

Evaluation of Antioxidant And Anti-Ulcerogenic Effects of *Eremurus Persicus* (Jaub & Spach) Boiss Leaf Hydroalcoholic Extract On Ethanol-Induced Gastric Ulcer In Rats

Mohammad Beiranvand

Lorestan University Faculty of Basic Science

Seifollah Bahramikia (✉ bahramikia.s@lu.ac.ir)

Lorestan University <https://orcid.org/0000-0002-9482-6828>

Omid Dezfoulian

Lorestan University Veterinary Faculty

Research Article

Keywords: Antioxidant, Oxidative stress, Eremurus persicus, Gastric ulcer, Phenolic compounds

Posted Date: June 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-653039/v1>

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Version of Record: A version of this preprint was published at Inflammopharmacology on August 25th, 2021. See the published version at <https://doi.org/10.1007/s10787-021-00868-x>.

Abstract

This study aimed to investigate the antioxidant and protective effect of *Eremurus persicus* (Jaub & Spach) leaf hydroalcoholic extract (EPE) in preventing gastric ulcers induced by ethanol in rats. Wistar rats weighing 180-220 g were randomly divided into five groups, included normal group, ethanol consumption group, ranitidine treatment experienced group, the recipient rats prepared by dose of 250 mg/kg plant extract, and the last group recipient the dose of 500 mg/kg plant extract. The gastric ulcer was induced by feeding 1 ml of 96% ethanol to each animal. The gastric ulcer index (UI), pH, oxidative stress parameters, and histopathological changes in all stomachs were measured. Pre-treatment of ethanol-induced rats with the EPE reduced ($P < 0.05$) the ulcer index and gastric juice pH, compared to ethanol intake rats. Additionally, pre-treatment with EPE at a dose-dependent manner alleviated the gastric oxidative stress injury in rats by increasing CAT activity, tissue NO[•] and GSH levels. EPE also was able to decrease the levels of ROS, MDA, PCO and serum NO[•]. According to the results, it can be concluded that pre-treatment with EPE prevents the formation of gastric ulcers caused by ethanol, which can be attributed to the antioxidant activity of plant polyphenols compounds.

Introduction

A peptic ulcer is a lesion of the gastrointestinal mucosa that spreads to the mucosal muscle layer. It is usually characterized by various necrosis stages, neutrophil infiltration, decreased blood flow, increased oxidative stress, and inflammation [1]. This disease does not have a high mortality rate in Iran, but statistics show more than 40% prevalent in the country [2]. An imbalance between aggressive agents (especially acid and pepsin) and mucosal defence agents (especially blood flow and prostaglandins) cause peptic ulcers. Its pathophysiology is a multifactorial process. Factors that may increase the incidence of peptic ulcer disease including stress, alcohol consumption, smoking, *Helicobacter pylori*, and Nonsteroidal anti-inflammatory drugs (NSAIDs) [3]. Since alcohol's high consumption is the most significant cause of gastric mucosal damage, the ethanol-induced gastric ulcer model is often employed to study the anti-ulcer compounds [4]. The most common drugs used to treat and prevent peptic ulcers include H₂ receptor blockers (such as cimetidine, ranitidine, etc.) and proton pump inhibitors (such as omeprazole, lansoprazole, etc.), antacids, and cell protective factors. Since these drugs cause various adverse effects such as impotence, arrhythmia, hematopoietic alterations, hypersensitivity, and Gynecomastia, it is needed to a natural anti-ulcer drug with fewer side effects [5].

Traditionally, medicinal plants have been used for the relief of symptoms of the disease. Despite the dramatic advances in modern medicine in recent decades, plants are still involved in health care. Although the toxicity of most plants has not been thoroughly evaluated, it is generally accepted that herbal medicines are safer than synthetic ones [6].

The species *Eremurus persicus* (Jaub & Spach) Boiss, known as serish, belongs to Xanthorrhoeaceae and the genus *Eremurus*. This genus is one of the most important genera of the Xanthorrhoeaceae family, with more than 40 species reported in the world and seven species in Iran. Natural habitats of *E. persicus*

are widely distributed in arid and semi-arid regions, mountain slopes, especially in central and middle Asia countries such as Afghanistan, Iran, Iraq, Tajikistan, Lebanon and Turkey. The slender and elongated leaves of this species are used as a vegetable in Central Asia. In earlier times, the roots of the serish have been used for bookbinding due to their glazed materials [7].

Serish is used in traditional medicine to treat liver, stomach, constipation, and diabetes disorders. Decoction and root Poultice of this plant has been used in the past to treat ulcers and scabies. Its cooked leaves, along with rice, are currently used as traditional Iranian food [8]. In a research on *E. persicus* extract, antiglycation [9], antibacterial and cytotoxic [10], antimalarial and anticancer activities of this plant have been positively evaluated [11]. On the other hand, the presence of terpenes and oxygenated terpene derivatives such as limonene, geranylgeraniol, n-nonanal, geranyl acetone, benzene acetaldehyde, linalool, α -pinene and 1,8-cineol have been confirmed in the essential oil of this plant. All studies of these abundant volatile compounds have shown that these compounds could have significant antioxidant activity, antimicrobial, antifungal, anticancer and acetylcholinesterase inhibitory [12]. Also, the presence of branched carbohydrates in the roots of this plant is confirmed [13].

A recent study on the anti-leishmaniasis properties of *E. persicus* root extract has shown that the compound aloesaponol III 8-methyl ether isolated from the plant extract can have a good therapeutic potential against leishmaniasis infections [14]. However, to date, there is no evidence of biological activity of *E. persicus* in vivo. Therefore, in the present study, phytochemical components of the hydroalcoholic extract of *E. persicus* aerial parts and its gastroprotective properties against ethanol-induced gastric ulcer rats have been evaluated.

Materials And Methods

Chemicals and drugs

Ranitidine was obtained by Kharazmi Pharmaceutical Company (Khorramabad, Iran). 2, 2- Diphenyl-1-picrylhydrazyl (DPPH), Gallic Acid, Catechin, 5,5-dithiobisnitro benzoic acid (DTNB), 2,7-dichlorofluorescein diacetate (DCFH-DA), 2,4-dinitrophenylhydrazine (DNPH), and guanidine hydrochloride from Sigma-Aldrich (St. Louis, MO, USA) were provided. Ethanol, Folin–Ciocalteu's reagent (FCR), hydrogen peroxide (H_2O_2), 5-sulfosalicylic acid ($C_7H_6O_6S.2H_2O$), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 1-naphtylethylenediamine, and Sulfanilamide were purchased from Merck Co (Germany). All other chemicals used were analytical grade.

Plant material and preparation of extract

The leaves of *E. persicus* plant were collected from Zagheh section, located 35 km from Khorramabad city (with geographical coordinates: 33°29'56"N 48°42'31"E) in mid-May 2019, and then registered by Dr. Hamed Khodayari with the herbarium code Hlukh 25031398 in the Biology Department of Lorestan University. Then, the plant was dried in the shadow, crushed and exhaustively extracted with 80% (v/v)

aqueous EtOH under reflux. The obtained eluent was dried under vacuum (using a rotary device (RV 06ML/German)) at 40°C. Then, the dried extract was weighed and stored for future use.

Total phenolic contents assay

The plant's total phenolic content was determined using the Folin–Ciocalteu's reagent (FCR) according to the method described [15]. At the first step, 0.5 ml of the extract with a concentration of 1 mg/ml was added with 2.5 ml of diluted FCR solution (with a proportion of 1 to 10). Subsequently, 2 ml of 7.5% Sodium carbonate (Na_2CO_3) solution was injected. Ninety minutes later, kept in the dark media, and then the samples' absorption at a wavelength of 765 nm was interpreted in the laboratory [16]. The phenolic compounds extracted amount was determined using the standard curve of gallic acid. It was expressed as milligrams equivalent gallic acid per gram of dry weight (mg GA/g dry).

