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Protective effect of *Juglans regia* L. (walnut) leaves extract against indomethacin induced gastric mucosal damage in rats

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Abstract

Indomethacin, an anti-inflammatory agent used for pain, fever and inflammation, causes severe lesions in the gastrointestinal tract in humans and animals. For this reason, there has been an increase in the search for new compounds that can help prevent and/or alleviate these lesions caused by indomethacin. The aim of the present study is to evaluate the gastroprotective effect of *Juglans regia* administration against indomethacin induced gastric lesion. A total of forty rats were divided equally among four groups for the experiments. Groups were designed as Control (C), Indomethacin (IND), *Juglans regia* + Indomethacin (JR+IND), and Pantoprazole + Indomethacin (PAN+IND). Following the 7-day adaptation period, substance applications (pantoprazol and *Juglans regia* extract) were made for 10 days. In the IND, JR+IND and PAN+IND groups were administered indomethacin 100 mg/kg single dose on day 11th. Indomethacin caused significant increases in the stomach IL-6 and TNF-α levels compared with the C group (respectively, $p<0.001$, $p<0.01$). In the PAN+IND and JR+IND groups showed a significant lower in the IL-6 levels compared with IND group (respectively, $p<0.001$, $p<0.01$). The C-reactive protein (CRP) and Cyclo-oxygenase-2R (COX-2R) levels of IND group were increased compared to C group ($p<0.001$). There was a significant decrease in CRP and COX-2R levels after PAN and JR treatment compared with IND ($p<0.001$). COX-2, CRP and proinflammatory cytokines is seen as the main target for the treatment of inflammatory diseases. As a result; it was determined that JR has a protective effect, which may be related to decrease inflammatory markers, against of gastric damage caused by indomethacin.

Keywords: Gastric ulcer; *Juglans regia*; Indomethacin; C-reactive protein; Cyclo-oxygenase-2R

1. Introduction

Indomethacin, an anti-inflammatory agent used for pain, fever and inflammation, causes severe lesions in the gastrointestinal tract in humans and animals [1]. Currently, proton pump inhibitors, histamine H2 receptor blockers, and antibiotics if bacterial origin are used for the treatment and/or prevention of progression of gastric lesions, depending on the underlying cause. However, it is stated that these treatment protocols also cause various side effects, thus limiting their clinical benefits [2, 3]. After it was determined that free radicals [4] proinflammatory cytokines, PGE2 and cyclooxygenase 2 [5] have an important role in the formation of mucosal lesions induced by indomethacin, interest in researching natural new compounds that can show cytoprotective effects through antioxidant and anti-inflammatory activities and their active substances is increasing day by day. As a result of the literature review, it has been determined that plant extracts such as *Ayapana triplinervis* leaf [6], *Albizia anthelmintica* leaf [7], which have strong antioxidant and anti-inflammatory effects, or various active substances such as safranal [3], caftaric acid [8] have positive effects on gastric ulcer. There is a growing interest in the use of plant-derived agents for treating diseases and/or preventing chronic health conditions. One of these plants is *Juglans regia* L. (Walnut) belonging to the Junglandaceae family [9]. The walnut is trees found primarily in the temperate areas and commercially cultivated in United States, western South America, Asia, and central and southern Europe [10]. Its seeds, bark, green husk and leaves are commonly used in folk

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medicine because of its antidiabetic, hypoglycaemic, antioxidant, keratolytic, antifungal, hypotensive antidiarrheic, antihelmintic, and sedative effects [11-13]. Phenolic compounds help oxidative stress to be reduced by inhibiting oxidation of macromolecules [14]. The main phenolic compounds of walnut leaves are thought to be naphthoquinones and flavonoids [15], but a powerful antioxidant with a very broad phenolic content (3-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside) [16,17]. We investigated the gastroprotective effects of JR administration against indomethacin induced gastric ulcer.

