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The protective effect of *Aspalathus linearis* against the experimental gastric ulcer induced by ethanol in rats

El efecto protector de *Aspalathus linearis* contra la úlcera gástrica experimental inducida por etanol en ratas

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ABSTRACT

This study aimed to investigate the effect of *Aspalathus linearis* on the expression levels of major histocompatibility complex (MHC) molecules and their ability to present antigens to CD8, CD68, and T cells in an experimental rat model of gastritis. Furthermore, changes in tumor necrosis factor-alpha (TNF-α), myeloperoxidase, superoxide dismutase, and malondialdehyde levels due to the antioxidant properties of the plant were evaluated. The study included 24 female Wistar albino rats (*Rattus norvegicus*). *A. linearis* was administered to the treatment groups of an ethanol-induced gastritis rat model via oral gavage for 5 days the blood and stomach tissues were sampled for analysis. Biochemical evaluation revealed that TNF-α and myeloperoxidase levels decreased and superoxide dismutase activity increased in the *A. linearis*-treated groups compared with the ethanol-treated group. Histochemical evaluation revealed that the severity of inflammation decreased in the rats with gastritis treated with *A. linearis*. In addition, the number of cells expressing CD8 and CD68 decreased. The number of MHC-II immunopositive cells was higher in the *A. linearis*-treated group compared with the control and other treatment groups. *A. linearis* exhibits a protective effect on gastric mucosa by reducing the severity of oxidative stress owing to its antioxidant, anti-inflammatory, and antiapopitotic properties.

Key words: *Aspalathus linearis*; CD8; CD68; MHC class molecule; gastritis

RESUMEN

Este estudio tuvo como objetivo investigar el efecto de *Aspalathus linearis* en la expresión de moléculas del complejo mayor de histocompatibilidad (MHC) que presentan antígenos a las células CD8, CD68 y T en un modelo experimental de gastritis en ratas (*Rattus norvegicus*). También tuvo como objetivo evaluar los cambios en los niveles de factor de necrosis tumoral alfa, mieloperoxidasa, superóxido dismutasa y malondialdehído desencadenados por las propiedades antioxidantes de la planta. En el estudio se utilizaron veinticuatro ratas albinas Wistar hembra. En un modelo de gastritis inducida por etanol, se administró *A. linearis* a los grupos de tratamiento por sonda oral durante 5 días. Se tomaron muestras de sangre y tejido estomacal para análisis. La evaluación bioquímica mostró que los niveles de TNF-α y mieloperoxidasa disminuyeron y la actividad de superóxido dismutasa aumentó en los grupos tratados con *A. linearis* en comparación con el grupo de etanol. La evaluación histoquímica reveló que la severidad de la inflamación disminuyó en las ratas con gastritis sometidas al *A. linearis*. También se detectó que disminuyó el número de células que mostraban expresión de CD8 y CD68. El número de células inmuno positivas MHC II fue mayor en el grupo tratado con *A. linearis* en comparación con el control y otros grupos de tratamiento. Se concluyó que *A. linearis* tiene un efecto protector sobre la mucosa gástrica al reducir la severidad del estrés oxidativo debido a sus propiedades antioxidantes, antiinflamatorias y anti apopitóticas.

Palabras clave: *Aspalathus linearis;* CD8; CD68; molécula de clase MHC; gastritis

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INTRODUCTION

Gastric mucosa inflammation associated with mucosal damage is known as gastritis, whereas the term gastropathy refers to epithelial cell damage without inflammation. The etiology of gastritis includes several agents and viruses that can induce mucosal damage in the stomach, especially *Helicobacter pylori*. If left untreated, acute gastritis can progress into chronic disease, leading to complications [1]. Gastritis constitutes one of the most common, lifelong, serious, and insidious diseases in humans. Considering that more than half the global population experiences gastritis to some degree, hundreds of millions of people Worldwide may be affected by gastritis in one form or another [2]. Gastritis involves gastric mucosal barrier damage, which leads to proinflammatory cytokine release. During this inflammatory process, endogenous free radicals known as reactive oxygen species (ROS) are produced by proinflammatory cytokines. ROS include chemically reactive oxygen–containing compounds that are central to various physiological functions of living organisms. At low and moderate concentrations, they are required for body activities, such as intracellular homeostasis, cell death, and immune defense against pathogens [3, 4]. In addition to their roles in maintaining normal physiological functions, ROS may be involved in the development of pathological processes and can be induced via exogenous sources, such as radiation, lifestyle, stress, smoking, and ultraviolet light. To regulate the levels of free radicals, living organisms possess enzymatic and nonenzymatic antioxidant defense systems. Impairment in the ability of antioxidant systems to easily detoxify ROS causes oxidative stress. Previous studies have reported that oxidative stress causes a wide range of conditions, including neurodegenerative disorders, multiple sclerosis, rheumatoid arthritis, cancer, as well as aging [5, 6].

