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## Exploring the potential protective and anti-inflammatory effects of the crude ethanolic extract of *Annona squamosa* Linn fruit against Ethanol-mediated gastric erosion

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### ABSTRACT

**Background:** *Annona squamosa* L. is a medically important fruit that belongs to the family Annonaceae and has many medical benefits, including antiulcer effects.

**Aim:** The present study explored the potential protection and anti-inflammatory effects of the crude ethanolic extract of *Annona squamosa* L. (EEA) against ethanol-mediated peptic severe erosion using a rodent model in comparison with a synthetic compound broadly used in clinics to tackle peptic disturbances, known as Omeprazole.

**Methods:** To do that, twenty-four both sex Wistar rats were assigned into four groups (six rats per group), namely control negative (C-) and control positive (C+) both received normal saline, Omeprazole (OM) administered OM at a dose of 20 mg/kg of body, and crude EEA received the extract at a dose of 200 mg/kg for two weeks. All groups except the C- group received absolute ethanol at a dose of 5 ml/kg of body weight for 1 hour. After euthanization, the gross ulcer index and protection ratio results exhibited a good percentage of protection in the EEA group compared with the C+ and OM groups.

**Results:** These results were correlated with histopathological examination, which indicated that the gastric lesion of the EEA showed erosion in the pits area and isthmus, including destruction of parietal cells, whereas the gastric lesion of the C+ group showed severe necrosis of epithelial cells of the gastric mucosa, resulting in the absence of all mucosal layers of the affected area. In addition, the results revealed the anti-inflammatory properties of EEA through its ability to control the regulation of TNF- $\alpha$  and IL-1 $\alpha$ . Both proteins were downregulated in response to the presence of EEA.

**Conclusion:** To our knowledge, this is the first study to detect high IL-1 $\alpha$  in ethanol-induced severe gastric erosion. These results suggested that EEA exhibited protective and anti-inflammatory effects against ethanol-mediated gastric erosion.

**Keywords:** *Annona squamosa* L. fruit, antiulcer, IL-1  $\alpha$ , Omeprazole, ulcer index.

### Introduction

A gastric ulcer is a disturbance impairing the mucosal layer integrity of the stomach. This condition occurs due to a hierarchical inflammatory process activity leading to local injury and excavation through gastric acid and pepsin secretion (Shivhare and Vinode, 2023). Gastric ulcers and hyperacidity are two common causes of discomfort in people. According to a new statistical study, this disease affects 1 out of 10 patients throughout their lifetime (Xu *et al.*, 2021). High mortality and morbidity rates are associated with

gastric ulcers as a result of hemorrhage, perforation, and obstruction (Bertleff and Lange, 2010). Globally, one of the most common health issues is the gastric ulcer, especially with increasing risk factors, such as smoking, nutritional deficiency, stress, and alcohol drinking (Ravisankar *et al.*, 2016). At the molecular level, gastric ulcers are characterized by increased proinflammatory cytokines expression and oxidative stress (Mei *et al.*, 2012; Paikray *et al.*, 2024). Different cytokines are involved in the inflammatory response

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as a result of gastric ulcer, including TNF- $\alpha$  and IL-6 (Aziz *et al.*, 2018).

Through the years, efforts have continued to develop new medications for treating gastric ulcers successfully. One of these drugs, a proton pump inhibitor (PPI), known as Omeprazole (OM), is a medication considered a first-line treatment for acute gastric ulcers by reducing the secretion of gastric acid (Malfertheiner *et al.*, 2007; Sharma, 2023). However, therapy that is already used in the clinic may fail due to a direct or indirect cause, such as arrhythmia, impotence, hypersensitivity, gynecomastia, and hematopoietic changes (Kuna, 2019). Therefore, the global trend toward the utilization of alternative medicine, or what is known as plant-derived medicine, is due to an increase in up-to-date knowledge regarding medical properties, including the safety, efficacy, and quality of medicinal products of such types of medication (Falcão *et al.*, 2008; Naji *et al.*, 2018).

One of these medically important plants, *Annona squamosa* L., is a tropical, domestic species of India, Bermuda, the Bahamas, Mexico, Brazil, Peru, Ecuador, the West Indies, and Central and South America (El-Chaghaby *et al.*, 2014; Salmerón-Manzano *et al.*, 2020; Al Kazman *et al.*, 2020; Safira *et al.*, 2022). *A. squamosa* L. belongs to the Annonaceae family, and the tree grows as a small shrub, with large randomly organized branches having a light brownish to brownish coat with thin leaves (Kalidindi *et al.*, 2015). The *A. squamosa* L. is an edible fruit that contains vitamins (such as vitamin B1 (thiamine) and vitamin C), minerals, and dietary fiber. It has also been used in different food applications (Zahid *et al.*, 2018).