2. Material and methods

2.1. Extraction of Plant Material

The leaves of the plants were collected from located at Elazığ city of Turkey (38°29'06.2"N 39°18'47.4"E). 25 g of the sample was extracted with 500 ml of ethanol using a soxhlet apparatus for 2 h. It was evaporated under pressure at 50 °C. We obtained 7, 29 g of the extract from 25 g of leaves.

2.2. Animals

A total of fourty female Wistar albino rats were used which aged between 2 and 3 months. The rats were divided equally among four groups for the experiments. The animals were maintained under standard conditions (in constant temperature and ventilated rooms, 12 hours of daylight and 12 hours of darkness). They were fed ad libitum with standard laboratory chow (crude protein: 18.5%, crude fibre: 4.2%, crude fat: 4.5%, crude ash: 7%, lysine 1.00%, methionine: 0.42%, calcium: 1.00%, phosphorus: 0.60%, sodium: 0.25%, magnesium: 0.20%, and copper: 20mg/kg) and tap water throughout the experiment.

2.2.1. Control group (C)

This group was administered physiological saline solution (300µL/day) orally for 10 days.

2.2.2. Indomethacin group (IND)

This group administered physiological saline solution throughout 10 days + 11th day 100 mg/kg single dose indomethacin via orally.

2.2.3. Juglans regia + Indomethacin group (JR+IND)

This group administered *Juglans regia* leaf extract 200 mg/kg throughout 10 days + 11th day 100 mg/kg single dose indomethacin via orally.

2.2.4. Pantoprazole + Indomethacin group (PAN+IND)

This group administered 5 mg/kg pantoprazole throughout 10 days + 11th day 100 mg/kg single dose indomethacin via orally.

Following the 7-day adaptation period, substance applications were made for 10 days in accordance with the above-mentioned procedure. Physiological saline was also administered to the control and indomethacin groups to equalise the stress induced by oral gavage in all other groups.

2.3. Biochemical and Macroscopic Analysis

Stomachs of the animals under anesthesia were opened following cervical dislocation 24 h after IND administration and evaluated macroscopically. The rat stomach tissues were removed quickly and cleaned with physiological saline solution. These tissues were homogenized in phosphate buffer with pH 7.4 (1:9 ratio) using a homogenizer. These homogenates were then centrifuged at 5000 x g for 5 minutes in accordance with the kit procedures and the supernatants were taken and prepared for analysis. In calculating the gastric ulcer score, the ulcerated areas in all stomach tissues and the total area were measured in mm^2. Then, the ulcer index was determined as % according to the formula below [18].

$$\text{Ulcer index} = [\text{ulcer area} / \text{total stomach area}] \times 100$$

Later, some part of the stomach were homogenised in a 9-fold volume of phosphate buffered saline. ELISA kits were used to determine TNF-α, IL-6, CRP, COX-2R levels in tissue homogenates (Elabsciene WuHan-P.R.C).

2.4. Statistical analysis

One-way ANOVA was used to determine whether there are significant differences between the groups. Tukey multiple range test was used to detect significant differences pairwise between the groups. A value of $p<0.05$ was considered as significant. All statistical tests were performed with SPSS 18.

3. Results

The gastric photographs and ulcer index of all groups are presented in Figure 1-2. When ulcerated areas were measured, in the indomethacin applied groups had an increase in ulcerated areas compared to control group (IND, JR+IND $p<0.001$, PAN+IND $p<0.01$). There was a significant decrease in ulcerative areas in PAN+IND and JR+IND groups compared with IND group ($p<0.001$).

