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ORIGINAL ARTICLE

Gastroprotective Effect of Formononetin against Ethanol-Induced Gastric Ulceration in Rats via Augmentation of Cytoprotective Markers and Curtailing Apoptotic Gene Expression

Alauddin[1,2], Swati Chaturvedi[3,4], Lubna Azmi[2], Ila Shukla[2], Zaiba Naseem[5], Chandana Venkateswara Rao[2], Naresh Kumar Agarwal[1]

[1]Department of Zoology, HNB Garhwal Central University, Campus Badshahithaul Tehri Garhwal, Uttarakhand, [2]Division of Pharmacognosy and Ethnopharmacology, CSIR-National Botanical Research Institute, [3]Department of Pharmaceutics, Babasaheb Bhimrao Ambedkar University, [4]Division of Pharmaceutics and Pharmacokinetics, CSIR Central Drug Research Institute, [5]Department of Pharmaceutics, Faculty of Pharmacy, Integral University, Lucknow, Uttar Pradesh, India

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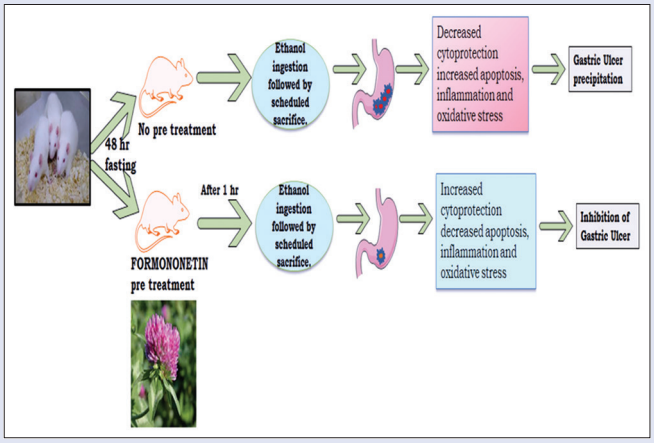
ABSTRACT

Background: Formononetin (FMN), one of the major isoflavones in red clover, has been shown to possess antioxidant, anti-inflammatory, antitumor, neuroprotective, and cytoprotective activities. However, there is no report on the gastroprotective effect of FMN against ethanol-induced gastric ulcer. **Objective:** Excessive alcohol consumption can lead to gastric ulcer, and the purpose of the present study was to examine the protective effect of FMN on mucosal lesions induced by ethanol. **Materials and Methods:** Fasted rats were orally administered with FMN at different doses, omeprazole (20 mg/kg), followed by intragastrical ingestion of ethanol (5 ml/kg) after 1 h and sacrificed after 1 h of exposure. Gross microscopic, macroscopic, and biochemical assays were scrutinized. **Results:** Compared with ethanol, FMN pretreatment showed a significant increase in the gastric levels of glutathione while decreased the malondialdehyde content remarkably. FMN pretreatment also bestowed the cytoprotective efficacy against ethanol-induced ulceration by reestablishing the decreased level of nitrite (NO). Furthermore, in histopathological sections, reduced pathological changes of gastric lesions were markedly observed in the FMN-pretreated groups compared with those in the ethanol group. Western blot analysis showed upregulation of BcL_2 while downregulation of Bax in FMN-pretreated gastric tissue of rats. **Conclusion:** These results indicate that FMN exerts gastroprotective effects through the antioxidative, anti-inflammatory, and antiapoptotic that are probably mediated by enhanced NO release, suggesting its therapeutic use to treat gastric ulceration by preserving mucosal glycoproteins and diminishing oxidative stress.

Key words: Apoptosis, cytoprotection, formononetin, gastric ulcer, oxidative stress

SUMMARY

- FMN is found to be highly potent against ethanol-induced gastric ulcer
- FMN decreased the oxidative stress and increased the cytoprotection through enhancement of nitrite levels
- The isoflavone is also found to decrease both inflammation and apoptosis in gastric tissue after ethanol ingestion
- Therefore, FMN exerts anti-inflammatory and cytoprotective effect along with acting as an antioxidant and depletion of apoptosis in gastric tissue.



Abbreviations used: NSAIDs: Nonsteroidal anti-inflammatory drugs; FMN: Formononetin; CMC: Carboxymethylcellulose; UI: Ulcer index; MDA: Malondialdehyde; GSH: Reduced glutathione; NO: Nitrite; TNF-α: Tumor necrosis factor-alpha; Hgb: Hemoglobin; T-RBC: Total red blood cells; Hct: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; TLC: Total leukocyte count

Correspondence:

Dr. Chandana Venkateswara Rao,
Division of Pharmacognosy and Ethnopharmacology,
CSIR-National Botanical Research Institute,
Lucknow, Uttar Pradesh, India.
E-mail: chvrao@yahoo.com
Prof. Naresh Kumar Agarwal,
Department of Zoology, HNB Garhwal Central University, Campus Badshahithaul, Tehri Garhwal, Uttarakhand, India.
E-mail: agarwalnareshk3@rediffmail.com
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INTRODUCTION

