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Gastroprotective Effects of Jujuba (*Ziziphus jujuba*) Fruit Extract Against the Ethanol-Related Gastric Ulcer in Rats

Jujuba (*Ziziphus jujuba*) Meyve Ekstraktının Ratlarda Etanol ile Oluşturulan Mide Ülserine Karşı Gastroprotektif Etkileri

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Abstract

Aim: Gastric ulcer is a global health problem. Alcohol consumption and nonsteroidal anti-inflammatory drugs may lead to gastric ulcers. Here, we examined the possible gastroprotective effects of *Ziziphus jujuba* (Zj) fruit extract on a rat model of ethanol-induced gastric ulcer.

Material and Method: Chemical properties of Zj were specified using performance liquid chromatography coupled to mass spectrometry. The animals were administered Zj extract (4-8 ml/kg) before administering absolute ethanol. Oxidative stress and proinflammatory markers, immunoexpression of NF-κB and caspase-3 were measured to evaluate Zj's effects.

Results: Zj fruit extract significantly reduced values of cytokines such as TNF-α, IL-1β, and IL-6 and the expressions of NF-κB and caspase-3 in the immunohistologic analysis. We found out ulcerative foci in the ulcer group in the histopathological analysis, while we didn't observe ulcerative foci in the treatment groups.

Conclusion: This experimental study showed that Zj fruit ameliorated the ethanol-induced gastric ulcer model in rats.

Keywords: Ethanol, gastric ulcer, proinflammatory cytokines, caspase-3, *Ziziphus jujuba*

Öz

Amaç: Gastrik ülser global bir sağlık sorunudur. Alkol tüketimi ve nonsteroid anti-inflamatuar ilaç kullanımı mide ülserine yol açabilmektedir. Bu çalışmada, *Ziziphus jujuba* (Zj) meyve ekstraktının ratlarda oluşturduğumuz etanol kaynaklı mide ülseri modeli üzerindeki olası gastroprotektif etkilerini inceledik.

Gereç ve Yöntem: Zj'nin kimyasal özellikleri kütle spektrometresi ile birleştirilmiş performans sıvı kromatografisi kullanılarak belirlendi. Hayvanlara saf etanol verilmeden önce Zj ekstresi (4-8 ml/kg) uygulandı. Zj'nin etkilerini değerlendirmek için oksidatif stres ve proinflamatuar belirteçler, NF-κB ve kaspaz-3'ün immünoekspresyonu ölçüldü.

Bulgular: Zj meyve ektraktı, immünohistolojik analizde TNF-α, IL-1β ve IL-6 gibi sitokinlerin değerlerini ve NF-κB ve kaspaz-3 ekspresyonlarını önemli ölçüde azaltmıştır. Histopatolojik analizde ülser grubunda ülseratif odaklar tespit edilirken, tedavi gruplarında ülseratif odaklar gözlenmedi.

Sonuç: Bu deneysel çalışma, Zj meyvesinin sıçanlarda etanol ile indüklenen gastrik ülser modelini iyileştirdiğini göstermiştir.

Anahtar Kelimeler: Etanol, gastrik ülser, proinflamatuar sitokinler, kaspaz-3, *Ziziphus jujuba*

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INTRODUCTION

Gastric ulcers are among the most frequent gastrointestinal disorders worldwide,[1] and they don't have a definitive treatment.[2] Gastric ulcer is the erosion of gastric mucosa caused by nonsteroidal anti-inflammatory drugs, Helicobacter pylori, alcohol consumption, and stressful conditions.[3,4] Alcohol consumption causes necrosis and vascular damage, resulting in ulceration in the gastric cells;[4,5] thus, it increases the gastrointestinal bleeding risk.[6] Besides, the administration of alcohol is linked to the regulation of the nitric oxide pathway and disequilibrium of proinflammatory cytokines.[5] The signaling pathway of nuclear factor kappa B (NF-κB) is among the most well-known intracellular signaling pathways of the inflammatory response.[7] NF-κB acts in the arrangement of several genes that play a key role in immune responses and inflammatory processes,[8] causing activation of various proinflammatory cytokines, like interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6).[9] Ethanol-induced gastric ulcer raises the NF-κB expression and proinflammatory cytokine production.[10,11] Ethanol stimulates epithelial cell apoptosis in the gastric tissue,[12] whereas the apoptosis cascade is primarily initiated by caspase-3 activation.[13]