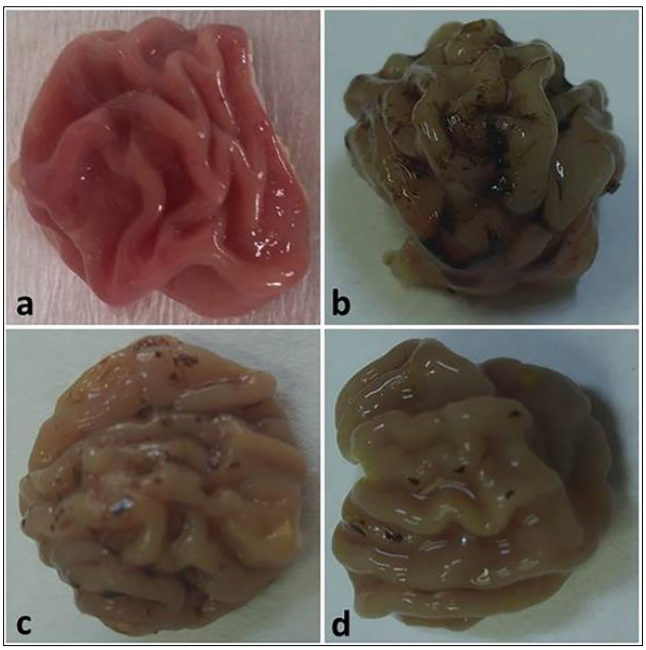

Figure 1 Macroscopic views of stomach tissues of control and experimental group animals (a: C, b: IND, c: JR+IND, d: PAN+IND)

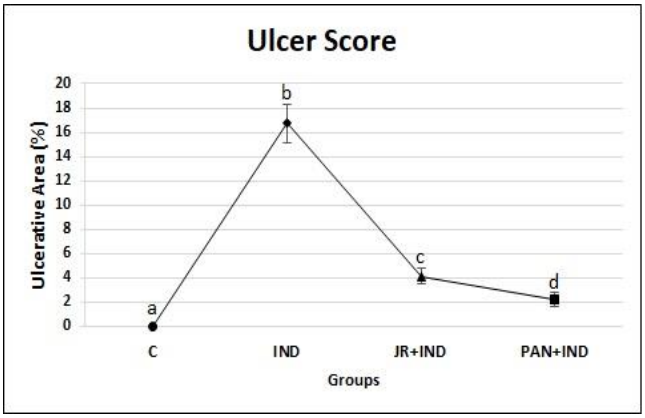

Figure 2 Total ulcerative areas % of control and experimental groups, mean ± SD. a-b, a-c, b-c, b-d: $p<0.001$, a-d: $p<0.01$, c-d: $p<0.05$

Effect of oral administration of IND, JR+IND and PAN+IND on CRP levels are shown in figure 3. The CRP levels of IND group were increased compared to C group ($p<0.001$). There was a significant decrease in CRP levels in PAN+IND and JR+IND groups compared with IND group ($p<0.001$). Also, figure 3 shown that the COX-2R levels were significantly higher in the IND group than in the C group ($p<0.001$). The PAN and JR markedly decreased stomach COX-2R levels compared to IND group ($p<0.001$).

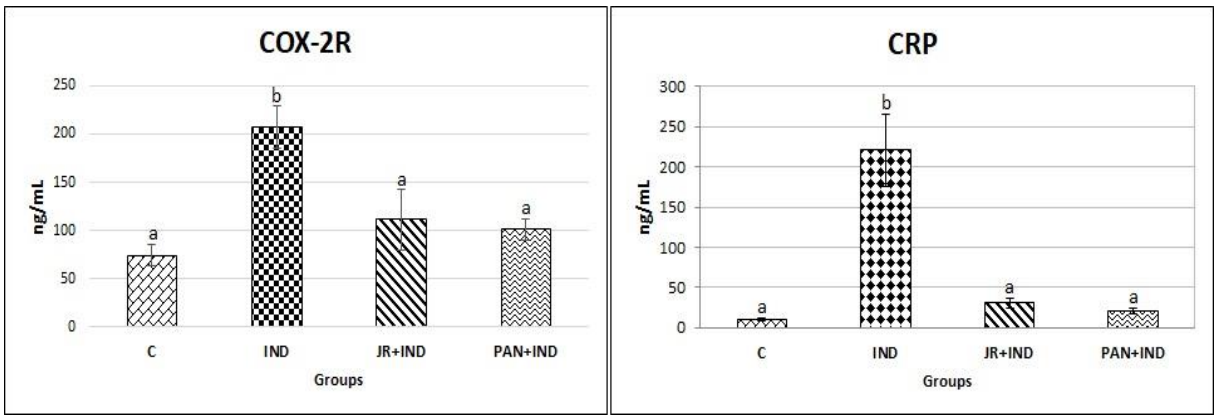

Figure 3 COX-2R and CPR levels of gastric tissues of control and experimental groups, mean ± SD. a-b: $p<0.001$.

