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Pomegranate peel boosts the potential of Manuka honey to protect against ethanol-induced gastric ulcers in rats by enhancing its anti-inflammatory and antioxidant mechanisms

Osama AH Abuzinadah[1*], **Soad S Ali**[2,3,4], **Ghadeer M Alsrehy**[1], **Fatma A Al-Nefeiy**[5], **Naser A Alkenani**[1], **Daklallah Ahmed Almalki**[6]

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Authors' Affiliation:
[1]Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[2]Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[3]Yousef Abdullatif Jameel Scientific Chair of Prophetic Medicine Application, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[4]Department of Histologyand cell biology, Faculty of Medicine, Assiut University, Assiut, Egypt
[5]Department of Biology, Faculty of Science, University of Jeddah, Jeddah, Saudi Arabia
[6]Department of Biology, Faculty of Science and Arts (Qelwah), Albaha University, Albaha, Saudi Arabia

[*]**Corresponding Author**
Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, Jeddah,
Saudi Arabia
Email: oabozenada@kau.edu.sa

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ABSTRACT

Discovering alternative safe medicine for the management of gastric ulcers (GU) is a vital issue. This study aimed to investigate the possible protective effect of adding pomegranate peel (PP) to the Manuka honey (MH) against ethanol (EtOH)-induced GU in rats, highlighting the anti-oxidative and anti-inflammatory mechanisms. Twenty four rats were randomly divided into 6 groups; C (control), GU (EtOH 0.5 mL/100 g), OM (omeprazol 40 mg/kg), MH (2.5 g/kg), PP (PP 250 mg/kg), and PP+MH (PP 250 mg/kg and MH 2.5 g/kg). Stomach sections were stained with H&E, PAS, and tumor necrosis factor α (TNFα) (immunohistochemical staining). Gastric contents of malondialdehyde (MDA), interleukin 2 (IL-2), IL-6, and nitric oxide (NO) were estimated. In comparison with the C group, EtOH caused marked damage of stomach surface mucous epithelium with degenerative changes, and hemorrhage of glandular elements, gastric MDA, TNFα, IL-2, and IL-6 were elevated while reduced gastric contents of mucus and NO. Immunohistochemical staining of stomach mucosa showed marked increased TNFα expression. Oral administration of OM, MH, and PP+MH improved all biochemical, histological, and immunohistochemical alterations. The best effect owes to the PP+MH combination. The combination of PP+MH had a protective effect on EtOH-induced GU, as it boosted the potential of MH to act as an anti-inflammatory and antioxidant.

Keywords: Gastric ulcer, ethanol, pomegranate peel, Manuka honey, anti-inflammatory, antioxidant

1. INTRODUCTION

Gastric ulcer (GU), also called stomach ulcer, is a disease that affects millions of individuals worldwide, with many of them developing stomach cancer as a result. Globally, the incidence of GU is expected to increase due to the unhealthy lifestyle and increased psychological stress (Lin et al., 2019). GU disease is defined by a wound in the stomach's mucous membranes that may extend to the submucosal layer (Bamji and Benson 2014; Asali et al., 2018). The disease is associated with many complications such as internal bleeding, gastric perforation, and obstruction of the gastric outlet (Karaboğa et al., 2018). GU lesion has been linked to numerous pathophysiologic factors; the most important is the disruption in the balance between elements that strive to safeguard or destroy the mucosal epithelium (Song et al., 2016).

GU is most commonly caused by infection with the bacterial Helicobacter pylori or by long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, and naproxen, alcohol intake, psychological, and physiological stress (Drini 2017; Park et al., 2021). The experimental model of GU developed with ethanol (EtOH) is one of the most important laboratory models for the study of the efficiency and mechanism of action of medicines and pharmaceuticals in the treatment of gastric ulcer. EtOH causes acute inflammation of the gastric membrane characterized by a large amount of neutrophil infiltration. It continues to form oxygen free radicals (OFR) that initiate cell membrane lipid peroxidation (LPO) (Balan et al., 2015; Boutemine et al., 2018). In addition, EtOH induces the formation of the inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL1β). Apoptosis also plays an important role in EtOH-induced gastric ulcer (Hussein et al., 2020).

Proton pump inhibitors, histamine type-2 blockers (medicines that limit acid production), antibiotics (medicines that eradicate Helicobacter pylori), antacids (medicines that neutralize acid), and cytoprotective medicines like sucralfate and misoprostol are among the current treatments for GU. However, many of these medications might have unwanted side effects and put a financial strain on GU patients. As a result, scientists are still looking for new drugs that can cure stomach ulcers with more excellent safety, fewer side effects, more efficacy, and lower cost (Chan and Leung 2002; Jain et al., 2007; Lanas and Chan 2017; Park et al., 2021).

