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The protective effect of Pomegranate extract against the experimental gastric ulcer induced by ethanol in rats

El efecto protector del extracto de Granada contra la úlcera gástrica experimental inducida por etanol en ratas

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ABSTRACT

The Pomegranate (*Punica granatum*) is a commonly found fruit in the Mediterranean and Iran, which has a variety of uses including medicinal purposes, cosmetics, and as a spice in culinary applications. Pharmacological functions of Pomegranate include antioxidation, anti-tumor, anti-hepatotoxicity, anti-lipoperoxidation and antibacterial properties. The aim of this study was to evaluate the therapeutic efficacy of Pomegranate extract by utilizing its antioxidant activity in an experimental rat model of gastritis induced by ethanol. In the study, 24 female Wistar albino rats (180–200 g) were used. Gastritis in rats was induced using Ethanol. In experimental groups, Tumor necrosis factor-alpha, Myloperoxidase, Superoxide Dismutase and Malondialdehyde were examined for biochemical analyzes. Streptavidin peroxidase immunohistochemistry method was applied to gastric tissues with gastritis. A statistically significant difference was observed between Superoxide Dismutase and Meloperoxidase levels. CD8 and CD68 immunoreactivity was higher in the Ethanol group compared to the other groups. A decrease was observed in CD8 and CD68 positive immunoreactivity in Ethanol+Pomegranate extract group compared to Ethanol group. The study found that the immunoreactivity of MHC-I and MHC-II was found in specific locations, namely intraepithelial lymphocytes located in the epithelium, some capillary vessel endothelium, and connective tissue. Changes in anti-oxidative stress markers such as Superoxide Dismutase and Myloperoxidase contributed to the mucosal protective effect of Pomegranate extract in Ethanol-induced gastritis.

Key words: Gastritis; immunohistochemistry; MHC class molecules; pomegranate extract; rat

RESUMEN

La granada (*Punica granatum*) es una fruta que se encuentra comúnmente en el Mediterráneo e Irán, y que tiene una variedad de usos que incluyen fines medicinales, cosméticos y como especia en aplicaciones culinarias. Las funciones farmacológicas de la granada incluyen propiedades antioxidantes, antitumorales, anti hepatotoxicidad, anti lipoperoxidación y antibacterianas. El objetivo de este estudio fue evaluar la eficacia terapéutica del extracto de granada mediante la utilización de su actividad antioxidante en un modelo experimental de gastritis inducida por etanol en ratas. En el estudio se utilizaron 24 ratas albinas Wistar hembra (de 180 a 200 g). La gastritis en ratas se indujo utilizando etanol. En los grupos experimentales, se examinaron el factor de necrosis tumoral alfa, la mieloperoxidasa, la superóxido dismutasa y el malondialdehído para análisis bioquímicos. Se aplicó el método de inmunohistoquímica de estreptavidina peroxidasa a tejidos gástricos con gastritis. Se observó una diferencia estadísticamente significativa entre los niveles de superóxido dismutasa y mieloperoxidasa. La inmunorreactividad de CD8 y CD68 fue mayor en el grupo de etanol en comparación con los otros grupos. Se observó una disminución en la inmunorreactividad positiva para CD8 y CD68 en el grupo de etanol + extracto de granada en comparación con el grupo de etanol. El estudio encontró que la inmunorreactividad de MHC-I y MHC-II se encontró en ubicaciones específicas, a saber, linfocitos intraepiteliales ubicados en el epitelio, algunos endoteliales de vasos capilares y tejido conectivo. Los cambios en los marcadores de estrés antioxidante, como la superóxido dismutasa y la mieloperoxidasa, contribuyeron al efecto protector de la mucosa del extracto de granada en la gastritis inducida por etanol.

Palabras clave: Gastritis; inmunohistoquímica; moléculas de clase MHC; extracto de granada; rata

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INTRODUCTION

Gastrointestinal hemostasis, which is crucial for human well-being and a long life, is one of several pathways that play a vital role. Gastritis can be caused by various factors including non-steroidal anti-inflammatory drugs, burns, brain injury, autoimmunity, and *Helicobacter pylori* infections. However, contributing factors such as obesity, smoking, heavy alcohol intake, and fast food consumption originated from modern lifestyle and dietary habits also play a significant role in the development of gastric diseases [1]. The pathogenesis of gastritis involves inflammation, gastric mucosal cell changes (degeneration, apoptosis and necrosis), barrier damage and bleeding. Furthermore, oxidative-induced gastric disorders are significantly influential in the development of pathogenesis [1].

