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Research Article
Magnolin Alleviates Gastric Ulcer Induced by Ethanol/HCl in Mice Model via Oxidative Stress and NF-κB Pathway

[1]Ali Tureyen and [2]Sinan Ince

[1]Department of Gastroenterology, Ministry of Health Eskisehir City Hospital, Eskisehir, Turkey
[2]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

Abstract

Background and Objective: The gastric ulcer arising from many reasons is an important disease and is developed too often by the excessive consumption of alcohol. In this study, the potential gastric protection effect of the magnolin having significant pharmacological effects against the acute gastric damage induced by the ethanol/HCl in mice is examined. **Materials and Methods:** The study was carried out with a total of 49 Swiss albino mice, control, dimethylsulfoxide, omeprazole, ethanol/HCl and in addition on the groups given 3 different doses of magnolin (2.5, 5 and 10 mg kg^{-1}). The effect of magnolin was examined on gastric damage with the macroscopic, biochemical and proinflammatory cytokines mRNA expression analysis of the gastric tissue. **Results:** As a result of the study, it was observed that orally administered magnolin was significantly effective in alleviating macroscopic and histopathological lesions caused by ethanol in the gastric mucosa, improved antioxidant activity, prostaglandin E2 and nitric oxide levels and decreased lipid peroxidation in the gastric mucosa. However, it was also found that pro-inflammatory cytokine expressions induced by ethanol were decreased by the application of magnolin. **Conclusion:** The findings of the study showed that the magnolin represses the lipid peroxidation and inflammatory response, on the other hand, can exhibit a protective effect against *in vivo* ethanol-derived gastric tissue by increasing activation of the antioxidant system.

Key words: Magnolin, ethanol, gastric ulcer, inflammation, antioxidant, lipid peroxidation

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Corresponding Author: Sinan Ince, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey Tel: +902722282700-2764

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The gastric ulcer is a digestive system disease considered at the rate of 5-10% amongst the disorders[1]. There are factors like *Helicobacter pylori* infection, excessive use of Non-Steroid Anti-Inflammatory medicines (NSAID), alcohol consumption, smoking and stress amongst the etiological factors. Especially, alcohol consumption and use of NSAID medicines increase the bleeding risk in the upper parts of the gastrointestinal system[2]. Gastric ulcers are, in general, revealed because of the unbalancing factors between mucosal protective factors and abrasive aggressive factors to which mucus is exposed. Gastric Hydrochloric Acid (HCl) secretion, reactive oxygen radicals, mucosal hypoperfusion and alcohol consumption may be thought of as samples[3]. Alcohol consumption is among the most essential factors contributing to gastric ulcer formation and excessive alcohol consumption increases gastric mucosal damage risk[4]. For this reason, ethanol-induced gastric mucosal damage models is a model used frequently in an investigation of the factors leading to human gastric ulcers and materials having antiulcer activity[5].

Magnolin is a natural compound extracted from *Magnoliae flos* with biological effects of antioxidant and anticancer nature and anti-inflammatory activity[6,7]. The studies have exhibited that the magnolin prevents cell growth and conversion by targeting ERK pathway activation[8]. In addition, it was reported that the magnolin prevents Tumour Necrosis Factor-α (TNF-α) and Prostaglandin E2 (PGE2) production[9]. Kim *et al.*[10] showed that magnolin inhibits Nitric Oxide (NO) and PGE2 production and inducible Nitric Oxide Synthase (iNOS) and cyclooxygenase-2 (Cox-2) expression, by suppressing I-kappa B-alpha degradation and p65 Nuclear Factor-κB (NF-κB) translocation respectively. It was also reported that, in other studies, it showed essential anti-inflammatory bioactivity on chondrocytes through suppression of NF-κB pathway activation of magnolia[11] and increased antioxidant activities in rats with hind limb ischemia-reperfusion damage[12].

This study, it was aimed to determine the effects of magnolin application on lipid peroxidation, antioxidant activity, inflammation and histopathologic changes in mice.

MATERIALS AND METHODS

Study Area: The study was carried out at the laboratories of the Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey from February-May, 2021.

Chemicals: Magnolin used in the study was purchased from ChemFaces (Wuhan, Hubei, China), omeprazole from Sandoz Drug Industry (Istanbul, Turkey), dimethyl sulfoxide (DMSO), HCl and ethanol from Sigma-Aldrich (MO, USA) companies. Other chemicals to be used to determine the parameters to be analysed were purchased from commercial companies.

Experimental design: In this study 3 months (30-40 g) 49 Swiss albino male mice were used. The mice were kept and fed under the conditions of 22±2°C room temperature and 55-60% humidity and with a photoperiod of 12:12 hrs, during the study. The mice in the study group were fed with standard rodent food and fresh drinking water ad libitum. In addition, the animals were kept without food before 24 hrs of alcohol-induced ulcer formation. The procedures used and the care of animals was approved by the Institutional Animal Care and Use Committee in Afyon Kocatepe University (Approval No. 49533702/17).

