Humic Acid Has Protective Effect on Gastric Ulcer by Alleviating Inflammation in Rats

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Abstract—The new agents are needed in treatment of gastric ulcer that have less side effects, adequate efficacy, and no drug interactions. In this study, we aimed to investigate the potential protective effects of humic acid on experimental gastric ulcer. Wistar Albino male rats ($n = 48$) were randomly divided into 8 groups as follow; Control (without any applications), Humic acid (50 mg/kg), ethanol group (1 mL/rat), and indomethacin group (25 mg/kg). In the treatment groups, both gastric ulcer model and humic acid 50 mg/kg were applied. In addition, famotidine the antiulcer drug was used as positive control. All medications were administered by oral gavage. Levels of ADAM10 and ADAMTS12 in gastric mucosa were determined by ELISA method. Hematoxylin-Eosin (H&E) staining, iNOS, and PCNA immunohistochemical staining were performed for histopathological investigations. Apoptosis was demonstrated by using the TUNEL method. In addition, the levels of inflammatory cytokines (TNF-α, IL-6, IL-10) and caspase-3 gene were determined by qRT-PCR. ADAM10 and ADAMTS12 levels significantly increased in the treatment groups compared to the ulcer groups ($p < 0.05$). The experimental groups showed mucosal erosion, bleeding, leukocyte infiltration and edema. Treatment with humic acid and famotidine was found to suppress iNOS activity, thereby decreasing proinflammatory activity and preventing damage to the gastric mucosa, while reducing the number of apoptotic cells. IL-6, IL-10, TNF-α and caspase-3 levels were significantly decreased in the treatment groups compared to damaged gastric mucosa. As a result, humic acid may be defined as a potential protective agent with its anti-inflammatory effect in gastric ulcer.

Keywords: gastric mucosa injury, humic acid, ADAM10, ADAMTS12, inflammation, famotidine

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INTRODUCTION

Gastritis and gastric ulcer are the most common forms of gastrointestinal diseases that have increasing rate of morbidity and mortality (Chow and Sung, 2009). The main pathogenesis of ulcer is the disruption of the integrity of the gastroduodenal mucosa due to the changes in balance between aggressive and protective/restorative factors. The most important aggressive factors are acid and pepsin formation, while prevention/repair mechanisms of gastroduodenal mucosa are disrupted due to genetic, environmental and infectious factors with the help of aggressive factors and ultimately ulcer occurs (Takeuchi et al., 1991; Pan et al., 2008). Today, proton pump inhibitors, histamine receptor antagonists, anticholinergics and antibiotics are used in combination for the treatment of gastritis and gastric ulcer (Stewartand and Ackroyd,

2011). Medicines for gastric ulcer treatment are mainly used to relieve symptoms, promoting ulcer healing, preventing ulcer relapse, and avoiding complications. So far, two main kinds of treatment methods are used in clinical practice: those for treating hyperacidity and for protecting the gastric mucosa (DeVault and Talley, 2009; Sen et al., 2009; Baraka et al., 2010; Liu et al., 2012; Chang et al., 2015). However, the potential side effects of these drugs, their limited effects and their interactions with other drugs appear to be shortcomings of these medications (Fisher and Le Couteur, 2001; Wedemeyerand and Blume, 2014). Therefore, there is a need for novel medications that have less side effects and less toxic.

For this purpose, herbal medicine applications have gained importance in recent years and have found widespread application areas. Most recent stud-

ies about the treatment of gastrointestinal disorders have focused on the potential role of natural medicine due to their availability, better protection, lower cost, and lower toxicity (Lee et al., 2009; Bansal and Goel, 2012). There are many studies about natural molecules such as safranal (Tamaddonfard et al., 2019), *Cuphea ignea* extract (Mousa et al., 2019), oxyresveratrol (Aziz et al., 2019), Kangfuxin Periplaneta Americana L. extract (Lu et al., 2019), spirulina (Mahmoud and El-Ghffar, 2018), a natural pigment sodium copper chlorophyllin (Lv et al., 2019) and crocin (El-Maraghy et al., 2015) for the treatment of gastric ulcer. In these studies, the agents used in the protection for gastric mucosal damage have anti-inflammatory, antioxidant and antiapoptotic effects, and they have a relevant effects on wound healing.

The ADAM protein family have a-secretase activity and act on inflammation, cell proliferation, angiogenesis and wound healing (Eto et al., 2000; Seals and Courtneidge, 2003). ADAM10 has a wide range of functions, such as ECM (extracellular matrix) degradation, local shedding of various cell surface proteins and affecting cell signaling patterns. ADAM10 mediates the migration of epithelial cells that are critical for wound healing (Lemjabbar and Basbaum, 2002; Yan et al., 2002). It was reported that ADAM10 levels decrease in gastroduodenal mucosa samples taken from patients with gastritis and ulcers (Erin et al., 2018). ADAMTSs are closely related to ADAM proteinases that play a role in ectodomain shedding or activation of various cell surface molecules, including growth factors and adhesion receptors (Seals and Courtneidge, 2003). ADAMTS12 was associated with osteoarthritis of cartilage components due to its ability to disrupt aggrecan and cartilage oligomeric matrix protein (Somerville et al., 2004). Tissues with ADAMTS12 deficiency were found to show a significant increase in several inflammatory parameters at both RNA and protein levels (Kurz et al., 2006). ADAMTS12 plays a protective role in inflammatory pathogenesis (Moncada-Pazos et al., 2012). However, there is no study that reveals the role of ADAM10 and ADAMTS12 in the development of gastric ulcer and the effects of the agents used for treatment on such metalloproteases.

Humic acids have medical importance and are often found in peat, sapropel, mummies and other humus substances (Archarya et al., 1998; Yudina et al., 1998). Humic acids were used as externally applicable drugs for the clinical treatment of diseases such as hematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis and osteochondrosis (Laub, 1999). Daily doses of humic substance extract were determined as 0.05, 0.1, 0.15, 0.2, 0.3, 0.4 and 0.5 g/kg for rabbits and mice. The researchers did not observe any morphological and histological changes in animals compared with the control group at all doses (Kel'ginbaev et al., 1973).

In our study, the effects of humic acid at dose of 0.05 g/kg administered to animals were examined, as given in the literature. Studies about ulcers with substances containing humic acid are available in the literature. There are no studies showing the protective effects of humic acid, which is a common area of use, directly on gastric ulcers.

Therefore, in our study; in gastric ulcer models created using ethanol and also indomethacin, the aim was to investigate the protective effects of humic acid with biochemical, histopathological, immunohistochemical and genetic analysis. Another aim of this study was to determine whether the effects of humic acid have a relationship with changes in stomach ADAM10 and ADAMTS12 levels.

MATERIALS AND METHOD

Study Design

Procurement of animals and the experimental stage was carried out in Canakkale Onsekiz Mart University Experimental Research Application and Research Center (COMUDAM). In our study, 48 adult Wistar Albino male rats with the same biological and physiological characteristics were used and kept in standard laboratory conditions (22 ± 1°C, 12 h light/dark cycle). The animals were randomly divided into 8 groups ($n = 6$). The grouping of subjects was planned as follows. The drugs were administered to the rats orally. Humic acid was obtained from Sigma-Aldrich, CAS Number 1415-93-6.

Group 1: Control group—There was no application other than distilled water, rats were sacrificed at the end of 24 h of fasting and stomachs were removed.