Total flavonoid contents assay

The total flavonoids content from plant was measured by aluminium chloride (AlCl_3) Colorimetric method [17]. 0.5 ml of a plant extract with a 1 mg/ml concentration was mixed to 2 ml of distilled water and 0.15 ml of 15% sodium nitrite (NaNO_2) solution. After 6 minutes, 0.15 ml of 10% AlCl_3 solution and 2 ml of 4% Sodium hydroxide were injected into the sample, and eventually, its volume with distilled water reached 5 ml. The absorbance was read after 15 minutes against the freshly prepared sample blank at a wavelength setting of 510 nm. The catechin compound was used to draw the standard curve, and the results were expressed as equivalent to catechin (mg catechin/g dry) [18].

Determination of radical scavenging capacity

Antioxidant activity of extract was measured by using DPPH free radical based on the Blois (1958) method [19]. In summary, 0.2 mM DPPH solution was prepared in ethanol, and 4.925 ml of it was added to 1.075 ml of various extract concentrations (400–25 $\mu\text{g}/\text{mL}$). The samples were then shaken vigorously and placed at Laboratory temperature for 30 minutes away from light and following this, their absorption at a wavelength of 517 nm was read by the Epoch microplate reader (Biotek model/USA). In this test, ascorbic acid (vitamin C) was used as a positive control [20]. The percentage of inhibition of DPPH free radicals was calculated using the following formula:

$$\text{Inhibition of free radicals (\%)} = [(A_0 - A_1 / A_0) \times 100]$$

In this formula, A_0 absorption of DPPH and A_1 , absorption samples in the presence of DPPH. The results were compared with ascorbic acid as a positive control. Sample ability to inhibit (or scavenge) 50% of free DPPH radicals was determined as IC50.

FTIR spectrum analysis

Fourier Transform Infra-red (FTIR) spectroscopy analysis, to determine organic compounds and identification of functional groups in chemical compounds of plant extract for antioxidant activity, was used. Combine the powder of the plant's completely dry extract and the potassium bromide seeds (KBr) in

a ratio of 1 to 100, and after grinding them, pour some of their mixture into a special metal mold and press it with a hydraulic press machine until it became a transparent pill. The resulting tablet was analyzed by FTIR spectrophotometer (Shimadzu 8400S model/Japan) in the range of 4000 – 400 cm^{-1} wavelength with a resolution of 4 cm^{-1} [21].

Animals

Thirty adult albino male Wistar rats weighing 180–220 g were purchased from the Animal Sciences Department of Kermanshah University of Medical Sciences. The rats were caged for 3 days in the animal house of the School of Veterinary Medicine of Lorestan University with controlled conditions for laboratory animals, including polypropylene cages with wood shaving and temperature of 22–24°C with 12 h dark/light cycles and open access to pellet and water. Distilled water was used for the oral administration of the standard drug and plant extracts in all animals. All protocols were conducted in accordance with the ethical guidelines for lab animals' research with approval number LU.ECRA.2020.32 was confirmed by ethical board of the School Veterinary Medicine of Lorestan University (at date of Ethical Approval: 11 April 2020).

Experimental Design

Rats were weighed on day one and remained under 12 h day-night cycles for environmental adaptation with supply water and food. The next day, rats were incidentally divided into 5 groups, except the negative control stayed hungry for 24 hours (food deprivation). During 24 hours, to prevent dehydration, drinking water was free for all groups. On the third day, all rats were weighed again, and according to the designed model groups, all, except the first, received the extract and the drug with a gavage needle [22]. The rats were divided into 5 groups, as follows:

- Negative control or normal group (normal healthy rats that didn't receive extract, drug, or ethanol during the research)
- Ethanol intake rats (received only 1 ml of ethanol per rat)
- Ranitidine treatment experimented group (recipient ranitidine 50 mg/kg + 1 ml ethanol per rat)
- Experimental group 1 (received plant extract at a dose of 250 mg/kg + 1 ml ethanol per rat)
- Experimental group 2 (received plant extract at a dose of 500 mg/kg + 1 ml ethanol per rat)

Ranitidine and extract pre-treatments were given orally only once/day for 1-day prior to ulcer induction. During the gavage of the extract and drug from third to fifth groups, to create the same stress conditions caused by the gavage, first and second groups, 1 ml of gavage distilled water were given.

With the exception of the first group (negative control), gastric ulcers were induced by 1 ml of 96% ethanol per gavage and followed with administration of drug or extract gavage in three other treatments 1 hour after alcohol ingestion. Thereafter, animals were anesthetized for 2–5 min and euthanized by cervical dislocation. Blood collection tubes were centrifuged (3000 g/10 min), allowed serum separated

from cellular contents, and stored at - 20°C for further investigation. At the same time, animal stomachs were pulled out, opened along the greater curvature, and collected for volume and pH determination. After that, gastric tissue specimens were rinsed gently with a physiology saline solution to remove blood clots and gastric contents, then for gross examination, scanned to calculate peptic ulcer index [4]. Subsequently, the stomach was incised longitudinally and shared by dividing it in half. One section was reserved for a sterile tissue container containing 10% neutral-buffered formalin for histopathological examination, and the rest was stored at -20 °C for determination of antioxidant and stress oxidative parameters.

Measurement of peptic ulcer index (UI) and inhibition percentage (%I)

According to the Takagi and Okabe (1968) method, the gastric ulcer index (UI, mm²) and ulcer index inhibition (%I) were determined [23]. Based on this method, at first, using of a ruler, the ulcerated surface area was measured. Then, the ulcer's degree was evaluated based on ulcer intensity using Table 1. Then the UI and %I were calculated with the following formulas [24]:

$$\text{Ulcer index (UI)} = \frac{\text{Total degrees of Ulcers in each group}}{\text{Number of animals in each group}}$$

$$\text{Inhibition of ulceration (\%I)} = \frac{\text{Positive control group (ethanol)UI} - \text{Pretreatment group UI}}{\text{Positive control group (ethanol)UI}}$$

Table 1
Gastric ulcer scoring system based on the severity of the ulcer.

Ulcer score	Gastric Lesions
0	No lesion
1	Mucosal oedema and petechiae
2	One to five small lesions (1-2mm),
3	More than five small lesions or one intermediate lesion (3-4mm)
4	Two to more intermediate lesions or one gross lesion (> 4 mm)
5	Perforated ulcers

Measurement of stomach juice volume and pH

The pH and volume of gastric acid juice were evaluated by the published studies [25]. The stomach juice of each rat for 10 minutes at 2000 g was centrifuged. The pellet was removed, and the volume of the supernatant was determined. Then, to 1 ml of Supernatant, 1 ml of distilled water was added, and the pH was measured by a pH meter (Hanna pH meter model 211/USA).

Histopathological analysis

A gastric specimen was fixed at 10% formalin, dehydrated by ethanol ascending concentrations, and embedded in paraffin. Then 5 µm sections were prepared and stained with hematoxylin and eosin (H&E). The pathological changes were examined using an optical microscope [26].

Biochemical analysis

In order to measure biochemical parameters, gastric tissue was homogenized [27]. The gastric tissue was first weighed using a calibrated scale, then 9 ml of PBS buffer was added to 1 gram of it, and the resulting mixture was homogenized on ice with the help of a homogenizer. At this stage, ice was used to prevent proteins' damage and reduce activity and inactivation of enzymes within tissue during homogenization. Next, the homogeneous mixture was transferred to the falcon tube and centrifuged by refrigerated centrifuge for 20 minutes at a rate of 12000 g at 4°C. The supernatant (surface liquid) was collected, and the resulting sediment was discarded. Supernatants were divided into separate micro-tubes and kept at -20 °C until evaluating the oxidative stress biomarkers.

Reactive oxygen species assay

The reactive oxygen species (ROS) level in the reaction mixture was determined by following the oxidation of 2, 7-dichlorofluorescein diacetate (DCFH-DA) to fluorescent 2, 7- dichlorofluorescein (DCF) compound, according to the documented literature as modified [28]. In summary, the reaction mixture consisting 1.7 ml of phosphate buffer solution (50 mM, pH = 7.4), 0.2 ml of gastric homogenate, and 100 µl of DCFH-DA solution (10 µM). Then, the samples were incubated at 37 °C for 15 minutes. The level of ROS formation was measured by DCF formation using a Cary-Eclipse fluorescence spectrophotometer (Agilent/USA) with excitation wavelength and emission at 488 and 521 nm.