The animals were divided into 7 groups each consisting of 7 male mice. Ethanol/HCl[13] and magnolin[14] doses to be used in the experimental stage were determined by taking previous studies into account. The groups for the experimental study were established as follows:

Group I is the only physiologic saline administered control group, Group II is the 0.5 ml gastric gavage of 0.1% DMSO, Group III is the 0.15 M HCl and 98% ethanol mixture in 0.2 mL/40 g volume, Group IV is the 20 mg kg^{-1} omeprazole gastric gavage solved in physiologic saline, Group V is the 2.5 mg kg^{-1} magnolin dissolved in DMSO, Group VI is the 5 mg kg^{-1} magnolin dissolved in DMSO and Group VII is the 10 mg kg^{-1} magnolin dissolved in DMSO.

DMSO and magnolin combination were orally applied for 7 days and physiologic saline was orally applied to control, omeprazole and ethanol groups within the same period. After 1 hr from the last administration ethanol/HCl was administered in designated quantity to other groups other than the control group and after 1 hr from this administration intracardiac blood and stomach tissue samples were taken under general anaesthesia (isoflurane inhalation anaesthesia 4% in air) from animals. To obtain serum from blood samples, the blood was centrifuged for 15 min at 600 g. In addition, for the homogenization of stomach tissues, the tissues were homogenized in 0.15 M Tris-HCl buffer (pH 7.4). Thereafter, the tissues were centrifuged for 10 min at 4°C and 2500 rpm. The supernatants obtained after centrifugation were kept until analyzed at -20°C.

Macroscopic investigation of stomach and determination of damage: After stomach tissue was extracted it was opened by cutting along the curvature line and was macroscopically displayed after rinsed in physiologic saline. Gastric lesions were assessed by blinded observers and scoring was (0, no lesion, 1-2 small lesions, 3-4 small ulcer, 5-6 big ulcer, 7 full ulcers)[15]. Digital images of the ulcer were obtained for the determination of gastric damage alterations and analysis was obtained by using a computer-based image analyser (iSolution FL ver 9.1, IMT i-solution Inc., Vancouver, BC, Canada) and total areas of ulcerated stomach parts were calculated as a square millimetre of the stomach mucosa.

Determination of gastric mucus: The methods of Rujjanawate *et al.*[16] and Ribeiro *et al.*[17] were taken as the basis for the determination of gastric mucus. For this purpose, some part of the stomach was immersed into the solution (pH 5.8) including 10 mL 0.02% Alcian blue and 0.16 M sucrose/0.05 M sodium acetate and was incubated at 25°C for 24 hrs and thereafter was centrifuged for 10 min at 3000 g and 4°C. The absorbance of supernatant taken was identified at 620 mm with a spectrophotometer and free mucus in the stomach content was calculated according to binding quantity (mg g^{-1} tissue) of Alcian blue.

Biochemical analysis: In the stomach tissue taken, Malondialdehyde (MDA)[18], reduced Glutathione (GSH)[19] levels, Superoxide Dismutase (SOD)[20] and Catalase (CAT)[21] activities and protein[22] levels were measured with spectrophotometrically by Shimadzu 1601-UV spectrophotometer (Tokyo, Japan). To identify PGE2 and NO the commercial BT-LAB (Shanghai, China) and Cayman (Michigan, USA) ELISA kits were used, respectively.

mRNA expressions of proinflammatory cytokines: Total RNA of the gastric was extracted and reversed transcribed using ABT™ Blood/Tissue RNA Purification Kit (Atlas Biotechnology, Ankara, Turkey), respectively following the manufacturer's protocols. Expression levels of NF-κB, Cox-2, iNOS, TNF-α, interleukin 1β (IL1-β) and interleukin-6 (IL-6) mRNA inside the prepared gastric tissues were determined by real-time PCR (StepOnePlus, Applied Biosystems). Primers were ordered from Sentegen Biotechnology (Ankara, Turkey) and shown in Table 1. Each sample was analyzed in triplicate. Also, normalization was performed according to the housekeeping gene β-actin expression level. The results are stated as relative gene expression using the delta-delta CT method[23].

Table 1: Description of polymerase chain reaction primers (NF-κB, Cox-2, iNOS, TNF-α, IL1-β, IL-6, and β-actin) and product size

Genes	Primer sequences	Product size (bp)
NF-κB	F: GTAGACAGCCCCATTCAGGG	
	R: CAGACCTGCTTGGAGAAGGG	179
Cox-2	F: TCGGAGCCCCAGATATAGCA	
	R: TTTCCGGCTAGAGGTGGGTA	184
iNOS	F: AACTTGTTTGCAGGCGTCAG	
	R: CACATTGCTCAGGGGATGGA	174
TNF-α	F: ATCCATCTCTTTGCGGAGGC	
	R: GGGGGAGAGGTAGGGATGTT	116
IL1-β	F: TGCCACCTTTTGACAGTGATG	
	R: TGATGTGCTGCTGCGAGATT	185
IL-6	F: CCCCAATTTCCAATGCTCTCC	
	R: CGCACTAGGTTTGCCGAGTA	195
β-actin	F: ACTGTCGAGTCGCGTCCA	
	R: TCATCCATGGCGAACTGGTG	164

Histopathological examination: Collected stomach tissues were fixed in 10% (v/v) solution and embedded in paraffin. Paraffin blocks were sectioned at 5 μm thickness using a microtome (RM-2125 RT, Leica, Nussloch, Germany). Tissue damage was investigated under the microscope (Nikon DS Fİ3, microscope digital camera systems, Tokyo, Japan) by Hematoxylin and Eosin (H and E) staining.