Group 2: Indomethacin group (Indo) Negative control—After 24 h of fasting, 25 mg/kg dose of indomethacin was administered, stomachs were taken after 6 h.

Group 3: Ethanol group (Eth) Negative control—After 24 h fasting, 1 mL/rat dose of absolute ethanol (>99.5%) was given using oral gavage. After 90 minutes, the stomachs were taken.

Group 4: Humic Acid group (Hum)—After 24 h of fasting, 50 mg/kg dose of humic acid was administered. After 90 min, stomachs were taken.

Group 5: Humic Acid + Indomethacin group (Hum + Indo) − Rats were given a dose of 50 mg/kg humic acid after 24 h of fasting. After 5 min, 25 mg/kg of indomethacin was administered and stomach was removed after 6 h.

Group 6: Humic + Ethanol group (Hum + Eth)—Following 24 h of fasting humic acid was administered at a dose of 50 mg/kg. After 120 min, absolute ethanol with a dose of 1 ml/rat (>99.5%) was given and 90 minutes later the stomachs were removed.

Group 7: Famotidine + Indomethacin group (Fam + Indo) Positive control—40 mg/kg famotidine was
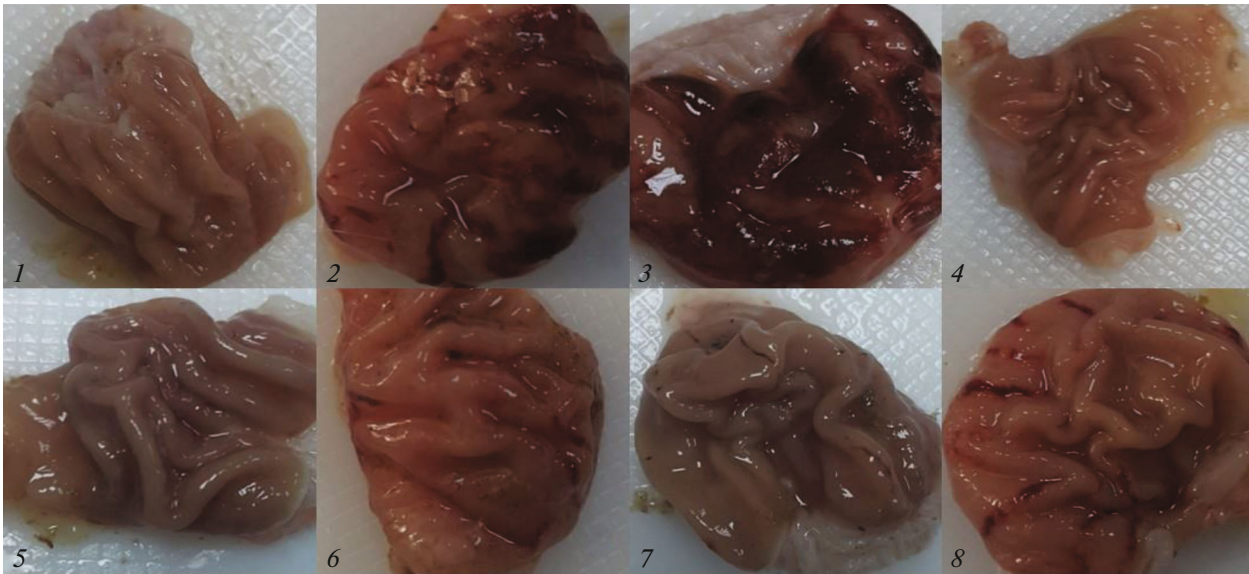
Fig. 1. Macroscopic images of groups. (*1*) Control; (*2*) Indomethacin; (*3*) Ethanol; (*4*) Humic acid; (*5*) Humic acid + Indomethacin; (*6*) Humic acid + Ethanol; (*7*) Famotidine + Indomethacin; (*8*) Famotidine + Ethanol.

administered after 24 h of fasting. After 5 min, 25 mg/kg of indomethacin was administered and stomachs were taken after 6 h.

Group 8: Famotidine + Ethanol group (Fam + Eth) Positive Control—After 24 h fasting, 40 mg/kg dose of famotidine was applied. Then 120 min later, 1 mL/rat dose of absolute ethanol (>99.5%) was applied and stomachs were removed after 90 min).

At the end of the experiment, animals were sacrificed under ketamine-xylazine anesthesia by collecting intracardiac blood. The results obtained at the end of the study were compared with famotidine, which is currently used for antiulcer treatment.

Determination of Gastric Ulcer Index

Gastric ulcer index was determined based on a previous study by a researcher unaware of the study groups (Saviç et al., 2011). Gastric mucosal damage scored as follow; 0: no lesions; 0.5: slight hyperemia or ≤5 petechiae; 1: ≤5 erosions ≤5 mm in length; 1.5: ≤5 erosions ≤5 mm in length and many petechiae; 2: 6−10 erosions ≤5 mm in length; 2.5: 1−5 erosions > 5 mm in length; 3: 5−10 erosions > 5 mm in length; 3.5: >10 erosions > 5 mm in length; 4: 1−3 erosions < 5 mm in length and 0.5−1 mm in width; 4.5: 4−5 erosions < 5 mm in length and 0.5−1 mm in width; 5: 1−3 erosions > 5 mm in length and 0.5−1 mm in width; 6: 4 or 5 grade 5 lesions; 7: ≤6 grade 5 lesions; 8: complete lesion of the mucosa with hemorrhage. The mean gastric ulcer index of the groups was obtained by dividing the total score by the number of animals in the group.

Histopathological Analysis

Photographs of the samples were taken for macroscopic examination (Fig. 1). Gastric tissue samples were then fixed in 10% neutral buffered formalin for 48 h. After fixation, paraffin blocks were prepared using routine histological methods. Sections of 5 micrometer thickness were stained with hematoxylin and eosin (H&E). Histopathological lesions were scored on a scale of 0−14 according to the criteria described by (Laine and Weinstein, 1988) and gastric injury score (GIS) was calculated. A total of 1 cm gastric area on each histological section was examined for epithelial cell loss (score: 0−3), edema in the mucosa (score 0−4), hemorrhagic damage (score: 0−4) and the presence of inflammatory cells (score: 0−3). All histological examinations were performed using an Olympus CX41 (Olympus, Japan) light microscopy and image analysis system (Image Analysis Software Camera Gen III, Istanbul, Turkey).

Immunohistochemical Analysis

Inducible nitric oxide synthase (iNOS) and proliferating cell nuclear antigen (PCNA) immunohistochemical markers were used for the avidin biotin complex (Hsu et al., 1981). Primary antibodies (INOS, 1 : 100, NB300-605, Novus Biologicals, Littleton, CO; PCNA, 1 : 100, Abcam 2426, Cambridge, UK) were incubated in the humidified chamber for 60 min. Secondary antibody and streptavidin peroxidase (Ultra Vision Detection System-HRP kit, Thermo Scientific/Lab Vision, Fremont, CA, USA) were used according to the manufacturer's instructions. In addition, 3-amino-9-ethylcarbazole (AEC) was used as a chromogen for iNOS and PCNA staining to create contrast. Counter-

stains were made with Mayer's hematoxylin. INOS and PCNA positive stained cell numbers were expressed as positive stained cells/mm^2 in the stomach tissue for each group.