Measurement of catalase enzyme activity

The activity of Catalase (CAT) in all study groups was measured with a slight modification by Aebi (1984) method [29]. 100 µl of stomach homogenate supernatant was added to the cuvette containing 1900 µl of phosphate buffer (50 mM, pH = 7). The reaction mixture was prepared by adding 1 ml of hydrogen peroxide (H₂O₂) and absorbance changes were measured at 15-second intervals within 60 seconds. H₂O₂ decomposition rate was measured by a UV/VIS spectrometer (T80+/England) at 240 nm. The expression of the CAT activity was calculated based on the following formula.

$$\text{activity enzyme} = \frac{\Delta A \times \text{total volume}}{\epsilon \times \Delta t \times \text{enzyme volume} \times \text{mg Pr}}$$

ΔA =Absorption difference; Δt = time difference; ϵ =Molar absorption coefficient H₂O₂ = 43.6 cm⁻¹ M⁻¹

Reduced glutathione assay

Glutathione (GSH) level, to calculate its differences among all groups was measured by Jollow et al. (1974) with a little change [30]. To the 0.5 ml of stomach homogenate, 1 ml of sulfosalicylic acid (4%) was added and then placed at 4 °C. After 1 h, the samples were centrifuged for 15 minutes at 3000 rpm at 4 °C. To 1 ml of each sample's supernatant, 0.1 ml of DTNB (4 mg/ml) and 0.9 ml of phosphate buffer (0.1 M, PH = 7.4) were added. With yellow color formation, the samples' absorption was read at a wavelength of 412 nm. Reduced glutathione was expressed as µg/mg of protein.

Measurement of lipid peroxidation

Malondialdehyde (MDA) levels were measured to determine the evidence of membrane lipid peroxidation probably increased after tissue damages by double heating method with a slight change [31]. In short, to 0.5 ml of each homogenized sample of the stomach, 2.5 ml of trichloroacetic acid (TCA, 10%) was added, and for 15 minutes placed in a boiling water bath. With gradual cooling, at room temperature, and 4 °C, the test tubes were centrifuged at 3000 rpm for 10 minutes. The following 0.3 ml of supernatant per tube to new tubes containing 0.3 ml were transferred from the TBA solution (0.67%). Each tube was placed in a boiling water bath (95 °C) for 20 minutes in the next step. Ultimately, the samples' absorption was measured at 532 nm against the blank solution after cooling tubes were placed at room temperature and at 4 °C. the value of MDA was designed based on the molecular absorption coefficient of MDA-TBA complex ($\epsilon = 1/56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$) and was expressed based on nmol/mg Protein.

Protein carbonyl content assay

Protein carbonyl (PCO) content as an indicator of protein oxidation was measured using Reznick and Packer methods. To the reaction mixture (2 mg protein), 1 ml of DNPH (10 mM in HCl) was added and the samples were incubated at room temperature for 1 h and were vortexed every 15 minutes. Then, 1 ml of trichloroacetic acid (TCA) (10%, w/v) was added to each reaction mixture and were centrifuged at 3000 g for 10 min.. The pellet (protein) was washed three times with 2 ml of ethanol/ethyl acetate solution (v/v, 1: 1) and dissolved in 1 ml of guanidine hydrochloride solution (6M, pH = 2.3). After 10 minutes, their absorbance at a 370 nm wavelength was read against the blank solution. The PCO value was calculated based on the molar extinction coefficient of DNPH ($\epsilon = 2.2 \times 10^4 \text{ cm}^{-1} \cdot \text{M}^{-1}$). The data were expressed as nmol/mg protein [28].

Determination of total nitric oxide levels in serum and tissue

Nitric oxide (NO[•]) acts as an important radical molecule in the intracellular signalling pathway and has a protective role with the effect of vascular dilation. However, its elevation, which principally induced after inflammatory cells activation, can become destructive. Therefore, concerning to the importance of NO[•], the level of this molecule was determined in the serum and gastric tissue of rats by measuring nitrite and nitrate accumulation, as its stable degradation products. The serum and tissue nitrite levels were bio-assayed using the Griess reagent, according to Hortelano et al. (1995). Griess reagent in the presence of nitrite produce a red diazo complex. The reaction product's color intensity was measured at 540 nm, and the results were expressed as µmol/L using the standard NaNO₂ curve [32].

Statistical analysis

The results were expressed as the means \pm SD. The Student's t-test detected statistical differences between the two groups. When more than two groups are compared (or analyzed), a one-way analysis of variance (ANOVA) was used. Values of $P < 0.05$ were considered statistically significant. The data were analyzed using GraphPad Prism 8.0 software (San Diego, CA, USA).

Results

Phytochemical examination

The phytochemical screening of aqueous ethanolic extract of *E. persicus* plant revealed polyphenols, flavonoids, and glycosides. The quantitative phytochemical investigation of total phenolic and flavonoid contents in the extract was shown in Table 2.

Table 2
Total phenol and flavonoids in the EPE.

Sample	Total phenolic content ¹	Total flavonoid content ²
EPE	235 \pm 0.02	72.2 \pm 0.01
The results of the three independent tests were expressed as mean \pm SD. (<i>Eremurus persicus</i> (Jaub & Spach) Boiss extract = EPE)		
¹ The phenolic content expressed as milligrams of gallic acid equivalents per gram of dry weight.		
² The flavonoid content was expressed as milligrams catechin equivalents per gram of dry weight.		

Radical scavenging activity of EPE

As indicated in Fig. 1, with increasing concentration, the percentage of free radical scavenging (Inhibitory) was increased. The 50% inhibition concentration (IC₅₀) of free radicals by the aqueous ethanolic extract was estimated to be 79.80 \pm 0.03 μ g/ml.

Investigation of the FTIR spectrum of EPE

As shown in Fig. 2, the main peaks of the aqueous ethanolic extract can be seen in the range of 3340, 2937, 1625, 1409, that with the stretching vibration of alcohol O-H, phenols or N-H amines, the stretching vibration C-H in CH₃, stretching vibration C = O in carboxyl or amide group, strong stretching vibration C-C at aromatic compounds correspond. The range peaks of 1076cm⁻¹ and 1267cm⁻¹ are related to the vibrational tension of C-O ethers and esters or C-N amines [33–35]. According to the observed peaks, it can be concluded that the EPE contains phytochemicals, including phenolic and flavonoid compounds, glycosides, coumarin derivatives, and naphthoquinones [36]. As a result, the antioxidant activity of this plant extract is also dependent on these compounds.

Effect of EPE on ulcer index, percentage of inhibition of ulceration and gastric juice pH

According to Table 3, the ethanol intake rats' pH of stomach juice was significantly reduced compared to the negative control animals. This (pH = 2.6) is one reason for the greater value of the ulcer index in ethanol rats. Meanwhile, rats of the ranitidine treatment experienced group, as a standard group, had a significant increase in stomach juice pH, indicating drug effectiveness in reducing acidity (secretion of acid) caused by ethanol gavage. Also, this group's ulcer index was mild, and the ulcer inhibition percentage by this drug is obtained at the highest level. Rats of experimental groups 1 and 2 that received doses of 250 and 500 mg/kg of EPE had a pH of 4.5 and 6.4 gastric juice, and an inhibition percentage of ulcers by 37 and 66. The dose of 500 mg/kg of EPE was more efficient than the 250 mg/kg. EPE indicates has a relatively better effect in contrast to ranitidine ulcer-related concerns.

Table 3

The effects of different doses of EPE and ranitidine drug on gastric ulcer caused by ethanol.