As shown in figure 4, indomethacin caused significant increases in the stomach IL-6 and TNF-α levels compared with the C group (respectively, $p<0.001$, $p<0.01$). Treatment with PAN and JR showed a significant lower in the IL-6 levels compare with IND group (respectively, $p<0.001$, $p<0.01$) (Figure 4). However, the administration of PAN and JR did not change TNF-α levels when compared with IND group (Figure 4).

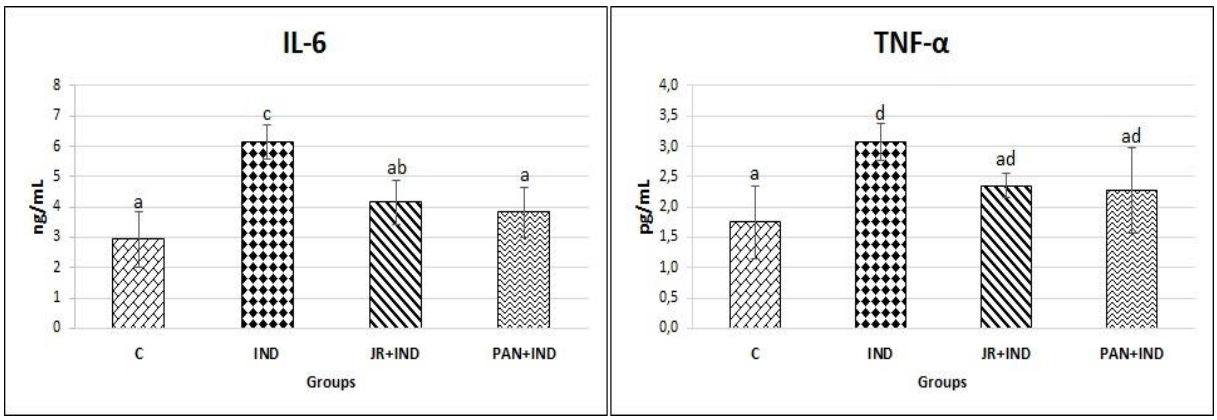

Figure 4 IL-6 and TNF-α levels of gastric tissues of control and experimental groups, mean ± SD. a-c: $p<0.001$, a-d, ab-c: $p<0.01$

4. Discussion

Ulcer is induced due to various factors, and it is known that gastric ulcer caused by indomethacin is associated with inhibition of prostaglandin and mucus synthesis [5, 19]. Therefore, the gastric ulceration model induced by indomethacin is widely used both to investigate the pathogenesis of ulcer and to evaluate the gastric protective effect of various natural products and their active ingredients. In this study, when the ulcer index was calculated, we determined that severe lesions occurred in the IND group, and we also showed in figure 2 that there were significant reductions in the lesioned areas of the stomach when the treatment groups (PAN+IND and JR+IND) were compared with the IND group.

It has been reported that the role of TNF-α signaling pathway and immune cell infiltration in the initiation of pathogen-related immune response in gastric mucosal immunity is quite large and even IL-11/STAT3 inhibits pathogen colonization [20]. However, as a result of gastric ulcer, there is an increase in the level of free oxygen radicals and an increase in the expression of TNF-α and NF-kB from macrophages, and it is stated that the formation of tissue damage