Apoptosis

Apoptotic cells in the gastric mucosa were determined with the terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) method (S7100 ApopTag Plus Peroxidase Onsite, Merck Millipore, Darmstadt, Germany), according to the previous study (Karaboga et al., 2018). Apoptotic cells determined using intense brown nuclear staining were calculated as positive cells/mm^2 for each group.

Total RNA, cDNA, and Real-Time PCR (qRT-PCR) Analysis

It was reported in previous studies that cytokine expressions are altered in experimental acute gastric mucosal damage. IL-6, IL-10, TNF-α and Casp-3 gene expressions were determined in stomach tissue. At the end of the experimental process, approximately 10−30 mg of stomach tissue samples were taken and homogenized in the homogenizer (RETSCH brand MM 400 model). Total RNA isolation (Ambion Pure-Link RNA MiniKit) was performed from the obtained homogenates. Then, the concentrations of the samples were equalized by measuring the RNA concentrations and purities with the Nanodrop device. cDNA synthesis (High Capacity cDNA Reverse Transcription Kit) was performed from the RNA samples. PCR conditions are in order; Step 1: 25°C, 10 min; Step 2: 37°C, 120 min; Step 3: 85°C, 5 min. The obtained cDNAs were increased in StepOne (Thermo Scientific, USA) real time PCR in accordance with the Tagman qPCR Mastermix protocol. B-actin was used as housekeeping gene. Gene expressions were determined using Ct values and floor changes were evaluated by the $2^{-(\Delta\Delta CT)}$ method. The primary ID numbers for IL-6, IL-10, TNF-α and Casp-3 are Rn01410330_m1, Rn01483988_g1, Rn01525859_g, and Rn00563902_m1, respectively.

Biochemical Analysis

ADAMTS12, which is one of the indicators of the inflammatory process, and ADAM10 activities, which are expressed in patients with gastritis, were investigated in rat stomach tissues with the ELISA method. Tissue ADAMTS12 and ADAM10 concentrations were determined in accordance with the manufacturer's instructions using commercially available enzyme-linked immunosorbent assay kits (ELISA) (Mybiosource Inc., San Diego, CA 92195-3308 USA). The color intensity was read at 450 nm via a multiplate reader. The results were determined in picograms per milliliter. The results that were fully compatible with the standard curve were evaluated comparatively.

Statistical Analysis

The SPSS 22 program was used for the statistical analysis of the values obtained after biochemical evaluations. Firstly, it was seen that group distributions were not normal (Shapiro-Wilk, $p > 0.05$). One-Way ANOVA Tukey's test was carried out for Post Hoc Test. The data, where the difference between the groups was $p < 0.05$, was considered significant. All results were evaluated as mean ± standard deviation.

Data for histological evaluations were evaluated using the SPSS (PASW Statistics 21, SPSS Inc., IL, USA) statistics program. The numerical parameters of the groups were evaluated using a nonparametric test (Kruskal−Wallis) and the significance of the values obtained in the two-way comparison was measured using the Mann−Whitney U test. $p < 0.05$ value was considered statistically significant.

RESULTS

Macroscopic Review

As can be seen from the photos of the stomach taken for macroscopic inversion, the treatment effect of the humic acid used as the therapeutic agent is visible, while the wound and necrosis areas are visible in the models with gastric ulcer (Fig. 1). Also, gastric ulcer index of groups was expressed in Table 1. Humic acid treatment provided a significant decrease in Hum + Eth and Hum + Indo groups.

Histopathological and Immunohistochemical Analysis Findings

Microphotographs of H&E-stained gastric histopathological examination are presented in Fig. 2. Microscopic examination revealed normal gastric structure in the control group (Fig. 2a). Mucosal erosion, bleeding, leukocyte infiltration and edema were seen in the indomethacin and ethanol groups (Figs. 2b, 2c). Pretreatment with humic acid and famotidine resulted in a marked decrease in CBS in the gastric mucosa. The GIS scores of the groups are presented in Table 1. The data obtained as a result of immunohistochemical analysis show immunohistochemical iNOS activation in the gastric mucosa, as shown in Fig. 3. Ethanol and indomethacin administration increased gastric iNOS expression. Treatment with humic acid and famotidine suppressed iNOS activity; thereby reducing proinflammatory activity and preventing damage to the gastric mucosa. The distribution of iNOS-positive cell numbers belonging to the groups is shown in Table 1. Anti-PCNA labeled gastric mucosa sections are given in Fig. 4. In the control group, PCNA was found to be highly expressed and it was significantly reduced in the ethanol and indomethacin groups. Pretreatment with

Table 1. Effect of humic acid and famotidine on gastric ulcer index, GIS, iNOS, PCNA and apoptotic cell count in gastric mucosa

Groups	Gastric ulcer index	GIS (0—14)	iNOS, mm^2	PCNA, mm^2	Apoptotic cell, mm^2
Control	1.33 ± 1.03	0.33 ± 0.51	0,66 ± 0.81	29.16 ± 2.56	3.16 ± 1.47
Indomethacin	61.16 ± 5.84	9.83 ± 1.47[a]	17.83 ± 2.31[a]	13.6 ± 2.5[a]	35.83 ± 5.11[a]
Ethanol	83.50 ± 5.50	11.83 ± 0.75[a]	28.83 ± 3.31[a]	12 ± 1.78[a]	40.66 ± 6.5[a]
Humic acid	1.16 ± 0.75	0.16 ± 0.40	0.16 ± 0.40	29.66 ± 3.55	2.66 ± 1.21
Hum + Indo	28.66 ± 4.41	5.83 ± 1.16[b]	8.66 ± 1.03[b]	20.16 ± 2.13[b]	20.5 ± 2.58[b]
Hum + Eth	34.83 ± 5.56	6.50 ± 1.51[c]	11 ± 1.41[c]	18.5 ± 3.50[c]	22.16 ± 3.43[c]
Fam + Indo	26.16 ± 3.71	5.16 ± 1.16[b]	6.16 ± 1.47[b]	23.66 ± 1.75[b]	18.33 ± 2.50[b]
Fam + Eth	36.50 ± 5.64	6.83 ± 0.98[c]	9.33 ± 1.21[c]	21.66 ± 2.87[c]	19.6 ± 3.55[c]

[a] $p < 0.01$ compared to the control group, [b] compared to the indomethacin group $p < 0.01$, [c] $p < 0.01$ compared to ethanol group, GIS; stomach damage score, iNOS; inducible nitric oxide synthase, PCNA; Proliferative cell nuclear antigen.

humic acid and famotidine increased PCNA activity in gastric mucosa. Treatment with humic acid and famotidine increased the number of PCNA-positive cells, accelerating epithelial regeneration and mucosal healing. PCNA-positive cell numbers in the gastric mucosa of the groups are shown in Table 1.

Apoptosis in the control group was observed as several apoptotic cells seen as nuclei drowning in the gastric mucosa (Fig. 5). The number of apoptotic cells in the ethanol group increased significantly compared to the control group. Pretreatment with humic acid and famotidine reduced the number of apoptotic cells in the gastric mucosa. The apoptotic cell numbers for the groups are shown in Table 1.

Gene Expression Findings

In groups where ulcers were induced by applying indomethacin and ethanol, cytokine expressions were increased compared to control ($p < 0.05$). Figure 6 shows that humic acid administered for therapeutic purposes reduces cytokine gene expressions. Humic acid has similar effect on genes as famotidine.