The Pomegranate *(Punica granatum)* is a commonly found fruit in the Mediterranean and Iran, which has a variety of uses including medicinal purposes, cosmetics, and as a spice in culinary applications. Pharmacological functions of Pomegranate include antioxidation, anti-tumor, anti-hepatotoxicity, anti-lipoperoxidation and antibacterial properties [2, 3]. It has been shown that Pomegranate juice has much higher antioxidant activity than red wine and green tea (*Camellia sinensis)* [4]. Extracts from Pomegranate are used as potential therapeutics in inflammation, bacterial infections, healing wounds, neurodegenerative disorders, obesity, diabetes mellitus and also protect against cancer [5].

Living organisms have enzymatic and non-enzymatic antioxidant defense systems to regulate the levels of free radicals [5]. When reactive oxygen species (ROS) are not eliminated by antioxidant systems, it results in a state of oxidative stress. Previous studies have shown that oxidative stress causes several diseases, including neurodegenerative disorders, multiple sclerosis, rheumatoid arthritis and cancer, as well as the natural aging process [6, 7, 8].

For the immune system to generate a targeted response against a protein antigen, it is essential that antigen-presenting cells process the antigen and display specific peptides through major histocompatibility complex (MHC) molecules on their surface, which can then be recognized by T cells. MHC class I (MHC-I) molecules activate $CD8^+$ cytotoxic T cells, which are responsible for eliminating intracellular pathogens. On the other hand, MHC class II (MHC-II) molecules activate $CD4^+$ helper T cells, which can target both intracellular and extracellular pathogens. Hence, certain functions, such as cytokine production and antibody synthesis required for leukocyte activation occurs [9]. CD68 is a protein highly expressed by circulating macrophages and tissue macrophages that contain specialized elements called lysosomes [10]. It is commonly used as a macrophage marker. CD8 is expressed on the cell surface membranes of functionally different T cell populations and is a cytotoxic T lymphocyte cell surface receptor [11].

The aim of this study was to evaluate the therapeutic efficacy of Pomegranate extract by utilizing its antioxidant activity in an experimental rat model of gastritis induced by Ethanol. In the presedent study, it was aimed to determine the possible changes in the expressions of critical molecules such as CD8, CD68, MHC-I and MHC-II during the healing process by benefiting from the antioxidant activity of Pomegranate extract in gastritis experimentally induced in Wistar albino rats (*Rattus norvegicus*). Additionally, it was aimed to reveal changes in the serum levels of Tumor necrosis factor-alpha (TNF-α), Myeloperoxidase (MPO), Superoxide Dismutase (SOD) and Malondialdehyde (MDA), which are components of the enzymatic antioxidant system.

MATERIALS AND METHODS

Herbal extract

The Pomegranates used in the study were obtained from Pomegranate producers registered in Siirt in Turkey. After the Pomegranates were thoroughly washed and dried, they were cut in half and Pomegranate juice was extracted with a juicer. The resulting Pomegranate juice was filtered and lyophilized (Christ, 0.21 mm Hg, -80°C [Panasonic-MDF-U53865-PE/Japon]) until dry. The extract obtained was stored in a dark bottle at +4°C (TSX series/Thermo Fisher Scientific/USA).

Ethical statement

The research was carried out in compliance with the animal experimentation regulations set by the Local Ethics Committee of Dicle University Health Sciences Application and Research Center Animal Experiments (Protocol no: 2022-08).

Experimental design

In the study, 24 female Wistar albino rats (*Rattus norvegicus*) (180-200 g) were used. Animals were housed under conditions of constant temperature (22 ± 3°C) and humidity (50-55%), a 12 h light/dark cycle and free access to food and water. In order to create the experimental gastritis model, the rats were deprived of food for a period of 24 h prior to the experiment, with unrestricted access to water during this time. Gastritis in rats was induced using ethanol. The animals were randomly divided into 4 groups.

- Group 1 (control): healthy rats were given 1 mL saline by oral gavage during the study period.
- Group 2 (Pomegranate extract): healthy rats were given 0.5 mL (100 mg·kg^{-1}) by oral gavage during the study period [12].
- Group 3 (Ethanol): healthy rats were given 2 mL Ethanol by oral gavage on the first day.
- Group 4 (Ethanol+Pomegranate extract): healthy rats were given 2 mL Ethanol on the first day and then 0.5 mL (100 mg·kg^{-1}) Pomegranate extract by oral gavage during the study period [12].

