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Essential oils of *Marrubium peregrinum* resisted ethanol-induced gastric toxicity *in vivo*: biochemical and histopathological approaches

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ORIGINAL ARTICLE

Abstract

Marrubium peregrinum is acknowledged as a traditional therapeutic plant that relieves stomach aches and gastrointestinal disorders. The present investigation evaluates the acute toxicity and gastroprotective potential of the essential oil of *Marrubium peregrinum* (EOMP) in absolute ethanol-mediated ulceration. It identifies molecular pathways that underlie its bioactivities. The anti-ulcer potential of EOMP (100 and 200 mg/kg) was evaluated both macroscopically and microscopically. Moreover, toxicological evaluation included behavioral, biochemical, and histological alterations that occurred due to EOMP ingestion. Pretreatment with EOMP (100 and 200 mg/kg) noticeably reduced the severity of gastric injury mediated by absolute ethanol and lowered hemorrhagic and gastric tissue disruptions. Ingestion of OEMP (100 and 200 mg/kg) 1 h prior to ethanol initiation significantly inhibited lesion incidence by 73.72% and 77.65%, respectively. EOMP supplementation resisted ethanol-induced histological alterations and restored gastric defensive factors (increasing mucin secretion and pH). Moreover, EOMP-treated mice exhibited higher heat shock protein 70 (HSP 70) and lower Bax proteins, matching with increased prostaglandin E2, catalase, and superoxide dismutase contents. The ethanol-mediated oxidative stress (MDA) and gastric inflammation (tumor necrosis factor-α and interleukin-6) were significantly mimicked because of EOMP pretreatments. The toxicity evaluation evidenced the safe ingestion of up to 2 g/kg EOMP in mice without any noticeable morbidity throughout the 2-week toxicity trial. These outcomes present the prophylactic effect

of EOMP against ethanol-mediated stomach toxicity by strengthening gastric defense factors, increasing endogenous antioxidants, lowering inflammatory mediators, and reducing apoptotic actions. Altogether, the outcomes contributed to faster gastric tissue recovery because of EOMP pretreatment, without any incidence of toxicity.

Keywords: antioxidant; gastric ulcer; immunohistochemistry; inflammation; *Marrubium peregrinum*

Introduction

Gastric ulcer (GU) is considered one of the most prevalent gastric diseases; even with current drug innovation, its incidence has not decreased, yet in some cases, it may have even increased. A 2019 Global Burden of Disease study unveiled that the prevalence of gastric ulcer increased from 6,434,103 in 1990 to 8,090,476 in 2019, while the age-standardized prevalence proportion dropped from 143.4/100,000 to 99.4/100,000 individuals (Ren *et al.*, 2022). As a sophisticated, multi-faceted process, gastric ulcer develops as an outcome of an imbalance between aggressive factors (excessive use of nonsteroidal anti-inflammatory drugs, stress, alcohol abuse, and irregular dietary habits) and gastric mucosal defense factors (e.g., blood flow, prostaglandin secretion, and mucin/peptide secretions).

Ethanol is considered a corrosive agent that disrupts gastric mucosa by provoking neutrophil aggregation, oxidative stress initiation by up-regulation of reactive oxygen species (ROS) generation and inflammatory cytokines, and inflammatory cell infiltration, consequently damaging gastric mucosa. Pro-inflammatory mediators and oxidative homeostasis are major associates of alcohol-induced gastric ulceration. Moreover, pro-apoptotic proteins, such as Bax and Bak (pro-apoptotic Bcl-2 family proteins), as well as protein c-Myc, are activated, thereby provoking apoptosis and further gastric tissue injury (Al-Qaisi *et al.*, 2025b). Signaling pathways, such as nuclear factor-erythroid 2 related factor 2 (Nrf2), are considered cellular defense mechanisms against oxidative damage through enhancing antioxidant production, including endogenous antioxidants, such as glutathione peroxidase (Gpx) and superoxide dismutase (SOD) (Akcakavak *et al.*, 2024). Nrf2 is linked with negative regulator KEAP1 during normal physiological state, which stays inactive inside the cytoplasm or is broken down by proteases. Under pathological state or oxidative stress, Nrf2 was disaffiliated from KEAP1 and moved toward the nucleus, resulting in the transcriptional activation of antioxidant phase II genes such as *heme oxygenase-1* (HO-1) (Mohammed *et al.*, 2025). Despite numerous drug discoveries and their FDA approvals, there are no FDA-approved drugs for management of gastric ulcers. Nevertheless, most of their advantages are held back by their diverse adverse effects, such as elevated risk of gynecomastia, hospital/community-acquired pneumonia, arrhythmia, and hematopoietic alterations (Schubert, 2017). Thus, searching for alternatives became a continuous mission of leading scientists by exploring natural products from medicinal plants because of their medicinal effects, mitigation of stomach inflammation, and antioxidant potential, not to mention their strengthening gastric defense barriers due to their enriched phytoconstituents (phenolics, flavonoids, and terpenoids) (Al-Qaisi *et al.*, 2025b).

According to the World Health Organization (WHO), plants and their bioactive constituents, including aromatic compounds, are a primary therapeutic source used globally by more than 75% of the population. Patients in industrialized/developed nations use herbal medicines confidently as prophylaxis or curatives for numerous health disorders (Parvin *et al.*, 2023). For centuries, aromatic plants have been a significant source of flavor and organoleptic properties in foods. The therapeutic features of aromatic plants are primarily linked to the bioactivities of their essential oils, including antimicrobial, hepatoprotective, antiviral, anticarcinogenic, spasmolytic, and antioxidant properties, among others. Lamiaceae is a large and diverse plant family comprising 6,900–7,200 species across 233–263 genera. Genus *Marrubium* L is recognized with more than 30 species dispersed across Europe, Asia, and the Mediterranean region (Akgül *et al.*, 2008; Göğer *et al.*, 2019; Meyre-Silva and Cechinel-Filho, 2010). *Marrubium peregrinum* is a well-known aromatic species with diverse phytoconstituents, including various common lipid classes in its essential oils, which are utilized in pharmaceutical formulations (Gyuzeleva *et al.*, 2023).

Chromatographic characterization of *M. peregrinum* essential oil revealed its major constituents as monoterpenes, limonene, camphene, β-pinene, sabinene, and terpinolene. Essential oils of *M. peregrinum* of the Greek region were recognized due to their three major compounds: b-phellandrene, bicyclogermacrene, and epi-bicyclosesquiphellandrene (Kaurinovic *et al.*, 2010). While those of Central Europe are enriched with b-caryophyllene, germacrene D, and bicyclogermacrene (Nagy and Svajdlenka, 1998). Phytochemical fractioning of *M. peregrinum* showed different compounds as flavonols (kaempferol), flavones (apigenin and luteolin),

glycosylated flavonoids, caffeic acid derivatives, and four diterpenoids (peregrinin, peregrinol, marrubiin, and pre-marrubiin) (Demiroz Akbulut *et al.*, 2020; Kaurinovic *et al.*, 2010; Yabrir, 2018). Ethnobotanists reported different traditional uses of *M. peregrinum,* such as vascular diseases (antispasmodic and antihypertensive potential) and digestive disorders (Kaurinovic *et al.*, 2010). Previous studies evidenced the vast biopotential of *Marrubium* extracts and their application as plant supplements. Bioactivities, such as cytotoxic, antioxidant, and antimicrobial effects, as well as their modulatory actions on pathological intermediates, have been detailed in recent decades. Marrubenol, a common constituent of *M. peregrinum*, has been correlated with suppressing action on mice artery contraction potential in blocking L-type calcium (Ca^{2+}) channels in smooth muscle cells (El Bardai *et al.*, 2004). A common flavonoid of *M. peregrinum*, apigenin, is labeled as a remarkable anti-ulcer compound because of its modulatory effects on oxidative stress, apoptotic, and inflammatory molecular mechanisms (Alamri, 2024). Moreover, two major components (b-caryophyllene and germacrene-D) of *M. peregrinum* essential oils have been shown as effective anti-ulcer compounds because of their positive alterations on gastric defense barriers (Espinosa-Juárez et al., 2024; Sharma *et al.*, 2016).

