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Gastroprotective effect of the flavonoid fustin isolated from *Cotinus coggygria* heartwood in a rat model of indomethacin-induced gastric ulceration

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Abstract: *Gastric ulcer is a common health issue. Cotinus coggygria heartwood extracts rich in the flavonoids fustin and sulfuretin have shown protective effects in a rat model of indomethacin-induced gastric ulceration. Fustin itself has been protective in a rat model of ethanol-induced gastric ulcer. In the present study we aimed to reveal the effect of the flavonoid fustin isolated from Cotinus coggygria heartwood in a rat model of indomethacin-induced gastric ulceration.*

Fustin was isolated from Cotinus coggygria heartwood and purified. The experiment was performed on 30 male Wistar rats allocated to three groups – Control, Indo and F10. F10 group was pretreated with

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fustin (10 mg/kg b.w. for 7 days). Rats were sacrificed and macroscopic, histopathological and immunohistochemical investigations of the stomachs were performed.

Indomethacin caused severe mucosal damage, proven by the macroscopic, microscopic and immunohistochemical gastric mucosa investigation. Fustin pretreatment slightly reduced the macroscopic indices for gastric damage (ulcer number, area, score and index), and significantly alleviated the severity of the microscopic indices (mucosal erosions, necrosis, hemorrhages and inflammation). In fustin-pretreated rats, the expression of NF-κB was reduced in comparison with indomethacin-treated animals.

In conclusion, fustin exerted a gastroprotective action in an indomethacin-induced gastric ulceration model, probably due to its anti-inflammatory activity.

Keywords: gastric ulcer, fustin, flavonoid, *Cotinus coggygria* heartwood, Wistar rats

Introduction

Gastric ulcer is a common health problem with relatively limited treatment options [1]. Indomethacin-induced gastric ulceration (IIGU) is an animal model, highly utilized in the evaluation of potentially gastroprotective substances. The most common mechanisms that contribute to the mucosal damage in this model are oxidative stress, inflammation and apoptosis [2, 3]. *Cotinus coggygria* heartwood (CCH) extracts have shown protective effects in a rat model of IIGU. This extract has revealed also strong antioxidant, cytoprotective and anti-inflammatory properties *in vitro* and *in vivo*. There is vast scientific evidence that the effects of isolated plant substances may differ from those of the plant extracts from which they originate. The main phenolic components of CCH extracts are the flavonoids fustin and sulfuretin [4–6]. Significant antioxidant, anti-inflammatory and antiapoptotic properties have been attributed to fustin [7–10]. Recently, fustin had demonstrated a gastroprotective effect in a model of ethanol-induced gastric ulceration in rats. In this experiment, higher doses of the flavonoid were used [11]. We decided to use a dose of 10 mg/kg in order to approach the fustin content in CCH extract used in our previous experiments. The aim of the present study was to determine the effect of fustin isolated from CCH in a rat model of IIGU. Our results showed that fustin was able to exhibit weak pro-tective effect against the gastric-mucosa damage in this model, probably due to its anti-inflammatory activity.

Materials and methods

Isolation and purification of fustin

Fustin was isolated according to the method described by Novakovic et al. [12].

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Plant material and extraction

CCH was collected in May 2021 at Deliblatska Peščara (Deliblato Sand), Vojvodina province, Serbia. Prof. Milan Veljic (Faculty of Biology, University of Belgrade) identified the plant material. Then the heartwood was air-dried and grinded to fine powder. After that, 1 kg wood powder underwent triple extraction with 10 L of methylene chloride/methanol 1:1 for 24 h at room temperature, which resulted in the production of 76 g crude extract. This extract was further fractionated by Si gel column chromatography (CC).