At the end of the study period (6 days), blood sample was obtained from the heart under Xylazine-Ketamine (10-90 mg·kg^{-1}) anesthesia and the animals were sacrificed.

Tissue harvesting

Gastric tissue sample were obtained from the euthanized rats. The tissue samples taken were fixed in formalin-alcohol solution for 18 h and were embedded in paraffin after undergoing routine histological processing [13]. Five-micron-thick serial sections were cut from (Leica RM2235 Rotary microtome/Germany) the paraffin blocks at 100-lm intervals. The slides were stained with Crossman's triple stain for the determination of the histopathologic changes.

Immunohistochemical procedure

Sections from paraffin blocks were placed on adhesive slides coated with Aminopropyltriethoxysilane and were then deparaffinized, rehydrated, and washed in distilled water. The sections were treated with 3% H_2O_2 prepared in Methanol for 20 min to remove endogenous peroxidase activity. The sections were washed using 0.01 M phosphate buffer saline (PBS, 4×5). Next, citrate buffer

(0.01 M, pH 6.0) was prepared for retrieving antigens by boiling the sample at 95°C and allowed to cool. Slides were incubated in protein blocking solution (Ultra V Block, Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, USA; TA-125UB) for 15 min at room temperature (25°C) to prevent nonspecific staining. Next, sections were immediately incubated (Arçelik 5223 NHEY Fridge/ Turkey) 4°C overnight with mouse monoclonal CD8 [CD8 (UCH-T4), sc-1181; Santa Cruz Biotechnology], CD68 (CD68, Clone KP1; cat.no: CM 033 A,B,C; Biocare Medical), MHC-I [HLA class I (B-D11), sc-65319; Santa Cruz Biotechnology], and MHC-II [MHC-II (HLA-DR) Ab-1 (Clone LN3); Thermo Fisher Scientific Lab Vision Corporation), and primary antibodies were diluted at 1/200 ratios. After incubation, sections were washed using 0.01 M PBS (4×5). To visualize the colored reaction, slides were immersed in 3,3'-diaminobenzidine (DAB) (Thermo Fisher Scientific Lab Vision Corporation, Fremont, CA, USA), a chromogen, for 4–10 min. The sections were counterstained using Gill's Hematoxylin for 1 min and then washed in tap water until they turned blue. Sections were passed through graded alcohol series and xylol and then covered with entellan [13, 14].

Negative and positive controls were used to exhibit specificity of immunohistochemical staining. Human tonsil tissue retrieved from tissue archives of Dicle University Department of Pathology with cells with known possession of CD8, CD68, MHC-I, and MHC-II surface receptors was used as positive control (FIG. 1). As for negative controls, normal mouse IgG (Santa Cruz sc-2025) was used. As a result, nonspecific staining was not detected in negative controls. Tissue sections were examined by conventional light microscopy (Nikon Eclipse E400; Nikon, Tokyo, Japan) and were photographed with a digital camera (Nikon Coolpix 4500).

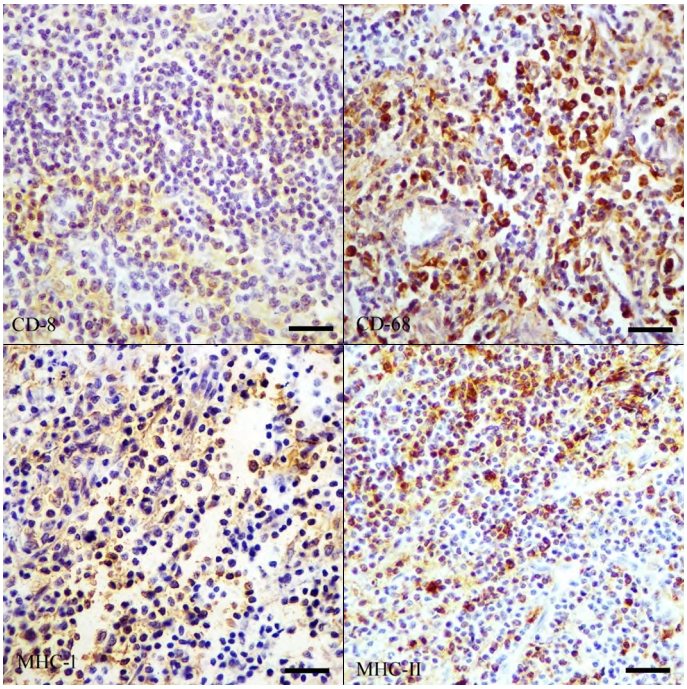

FIGURE 1. Positive control for CD8, CD68, MHC-I and MHC-II in the stomach in rats. Bar: CD8 and MHC-I figures 50 µm, CD68 and MHC-II figures 100 µm