Ziziphus jujuba (Zj) is a fruiting plant-specific (jujube) that belongs to the Rhamnaceae family.[14] Different parts of Zj have various medicinal features.[15] Zj is a species of China with a history extending back over 4000 years.[16] Zj extract decreases oxidant and inflammation levels.[17-19] while increasing the antioxidant response.[18,20,21] Zj polysaccharides reduce various proinflammatory cytokine levels.[19,22] In a previous study, Zj stem bark extract prevented gastric mucosal injury created by ethanol in rats.[23] Zj lam leaves extract demonstrated anti-ulcer activity.[24]

Here, the possible effects of Zj fruit extract on oxidant/antioxidant parameters, inflammatory markers, and apoptosis in the gastric ulcer model were investigated using biochemicals and histopathological methods. This study's justification is finding a new molecule for the gastric ulcer treatment, which has fewer side effects and performs preventive properties comparing the similar drugs used for this purpose worldwide.

MATERIAL AND METHOD

Ziziphus jujuba Fruit Extract

The jujube fruits (*Ziziphus jujuba* Mill.) were obtained in the harvest season (last week of September) from a commercial orchard in Amasya province in Turkey. Jujube fruits belong to the commercial standard cultivar "Li". After the fruits were washed, the tiny seeds were taken out. The pulpy jujube juices were extracted using a homogenizer[25] and given to the rats intragastrically at 4 ml/kg and 8 ml/kg.

Animals and Ethical Approval

All animal applications, including surgical and medical procedures, were accepted by the Animal Experiments Local Ethics Committee, Atatürk University (Protocol no: 19.04.2016/70). The rats were supplied from the Animal Research and Application Center, Atatürk University. They were exposed to a 12 h light/dark cycle at 21°C temperature and %55 humidity. The animals were placed in cages measuring 470×312×260 mm and allowed to feed freely.

Induction of Gastric Ulcer by Ethanol and Treatments

In this study, 32 Wistar albino female rats, 20-24 weeks of age, 257±4 g weighing, were used. 4 random groups were formed (n=8). The control group was intragastrically administered with distilled water for ten days. Ulcer+Zj fruit extract groups were intragastrically administered 4 ml/kg and 8 ml/kg Zj extracts for ten days. The animals in the ulcer group and Zj fruit extract groups were fasted for 24 hours, but they were allowed to reach water before the ulcer model was performed. On the 11th day of the experiment, an ulcer model was created by intragastric 5 ml/kg absolute ethanol (99%) administration. After 90 mins, rats were sacrificed. The Stomachs were incised over the greater curvature and washed with saline to clean the blood clots. Later, the samples were fixed on a surface and photographed.

The High-Performance Liquid Chromatography (HPLC) Analysis of Fruit Extract Phenolic Profile

HPLC coupled to a photodiode array (HPLC-PDA) to able to determine the phenolic properties of the samples. HPLC-PDA levels were demonstrated as mg/100 ml. Standard calibration curves were prepared via gallic acid, caffeic acid, 4-hydroxy benzoic acid, vanillic acid, p-coumaric acid, catechin, chlorogenic acid, syringic acid, ferulic acid, cyanidin-3-glucosidase, and delphinidin-3-glucosidase. A membrane filter (0.45 µm) was used for samples and stock solutions. A filtered sample (1 ml) was used in vials for analyzing with Waters W600 HPLS system with PDA (Waters 996) detector. Luna C18 column (Phenomenex, Utrecht, The Netherlands), which is heated to 40°C, was preferred as the stationary stage. Solvent A content is distilled water with 0.1% (v/v) trifluoroacetic acid (TFA). Then, solvent B content is acetonitrile with 0.1% (v/v) TFA, acetonitrile with 0.1% (v/v) TFA formed the mobile phase. The flow rate was 1 ml/minute. The chromatograms were measured at 280, 312, 360, and 520 nm. Retention times and characteristic UV spectra determined the identification, and standard external curves formed quantification.[26]