Many studies have revealed that *A. squamosa* L. has medical properties. Extracts yielded from different parts of the *A. squamosa* L. plant are used to treat a variety of diseases, including tumors, fever, dysentery, hemorrhage, and epilepsy (Anaya-Esparza *et al.*, 2020). Therefore, the present study aimed to evaluate the protective and anti-inflammatory effects of the crude ethanolic extract of *A. squamosa* L. on ethanol-induced gastric erosion using a rodent model.

## Methods

### Animals

Twenty-four healthy, both sexes, and adult Wistar rats with a weight range of 150-200 g were obtained from the Faculty of Science/University of Kufa. Rats were placed in plastic cages (two rats per cage) with wood chips for bedding and housed at an animal house, Faculty of Science/University of Kufa, under controlled conditions. Rats were provided with free access to standard rat pellets and drinking water. Rats were grouped randomly (using the matched pairs method, according to the sex) and adapted for a one-week experimental period.

### Experimental design

The animals were divided into four groups: each group consisted of six rats, namely control negative (C-), control positive (C+), OM (Germany), and crude ethanolic extract of *Annona squamosa* L. (EEA). Each group was subjected to receiving the compounds by gavage daily for 2 weeks at a specific dose as follows: the C- group received normal saline, while the C+ group received normal saline before receiving absolute ethanol (Spain) at a dose of 5 ml/kg of body weight (Park *et al.*, 2021). The OM group received OM at a dose of 20 mg/kg (Nworgu *et al.*, 2019), and the EEA group received EEA at a dose of 200 mg/kg (Hamid *et al.*, 2012) before receiving absolute ethanol at a dose of 5 ml/kg of body weight. All rats were euthanized 1 hour after receiving absolute ethanol via exsanguination under general anesthesia using ketamine and xylazine.

### Collection and preparation of crude ethanolic extract of *A. squamosa* L. fruit

A fresh of *Annona squamosa* L. fruit was purchased from the local market in Al-Najaf. The plant was classified by a professional plant taxonomist in the Faculty of Science/University of Kufa according to the formal document No. 1,315 on April 2, 2023. For the extract preparation, the fruit *Annona squamosa* L. was washed in water, cut into small pieces, dried in shade, and then converted to fine powder using a grinder; 50 mg of the produced fruit powder were mixed with 500 ml of 70% ethanol for 24 hours on a magnetic stirrer at 45°C. The extract was filtered using Whatman No.1 filter paper twice, which was then concentrated under reduced pressure using a rotary evaporator at 40°C and 90 rpm (Harborne, 1998). The final concentrated extract was refrigerated in sterile sample bottles for further investigation.

### Induction of gastric ulcer

The ulcer was induced after 24 hours of fasting by administration of 99.9% absolute ethanol at a dose of 5 ml/kg of body weight by gavage. One hour later, the animals were anaesthetized with ketamine and xylazine. The stomach samples were dissected and opened along the greater curvature to determine the number and length of gastric lesions (Morsy *et al.*, 2012).

### Evaluation of gross gastric lesions

Gastric ulcers were measured following the induction of each experimental group. First, the dissected stomach was placed on transparent glass or a board and then scanned using a camera. The ulcer scoring was made according to Takagi and Okabe (Takagi and Okabe, 1968): 0 no lesion, 1-2 mucosal petechial, 1-5 small lesions size 1-2 mm, and < 5 small lesions or one intermediate lesion 3-4 mm, 2 or more intermediate lesions or one gross lesion > 4 mm and perforated ulcers. The equations used to calculate the ulcer index and percentage of protective ratio are as follows:

$$\text{Ulcer Index (UI)} = \frac{\text{Total ulcer index}}{\text{number of animal ulcerated}}$$

$$\% \text{ Protective ratio} = \frac{\text{UI of Ulcerogen treated group}}{\text{UI of Ulcerogen treated}} - \frac{\text{UI of treatment group}}{\text{UI of Ulcerogen treated}}$$

#### Histopathological preparing study

Stomach samples were collected, washed once with PBS, and fixed within 10% formalin (Chemanol, KSA) for (48 hours) with changing the formalin of the collected samples 24 hours later. The stomach samples were then sliced into pieces measuring 0.5 cm in thickness each and excised from the affected tissue. After fixation steps, several steps were performed for preparing samples, namely the dehydration process (done by serially increasing concentrations of ethanol (Retouch, China), paraffin embedding (Chemact Petrochemicals, China) blocks step, sectioning and mounting on the slide, and staining step using hematoxylin and eosin (Leica Biosystems, USA). Finally, histopathological changes and imaging were detected using a light microscope at (10X and 40X) magnifications (Luna, 1968).