Biochemical analysis

Before the animals were sacrificed, blood samples were collected from the heart into serum tubes under general anesthesia. TNF-α (tumor necrosis factor-alpha), MPO (myeloperoxidase), SOD (Superoxide Dismutase) and MDA (Malondialdehyde) values were determined using Eliza kit from the blood which was transported to the laboratory in accordance with the cold chain procedure.

All blood samples were analyzed in duplicate, the obtained values were displayed on 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 used to test the homogeneity of the data. Differences between groups were evaluated by one-way analysis of variance (ANOVA) test and Kruskal Wallis was used as a post hoc test. $P<0.05$ value was considered statistically significant.

RESULTS AND DISCUSSIONS

Histopathologic findings

The results showed that both the control group and the group treated with Pomegranate extract had mucosa layers that were consistent with the typical histological structure. The gastric mucosa consisted of a single layer of prismatic epithelium, the lamina propria was filled with gastric glands, and the mucosal layer was covered with a thin lamina muscularis (FIG. 2).

In the Ethanol group, most of the single layer prismatic epithelial cells were degenerated and epithelial integrity was disrupted. The disrupted epithelial cells were found to form extensions towards the lumen. Gastric glands located under the mucosa were found to have dilatations. The glandular cells exhibited disorganization, and there were areas of hemorrhage interspersed between them. Intense inflammatory cell infiltration was found in the lamina propria. The level of cell infiltration was higher in the cutaneous mucosa. When the Ethanol+Pomegranate extract group was examined, It has been observed that damage to the apical part of the mucosa is significantly reduced by treatment with Pomegranate extract compared with the ethanol-only group. Damage to single-layer prismatic epithelial cells was also significantly reduced. There was a marked decrease in dilatation of the gastric glands with mild hemorrhagic areas. It was determined that mitotic activity was higher in the glands located in the basal part of the lamina propria compared to the other groups. The distribution of inflammatory cells within the lamina propria was noted to decrease, scattering towards the epithelial tissue (FIG. 2).

Immunohistochemical findings

Immunohistochemical findings of CD8, CD68, MHC I and MHC II in the stomach are given in TABLE I.

CD8 and CD68 immunoreactivity was positive in numerous inflammatory cells localized in connective tissue where cell infiltrations were intense. CD8 and CD68 immunoreactivity was higher in the Ethanol group compared to the other groups. A decrease was observed in CD8 and CD68 positive immunoreactivity in Ethanol+Pomegranate extract group compared to Ethanol group (FIG. 3).