Persistent exposure of the gastric tract to a number of substances such as hydrochloric acid and digestive enzymes causes epithelial damage.[1] Mucosal injury occurs when these noxious factors destroy an intact mucosal layer or when it gets impaired.[2] Peptic ulcer, one of the most common and life-threatening diseases of the gastrointestinal tract, occurs due to imbalance between the offensive (e.g., acid, pepsin,

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Helicobacter pylori infection, smoking, and excessive alcohol intakes) and defensive mechanisms (e.g., secretion of bicarbonate, mucus, nitric oxide, growth factors, heat-shock proteins, and continuous blood flow and prostaglandins) of the body.[3] The complexity of the disease involves bleeding, penetration, perforation, and gastric outlet obstruction, among which the most common complication is hemorrhage affecting about 15% of the population with peptic ulcer.[4,5]

Various models have been developed for the induction of gastric ulcer including acetic acid, anti-inflammatory drugs, and ethanol.[6] Excessive ethanol ingestion may also develop gastric ulcer[7] by damaging the vascular endothelium lining of the stomach and aggravates inflammatory responses causing ischemia.[8]

Nonsteroidal anti-inflammatory drugs and smoking have been considered to be the main cause of peptic ulcer; hence, limited research has been focused on the lead molecules to be developed as an antiulcer agent.[9] Drugs such as antacids, anticholinergics, H_2-receptor antagonists, and proton pump inhibitors have been developed synthetically that act specifically for preventing or treating gastric ulcers. However, nowadays, these drugs show symptomatic relief with many side effects such as diarrhea, dry mouth, gastric discomfort, indigestion, flatulence, back pain, pruritis, skin rash, sleepiness, and dizziness. Therefore, there is a need of novel nontoxic, natural antiulcer candidates from medicinal plants.[10]

In recent years, flavonoids have gained the interest of researchers because they have promising powerful antioxidant properties and improve mitochondrial bioenergetics that protects the human body from free radicals by their hydrogen-donating abilities.[11] Formononetin (FMN), chemically known as 7-hydroxy-3-(4-methoxyphenyl) chromen-4-one [Figure 1], is one of the major methoxylated isoflavones found in red clover and in commercially available extracts of this plant. Apart from red clover, it also occurs in many leguminous and *Fabaceae* plants (e.g., green beans, soy, and lima beans).[12] It is well known that polyphenols such as flavones and isoflavones possess potential antioxidant activity and free radical scavenging capacity.[13,14] Various researches revealed the antioxidant, anti-inflammatory, antitumor, gastroprotective, neuroprotective, and cytoprotective activity of isoflavones such as morin, diosmin, and quercetin and its glycoside.[15-18] FMN has also been shown to exhibit anti-inflammatory and antioxidant activity as it has been revealed through various *in vitro* and *in vivo.*[13,19,20] The hydroalcoholic extract of *Andrographis paniculata* containing various flavonoids, including FMN in higher proportion, has been shown to inhibit the acid-secreting hydrogen potassium pump, indicating the antisecretory and gastroprotective efficacy of FMN.[21]

Furthermore, the antioxidant activity of FMN has been studied in some organs and serum, but its activity on gastrointestinal tract has been inadequately explored. Hence, the present study aims to evaluate the pharmacological efficacy of FMN as a gastroprotective agent against ethanol-induced gastric ulcer.

MATERIALS AND METHODS

Drugs and chemicals

FMN was procured from TCI Chemicals (India) Pvt. Ltd. Omeprazole reference drug was purchased from Alfa Aesar (India). The rat tumor necrosis factor (TNF-α) ELISA Kit (catalog No. EZRTNFA) was procured from Merck.

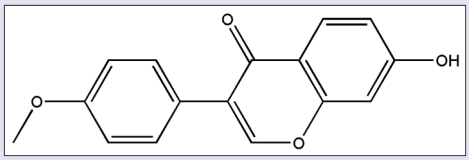

Figure 1: Chemical structure of formononetin

Animals

Adult female Sprague–Dawley rats (200–240 g) obtained from the Institutional Animal Facility (National Laboratory Animal Centre) were used in this study. The animals were housed in pathogen-free and climate-controlled environment conditions; room temperature maintained at 24°C ± 2°C and relative humidity at 40%–60% with *ad libitum* access to food and water. All animal studies were approved by the Institutional Animal Ethics Committee following the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals.

Experimental design

Acute toxicity

The acute toxicity studies were performed according to the fixed-dose method of Organization for Economic Co-operation and Development (2002) Guideline No. 420.[22] A single dose of FMN (2 g/kg body weight) was administered orally to groups of animals after a 12-h fasting. Animals receiving vehicle (0.25% carboxymethylcellulose [CMC]) served as control. The general, behavioral, neurological, and autonomic behavior of the animal models was observed at 0, 30, 60, 120, 180, and 240 min after and then once a day for the next 14 days. At the end of the period, the number of survivors was recorded and the acute toxicological effect was estimated.