Manuka honey (MH) is a single-flowered honey produced from Leptospermum scoparium tree (family Myrtaceae) in New Zealand and eastern Australia. Black honey has received much attention for its antibacterial, antioxidant and potential role in wound healing. MH has the highest concentrations of phenol and flavonoid chemicals (pinobanksin, pinocembrin, and chrysin) associated with OFR scavenging activity compared to other honeys (Almasaudi et al., 2016; Mărgăoan et al., 2021). Previous studies reported a significant antiulcer effect of MH against EtOH-induced GU in rats (Almasaudi et al., 2016). The peel of the pomegranate (*Punica granatum* L.) has a wide range of pharmacological properties, including antioxidant, antibacterial, anticancer, antinociceptive, and anti-diabetic activities (Thangavelu et al., 2017; Qusty et al., 2021; Mohamed and Mabrok, 2022). All EtOH, indomethacin, and aspirin-induced rat GU are protected by the aqueous and EtOH extracts of PP (Alam et al., 2010; Moghaddam et al., 2013; Ifora et al., 2020; Mohamed and Mabrok 2022). So far, the mechanism of action of PP as an anti-ulcer agent on EtOH-induced GU, including antioxidants, anti-inflammatory agents, and histological changes, has not been studied.

This study aimed to investigate the mechanism (s) of PP as a protective agent against EtOH-induced GU in rats, highlighting the anti-oxidative and anti-inflammatory mechanisms and also the study of the possible protective effect resulting from adding PP to the MH.

2. MATERIALS AND METHODS

Therapies

Omeprazole (OM, Sigma, USA), MH (New Zealand), and PP powder (Abazeer, Saudi Arabia). OM was suspended in 3% tween 80, MH and PP were prepared in distilled water.

Animals

This study used 24 male albino rats weighing 200-250 grams. Throughout the experiment, rats were kept under a 12 hour light-dark cycle at room temperature and a relative humidity of 60-70%. Rats had unlimited access to food and water ad libitum. The experiment was conducted in strict accordance with the guidelines and laws of the Research Ethics Committee of King Abdulaziz University in Saudi Arabia. This study was conducted from April 2020 to October 2021.

Induction of GU

GU was induced by gavage of 70% EtOH at a dose of 0.5 ml / 100 g body weight (BW) (Boligon et al., 2014).

Study groups

Rats were randomly divided into 6 groups (n=4) as follows: C: control rats gavage tween 80 (3%), GU: ulcer rats gavage EtOH (0.5 mL/100 g BW), OM: rats gavage OM (40 mg/kg BW) (Raish et al., 2021), MH: rats gavage MH (2.5 g/kg BW) (Almasaudi et al., 2016), PP: rats gavage PP (250 mg/kg BW) (Ahmed et al., 2020), and PP + MH: rats gavage PP (250 mg/kg BW) and MH (2.5 g/kg BW). All therapies were gavage 10 days before induction of GU. One hour after GU induction, rats were anesthetized with ether and sacrificed by cervical dislocation. The stomachs were removed and rinsed with a 0.9% saline solution to clean the blood. Stomachs were either preserved in 10% buffered formalin solution (pH=7.4) for hematoxylin and eosin staining (H&E), periodic acid Schiff (PAS) staining, and tumor necrosis alpha (TNFα) immunohistochemical staining. Other stomachs were stored at −80◦C for the biochemical analyses.

Assessment of gastric mucosa histopathological lesions (H&E stain)

Formalin-fixed stomachs were embedded in paraffin wax and serially sectioned (3–5 μm), stained with H&E, examined and photographed under a light microscope.

Assessment of gastric mucosa glycoprotein contents (PAS stain)

Formalin-fixed stomachs were embedded in paraffin wax and serially sectioned (3–5 μm), stained with PAS, examined and photographed under a light microscope.

Preparation of stomach homogenate

In 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, and 2% Triton X-100, one gram of stomach was homogenized. At four °C, homogenized tissues were sonicated twice at 30-second intervals. After sonication, homogenized tissues were centrifuged for 10 minutes at four °C at 4000 rpm/min.

Assessment of stomach homogenates antioxidant / oxidative stress markers

Homogenized stomachs were used to measure enzymatic antioxidants, including catalase (CAT) and superoxide dismutase (SOD). Besides, the contents of malondialdehyde (MDA, LPO marker), an oxidative stress marker, were measured in the stomach homogenates. According to the manufacturer's instructions, these oxidative stress / antioxidant markers were determined using the Biodiagnostic Egypt colorimetric kits.

Assessment of stomach homogenates nitric oxide (NO)

According to the manufacturer's instructions, homogenized stomachs were used to measure NO, a protective factor for stomach mucosa, using the Biodiagnostic Egypt colorimetric kits.

Assessment of stomach homogenates TNFα

According to the manufacturer's instructions, homogenized stomachs were used to measure the inflammatory cytokines, TNFα, IL2, and IL6 using the ELISA kits of Bioassay Technology Laboratory (Shanghai, China).

Assessment of gastric mucosa TNFα (immunohistochemical stain)

The immunoperoxidase (PAP, peroxidase/antiperoxidase) was adopted using Lab Vision (Fremont, CA) TNFα antibody. The slides were then examined and photographed under a light microscope.