Groups	Gastric juice volume (mL)	Gastric juice pH	Ulcer index (UI)	Inhibition of ulceration (%)
Normal	1.61 ± 0.40	6.5 ± 0.08	0	-
Ethanol (%96)	2.62 ± 0.82	2.6 ± 0.04	0.00 ± 4	0
RAN (50 mg/kg) + Ethanol	*1.58 ± 0.46	**8.5 ± 0.65	0.43±*1.16	70
EPE (250 mg/kg) + Ethanol	3.15 ± 1.15*	4.5 ± 0.20**	0.50±*2.5	37
EPE (500 mg/kg) + Ethanol	**1.72 ± 0.96	6.4 ± 0.12*	0.70 ± 1.33 **	66
The results were expressed as means ± SD for 6 rats in each group. (<i>Eremurus persicus</i> (Jaub & Spach) Boiss extract = EPE; Ranitidine = RAN) * Significant difference with P < 0.01 compared to positive control group, ** Significant difference with P < 0.05 compared to positive control group.				

Pathological findings on the gastric mucosa

Macroscopic findings

Acute stomach ulcers were induced by intra-gastric administration of ethanol. As depicted in Fig. 3, the peptic ulcer surface was more extended significantly in the ethanol-stimulated rats than in normal rats. Pre-treatment with EPE or RAN could effectively be prevented the acute form of ethanol caused diffuse mucosal damage.

Histopathologic findings

Results of observation of microscopic lesions in various groups are as follows:

- Ethanol intake: extensive destruction and necrosis of mucosal tissue, severe hemorrhage, edema, and infiltration of leukocyte cells (Fig. 4B).
- Ranitidine 50 mg/kg group: Mild degeneration and necrosis at the apex of mucosa tissue, very small amounts of edema, hemorrhage, and infiltration of inflammatory cells (Fig. 4C).

- Group 250 mg/kg plant extract: moderate destruction and necrosis of mucosal tissue (One-third to one-fourth the mucosa), low edema values, hemorrhage, and leukocyte infiltration (Fig. 4D).
- Group 500 mg/kg plant extract: Mild degeneration and necrosis at the apex of mucosal tissue, very small amounts of edema, hemorrhage, and infiltration of inflammatory cells (Fig. 4E).

Effect of EPE on biochemical analysis results

Level of ROS

According to Fig. 5, induction of gastric ulcer by ethanol increased the ROS formation rate from 53.09 (a.m.u) in the normal group to 288.67 (a.m.u) in the ethanol intoxicated rats. DCF fluorescence intensity in the group receiving ranitidine decreased from 288.67 to 90.12 (a.m.u) compared to the ethanol intake group. Also, fluorescence intensity decreased in the pre-treatment groups with doses of 250 and 500 mg/kg of EPE, to 142.19 and 107.92 (a.m.u).respectively.

CAT enzyme activity

Changes in CAT enzyme activity in this study are shown in Fig. 6. Induction of gastric ulcer by ethanol reduced the activity of this enzyme in the ethanol intoxicated rats to the amount 0.266 (IU/mg Pr). On the other hand, administration of ranitidine increased the activity of this enzyme to the amount 0.673 (IU/mg Pr). Moreover, administration of the 250 and 500 mg/kg doses of EPE to rats increased the activity of this enzyme by 0.362 and 0.529 (IU/mg Pr), respectively.

Level of GSH

As depicted in Fig. 7, GSH levels in the gastric tissue of ethanol intoxicated rats decreased to the amount 4.22 ($\mu\text{g}/\text{mg Pr}$). However, administration of ranitidine and administration of 250 and 500 mg/kg doses of plant extract increased the levels of this non-enzymatic antioxidant to 5.01, 4.83 and 5.22 ($\mu\text{g}/\text{mg Pr}$), respectively.

Lipid peroxidation measurement

As be displayed in the Table 4, the amount of MDA in the ethanol intake group was increased to the amount of 0.55 ± 0.02 (nmol/mg Pr) compared to the negative control group. While pretreatment with ranitidine and plant extract at doses of 250 and 500 mg/kg reduced MDA levels to the amount of 0.41 ± 0.01 , 0.45 ± 0.01 and 0.4 ± 0.01 (nmol/mg Pr), respectively.

Table 4

Effect of EPE on ethanol-induced changes in gastric mucosal oxidative stress markers: malondialdehyde (MDA) level, inhibition of protein carbonyl (PCO) formation, serum nitric oxide (NO[•]) level and tissue nitric oxide (NO[•]) level in rats.

Groups	MDA (nmol/mg Pr)	PCO (nmol/mg Pr)	Serum NO [•] (μmol/L)	Tissue NO [•] (μmol/mg Pr)
Normal	0.27 ± 0.010	-	23.8 ± 1.6	1.42 ± 0.060
Ethanol (%96)	0.55 ± 0.022 *	14.98 ± 0.4	43.1 ± 2.1 [§]	0.94 ± 0.040 @
RAN (50 mg/kg) + Ethanol	0.41 ± 0.017 **	12.99 ± 0.2 [#]	25.7 ± 2.7 ^{\$\$}	1.39 ± 0.085 @@
EPE (250 mg/kg) + Ethanol	0.45 ± 0.015**	[#] 13.58 ± 0.43 [#]	33 ± 2 ^{\$\$\$}	1.02 ± 0.081 @@
EPE (500 mg/kg) + Ethanol	0.4 ± 0.010**	12.88 ± 0.24 ^{##}	29 ± 1.8 ^{\$\$\$}	1.37 ± 0.069 @@@
The results were expressed as means ± SD for 6 rats in each group. (<i>Eremurus persicus</i> (Jaub & Spach) Boiss extract = EPE; Ranitidine = RAN)				
* Significant difference with P < 0.02 compared to normal group, ** Significant difference with P < 0.05 compared to ethanol group.				
[#] Significant difference with P < 0.001 compared to ethanol group, ^{##} Significant difference with P < 0.05 compared to ethanol group.				
[§] Significant difference with P < 0.001 compared to normal group, ^{\$\$} Significant difference with P < 0.05 compared to ethanol group, ^{\$\$\$} Significant difference with P < 0.001 compared to ethanol group.				
@ Significant difference with P < 0.001 compared to normal group, @@ Significant difference with P < 0.001 compared to ethanol group, @@@ Significant difference with P < 0.05 compared to ethanol group.				

Measurement of PCO

According to Table 4, ethanol-induced oxidative damage increased PCO levels in the ethanol consumption group to the amount 14.98 ± 0.4 (nmol/mg Pr). However, pretreatment with ranitidine and doses of 250 mg/kg and 500 mg/kg of plant extract inhibited the level of PCO formation by 12.99 ± 0.2, 13.58 ± 0.43 and 12.88 ± 0.24 (nmol/mg Pr), respectively.

Level of serum NO[•]

As be exhibited in the Table 4, the serum NO[•] level in the ethanol intoxicated group was 43.1 ± 2.1 (μmol/L) compared to the normal group. However, administration of ranitidine reduced serum NO[•] levels to the amount 25.7 ± 2.7 (μmol/L). Pretreatment with doses of 250 mg/kg and 500 mg/kg of plant extract also reduced the level of this factor to amount 33 ± 2 and 29 ± 1.8 (μmol/L), respectively.

Level of tissue NO[•]

With refer to the Table 4, induction of gastric ulcer by ethanol in the ethanol intake group led NO⁺ tissue level to reach 0.94 ± 0.04 ($\mu\text{mol}/\text{mg Pr}$). Whereas, pretreatment with ranitidine and doses of 250 mg/kg and 500 mg/kg of plant extract increased the level of NO⁺ tissue in these groups to the amount 1.39 ± 0.08 , 1.02 ± 0.08 and 1.37 ± 0.06 ($\mu\text{mol}/\text{mg Pr}$).

Discussion

Peptic ulcer disease (gastric and duodenal ulcers) is a serious and growing health problem around the world that is estimated to affect 10% of the world's population with an annual increase of 4 million people. Furthermore, it is the most common gastrointestinal disease, involving 40% of developed countries and 80% of developing countries because of overuse of NSAIDs and *Helicobacter pylori* infection [37]. Current clinical treatment of gastric ulcer indicates a high recurrence rate and low recovery. Therefore, gastric ulcers treatment is still a major challenge that has made the development of new drugs and alternative therapies as a necessary and urgent need. Previous studies have reported the anti-ulcer properties of many medicinal plants as an excellent potential source for drugs production [38]. In recent years, many attempts have been made to detect gastroprotective and anti-ulcer drugs [27]. In this regard, several studies have been widely performed using ethanol-induced gastric ulcer model in animals to investigate the gastroprotective activity of plants and drugs. The effect of ethanol generated gastric ulcer begins with its rapid penetration into the gastric mucosa, thereby increasing mucosal permeability and secretion of vasoactive mediators such as endothelin-1, leukotrienes C₄, and histamine. These vasoactive agents increase gastric mucosal lesions by reducing blood flow to the mucosal membranes.