#### Immunohistochemistry study

Stomach samples in paraffin-embedded form were sliced into 4 µm thick sections, carefully placed in a water bath (FALC BI, Italy), and then mounted on positively charged glass slides (CrystalCruz® Electro-Statically Charged Micro Slides, sc-363562, SANTA CRUZ BIOTECHNOLOGY, USA). The stomach tissue section was deparaffinized (using a hot air oven at 55°C for 1 hour) and rehydrated (using xylene and gradually decreasing concentrations of ethanol for 3 minutes/concentration). Immunohistochemistry was performed according to the manufacturer's protocol using a Dako EnVision detection immunohistochemistry kit (Envision FLEX, Dako, K8000, Denmark). In the following step, slides were incubated with the primary antibodies, namely anti-necrosis factor-α primary antibody (Polyclonal Rabbit Antibody: E-AB-40015, Elabscience, China) (TNF-α) and anti-interlukin-1α primary antibody (Polyclonal Rabbit Antibody: E-AB-40407, Elabscience, China) (IL-1 α). The sections were incubated with a secondary antibody labelled horseradish peroxidase (EnVision FLEX /HRP, SM802) and were then examined using DAB+ chromogen. All tissue sections were stained with Mayer haematoxylin (Bio-Optica, 05-06002/L, Italy), dehydrated, and cleared. Finally, all slides were mounted with a cover slide, the protein signal was detected using a light microscope, and the image was captured using 10X and 40X.

#### Statistical analysis

The one-way F test with multiple comparisons (ANOVA) and the Fisher test (the least significant difference)  $P \leq 0.01$  were achieved to compare the control positive group (C+), OM group, and the crude ethanolic extract of *A. squamosa* L. (EEA). The statistical analysis was carried out using GraphPad Prism 6 software for Windows (La Jolla, CA, USA).

#### Ethical approval

According to ethical instructions, this study was approved by the Ethical Committee of Animal Care and Use/University of Kufa, as documented through formal application No. 11203 on 7 May 2023.