Induction of gastric ulcer

Animals were randomly allocated to six groups (n = 6) deputed as I–VI fasted 48 h before receiving the treatment. Group I served as sham control (0.25% CMC suspension, 1 ml/kg p.o.), Group II (ulcer control receiving absolute ethanol, 5 ml/kg p.o.), Group III (positive control treated with absolute ethanol, 5 ml/kg p.o + omeprazole, 20 mg/kg in 0.25% CMC suspension), Group IV (absolute ethanol, 5 ml/kg p.o. + FMN, 25 mg/kg p.o.), Group V (absolute ethanol, 5 ml/kg p.o. + FMN, 50 mg/kg p.o.), and Group VI (absolute ethanol, 5 ml/kg p.o. + FMN, 100 mg/kg p.o.). The doses were selected after the observed results of acute toxicity study. Previous studies revealed that FMN showed rapid systemic absorption after oral administration to rats at a dose of 50 mg/kg.[12] Hence, authors decide to administer FMN at three different doses, <50 mg/kg (i.e., 25 mg/kg), 50 mg/kg, and >50 mg/kg (i.e., 100 mg/kg). Ethanol was administered after 60 min of treatment with FMN. After 1 h, rats were anesthetized under light ether anesthesia and blood samples were collected through the retro-orbital plexus into heparinized microcentrifuge tubes. Plasma was harvested from the blood samples followed by centrifugation at 13,000 g for 10 min on Sigma 1-15K (Frankfurt, Germany) and stored frozen at −70°C ± 10°C till analysis. Eventually, animals were sacrificed using light ether anesthesia followed by cervical dislocation and the stomach tissues were evacuated safely.

Estimation of gastric acid secretion: pH and ulcer index

The determination of gastric ulcer index (UI) and pH was performed using the method described previously.[23] The stomach samples were removed subsequently collected the gastric content and drained into a graduated microcentrifuge tube and centrifuged at 2000 ×g for 15 min. The supernatant volume and pH were recorded with a digital pH meter. The degree of gastric mucosal damage was evaluated for gross pathology according to a 0–5 scoring system based on the number and severity of gastric lesions as previously described.[24] The mean score was calculated and expressed as the UI. The percentage of inhibition was calculated by the following formula:

% inhibition = $[(UI_{ethanol\ control} - UI_{treated})/UI_{ethanol\ control}] \times 100$.

Estimation of in vivo oxidative stress markers

Rat stomach was homogenized in ice-cold Tris-EDTA buffer, pH 7.4, and tissue homogenate was used for further *in vivo* biochemical estimation described below.

Estimation of malondialdehyde

The quantification of malondialdehyde (MDA) was done according to the method described by Mihara and Uchiyama, 1978 with slight modifications.[25] Briefly, homogenate was mixed with 30% trichloroacetic acid and 2% thiobarbituric acid, and mixture was boiled in water bath at 90°C for 15 min. After centrifugation (1500 g, 10 min), the supernatant was read at 532 nm ELISA plate reader (BIOTEK, USA) and concentration of MDA was expressed as nanomole per microgram protein (nm/μg).

Estimation of reduced glutathione content

Estimation of reduced GSH was done according to the protocol described earlier.[26] Briefly, the homogenate was mixed with 0.1 M sodium phosphate buffer (pH 8.0) and 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid and incubated at room temperature for 10 min, resulting in an intense yellow color product that was read at 412 nm using ELISA plate reader (BIOTEK, USA). The GSH concentration (μM of GSH/μg protein) was enumerated from the standard curve prepared with GSH.

Estimation of nitrite content in stomach tissue

Levels of nitrite (NO) were arbitrated by measuring nitrite accumulation using Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylene diamine dihydrochloride in 5% H_3PO_4). Supernatant of homogenate samples and Griess reagent were mixed in equal amount (150 μl) and incubated at 37°C for 20 min. The test mixture was subsequently read on an ELISA plate reader (BIOTEK, USA) at 540 nm; using sodium nitrite was used to prepare a standard curve.[27]

Estimation of nitrite content in plasma

Levels of NO content in the plasma were measured using Griess reagent according to the method described above. In brief, the plasma and Griess reagent were mixed in equal quantity followed by incubation at 37°C for 20 min. The test mixture was subsequently read on an ELISA plate reader (BIOTEK, USA) at 540 nm; using sodium nitrite was used to prepare a standard curve.

Estimation of tumor necrosis factor-α in stomach tissue

The levels of TNF-α were assessed using a rat TNF-α ELISA kit. The methodology was according to the same. Briefly, the microtiter plate was washed four times using diluted wash buffer. TNF-α standard solution and samples to be assayed were added at the quantity described. The plate was incubated for 2 h with constant shaking and then washed four times. Detection antibody was affixed (1 h) and the plate was again incubated for 30 min after addition of avidin-HRP D with constant shaking succeeded by washing in the similar pattern. Again the plate was washed and substrate solution F was enumerated, after 15 min, the stop solution was added. The absorbance was read within 30 min at 450 nm.

Histopathological analysis

Four percent formalin-fixed gastric mucosal tissues were embedded in paraffin after gradient dehydration. Paraffin wax tissue blocks were cut at 5 μ thickness by sledge microtome. The obtained tissue sections were deparaffinized and stained by hematoxylin and eosin (H and E) for histopathological examination through the light microscope.