Since infusing medicinal plants' aerial parts is a common traditional route to managing stomach disorders, preparing essential oil and exploring its bioactivities undoubtedly contribute to this species' pharmacological potential. In this study, we investigate the acute toxicity and gastric-healing potential of EOMP with the aim of exploring its underlying molecular mechanisms.

Materials and Methods

Plant collection and EO isolation

The aerial parts of *M. peregrinum* were collected from Safeen Mountain during the spring of 2023 (altitude: 36.30378, longitude: 44.41983) and authenticated by taxonomist Dr. Abdulla Sardar Shakur (Figure 1). The plant parts were air-dried in the shade (25°C), while the green cladodes were crunched to create a fine powder and stored in a freezer for future use. In all, 5 g of powder was used for hydrodistillation for 2.5–3 h via a Clevenger-type machine, resulting in a 0.15% (w/w) yield. The distillate was collected in ethyl ether and dried with Na_2SO_4. The solvent was separated using vacuo, and the resultant was kept in dark vials at a cool temperature throughout the study.

Acute toxicity test

Sprague Dawley mice (6–7 weeks old; 180–200 g) were provided by the Research Centre of Erbil Polytechnic University and used after a 1-week adaptation procedure. The animals were kept in an environmentally controlled condition (22±2°C; relative humidity [RH]: 50±5%) with

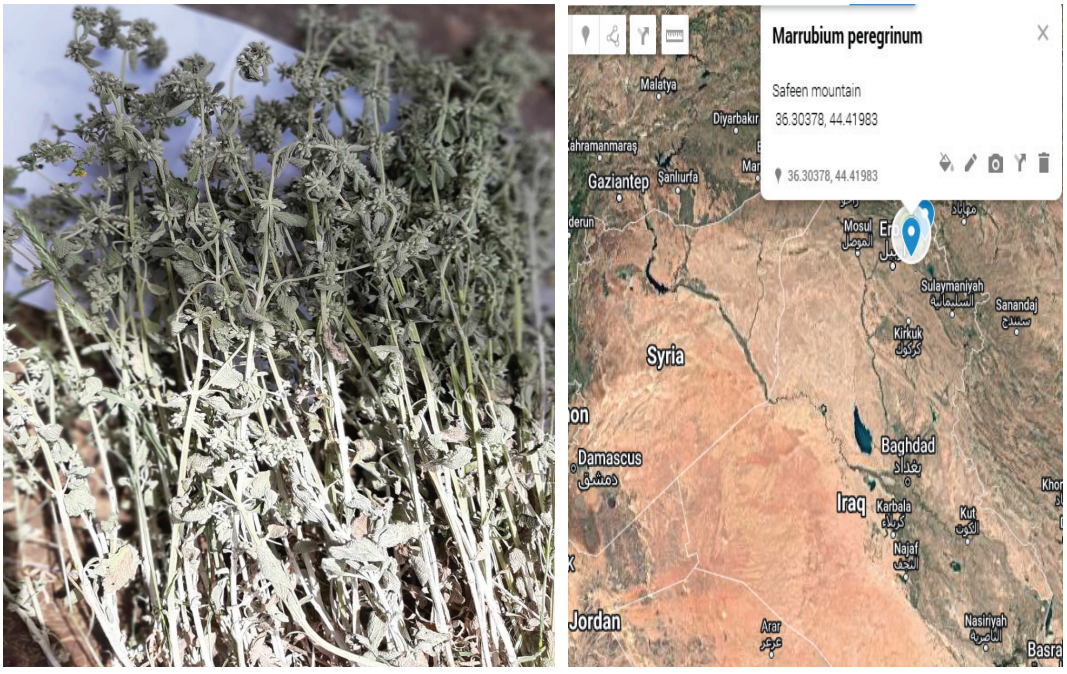

Figure 1. Aerial parts of *Marrubium peregrinum* on Safeen mountain, Erbil, Iraq.

a 12-h light–dark cycle and a specific pathogen-free (SPF) area. The mice had free access to sterilized tap water *ad libitum* and standard rodent chow. Animal trials were performed in compliance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) and Organization for Economic Co-operation and Development (OECD, 2025) guidelines (Percie du Sert *et al.*, 2020). After that, 36 animals (18 males and 18 females) were grouped in three cages, fasted overnight, and ingested the following:

1. Group A mice received 1 mL of normal saline
2. Group B mice received 1 mL of 1 g/kg of EOMP
3. Group C mice received 1 mL of 2 g/kg of EOMP

Following the treatments, all mice were fasted for an additional 3 h and observed occasionally every 8 h for the next 1 week for any possible toxicity incidence or physiological changes (tremors, diarrhea, lethargy, salivation, eye/fur changes, or skin biting) or even death. After 2 weeks, all mice received an intraperitoneal anesthetic injection prepared from 3-mg/kg xylazine and 300 mg/kg ketamine; finally, the animals were euthanized humanely. The liver and kidneys were dissected for histopathological screening, and serum samples were analyzed using different biochemicals (A.j. Jabbar and Abdul-Samad Ismail, 2025).

Gastroprotective experiment

Experimental design

In all, 30 male mice (7–8 weeks, weighing 190–200 g) were provided by Erbil Polytechnic University, and the overall animal procedures complied with the ARRIVE guidelines and were approved by the Ethical Committee of the Erbil Polytechnic University (registration #23) (Percie du Sert *et al.*, 2020). Then, the animals were fasted for 16 h and randomly allocated into the following five groups (n=5), in which they received oral treatments via gavage: normal control, A (1% CMC+normal saline); positive control, B (1% CMC+ethanol); reference drug, C (30-mg omeprazole+ethanol); low dose group, D (100 mg/kg EOMP+ethanol); and high dose group, E (200 mg/kg EOMP+ethanol). After 1 h, the normal control received 1 mL of normal saline, and groups B–E received 1 mL of absolute ethanol to induce ulceration. After 3 h, mice underwent euthanasia for relevant biochemical and histological investigations (Figure 2).

Gross and ulcer score study

Stomach samples of all mice were collected for macroscopical evaluation, and were opened at a greater curvature, mucus collected, then washed, and examined for total stomach acidity (TSA), gastric pH, lesion areas, Alcian blue binding capacity, and finally ulcer score calculated as mentioned (Al-Qaisi *et al.*, 2025a).

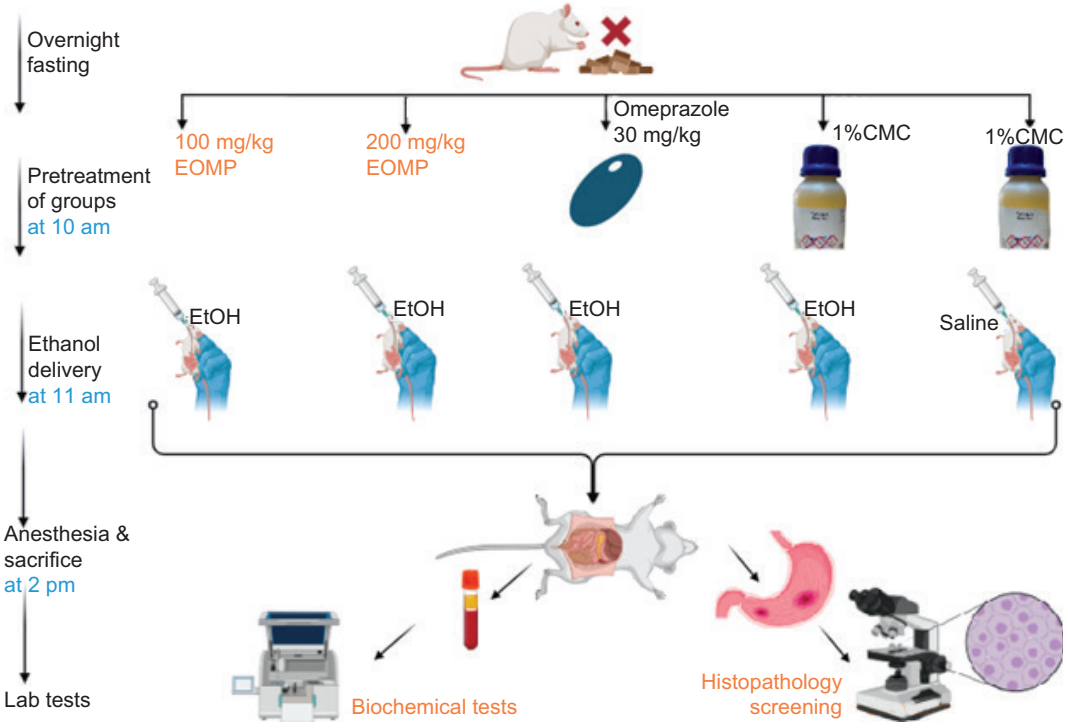

Figure 2. Experimental design for gastroprotective trial.

