.3, 50.0 and 16.66 %, respectively (Table 2).

Under dissecting microscope, colonic sections of rats in the control group exhibited typical normal features (Plate 1). On the other hand, colonic sections of the colitis rats appeared ulcerated, oedematous and haemorrhagic (Plate 1B). However, the 5-day pretreatment with DEX and ethanol extracts of *A. gummifera* (at 400 mg/kg) and *A. kurdicus* (at 200 and 400 mg/kg), significantly decreased the macroscopic lesions produced by the intra-rectal administration of acetic acid (Plate 1C and 1D).

The wet weight/length ratio (an indicator of inflammation) increased more than 3-fold in colitis rats (0.94 ± 0.06 g/cm) compared to normal control rats (0.29 ± 0.02 g/cm). This ratio was greatly improved in rats pre-medicated with DEX (0.33 ± 0.02 g/cm), *A. gummifera* at 400 mg/kg (0.62 ± 0.04 g/cm) and *A. kurdicus* at 200

and 400 mg/kg (0.71 ± 0.05 and 0.54 ± 0.03 g/cm, respectively). Severity of mucosal damage was scored on a 0–6 scale in a randomized, blinded fashion. In the colitis control group, the level of damage was 5.62 ± 0.37 . Administration of *A. gummifera* (400 mg/kg) and *A. kurdicus* (200 and 400 mg/kg) 5 days before induction of UC significantly decreased inflammatory damage score from 5.62 ± 0.37 to 2.60 ± 0.17 , 3.13 ± 0.27 and 2.57 ± 0.15 , respectively (Table 2).

Ulcer area (3.10 ± 0.11 cm²) and ulcer index (8.72 ± 0.57) values in colitis control rats were significantly increased after intra-rectal infusion of 4 % acetic acid, when compared to normal rats. Pre-treatment with *A. gummifera* (400 mg/kg) and *A. kurdicus* (200 and 400 mg/kg) extracts for 5 days significantly and dose dependently attenuated the increases in ulcer area and ulcer index compared to colitis control rats (Table 2 and Figure 2). The 200 mg/kg dose of the ethanol extract of *A. gummifera* did not offer protection against the colonic lesions induced by acetic acid.

Colonic MPO activity

The activity MPO in the colonic tissue was estimated by a standard enzymatic assay. Figure 3 depicts elevated levels of MPO in colonic mucosa of rats following intra-rectal infusion of 4 % acetic acid compared to normal control group (16.40 ± 0.85 U/g and 4.27 ± 0.18 U/g, respectively). Pretreatment with either 400 mg/kg of *A. gummifera* or 200 and 400 mg/kg of *A. kurdicus* extracts for 5 days produced significant decreases in MPO activity (9.33 ± 0.11 , 11.70 ± 0.14 and 9.18 ± 0.10 U/g, respectively) compared to colitis control animals (16.40 ± 0.85 U/g).