Western blot analysis

Western blot analysis was carried out for selected markers Bax and Bcl_2. Briefly, tissues were lysed using CelLytic M Cell Lysis Reagent (catalog no. C2978, Sigma, USA) containing 1X protein inhibitor cocktail (catalog no. P8340, Sigma, USA). Protein estimation was done by Bradford Protein Assay (catalog no. 23236, Thermo Scientific, USA). Equal amount (50 μg/well) of proteins was loaded in 10% Tris-SDS gel and blotted on polyvinylidene fluoride membranes using wet transfer system. After blocking for 2 h at 37°C, the membranes were incubated overnight at 4°C with anti-protein primary antibodies specific for Bax and Bcl_2 (1:1000 Millipore, USA) and β actin (1:5000 Millipore, USA). β-actin was used for normalization of data.

Statistical analysis

All data were presented as mean ± standard error mean and analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests for the possible significance identification between the various groups. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered as statistically significant. Statistical analysis was performed using GraphPad Prism software (5.02) (Trial Version), San Diego, CA, USA.

RESULTS

Acute toxicity evaluation

During the 14 days, there were no physiological or behavioral changes or mortality was observed in rats treated with 2 g/kg FMN. The administered dose (2 g/kg) did not produce any visible signs or symptoms of toxicity such as diarrhea, weakness, tremors, seizures, or loss of controlled movement in the treated animals. The effect of

Table 1: Effect of formononetin on hematological parameters in rats

Groups	Red blood cells						TLC ($\times10^3$/mm^3)	Platelets ($\times10^3$/mm^3)
	Hgb (g %)	T-RBC ($\times10^6$/mm^3)	Hct (%)	MCV (m^3)	MCH (pg)	MCHC (g %)		
Control	14.5±0.13	7.56±0.07	43.70±0.49	57.83±0.26	19.25±0.11	33.25±0.23	15.95±1.4	849.00±45.13
FMN (2000 mg/kg)	14.60±0.26	7.53±0.1	43.14±0.92	57.30±0.34	19.40±0.24	33.86±0.29	14.65±0.76	877.75±28.17

FMN: Formononetin; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; TLC: Total leukocyte count; Hgb: Hemoglobin; T-RBC: Total red blood cells; Hct: Hematocrit

Table 2: Effect of formononetin on renal function biochemical parameters in rats

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Phosphorus (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)
Control	47.97±2.26	0.60±0.02	12.34±0.31	4.47±0.13	130.67±2.03	0.99±0.08
FMN (2000 mg/kg)	53.64±2.35	0.61±0.01	10.37±2.01	4.77±0.12	130.4±1.03	1.04±0.02

FMN: Formononetin

FMN on various serum and plasma biochemical parameters was also examined, and no significant changes were observed in comparison to control [Tables 1 and 2].

Effect of formononetin on gastric acid secretion: Gastric ulcer index and pH

The prime role of gastric acid is to act as a defensive agent against numerous gastrointestinal diseases. Considering the same, gastric acid secretion, i.e., gastric pH and gastric content, was evaluated. As illustrated in Table 3 and Figure 2, pretreatment with FMN restored the normal strength of stomach and gastric UI due to exposure to ethanol in different degree. The pH in the positive model was declined to 1.9 (units) which were elevated by the administration of FMN at different doses by 2.37, 2.44, and 3.47 folds when compared to the ulcer model.

Effect of formononetin on various oxidative stress markers in ethanol-induced gastric ulcer model

The stomach tissue was further scrutinized for the oxidative stress markers [Table 4 and Figure 3]. Ethanol made evident a compelling upsurge in fructification of MDA (5.21 ± 0.17 nM of MDA/mg of protein). The pretreatment with FMN (25, 50, and 100 mg/kg) provided