Histological, histochemical, and biochemical analyses

The stomachs were cleaned and sliced into small pieces (1–2 cm) and fixed in formalin 10% (v/v). After tissue processing and paraffinization, the slides were prepared for histopathological examinations, as detailed by researchers (Mohammed *et al.*, 2025). Then, tissue homogenates were prepared for determining immunohistochemical proteins (Bax and heat shock protein 70 [HSP 70]) and oxidative stress indicators (SOD, catalase [CAT], malondialdehyde [MDA], and prostaglandin E2 [PGE2]) by using available enzyme-linked-immunosorbent serologic assay (ELISA) kits (Al-Qaisi *et al.*, 2025a). To determine inflammatory conditions, serum samples were screened for tumor necrosis factor-α (TNF-α), interlukin 6 (IL-6), and IL-10 cytokines by following previous protocols (Mahmoud Al-Adwan *et al.*, 2025).

Statistics

The obtained data were processed using SPSS, One-way analysis of variance (ANOVA), and computes were created via GraphPad Prism (version 9.0). Results were expressed as mean ± standard error of mean (SEM), $p < 0.05$ was considered as statistically significant.

Results

Acute toxicity

The 2-week toxicity trial showed an increased safety margin of EOMP in mice ingested with up to 2 g/kg according to biochemical and histological evaluations. The observational process did not find any abnormal behavior/physiological changes or mortality during or after the trial. Mice ingested with oral dosages of 1 g/kg and 2 g/kg EOMP did not exhibit any toxicity, such as gastrointestinal issues (vomiting, diarrhea, or saliva leakage), respiratory distress, behavior changes (lethargy, tremors, or seizures), physical changes (eye/fur color), convulsions, or any movement disabilities. Throughout the trial, the water and food intake of supplemented mice was similar to that of normal saline-treated mice. The histopathological evaluation of the obtained liver and kidneys denoted regular tissue architecture without any signs of tissue layer distortion, hemorrhage, tubular necrosis, vacuolation, hydropic degeneration, or necrotized fatty hepatic changes. Moreover, liver and kidney functional parameters in serum samples were found to vary insignificantly between EOMP and normal saline-treated mice (available on request). Overall, the above data can serve as scientific evidence to support the safe ingestion of EOMP up to 2 g/kg in animal models (Figure 3).

Assessment of gastroprotective activity

Macroscopical views

The macroscopic examination of dissected stomachs unveiled significant variation in the number of gastric folds or rugae, as well as mucosal/submucosal layers, between normal saline and EOMP-treated mice. The normal saline-treated mice showed increased gastric folds or rugae without any signs of lesions/bleeding areas. The stomachs of ulcer-control mice were denoted with clear mucosal lesions (oval/round-shaped with defined edges), which were extended as injury hemorrhage bands along gastric axis (Figure 4). Moreover, ulcer-control mice had a few gastric folds or rugae, with a more flattened stomach surface layer and several swollen/edematous areas in the stomach lining. In contrast, pretreatment with omeprazole or EOMP (100 and 200 mg/kg) ameliorated ethanol-mediated gastric tissue damage, indicated by fewer lesions/hemorrhagic areas, less mucosal injury, lower congested/swollen areas, and higher gastric folds, compared to ulcer-control mice. In a dose-related manner (200 mg/kg being more effective), EOMP supplementation resisted ethanol-mediated gastric toxicity according to early gross evaluations.

Histopathological examination using H&E staining

The microscopic views of gastric tissues varied significantly between experimental mice because of different pretreatments and ethanol delivery. Normal control mice exhibited regular gastric tissue layers, shown by the usual organization of tissue layers without any obvious signs of tissue deterioration. The ulcer-control mice exhibited the highest gastric tissue modifications, shown by increased epithelial deterioration, numerous cell necrosis, increased migration of inflammatory cells to submucosal layer, enlarged edema, and higher mucosal erosions, compared to ulcer controls. Both omeprazole and EOMP pretreatment showed significant resistance against ethanol-mediated gastric tissue penetrations, evidenced by slight-to-moderate mucosal damage, lower epithelial cell damage, and less inflammatory cell infiltrations, as well as narrower submucosal edema areas, than ulcer controls (Figure 5).

Influence of EOMP on some gastric acidity and ulcer

The ethanol oral delivery caused a significant weakening of gastric defense barriers, reducing mucus secretion, altering gastric pH, and acidity. Normal control mice exhibited sufficient mucus content, maintained gastric pH, and increased Alcian blue binding capacities (ABC), compared to treated mice. Ulcer controls exhibited severe gastric tissue injury because of altered gastric barriers, including reduced mucus secretion (0.76 g), the lowest gastric pH (2.89), the highest gastric ulcer areas (704.3 mm), lowered Alcian binding capacities