Tissue Collection and Homogenization

Gastric tissues were excised and washed with saline to get rid of the stomach's content. Then, photographs were taken macroscopically. The entire stomach was cut into two pieces from minor and major curvatures. One part was kept in 3,7 % formaldehyde for further immunohistochemical and pathological analysis, and the other part was kept in -80°C for

further biochemical analysis. Gastric tissue with all layers was homogenized in the ice-cold phosphate buffer (50 mM, pH 7,4). Supernatants were acquired centrifuging homogenates at 3,000 rpm for 15 min at 4°C.

Spectrophotometric Assays (Evaluation of the content of *Ziziphus jujuba* Fruit Extract)

Free radical clearance activity was evaluated with ABTS (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid)). The ABTS activity measurement was the modified version of Re et al.[27] Antioxidant features of Zj fruit extract content were assessed with cupric reducing antioxidant capacity (CUPRAC) analysis.[28] The total phenolic content was examined with Folin–Ciocalteu reagent (FCR) and the method by Folin and Singleton.[29,30]

Cytokines Analyses

An enzyme-linked immunosorbent test (ELISA, BioTEK Powerwave XS Winooski, UK) was performed to examine ethanol's effect on cytokine levels in stomach homogenate. For TNF-α levels, Rat TNF-α ELISA Kit (Cat No:E-EL-R0019, Elabsience); for IL-1β levels, Rat IL-1β ELISA Kit (Cat No:E-EL-R0012, Elabsience); and for IL-6 levels, Rat IL-6 ELISA Kit (Cat No:E-EL-R0015, Elabsience) were used. The measurements were performed according to the manufacturer's instructions.

Hematoxylin and Eosin Analyses

All gastric tissues were fixed in 3,7% solution formaldehyde for 48 hours. After the fixation, all stomach samples were routinely examined for the histological tissue processing, as described previously.[31] According to this tissue follow-up procedure, all tissues were kept in increasing alcohol series (50%, 60%, 70%, 80%, 96%, 99% alcohol - 1 hour). It was then kept in 3 different xylene for 15 minutes. Finally, after waiting for 2 hours in melted paraffin, blocking was performed. After the tissue was processed, 5 micrometer thick sections were taken from each paraffin block and staining protocol was passed. Accordingly, all slides were kept in at 60 degrees for 20 minutes, and all slides were kept in xylene for 5 minutes and 3 times due to paraffin was removed. Subsequently, the slides were kept in decreasing alcohol series (99%, 96%, 80%, 70%, 60%, 50% alcohol - 5 minutes), washed for 5 minutes in running water after alcohol and stained in Mayer's hematoxylin for 5 minutes. It was kept in running water for 5 minutes to remove the excess dye. Dyeing was done in alcoholic eosin for 2 minutes. In order to remove the excess dye, it was gently shaken in 96% alcohol 10 times and kept in 99% alcohol for 5 minutes. Finally, it was passed through 3 xylene series (5 minutes), and the slides were covered with a coverslip with adhesive medium.

Immunohistochemical Analysis (IHC staining)

Inflammatory and apoptotic features of the groups were examined using caspase-3 (Novus Biological, USA) and NF-κB (Abcam, UK).[32] Immunohistochemical staining was performed by the Ventana BENCHMARK GX automatic immunohistochemistry staining system.[33] The sections were investigated by a light microscope (Olympus BH-40). Semi-quantitative scoring was made based on the immunohistochemical staining intensity and dyeing cells number.

Statistical Analysis

Statistical analysis was established using the one-way ANOVA and Tukey test. The results were demonstrated as mean±standard deviation (SD). A p-value of less than 0.05 was considered significant.