Gastritis is a histological description of mucosal inflammation. When inflammation occurs, in order for T cells, one of the most important components of the acquired immune system, to produce a specific response against the antigen in a protein structure, this antigen must be processed and presented to the T cells by antigen–presenting cells. The recognition of the antigen by T cells requires that the antigen–presenting cells bind to the antigen via the protein molecules on its surface. These surface peptides include major histocompatibility complex (MHC) molecules. The acquired immune system depends on the cell surface display of the host proteome in the form of peptides on MHC–I molecules. MHC–I molecules are specialized to present antigens to CD8 T lymphocytes and MHC–II molecules are specialized to present antigen to CD4 T lymphocytes, and both are crucial for initiating and expanding the immune response [7]. CD68 is a highly expressed protein by circulating macrophages and tissue macrophages that contain specialized parts known as lysosomes; whereas, CD8+ is a cytotoxic T lymphocyte cell surface receptor. CD8+ cytotoxic T lymphocytes are activated by antigenic stimulus via the infected cell presented by MHC–I to destroy the infected cell. CD4+ helper T cells recognize antigens presented by MHC–II molecules in the presence of a costimulus and are targeted for Th1 or Th2 responses [8].

There has been an increase in the trend of studying medicinal plants in global research. Rooibos (*Aspalathus linearis*), a legume shrub native to the Cederberg Mountains in South Africa, possesses numerous health–promoting properties, such as antihypertensive, hepatoprotective, antidiabetic, anti–inflammatory, antioxidant, antimutagenic, anticancer, and antiobesity properties [9, 10]. It also exhibits radical scavenging activity, especially against hydrogen peroxide and superoxide anions [9]. Numerous agents with antioxidant and anti–inflammatory effects on gastritis have been previously studied [11, 12, 13]. Although several studies have been conducted to examine the effect of *Aspalathus* on organ systems and diseases, to the best of the knowledge, there have been no studies regarding its effect on stomach ulcers. Consequently, this study aimed to evaluate the efficacy of *A. linearis* in treating experimental ethanol–induced gastritis in rats (*Rattus norvegicus*) owing to its anti–inflammatory, antioxidant, and microbial modulating activity. No studies regarding the distribution and expression of MHC class I and II molecules in experimentally induced ulcer or gastritis was found in the literature. Herein, the protective effects of *Aspalathus* against gastritis owing to its anti–inflammatory, antioxidant, and antiapopitotic activities and possible changes in the expression of MHC complex molecules, which are critical for the healing process, and the levels of free oxygen radicals were evaluated.

MATERIALS AND METHODS

Herbal extract

A. linearis was obtained from Nutrex Hawaii, USA. During the study period, daily doses were freshly prepared and stored at 4°C (TSX series/Thermo Fisher Scientific/ABD, USA).

Animal material

The study was conducted according to the experimental animal guidelines of the Dicle University Health Sciences Research and Application Center Experimental Animals Local Ethics Committee (Protocol no: 2022/36). Herein, 24 female Wistar albino rats (180–200 g) were used. The rats were housed under constant temperature (22 ± 3°C) and humidity (50–55%), 12–h light/dark cycle, and free access to food and water. To establish the experimental gastritis model, the rats were fasted for 24 h and only allowed access to water. Gastritis in rats was induced via ethanol. The rats were randomly divided into four groups.

Experimental design

Group 1 (control): Healthy rats were given 1 mL saline via oral gavage during the study period.