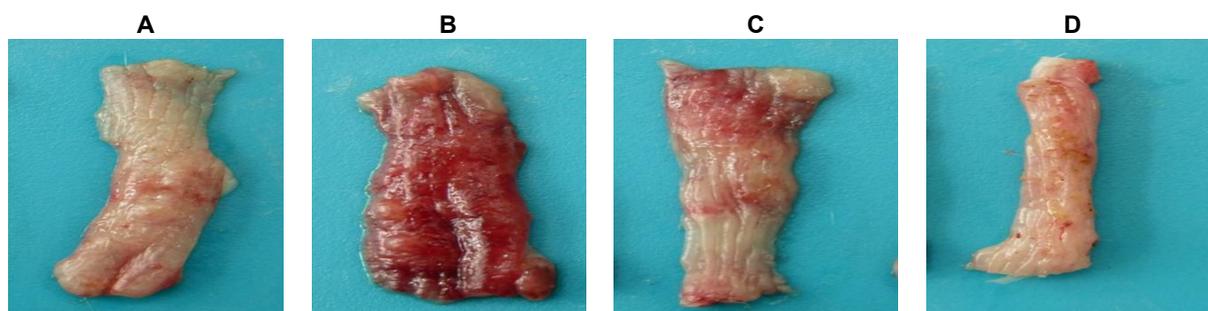
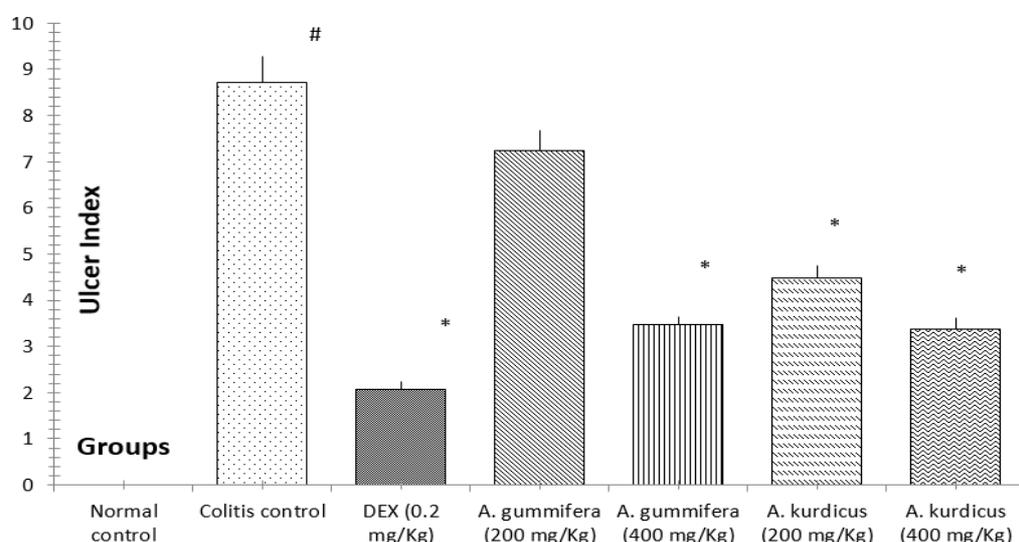
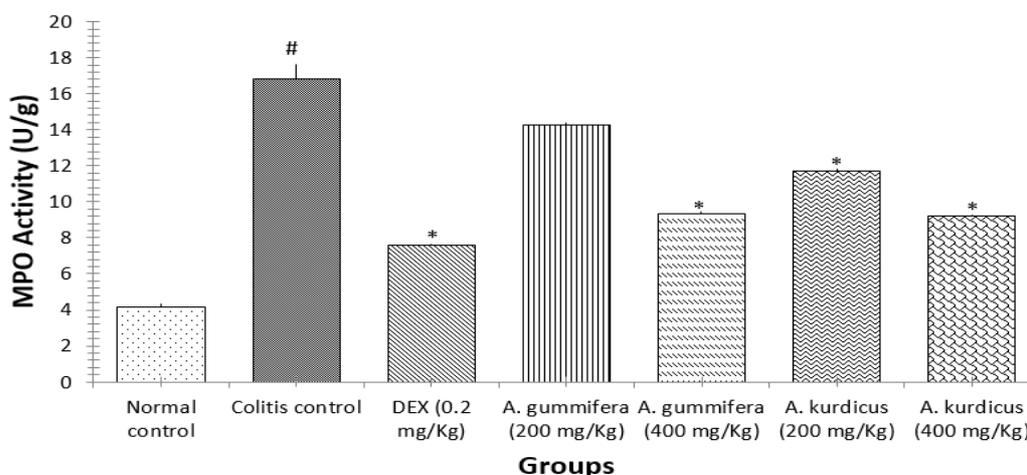


Plate 1: Photographs of colons. (A) Normal control rat. (B) Colitis control rats. Note the extensive hyperemia, edema, and ulceration of the colon. This colon was given a damage score of 5.62 ± 0.37 . (C and D) colitis rat administered 400 mg/kg of *A. gummifera* or *A. kurdicus* extracts, respectively 5 days before the acetic acid challenge. Note the improvement in the level of hyperemia, edema, and ulceration of the colon. These colons were given damage scores of 2.60 ± 0.17 and 2.57 ± 0.15 , respectively

Table 2: Effects of DEX and ethanol extracts of *A. gummifera* and *A. kurdicus* on the macroscopic parameters of ulcerative colitis induced by acetic acid in rats

Group	Dose (mg/kg)	Incidence of diarrhea	Colon weight/length (g/cm)	Macroscopical score (0-6)	Ulcer area (cm ²)
Normal control	0.0	0/6 (0%)	0.29±0.02	0.00±0.00	0.0±0.00
Colitis control	0.0	5/6 (83.33%)	0.94±0.06	5.62±0.37	3.10±0.11
DEX	0.2	1/6 (16.66%)	0.33±0.02*	1.61±0.15*	0.45±0.02*
<i>A. gummifera</i>	200	4/6 (66.66%)	0.89±0.04	4.64±0.30	2.60±0.21
	400	2/6 (33.33%)	0.62±0.04*	2.60±0.17*	0.87±0.05*
<i>A. kurdicus</i>	200	3/6 (50.0%)	0.71±0.05*	3.13±0.27*	1.35±0.08*
	400	1/6 (16.66%)	0.54±0.03*	2.57±0.15*	0.80±0.06*