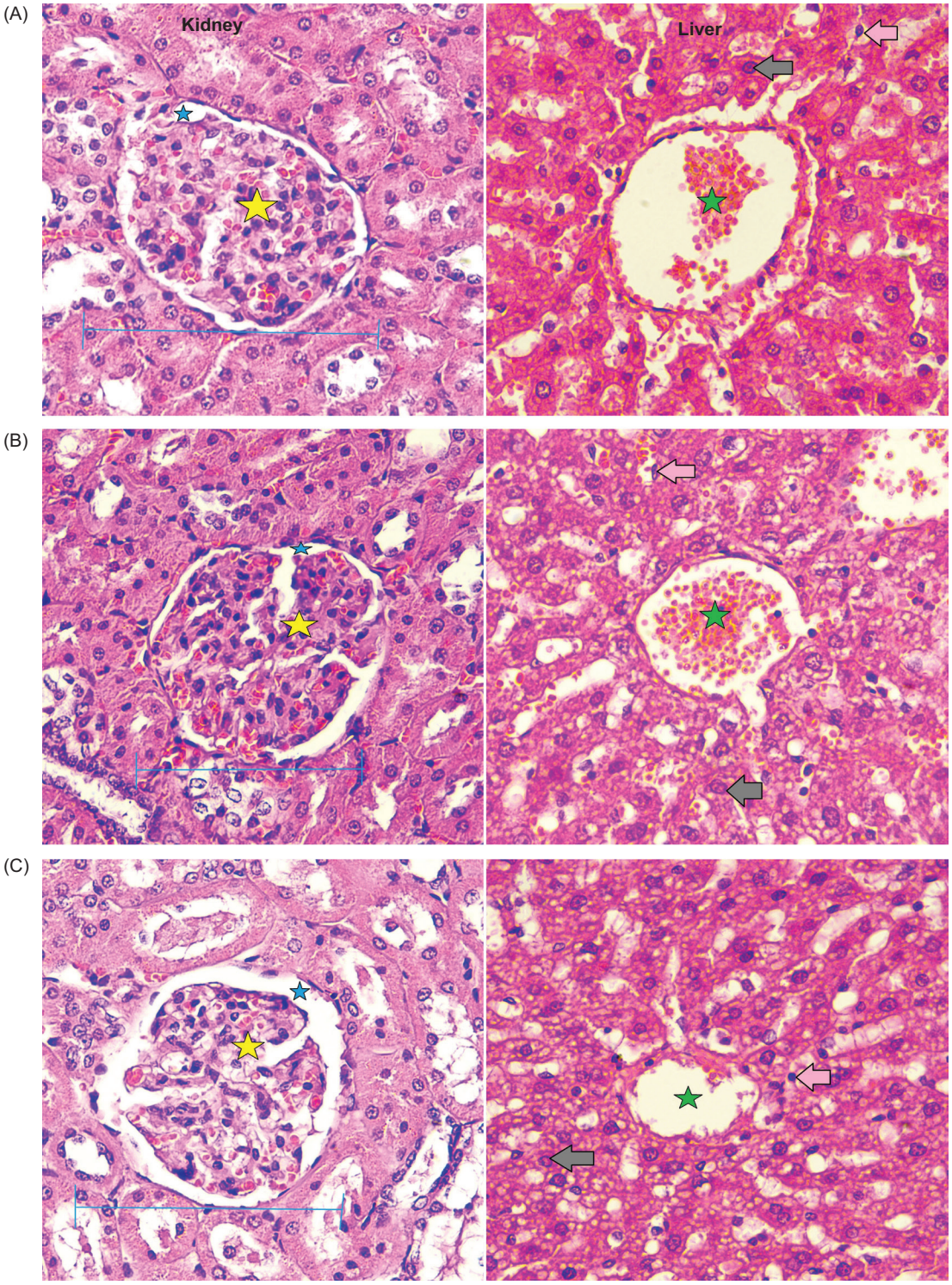

Figure 3. Histological screening of the liver and kidneys collected from different experimental groups. Group A had normal saline; group B had ingested 1 g/kg of EOMP; group C had ingested 2 g/kg of EOMP. Yellow star: glomerulus; blue line: glomerulus and Bowman's capsule; blue star: Bowman's space; green star: central vein; pink arrow: Kupffer cells; gray arrow: hepatocyte (hematoxylin and eosin [H&E] staining, 40×). PCT: proximal convoluted tubules; DCT: distal convoluted tubules.

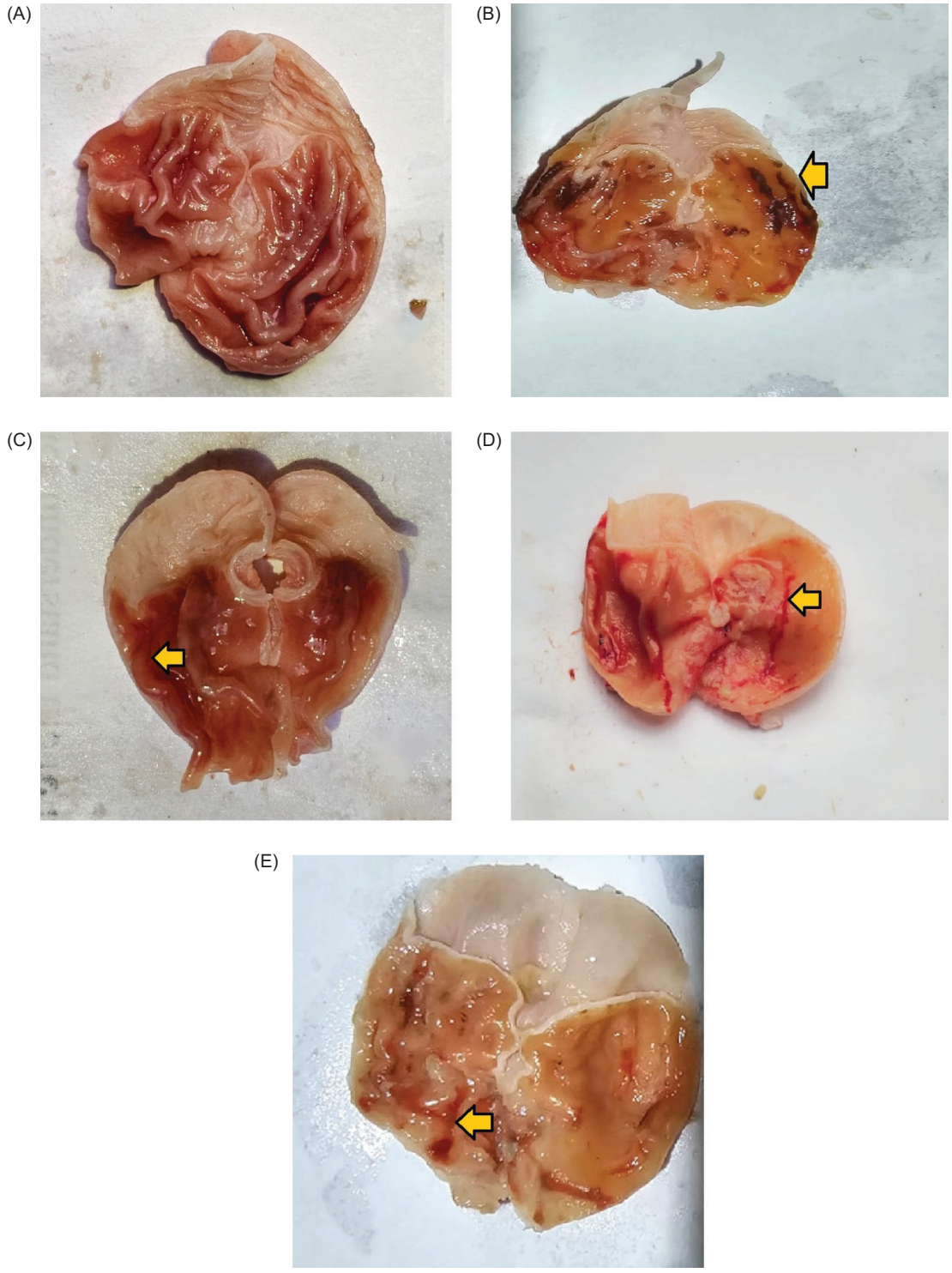

Figure 4. Effect of EOMP pre-ingestion on the macroscopic appearance of the stomachs exposed to ethanol-induced gastric ulcer in mice. Group A mice had 1% CMC+normal saline; group B mice received 1% CMC+ethanol; group C mice had omeprazole+ethanol; groups D and E mice had pre-ingested EOMP (100 and 200 mg/kg, respectively)+ethanol. The gross views of the stomachs from ulcer-control mice showed increased mucosal injury and black/red hemorrhagic areas, which were noticeably lower in mice pretreated with EOMP.