Group 2 (*A. linearis* group): Healthy rats were given 2 g·100 mL^{-1} of aqueous extract during the study period; the administered dose was 10 mL aqueous extract/100 g via oral gavage (Hong *et al.*) [14].

Group 3 (ethanol group): Healthy rats were given 2 mL of ethanol via oral gavage on the first day.

Group 4 (ethanol + *A. linearis*): Healthy rats were given 2 mL of ethanol on the first day and then 2 g·100 mL^{-1} of aqueous extract during the study period; the administered dose was 10 mL of aqueous extract/100 g via oral gavage [14].

At the end of the study period (6 d), cardiac blood was sampled and the rats were euthanized under Xylazine–Ketamine (10–90 mg·kg^{-1}) anesthesia.

Tissue harvesting

Gastric tissue samples were obtained from the euthanized rats. The specimens were fixed in formaldehyde solution for 18 h and paraffin–embedded blocks were prepared following routine histological procedures (Leica RM2235 Rotary microtome / Germany) Serial sections of 5 µm thickness were sliced from the paraffin blocks. Crossman's triple staining was performed to determine histopathologic changes.

Immunohistochemical procedure

The paraffin-embedded sections were placed on adhesive slides coated with aminopropyltriethoxysilane and subjected to deparaffinization and rehydration and washed using distilled water. The sections were treated with 3% hydrogen peroxide prepared in methanol for 20 min to remove endogenous peroxidase activity and washed with 0.01 M phosphate-buffered saline (PBS) (4 × 5). After washing, citrate buffer (0.01 M, pH 6.0) was prepared for antigen retrieval, boiled at 95°C for 30 min, and then left to cool. To prevent nonspecific staining in the sections, they were incubated with a protein blocking solution (Ultra V Block, Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, USA; TA-125UB) at room temperature for 15 min.

Immediately afterward, the sections were probed with mouse monoclonal MHC-I (MHC-I [HLA] Ab-1 [Clone B-D11]; Thermo Fisher Scientific Lab Vision Corporation), MHC-II (MHC-II [HLA-DR] Ab-1 [Clone LN3]; Thermo Fisher Scientific Lab Vision Corporation), CD8 (UCH-T4 Santa Cruz), and CD68 (KP1 Biocare Medical) primary antibodies diluted at 1/200 ratios at 4°C overnight. After incubation, the sections were washed with 0.01M PBS (4 × 5). To visualize the color reaction, the sections were incubated with 3,3'-diaminobenzidine (DAB) (Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, USA), a chromogen substrate, for 4–10 min [15]. Then, the sections were placed for 1 min for contrast staining in Gill's hematoxylin and subsequently washed with tap water until they turned blue. The sections were passed through graded alcohols and xylol and covered with entellan.

For negative controls, primary antibodies were substituted with PBS or normal mouse IgG (Santa Cruz Biotechnology, sc-2025) and the same immunohistochemical procedure was applied for the remaining staining. After staining, the preparations were examined, evaluated, and photographed using a Nikon-Eclipse 400 DSRI (Japan) microscope with an attached Nikon digital camera (NIS Elements Imaging Software (version 3.10).

Immunohistochemical evaluation

Immunohistochemical staining was semiquantitatively evaluated via intensity score. The staining intensity of the cells was determined as (-) no staining, (+) weak, (++) moderate, and (++++) strong staining [16]. The staining intensity of immunostaining reactions in cells was assessed by two independent investigators (MAK and ZK), and the average score of the two observers was considered. MHC-I, MHC-II, CD8, and CD68 localization in the stomach were evaluated under light microscopy at ×20, ×40, and ×100 magnifications.

Biochemical analysis

Before the animals were euthanized, cardiac blood samples were collected under general anesthesia in serum tubes. TNF-α, malondialdehyde (MDA), superoxide dismutase (SOD), and myeloperoxidase (MPO) levels in the blood samples were determined using an enzyme-linked immunosorbent assay kit. The blood samples were transported to the laboratory according to the cold chain procedure.

The blood samples were analyzed in duplicates via a BS-400 automated spectrophotometer (Mindray, Shenzhen, China).

Statistical analysis

SPSS software version 24.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Kolmogorov–Smirnow test was performed to assess the homogeneity of the data. Differences between the groups were evaluated via one-way analysis of variance, and Kruskal–Wallis test was used as a post hoc test. A *P*-value of <0.05 was considered significant.