Statistical analysis

The obtained results were analyzed using Prism® (version 8.4.0, GraphPad Software Inc., La Jolla, CA, USA). The results were exhibited as mean ± standard derivation (SD). Comparison between groups was determined by conducting ANOVA analysis followed by Tukey's multiple comparisons test. Results were considered significant if p values were < 0.05.

3. RESULTS

Stomach histopathological lesions (H&E stain)

The C group stomachs showed healthy intact surface mucosa lined by mucous cells and covered with protective mucous film; the gastric gland showed normal glandular epithelium (Figure 1a, b). The GU group stomachs showed marked damage of surface

epithelium, loss of mucous film, degeneration and hemorrhage of deep glandular tissue, and edematous submucosa with congested vessels (Figure 1c, d). OM pretreated group showed marked preservation of surface mucous protective epithelium and glandular elements; slight edema and prominent blood vessels were still observed in the submucosa (Figure 1e, f). MH pretreated group showed intact non-ulcerated surface mucous cells; the glands are lined by small size HCl-secreting oxyntic cells beside peptic cells (Figure 2a, b). PP pretreated group showed potential protection against EtOH-induced GU; few regions showed surface desquamated cells, congested capillaries were observed among gastric glands (Figure 2c, d). PP+MH pretreated group showed marked preservation of surface mucous protective epithelium and glandular elements (Figure 2e, f).

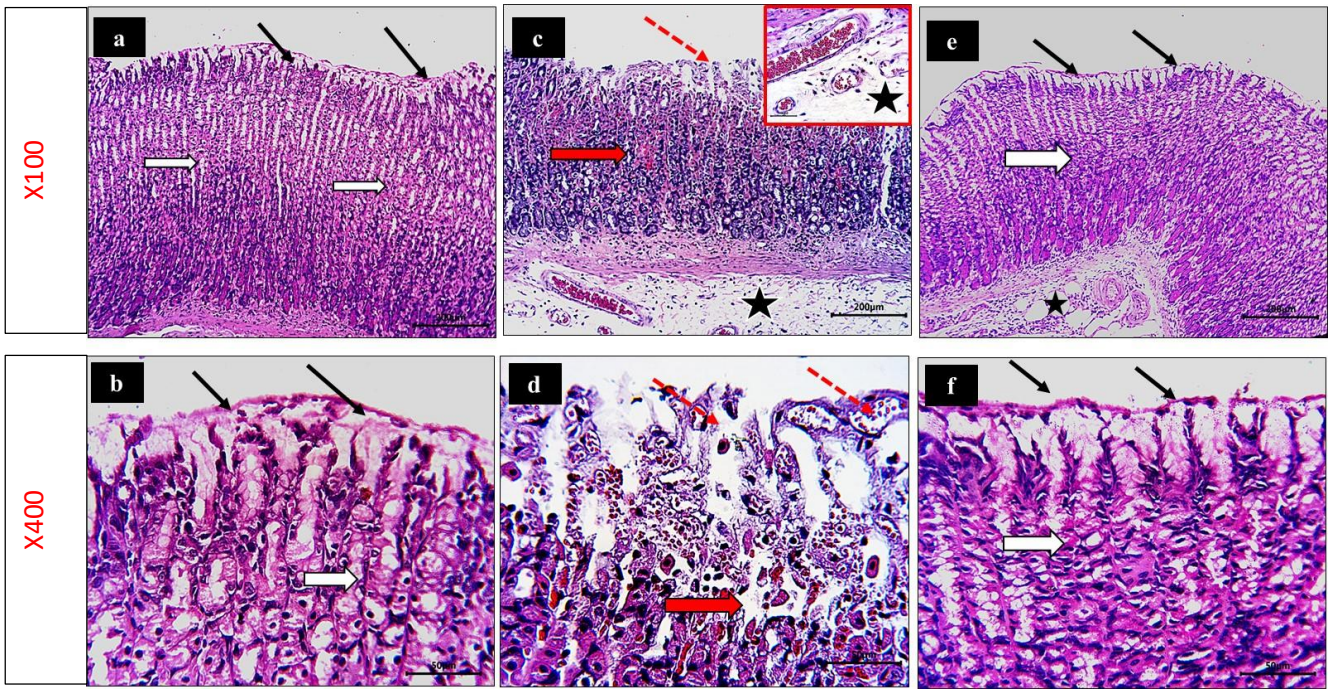

Figure 1 Effect of GU and OM on stomach histopathology stained by H&E and photographed at x100 bar mμ&400 bar=50 mμ. Photo a & b represent normal C group stomach with healthy intact surface mucosa lined by mucous cells and covered with protective mucous film (black arrows); gastric glands show normal glandular epithelium (white arrows). Photo c & d represent normal GU group stomach that show marked damage of surface epithelium, loss of mucous film (dotted red arrows), degeneration and hemorrhage of deep glandular tissue (thick red arrows); bedsides the submucosa is edematous and show congested vessels (star, insert). Photo e & f represent normal OM pretreated group stomach that show marked preservation of surface mucous protective epithelium (thin black arrows) and glandular elements (white arrows); besides submucosa slight edema and prominent blood vessels congestion (star).