### Results

#### EEA reduces stomach hemorrhage following absolute ethanol treatment

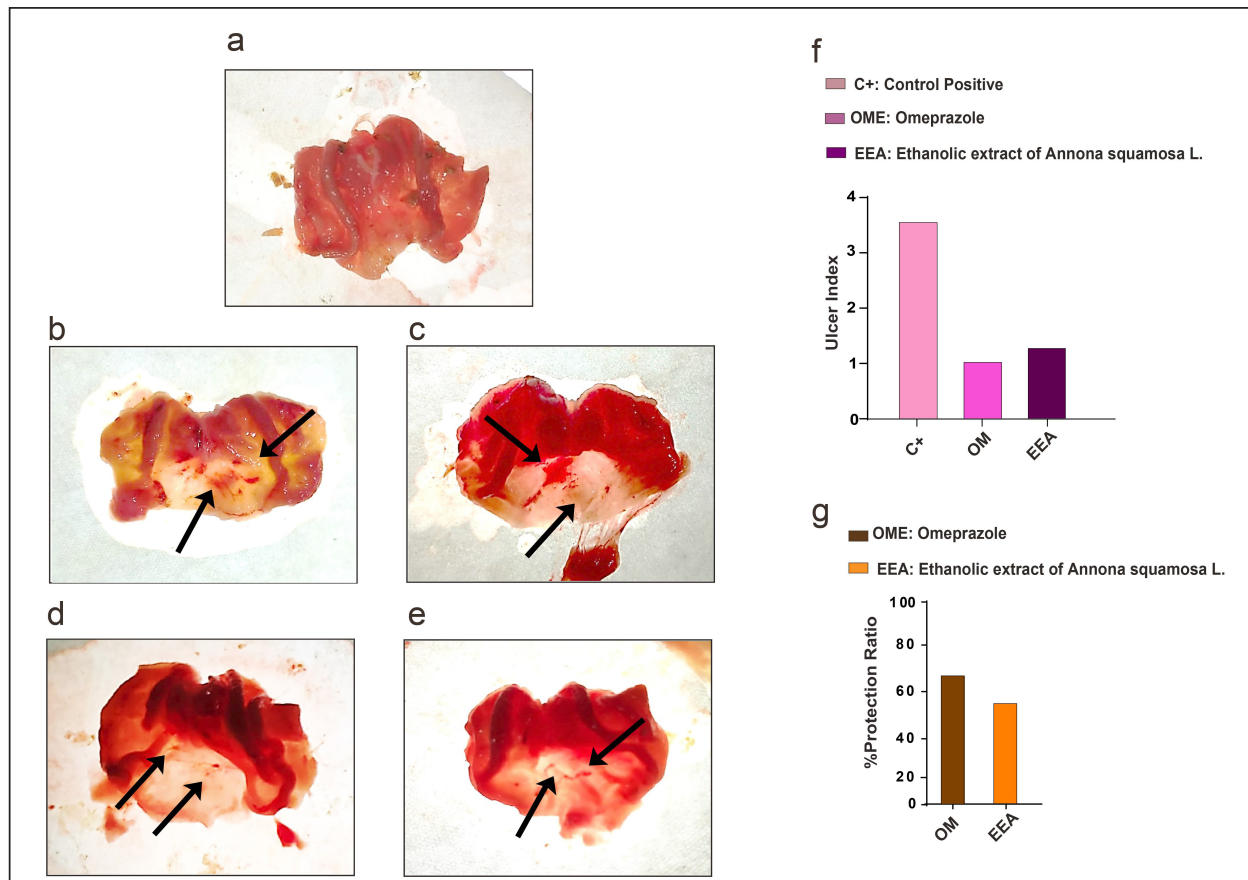
In order to investigate the protective effect of the ethanolic extract of *A. Squamosa* L. (EEA), a gross examination took place and the observation revealed hemorrhagic lesions in the stomachs of all rats that received absolute ethanol. However, the severity of hemorrhagic lesions was varied, where hemorrhagic patches with the presence of intermediate-sized lesions were observed in the C+ group (Fig. 1b and c). While the petechial hemorrhagic lesions observed in the EEA group (Fig. 1d) compared with OM group (Fig. 1e) showed convergent results. In correlation with the gross results, the ulcer index showed a lower number of stomach ulcers in the EEA group compared with the C+ group. The OM-treated group indicated similar results to the EEA group (Fig. 1f). Along with these results, the protection ratio revealed a good protective percentage in the EEA group compared with the OM group (Fig. 1g).

Further investigation, histopathological examination showed that in the C+ group, the lesion was characterized by severe erosion in gastric mucosa due to the necrosis of epithelial cells in pits, isthmus, and neck of mucosal layer accompanied with infiltration of neutrophils (Fig. 2 bi and bii) compared with the C- group did not exhibit any pathological changes (Fig.2a). While the OM group showed a small area of erosion and necrosis of epithelial cells observed in the surfaces of gastric mucosa involving the upper pits layer with the presence of inflammatory cell infiltration (Fig. 2ci and cii). In the case of EEA, necrotic areas of epithelial cells were observed in the pits of gastric mucosa that involved all pits with the presence of inflammatory cell infiltration in the affected area. These results indicated that EEA reduced the effect of ethanol-mediated stomach erosion.

#### Downregulation of TNF-α and IL-1 α correlates with anti-inflammatory effect of EEA

For further confirmation and to check the anti-inflammatory properties of EEA, the expression levels of TNF-α and IL-1α were examined. The results demonstrated that TNF-α was highly expressed in the





**Figure 1.** EEA showed high protection ability against ethanol-mediated ulcer formation. (a) C- group (b and c). C+ group: hemorrhagic patches and intermediate hemorrhagic lesions (black arrows) were observed in the body area of the affected stomach. (d) OM group: Two petechial hemorrhagic lesions (arrows) were observed in the body area of the affected stomach. (e) EEA group: three petechial hemorrhagic lesions were observed in the body area of the affected stomach. (f) Ulcer index. (g) Percentage of protection ratio, mean,  $n = 6$ .

C+ group (Figs. 3b, 4b) compared with the C- group (Figs. 3a and 4a). Interestingly, the expression level of IL- $\alpha$  was high as well. In contrast, a significant decrease in the expression levels of both TNF- $\alpha$  and IL-1 $\alpha$  was observed in the OM group (Figs. 3c, 4c) and EEA group (Figs. 3d, 4d) compared with the C+ group (Figs. 3e, 4e). Taken together, these results confirmed the anti-inflammatory properties of EEA following the induction of an ethanol-mediated stomach erosion.