RESULTS

Determination of Phenolic Substance Content of Zj Extract

The HPLC chromatogram (360 nm) of the phenolic substances in the Zj extract is shown in **Figure 1**. The phenolic and antioxidant substances in the Zj samples are shown in **Tables 1** and **2**, respectively. Xie et al. found phenolic compounds in jujube and identified them as coumarin, gallic acid, catechin, caffeic acid, chlorogenic acid, quercetin, p-coumaric acid, epicatechin, ferulic acid, and rutin.[34] Gallic acid content of pulp samples taken from different growing stages was 3.19-81.27 ppm, catechin content was 3.81-56.62 ppm, p-coumaric acid content was 1.21-22.94 ppm, ferulic acid content was 6.16-44.39 ppm, and rutin content was 5.12-47.93 ppm. When these values were compared to this study, the contents of gallic acid, catechin, and p-coumaric acid were similar; ferulic acid and rutin contents were higher than current results.

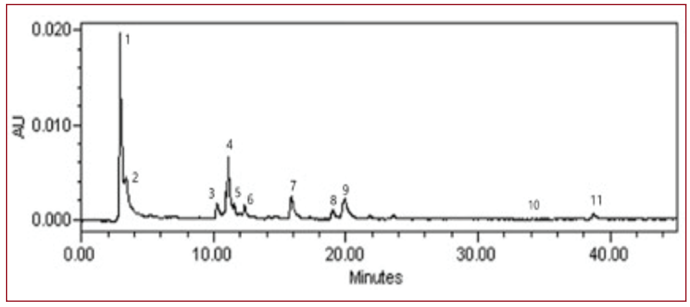

Figure 1. The HPLC chromatogram (360nm) of the phenolic substances in the Zj fruit extract. Peak identification;1 Gallic acid; 2 protocatechuic acid; 3 syringic acid; 4 catechin; 5 epigallocatechin; 6 p-coumaric acid; 7 ferulic acid; 8 ellagic acid; 9 rutin; 10 t-cinnamic acid; 11 kaempferol

Table 1. Content of antioxidant substance of jujube samples according to two different methods (CUPRAC and ABTS), Total phenolic substance content according to Folin Ciocalteu reactif (FCR) method.

Parameters	Average±St-dev (mg TEAC/100 ml)
CUPRAC	640.93±93.67
ABTS	4257.45±154.00
FCR	92.55±8.20

Table 2. Phenolic substance content of jujube samples

Parameters	Average (ppm)±St-dev
Gallic acid	3.20±1.26
Catechin	6.75±0.71
Syringic acid	1.08±0.26
EGC	2.09±0.55
Ellagic acid	1.28±0.50
t-Cinnamic acid	1.83±0.33
Protocatechuic acid	0.75±0.17
p-Coumaric acid	0.98±0.22
Ferulic acid	0.26±0.04
Kaempherol	1.09±0.46
Rutin	1.97±0.72

Oxidative Stress and Cytokine Results

Total antioxidant status (TAS), oxidative stress index (OSI), and total oxidant status (TOS) values were represented in **Table 3**. No significant difference was observed between the all-experiment groups ($p>0.05$). In the **Figure 2**, IL-1β, TNF-α, and IL-6 levels elevated in the ulcer group compared to the control group ($p<0.05$). The same parameters declined in the Ulcer+Zj 8 ml/kg group ($p<0.05$). Results are given in **Figures 2a**, **2b**, and **2c**, respectively.

Table 3. TAS, TOS and OSI levels between groups

Groups/Parameters	TAS	TOS	OSI
Control	3.5099±1.50	35.0274±9.25	2.0101±0.15
Ulcer	3.4644±2.06	55.0556±13.69	1.9880±0.36
Ulcer+Zj 4 ml/kg	3.1237±1.45	43.5756±18.78	1.6372±0.89
Ulcer+Zj 8 ml/kg	4.5172±2.77	49.1317±20.31	1.2653±0.83

Data were analyzed by one-way analysis of variance followed by Tukey test. Values are expressed as mean values±SD. N= 8 per study group. There is no statistical significance between all experimental groups.