Isolation and identification of fustin

Fustin was isolated from the crude extract using combination of Si gel column chromatography (Merck silica gel particle size 0.063-0.200 mm) with methylene chloride/methanol mobile phase and sempipreparative reversed phased high performance liquid chromatography (HPLC, Agilent Technologies 1100 Series HPLC-DAD and Zorbax Eclipse XDB C18 column, 250 × 9.4 mm, i.d. 5 μm). Fractions from CC containing fustin were those eluted methylene chloride/methanol approximately 80:20 and screened by thin layer chromatography (TLC). Pure fustin was isolated from these fractions by reversed phase semi-preparative HPLC using water/acetonitrile system, 254 nm for detection and the following program: 0-20 min, 20-37% CH_3CN; 20-21 min, 37-50% CH_3CN; 21-27 min, 50% CH_3CN; and 27-30 min, 50-100% CH_3CN. Fustin was determined using nuclear magnetic resonance (NMR) spectra (Bruker Avance III 500, 500 MHz for ^{1}H; 125 MHz for ^{13}C), in CD3OD as solvent. The purity was determined using HPLC and NMR.

Animals and treatment

30 male Wistar rats were used (225 ± 25 g). Animals were housed in plastic cages at room temperature (22 ± 1°C), with a free access to food and water and under a 12-hour light/dark cycle. Rats were deprived of food for 24 h before ulcer induction. All experimental procedures were carried out in accordance with the national and international laws and policies (EU directive 2010/63/EU for animal experiments) and were approved by the Bulgarian Food Safety Agency (Protocol No.23/April 15th, 2021; Permission No.305/June 28th, 2021).

There were three groups, each consisting of 10 rats: Control, Indo and F10. The animals were treated daily for 1 week by an orogastric tube. Fustin was prepared as a suspension in a vehicle (50 μL of Tween 80 per 10 mL distilled water). Control and Indo rats received the vehicle (10 mL/kg). F10 animals received fustin (10 mg/kg) as a 10 mL/kg suspension.

Induction of gastric ulcer

On the 7th day of the experiment, 1 h after the pretreatment, rats from groups Indo and F10 received orally 100 mg/kg indomethacin, suspended with 50 μL Tween 80 in a total volume of 10 mL/kg. Control rats were applied 10 mL/kg of the vehicle. The animals were anaesthetized with diethyl ether 4 hours after the treatment. Blood was collected from the sublingual veins for biochemical analysis. After the animal

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decapitation, the stomachs were removed, opened along the great curvature, washed and inspected macroscopically. Then gastric tissue samples were preserved for microscopic, biochemical and immunohistochemical evaluation.

Macroscopic evaluation of the ulcers

Ulcers were counted and measured and their area was calculated. Five petechiae were considered a single 1 mm^2 lesion. Ulcer score defining the severity of ulceration was assessed following the method of Dekanski et al. [13] as follows: 0 – no damage; 1 – blood in the lumen; 2 – pinpoint erosions; 3 – 1-5 small erosions < 2 mm; 4 – more than 5 small erosions < 2 mm; 5 – 1-3 big erosions > 2 mm; 6 – more than 3 big erosions > 2 mm. Then the average ulcer score for each group was calculated [14]. Ulcer index (UI) for each group and percent protection (PP) were determined by the following formulae:

UI = total ulcer score/number of animals PP = (UI Indo – UI F10) x 100/UI Indo

Histopahological investigation

Stomach tissue samples were fixed in 10% neutral-buffered formaldehyde solution. Later, they were embedded in paraffin, cut into sections, placed on microscope slides and stained with hematoxylin and eosin, followed by light microscopy investigation. The presence of deep erosions, hemorrhages, epithelial necrosis and inflammatory cells infiltration were evaluated using the following scale: 0 – no; 1 – low; 2 – moderate; 3 – high.