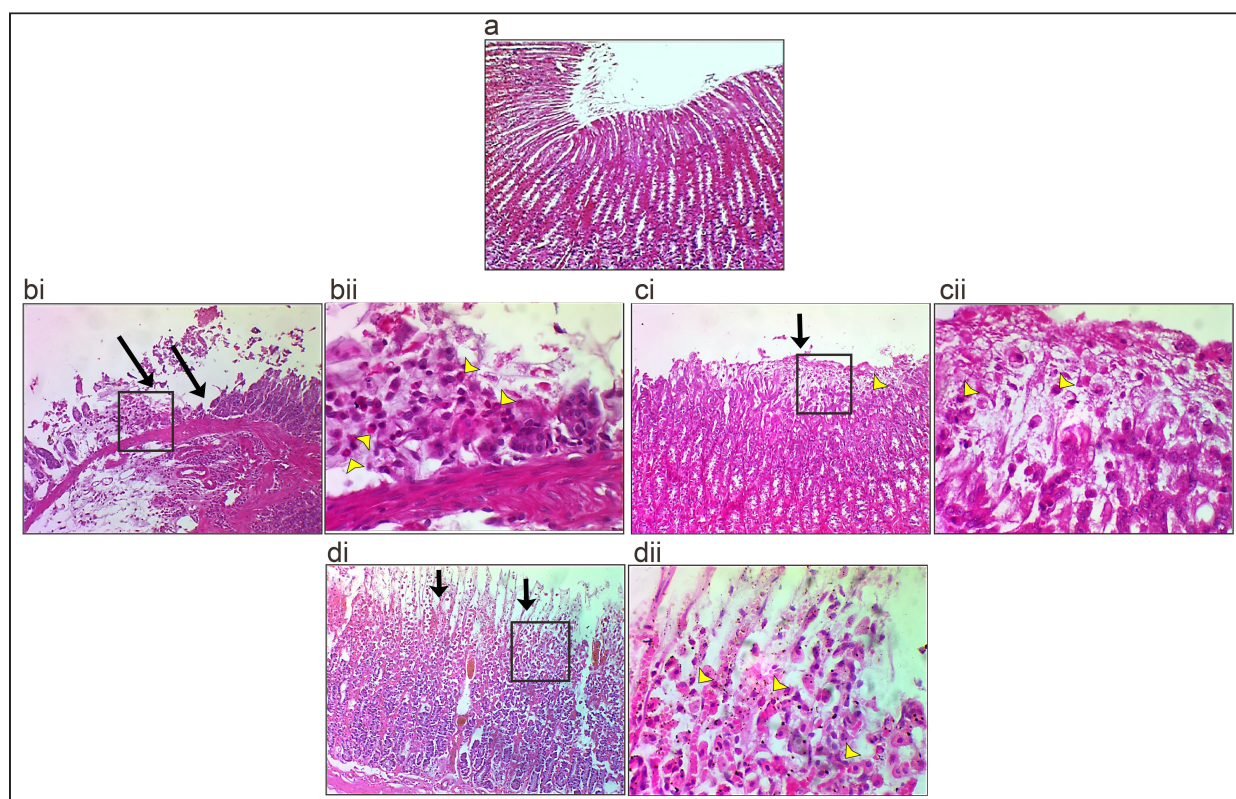
### Discussion

Gastric ulcers are well-described and commonly occur in clinics due to a variety of causes. It has been found that gastric ulcers result from an imbalance in the secretion of pepsin and gastric acid in addition to defensive factors of the gastric mucosal layer (Pélico *et al.*, 2020). The stomach defense lines can be debilitated and penetrated by chemical compounds, such as alcohol, though, creating lesions in the epithelial layer and increasing edema formation due to increasing the permeability of the vascular layers. Such lesions can be induced by

ethanol by different mechanisms, including a decrease in gastric PH, resulting in lowering gastric motility, and reducing mucus and bicarbonate secretions (Abd-Alla *et al.*, 2022).