Moreover, ethanol damages blood vessels by reducing mucus production (by damaging its constituent components) and increasing the production of ROS, which leads to bleeding, tissue necrosis, and ultimately disruption of the protective barrier. Previous studies suggest that ethanol-induced gastric mucosal lesions are significantly associated with increased oxidative stress and the free radicals derived from oxygen, reducing antioxidant enzymes' activity. In this context, the infiltration and activation of neutrophils directly contribute to the increased ROS production. Therefore, it is likely that the infiltration and accumulation of neutrophils in the gastric mucosa result in free radical formation, which damages cellular components, including lipids and proteins [39, 40]. PCO is involved in many diseases, and it can be used as a diagnostic marker for oxidative stress. Therefore, the detection and reduction of PCO play a pivotal role in recovering these diseases [41, 42]. Importantly, in our study, the pre-treatment of the ethanol-induced rats with EPE and ranitidine ameliorate the ROS levels and PCO production. These effects are likely through the strengthening the antioxidant defense system of gastric cells.

Several studies have reported that ethanol can stimulate lipid peroxidation in cell membranes through ROS production, resulting in cell damage. Many studies have shown that ethanol can stimulate lipid peroxidation in cell membranes via ROS [43]. The increased lipid peroxidation by ethanol accelerates gastric ulcers by destroying membrane integrity and increasing cell permeability of gastric epithelial cells. These events lead to MDA production as the end product of the oxidation of unsaturated fatty acids in cell membranes. MDA is commonly used as a reliable indicator of lipid peroxidation, which its level can

be used to estimate the extent of ethanol-induced gastric tissue damage. In agreement with previous studies, our data showed that ethanol gavage significantly increases MDA production in gastric tissue [44–46]. Significantly, pre-treatment of ethanol-induced rats by EPE at doses of 250 mg/kg and 500 mg/kg reduced MDA levels. Different studies have shown that the generation of hypochlorous acid (HOCL), as one of the dangerous species resulting from the enzyme myeloperoxidase (MPO), increased following the oxidation of chloride ions by hydrogen peroxide in neutrophils. At this time, antioxidant defenses of gastric cells are activated and destroy H_2O_2 , with the purpose to suppress this process. The antioxidant enzyme CAT mediates the conversion of H_2O_2 into water and oxygen. GSH can also neutralize H_2O_2 by functioning as a cofactor for the glutathione peroxidase (GPx) enzyme. Many evidence shows the reduction of CAT activity and GSH content in gastric tissue resulted from ethanol-induced gastric mucosal damage. This reduction leads to elevated ROS levels, resulting in increased lipid peroxidation and PCO formation [47, 48]. In this context, Amaral et al. [49] have demonstrated that the ethanol-induced mucosal damage increases the production and accumulation of ROS and MDA. The authors further showed that this occurs by enhancing the MPO enzyme activity that resulted from decreased CAT activity and GSH levels in gastric tissue. As well, Liu et al. [50] have reported that ethanol administration, damages gastric tissue by increasing MDA and PCO levels. The results of our study showed that EPE has high antioxidant properties by reason of its relatively high levels of phenolic and flavonoid compounds, glycosides, coumarin derivatives, and naphthoquinones [51, 52]. Polyphenols, as one of the plant antioxidants, can terminate the chain reaction by reacting with free radicals so that their phenolic hydroxyl group reduces this radical by giving an electron to free radicals. As a result, the aromatic free radicals formed will be stable owing to the resonance effect. On the other hand, polyphenols can enter the lipid bilayer of the gastric mucosa, protecting the entire lipid layer from oxidation [53, 54]. Our results indicated that EPE has a gastroprotective effect against gastric ulcers ethanol-induced. Because phenolic compounds play a major role in plants' antioxidant activity, the EPE's anti-ulcer activity may be related to these compounds. With their synergistic effect with antioxidants such as GSH, CAT, etc., these compounds can inhibit the chain reaction of free radicals and increase the antioxidant level of gastric tissue to protect it against ethanol-induced damage.

Here, we used ranitidine, which inhibits gastric acid secretion by blocking H_2 receptors. Further, ranitidine has antioxidant properties [55], and therefore, it functions more efficiently than EPE. We could show the effects of ranitidine by biochemical, macroscopic, and microscopic analysis. Further, ranitidine increased the pH of gastric juice, which led to a decrease in the ulcer index. Many evidence shows even after gastric mucosal injury, the stomach is stimulated; thus, gastric juice does not affect gastric mucosa. It secretes a large amount of stomach acid, which increases the volume of gastric juice and reduces its pH. These events worsen gastric mucosal damage and, as a result, intensify gastric injury [56]. It is manifested in our results that EPE effectively reduces gastric juice volume and increases gastric juice pH in rats that receive alcohol, thereby protecting the gastric tissues and preventing ethanol-induced gastric injuries. Yet, herbal extracts might act as H_2 -receptor blockers and prevent histamine from binding to its receptor [57]. Therefore, a mechanism of action of EPE is related to a further reduction of gastric juice pH. In addition,

macroscopic and microscopic evaluations and the inhibition percentage of ulcers also confirmed this effect.

NO^{\bullet} , as a gaseous free radical, plays a role in physiological processes and in pathophysiological conditions. The level of NO^{\bullet} is increased due to the activity of inducible nitric oxide synthase (iNOS), anion superoxide ($\text{O}_2^{\bullet-}$) resulting from ethanol metabolism and neutrophils [58, 59]. Thus, NO^{\bullet} is prone to react with $\text{O}_2^{\bullet-}$ that results in proxy nitrite (ONOO^-). This later free radical is a species with cellular toxicity that can oxidize various cellular components, disrupt cellular processes, disrupt cellular signaling pathways, and induce cell death. Interestingly, ethanol consumption is associated with increased expression of iNOS, which leads to increased NO^{\bullet} levels.

Along with this evidence, in response to ethanol-induced gastric ulcer increased the NO^{\bullet} production. The increase probably is due to the increased induction of iNOS expression, which our results are in accordance to the studies of Yu et al. [60] and Li et al. [61].

On the other hand, NO^{\bullet} as a vasodilator factor with a short half-life regulates vascular and nutrient blood flow. It maintains the stomach and mucus barrier's epithelial integrity and, most importantly, has a pivotal role in angiogenesis, tissue regeneration, and ulcer healing [62]. As a result, in the present study, its reduction in the gastric tissue of the ethanol group may be caused by increased ONOO^- and oxidative damage and the decreased endothelial nitric oxide synthase (eNOS) expression as a protective factor. Besides, direct-ethanol damage on epithelial cells and sub-mucosal endothelial vessels might also contribute to the reduction in the gastric tissue. According to Zhang et al., [63] this event leads to the rupture of blood capillaries and reduced blood flow in these vessels [42]. Numerous studies have also confirmed that NO^{\bullet} level in rats' gastric tissue under ethanol gavage was significantly decreased [64–67]. In this study, EPE improves the antioxidant defense system (by participating in free radical scavenging). It probably leads to a change in the nitric oxide system (by reducing iNOS expression and increasing eNOS expression) during ethanol's oxidative damage.

Conclusion

Based on the results, it can be concluded that the mechanisms which are involved in damaging the gastric mucosa after ethanol administration include the overproduction of free radicals, reduction of internal antioxidants, and increase of acid secretion. In this study, EPE's protective effect on the prevention of ethanol-induced gastric ulcers depends on the antioxidant effect of phenolic, flavonoid compounds, and possibly glycosides and coumarin derivatives available in the plant. With an increasing dose of EPE, the amount of these compounds in the extract increases; thus, the protective effect of EPE is subsequently increased. Therefore, EPE's main mechanism in preventing gastric ulcers is due to the scavenging of free radicals caused by ethanol and its synergistic effect, which causes increased levels of CAT, GSH, and tissue nitric oxide and decreased levels of ROS, lipid peroxidation, and PCO.