Statistical analysis: Statistical analyses of data were performed using the SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA) and data are expressed as mean±standard deviation (n: 7 per group). Data were analyzed by one-way analysis of variance (ANOVA) followed by *post hoc* Duncan's multiple range test. The p-values less than 0.05 were considered significant. Significant differences were pointed with different alphabetical letters.

RESULTS

Changes in gastric mucosa: Macroscopic changes were not observed in the stomach of the mice in control and DMSO groups Fig. 1(a-b) while in stomach mucosa of the mice exposed to ethanol/HCl treatment focal hemorrhagic ulcerative lesions (necrobiotic changes and epithelium cell loss in stomach mucosa) was found (Fig. 1c). When gastric mucosa of the group treated with omeprazole was investigated it was determined that the medicine has a protective effect on alcohol-induced gastric damage (Fig. 1d). However, when the stomach mucosa of the magnolin groups was investigated, it was observed Fig. 1(e-g) that the damage was decreased significantly ($p<0.001$) for the mice administered with magnolin in 3 different doses of (2.5, 5 and 10 mg kg^{-1}) compared to the ethanol group. By scoring

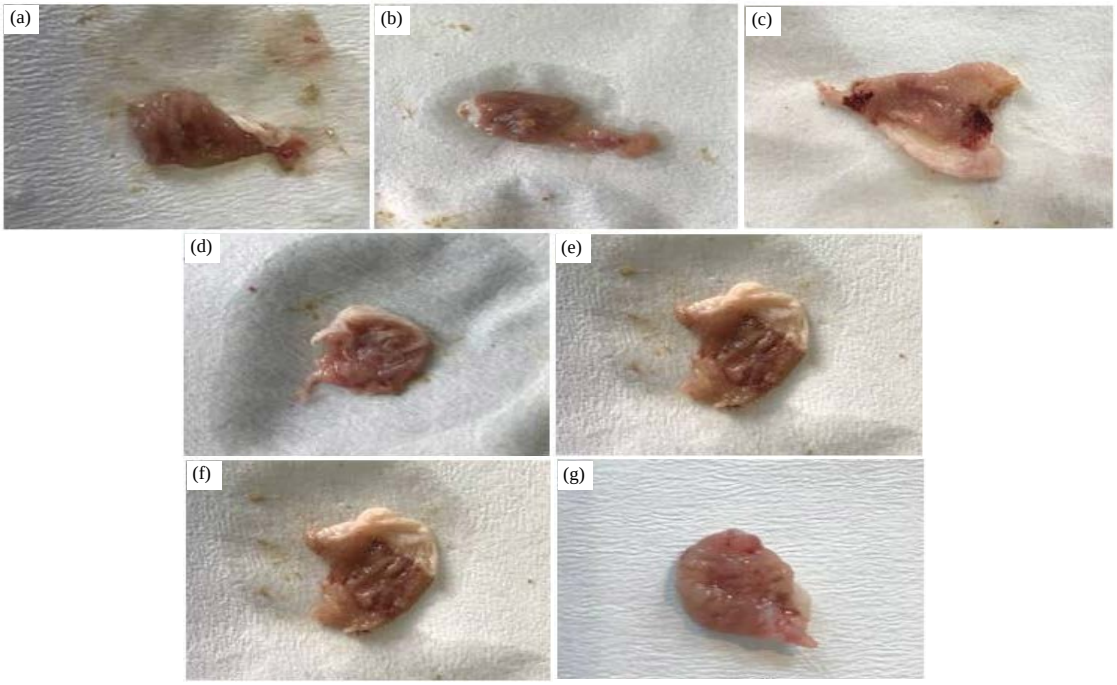


Fig. 1(a-g): Macroscopic evaluation of the effect of magnolin on ethanol/HCl-induced gastric ulcer in mice
(a) Mice were treated with physiologic saline (p.o.), (b) DMSO (0.1% p.o), (c) 1 hr before of the oral administration of 98% ethanol/0.15 M HCl (0.2 mL/40 g), (d) Omeprazole (20 mg kg^{-1}; p.o.), (e) 2.5, (f) 5 and (g) 10 mg kg^{-1} magnolin (p.o.)

(Fig. 2a) and damage investigation (Fig. 2b) it was found that gastric mucosal damage and lesion area in the alcohol group were more compared to the normal control group, however, the oral administration of magnolin in doses of 2.5, 5 and 10 mg kg^{-1} decreased the gastric mucosal damage and lesion areas when compared with alcohol group ($p<0.001$). When compared mucosal context (Fig. 2c) to normal control mice it was observed that gastric mucosal content of the alcohol group was significantly decreased ($p<0.001$), but gastric mucosal content for the groups administered with magnolin (2.5, 5 and 10 mg kg^{-1}) was higher than alcohol group ($p<0.001$).