Stereotypically, ethanol is the most convenient material for inducing gastric ulcers in rat models. It causes a progressive disturbance in the microcirculation of the mucosal layer as well as ischemia, resulting in free radical production. In addition to increased gastric acid secretion, hemorrhage and areas of ulcer (Abd-Alla *et al.*, 2022). In the current study and agreement with previous studies, using absolute ethanol only as a control positive showed the successful formation of gastric erosion, where gross observation showed the patches of hemorrhagic lesions in the affected stomach (Fig. 1b-e) and the histopathological observation showed the destruction of the gastric mucosa, which included all layers of gastric mucosa (Fig. 2bi and bii). Over the years, many chemical compounds have been discovered, developed, and used in clinics to tackle gastric ulcers. One of these well-known compounds,



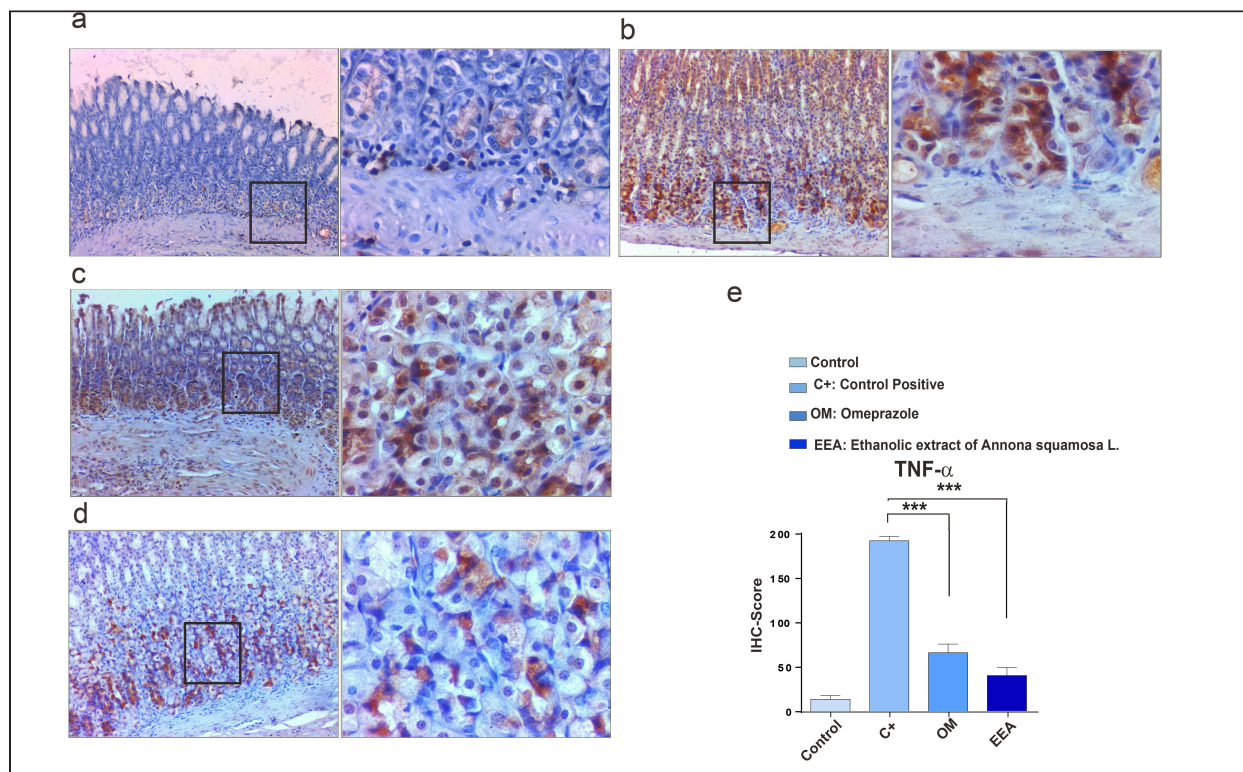


**Figure 2.** Histopathological section of stomach. (a) C- group showed normal gastric histological architectures. (bi and bii) C+ group showed severe necrosis (black arrows) of epithelial cells of gastric mucosa, where the necrosis of epithelial cells led to the absence of all mucosal layers of the affected area. In addition, infiltration of inflammatory cells (mostly neutrophil) (yellow arrowhead) was observed in the necrotic area. (ci and cii) OM group showed that the necrosis of epithelial cells (black arrows) was observed in surfaces of the gastric mucosa that involved the upper pits layer with the presence of inflammatory cell infiltration (yellow arrowhead) in the affected area. (di and dii) EEA group showed necrosis of epithelial cells (black arrows) was observed in the pits layer of the gastric mucosa that involved the all-pits layer with the presence of inflammatory cell infiltration (yellow arrowhead) in the affected area (H and E: 10X and 40X).

OM, is known as the principal and first-line medication used for peptic ulcer treatment through the reduction of gastric acid secretion (Malfertheiner *et al.*, 2007; Sharma, 2023). In spite of successful outcomes of medical compounds that used to cure peptic ulcer, unpredictable and unwanted side effects have been recorded (Lambert *et al.*, 2015; Kuna *et al.*, 2019; Paz *et al.*, 2020). Therefore, alternative or herbal medicine is becoming the most popular medicine for treating various diseases, including peptic ulcer (Naji *et al.*, 2018; Kuna *et al.*, 2019).