Biochemical Analysis Findings

Results are given as mean ± standard deviation (pg/ml). When ADAMTS12 levels are examined, the ADAMTS12 level decreased significantly in the ethanol and indomethacin groups compared to the control group ($p = 0.003$). Similarly, ADAM10 levels were decreased in rats with gastric mucosal damage caused by ethanol and indomethacin ($p = 0.000$). However, when only the humic acid-treated group is evaluated, the results seem to be close to controls. The results are statistically significant compared to the damaged group ($p < 0.05$). Famotidine is a commercially available H$_2$ receptor blocker used for the treatment of ulcers. The reason we used famotidine as a positive control is that, it proves the effectiveness of humic acid

by comparison. When the results are examined, humic acid has similar effects to famotidine (Figs. 7, 8).

DISCUSSION

With this study, the following findings were firstly revealed in gastric ulcer models created with both ethanol and indomethacin. (a) Humic acid has a protective effect on stomach damage at the macroscopic and histopathological level, (b) humic acid suppresses iNOS and apoptosis and increases PCNA activity, (c) humic acid reduces TNF-α and IL-6 levels that are proinflammatory cytokines in the gastric mucosa, while it increases IL-10 levels which is a member of anti-inflammatory cytokines, (d) humic acid increases ADAM10 and ADAMTS12 levels, and (e) these protective effects of humic acid are as strong as famotidine.

In addition to this data, it was also revealed for the first time that (f) ADAMTS12 was also present in gastric mucosa so ADAMTS12 levels were determined in healthy, gastric ulcer models and humic acid treated groups. These results show us that humic acid has potential for wound healing with its anti-inflammatory and antiapoptotic properties, as well as affecting ADAM10 and ADAMTS12 levels in experimental gastric ulcer models.

Gastric ulcers have widespread morbidity and mortality in worldwide. High concentrations of alcohol disrupt the bicarbonate release mechanism by overcoming the gastric mucosal barrier and act against hydrogen ions to cause acute gastric mucosal lesions characterized by mucosal bleeding. Alcohol also stimulates acid secretion (Peterson et al., 1986).

Non-steroidal anti-inflammatory drugs (NSAIDs) affect the amount of stomach acid, integrity of the mucosal barrier, bicarbonate and glutathione levels, and mucosal blood flow rate through inhibition of prostaglandin synthesis (Flower, 2003). The pathogenesis of symptomatic peptic ulcer disease caused by repeated exposure to NSAIDs is basically the result of
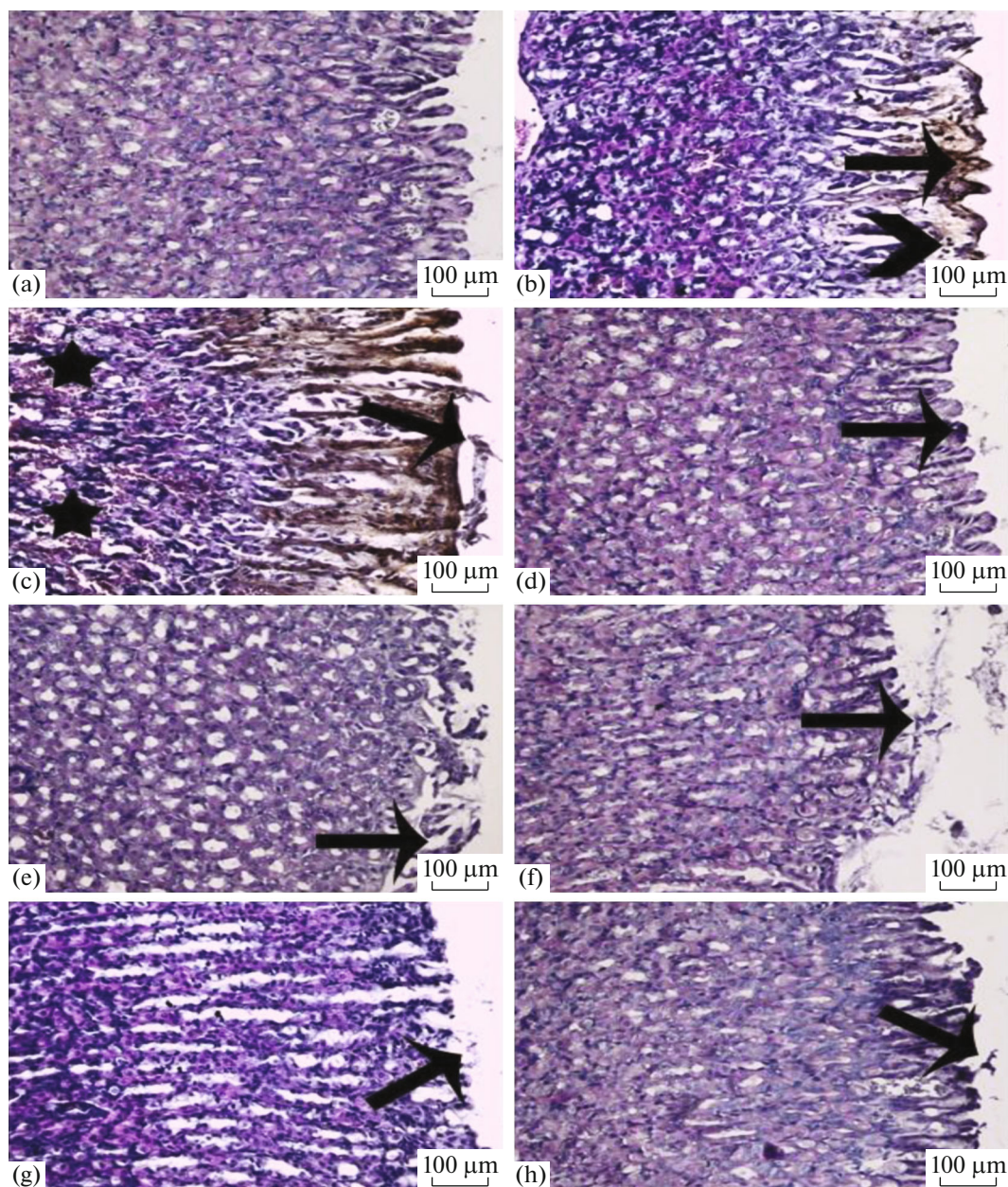
Fig. 2. Microscopic examination of H&E-stained gastric mucosa. Control group had normal histological structure (a). Histopathological changes such as bleeding (star), epithelial loss (arrow) and infiltration (arrowhead) were observed in indomethacin (b) and ethanol groups (c). The humic acid group (d) was similar to the control group. Hum + Indo (e) and Hum + Eth (f) had attenuated histopathological changes. Pre-treatment with famotidine retained the structure of the gastric mucosa (g and h) (Magnification; 200×, scale bar; 100 μm).

systemic (absorption) inhibition of gastrointestinal mucosal cyclo-oxygenase (COX) activity. Even the administration of intravenous or intramuscular NSAIDs can cause stomach or duodenal ulcers in animals and humans (Estes et al., 1993).