Changes in biochemical parameters: It was found that the alcohol treatment increased MDA levels (Fig. 3a) in gastric tissue compared with the control group ($p<0.001$). In contrast, the magnolin treatment decreased the MDA levels compared with the alcohol group ($p<0.001$) depending on the increasing dose. On the other hand, it was found that the alcohol treatment decreased ($p<0.001$) GSH levels in gastric tissue (Fig. 3b) when compared with the control group, nevertheless the magnolin treatment in a dose-dependent manner increased the GSH levels ($p<0.001$) when compared to the alcohol group. In addition to these, it was observed that the alcohol treatment decreased SOD (Fig. 3c) and CAT (Fig. 3d) activities in gastric tissue when compared with the control group ($p<0.001$), the magnolin treatment improved these enzyme activities when compared with the alcohol group ($p<0.001$). It was found that alcohol treatment decreased gastric PGE2 (Fig. 3e) and NO (Fig. 3f) levels when compared with control ($p<0.001$) but, the magnolin treatment increased PGE2 and NO levels when compared with alcohol depending on the increasing dose ($p<0.001$). In addition, it was observed that DMSO treatment merely didn't affect these parameters when compared with control, omeprazole treated as positive control returned these parameters to control.

mRNA expression levels in gastric tissue: When mRNA expression fold increases of proinflammatory genes in gastric tissue were investigated, NF-κB (Fig. 4a), Cox-2 (Fig. 4b), iNOS (Fig. 4c), TNF-α (Fig. 4d), IL1-β (Fig. 4e) and IL-6 (Fig. 4f) mRNA expression levels were found more in the alcohol group compared to control groups ($p<0.001$). However, it was found that mRNA expressions of these genes in groups administered with magnolin in doses of 2.5, 5 and 10 mg kg^{-1} were decreased compared to the alcohol group ($p<0.001$). It was determined that these values in the omeprazole group which is the positive group approached the control group values and that these parameters in the DMSO group were not statistically different than control group parameters.

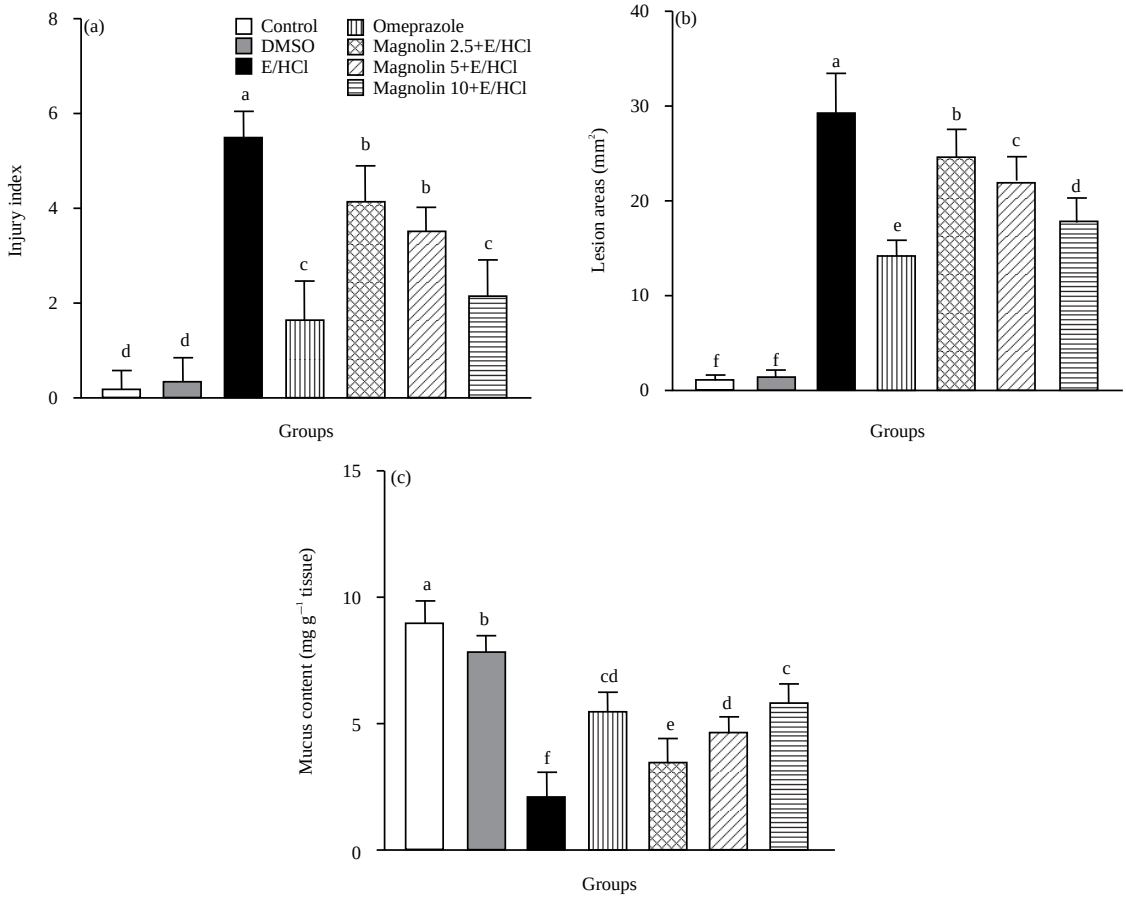


Fig. 2(a-c): (a) Effect of magnolin on gastric injury score, (b) Total lesion areas (mm^2) and (c) Mucus content against ethanol/HCl-induced gastric damage in mice

Mice were treated with physiologic saline (p.o.), DMSO (0.1% p.o), 2.5, 5, and 10 mg kg^{-1} magnolin; p.o., or omeprazole (20 mg kg^{-1}; p.o.)1 hr before of the oral administration of 98% ethanol/0.15 M HCl (0.2 mL/40 g). The results are presented as the Mean±SD 7 mice per group. Letters (a, b, c, d, e and f) show statistically significant differences between all groups and the control group ($p<0.001$)

Histopathological changes: Histopathological changes were not observed in the control (Fig. 5a) and DMSO (Fig. 5b) groups. However, acute superficial epithelial damage in gastric mucosa, bleeding, mononuclear cell infiltration and edematous changes in the mucosa ($p<0.001$) were determined after ethanol/HCl treatment as shown in Fig. 5c. These observed changes were decreased in the omeprazole group (Fig. 5d) comparing to the alcohol group and gastric mucosa was little affected by the damage. On the other hand, it was found that these microscopic lesions were decreased in magnolin treatments at doses of 2.5 (Fig. 5e), 5 (Fig. 5f) and 10 (Fig. 5g) mg kg^{-1} compared to the alcohol group ($p<0.001$).