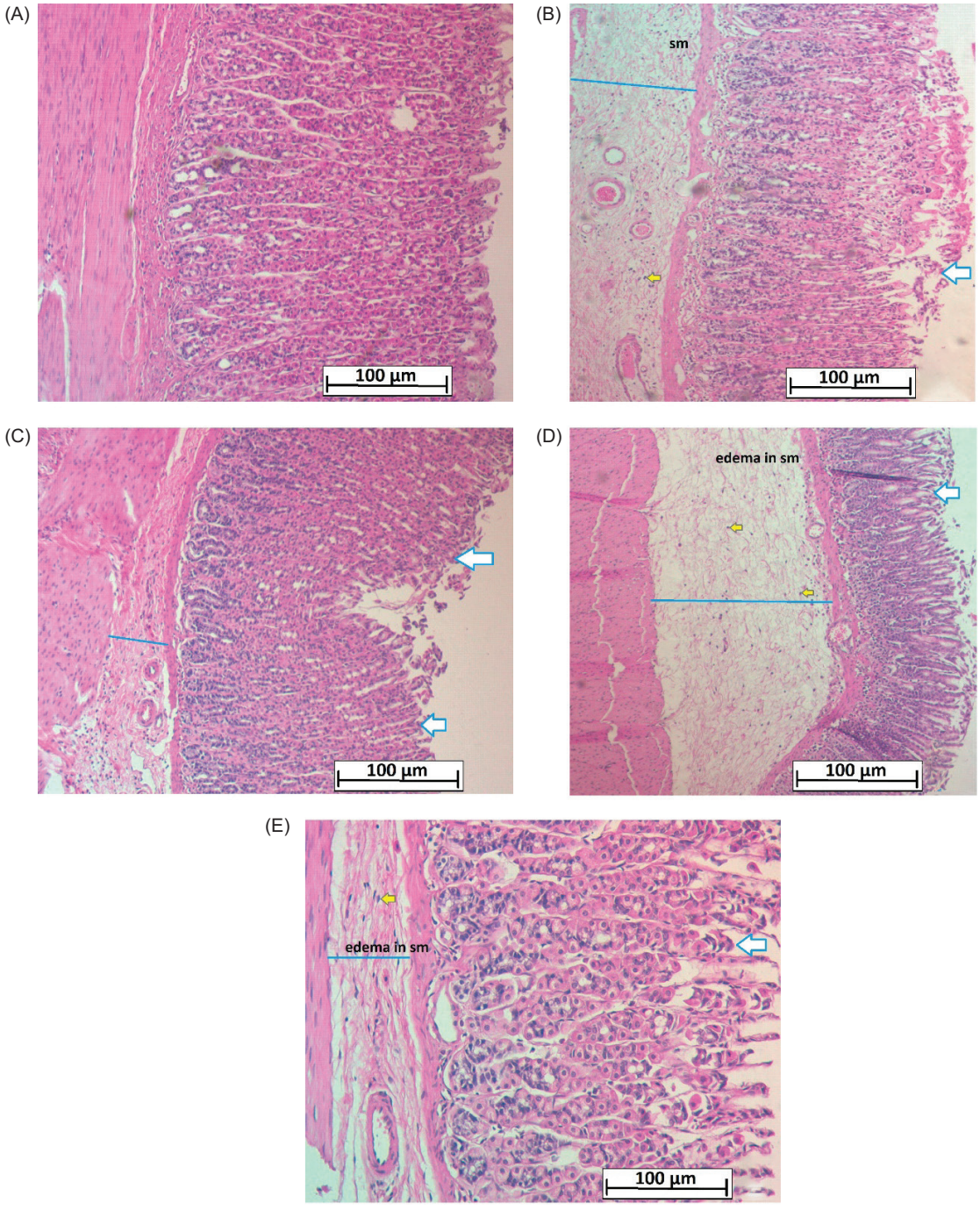

Figure 5. Histological assessment of stomach tissues from different experimental mice groups. Group A mice had 1% CMC+normal saline; group B mice received 1% CMC+ethanol; group C mice had omeprazole+ethanol; groups D and E mice had pre-ingested EOMP (100 and 200 mg/kg, respectively) + ethanol. Blue line: edema of the submucosa (SM) layer with glands; yellow arrow: inflammatory (leukocyte) cells infiltration; and white arrow: epithelial disruption. Ulcer-control mice were recognized with increased submucosal edema, inflammatory cells, and epithelial distortion in their gastric tissues. Such alterations were ameliorated in EOMP-pretreated mice.

(143.5 mg/g), and increased TSA (72.8 mEq/L/100 g). Omeprazole or EOMP pretreatment (100 and 200 mg/kg) strengthened gastric defense barriers, denoted by elevated mucus weight (1.70, 1.40, and 1.55 g), sustained gastric pH (6.8, 5.64, and 6.5), and less ulcerative areas (119.2, 184.3, and 157.4 mm), less total stomach acidity (34.3, 46.8, and 39.7 mEq/L/100 g), and heightened Alcian blue binding capacities (539.4, 324.5, and

438.4 mg/g). Pre-ingestion of omeprazole or EOMP (100 and 200 mg/kg) led to significant ulcer inhibitory proportions (83.07%, 73.72%, and 77.65%, respectively) relative to the ulcer control's zero value (Table 1). The present data results evidenced significant modulatory potential of EOMP on gastric defense factors against ethanol-mediated gastropathy in mice. The up-regulation of Alcian blue binding capacities in supplemented mice denoted increased modulatory potential of EOMP on muco-substances (glycoproteins) and mucin contents in gastric tissues that improved gastric barriers against aggressive factors as ethanol (Figure 5).

Results of immunohistochemical staining

The immunohistochemical concentration (HSP 70 and Bax) in gastric tissue homogenates varied significantly between experimental mice because of different pretreatments and ethanol ingestion. As shown in Figure 6, normal control mice had a regular amount of HSP 70 (155.3 µg/mg) in their gastric tissues, as expected. Ulcer-control mice showed the lowest concentration of HSP 70 (65.22 µg/mg) in their tissues, denoting increased cellular apoptosis and continuous gastric cell destruction. Omeprazole or EOMP (100 and 200 mg/kg) ingestion enhanced production of HSP 70 (510.8, 294.7, and 464.5 µg/mg), which acted as a molecular chaperone that facilitated proper folding of semi-partially damaged proteins, supplied protein precursors for mitochondria, and enhanced cell repair/survival. The outcomes indicated significant modulatory potential of EOMP on HSP 70 proteins, which strengthened gastric defense mechanisms by protecting key enzymes, improved gastric resistance against irritants, provoking cell proliferation and migration at ulcer margins, as well as promoting angiogenesis in granulation tissue, all of which aided in faster recovery of the ulcer.

The Bax protein intensity in stomach tissues was found to vary noticeably between pretreated groups and ulcer-control mice. Normal control mice showed minimal Bax protein levels (1.2 ng/mg) in their stomach tissues, denoting a lack of any stressful condition or apoptotic process. The ulcer-control mice showed the highest level (3.68 ng/mg) of pro-apoptotic Bax protein, indicating an increased proportion of apoptosis that worsened gastric tissue injury. Mice pretreated with omeprazole or EOMP (100 and 200 mg/kg) had significantly lower Bax protein intensity (1.51, 2.61, and 2.11 ng/mg) in their gastric tissues, denoting a lower stressful/apoptotic action and fewer cellular disruptions. Moreover, mice that received a higher dose of 200 mg/kg EOMP exhibited reduced apoptotic actions compared to a lower dose of 100 mg/kg, indicated by higher HSP 70 and lower Bax proteins in their stomach tissues, which evidenced a gastroprotection effect in a dose-related manner (Figure 6).

Effect of EOMP on oxidative stress

Ethanol-mediated gastric injury is commonly associated with oxidative stress, resulting from increased ROS production and depletion of tissue antioxidants. The present data clearly indicated oxidative stress in mice exposed to ethanol and different treatment strategies. Normal control mice showed efficient amounts of antioxidants (SOD, 21.33 U/mg; CAT, 52.75 nmol/min/mg; PGE2, 16.92 pg/mL) and reduced MDA levels (13.43 nmol/mg) in their gastric tissue homogenates. The ulcer positive controls demonstrated obvious oxidative stress-mediated tissue damage, shown by decreased antioxidant enzymes (SOD, 7.45U/mg; CAT, 15.20 nmol/min/mg; PGE2, 5.80 pg/mL) and increased MDA (lipid peroxidation indicator; 93.60 nmol/mg). In contrast, oral delivery of omeprazole, 100 mg/kg EOMP or 200 mg/kg EOMP led to significant resistance against ethanol-mediated gastric ulceration, denoted by elevated SOD (17.48, 12.62, and 15.71 U/mg, respectively), CAT (44.40, 31.63, 40.35 nmol/min/mg, respectively), and PGE2 (44.33, 28.31, 38.40 pg/mL, respectively). Moreover, as an oxidative stress indicator, the present study determined MDA levels in gastric tissue homogenates, which were significantly high in ulcer controls (MDA, 93.60 nmol/mg)