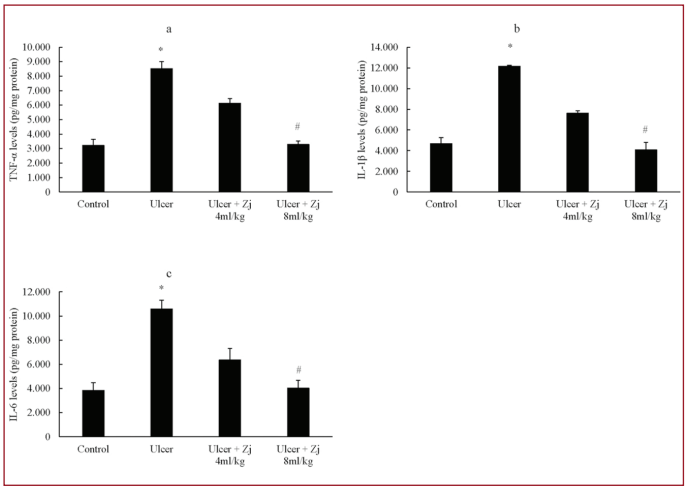

Figure 2. Effect of Zj fruit extract on gastric anti-inflammatory and proinflammatory cytokines levels decreased by ethanol. Values are mean ± SD. Data were analyzed via one-way ANOVA followed by Tukey test (n= 8 per group). Figure 2a, figure 2b, and figure 2c demonstrate TNF-α, IL-1β, and IL-6 levels, respectively. *p<0.05; compared to the control group, #p<0.05; compared to the ulcer group.

Histopathological Observations of Gastric Ulcer Tissues in Rats

Histopathological evaluation (hematoxylin and eosin staining) of groups is shown in **Figure 3**. There was no ulcerative damage in the gastric mucosa of the control group. In the ulcer group, intense neutrophil infiltration with degeneration of the surface epithelium, dilation of the gastric glands, and gastric pits were observed. In the ulcer+Zj 4 ml/kg group, partial irregularity in gastric pits and low neutrophil infiltration were observed. In the ulcer+Zj 8 ml/kg group, gastric mucosa was regular and generally resembled a healthy group. Low levels of neutrophil infiltration and necrotic cells were observed.

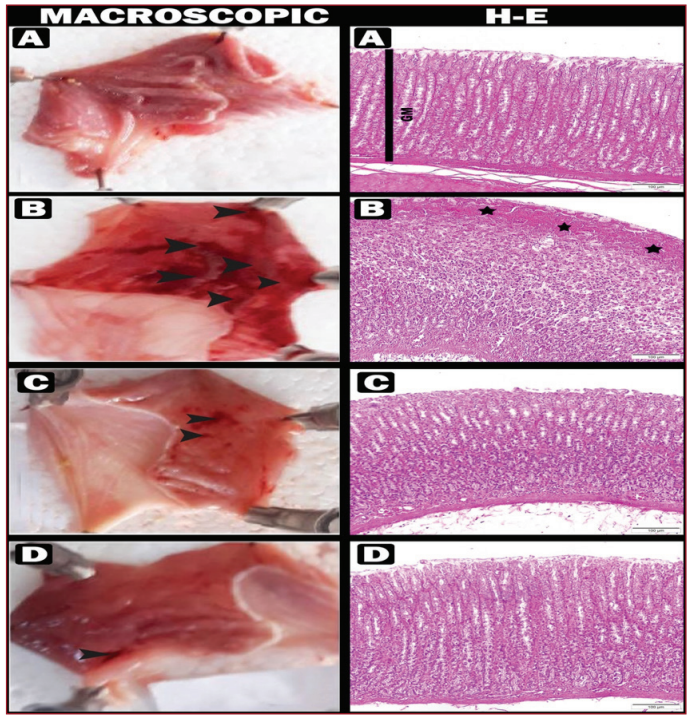

Figure 3. Histopathological evaluation of groups. A control group, B ulcer group, C and D Zj fruit extract treatment groups (4 ml/kg and 8 ml/kg, respectively). -Star: Ulcerative area, Arrow: Macroscopic Ulcer Area, GM: Gastric Mucosa, H-E: Hematoxylin and Eosin Staining

In the evaluation of NF-κB and caspase-3 immune reactivity (**Figure 4**), there was excessive immune reactivity in the ulcer group compared to the control group. Also, there was a dose-dependent low immune reactivity in the ulcer+Zj fruit extract groups compared to the ulcer group. The immunohistochemical evaluation was scored as; - (none), + (mild immune reactivity), ++ (moderate immune reactivity), and +++ (excessive immune reactivity) (**Table 4**).