RESULTS AND DISCUSSIONS

Histopathologic findings

Examining Groups 1 and 2 revealed that the gastric mucosa exhibited a normal histologic structure. In the gastric mucosa comprising a single layer prismatic epithelium, the lamina propria was filled with gastric glands (FIG. 1).

Examining Group 3 revealed that degeneration in the epithelial cells and deterioration in the epithelial integrity. The glands in the submucosa were had dilatations and hemorrhagic areas (FIG. 1). Intense inflammatory cell infiltration was found in the lamina propria and these infiltrations were more intense in the mucosal part of the cell.

Examining Group 4 revealed that the degenerations in the epithelial part of the mucosa significantly reduced because of *A. linearis*

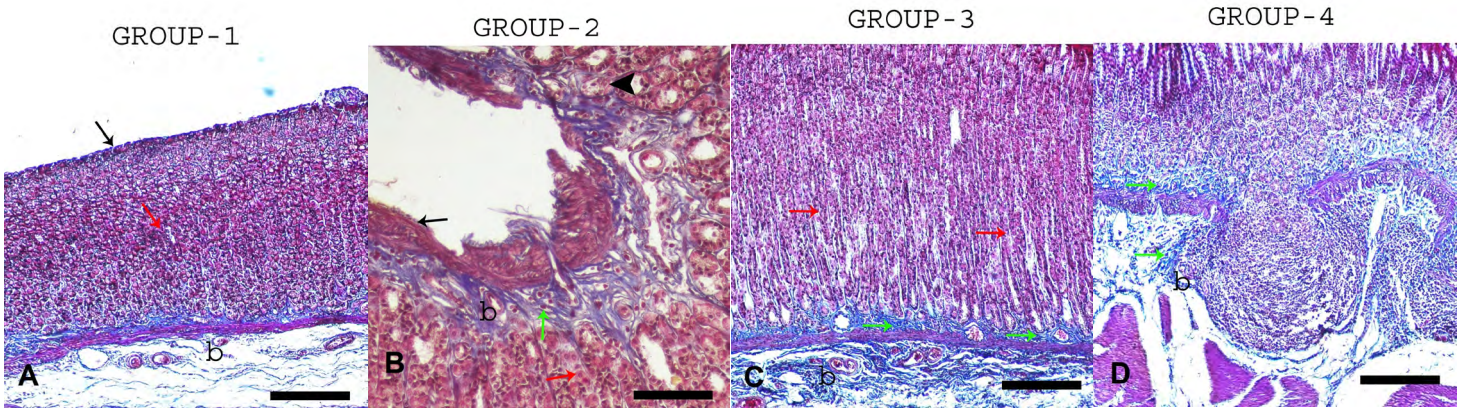

FIGURE 1. Sections of gastric mucosa of controls and treatment groups (Crossman's triple stain). Group 1, black arrow: normal mucosa, red arrow: Regular arrangement of tubular fundic glands which are lying perpendicular to the surface with short narrow pits. Group 2, black arrow: impaired mucosal integrity, red arrow: disintegrated glands, green arrow: intraepithelial cell infiltration, head arrow: epithelial cells. Group 3, red arrow: disintegrated glands, green arrow: subepithelial cell infiltration. Group 4, green arrow: cells infiltration in stroma. b: blood vessel. Group 1, 2 and 4 figures 25 µm, Group 3 figure 50 µm

treatment. The reorganization of the disrupted epithelial integrity was also observed. The dilatations in the gastric glands decreased, and no hemorrhagic areas were observed. Mitotic activity was higher in the basal-located glands in the lamina propria than that of the other groups (FIG. 1).

Immunohistochemical findings

The immunohistochemical findings of CD8, CD68, MHC-I, and MHC-II in the stomach are presented in TABLE I. CD8 and CD68 immunoreactivity was observed in connective tissue where cell infiltration was dense. Numerous inflammatory cells in this region were also positive for these factors. CD8 and CD68 immunoreactivity was higher in Group 3 compared with that in control and the other experimental groups. CD8 and CD68 positive immunoreactivity increased in Group 3 and decreased in Group 4 (FIGS. 2 and 3).