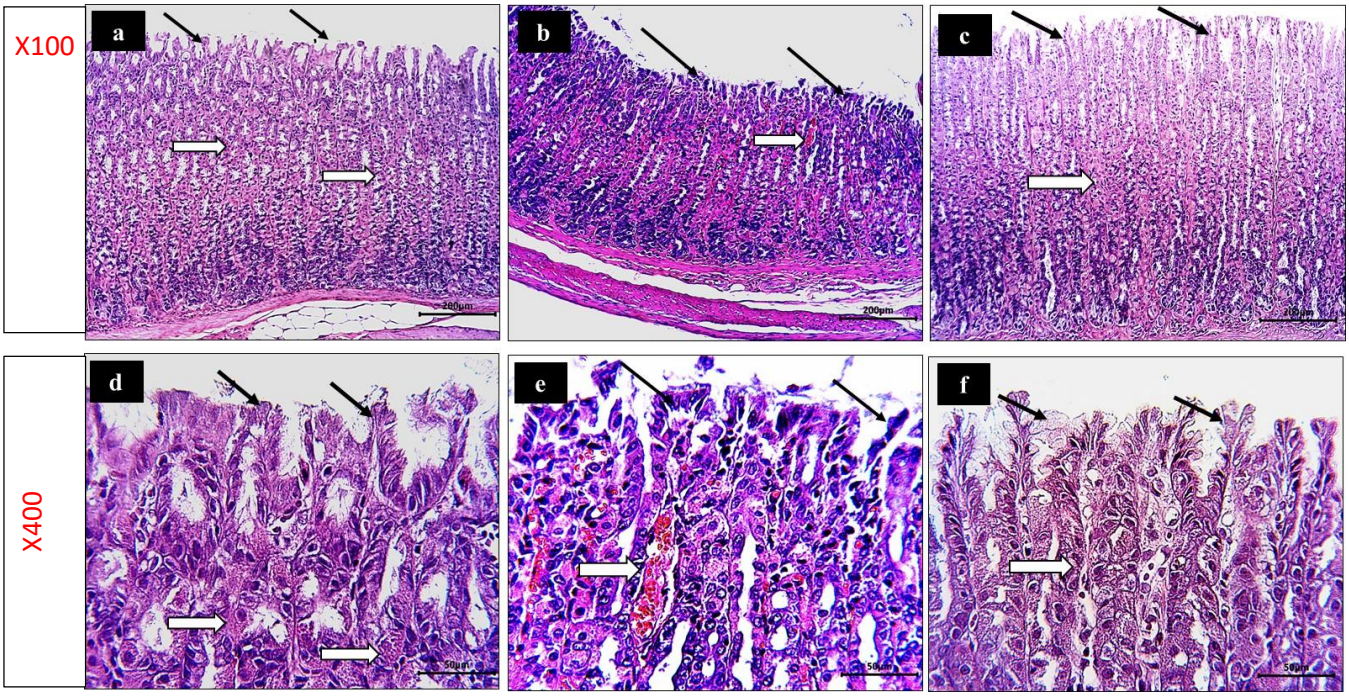

Figure 2 Effect of MH, PP, and their combination on stomach histopathology stained by H&E and photographed at x100 bar= 200mμ and x400 bar =50 mμ. Photo a & b represent MH group stomach show intact non- ulcerated surface mucous cells (black arrows) and the glands are lined by small size HCl-secreting oxyntic cells beside peptic cells (white arrows). Photo c & d represent PP group stomach that show potential protection against GU confirmed by the observed few regions that show surface desquamated cells (black arrows) and congested capillaries among gastric glands (white arrows). Photo e & f represent PP+MH pretreated group stomach that show marked preservation of surface mucous protective epithelium (thin black arrows) and glandular elements (white arrows).

Stomach glycoprotein contents (PAS stain)

The C group stomachs showed healthy intact surface and glandular epithelium highly stained by PAS (Figure 3a). The GU group stomachs showed degenerated surface and glandular epithelium with marked depletion of PAS positive protective mucous layer (Figure 3b). OM pretreated group showed potential preservation of PAS positive mucous material in the surface and glandular epithelium (Figure 3c). MH pretreated group showed marked preservation of PAS positive mucous material in the surface and glandular epithelium (Figure 3d). PP pretreated group showed preserved PAS positive material in the surface and glandular epithelium (Figure 3e). PP+MH pretreated group showed superior preservation of PAS positive surface mucous protective layer in the surface and glandular epithelium (Figure 3f).