Table 4. Scores of NF-κB and caspase-3 expressions of groups in ethanol-induced stomach ulcer model

Groups/Parameters	NF-κB	Caspase-3
Control	+	+
Ulcer	+++	+++
Ulcer+Zj 4 ml/kg	+	+
Ulcer+Zj 8 ml/kg	-	-

- none, + mild immune reactivity, ++ moderate immune reactivity, +++ excessive immune reactivity

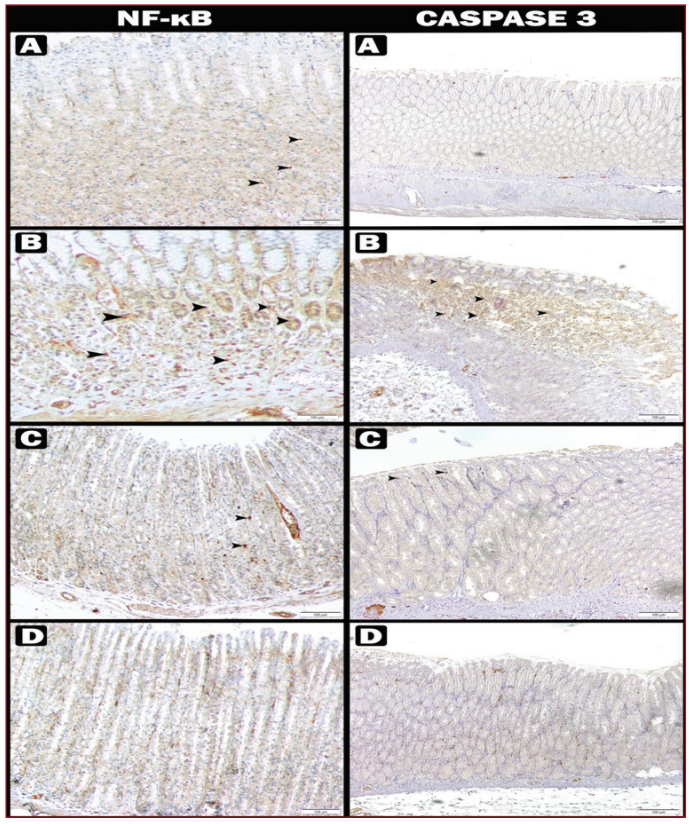

Figure 4. Immunohistochemical evaluation of groups. A control group, B ulcer group, C and D Zj fruit extract treatment groups (4 ml/kg and 8 ml/kg, respectively). -Arrowhead: Immune positive cells.

DISCUSSION

Alcohol-related gastric ulcer is the primary disorder of the gastrointestinal system.[35] Ethanol administration penetrates the gastric mucosa and causes inflammatory cell infiltration. Following, gastric blood flow diminishes, and oxidative stress occurs. Subsequently, bicarbonate decreases gastric mucus and nitric oxide secretion and induces necrotic mucosal damage, leading to cell death.[11,36-38] For the first time, this study shows that Zj extract at 4 ml/kg and 8 ml/kg attenuates ethanol-induced gastric ulcer by diminishing oxidative stress and suppressing inflammation.