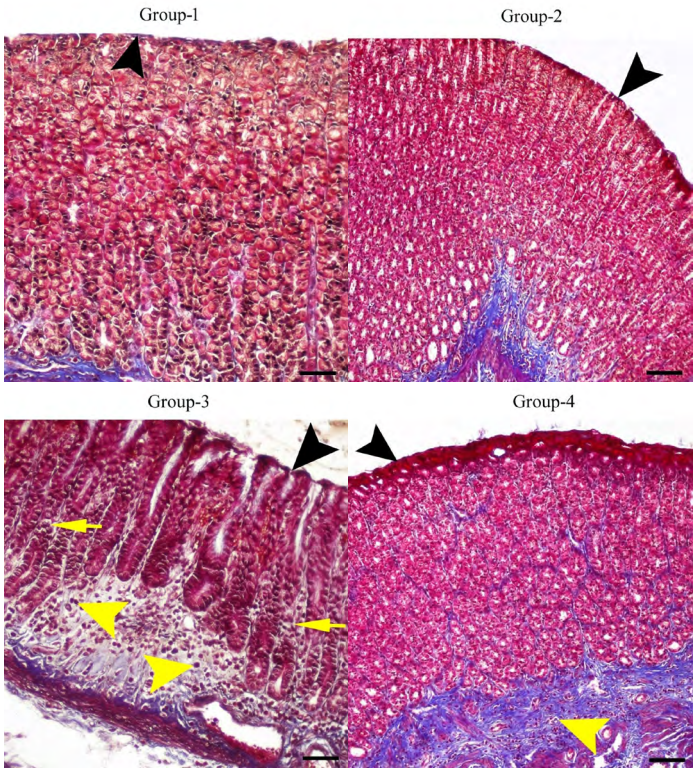

FIGURE 2. Sections of gastric mucosa of controls and treatment groups (Crossman's triple stain). Group 1 and Group 2, black arrow: normal mucosa. Group 3, black arrow: impaired mucosal integrity, yellow arrow: intraepithelial cell infiltration, yellow head arrow: hemorrhagic area. Group 4, yellow arrow: subepithelial cell infiltration. Group 2: figure 25 µm, Group 1, 3, 4: figures 50 µm

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

Groups	CD8	CD68	MHC I	MHC II
1 (control)	++	++	++	++
2 (*P. granatum* extract)	++	++	++	++
3 (Ethanol)	+++	+++	+	+
4 (Ethanol + *P. granatum* extract)	+	+	+	+

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

The study found that the immunoreactivity of MHC–I and MHC–II was found in specific locations, namely intraepithelial lymphocytes located in the epithelium, some capillary vessel endothelium, and connective tissue. When Ethanol and Ethanol+Pomegranate extract groups were examined, it was observed that positive immunoreactivity was distributed throughout the lamina propria (FIG. 4).