The side effects of the drugs used for the treatment of gastric ulcers are few but have led to the trials of some active substances in this area (Muazzam et al., 2015). Examples of molecules used for this purpose and understood to have beneficial effects include Rubus crataegifolius (black raspberry, RF), *Ulmus macrocarpa* (elm, UL) and Gardenia jasminoides (cape jasmine, GJ) (Park et al., 2019), *Jinlingzi* powder and extracts (Zhao et al., 2019), *Cuphea ignea* extract (Aziz et al., 2019), sodium copper chlorophyllin (Lv et al., 2019), safranal (Mousa et al., 2019) and Spirulina (Mahmoud and El-Ghffar, 2018). In these studies, wound healing, antioxidant, anti-inflammatory and antiapoptotic effects of the substances used were investigated in general. Therefore, in our study, we
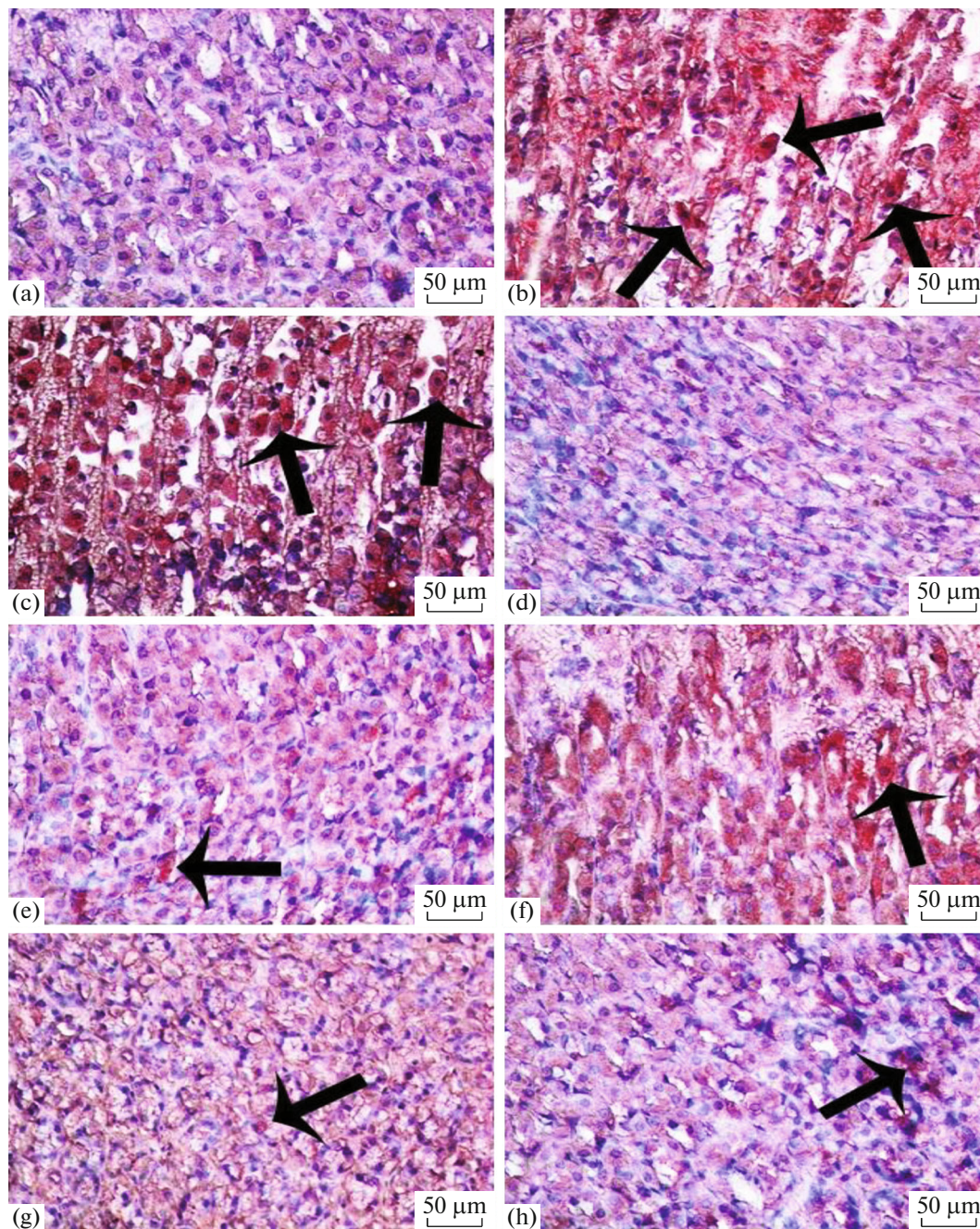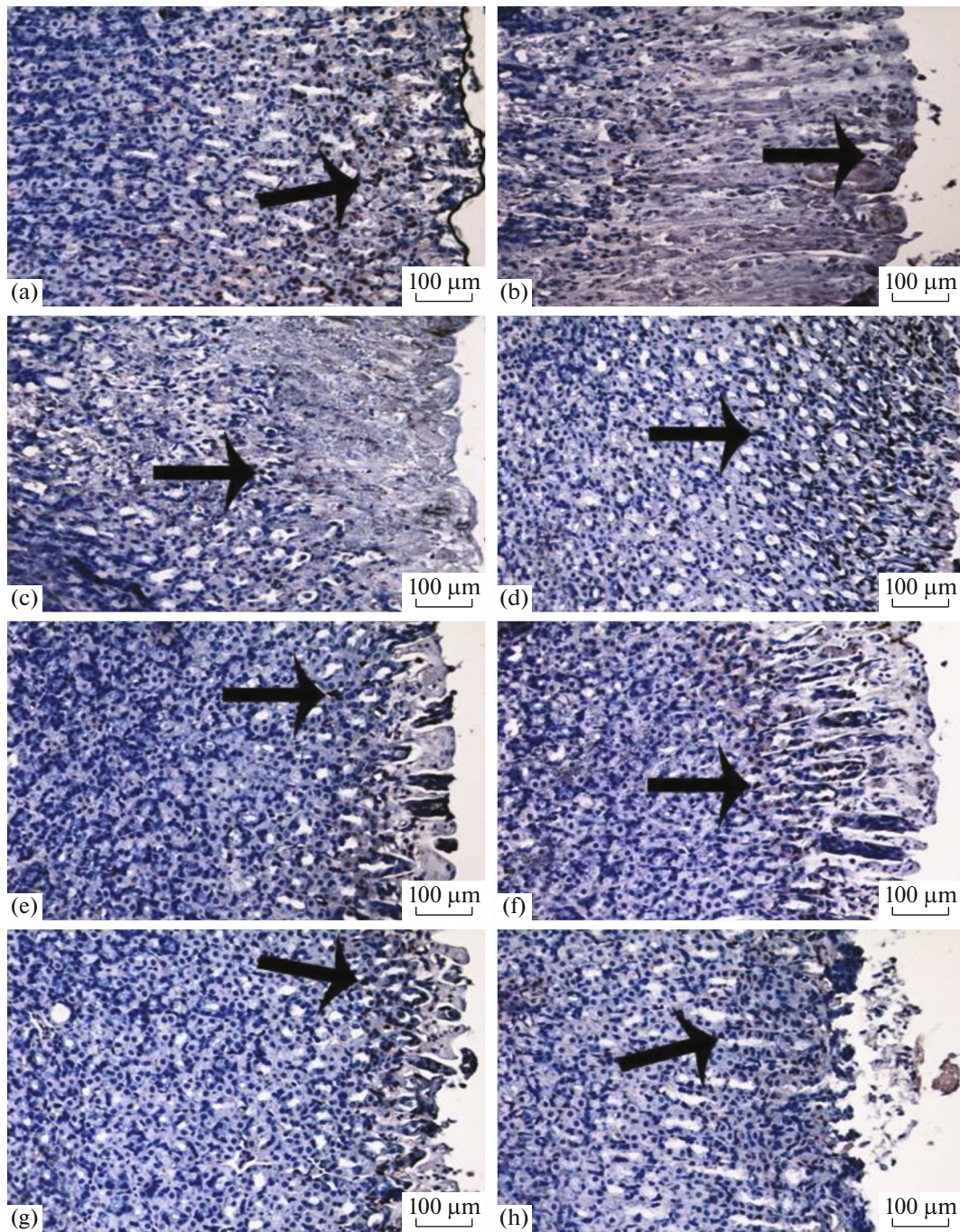
Fig. 3. Immunohistochemical iNOS activity in gastric tissue. Control (a), Indomethacin (b), Ethanol (c), Humic acid (d), Hum + Indo (e), Hum + Eth (f), Fam + Indo (g) and Fam + Eth (h) group (magnification; 400×, scale bar 50 μm, counter-curtain; Mayer hematoxylin).