Abbreviations

EPE: *Eremurus persicus* (Jaub & Spach) Boiss extract; RAN: Ranitidine; FCR: Folin–Ciocalteu’s reagent; DPPH: 2, 2-Diphenyl-1-picrylhydrazyl; FTIR: Fourier Transform Infra-red Spectroscopy; PBS: Phosphate-buffered saline; DCF: 2, 7-dichlorofluorescein. NO[•]: Nitric oxide; MDA: Malondialdehyde; PCO: protein carbonyl; GSH: Glutathione; NSAIDs: Nonsteroidal anti-inflammatory drugs; ROS: reactive oxygen species; CAT: Catalase; H₂O₂: Hydrogen peroxide; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase.

Declarations

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by a grant from the Faculty of Basic Sciences, Lorestan University. The founding sponsor had no role in the study design, performance, data collection and analysis, decision to publish, or preparation/writing of the manuscript.

Authors’ contributions

MB conducted the experiments and wrote the manuscript and all images were drawn. SB participates in the design and interpretation of the studies, and the review of the manuscript. OD participated in the preparation of tissue samples and pathological analysis. All authors approval for publication.

Acknowledgements

The authors appreciate the financial support of this investigation by Lorestan University.

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Figures

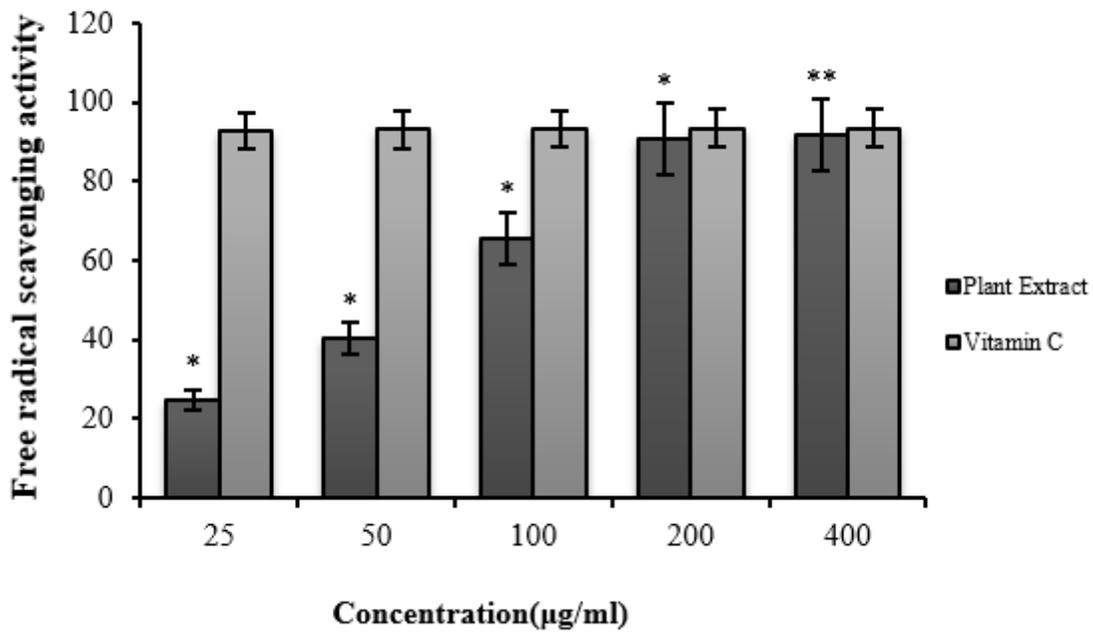


Figure 1

Antioxidant activities of the EPE and vitamin C as standard compound based on DPPH radical scavenging assay. Results were expressed as mean \pm SD for 5 concentrations in plant extract and vitamin C. * Significant difference with $P < 0.001$ compared with vitamin C, ** Significant difference with $P < 0.01$ compared with vitamin C.

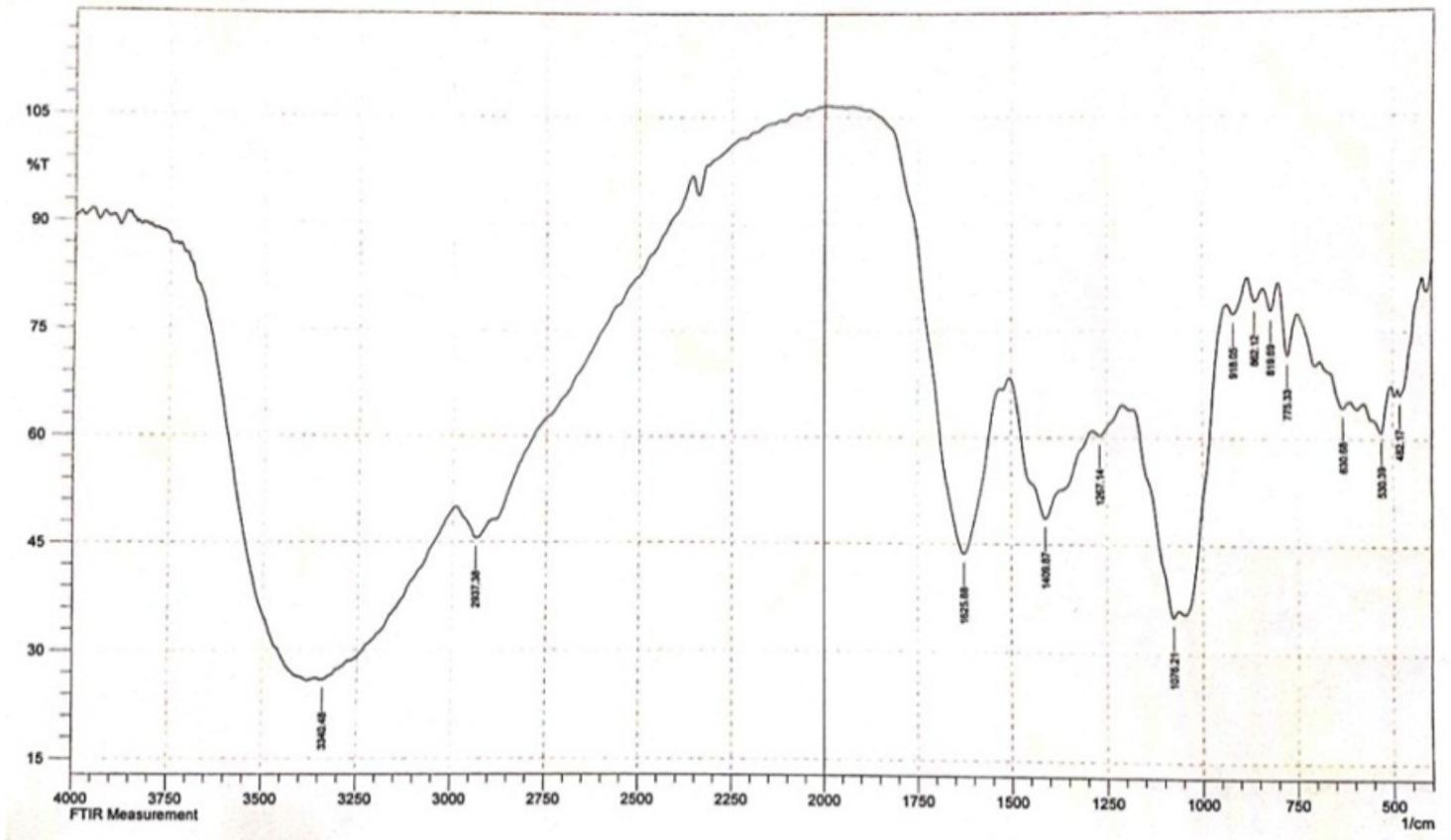


Figure 2

FTIR spectrum of EPE.