Table 1. ***Marrubium peregrinum*** **(EOMP) effects on some gastric estimations in mice.**

Animal groups	Mucus weight (g)	pH	Ulcer area $(mm)^2$	Inhibition	TSA (mEq/L/100 g)	Alcian blue binding capacities (mg/g) of tissues
A	2.45 ± 0.57^{a}	6.5 ± 0.40^{a}	–	–	22.3±0.7	612.30±10.8
B	0.76 ± 0.24^{b}	2.89 ± 0.60^{b}	704.3 ± 7.33^{a}	–	72.8±1.5	143.5±7.4
C	1.70 ± 0.65^{a}	6.8 ± 0.49^{a}	119.2 ± 5.49^{b}	$83.07\%^{a}$	34.30±3.8	539.4±7.4
D	1.38 ± 0.30^{c}	5.64 ± 0.72^{d}	184.3 ± 5.4^{c}	$73.72\%^{b}$	46.8±4.5	324.5±8.2
E	1.55 ± 0.38^{d}	6.5 ± 0.74^{a}	157.4 ± 6.8^{d}	$77.65\ \%^{c}$	39.7±2.9	438.4±9.3

Notes: The same superscript alphabets on values in the same column indicate insignificance at 5%. Group A mice had 1% CMC+normal saline; group B mice received 1% CMC+ethanol; group C mice had omeprazole+ethanol; groups D and E mice had pre-ingested EOMP (100 and 200 mg/kg, respectively) + ethanol. TSA: total stomach acidity.

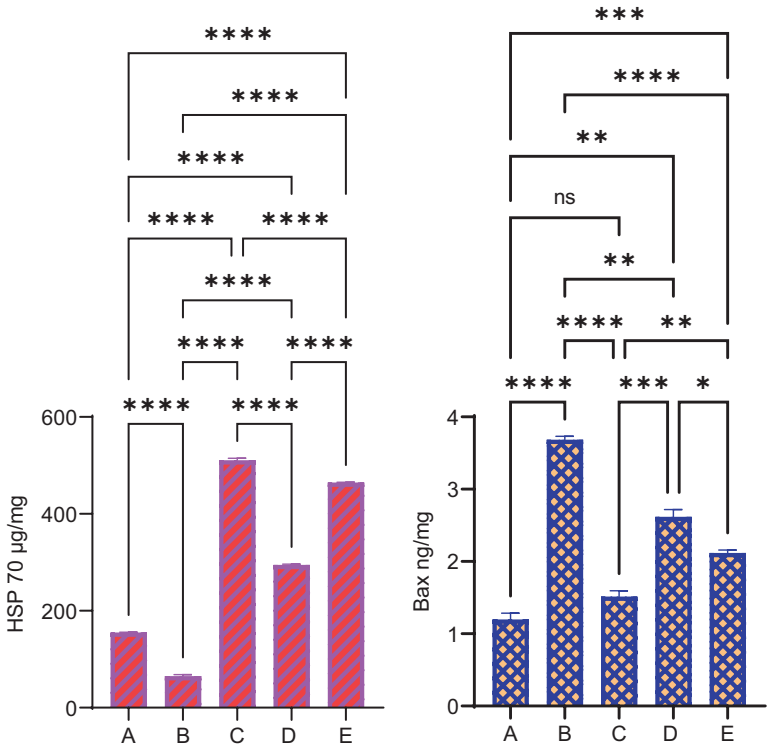

Figure 6. Effect of EOMP on HSP 70 and Bax intensity in the gastric tissues of mice administered with ethanol. Group A mice had 1% CMC+normal saline; group B mice received 1% CMC+ethanol; group C mice had omeprazole+ethanol; groups D and E mice had pre-ingested EOMP (100 and 200 mg/kg, respectively) + ethanol. The ulcer control had low HSP 70 protein and the highest Bax intensity in their gastric tissue homogenates, while pretreatment with omeprazole or EOMP resulted in the up-regulation of HSP 70 and Bax proteins in stomach tissues, thereby decreasing ethanol-mediated apoptotic tissue injury and enhanced gastric tissue repair by supporting refolding of semi-disrupted proteins. ns: nonsignificant; *p > 0.05; **p > 0.01; ***p > 0.001; ****p > 0.0001.

and notably lower (MDA, 17.48, 25.42, 21.44 nmol/mg, respectively) in omeprazole or 100 mg/kg EOMP or 200 mg/kg EOMP-treated groups (Figure 7).

Effect of EOMP on serum inflammatory cytokines

Inflammatory cytokines are key players in the recovery of gastric ulcer; however, increased production of pro-inflammatory cytokines halts healing action, as in the case of ethanol-mediated gastropathy. In the present study, normal control mice had the lowest inflammatory mediators (TNF-α, 95.6; and IL-6, 28.3 pg/mL) and increased anti-inflammatory cytokine (43.8 pg/mL). Ulcer vehicle group exhibited a prolonged inflammatory stage, shown by elevated pro-inflammatory chemicals (TNF-α, 276.7 pg/mL; and IL-6, 57.5 pg/mL) and reduced IL-10 (18.54 pg/mL) level. In contrast, omeprazole or EOMP (100 and 200 mg/kg) pretreatment ameliorated ethanol-mediated inflammation, shown by the down-regulation of TNF-α (112.5, 167.5, and 121.9 pg/mL, respectively) and IL-6 (31.40, 42.8, and 37.45 pg/mL, respectively) and up-regulated IL-10 cytokines (45.78, 32.81, 39.40 pg/mL, respectively), all of which reduced neutrophil infiltration, microcirculatory alterations, and lessen gastric hemorrhage. TNF-α and IL-6 were found to increase by 189.4% and 103.1%, respectively, in ulcer controls, compared to normal control mice, but supplemented (100 and 200-mg/kg EOMP) mice had lower TNF-α (39.46% and 55.76%, respectively) and IL-6 (25.56% and 34.86%, respectively) values relative to ulcer-control mice. Moreover, IL-10 was up-regulated by 76.96% and 112.51% in EOMP-treated mice (100 and 200 mg/kg, respectively), compared to ulcer-control mice (Figure 8).

Discussion

Plants and their aromatic compounds can have toxic effects, especially if they are consumed in large amounts in repeated doses; therefore, safety evaluation of these natural resources is crucial before considering them as therapeutic agents for human ailments. The present trial evaluated the safety of single-dose delivery of up to 2 g/kg EOMP in male and female mice by following OECD