aimed to determine the wound- protective effects of humic acid at the histopathological level. In addition, we determined the antiinflammatory and antiapoptotic effects at the molecular level. Furthermore, we identified the effects on iNOS, PCNA, ADAM10 and ADAMTS12. Thus, we aimed to understand the mechanisms underlying the protective effect of humic acid in gastric ulcers.

In our study, we used two different methods to create a gastric ulcer model. Both the indomethacin-induced gastric ulcer model and the ethanol ulcer model are widely used in gastric ulcer research in pathophysiological studies and drug therapy studies (Aziz et al., 2019; Mousa et al., 2019; Park et al., 2019). We aimed to determine the protective effect of humic acid with both methods. We also administered famotidine, one of the agents commonly used in the treatment of gastric ulcers, and compared it with humic acid. Thus, it was possible to compare the protective effects of humic acid with a adjuvant agent whose

Fig. 4. Effect of PCNA immunoreactivity of Humic acid and Famotidine in gastric tissue. Control (a), Indomethacin (b), Ethanol (c), Humic acid (d), Hum + Indo (e), Hum + Eth (f), Fam+ Indo (g) and Fam+Eth (h) group (magnification; 200×, scale bar 100 μm, countersunk; Mayer hematoxylin).

effectiveness was determined for gastric ulcer. When we evaluate the protective effects of humic acid in the ulcer model, first, it turns out to be an effective agent in terms of wound healing. As a matter of fact, humic acid was shown to reduce mucosal erosion, bleeding, leukocyte infiltration and edema in the indomethacin and ethanol groups. In many studies, inflammation was identified as one of the factors responsible for gas-tric mucosal injury. Leukocyte and macrophage migration to ulcerated or damaged areas leads to the emergence of inflammatory functions (Aziz et al., 2019). The secretion of the proinflammatory cyto-kines of macrophages migrating to the injury area leads to a hike in the blood flow of ulcerated mucosa and thereby delays wound healing (Lu et al., 2019). Li and his colleagues examined the protective effect of
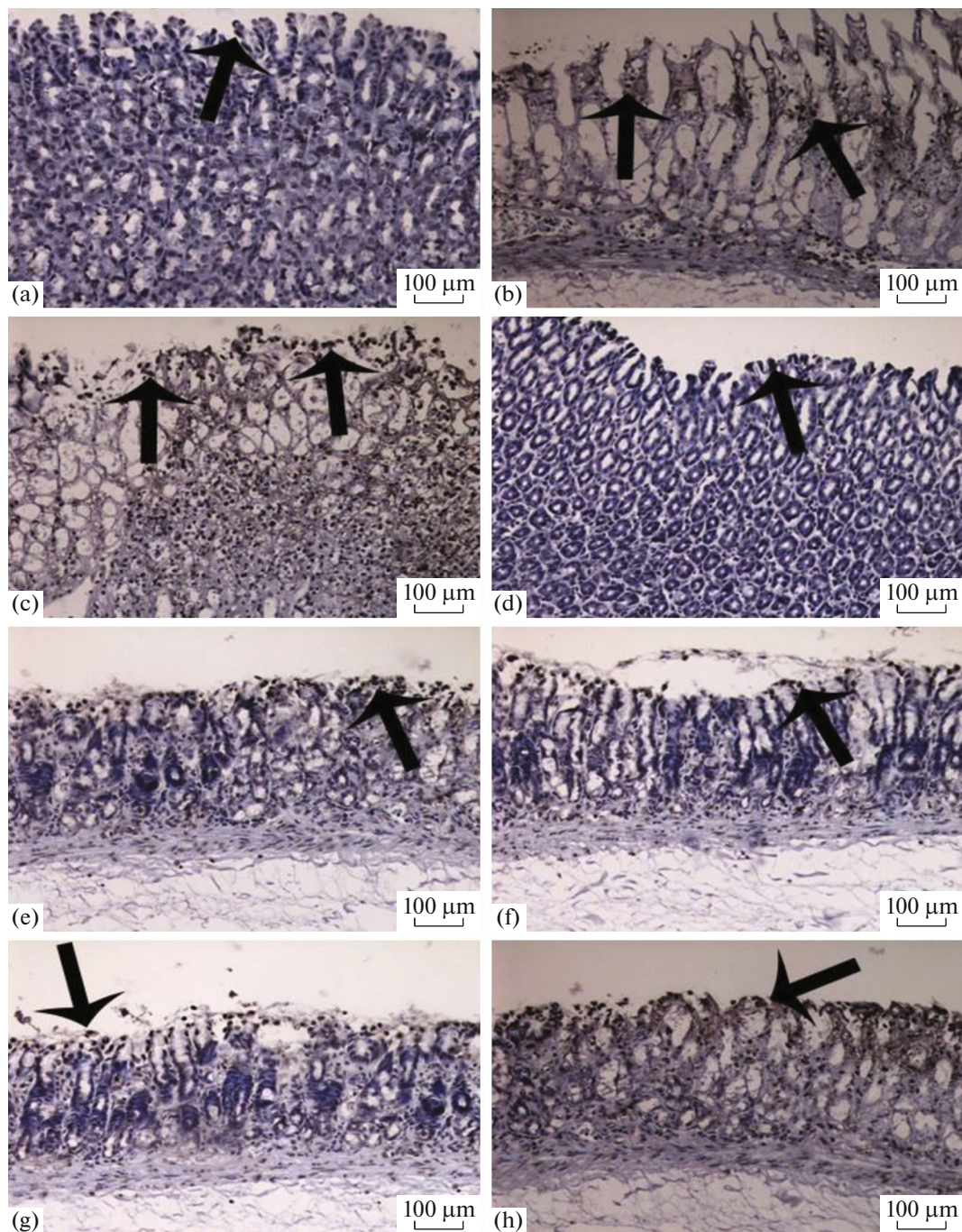
Fig. 5. Apoptotic examination of gastric tissue with the TUNEL method. There were several TUNEL positive cells in the control and humic acid groups (a and d). Indomethacin and ethanol applications caused an increase in the number of apoptotic cells (b and c). Pretreatment with humic acid and famotidine resulted in a marked reduction in the number of apoptotic cells (d, f, g and h) (magnification; 200×, scale bar 100 μm, plywood; hematoxylin).

tetrahydrocotyline (THC) in an ethanol-induced gastric ulcer model in mice (Li et al., 2013). Accordingly, it was found that pro-inflammatory cytokine (TNF-α and IL-6) levels and myeloperoxidase (MPO) activity increased and administration of THC at 10 and 20 mg/kg significantly decreased gastric lesions and proinflammatory cytokine levels compared to the ethanol group (Li et al., 2013).