Immunohistochemical study

NF-κB expression was determined by universal highly sensitive visualization system for antibody detection EnVision FLEX using rabbit anti NF-kB-p100 polyclonal antibody (E-AB-32222; Elabscience, USA)), diluted 1:200. Tissue sections, 4 mcm thick, were embedded in paraffin and placed on silanized slides. Saturation index of immune deposits was determined semi-quantitatively in 50 cells of each probe using the following score: 1 – lacking, 2 – weak, 3 – moderate, 4 – strong. The average intensity of the immune reaction was verified in the following way: number of cells of each type x corresponding coefficient (1, 2, 3 or 4) x total number of $cells^{-1}$.

Statistical analysis

The results were presented as mean ± S.E.M. Data were analyzed by GraphPad Prism statistical software using one-way ANOVA, followed by Dunnett's multiple comparison post hoc test. A p-value less than 0.05 was considered significant.

Results

Identification of purified fustin

The obtained ^{1}H and ^{13}C NMR spectral data of purified fustin are presented in Figure 1 and Table 1.

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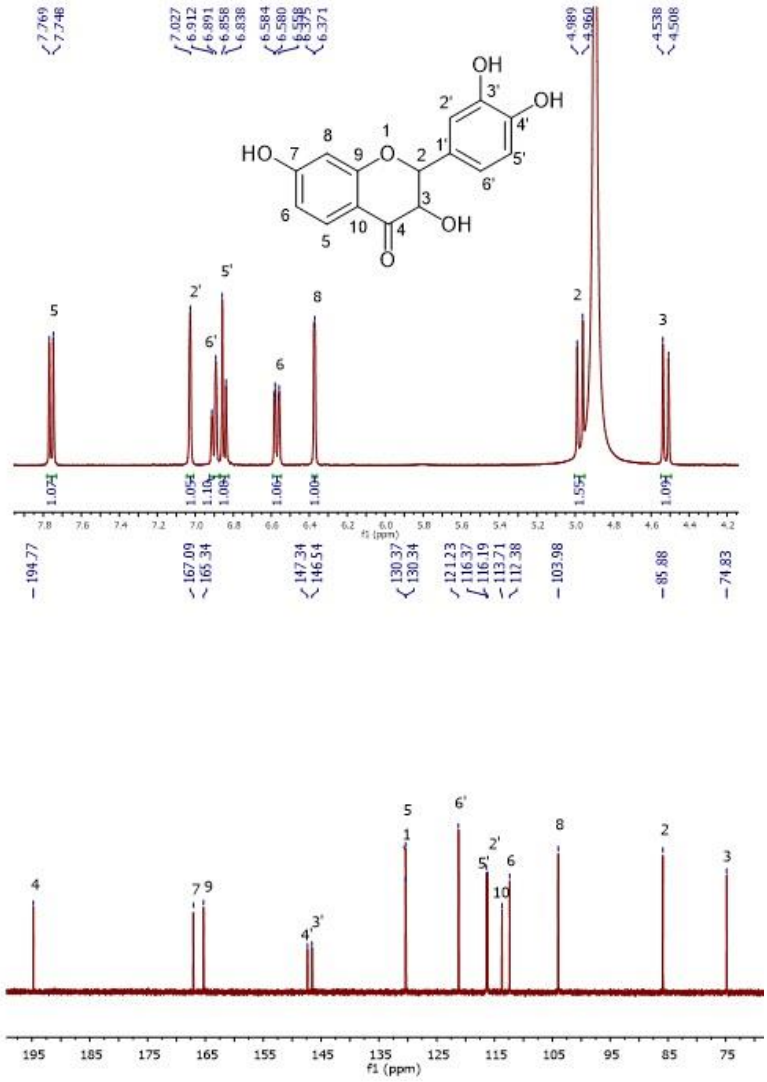

Figure 1. ^{1}H and ^{13}C NMR spectra of fustin

Table 1. ^{1}H and ^{13}C NMR data for fustin: CD3OD, 400 MHz for ^{1}H, 100 MHz for ^{13}C