DISCUSSION

Many incidences of the side effects of the medicines used in the treatment of gastric ulcers accompanies also this failure in this treatment process. It was reported the use of natural compounds (like hesperidin and quercetin) in the treatment of gastric ulcers because of the safety and lower side effects besides the use of traditional medicines[16]. But, the ulcer preventive activity of the magnolin having antioxidant[11] and anti-inflammatory effect[12] was not enlightened before. For this reason, for the first time, the current study illuminates the potential gastroprotective activity of the magnolin against an ethanol-induced acute gastric ulcer in the mice.

Alcohol intake is one of the most common reasons for the gastric ulcer for humans, ethanol/HCl induced gastric ulcer is a good model describing this instance[17]. It is possible to observe mucus secretion and decrease in antioxidant activity but it is also possible to observe mucosal ulceration, bleeding, lipid peroxidation and inflammation in ethanol/HCl induced gastric damage[24,25]. In this study presented it was found similarly that ethanol/HCl induced gastric ulcer model has

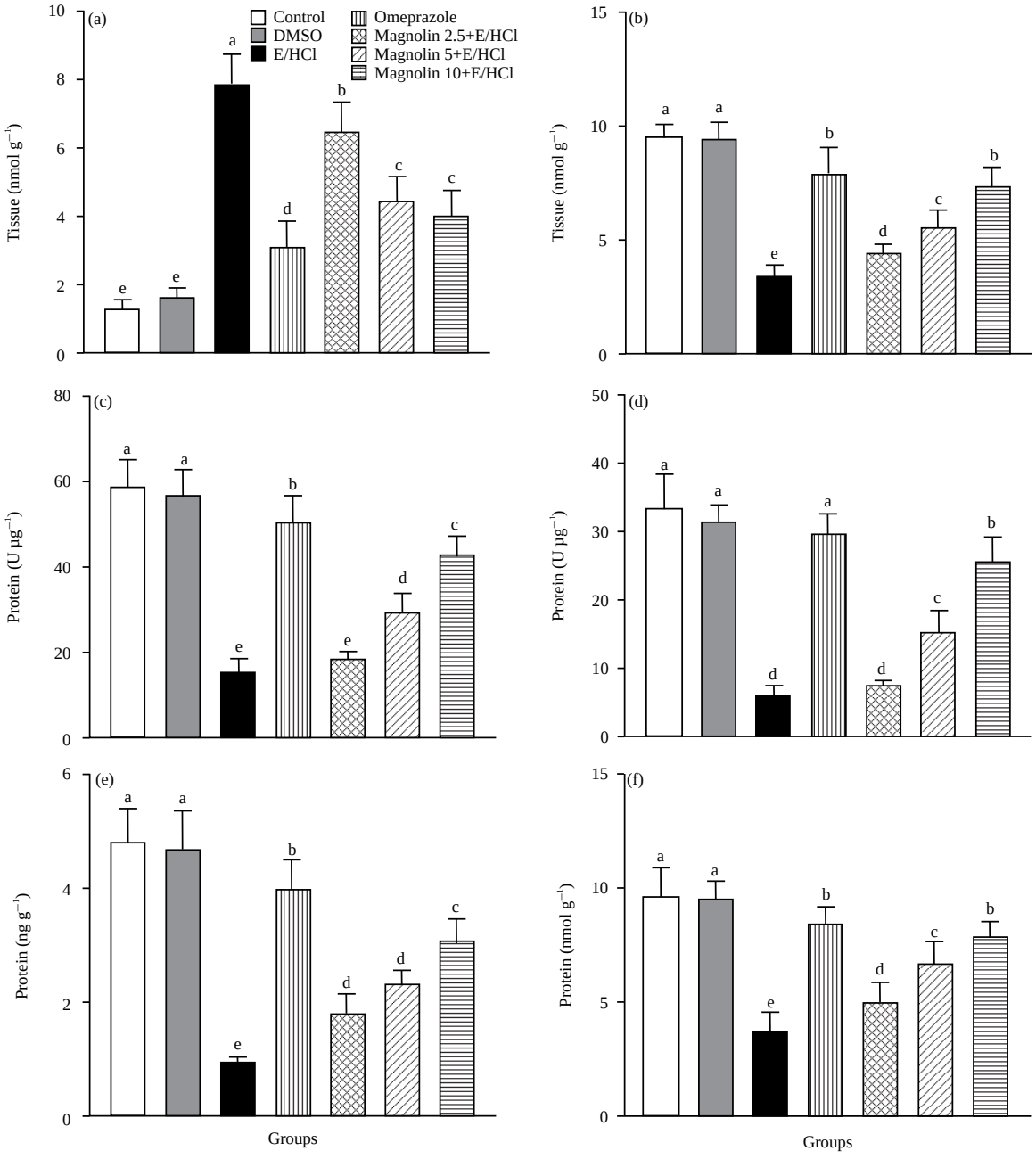


Fig. 3(a-f): Effect of magnolin on different levels

(a) Malondialdehyde (MDA), (b) Reduced glutathione (GSH), (c) Superoxide dismutase (SOD), (d) Catalase (CAT), (e) Prostaglandin E2 (PGE2) and (f) Nitric oxide (NO) levels against ethanol/HCl-induced gastric damage in mice. Mice were treated with physiologic saline (p.o.), DMSO (0.1% p.o), 2.5, 5 and 10 mg kg^{-1} magnolin (p.o.) or omeprazole (20 mg kg^{-1}; p.o.) 1 hr before of the oral administration of 98% ethanol/0.15 M HCl (0.2 mL/40 g). The results are presented as the Mean±SD of 7 mice per group. Letters (a, b, c, d, and e) show statistically significant differences between all groups and the control group ($p<0.001$)

indicated the designated effects in the mice, nevertheless, it was found that the magnolin known as having bioactivity has gastric protective and anti-ulcerative effects in mice. Obtained data showed that oral administration of ethanol/HCl to mice created necrobiotic areas in gastric mucosa. On the other hand, it was observed that magnolin treatment decreased ulcer area significantly as well as epithelium cell loss and oedema depending on the increasing dose in animals.

It is known that ethanol induces the production of free radicals stimulating inflammation by the secretion of proteolytic enzymes, lipoxygenases and releasing of signal

