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Thymoquinone Ameliorates Indomethacin-Induced Gastric Ulcers in Rats: A Dose Response Study

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Abstract: A peptic ulcer is painful sores in the lining of the stomach. Acute and chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) is considered a significant cause of peptic ulcers. Different doses (20-60 mg/kg body weight) of thymoquinone (TQ), the main constituent of *Nigella sativa* L. essential oil, have been shown to protect gastric tissue from NSAID-induced peptic ulcers. Researchers attributed the protective effect of TQ to its antioxidant properties. This study aimed to determine effective antiulcer dose range of TQ. In this study, we conducted a series of experiments to determine the optimal dosage of TQ in indomethacin-induced gastric ulcers in rats. Additionally, we investigated the effect of TQ on superoxide dismutase (SOD) activity, glutathione (GSH) levels, and malondialdehyde (MDA) levels. Our results showed that, when administered at 40 and 20 mg/kg body weight, TQ was ineffective in preventing indomethacin-induced gastric ulcers. Moreover, TQ itself induced gastric ulcers at 40 mg/kg dose. As the doses of TQ decreased, the protective effect increased. 0.5 mg/kg TQ provided the best protection in terms of gastric ulcer area and antioxidant parameters, having statistically the same result with famotidine. Low dose TQ is an efficient protector of indomethacin-induced gastric damage, and it significantly enhances antioxidant parameters of gastric tissue. High dose TQ administration did not produce any desirable effects, but increased ulcer index.
Keywords: Thymoquinone, Rats, Indomethacin, SOD, GSH, MDA.

1. Introduction

Peptic ulcer disease shows a great deal of variation among societies and regions (1). In the past, investigators estimated the lifetime prevalence of peptic ulcer disease to be about 5-10% in the general population, with an incidence of 0.1-0.3% per year. Although researchers noted a worldwide decline in uncomplicated peptic ulcer disease, it remains a common and costly condition (2, 3).

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Helicobacter pylori are the principal cause of peptic ulcers. A potentiating factor for gastric ulcers, mucosal ischemia, is induced by free radical generation, which is the result of lifestyle factors, such as diet, smoking, alcohol, stress, and consumption of fatty foods. Excess secretion of hydrochloric acid, which decreases local blood flow and mucus, is also implied in the etiology of peptic ulcers. The constant use of drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), anticoagulants, corticosteroids, and chemotherapeutic agents are also related to the formation of peptic ulcers (4).

NSAIDs induce gastric damage through inhibition of COX-1, which leads to a reduction of prostaglandin secretion in the gastrointestinal tract and its cytoprotective effects in gastric mucosa. COX-2 inhibition may also play a role in the mucosal injury (5). Increase in mucosal permeability, neutrophil infiltration, production of oxyradicals, and finally, lesions in the gastric mucosa follow the mentioned inhibition (6).

Throughout history, people have been trying various plants for the treatment of ulcers (4, 7, 8). One of the most frequently used plants for the treatment of ulcers is Nigella sativa L., which has been utilized for many diseases (9). Nigella sativa L. is a member of the Ranunculaceae family and is cultured in tropical and subtropical areas. Its seeds are known as black seeds, and the essential oil these seeds possess is considered to be the pharmacologically active part. The most abundant compound present in the essential oil is thymoquinone (TQ), with reported proportions ranging between 18 and 57% (10).

Numerous studies were conducted regarding the gastroprotective effects of Nigella sativa L. essential oil and TQ in different animal models (11-13). Researchers used different doses of TQ (up to 60 mg/kg body weight), and several vehicles (corn oil, water, Carboxymethyl cellulose). Earlier studies (14, 15) reported that TQ exerts its therapeutic actions by scavenging free radicals, however, recent reports suggest that TQ is anti-oxidant in low doses and pro-oxidant at high doses (16, 17).

Our aims in this study were to evaluate the effects of TQ in indomethacin (IND)-induced gastric ulcer in rats, to determine the effective dosage of TQ, and to assess antioxidant parameters, namely superoxide dismutase (SOD) activity, glutathione (GSH) amount and malondialdehyde (MDA) levels in ulcerous tissues.

2. Material and Methods

2.1. Animals

In this study, we used 84 male albino *Wistar* rats obtained from Ataturk University Medical Experimental Research Center (ATADEM). The animals weighed between 180 and 220 g. They were fed standard rat chow under normal temperature conditions (22±2 °C) and kept in separate groups before the experiment. Animal care and experiment protocols were approved by the Experimental Animal Ethics Committee, Ataturk University, Erzurum, Turkey (27.11.2014/1307).

2.2. Chemicals

Indomethacin was purchased from Deva Holding A.S. (Istanbul, Turkey) and famotidine from Sandoz a Novartis Drug Company (Turkey). We obtained thiopental sodium from IE Ulagay A.S. (Istanbul, Turkey) and TQ ($C_{10}H_{12}O_2$, 2-Isopropyl-5-methylbenzo-1,4-quinone, purity≥98%) from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Study Design

We performed three experiments to assess the influence of TQ in IND-induced gastric ulcer in rats (18, 19).

In the first experiment, 24 rats were randomly assigned to 4 groups (n=6) and fasted for 24 hours. The first group received water, the second group 40 mg/kg TQ, the third group 20 mg/kg TQ, and the fourth group 40 mg/kg TQ. After five minutes, the first, third and fourth groups received 25 mg/kg IND. TQ was suspended in water since it did not dissolve. Six hours later, the rats were sacrificed using high dose anesthesia. Their stomachs were removed and evaluated for damaged areas. This experiment was designated EXP1.