Atom number	δ_H J (Hz)	δ_C	Atom number	δ_H J (Hz)	δ_C
1	-	-	9	-	165.3
2	4.98 d (12.0)	85.9	10	-	113.7
3	4.52 d (12.0)	74.8	1’	-	130.4
4	-	194.8	2’	7.03 d (1.6)	116.2

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5	7.75 d (8.4)	130.3	3’	-	146.5
6	6.57 dd (8.8, 1.6)	112.4	4’	-	147.3
7	-	167.1	5’	6.85 d (8.0)	116.4
8	6.37 d (1.6)	104.0	6’	6.90 dd (8.0, 1.6)	121.2

Macroscopic evaluation of gastric lesions

The macroscopic appearance of rat stomachs is presented on Figure 2. In the control group no gastric erosions were observed. In Indo group multiple mucosal lesions were found in the glandular stomach area. They were widespread, linear and bleeding at the moment of observation. In F10 group the lesions were less in number and smaller, even lacking.

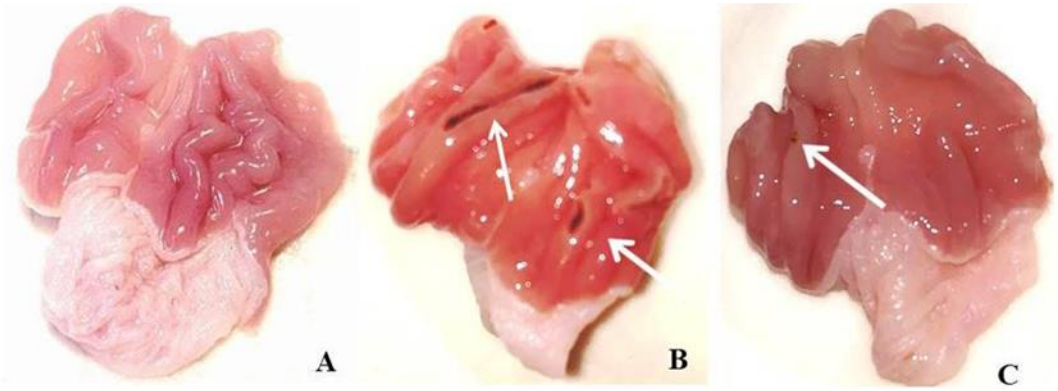

Figure 2. Macroscopic appearance of rat stomachs in a model of indomethacin-induced gastric ulceration: A. Control group – normal appearance; B. Group Indo – many long linear ulcerations; C. Group F10 – decreased number or no ulcers

The average number of lesions in Indo rats was 6.1 ± 1.6, and their average area was 16.9 ± 5.9 mm^2. The average number of ulcers in F10 rats was 4.8 ± 1.3 with an average area of 15.1 ± 6.2 mm^2. Compared to Indo group, in F10 group the ulcer number and ulcer area were reduced by 21% and 11%, respectively (Figure 3).

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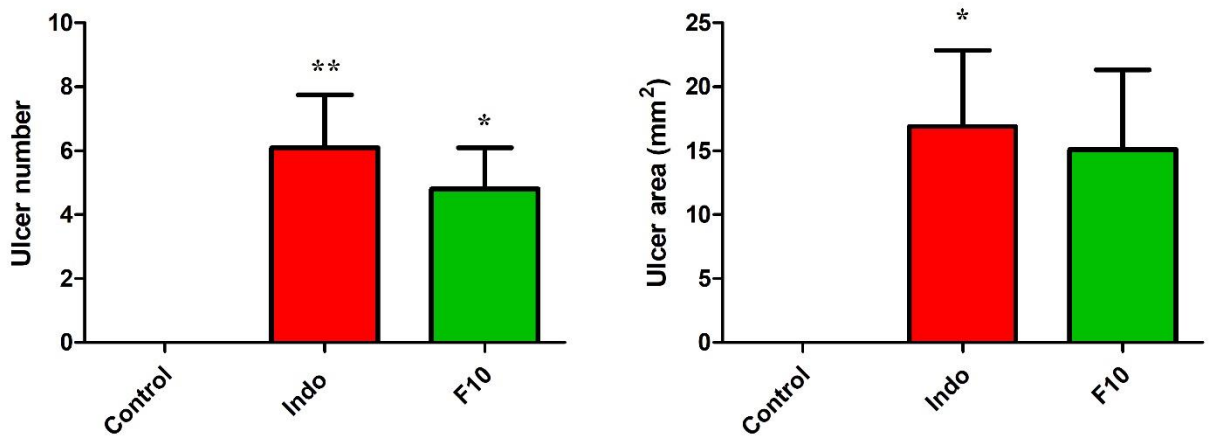