Numerous studies have illustrated that many plants belonging to different plant families have medical utilization. One of these Annonaceae is, a plant family that possesses a promising property regarding gastric ulcer treatment (Mahima *et al.*, 2020). The phytochemical screening of *A. squamosa* L. illustrated an excellent percentage of a wide variety of bioactive and antioxidant compounds, such as distinct metabolites, flavonoids, alkaloids, terpenoids, saponins, phytosterols, tannins, carbohydrates, phenolic

compounds, and fixed oil (Othman *et al.*, 2019; Avhad and Attarde, 2021). All these bioactive compounds have been used for their pharmacological properties to treat different medical conditions (Othman *et al.*, 2019). These medical conditions can be summarized as follows: anti-inflammatory, anti-diabetic, antipyretic, anti-diarrhea and antifertility (Mahima *et al.*, 2020). Other studies have demonstrated that extracts from *A. squamosa* L. twigs exhibit significant antiulcer activity. In the same context, it has been found that *A. squamosa* L. fruit manifested clear cryoprotection in the case of aspirin and alcohol models by controlling the mucin level (Yadav *et al.*, 2011). In the present study, EEA and OM showed a convergent protective effect against ethanol-induced gastric erosion compared with the C+ group (Fig. 1f and g). Similarly, the histopathological results of the EEA group (Fig. 2ci and cii) and the OM group (Fig. 2di and dii) exhibited necrosis only involving epithelial cells that lined the pits with infiltration of inflammatory cells (neutrophils). These results suggested that the protective features observed



**Figure 3.** Low expression of TNF- $\alpha$  in response to the EEA protection effect. (a) Control negative group, (b) C+ group, (c) OM group, (d) EEA group, and (e) Immunohistochemistry (IHC) score showed a significant decrease of TNF- $\alpha$  in OM and EEA groups compared with the C+ group. \*\*\* $p \leq 0.001$ , error bars = mean  $\pm$  standard error of mean ( $n = 3$ ).

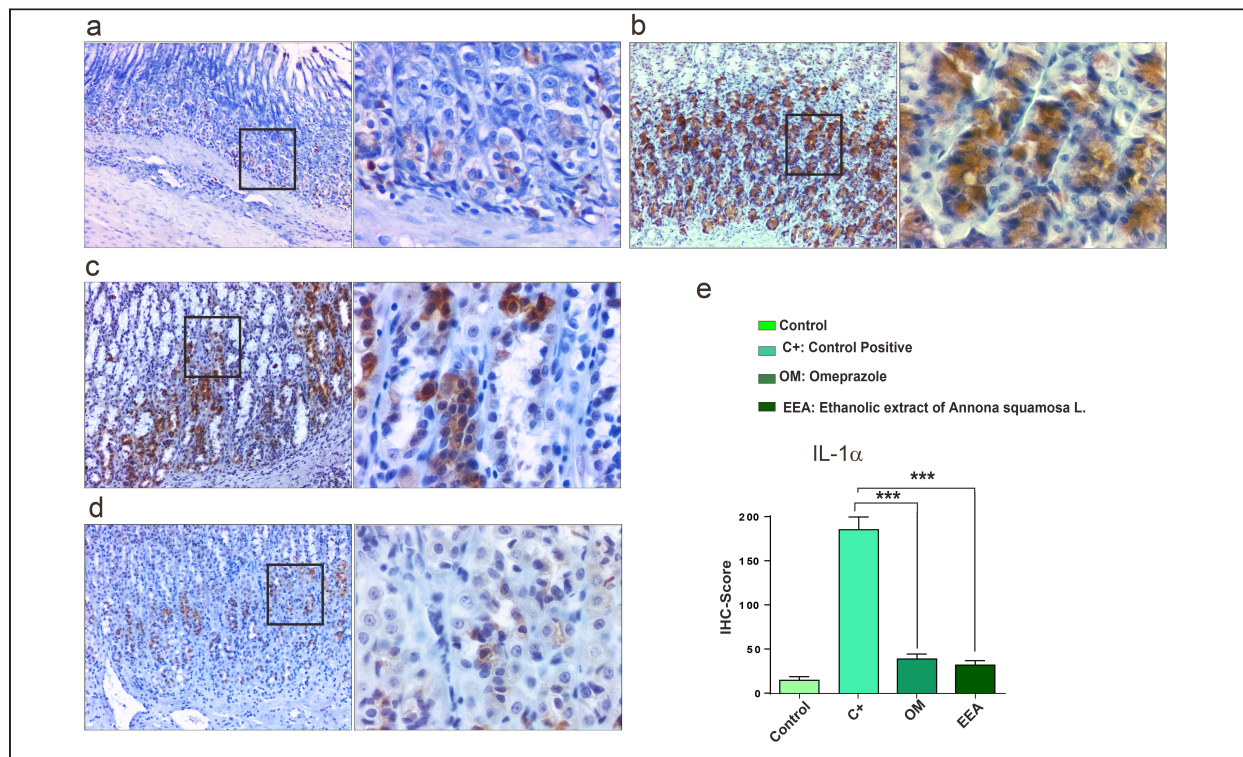
in EEA group were similar to those of the OM-treated group compared with the negative and positive control groups.