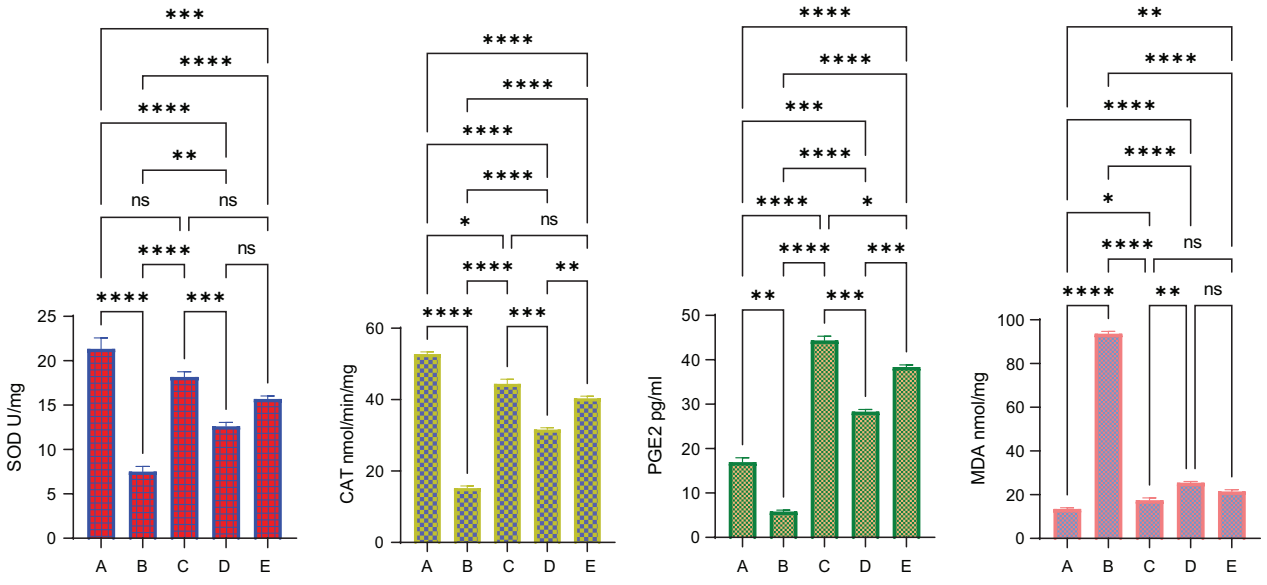

Figure 7. Effect of EOMP on oxidative stress indicators in gastric tissues of experimental mice in ulcer trial. Group A mice had 1% CMC+normal saline; Group B mice received 1% CMC+ethanol; group C mice had omeprazole+ethanol; groups D and E mice had pre-ingested EOMP (100 and 200 mg/kg, respectively)+ethanol. Ethanol delivery led to significant oxidative stress-mediated gastric injury, indicated by decreased endogenous antioxidants and increased MAD levels. In contrast, EOMP pretreatment resisted ethanol-mediated oxidative stress damage by promoting antioxidant generation and reducing lipid peroxidation rates, aiding in faster ulcer healing. ns: nonsignificant; *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$; ****$p > 0.0001$.

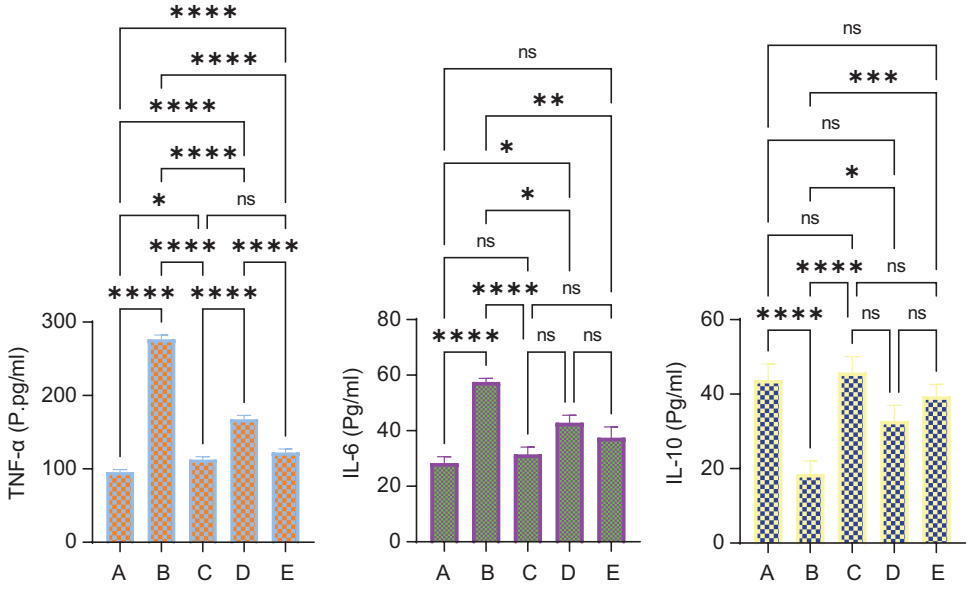

Figure 8. Effect of EOMP on serum inflammatory mediators in experimental mice of ulcer trial. Group A mice had 1% CMC+ normal saline; group B mice received 1% CMC+ethanol; group C mice had omeprazole+ethanol; groups D and E mice had pre-ingested EOMP (100 and 200 mg/kg, respectively)+ethanol. Administration of ethanol provoked inflammatory response, shown by up-regulated TNF-α and IL-6 levels and down-regulated IL-10 cytokine, thereby resulting in a prolonged inflammatory process that subsequently enhanced gastric hemorrhage and mucosal lesions. In contrast, omeprazole or EOMP pretreatment resisted ethanol-induced inflammation, limiting inflammatory cell infiltration into submucosal layers and promoting faster ulcer healing. ns: nonsignificant; *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$; ****$p > 0.0001$.

standards (Jonsson *et al.*, 2013); the evaluated dose did not result in any toxicity, abnormal behavior, or mortality. Similarly, researchers confirmed the nontoxic effects of *Marrabium* essential oil on *A. salina* (brine shrimp larvae), showing increased cell viability after 24-h exposure (Rezgui *et al.*, 2021). Accordingly, *M. persicum* extract supplementation displayed anti-inflammatory and nontoxic effects in carrageenan-induced inflammation in animal models (Çevik *et al.*, 2012). Fortunately, despite their diverse phytoconstituents, toxicity of *marrubium* essential oils was not observed. The present outcomes serve as the first detailed *in vivo* toxicity evaluation of *M. peregrinum* essential oil in an animal model.

Abuse of alcohol ingestion demolishes gastric defense barriers, making it more susceptible to gastric erosion and loss of rugae muscles. As the first line protection, the stomach secretes mucus/mucopolysaccharides that help to maintain gastric pH and reduce gastric acidity damage provoked by absolute ethanol (He *et al.*, 2022). Gastric mucosa, along with tight junctions between epithelial cells, serves as a protective semipermeable layer for digestive enzymes/pepsin to prevent deeper tissue penetration; however, such limited permeability can be altered by ethanol, which enhances the release of vasoactive products as well as vascular injury. Destruction of vasculature promotes necrotization, erosion of gastric rugae, and enhances the evacuation of bicarbonate and mucus production (gastric acidity; Liu *et al.*, 2021). In our study, EOMP pretreatment ameliorated ethanol-mediated gastric injury, reduced rugae erosion, and lowered histopathological alterations (mucosal injury, submucosal edema, inflammatory cell/fibroblast infiltration, vascular edema, epithelium disruption, and gastric erosion) by strengthening gastric defense systems (increasing production of mucus content/glycoproteins and maintaining gastric acidity/pH), dyspepsia, gastroesophageal reflux, and gastric dysmotility. This gastroprotective property of EOMP could be linked with its phytoconstituents (apigenin, b-caryophyllene, bicyclogermacrene, and germacrene-D), which were repeatedly mentioned as anti-ulcerative agents in different *in vivo* trials because of their regulatory actions on oxidative stress, inflammation, and apoptotic molecular pathways (Alamri, 2024; Esteves *et al.*, 2005).

Apoptosis plays a vital role in the initiation of gastric mucosal injury and epithelial cell death. The up-regulation of proapoptotic proteins (p53 and Bax) was correlated with gastric sore formation and progression into gastric tumor. Oral intake of ethanol provokes oxidative stress and inflammation that activate intrinsic apoptosis mechanisms, which increase Bax and decrease HSP 70 expressions. HSP 70 is a key regulator of apoptotic actions that can provoke adaptive and internal immune responses (Boonyong *et al.*, 2023). HSP 70 is considered as an apoptotic protein that stimulates or inhibits apoptotic process as a cytoprotective action to surpass stressful cellular conditions, thus classified as a cytoprotective molecule of the gastrointestinal tract (GIT) that accelerates stomach healing process in mucosal injuries (El-Shiekh *et al.*, 2021). In the present ulcer trial, oral delivery of ethanol down-regulated HSP 70, reducing cellular response, and further enhancing cellular irregularities, up-regulated protein denaturation, and increased partially unfolded proteins. EOMP supplementation limited apoptotic protein alterations associated with ethanol's oral delivery by increasing HSP 70 concentration in gastric tissues. Moreover, Bax proteins (a key regulator of mitochondrial membrane integrity and apoptosis induction) were expectedly increased in ulcer-control mice, while omeprazole or EOMP supplementation remarkably down-regulated Bax proteins in gastric tissues. Such modulatory potential of *M. peregrinum* essential oils on immunohistochemical proteins could be linked with *Marrubium*'s phytoconstituents, mainly apigenin, caryophyllene, spathulenol, caryophyllene oxide, bicyclogermacrene, and germacrene-D (Demiroz Akbulut *et al.*, 2023; Mannoubi *et al.*, 2023). These compounds were repeatedly reported as anticancer agents because of their regulatory actions on Bax and Bcl-2 apoptotic proteins (Delgado *et al.*, 2021). Moreover, apigenin (a major component of EOMP) exhibited remarkable cytotoxicity against human lung cancer A549 cells, which were mainly linked with its regulatory effects on immunohistochemical Bax- and Bcl-2-provoked mitochondrial mechanisms (Lu *et al.*, 2010).