Figure 3. Effects of fustin on the number and area of the gastic ulcers in a rat model of indomethacin-induced gastric ulceration; $^{*}p < 0.05$ vs. Control; $^{**}p < 0.01$ vs. Control

The ulcer score (US) and ulcer index (UI) in F10 group were reduced slightly but not significantly, compared to Indo group. The percent protection (PP) was 13.3% (Table 2).

Table 2. Macroscopic indices for gastric ulceration in a rat model of indomethacin-induced gastric ulceration and fustin pretreatment. UI - ulcer index; PP - percent protection

Group	Ulcer score (mean ± S.E.M)	Ulcer index	PP (%)
Control	0	0	-
Indo	4.5 ± 0.64	4.5	-
F10	3.8 ± 0.66	3.8	13.3

Histopathological investigation

The microscopic appearance of the stomach mucosa in Control rats was normal (Figure 4A, B). The ulcers in all animals in Indo group were characterized by severe epithelial necrosis (Figure 4C). In 20% of these animals, deep erosions and hemorrhages were present (Figure 4D). Inflammatory cell infiltration was found in 90% of cases. In F10 rats the damage caused by indomethacin was attenuated. There were no deep

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erosions and hemorrhages. Epithelial necrosis was present only in half of F10 animals and its severity was alleviated in comparison with that of Indo group (Figure 4E, and 4F). Only in one of these animals an inflammatory infiltrate was found.