In our study, we observed leukocyte infiltration microscopically in rats with a gastric ulcer model and found that TNF-α and IL-6 gene expressions, which are proinflammatory cytokines, increased in samples obtained from gastric mucosa. This increase in proinflammatory cytokines was inhibited by humic acid and the reduction in leakage of inflammatory cells and focal necrosis was confirmed with histological exam-
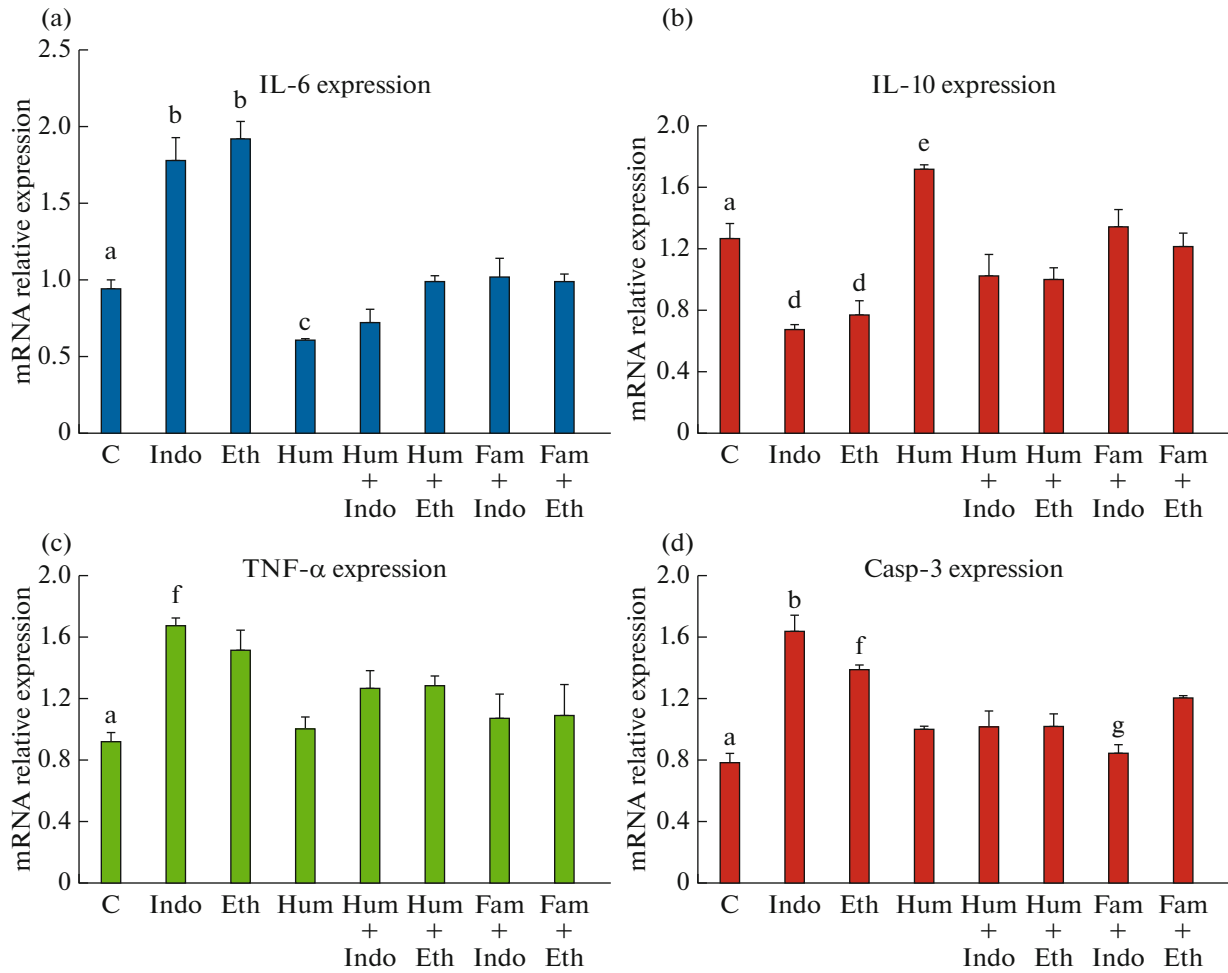
Fig. 6. Gene expression levels of IL-6 (a), IL-10 (b), TNF-a (c) and Casp-3 (d) in all groups of rat gastric tissue. After normalizing the mRNA level with P-Actin, it showed a $2^{-(\Delta\Delta CT)}$ relative expression. One-way ANOVA was used to compare gene expression levels between groups and results were evaluated according to the post hoc Tukey's test. a: different from Indo and Eth groups, b: different from Hum, Hum + Indo, Hum + Eth, Fam + Indo and Fam + Eth groups c: different from Fam + Indo group, d: different from Hum, Fam + Indo and Fam + Eth groups, e: different from Hum + Indo, Hum + Eth and Fam + Eth groups, f: different from Hum, Hum + Indo, Hum + Eth groups g: different from Fam + Indo group. $p < 0.05$ statistically different.
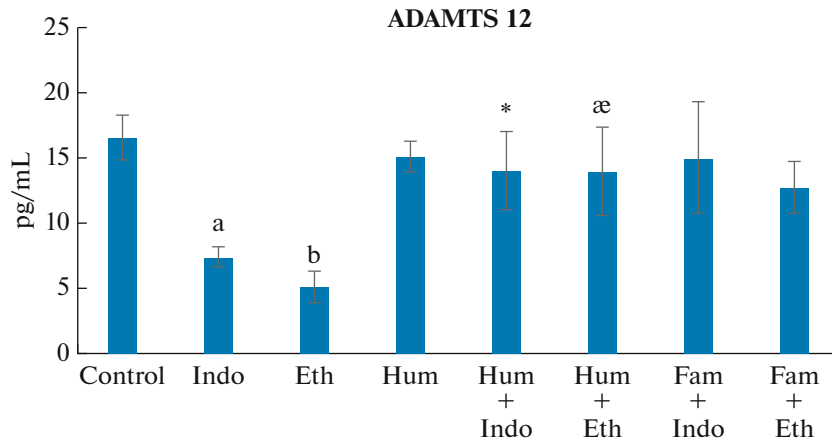
Fig. 7. Effect of humic acid and famotidine on ADAMTS12 proteins in gastric mucosa.
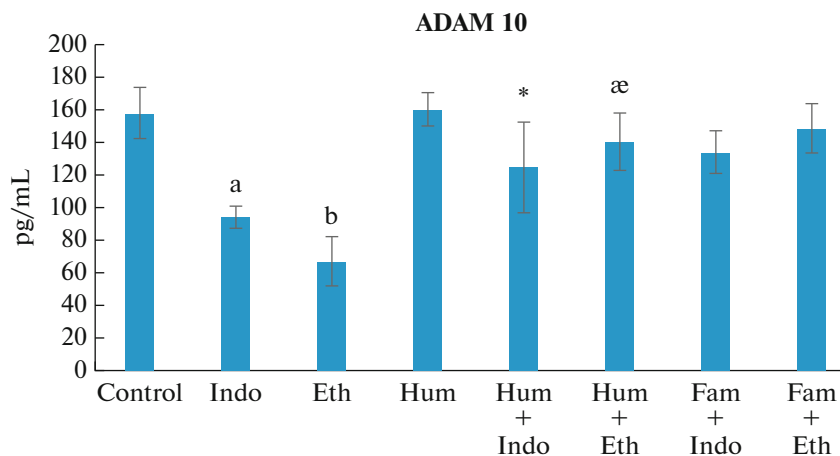
ADAM 10

Fig. 8. Effect of humic acid and famotidine on ADAM10 proteins in gastric mucosa.

ination (Figs. 2−5). We also observed that humic acid acts as an anti-inflammatory, with a increasing effect on IL-10 gene expression. IL-10, in turn, acts by selectively blocking the expression of genes encoding proinflammatory cytokines and chemokines while at the same time increasing the expression of anti-inflammatory molecules and thereby promotes gastric ulcer healing (Vinagre et al., 2018).