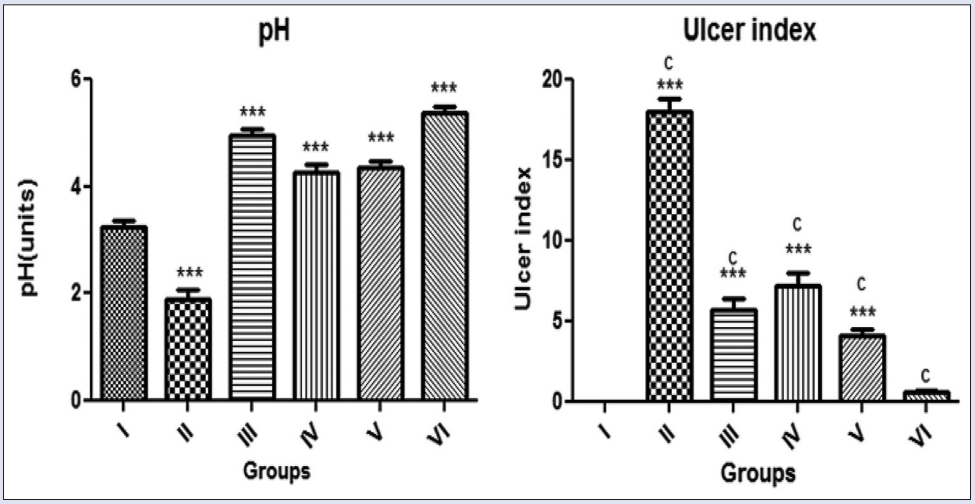

Figure 2: Effect of formononetin on pH and ulcer index. Group I (control; 0.25% carboxymethylcellulose), Group II (ethanol; 5 ml/kg), Group III (omeprazole; 20 mg/kg), Group IV (ethanol; 5 ml/kg + formononetin; 25 mg/kg), Group V (ethanol; 5 ml/kg + formononetin; 50 mg/kg), and Group VI (ethanol; 5 ml/kg + formononetin; 100 mg/kg). Results are expressed as mean ± standard error mean ($n = 6$). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ signify when all groups were compared to control group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ signify when all groups were compared to ulcer control group

Table 3: Effect of formononetin on pH and ulcer index

Group (treatment)	pH	Ulcer index	Percentage inhibition
I (Sham control; 0.25% CMC)	3.24±0.12	-	-
II (Ethanol; 5 ml/kg)	1.9±0.18***,c	18±0.81***	0
III (Ethanol; 5 ml/kg + OME; 20 mg/kg)	4.96±0.11***	5.75±0.62***,c	68.05
IV (Ethanol; 5 ml/kg + FMN; 25 mg/kg)	4.27±0.13***	7.25±0.75***,c	59.72
V (Ethanol; 5 ml/kg + FMN; 50 mg/kg)	4.34±0.14***,a	4.125±0.42***,c	77.08
VI (Ethanol; 5 ml/kg + FMN; 100 mg/kg)	5.37±0.13***	0.625±0.12c	96.53

*$P<0.05$, **$P<0.01$, ***$P<0.001$ signifies when compared to sham control group. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.00$ signifies when compared to ulcer control group. FMN: Formononetin; CMC: Carboxymethylcellulose. Results are expressed as mean ± SEM. Group I (control; 0.25%CMC),Group II (Ethanol; 5ml/kg), Group III (Omeprazole; 20mg/kg),Group IV (Ethanol; 5ml/kg + FMN; 25mg/kg), Group V (Ethanol; 5ml/kg + FMN; 50mg/kg), Group VI (Ethanol; 5ml/kg + FMN; 100mg/kg). Results are expressed as mean ± SEM ($n = 6$). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test

Table 4: Effect oxidative stress of formononetin on *in-vivo* markers

Group (treatment)	MDA (nM of MDA/µg of protein)	GSH (µM of GSH/µg of protein)
I (Sham control; 0.25% CMC)	3.40±0.12	10.73±0.41
II (Ethanol; 5 ml/kg)	5.21±0.17**	7.72±1.2***
III (Omeprazole; 20 mg/kg)	4.03±0.20	8.77±0.73a
IV (Ethanol; 5 ml/kg + FMN; 25 mg/kg)	5.28±0.65**	6.96±0.42c
V (Ethanol; 5 ml/kg + FMN; 50 mg/kg)	4.64±0.18	11.38±0.79c
VI (Ethanol; 5 ml/kg + FMN; 100 mg/kg)	3.24±0.19b	15.52±0.79c

*$P<0.05$, **$P<0.01$, ***$P<0.001$ signifies when compared to control group. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ signifies when compared to ulcer control group. FMN: Formononetin; CMC: Carboxymethylcellulose, MDA: Malondialdehyde, GSH: Reduced glutathione. Results are expressed as mean ± SEM. Group I (control; 0.25%CMC),Group II (Ethanol; 5ml/kg), Group III (Omeprazole; 20mg/kg),Group IV (Ethanol; 5ml/kg + FMN; 25mg/kg), Group V (Ethanol; 5ml/kg + FMN; 50mg/kg), Group VI (Ethanol; 5ml/kg + FMN; 100mg/kg). Results are expressed as mean ± SEM ($n = 6$). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test.