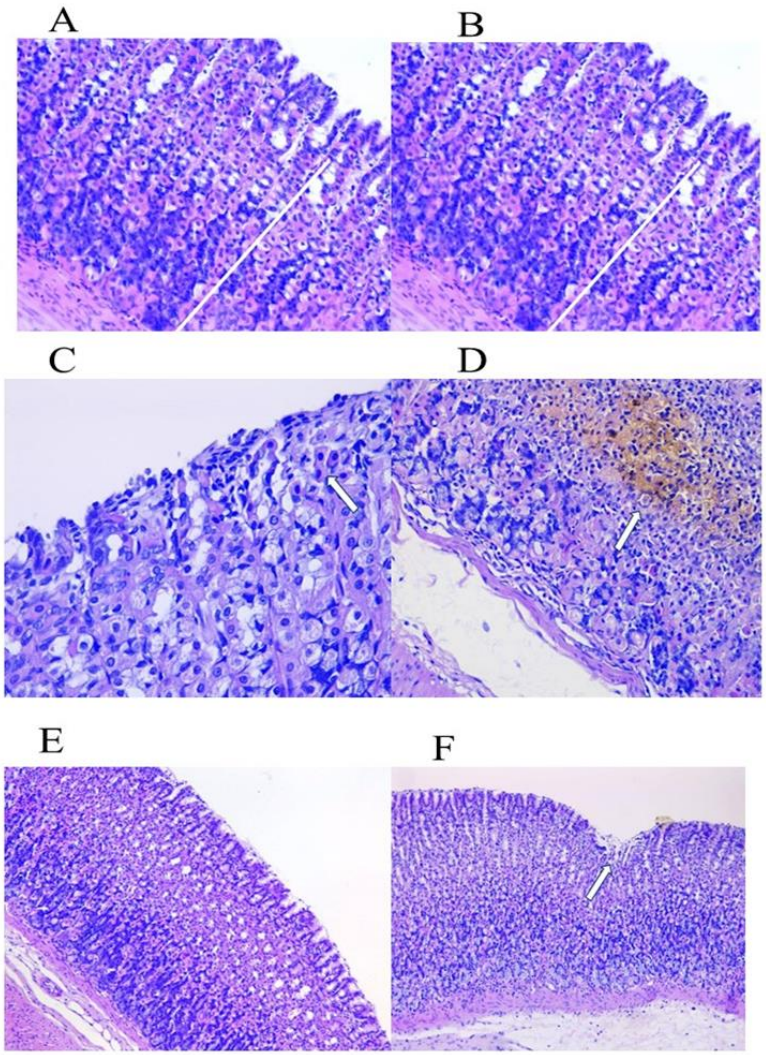

Figure 4. Microscopic appearance of gastric mucosa in a rat model of indomethacin-induced gastric ulceration and fustin pretreatment: A, B – Control group (preserved architectonics); C, D – group Indo (severe epithelial necrosis, deep erosions and hemorrhages); E, F – F10 group (normal architectonics, slight epithelial necrosis). Staining with hematoxylin and eosin; magnification x 100, x 200

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In the control and F10 groups, deep erosions and hemorrhages were lacking (average score 0). In Indo group, the average erosion score was 0.2 ± 0.13, and the hemorrhage score – 0.3 ± 0.21 (Figure 5A, and 5B). The necrosis score for the control group was also 0, for Indo group – 3, and for F10 group – 0.6 ± 0.22 (Figure 5C). The score for inflammatory infiltration in the control group was 0, in Indo group – 1.7 ± 0.26, and in F10 group – 0.1 ± 0.1 (Figure 5D).