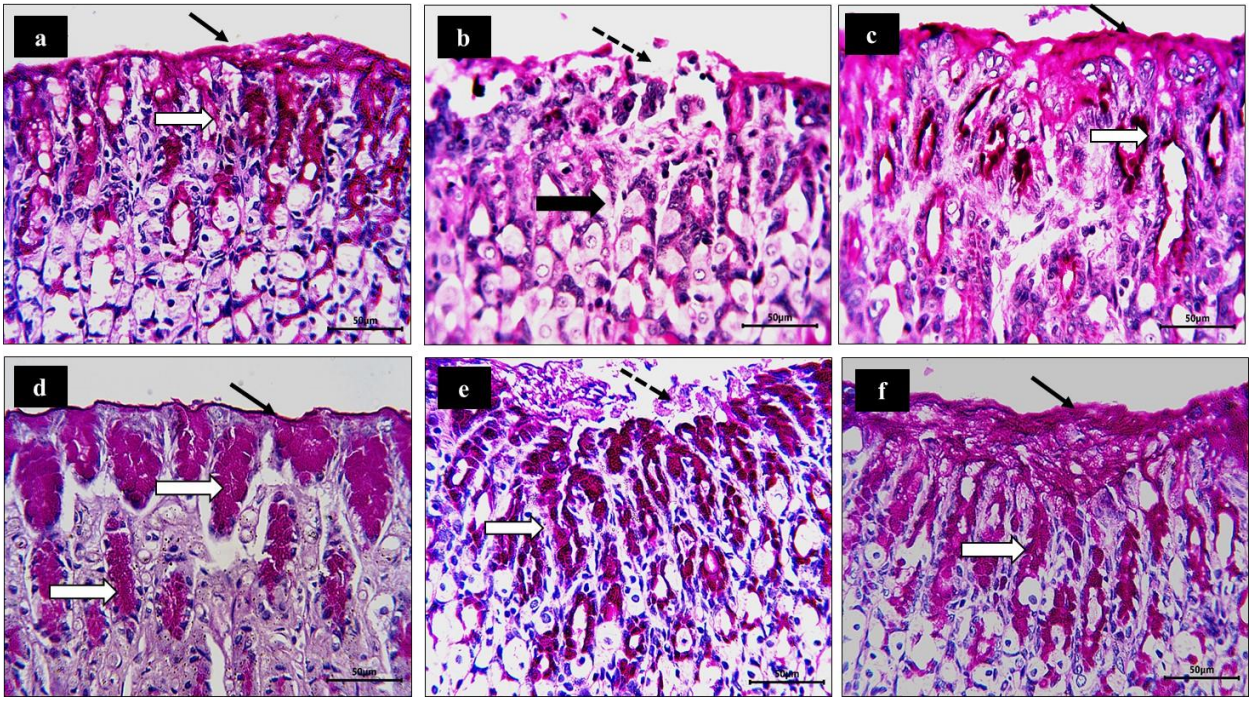

Figure 3 Effect of GU, OM, MH, PP, and their combination on stomach histopathology stained by H&E and photographed at x50 mμ. Photo a represents normal C group stomach with healthy intact surface (black arrow) and glandular (white arrow) epithelium; besides their highly stained by PAS. Photo b represents GU group stomach shows degenerated surface (dotted arrow) and glandular epithelium (black arrow) with marked depletion of PAS positive protective mucous layer. Photo c represents OM pretreated group stomach shows potential preservation of PAS positive mucous material at the surface (black arrows) and glandular (white arrow) epithelium. Photo d represents MH group stomach show marked preservation of PAS positive mucous material at the surface (black arrows) and glandular (white arrow) epithelium. Photo e represents PP group stomach that show preserved PAS positive material at the surface (black arrow) and glandular (white arrow) epithelium. Photo f represents PP+MH pretreated group stomach that show superior preservation of PAS positive material at the surface (black arrow) and glandular (white arrow) epithelium.

Stomach homogenates oxidative stress / antioxidant markers

The oxidative stress marker, MDA, was significantly increased in the stomach of the GU group compared to the C group ($p<0.001$). There were significant decreases in stomach MDA content in OM, MH, and PP+MH groups compared to the GU group ($p<0.01$, $p<0.01$, and $p<0.001$, respectively). There was also a significant decrease in stomach MDA content in PP+MH group compared to the PP group ($p<0.01$) (Figure 4a). The antioxidant markers, CAT and SOD, were significantly decreased in the stomach of the GU group compared to the C group ($p<0.001$ for both). There were significant increases in stomach CAT and SOD concentrations in OM, MH, and PP+MH groups compared to the GU group ($p<0.05$, $p<0.05$, and $p<0.001$, respectively for CAT and $p<0.05$, $p<0.01$, and $p<0.001$, respectively for SOD). There were also significant increases in stomach CAT and SOD concentrations in PP+MH group compared to the PP group ($p<0.01$ for both) (Figure 4b and c).