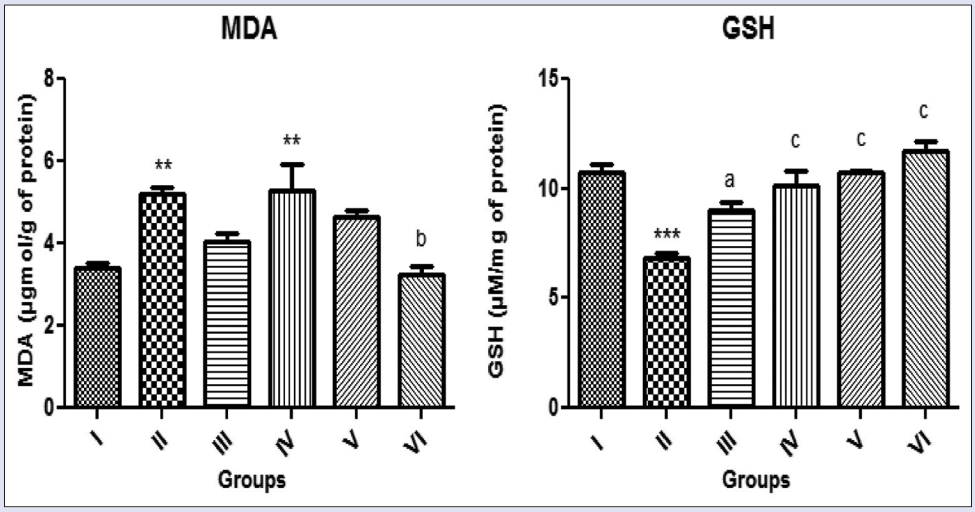

Figure 3: Effect of formononetin on biological antioxidants against ethanol induced gastric ulcer model. Group I (control; 0.25% carboxymethylcellulose), Group II (ethanol; 5 ml/kg), Group III (omeprazole; 20 mg/kg), Group IV (ethanol; 5 ml/kg + formononetin; 25 mg/kg), Group V (ethanol; 5 ml/kg + formononetin; 50 mg/kg), Group VI (ethanol; 5 ml/kg + formononetin; 100 mg/kg). Results are expressed as mean ± standard error mean ($n = 6$). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ signify when compared to control group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ signify when compared to ulcer control group

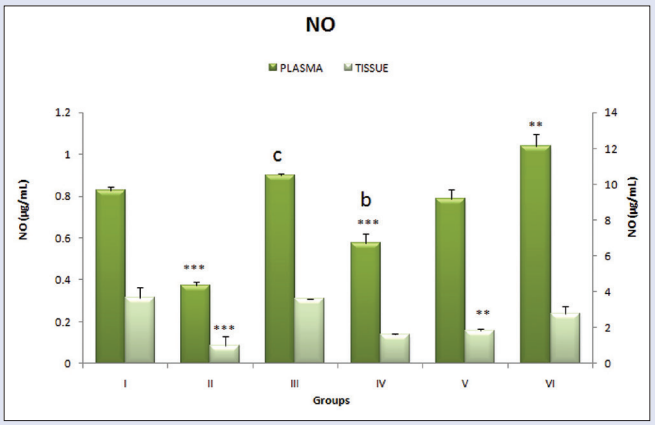

Figure 4: Effect of formononetin on cytoprotective biomarkers on ethanol induced gastric ulcer model. Group I (control; 0.25% carboxymethylcellulose), Group II (ethanol; 5 ml/kg), Group III (omeprazole; 20 mg/kg), Group IV (ethanol; 5 ml/kg + formononetin; 25 mg/kg), Group V (ethanol; 5 ml/kg + formononetin; 50 mg/kg), and Group VI (ethanol; 5 ml/kg + formononetin; 100 mg/kg). Results are expressed as mean ± standard error mean ($n = 6$). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ signify when compared to control group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ signify when compared to ulcer control group

a momentous protection from the same. Furthermore, downregulation of GSH was perceived by ethanol administration (7.72 ± 1.2 µM of GSH/µg of protein) which was significantly intensified by the treatment with FMN (6.96 ± 0.42, 11.38 ± 0.79, and 15.52 ± 0.79 µM of GSH/µg of protein) in a dose-dependent manner.

Effect of formononetin on levels of cytoprotective nitrite levels in ethanol-induced gastric ulcer

To assess the cytoprotective efficacy of FMN against ethanol-induced gastric ulcer, the levels of NO were estimated in stomach tissue of the treated rats. The level of NO decreased remarkably upon exposure to ethanol which is enhanced by FMN in a dose-dependent fashion [Figure 4].

Estimation of tumor necrosis factor-α in stomach tissue

Further, to evaluate the anti-inflammatory activity of FMN, we measured the levels of an important inflammatory cytokine in gastric tissue [Figure 5]. As expected, the levels of TNF-α were hiked up when exposed to ethanol. FMN treatment (25, 50, and 100 mg/kg) significantly lowers TNF-alpha levels in counterpart to toxic control.

Histopathological alterations in ethanol-induced gastric ulcer

Stomach tissues were preserved in 4% paraformaldehyde solution and kept it for 24 h. Tissues were then exposed to intensified isopropanol concentration (70%, 90%, and 100%) followed by dehydration with 100% xylene. The stomach samples were embedded in paraffin blocks. Samples were sliced of 5 µm thickness and stained with H and E for assessing mucosal damage in each of the stomach tissue sections [Figure 6].[28]

FMN pretreatment improves the pro-apoptotic and anti-apoptotic gene expression

The expression of pro-apoptotic gene (Bax) was increased after ethanol administration and *vice* versa results were achieved upon anti-apoptotic (BcL_2) protein marker [Figure 7]. Prophylactic treatment with FMN helps to restore the pro-apoptotic and anti-apoptotic gene expression significantly. These data indicate that apoptosis is involved in the pathology of ethanol-induced gastric ulcer model.

DISCUSSION

The present study unveiled the effect of FMN on gastric ulcer induced by oral administration of ethanol. Ethanol is considered to be one of the major agents for the development of gastrointestinal ulcer and damaging gastric mucosal surface.[6] In spite of having property to solubilize the protective mucous membrane, it also stimulates acid secretion and reduces blood flow leading to micro- and macro-vascular injuries.