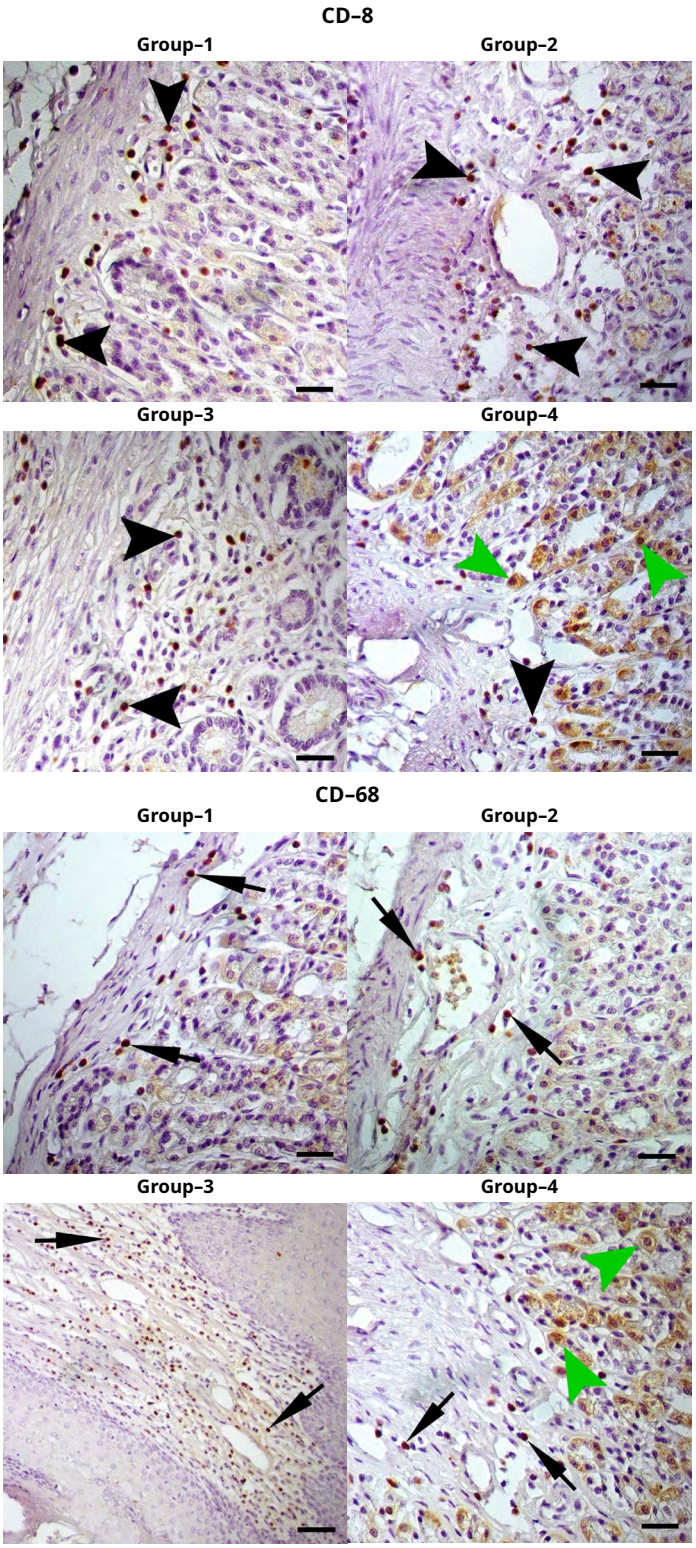

FIGURE 3. Immunohistochemical staining of CD8 and CD68 in the stomach. Black arrow: positive immunoreactivity in glands cell, red arrow: mitotic activity in cells, E: epithelium, S: stroma, G: glands. All figures 100 µm

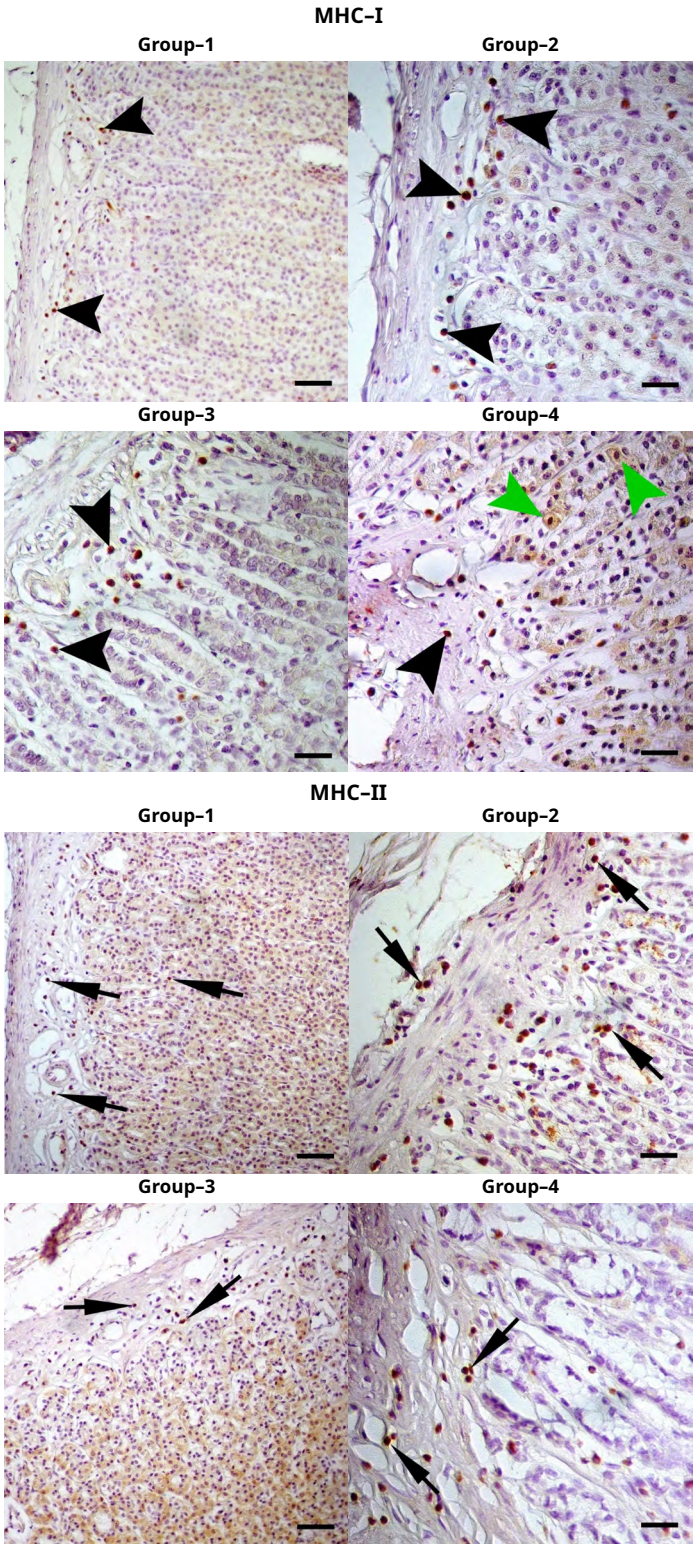

FIGURE 4. Immunohistochemical staining of MHC–I and MHC–II in the stomach. Black arrow: positive immunoreactivity in glands cell, red arrow: mitotic activity in cells, E: epithelium, S: stroma, G: glands, b: blood vessel, m: musculer layer. All figures 100 µm