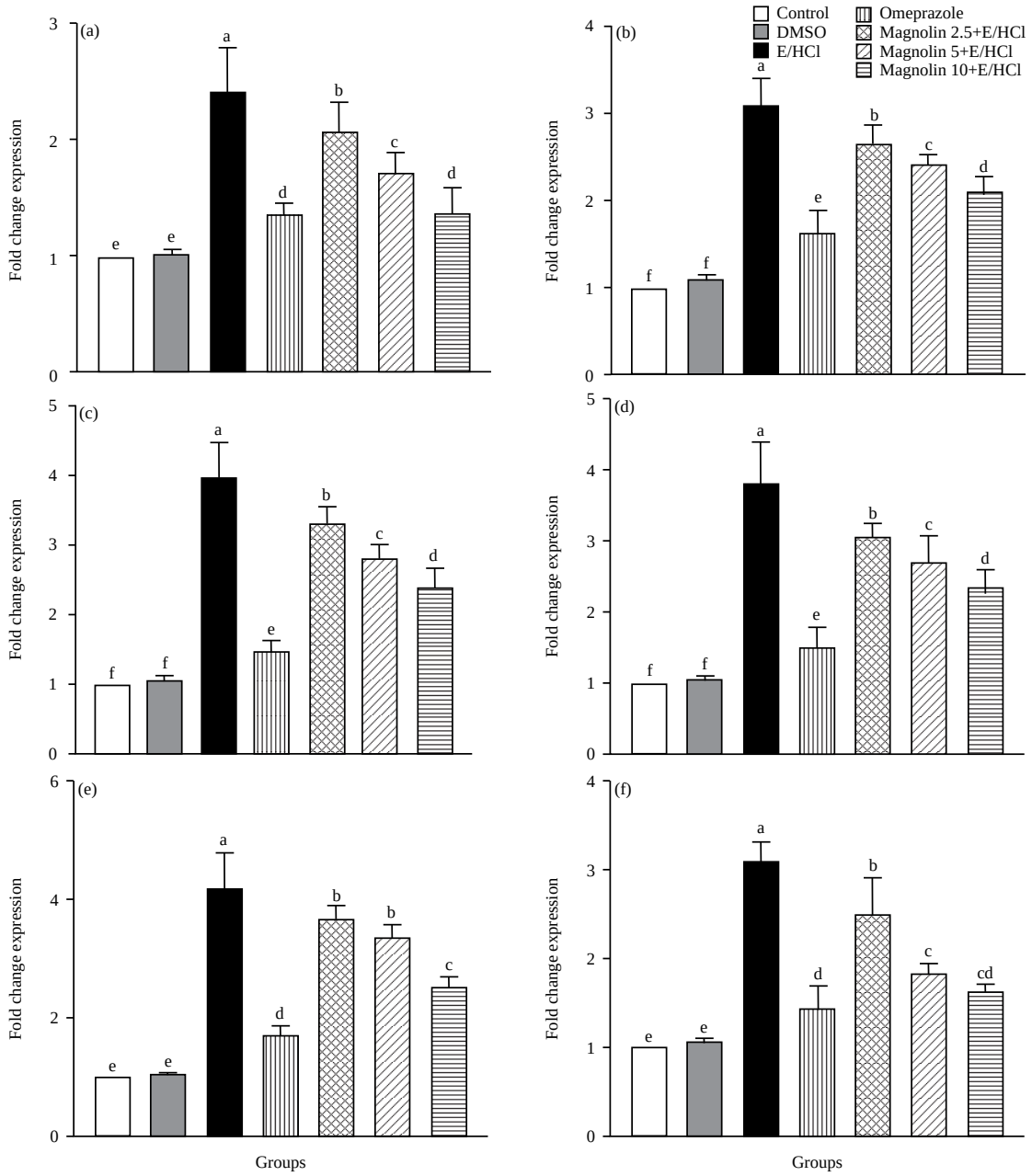
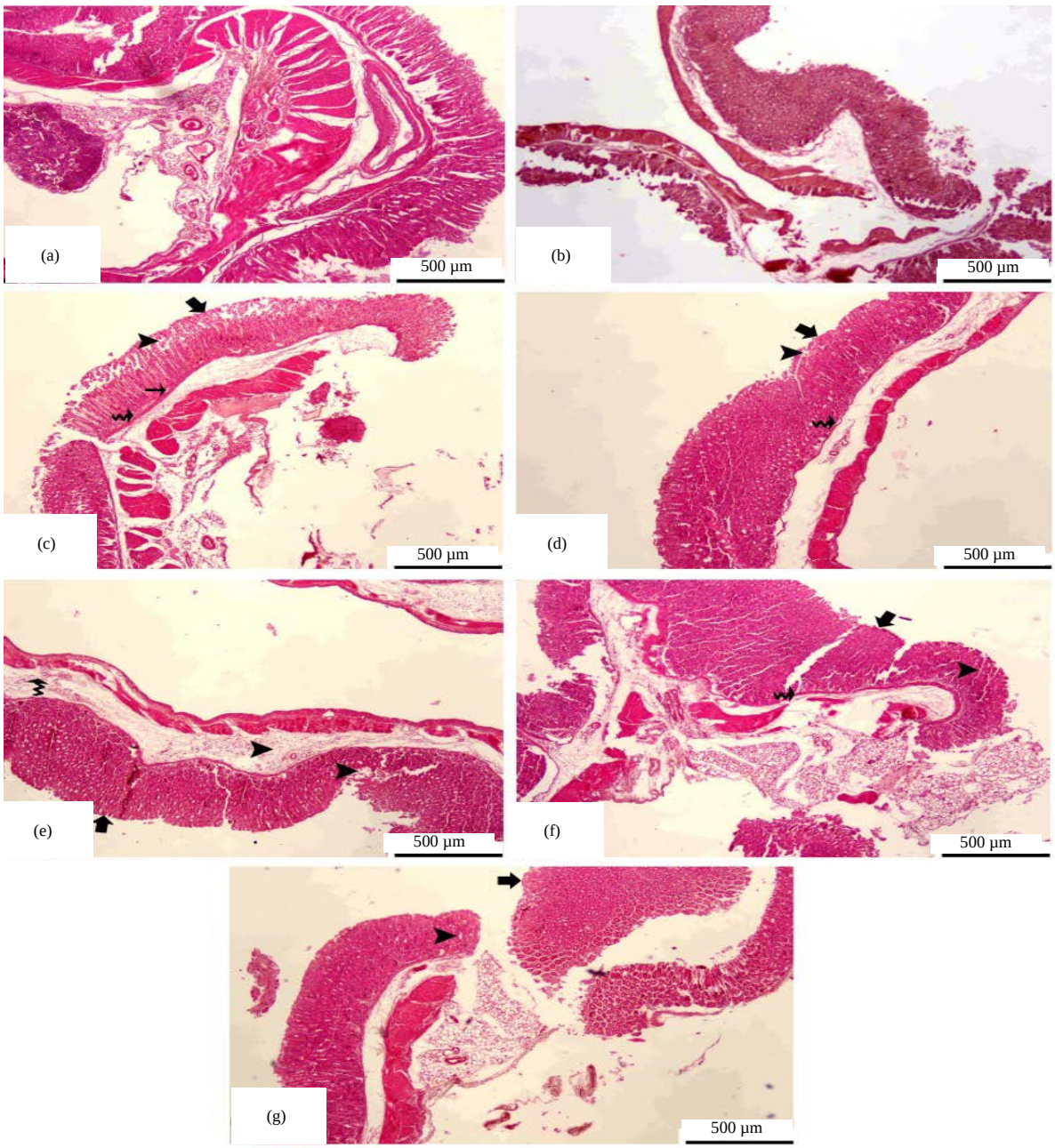


Fig. 4(a-f): Effect of magnolin on different expression level

(a) mRNA expression levels of NF-κB, (b) Cox-2, (c) iNOS, (d) TNF-α, (e) IL1-β and (f) IL-6 against ethanol/HCl-induced gastric damage in mice. Mice were treated with physiologic saline (p.o.), DMSO (0.1% p.o), 2.5, 5 and 10 mg kg^{-1} magnolin (p.o.) or omeprazole (20 mg kg^{-1}; p.o.)1 hr before of the oral administration of 98% ethanol/0.15 M HCl (0.2 mL/40 g). The results are presented as the Mean±SD 7 mice per group. Letters (a, b, c, d, and e) show statistically significant differences between all groups and the control group ($p<0.001$)

proteins[26]. It is observed that by the alcohol treatment, free radicals were produced in gastric tissue and GSH levels and antioxidants like SOD and CAT known as having protective effects against damages induced by oxidative stress together with increasing lipid peroxidation products such as MDA were decreased[25-28]. Similarly, in our study, it was found that MDA was increased in the mice administered with alcohol nevertheless GSH level and SOD and CAT activities were inhibited. But it was observed that the magnolin treatment improved increased lipid peroxidation and decreased oxidant effect induced by alcohol. These results show that antioxidant enzymes prevent partly oxidative damage and play an essential role in suppressing gastric damage. It is known that PGE2 increases mucosal blood flow by inducing gastric mucus