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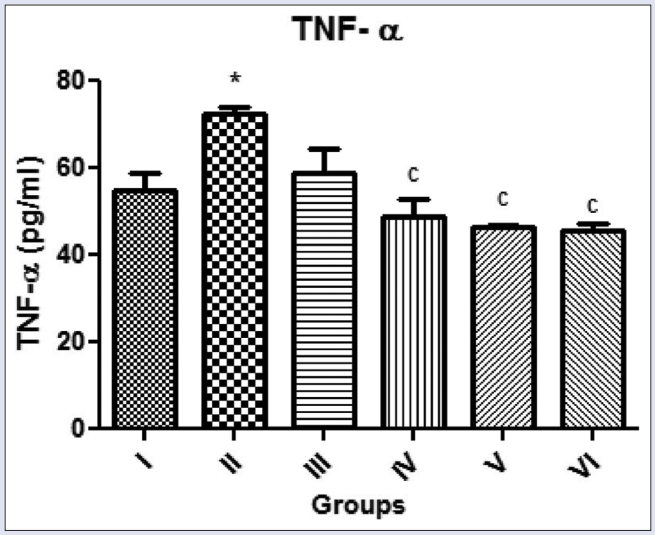

Figure 5: Effect of formononetin on inflammatory biomarker upon ethanol induced gastric ulcer model. Group I (control; 0.25% carboxymethylcellulose), Group II (ethanol; 5 ml/kg), Group III (omeprazole; 20 mg/kg), Group IV (ethanol; 5 ml/kg + formononetin; 25 mg/kg), Group V (ethanol; 5 ml/kg + formononetin; 50 mg/kg), and Group VI (ethanol; 5 ml/kg + formononetin; 100 mg/kg). Results are expressed as mean ± standard error mean (n = 6). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test. *P < 0.05, **P < 0.01, ***P < 0.001 signify when compared to control group. ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P < 0.001 signify when compared to ulcer control group

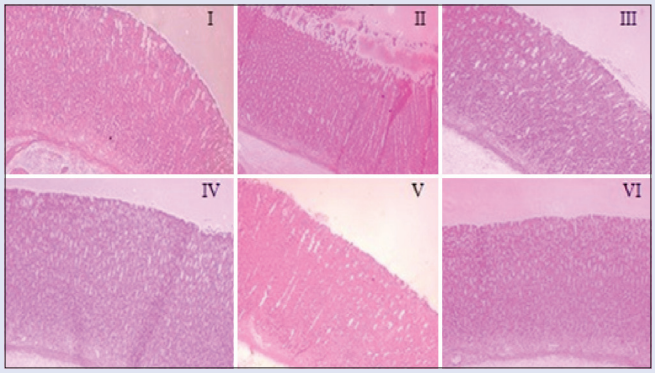

Figure 6: Histopathological alterations due to formononetin on ethanol induced gastric ulcer model. Group I (control; 0.25% carboxymethylcellulose), Group II (ethanol; 5 ml/kg), Group III (omeprazole; 20 mg/kg), Group IV (ethanol; 5 ml/kg + formononetin; 25 mg/kg), Group V (ethanol; 5 ml/kg + formononetin; 50 mg/kg), and Group VI (ethanol; 5 ml/kg + formononetin; 100 mg/kg)

acute toxicity study of FMN, to affirm the potential protective dose of the drug. Since FMN shows no symptoms of toxicity, we decided to continue the study at three doses. Herein, the authors showed that ethanol administration leads to the imbalance in oxidative stress parameters consequently causing tissue damage. Association between oxidative stress and gastric ulcer is a well-studied phenomenon,[29] thereby evaluating the levels of various antioxidant parameters such as MDA and GSH.[30]

FMN attenuates the increased levels of MDA induced by ethanol. Several literatures reported the relation between MDA and lipid peroxidation.[31-33] Increase in MDA concentration has been directly linked to lipid degradation in tissues subjected to toxicity oxidation stress.[34] Expectedly, the positive control group also decreased the hike up level of MDA and increased the consumed GSH due to ethanol administration. Omeprazole has been shown to be effective against gastric mucosal damage which is in line with the previous results[35] and is used as standard drug for comparing the new gastroprotective efficacy of any new agent. The present study unveils the same results in which FMN decrease the abruptly increased MDA concentration due to exposure to alcohol. GSH is considered to be an important marker for oxidation stress. Decreased concentration of GSH indicates the tissue damage due to its higher utilization during stress. FMN restores the level of GSH during treatment. Hence, it can be concluded that FMN has a potential to revert the biochemical markers of oxidative stress towards normal. Therefore, FMN can positively modulate the deleterious effect of ethanol in the experimental animal.