Ethanol-induced animal gastric ulcer is commonly used to investigate new anti-ulcer drugs.[39] Polyphenol and phytochemical compounds have essential roles in the prevention of gastric ulcer.[40,41] Ethanol causes gastric mucosal injury through the oxidative stress stimulation pathway in the gastric tissue.[42] The leading cause of gastric ulcer is an increase in the balance between the oxidant and antioxidant system in the stomach in favor of oxidant molecules.[43] Earlier reports have shown that eliminating free radicals with antioxidant compounds alleviates ethanol-mediated gastric ulcer, and scavenging free radicals with antioxidant compounds prevents ethanol-induced gastric ulcer.[11,43] In our study, we represented many phenolic molecules obtained from the Zj extract. Possible effects of these molecules on gastrointestinal system diseases have been reported. Catechin.[44] and gallic acid[45,46] have been proven to protect the gastric mucosa by increasing antioxidant levels. Syringic, gallic, cinnamic, p-coumaric, caffeic, ferulic, and protocatechuic acids prevented gastric ulcer through free radical scavenging, lipid peroxidation inhibiting, and antioxidant effects.[47,48] Ellagic acid[49] and kaempferol[50] accelerated ulcer healing. Rutin demonstrated anti-ulcer performance via gastric proton pump inhibition.[51] Zj extract has been shown to increase antioxidant levels in various brain regions,[18] liver injury[20] and nephrotoxicity models.[21] In this study, Zj fruit extract's gastroprotective features against ethanol-induced gastric mucosal damage may be related to the phenolic compounds of Zj extract. These results indicate that Zj extract has gastroprotective effects similar to Hamedi et al.[23]

Gastric cell and tissue damage associated with acute and chronic inflammation are caused by ROS overproduction in the stomach.[42] Ethanol prompts lipid peroxidation and NF-κB expression leading to mucosal hemorrhages and edema in vascular smooth muscle cells and endothelial cells.[52] NF-κB causes transcriptional activation of various proinflammatory-cytokines, such as TNF-α, IL-1, and IL-6 translocated into the nucleus.[9] NF-κB expression and TNF-α, IL-6 production increased significantly in ethanol-induced gastric ulcer.[10,11,53] In particular, the level of cytokines, including TNF-α and IL-6, is critical in determining the severity of ethanol-induced gastric mucosal damage.[10,38] TNF-α is secreted during gastric mucosal injury and acts in several mucosal damage steps.[54] These results suggest that NF-κB inhibition is vital in gastric ulcer pathogenesis. Molecules present in Zj extract such as gallic acid,[55] catechin,[56] ellagic acid,[57] syringic acid,[58] p-Coumaric acid,[59] and kaempferol[60] suppress inflammation by inhibiting NF-κB activity. Other reports have also shown that Zj extract suppresses lipopolysaccharide-induced NF-κB activity in RAW 264.7 cells[61] and inflammation in inflammatory bowel disease and liver injury.[19,22] These results are consistent with our data.

Apoptosis is essential for development and homeostasis in most tissue types.[62] The caspases are the center of the apoptosis pathway and induce various hydrolysis reactions which cause cell death when activated.[63] It is commonly accepted that caspase-3 is a vital effector, and protease, which performs alone or in relationship with apoptosis-related proteins, participates in hydrolysis.[64,65] Caspase-3 is an executive caspase in apoptosis.[66] Ethanol induces apoptosis in the gastric tissues and causes gastric mucosal damage.[12] Zj fruit extract has decreased neurotoxicity by reducing caspase-3 levels in culture cells.[17] In this study, severe immunopositivity was observed in the ulcer group compared to the control group. The groups treated with Zj fruit extracts performed a lower immunopositivity compared to the ulcer group. Thereby, we have determined that the antiapoptotic property of Zj fruit extract may prevent gastric ulcers.

CONCLUSION

The present study showed that Zj fruit extract demonstrated immunomodulatory, anti-ulcer, and anti-inflammatory features in the gastric ulcer model induced by ethanol in rats. These effects are related to the activity of a vast number of polyphenols in Zj fruit extract. Finally, further studies are recommended to make the study results more reliable.

ETHICAL DECLARATIONS

Ethics Committee Approval: All animal applications, including surgical and medical procedures, were accepted by the Animal Experiments Local Ethics Committee, Atatürk University (Protocol no: 19.04.2016/70).

Informed Consent: Not available.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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