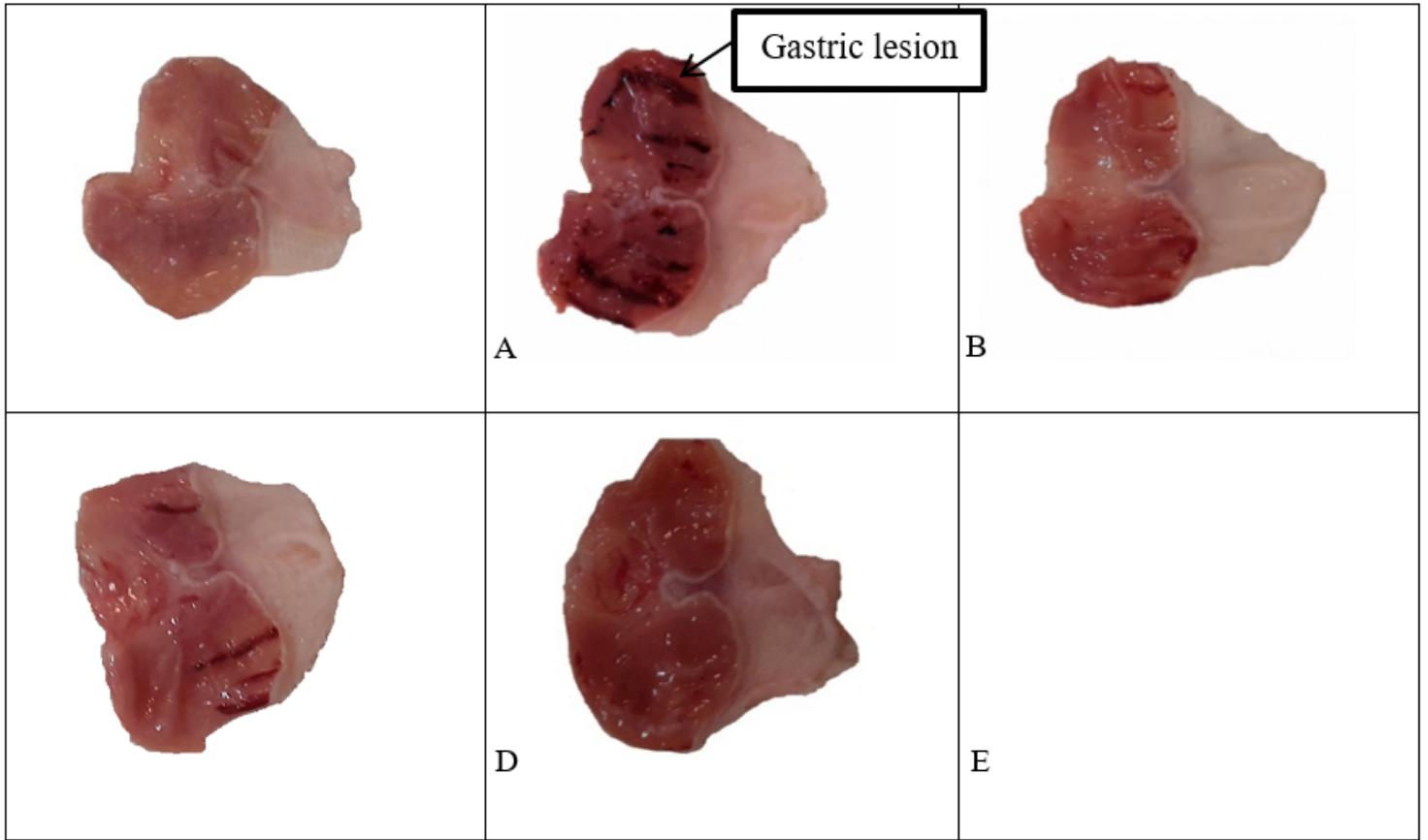


Figure 3

Macroscopic view of the stomach tissue in rats of normal group (A), ethanolic group (B), ranitidine 50 mg/kg recipient group (C), plant extract 250 mg/kg recipient group (D), plant extract 500 mg/kg recipient group (E).

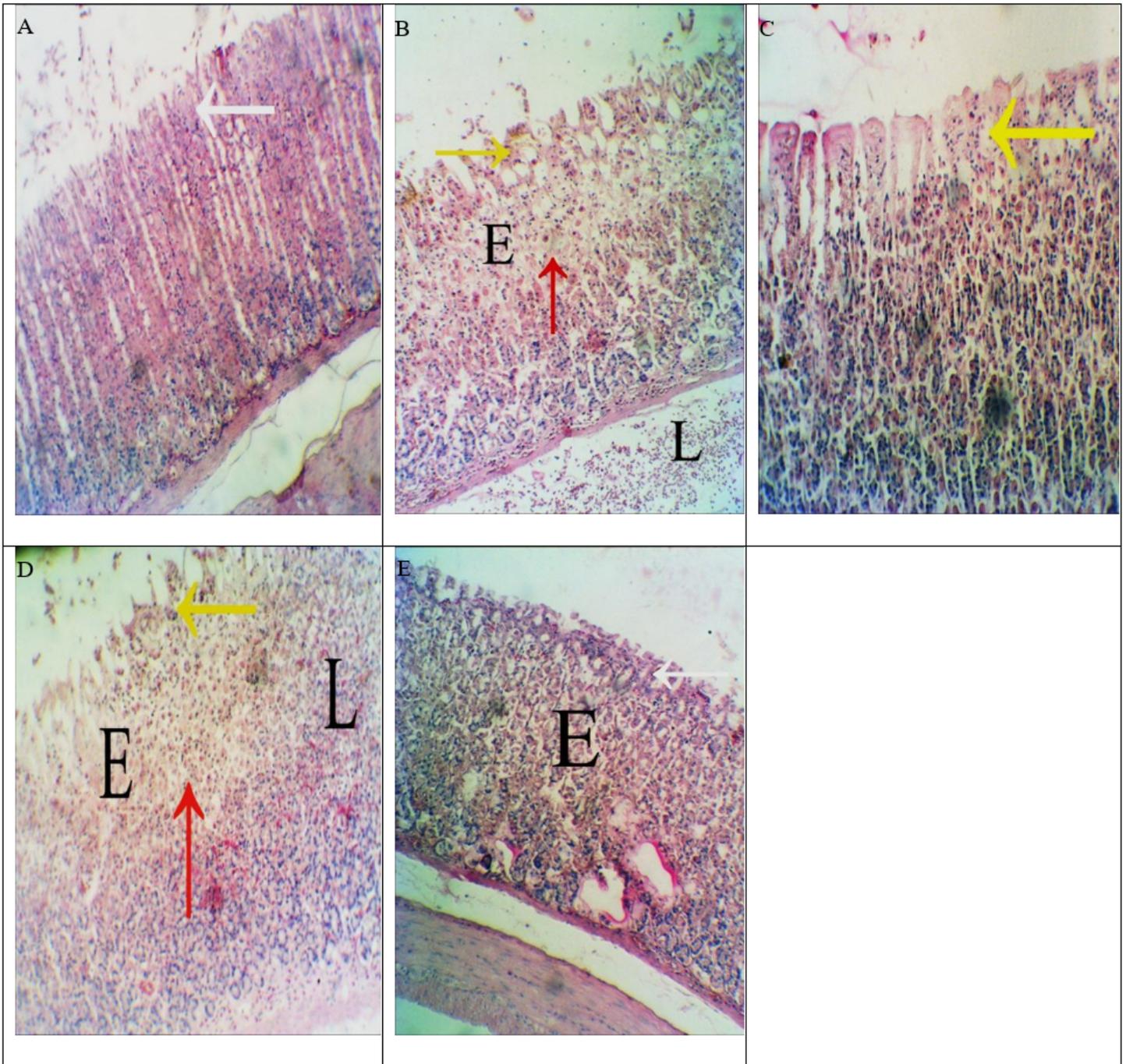


Figure 4

Microscopic view of the stomach tissue in rats of normal group (A), ethanolic group (B), ranitidine-pretreated group (C), plant extract group at a dose of 250 mg/kg (D) and plant extract group at a dose of 500 mg/kg (E). Yellow arrow: necrosis and destruction of the mucosa at the apex of the mucosa; Red arrow: necrosis and destruction of mucosa in the depth of the mucosa; White arrow: Healthy appearance or with minimal changes in the coating structure and mucosal layer; (E: edema, L: infiltration of inflammatory cells, H: Haemorrhage).