Values represent the mean ± SEM (n = 6); *significantly different from the values of control rats at $p < 0.05$

**Figure 2:** Effects of DEX and *A. gummifera* and *A. kurdicus* extracts on ulcer index of UC induced by acetic acid in rats. Values represent are mean ± S.E.M. for six rats for each group. Significantly different from the values of the normal control (#) and colitis control (*) rats, at $P < 0.05$ **Figure 3:** Effects of DEX and *A. gummifera* and *A. kurdicus* extracts on MPO activity in UC induced by acetic acid in rats. Values are mean ± S.E.M. of six rats for each group. *Significantly different from normal control and colitis control (*) rats ($P < 0.05$)

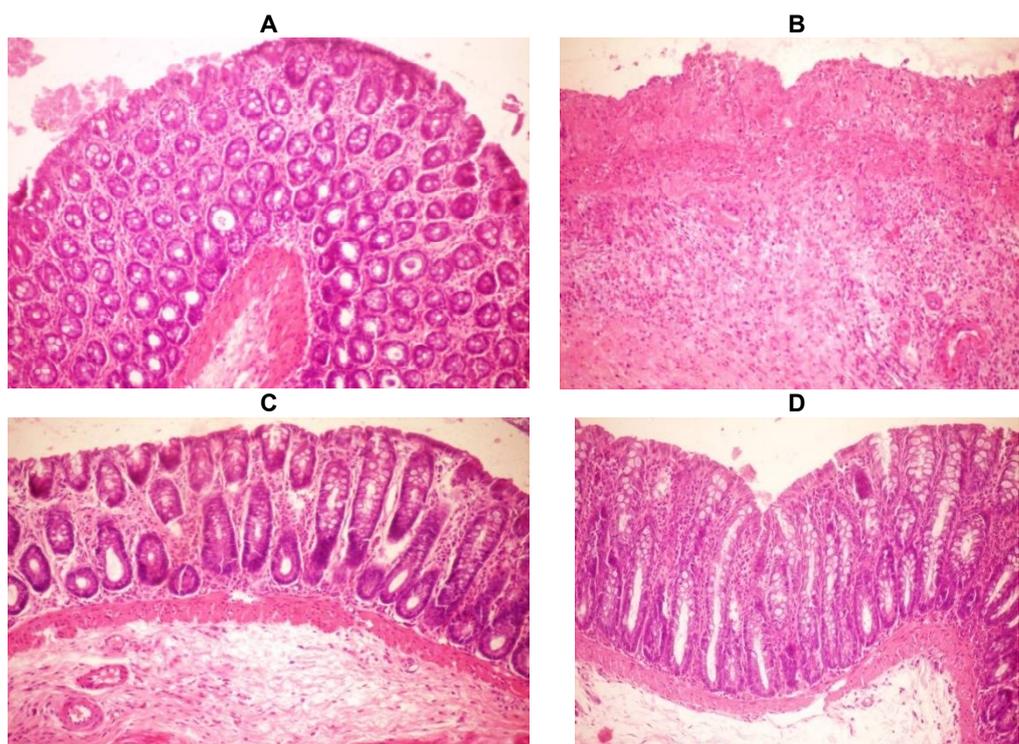


Plate 2: Photomicrographs of haematoxylin and eosin-stained paraffin sections of rat colonic tissues. Control group showing normal features (A), and colitis control group showing massive necrotic destruction of epithelium, sub-mucosal edema, and inflammatory cell infiltration in lamina propria, as well as loss of epithelium integrity (B). Pre-treatment with 400 mg/ kg of *A. gummifera* or *A. kurdicus* extract, attenuated the extent and severity of the histological lesions (C and D) (x 400)

Microscopic features of UC

The colon mucosa of normal control rats had a typical normal architecture with intact epithelium (Plate 2). In contrast, colon mucosa from the colitis control animals exhibited severe necrotic destruction of epithelium and inflammatory reactions (sub-mucosal edema, ulceration and loss of epithelium integrity as well as inflammatory cell infiltration in lamina propria (Plate 2B). Pretreatment with *A. gummifera* (Plate 2C) or *A. kurdicus* (Plate 2D) markedly reduced the severity of cellular lesions.