Tissue	Histopathological change	Control	DMSO	E/HCl	Omeprazole	Magnolin 2.5+E/HCl	Magnolin 5+E/HCl	Magnolin 10+E/HCl
Fundus	Epithelial loss	0.00±0.00^{c}	0.00±0.00^{c}	4.06±0.51^{a}	0.61±0.04^{c}	2.66±1.63^{b}	1.13±1.86^{c}	0.73±1.79^{c}
	Hemorrhage	0.00±0.00^{d}	0.00±0.00^{d}	3.56±0.98^{a}	0.33±0.05^{d}	2.01±0.97^{bc}	1.53±1.83^{c}	0.46±0.72^{d}
	Mononuclear cell infiltration	0.00±0.00^{d}	0.00±0.00^{d}	3.56±0.75^{a}	0.50±0.05^{cd}	2.16±1.13^{b}	1.03±1.18^{c}	1.86±2.17^{cd}
	Edema	0.00±0.00^{b}	0.00±0.00^{b}	2.90±0.54^{a}	0.66±0.06^{bc}	2.01±0.97^{a}	1.86±2.17^{a}	0.70±0.76^{b}

Fig. 5(a-g): Histopathologic and statistical evaluation of the effect of magnolin on ethanol/HCl-induced gastric ulcer in mice

(a) Mice were treated with physiologic saline (p.o.), (b) DMSO (0.1% p.o), (c) 1 hrs before of the administration of 98% ethanol/0.15 M HCl (0.2 mL/40 g), (d) Omeprazole (20 mg kg^{-1}; p.o.), (e) 2.5, (f) 5 and (g) 10 mg kg^{-1} magnolin (p.o.). Representative figures were stained with hematoxylin and eosin. The original magnification was ×4 and the scale bars represent 500 µm. Bold, thin and curved arrows and arrowheads indicate epithelial loss, haemorrhage, areas of mononuclear cell infiltration and oedema, respectively. In statistical evaluation, letters (a, b, c, and d) show statistically significant differences between all groups and the control group ($p<0.05$)

secretion, protects mucosal cells against ulcers and speeds up mucosal healing[29]. On the other hand, ethanol treatment decreases gastric mucosal PGE2 content[27,30]. In the study, ethanol/HCl treatment decreased gastric PGE2 levels. Nevertheless, magnolin treatment improved alcohol-induced effects in PGE2 levels. These results indicate that magnolin has gastric protective effects against gastric ulcers by increasing PGE2 quantity. It was reported that NO can protect the mucosa layer by increasing secretion of mucous and bicarbonate in the gastrointestinal mucosa, improving gastric blood flow and circulation and decreasing neutrophil infiltration[31]. In the study conducted in line with other studies[30-33] related to NO levels in gastric tissues, NO levels were decreased significantly by the alcohol treatment but NO level was increased significantly in the mice administered with magnolin. These results show that magnolin treatment can increase tissue NO level by improving the antioxidant defence system.

It is known that ethanol treatment activates the immune response and increases proinflammatory cytokine levels[34]. Relief of gastric ulceration is realized by inhibiting NF-κB activation in mucosal inflammation[35]. It was reported from some studies that Cox-2 regulating inflammatory reactions by mediating prostaglandins synthesis related with NF-κB gene and IL-1β, IL-6, TNF-α appearing during gastric damage and iNOS expressions were increased in the alcohol-induced gastric ulcer cases[15,30,36]. It was reported that neolignan compounds like honokiol, magnolol and obovatol isolated from *Magnolia* plant having similar compounds show their effects by inhibiting IκB phosphorylation of NF-κB[7,37], in addition, 4-O-methylhonokiol suppresses ear oedema and iNOS and Cox-2 expression accompany to this effect as well as suppression effect on NF-κB[7]. In our study, gastric NF-κB, Cox2, IL-1β, IL-6, TNF-α and iNOS mRNA expression levels significantly increased in mice exposed to alcohol. On the other hand, significant downstream regulation was found in gastric NF-κB, Cox2, IL-1β, IL-6, TNF-α and iNOS mRNA expression levels in groups treated with magnolin compared to the alcohol group. Our results show that magnolin treatment decreases inflammatory reaction by modulating the NF-κB signal pathway and hence may have an anti-inflammatory effect.

In line with other studies[15,16,30], after ethanol/HCl treatment, it was found histopathologically acute surface epithelium damage, bleeding, mononuclear cell infiltration in fundus and alterations with oedema in the mucosa. Contrary to this, it was found that alcohol-induced microscopic ulcerative lesions were decreased distinctively by the magnolin treatment. These results describe that the magnolin has a protective effect against alcohol-induced gastric ulcers in the mice model.

CONCLUSION

As a result, in the current study, it was determined that magnolin has a therapeutic effect in gastric damage by re-balancing oxidant/antioxidant production and by regulating downstream NF-κB mediated proinflammatory cytokine production against the ethanol/HCl induced gastric damage.

SIGNIFICANCE STATEMENT

Magnolin is a phytochemical compound and it exhibits anti-inflammatory and antioxidative activities. In this study, magnolin inhibited enhanced gastric ulcers in mice. In addition, by this study, it was proven that magnolin compound which is a biologically active substance may have a potential possibility for being used as a gastroprotective agent.

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