TABLE I
Immunohistochemical expression intensities of CD8, CD68, MHC I, and MHC II in stomach

Groups	CD8	CD68	MHC I	MHC II
Group 1 (Control)	++	+	++	++
Group 2 (*Aspalathus linearis*)	++	++	+++	+++
Group 3 (Ethanol)	+++	++	++	++
Group 4 (Ethanol + *A. linearis*)	+	+	+	++

Staining intensity; (-) no staining, (+) weak, (++) moderate, (++++) strong

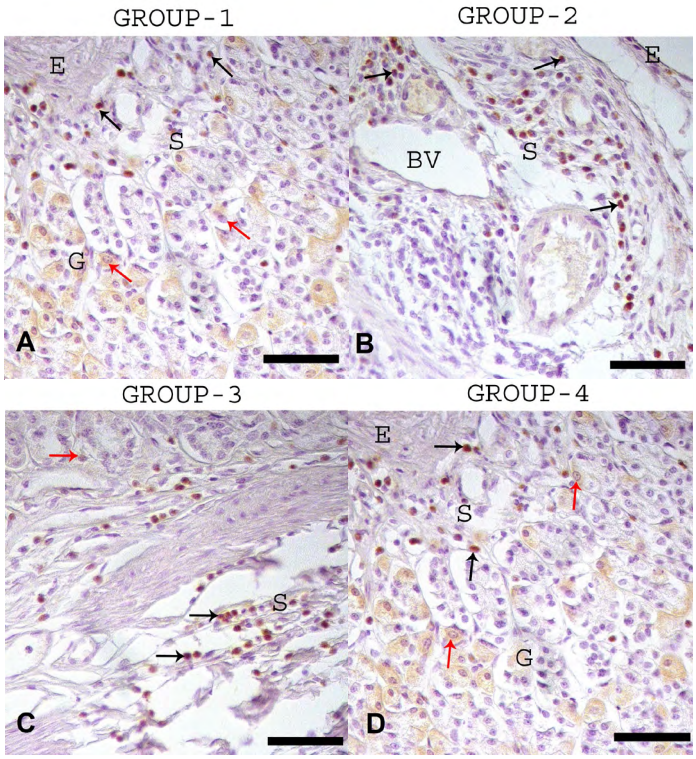

FIGURE 2. Immunohistochemical staining of CD8 in the stomach. Black arrow: positive immunoreactivity in glands cell, red arrow: mitotic activity in cells, E: epithelium, S: stroma, G: glands, BV: blood vessel. All figures 100 µm

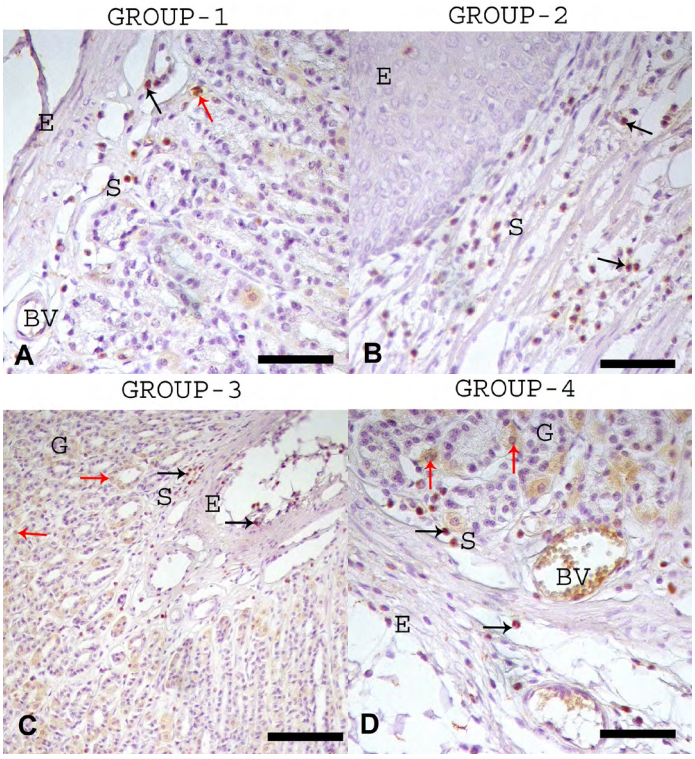

FIGURE 3. Immunohistochemical staining of CD68 in the stomach. Black arrow: positive immunoreactivity in glands cell, red arrow: mitotic activity in cells, E: epithelium, S: stroma, G: glands, BV: blood vessel. Group 2 figure 50 µm, Group 1, 3 and 4 Figures 100 µm