Biochemical findings

Serum TNF–α, MPO, SOD and MDA values of the groups are shown in TABLE II. There was no statistically significant difference between TNF–α and MDA levels between the groups. However, a statistically significant difference was observed between SOD and MPO levels (P<0.05). The highest SOD level was found in the Ethanol+Pomegranate extract group and the lowest in the Ethanol group. MPO levels were highest in the Ethanol group and lowest in the Ethanol+pomegranate extract group (FIG. 5).

TABLE II
Comparison of tumor necrosis factor–α, myeloperoxidase, superoxide dismutase and malondialdehyde levels in blood samples from all groups[a]

Groups	TNF–α	MPO	SOD	MDA
1 (control)	65,805±26,33	91,500±105,62	387,950±124,13	46,320±16,31
2 (*P. granatum* extract)	49,505±4,57	38,850±28,42	190,900±44,68	16,640±13,47
3 (Ethanol)	69,020±16,69	80,300±83,79	143,500±125,77	40,520±17,91
4 (Ethanol+extract)	61,790±34,30	19,900±7,39	410,150±91,26	41,265±13,27
P	0,540	0,019	0,013	0,061

TNF–α: Tumor necrosis factor–α, MPO: Myeloperoxidase, SOD: Superoxide Dismutase, MDA: Malondialdehyde. [a]*P*–values are for ANOVA tests, ranging from P=0.05 to P<0.001. Values are expressed as the mean standard deviation

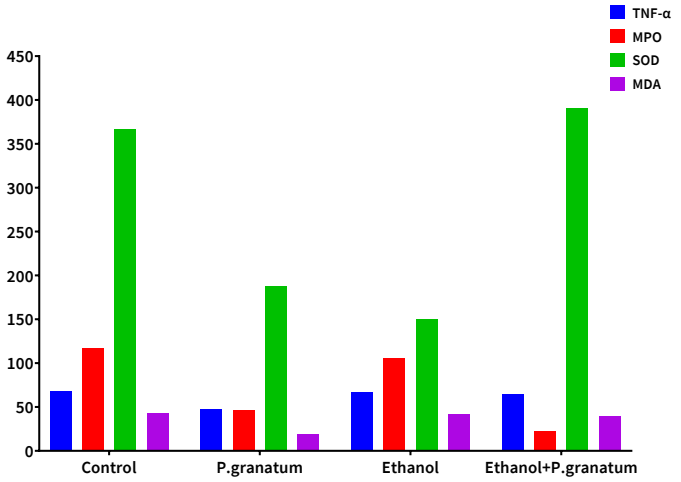

FIGURE 5. TNF–α (pg·mL^{-1}), MPO (IU·L^{-1}), SOD (IU·mL^{-1}) and MDA (mmol·L^{-1}) of ethanol–induced gastritis in rats

The Pomegranate fruit is recognized for its antimicrobial and antiviral properties. It has also been a focus of interest in cancer research. According to the evaluation of ability of fruit juices to reduce of iron capacity and clearance of free radicals, Pomegranate juice has been shown to contain higher levels of antioxidants than red wine and green tea [4]. Commercial Pomegranate juice, obtained by pressing the whole Pomegranate fruit and peels, contains significant amounts of antioxidants. Pomegranate fruit has been proven to lower blood pressure, affect enzyme activity, reverse vascular damage, contribute to healing of prostate cancer and arthritis, ameliorate diarrhea, protect phagocyte cells against autooxidative damage

through β-carotene, stimulate T cell functions, and support cytokine formation [15]. It was reported that Pomegranate peel extract used against experimental gastric ulcer in rats showed protective effect in gastric ulcer [16]. Significant reductions in the secretion of markers of vascular inflammation, thrombo spondinin (TSP) and cytokine transforming growth factor-b1 (TGF-b1) have been reported in obese rats fed an atherogenic diet supplemented with Pomegranate juice or Pomegranate extract. Pomegranate juice has also been shown to prevent oxidative degradation of nitric oxide [4].