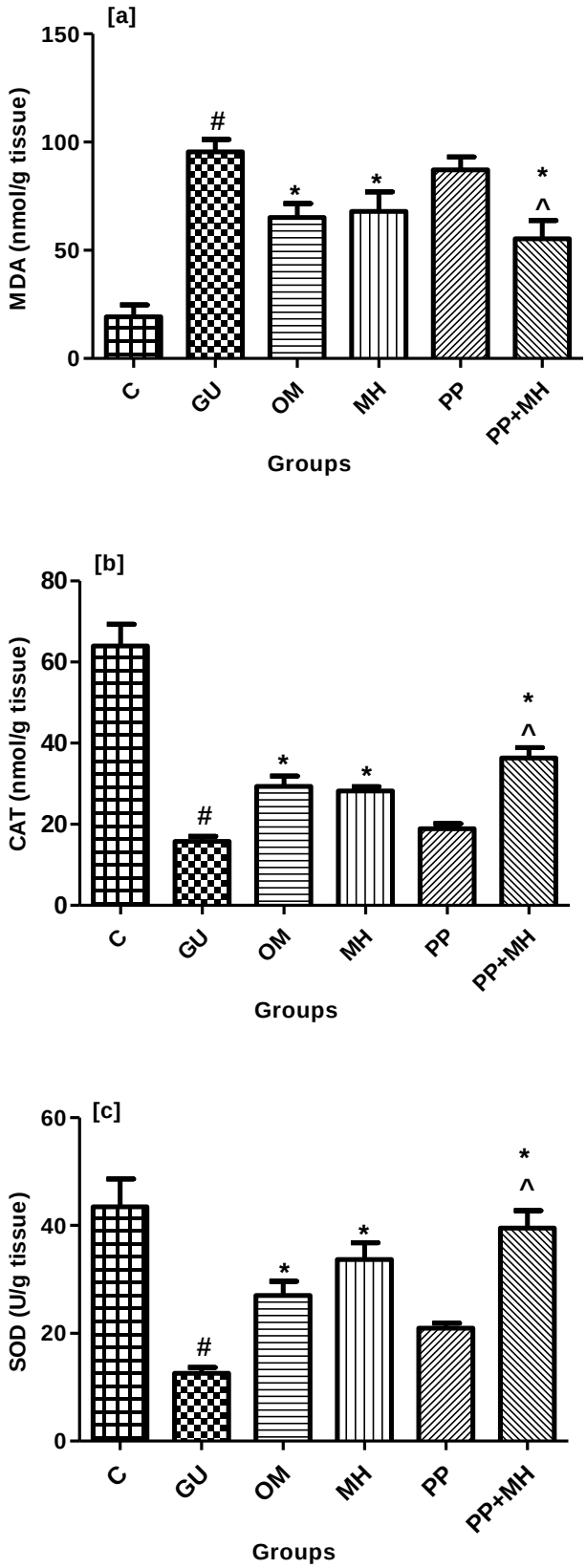

Figure 4 Effect of MH, PP, and their combination on stomach homogenates oxidative stress (MDA) / antioxidants (CAT and SOD) markers. Results are presented as mean ± SD (n=4). #Significant compared to C; *Significant compared to GU; ^Significant compared to PP. Significance level $p<0.05$.

Stomach homogenates NO contents

The gastric protective factor, NO, was significantly decreased in the stomach of the GU group compared to the C group ($p<0.001$). There were significant increases in stomach NO content in OM, MH, and PP+MH groups compared to the GU group ($p<0.05$, $p<0.05$, and $p<0.001$, respectively). There was also a significant increase in stomach NO content in PP+MH group compared to the PP group ($p<0.01$) (Figure 5).

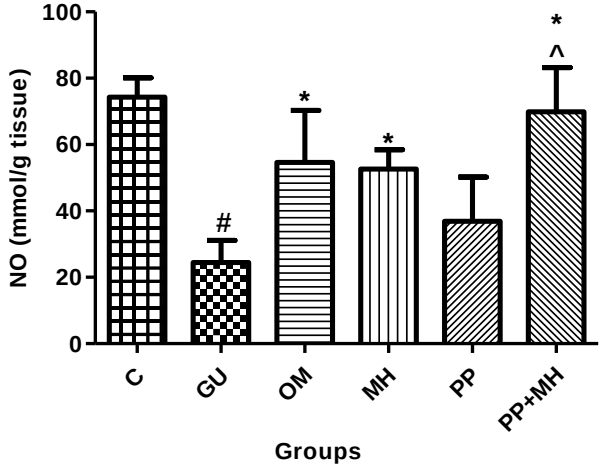

Figure 5 Effect of MH, PP, and their combination on stomach homogenates NO content. Results are presented as mean ± SD (n=4). #Significant compared to C; *Significant compared to GU; ^Significant compared to PP. Significance level $p<0.05$.

Stomach homogenates inflammatory cytokines contents

The inflammatory cytokines, TNFα, IL2, and IL6, were significantly increased in the stomach of the GU group compared to the C group ($p<0.001$ for all). There were significant decreases in stomach TNFα, IL2, and IL6 contents in OM, MH, and PP+MH groups compared to the GU group ($p<0.001$, $p<0.01$, and $p<0.001$, respectively for all). There were also significant decreases in stomach TNFα, IL2, and IL6 contents in PP+MH group compared to the PP group ($p<0.01$, $p<0.001$, and $p<0.01$, respectively) (Table 1).

Table 1 Effect of MH, PP, and their combination on stomach homogenates inflammatory cytokines content.