becomes easier [21]. Inflammatory response and proinflammatory cytokine production are induced in gastric damage induced by indomethacin [22]. Previous studies have stated that gastric ulcer causes in an increase expression of proinflammatory cytokine and in a decrease expression of anti-inflammatory cytokine [23, 24]. An increase in the level of TNF, which is one of the important indicators of indomethacin-induced gastric ulcer, is considered to be a negative sign for the gastrointestinal tract [25]. Numerous studies have previously reported that indomethacin significantly increases IL-6 and TNF-α levels [26, 27]. Antonisamy *et al.* [28] found that gastric ulcer causes production of TNF-α, IL-1β, IL-6 increases, while production of IL-4, IL-10 decrease. Jainu *et al.* [29] demonstrated that TNF-α and IL-1β levels were significantly increased after the administration of NSAID. Furthermore, JNK phosphorylation is accelerated in gastric ulcer caused by indomethacin, and NF-KB is caused MCP-1 expression which can lead formation proinflammatory cytokines and reactive oxidant. It is also stated that this event causes persistent neutrophil infiltration in ulceration areas [30, 31]. As a result, an increase in leukocyte accumulation in the areas of inflammation and disintegration of the cell membrane and mucosal damage are facilitated. Our findings are consistent with previous studies, and it was determined that proinflammatory cytokine levels (TNF-α, IL-6) were significantly increased in the IND group compared to group C. Ugan *et al*. [32] found an increase in the expression of proinflammatory cytokines such as, TNF-α and IL-1β in the ulcer group in their gastric ulcer model, and similarly, El-Demerdash *et al*. [22] found that there were an approximately 2-fold increase in TNF-α and IL-1β levels in the ulcer group. In this study, it was determined that there was a significant decrease in IL-6 levels when the treatment groups (PAN+IND, JR+IND) were compared with the IND group. We think that the decrease in IL-6 levels in the JR group is due to the strong antioxidant and anti-inflammatory activity of JR. Liu *et al*. [33] stated that gastric inflammation is suppressed by decreasing TNF-α, IL-6, IL-1β levels and increasing IL-10 levels of *Juglans regia* oligopeptides in gastric damage induced by ethanol.

In addition, aspirin and indomethacin are known as COX-1 selective inhibitors [34]. Kataoka *et al.* [35] stated that indomethacin induced COX-1 inhibition, which resulted in a decrease in PGE2 synthesis, increased gastric acid secretion, decreased mucus secretion consequently caused gastric damage. It is stated that COX-2 level is either very low or not detected in healthy human and animal gastrointestinal tract [36]. Moreover, it was suggested that the level of COX-2 increased within one hour after oral administration of aspirin or indomethacin [37]. In previous studies, significant increases in COX-2 and CRP levels have been reported in the indomethacin-induced ulcer model [26, 27]. In this study, it was determined that there was a significant increase in COX-2R and CRP levels in the IND group compared to the control group, whereas there was a decrease in COX-2R and CRP levels in the JR+IND and PAN+IND groups compared to the IND group. COX-2, considered as one of the pro-inflammatory enzymes, is seen as the main target for the treatment of inflammatory diseases [38]. It has also been reported that a selective COX-2 inhibitor following an inflammatory injury blocks edema and hyperalgesia without harming the gastric mucosa [39]. Considering this aspect, we think that *Juglans regia* has a strong anti-inflammatory effect and reduces COX-2R and CRP levels, and as a result of this effect, ulcerative areas in the JR+IND group decreased compared to the IND group.

5. Conclusion

The present study was determined that JR displayed gastroprotective effect against indomethacin-induced gastric ulcer in rats. Among the underlying mechanisms of its gastro-protective effect are antioxidative effect, suppression of COX-2R, CRP, IL-6 and TNF-α levels. JR appears to be a well alternative against indomethacin-mediated gastric ulceration, and we consider that an important step for further studies.

Compliance with ethical standards

Acknowledgments

Authors are acknowledged for their contributions.

Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

Statement of ethical approval

Experiments on animals were carried out in accordance with national guidelines and were approved by the Local Ethics Committee of Animal Experiments of Kafkas University (protocol no: 2017/57).

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