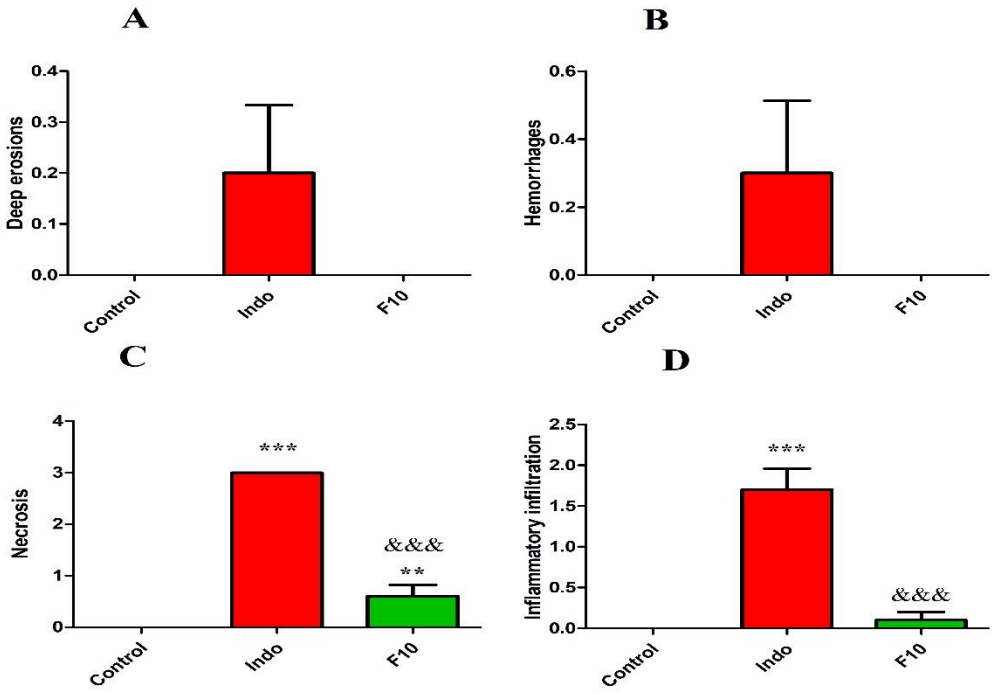

Figure 5. Microscopic assessment of the severity scores for erosions (panel A), hemorrhages (panel B), epithelial necrosis (panel C) and inflammatory cell infiltration (panel D) a rat model of indomethacin-induced gastric ulceration and fustin pretreatment; $^{**}p < 0.01$ vs. Control; $^{***}p < 0.001$ vs. Control; $^{\&\&\&}p < 0.001$ vs. Indo

Immunohistochemical study

As shown on Figures 6 and 7, NF-κB showed increased cytoplasmic expression in indomethacin-treated group. The expression was decreased in the stomach samples of fustin-treated animals.

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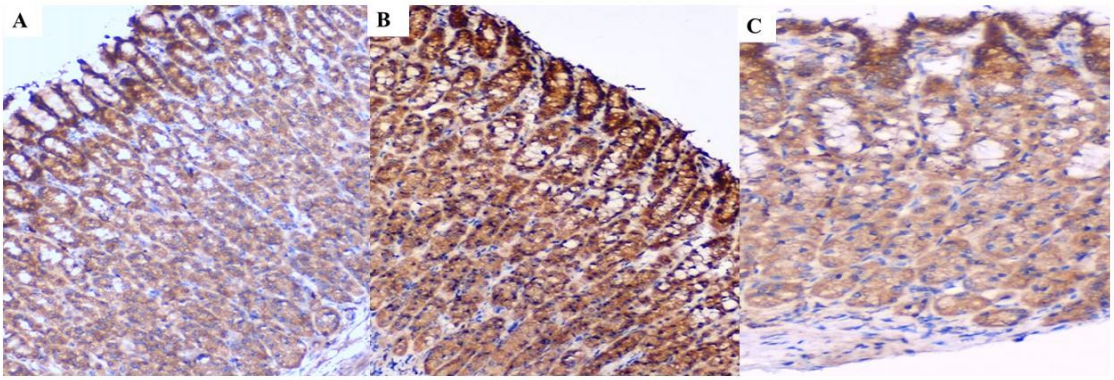

Figure 6. Immunohistochemical staining for NF-κB expression in a rat model of indomethacin-induced gastric ulceration and fustin pretreatment: A. Control group; B. Indo group; C. F10 group; magnification x 200

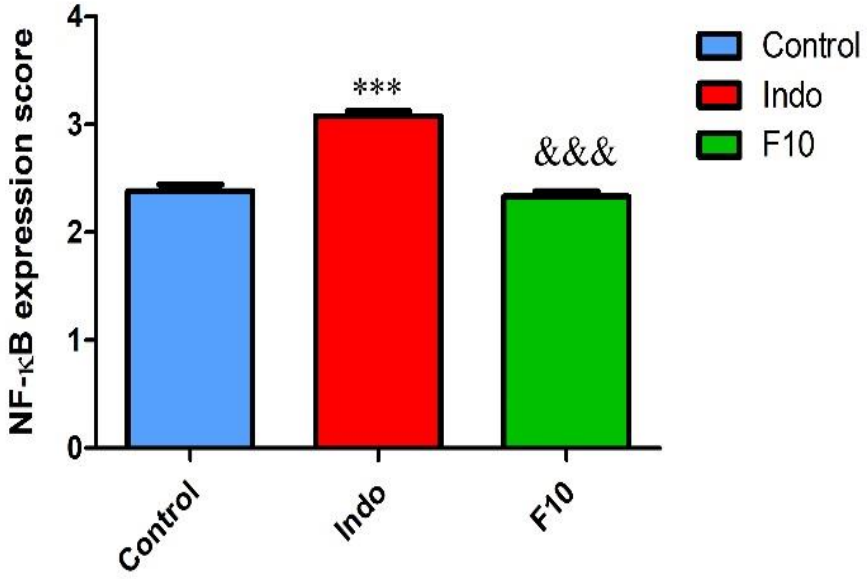

Figure 7. Immunohistochemical score for NF-κB expression in a rat model of indomethacin-induced gastric ulceration and fustin pretreatment. $^{***}p < 0.001$ vs. Control; $^{\&\&\&}p < 0.001$ vs. Indo