Oxidative stress in gastric tissues is an outcome of an imbalance between endogenous antioxidants and ROS molecules, which is exacerbated due to numerous stress factors, including absolute ethanol. Ethanol consumption can increase gastric acidity, a mere necrotizing factor weakens mucosal barriers and facilitates gastric epithelial injuries by down-regulating mitochondrial superoxide production.

Ethanol-mediated gastric acid up-regulation not only reduces the initiation of superoxide anions from mitochondrial electron transport chain proteins but also provokes the generation of more ROS molecules, subsequently increasing tissue oxidative stress (Moawad *et al.*, 2019). Scientists confirmed that gastric acids can modulate different pathways associated with cellular imbalance and oxidative stress, including mitogen-activated protein kinase (MAPK), signal transducer/activator of transcription 3 (STAT3), and nuclear factor-kappa B (NF-κB) (Gao *et al.*, 2022). In the first-pass alcohol metabolism, the stomach metabolizes ethanol via enzyme alcohol dehydrogenase (ADH), generating acetaldehyde, a toxic compound that further enhances the oxidative stress-mediated injury. To balance these, the microsomal

ethanol oxidizing system through cytochrome P450 family (CYP2E1) generates oxidized nicotinamide adenine dinucleotide phosphate (NADPH), which is used to localize in the cytoplasm. CYP2E1 provokes cyclooxygenase-2 (COX-2) enzyme production in the liver, which mediates prostaglandin synthesis and stimulates several inflammatory processes, leading to exacerbation of gastric lesions. Despite this, COX-2 has been linked also with natural gastric defense mechanism; therefore, its complete suppression can pose a detrimental effect on gastric ulcer recovery (Forsyth *et al.*, 2014). The present study found significant antioxidant potential of EOMP, which could be one of the mechanisms underlying its anti-ulcer effects against ethanol-mediated gastropathy. Similarly, researchers demonstrated *in vitro* an increase in antioxidant potential (IC_{50}: 8.63–16.41 mg/mL) of *M. peregrinum* essential oils, which were mainly linked with their phytochemicals, sesquiterpene (bicyclogermacrene, b-caryophyllene, and germacrene-D), and oxygenated sesquiterpenes (caryophyllene oxide and spathulenol) (Kaurinovic *et al.*, 2010). Moreover, previous studies have demonstrated the antioxidant potential of apigenin and germacrene-D (major components of *M. peregrinum* essential oils) in different *in silico* and animal trials, which were explained by their modulatory effects on several cellular and molecular pathways (NF-kB, P13/Akt, Nrf2, and MAPK) (Bektasevic *et al.*, 2023; Toplan *et al.*, 2022).

Overconsumption of alcohol changes mucosal permeability and facilitates gastric acid penetration as well as promotes vasoactive production from mast cells, leukocytes, and macrophages. Such cells are capable of generating numerous chemokines and cytokines (TNF-α and IL-6) that exacerbate inflammatory conditions via NF-κB activation in the already injured gastric tissues. NF-κB is recognized as a canonical and noncanonical mechanism that, in turn, stimulates pro-inflammatory genes, causing a continuous cycle of inflammation. Scientists confirmed that NF-κB is capable of modulating several cellular pathways, such as the survival and activation of immune cells, inflammasome regulation, T-cell differentiation, and maintaining a balance between pro- and anti-inflammatory gene expressions, all of which make this pathway a central mechanism in preserving immune homeostasis (Nam and Choo, 2021).

Pro-inflammatory mediators (TNF-α and IL-6) are the major players in the initiation of acute inflammatory response, parallel with neutrophil infiltration into gastric mucosa; they also provoke apoptotic actions via caspase 3 activation in gastric tissues. Moreover, increased pro-inflammatory cytokines compromise gastric microcirculation and mucosal injury through increased neutrophil recruitment/infiltration and up-regulation of nitric oxide synthase (iNOS) activity, thereby enhancing oxidative stress and inflammation of ulcer area (Abdel Aziz *et al.*, 2021).

The present study demonstrated significant anti-inflammatory potential of EOMP against ethanol-mediated gastropathy, demonstrated by lower oxidative stress and pro-inflammatory cytokines. Such modulatory effects of EOMP on estimated cytokines could be attributed to its phytochemicals (germacrene-D and apigenin), which are repeatedly reported as anti-inflammatory agents (Mohanty *et al.*, 2023). Similarly, researchers demonstrated the anti-inflammatory potential of *Marrubium* extracts as one of the pathways underlying its ameliorating actions on several human diseases in animal models, including arthritis (Aitbaba *et al.*, 2024) and diabetes (Demiroz Akbulut *et al.*, 2024). Such bioactivities are mainly correlated with its phytoconstituents, particularly phenolics, fatty acids, diterpenoid lactone, and flavonoids, namely apigenin-7-O-(3”-O-E-p-coumaroyl)-β-d-glucopyranoside, tiliroside, astragalin, and 4-(β-d-glucopyranosyloxy) benzoic acid (Demiroz Akbulut *et al.*, 2024; Velagapudi *et al.*, 2018).

Conclusion

Essential oils of *M. peregrinum* exhibited antiulcer activity in mice subjected to an acute toxicity and ethanol-mediated gastropathy model. We demonstrated that oral ingestion of up to 2 g/kg did not cause any toxic symptoms or mortality even after a 2-week trial. Pretreatment with EOM persisted ethanol-mediated stomach ulcers, indicated by reducing lesion formation and gastric mucus contents. Treatment with EOMP ameliorated gastric ulcers, parallel with lower inflammatory cytokines, leukocyte infiltration, reduced Bax proteins, and up-regulating HSP 70 proteins in gastric tissues. EOMP supplementation attenuated ethanol-induced oxidative stress and inflammation, as demonstrated by lower MDA and increased SOD, CAT, and PGE2 enzymes and fewer pro-inflammatory cytokines (TNF-α and IL-6). The findings demonstrated the gastroprotective actions of EOPM by modulating several molecular pathways. However, owing to some limitations of the study (poor laboratory facility, lack of funds, small animal house, and lack of specialized instruments/kits), further studies are required to elucidate and further characterize biomolecules underlying its bioactivity in larger animal trials prior to submitting it for clinical trials.

Data Availability

All relevant data are available in the article.

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Author Contributions

Khaled Abdul-Aziz Ahmed and Ahmed A.j. Jabbar: conceptualization; Ahmed A.j. Jabbar and Khalid M. Al-Qaisi: investigation; Jehan Y. Al-Humaidi, Goran Noori Salih, and Talal Salem Al-Qaisi: methods and registration; Muneera S.M. Al-Saleem, Rawaz Rizgar Hassan, Muzhda Haydar Saber, and Qosay A. Al-Balas: resources and validation; Mohammed M. Rahman, Noralhuda Ayad Ibrahim, Hanan Ibrahim Althagbi, and Ahmed Hameed Al-Dabhawi: software and design; Ahmed A.J. Jabbar: writing of manuscript. All the authors agreed on the final version of the manuscript.

Conflicts of Interest

None.

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