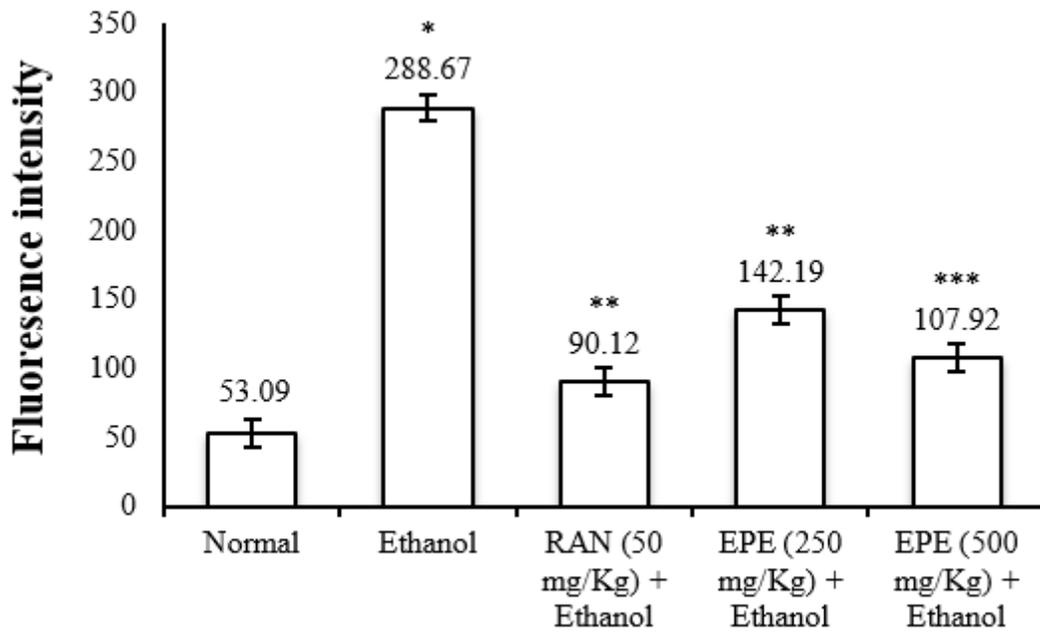


Figure 5

The effects of EPE and ranitidine drug on the level of ROS formation in studied groups. The results were expressed as means \pm SD for 6 rats in each group. (Eremurus persicus (Jaub & Spach) Boiss extract = EPE; Ranitidine = RAN). * Significant difference with $P < 0.01$ compared to normal group, ** Significant difference with $P < 0.01$ compared to ethanolic group, *** Significant difference with $P < 0.05$ compared to ethanolic group.

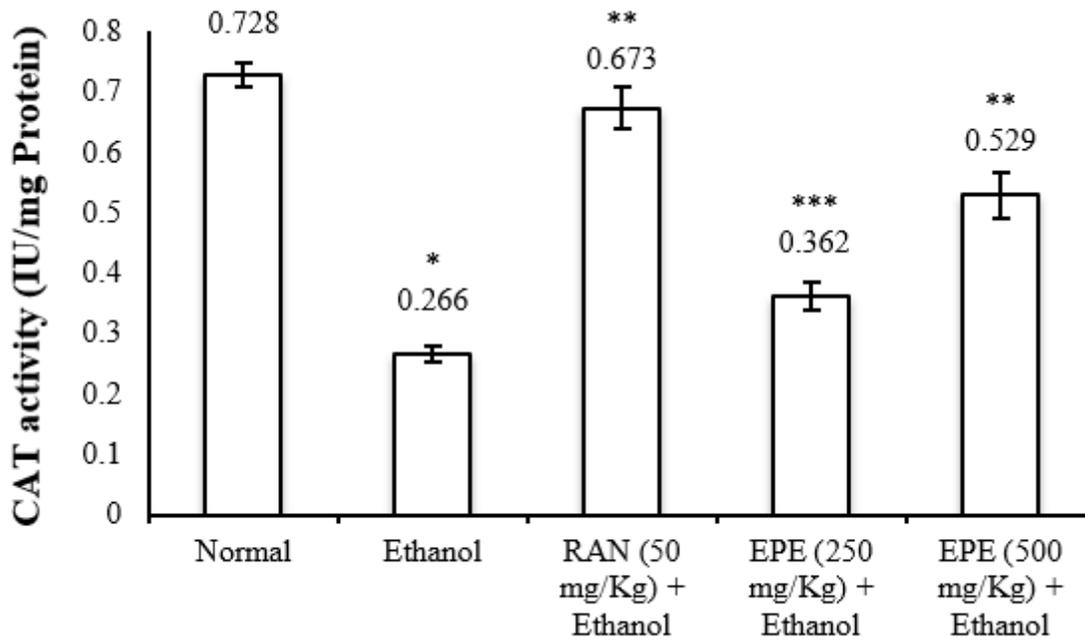


Figure 6

Changes in the activity of the CAT enzyme in gastric tissue of different groups. The results are shown based on the means \pm SD of the data for 6 rats in each group. (Eremurus persicus (Jaub & Spach) Boiss extract = EPE; Ranitidine = RAN). * Significant difference with $P < 0.05$ compared to normal group, ** Significant difference with $P < 0.05$ compared with ethanolic group, *** Significant difference with $P < 0.001$ compared with ethanolic group.

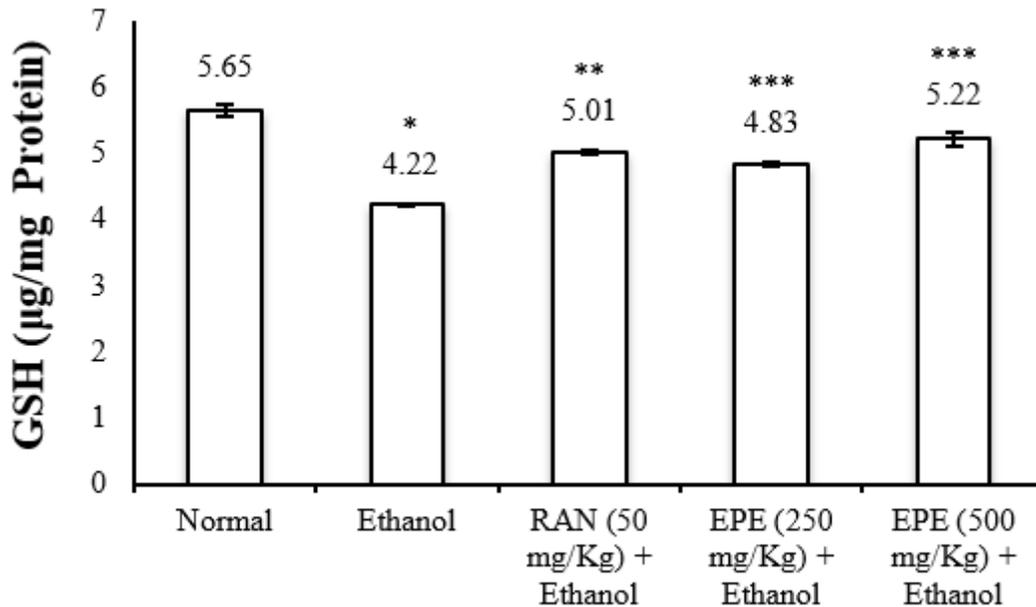


Figure 7

Changes of GSH level in gastric tissue of different groups. The results are shown based on the means \pm SD of the data for 6 rats in each group. (Eremurus persicus (Jaub & Spach) Boiss extract = EPE; Ranitidine = RAN). * Significant difference with $P < 0.001$ compared to normal group, ** Significant difference with $P < 0.05$ compared to ethanolic group, *** Significant difference with $P < 0.01$ compared to ethanolic group.

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