MHC-I and MHC-II immunoreactivity was localized in connective tissue via intraepithelial lymphocytes located in the epithelium. When Groups 3 and 4 were examined, positive immunoreactivity was observed to be distributed throughout the lamina propria. In addition, it was determined that the number of MHC-II immunopositive cells was higher in Group 2 than in the control and other experimental groups (FIGS. 4 and 5).

Biochemical findings

The serum TNF-α, MPO, SOD, and MDA levels of the groups are presented in TABLE II. No statistically significant difference was found between the groups regarding serum TNF-α, MPO, SOD, and MDA levels (FIG. 6).

Stress, smoking, alcohol use, and long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) can damage the gastric mucosa [17]. The mechanisms underlying ethanol-induced gastric damage have not been fully elucidated; however, it is well known that proinflammatory mediators, such as ROS, cytokines, and neutrophil infiltration, constitute major factors for gastric ulcer development [18, 19].

Lipid peroxidation has been used as an indicator of ROS-mediated cell damage in cell membranes. MDA is an end product generated via the peroxidation of polyunsaturated fatty acids and is often used to predict oxidative stress conditions [20]. SOD is an antioxidant enzyme that regulates ROS levels by catalyzing the conversion of superoxide to hydrogen peroxide and molecular oxygen [21]. Glutathione (GSH) peroxidase is another antioxidant enzyme that catalyzes the reduction of Hydrogen peroxide and lipid peroxides to lipid alcohols by oxidizing

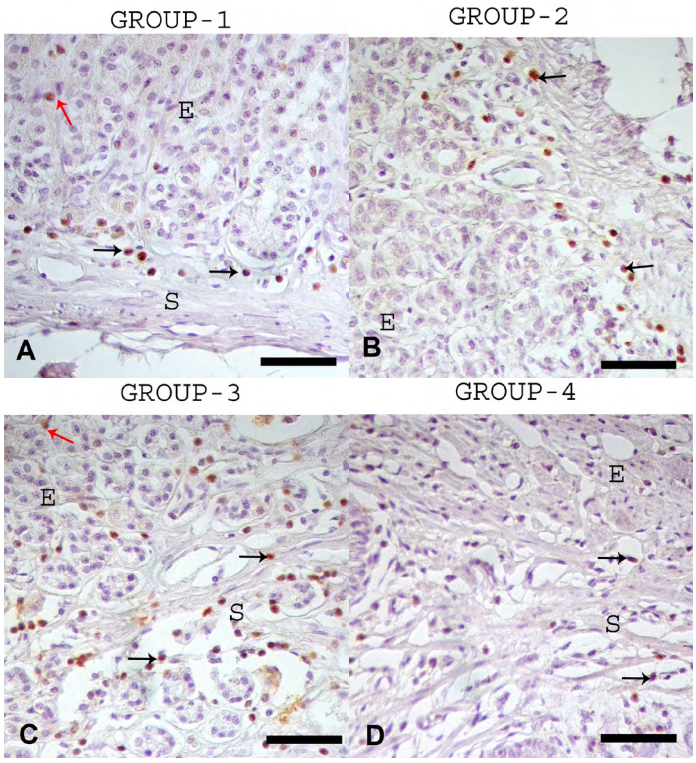

FIGURE 4. Immunohistochemical staining of MHC–I in the stomach. Black arrow: positive immunoreactivity in glands cell, red arrow: mitotic activity in cells, E: epithelium, S: stroma. All figures 100 µm