Groups	TNFα (pg/g tissue)	IL2 (pg/g tissue)	IL6 (pg/g tissue)
C	18.58±3.35	0.74±0.20	6.37±0.59
GU	81.39±13.92[a]	5.67±1.35[a]	29.15±9.17[a]
OM	30.69±10.63[b]	1.79±0.58[b]	11.82±1.98[b]
MH	35.58±4.20[b]	3.36±0.66[b]	19.32±3.17[b]
PP	62.32±25.15	4.43±0.78	24.96±5.74
PP+MH	24.31±5.20[a,c]	1.28±0.70[a]	10.13±0.89[a]

Results are presented as mean ± SD (n=4). [a]Significant compared to C; [b]Significant compared to GU; [c]Significant compared to PP. Significance level $p<0.05$.

Stomach TNFα protein expression

The inflammatory cytokines, TNFα protein expression was markedly increased in the stomach of the GU group compared to the C group Decreased TNFα protein expression in all treated groups (OM, MH, PP, and PP+MH) with superior response in MH group and PP+MH (Figure 6).

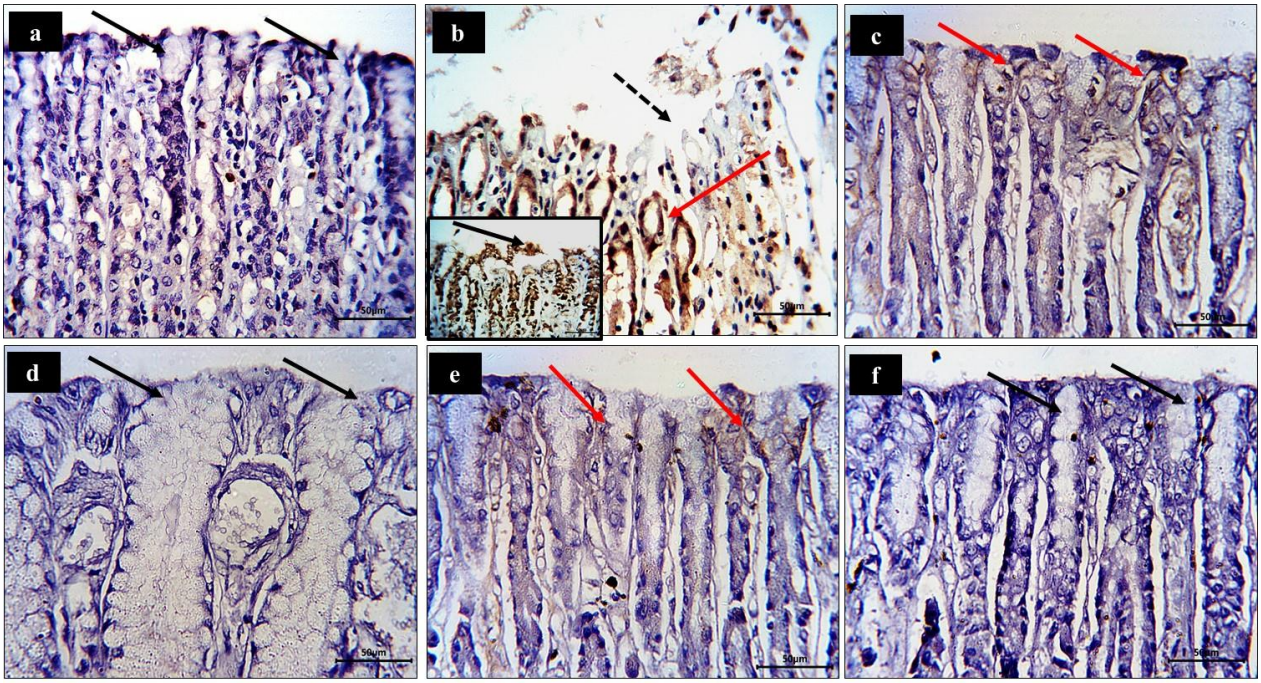

Figure 6 Effect of GU, OM, MH, PP, and their combination on stomach TNFα immunohistochemical stain (photographed at x50 mμ). Photo a represents normal C group stomach with no TNFα immuno-expression at the surface epithelium (black arrow). Photo b represents GU group stomach shows marked immuno-expression at the surface (red arrows) and glandular epithelium (dotted arrow). Photos c-f represents OM, MH, PP, and PP+MH pretreated groups stomachs show decreased immuno-expression (black and red arrows) in all treated groups with superior response in MH (d) and PP+MH(f) groups.

4. DISCUSSION

In the current study, the gastric protective effects of MH and PP were investigated against EtOH-induced GU in rats, either separately or in combination. EtOH directly damages the gastric mucosa, produces OFR, erodes gastric mucus, causes bleeding, and depletes bicarbonate (Fu et al., 2022). Histological evaluations showed that EtOH caused marked damage of surface mucous epithelium with degenerative changes and hemorrhage of glandular elements. These findings are consistent with previously published studies (Wang et al., 2018; Rahman et al., 2020; Fu et al., 2022).