DISCUSSION

Acute toxicity results revealed the non-toxic nature of *A. gummifera* and *A. kurdicus* extracts. Rats treated with doses up to 4000 mg/kg of *A. gummifera* and *A. kurdicus* did not exhibit physical symptoms of toxicity throughout the experimental period, and no mortalities were detected. Thus, the LD₅₀ values of these extracts were higher than 4 g/kg. In general, the higher the LD₅₀ value, the lower the toxicity of the test compound under investigation. Therefore, the tested extracts can be categorized according to Buck *et al*, [20] as highly safe since drugs with LD₅₀ values higher than 50 mg/kg are considered non-toxic. Moreover, drugs with oral LD₅₀ values

higher than 4000 mg/kg are classified as safe or practically non-toxic [21].

In this study, we investigated potential anti-inflammatory activities of *A. gummifera* and *A. kurdicus* extracts in a carrageenan model. This model has been accepted as an adequate inflammatory tool for investigating the anti-inflammatory effect of extracts and chemical compounds. Injection of 1.0 % λ-carrageenan induced edema in the foot pad of the rat hind paw. Progression of edema in rat paw as a result of carrageenan injection is a biphasic event [22]. The first phase of edema that lasts for 1 h is due to the release of serotonin and histamine. The second phase is related to the release of prostaglandins, protease and lysosome. The results of our study indicate that the ceiling volume of foot pad was observed 3 h following carrageenan-injection. Pretreatment with DEX, 400 mg/ kg of *A. gummifera* and 200 and 400 mg/kg of *A. kurdicus* protected against the carrageenan-induced acute inflammation over a period of 3 h. The observed anti-inflammatory effect of both extracts might be due to the presence of flavonoids. It has been suggested that the *in vivo* anti-inflammatory activity of *Astragalus* plants may be mediated by inhibition of NF-kappa B activation and mRNA expression [23].

Ulcerative colitis induced by rectal infusion of 4 % acetic acid in rats is a well-established animal model, which is phenotypically similar to human colitis [24]. The mechanism of induction of colitis by acetic acid appears to involve entrance of the protonated form of the acid to the epithelium, where it dissociates to liberate protons, thereby bringing about intracellular acidification which results in massive epithelial damage [25].

Different drugs have been used in the control of UC. However, their side effects pose serious clinical problems. Thus, attention is shifting towards use of naturally-occurring alternative treatments in combination with conventional medication. For this purpose, plants of *Astragalus* species are valuable due to their anti-inflammatory and antimicrobial effects [26].

In this present study, infusion of acetic acid into rat rectum produced a marked increase in the incidence of diarrhea as well as increase in colon weight which were correlated with inflammation and ulceration. Comparable pathological impairments were earlier reported in the same animal model [27]. Pretreatment with DEX, *A. gummifera* and *A. kurdicus* resulted in marked protection as demonstrated by decreases in acetic acid-induced diarrhea, inflammation and ulceration. Although the exact mechanism by which *A. gummifera* and *A. kurdicus* extracts protected against UC is not fully understood, it could be attributed to their inhibitory effects on the release or synthesis of inflammatory mediators generated by acetic acid. It has been suggested that flavonoids may have beneficial effects in the treatment of disorders associated with oxidative stress, such as inflammatory diseases [28].

The anti-inflammatory effects showed by *A. gummifera* and *A. kurdicus* extracts were also assessed biochemically. Measurement of MPO activity has been used as an index of inflammation in several tissues, including the colon [19]. MPO (a marker of neutrophil activity) is an enzyme found predominantly in neutrophils, which are the main inflammatory cell types that infiltrate injured mucosa during acetic acid exposure [29]. In this study, colonic MPO activity was very low in normal (control) animals but was markedly increased in colitis rats, which is strongly suggestive of massive neutrophil infiltration. Activated neutrophils enter inflamed mucosa of the large intestine during acute inflammation, leading to excess generation of reactive oxygen-derived free radicals and/or proteases such as elastase and collagenase, resulting in intestinal injury [30]. A decrease in

the activity of MPO is a manifestation of anti-inflammatory activity. In present study, pretreatments with *A. gummifera* (400 mg/kg) and *A. kurdicus* (200 and 400 mg/kg) resulted in attenuation of acetic acid-induced increases in MPO. This indicates a decrease in the neutrophil infiltration of colonic mucosa, since MPO activity is considered a biochemical marker of neutrophil infiltration [19].

CONCLUSION

The results of present study suggest that ethanol extracts of *A. gummifera* and *A. kurdicus* have protective activities against UC in rats due to their anti-inflammatory properties.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors 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 them.

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