TNF-α, which is a type of cytokine, is released by macrophages and plays a crucial role as an activator. Its cytotoxic effect plays an important role in regulating the inflammatory reaction and inflammation [17]. In the pathogenesis of diseases, it has been reported that TNF-α plays a key role in cell activation and regulation of MHC-I and MHC-II expression, and recent studies have shown an increase in the expression of TNF-α and its receptors in chronic inflammation sites under *in vivo* conditions [18]. Pomegranate extract has been reported to contribute to the healing of gastric and duodenal mucosa by causing a decrease in TNF-α and IL-1β activities and to show gastroprotective effect [19, 20]. According to the current study, the analysis of serum TNF-α levels showed that the group given Ethanol had an increase, while the groups treated with Pomegranate extract showed a decrease. This suggests that Pomegranate extract may have a therapeutic effect on the gastric mucosa in gastritis.

MPO is a major determinant of neutrophil infiltration in gastric mucosal tissues and is widely implicated as an indicator of neutrophil infiltration in experimental gastric injury [21]. Elevated MPO concentrations in both tissues and serum are frequently used as markers of polymorphonuclear leukocytes under conditions of inflammation and sepsis, and MPO has been shown to more practical and reliable marker of inflammation than IL-6 [22]. In this research, it was found that the Ethanol group showed an increase in serum MPO level, whereas the groups treated with the extract showed a decrease in serum MPO level. Additionally, it was observed a reduction in inflammation in the gastric mucosa based on histopathologic evaluation. The significant decrease observed provides evidence that MPO serves as an inflammation marker, which is in line with previous studies.

SOD protects the gastric mucosa against ROS formation by converting superoxide radicals containing hydroxyl peroxide and molecular oxygen [23]. Decreased anti-oxidant levels and overproduction of free oxygen radicals, especially super oxide and hydrogen peroxide, play a crucial role in ethanol-induced gastric damage and also induce gastric inflammatory response [1]. Sudheesh and Vijayalakshmi [24] reported that flavanoids in Pomegranate have antiperoxidative effect by causing an increase in both SOD and Catalase and Glutathione peroxidase activities. The results of the presedent study showed a decrease in serum SOD level in the Ethanol group, while an increase in the extract groups, suggesting a reduction in inflammation in the gastric mucosa. The histopathological results indicated that the reduction in inflammation observed in the groups receiving the extracts was likely a result of an increase in the activity of SOD.

In acute gastritis, inflammation, consisting mainly of mononuclear inflammatory cells and plasma cells, is superficial and mostly in the upper layers of the mucosa [25]. Studies in human and experimental animal models have reported that CD4 T lymphocytes are the main component of gastritis cell infiltrations, while CD8 T lymphocytes have the ability to initiate and sustain gastric inflammation [26]. This study found that the intensity of CD8 T lymphocyte positive immunoreaction was greater in the Ethanol group compared to the control groups. Additionally, the positive immunoreaction decreased in the treatment group, suggesting the involvement of mononuclear cells in the experimentally induced acute gastritis. In this study found evidence that CD8 T lymphocytes were present in the gastric mucosa during gastritis, which is consistent with the findings of Ohtani *et al*. [27] who also observed the infiltration of CD8 T lymphocytes in gastritis along with CD4 T lymphocytes.

CD68 is a transmembrane glycoprotein highly expressed by tissue macrophages [28]. The current study CD68 positive immunoreaction was higher in the Ethanol group compared to the other groups, while immunoreaction decreased in the treatment group. This suggests that Pomegranate extract may reduce inflammatory response-associated protein expression to attenuate gastric mucosal injury.

MHC-I and MHC-II class gene products encode cell surface glycoproteins involved in the binding and presentation of T lymphocytes to T cell receptors [29, 30]. This study revealed that positive immunoreactivity in MHC I and MHC II expressing cells decreased in Ethanol and treatment groups compared to control and Pomegranate extract groups. This suggests that Pomegranate's antioxidant properties may contribute to an increase in TNF-α and SOD levels, as well as changes in MHC class molecules that are consistent with changes in CD8 immunoreactivity, with increased levels during inflammation and decreased levels during healing.

CONCLUSIONS

Compounds such as catechin derivatives, flavonoids, phenolics and oligomeric proanthocyanidins classified as medicinal plant active compounds are known to have a mechanism of action related to the reduction of free radicals against gastric mucosal lesions. In this study, changes in anti-oxidative stress markers such as MPO and SOD suggest that pomegranate extract has a mucosal protective effect in ethanol-induced gastritis, but further in vivo studies are needed.

Conflict of interest

The authors declare that they no conflict of interest.

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