As indicated by pathological assessment of the stomach, pretreatment of rats with OM, MH, and PP+MH reduced gastric mucosal damage compared to EtOH-induced GU. The combination (PP+MH) provided the best protection. On the other hand, the stomachs of PP (aqueous suspension, 250 mg/kg) pretreated rats still had histopathological features of sporadic regions of superficial ulceration. Similar to our results, pretreatment of EtOH-induced GU rats with MH protected the gastrointestinal mucosa (Almasaudi et al., 2016). Moreover, in line with our findings, administration of black PP methanolic extract (50 mg/kg) appeared to reduce but not prevent inflammatory cell infiltration and bleeding in gastric mucosa after EtOH-GU induction. The same authors also reported that sour summer PP (50 mg/kg) and north white PP (50 mg/kg) were close to the EtOH-inducible state. In both groups, the submucosa was edematous and inflammatory leukocyte infiltration was visible (Moghaddam et al., 2013).

EtOH is commonly utilized in GU induction. Several studies have shown that EtOH can harm blood vasculature by lowering mucus formation and rising the generation of OFR. Furthermore, EtOH-induced oxidative stress is linked to increased OFR synthesis and decreased antioxidant enzyme activity (Liu et al., 2019; Bin Jardan et al., 2020). MDA is a reliable biomarker of LPO, produced by the oxidation of unsaturated fatty acids in cell membranes (Chen et al., 2019). Antioxidant inadequacy is another trigger of EtOH-induced GU. Antioxidant defenses against OFR are provided by SOD and CAT. According to our findings, GU-induced by EtOH reduced gastric mucus content, increased gastric MDA content, and lowered gastric CAT and SOD activities which is consistent with previous literature (Fu et al., 2022). OM, MH, PP, and PP+MH preserve the gastric mucus content. Similarly, PP powder restored the gastric mucus to nearly normal level and consequently protected gastric tissue (Mohamed and Mabrok 2022). Tannin, a therapeutic ingredient in PP, stimulates the deposition of microproteins at the mucosal,surfaces creates a

shield against irritants over the gastric mucosa, restricts acid secretion in the injured area, and promotes GU healing (Chang et al., 2005).

In the present study, OM, MH, and PP+MH all reduced stomach MDA levels while increasing CAT and SOD activity, with PP+MH having the best results. Similar results have been reported with MH regarding MDA and SOD activities (Almasaudi et al., 2016). The phenolic and flavonoid chemicals (pinobanksin, pinocembrin, and chrysin) found in MH have exhibited significant OFR scavenging activity (Jubri et al., 2013). PP alone had no or weak effect on gastric MDA, CAT, and SOD levels. The elevated MDA level associated with indomethacin-induced GU was also unaffected by the PP methanolic extract (Abd el-Rady et al., 2021). Despite PP's high ellagic acid content (Patel et al., 2019), it could be suggested that according to their slight water solubility, the water suspension of PP used in our study is not suitable for its absorption and action.

Inflammation is another major factor associated with increased OFR generation associated with EtOH-induced gastric injury. Previous research has shown that inflammatory cytokines play a role in the development of GU (Katary and Salahuddin 2017; Gomaa et al., 2018). TNFα is one of the most potent agents of inflammation and tissue damage, including the formation of GU. Previous research has revealed that TNFα elevation is linked to EtOH-induced GU and apoptosis (Badr et al., 2019). NO modulates gastrointestinal mucosal defenses, gastric blood flow, and epithelial barriers (Serafim et al., 2020). Our data presented that GU-induced by EtOH reduced gastric NO level, increased gastric TNFα, IL2, and IL-6 content, and TNFα gene expression which are consistent with previous literature (Almasaudi et al., 2016; Fu et al., 2022). OM, MH, and PP+MH increased gastric NO generation while decreasing TNFα, IL2, and IL-6 levels; in addition, gastric mucosal TNFα immune-expression in gastric cells was dramatically reduced which is similar with many researches (Almasaudi et al., 2016; Mohamed and Mabrok, 2022). PP alone had no or weak effect on gastric TNFα, IL2, and IL-6 levels. The elevated IL-2 level associated with indomethacin-induced GU was also unaffected by the PP methanolic extract (Abd el-Rady et al., 2021).

5. CONCLUSION

In comparison to OM and MH, the current study found that the aqueous suspension of PP alone could not protect stomach tissues against EtOH-induced GU. The water suspension of PP showed no antioxidant or anti-inflammatory effects. It also did not affect NO levels. PP's only was found to augment the protective mucous layer of stomach tissue against any detergents effects. The combination of PP+MH had a protective effect on EtOH-induced GU, as it boosted the potential protection of MH to act as an anti-inflammatory and antioxidant supplement.

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Ethical approval

This design was certified by College of Medicine Ethical Committee, KAU, Jeddah, SA (Reference No 502-19).

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Conflicts of interest

The authors declare that there are no conflicts of interests.

Data and materials availability

All data associated with this study are present in the paper.

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