The cytoprotection might be another possible factor responsible for gastric ulcer healing property of FMN. For the effective treatment of gastric ulcers, not only the prevention of further ulcer formation but also the enhancement of ulcer healing is important.[36] Ethanol damages the protecting layer of gastrointestinal tract, i.e., mucosal layer, which acts as a barrier between digestive enzymes and the tract. The present investigation is in accordance with previous results manifesting the cytoprotective efficacy of FMN against ethanol-induced gastric ulcer.[37]

Furthermore, be questing of NO plays an important role against ethanol-induced gastric ulcer. In several investigations, NO level was found to be decreased in gastric tissue exposed to ethanol. NO was also reported to be a crucial aspect in improving gastric tissue injury and increasing gastric mucosal defensive factor level and blood flow.[38] In this context, the results obtained for NO are consistent with the previous results. With the aforementioned investigation, it could be concluded that FMN exhibits cytoprotective as well as antisecretory activity.[39]

Moreover, inflammation has been regarded as the pivotal marker for ulcer. One of the pathogenesis features of peptic ulcers is the imbalances between offensive factors, such as gastric acid and protective factors, including inflammatory cytokines. Ethanol has been known to upregulate the pro-inflammatory markers and to downregulate anti-inflammatory biological facets.[40] To put into affirmations, the authors examined the levels of TNF-α and in gastric tissue. Expectedly, the TNF-α was found to be highly expressed in ethanol-induced ulcer model while the FMN-pretreated rats' gastric tissue exhibited significant decrease in the expression of TNF-α.

Apoptosis is a paramount mechanism that bestows the maintenance of normal cell turnover, proper embryonic development, and xenobiotic-induced cell death. The apoptotic process is pathophysiologically characterized by several pro-apoptotic and anti-apoptotic biological markers. Release of reactive oxygen species has been associated with the activation of Bax/Bcl_2-ratio, an important aspect to be considered during apoptosis study. Herein, the study also reported the aforesaid outcome that ethanol administration leads to the downstream gene expression of Bcl_2 and upsurge the genetic level of Bax. Consequently, the current investigation is in accordance with the previous results. Hence, the authors would like to conclude that the gastroprotective effect of FMN is due to its anti-inflammatory, cytoprotective, and antiapoptotic property.

CONCLUSION

From the above set of results, the authors would like to culminate that the prophylactic administration of FMN could suppress the ethanol-induced mucosal damage in gastric tissue by ameliorating mucosal hemorrhagic injuries, inflammation, and apoptosis in

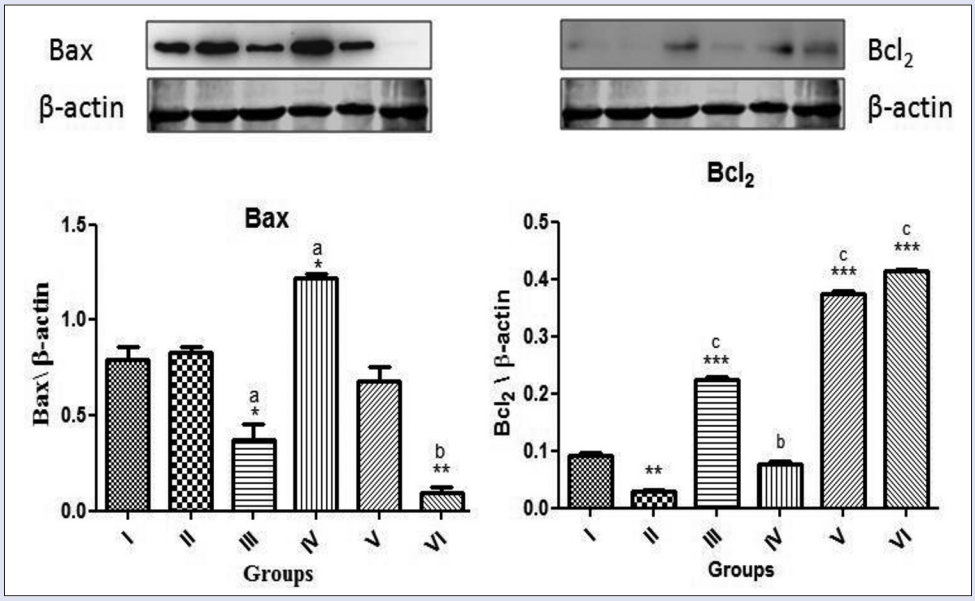

Figure 7: Representative blots and bar graph shows the quantification of Bax, Bcl_2 normalized with β-actin. Group I (control; 0.25% carboxymethylcellulose), Group II (ethanol; 5 ml/kg), Group III (omeprazole; 20 mg/kg), Group IV (ethanol; 5 ml/kg + formononetin; 25 mg/kg), Group V (ethanol; 5 ml/kg + formononetin; 50 mg/kg), and Group VI (ethanol; 5 ml/kg + formononetin; 100 mg/kg). Results are expressed as mean ± standard error mean ($n = 6$). Comparisons were made on the basis of the one-way ANOVA followed by the Bonferroni test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ signify when compared to control group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ signify when compared to ulcer control group

augment of mucin secretion and cytoprotection. This is the first time that gastroprotective mechanism of FMN has been elucidated. Effect of FMN upon gastric tissue apoptotic gene expression has also been shown for the first time. Considering the current study, FMN can be considered as a promising alternative antiulcer therapy to be used against irritant-induced gastric ulcer.

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Conflicts of interest

There are no conflicts of interest.

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