TNF- $\alpha$  is an important proinflammatory cytokine that plays a vital role in initiating and developing gastric ulcers. The development of this disorder occurs by controlling the primary acute inflammatory response. This is illustrated by increasing infiltration of neutrophils into the gastric mucosa (Fiorucci *et al.*, 1998). In addition, TNF- $\alpha$  is modulating cell death in the gastric mucosa. Therefore, downregulation of TNF- $\alpha$  might facilitate the gastric ulcer healing process. Consequently, in the present study, we examined the expression level of TNF- $\alpha$  and the results revealed a significant increase in the C+ group, while the EEA group showed a significant reduction in TNF- $\alpha$  expression level (Fig. 3). These results correlate with a previous study done by Aziz *et al.* (2018), which suggested the amelioration of gastric ulcers and reduction in TNF- $\alpha$  expression levels following treatment with oxyresveratrol.

IL-1 $\alpha$  is another crucial and ubiquitous proinflammatory cytokine. Constitutively, IL-1 $\alpha$  precursor is normally expressed in almost all types of healthy cells. However, it is released as a bioactive mediator due to the inflammatory process of necrosis (Cavalli *et al.*,

2021). It has been found that the expression levels of TNF- $\alpha$  and IL-1 $\beta$  were high in the case of ethanol-mediated gastric ulcer (Amirshahrokhi and Khalili, 2015). Surprisingly, our findings revealed a significant increase in the expression level of IL-1 $\alpha$  following ethanol induction, whereas the EEA treated group indicated a significant decrease in the expression level of IL-1 $\alpha$  (Fig. 4). These results suggested a brand-new vital role of involvement of the IL-1 $\alpha$  in the inflammatory response of gastric erosion induced by ethanol. IL-1 $\alpha$  and IL-1 $\beta$  have been encoded through distinct genes with very low homology. However, both of these cytokines are binding the same IL-1 family receptor known as IL-1R. Therefore, they induce similar pro-inflammatory effects (Rider *et al.*, 2013; Dincă *et al.*, 2022). Interestingly, the intervention of IL-1 $\alpha$  in the development of inflammatory diseases has relatively remained unnoticed, which may be because the records of different patients with inflammatory diseases illustrated that IL-1 $\alpha$  is hardly detected in the circulation (Cavalli *et al.*, 2021), which may explain the upregulation of IL-1 $\alpha$  in the tissue. The current study investigated the protective features of EEA against ethanol-mediated gastric erosion in different aspects. The EEA exhibited a very good degree of protection with anti-inflammatory properties. The take-





**Figure 4.** Low expression level of IL-1 $\alpha$  in response to the EEA protection effect. (a) Control negative group, (b) C+ group, (c) OM group, (d) EEA group, and (e) Immunohistochemistry (IHC) score showed a significant decrease of IL-1 $\alpha$  in OM and EEA groups compared with the C+ group. \*\*\* $p \leq 0.001$ , error bars = mean  $\pm$  standard error of mean ( $n = 3$ ).

home message is the administration of crude EEA *L.* reduced ethanol-induced acute erosion in Wistar rats with relatively high protection and anti-inflammatory effects. In the context the inflammatory response, and to our knowledge, this is the first record for up-regulation of IL-1 $\alpha$  in C+ and downregulation in protected groups with EEA.

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#### Conflicts of interest

The authors declare no conflicts of interest regarding this publication.

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#### Author's contribution

HJA. and MTM. designed the study and performed the experiment. AA analyzed data, wrote the manuscript, and prepared the figures. AAA. and WRH. processed tissues and prepared slides.

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