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Discussion

Non-steroidal anti-inflammatory drugs (NSAIDs)-induced ulcer disease is characterized by a high prevalence, which determines the widespread use of such a model in experimental pharmacology. This was our main consideration to choose IIGU model for the experiment. The mechanisms for ulcer induction by NSAIDs like indomethacin is complex. It includes both local and systemic effects. Systemic effects can be classified into prostaglandin (PG)-dependent and PG-independent. The topical damage takes place by generation of reactive oxygen species, disruption of hydrophobic properties of the superficial mucus layer, membrane phospholipids damage, allowing retrograde diffusion of acid, leading in turn to apoptosis and necrosis. A well-known systemic mechanism in NSAID-induced gastric damage is the depletion of physiological gastroprotective PGs like PGE_2 and PGI_2, caused by cyclooxygenase (COX) inhibition. COX-1 inhibition causes decreased mucosal blood flow, gastric hypermotility, increased basal gastric acid secretion (in gastritis), increased gastric acid concentration, and COX-2 inhibition promotes leukocyte adherence. NSAIDs are shown also to activate the inducible form of the enzyme nitric oxide synthase (NOS) and to inhibit its constitutive form, leading to decreased levels of endothelial NO and loss of mucosal integrity. Stress proteins are up-regulated as well. The action of extracellular-matrix modifying enzymes metalloproteinases (MMPs) is altered by increasing MMP-9/MMP-2 ratio. The gastroprotective action of melatonin is also disrupted [15].

Generation of reactive oxygen species causing significant oxidative stress is a key mechanism in the development of NSAID-induced gastropathy. Oxidative stress in turn causes production of pro-inflammatory cytokines like IL-1β, IL-6, TNF-α and leucocyte attraction, adhesion to the gastric mucosa and infiltration. Activated neutrophils produce more pro-inflammatory and pro-oxidative enzymes leading to a formation of a vicious cycle [16]. Reactive oxygen species also cause activation of nuclear factor-kappa B (NF-κB), a major inflammatory genes-expression regulator that plays a significant role in the development of NSAID-induced gastropathy [17, 18]. Natural substances were able to prevent NF-κB activation, probably by inhibition of oxidative stress and thus to reduce the production of proinflammatory cytokines and the related apoptosis and damage of the stomach mucosa [18].

The results from this experiment showed that in F10 group, the ulcers were decreased in number by 21% and area by 11%, and also the ulcer score and ulcer index were insignificanly reduced in comparison with those of Indo group. In spite of the insignificant effect of fustin on the macroscopic indices, the histopathological investigations showed that the gastric ulcerations in fustin-pretreated rats were more superficial, there were no deep necrotic lesions, and the hemorrhages and inflammatory infiltration were significantly reduced. The possible mechanism of the gastroprotective effect exerted by fustin might be linked

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to its anti-inflammatory action, as there was a decreased expression of NF-κB in the gastric mucosa of fustin-pretreated rats. NF-κB is an important modulator of pro-inflammatory responses involving TNF-α- and IL-1-driven pathways. It is also involved in the regulation of cell proliferation and apoptosis [19]. Recently, other authors have demonstrated an anti-inflammatory effect of higher doses of fustin, proven by decreased levels of the cytokines TNF-α and IL-1β, in a model of ethanol-induced ulcerogenesis [11].

Conclusion

The flavonoid fustin was able to exert a gastroprotective effect in a rat model of indomethacin-induced gastric ulceration, probably due to its anti-inflammatory properties.

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