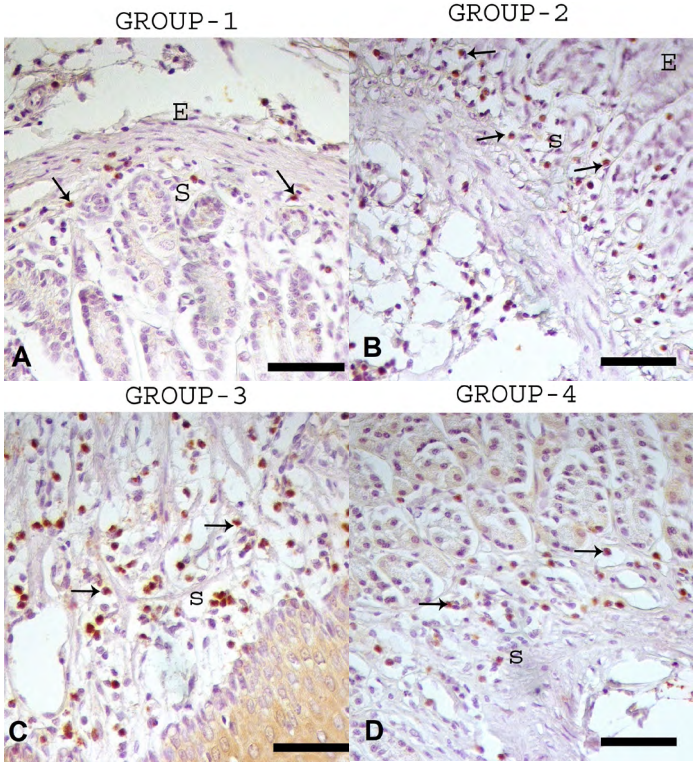

FIGURE 5. Immunohistochemical staining of MHC–II in the stomach. Black arrow: positive immunoreactivity in glands cells. E: epithelium, S: stroma. All figures 100 µm

TABLE II
Serum TNF–α, MPO, SOD, and MDA values of the groups (mean ± standard deviation)

Groups	TNF–α	MPO	SOD	MDA
Group 1 (Control)	65,805 ± 26,33	91,500 ± 105,62	143,500 ± 125,77	46,320 ± 16.31
Group 2 (*Aspalathus linearis*)	69.020 ± 16,69	80,300 ± 83,79	387,950 ± 124,13	40,520 ± 17,91
Group 3 (Ethanol)	50,100 ± 10,40	39,500 ± 45,37	199,700 ± 145,67	48,760 ± 14,38
Group 4 (Ethanol + *A. linearis*)	53,690 ± 26,99	37,650 ± 30,48	301,150 ± 118,17	43,670 ± 14,29
P–value	0,316	0,342	0,171	0,993

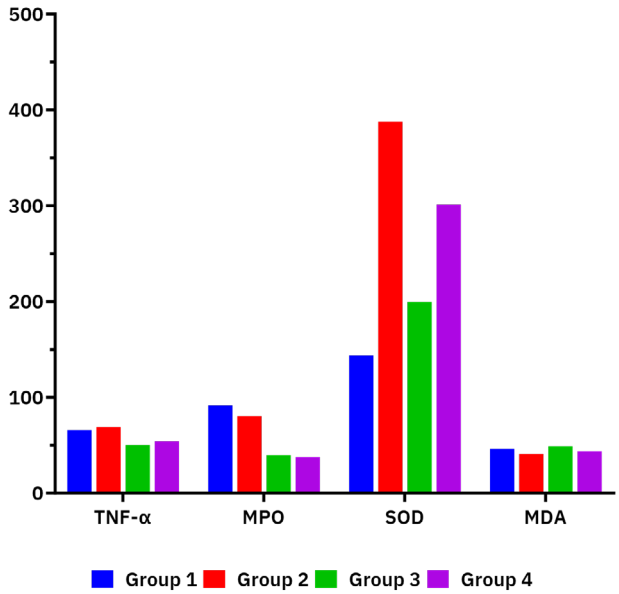

FIGURE 6. TNF–α (pg·mL^{-1}), MPO (IU·L^{-1}), SOD (IU·mL^{-1}) and MDA (mmol·L^{-1}) of ethanol–induced gastritis in rats

reduced GSH to Glutathione disulfide [22]. When ROS levels rise, catalase, GSH, GSH peroxidase, SOD, and vitamins A, E, and C, which comprise the antioxidant defense system of the human body, remove ROS via enzymatic and nonenzymatic pathways. If the oxidation system homeostasis is disrupted, free oxygen radicals are released which induce cell damage, leading to numerous chronic diseases, including ischemic stroke, asthma, and malignancies [23, 24].