The findings we obtained are compatible with the findings obtained after the application of *Cuphea ignea* (Aziz et al., 2019), Jinlingzi powder and its extractive components (Zhao et al., 2019), sodium copper chlorophyllin (El-Maraghy et al., 2015), and hesperidin (Elshozly et al., 2018) which were previously used as preservatives in different gastric ulcer studies.

It was reported that iNOS expressions increases in gastric ulcers, and TNF-α causes this increase (Yildirim et al., 2015). While increased iNOS expression induces NO formation that reacts with superoxides forming peroxynitrites (Lanas, 2018), this was shown to occur in rats treated with hesperidin (Elshozly et al., 2018), which is responsible for gastric mucosal injury. In our study, it was determined that in groups with high TNF-α levels, iNOS activity was also high and humic acid decreased iNOS activity. This suggests that the protective role of humic acid in ulcer healing may also be related to iNOS.

In our study, we observed that humic acid also increased the number of PCNA-positive cells. It can be said that this is a factor that reveals the epithelial regeneration and mucosal healing effect of humic acid. PCNA is known as an important tissue proliferation marker and PCNA activity was reported to be important for gastric mucosal healing (Caldas et al., 2014). Cellular proliferation rises with increasing PCNA reactivity and this is an indicator of ulcer re-epithelialization (Polo et al., 2012). Caldas et al. determined that *Hyptis martiusii* Benth oil extract increased immunohistochemical PCNA activity in the regener-

ation zone in rats (Lanas, 2018). Similarly, Elsaed et al. observed that increases in PCNA activation were in parallel to ulcer healing (Elsaed et al., 2015).

Humic acid also showed positive effects on apoptosis in the gastric mucosa. This effect of humic acid was seen both with a decrease in the number of apoptotic cells in TUNEL staining and a decrease in caspase-3 gene expression in the gastric mucosa. Various studies were conducted on agents that reduce apoptosis in stomach ulcers. A few of these studies are listed below, and it can be seen that the findings obtained are in line with our study. Arab et al. examined the effects of anti-oxidant and anti-inflammatory natural citrus diosmin, which has protective properties against cardiac, hepatic and kidney damage, in a rat model with gastric damage caused by ethanol (Arab et al., 2015). Diosmin reduced caspase-3 activity 2.4 times, which is a reliable indicator of apoptosis, and suppressed cytochrome C (Cyt C) levels by increasing anti-apoptotic Bcl-2 in favor of cell survival, as well as increasing anti-inflammatory IL-10 levels. Antonisamy et al. investigated the protective effects of friedelin from the *Azima tetracantha* plant in the ethanol-induced gastric ulcer model, and found that the apoptotic index of the group given ethanol was 16.64 times higher than that of the control group, and that the DNA fragmentation in the group pre-treated with friedelin decreased 4.62 times compared to the ethanol group and caspase-3 activity decreased by 4.62 time (Antonisamy et al., 2015). In the applied group, caspase-3 activity was 4.63 times higher, while the caspase-3 activity decreased by 3.62 times in the group given friedelin.

Lv et al. applied micsodium copper chlorophyllin as a preservative to mice with ethanol-induced gastric ulcer model and determined reductions in molecules that support apoptosis (Lv et al., 2019). They said this finally maintained the integrity of the normal gastric mucosal barrier. Another study revealed that activa-

tion of caspase-3 increases apoptotic cell death in gastric mucosa (Almasaudi et al., 2016).

When ADAMTS12 levels are examined, the rats with gastric mucosal damage had low levels. The low level of ADAMTS12 in lesion tissue causes the initiation of inflammatory processes. As a result, hematoma and various wounds occur with necrosis in the tissue. In a study by Moncada-Pazos et al. ulcerative colitis and sepsis were induced in transgenic mice without ADAMTS12 (Moncada-Pazos et al., 2012). It was revealed that mice without ADAMTS12 had severe inflammation. Since ADAM10 is also an indicator of inflammation, reductions in gastritis levels were observed in the presence of inflammatory factors, as determined in the literature (Erin et al., 2018). In our study, both ADAMTS12 levels decreased in damaged tissue and ADAM10 levels decreased significantly due to inflammation. In another study, it was stated that ADAMTS12 values played an important role in inflammation processes in arthritis and that their levels decreased significantly (Wei et al., 2014). Oztas et al. found that ADAMTS12 levels decreased significantly compared to controls in their study about pre-eclampsia and intrahepatic cholestasis of pregnancy (Oztas et al., 2016). In our study, we also showed humic acid decreased inflammation and increased ADAM10 and ADAMTS12 levels in humic acid-treated groups, however, it plays a protective effect at least as famotidine.

This study demonstrated the gastroprotective effect of humic acid using models of ethanol and indomethacin-induced ulcers. Humic acid exerts double beneficial protective effects by suppressing gastric aggressive factors, reactive oxygen species, proinflammatory cytokines, proapoptotic protein and developing gastric cytoprotective factors. The antiulcer activity of humic acid was confirmed by macroscopic and histological examination of the number and severity of ulcers, mucosal edema, epithelial abrasion of mucosal tissue, infiltration of inflammatory cells and bleeding. Consequently, humic acid was proven to have a healing effect on stomach tissues with ulcer and gastric mucosal damage, as well as increasing levels of ADAMTS12 and ADAM10, which are important metalloproteinases; thereby reducing inflammation, as well as decreasing inflammatory cytokine levels determined by gene expression. As seen in histopathological evaluations, humic acid has the protective effect against gastric mucosa damage at least as effectively as famotidine. The use of natural antioxidant products instead of chemical production drugs, which have a lot of side effects, is becoming more common. The results of this study showed that humic acid has potential as an protective agent that can be used for gastric ulcer.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. Our study was approved by Canakkale Onsekiz Mart University Animal Experiments Local Ethics Committee with decision 2018/05-02.

Highlights

Humic acid has a healing effect on stomach damage at the histopathological level, Humic acid suppresses iNOS and apoptosis and increases PCNA activity,

Humic acid reduces TNF-a and IL-6 levels that are proinflammatory cytokines in the gastric mucosa, while it increases IL-10 levels which is a member of anti-inflammatory cytokines, Humic acid increases ADAM10 and ADAMTS12 levels, these healing effects of humic acid are as strong as famotidine.

ADAMTS12 was also present in gastric mucosa for the first time, so ADAMTS12 levels were determined in healthy, gastric ulcer models and humic acid treated groups.

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