Reportedly, a low dose of eugenol, a substance present in *A. linearis*, enhances the healing process in experimentally induced chronic gastritis ulcer in rats [25]. Studies regarding the effects of *A. linearis* on rats have reported a wide range of physiological effects, including antimicrobial activity in cells, inhibition of oxidative stress in diabetic rat models, hepatoprotective effect, reduction of skin inflammation, increased regeneration, and supportive therapy in cancer [26, 27]. The antioxidant activity of *A. linearis* was reported in a study involving a streptozotocin–induced diabetic rat model. *A. linearis* can reportedly reduce advanced glycation end products and MDA in plasma and different tissues [28]. Inflammatory mediators, such as

free oxygen radicals, TNF-α, and platelet activating factors IL-1, IL-3, and IL-6, which are increasingly produced in the body, are considered to increase mucosal damage and cause ulceration and necrosis [28]. Plants with antioxidant activity can reduce or eliminate the negative effects of inflammatory mediators on the mucosal surface by inhibiting free oxygen radical formation. A study investigating the protective effects of *A. linearis* on the cardiovascular effects of diesel exhaust particles reported that it regulated inflammation by reducing the gene expression levels of TNFα, IL-1β, IL-8, IL-10, NF-κB, and IBKB induced by diesel exhaust particles [29]. In our experimental rat gastritis model, the TNF-α and MPO levels decreased in the groups treated with *A. linearis* and ethanol + *A. linearis* compared with the ethanol-treated group, probably owing to the strong antioxidant activity of *A. linearis*. Although this decrease was not significant, it suggested that *A. linearis* inhibited the formation of free oxygen radicals in the treated groups and reduced the negative effects of inflammatory mediators on the mucosal surface. Moreover, increase in SOD activity in the groups treated with *A. linearis* and ethanol + *A. linearis* compared with the ethanol-treated group has been previously reported [27, 30], indicating that *A. linearis* stimulates SOD activity.

Neutrophil infiltration into the gastric mucosa constitutes a critical process in the pathogenesis of gastric ulcers. Ethanol-induced neutrophil infiltration has been associated with the formation of gastric lesions, which can be determined by MPO activity [31]. The gastric mucosa has a rich innate immune system that includes CD4 and CD8 T cells as well as macrophages, mast cells, and eosinophils [32]. In the innate immune system, mammalian macrophages play an important role by producing cytokines and phagocytic function. M1-like macrophages are involved in eliminating proinflammatory (IL1 beta and TNF-α) and chemoattractant (CXCL-3, -8, and -10) cytokines, pathogens, damaged or transformed cells, pathologically healing other immune system cells, and in the case of prolonged inflammation, attracting CD8 T and B lymphocytes to attack the surrounding tissues [33]. Tissue macrophages are identified via the immunohistochemical detection of the CD68 molecule, a glycoprotein specific to monocytes/macrophages [34]. Herein, we evaluated the effects of *A. linearis* on CD8 and CD68 expression in inflammatory cells in an experimental ethanol-induced gastritis rat model. Ethanol + *A. linearis* treatment decreased the severity of inflammation, consequently decreasing the number of cells expressing CD8 and CD68.

Studies have reported that the expression of MHC molecules is considerably increased via activated innate and acquired immune system cytokines (such as interferons and TNF) [35, 36]. Herein, the immunoreactivity of cells displaying MHC class I and II molecule expression decreased in the groups treated with ethanol and ethanol + *A. linearis* compared with the control and the groups treated with only *A. linearis*. This finding supports the conclusion that owing to the antioxidant properties of *A. linearis*, changes in the expression of MHC class molecules increase during inflammation and decrease during recovery, especially in parallel with the changes in TNF-α and SOD levels and CD8 immunoreactivity.

CONCLUSIONS

In conclusion, the present study revealed the protective activity of *A. linearis* for the gastric mucosa as it reduces the severity of oxidative stress in acute gastritis. This result needs to be supported by *in vitro* and *in vivo* studies.

Conflict of interest

The authors declare that they have no conflict of interest.

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