Results of EXP1 led us to perform a second experiment. Twenty-four rats were randomly assigned to 4 groups (n=6). The first group received water, the second 10 mg/kg TQ, the third 5 mg/kg TQ, and the fourth 3 mg/kg TQ. Five minutes later, all groups received 25 mg/kg IND. Six hours later, the rats were sacrificed with a high dose of thiopental sodium. The rats' stomachs were removed and evaluated for damaged areas. This experiment was designated EXP2.

After observing a substantial decrease in ulcer areas in EXP2, a third experiment was performed using 36 rats. The animals were fasted for 24 hours and randomly assigned to 6 groups (n=6). The first and second groups received water. The third group received 40 mg/kg famotidine (FAM), the fourth group 2 mg/kg TQ, the fifth group 1 mg/kg TQ, and the sixth group 0.5 mg/kg TQ. Five minutes later, all groups except the first received 25 mg/kg IND. Six hours later, rats were sacrificed with high dose anesthesia. Stomachs were removed and assessed for ulcer areas. This experiment was designated EXP3. The stomachs obtained in this experiment were used to assess SOD activity and GSH and MDA levels.

2.4. Macroscopic analyses of stomach tissues

The rat stomachs were opened along the greater curvature and washed with serum physiologic. The ulcer areas were determined using a magnifier and a millimeter paper. The sum of the ulcerous areas was expressed in square millimeters (mm^2) as the ulcer score. Antiulcer effects were calculated using the following formula:

$$\text{Antiulcer effect}=\%\ \text{protection}=\left(1-\frac{\text{ulcer score of the treatment group}}{\text{ulcer score of control group (indomethacin)}}\right)\times 100$$

After macroscopic analyses, all rat tissues from EXP3 were kept at -80 °C and further used in biochemical assays. All other tissues were discarded.

2.5. Biochemical investigation of stomach tissues

All stomach tissue samples were first perfused with PBS/heparin and then ground in liquid nitrogen using a tissue lyser II grinding jar set (Qiagen, Hilden, Germany). For each sample, 100 mg was weighed and then treated with 1 mL of PBS buffer. This mixture was homogenized on ice in an Eppendorf tube, using a tissue lyser II adapter sets 2x24 homogenizer. Homogenates were centrifuged at 4 °C using a refrigerator centrifuge. SOD (20), GSH (21) and MDA (22) levels from the samples' supernatants and standards were measured at room temperature, in duplicate, according to the modified methods of the ELISA reader as previously described. The average absorbance of each sample and standard were measured. A standard curve was plotted, and an equation obtained. This equation was employed to calculate SOD activity and GSH and MDA concentrations. The SOD activity was expressed as U/mg protein, while GSH and MDA levels as nmol/mg protein.

2.6. Protein determination

Protein concentrations were determined using a commercial total protein kit (Sigma Aldrich, St. Louis, MO), according to the Lowry method (Peterson's modification).

2.7. Statistical analyses

IBM SPSS program (version 19.00) was used to conduct statistic comparisons between groups. The results are presented as Mean±SD. Group comparisons were performed using one-way ANOVA and Duncan multiple comparison tests. $P<0.05$ was considered significant.

3. Results

3.1. Indomethacin-induced gastric ulcers

Results of EXP1 and EXP2 are summarized in figure 1 The indomethacin-induced gastric ulcer model is well established, as can be seen from the ulcer areas of IND groups in EXP1 and EXP2. The amount of ulcer area in the TQ groups decreased as the amount of TQ decreased.

Figure 2 presents the results of the EXP3. Famotidine (40 mg/kg) produced 96.15% protection from the ulcer, while 0.5 mg/kg TQ decreased the ulcer area by 85.48%. 1 and 2 mg/kg doses of TQ ameliorated the gastric damage by 77.35 and 47.17%, respectively.

Figure 3 presents the macroscopic evaluation of tissues. 40 mg/kg TQ induced hemorrhages in gastric tissue. IND induced gastric ulcer model was successfully established.

3.2. Biochemical investigation of stomach tissues

The antioxidant levels (SOD, GSH, and MDA) in the stomach tissues obtained from EXP3 were evaluated to investigate the influence of antioxidant defenses on the ulceration process. Figure 4 presents the results. These results show that indomethacin reduced the activity of the SOD enzyme, decreased the GSH concentration, and increased the MDA concentration. Famotidine and all doses of TQ significantly ($P<0.05$) improved the SOD activity, increased GSH levels, and decreased MDA levels when compared with the IND group. 0.5 mg/kg dose TQ performed best in all three parameter tests amongst TQ doses.

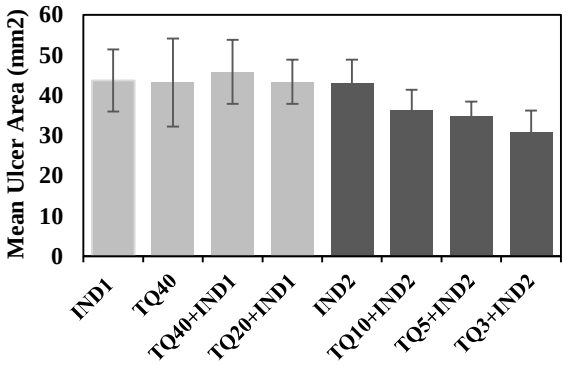

Figure 1: Results of EXP1 and EXP2. Light bars represent EXP1 and dark bars represent EXP2. IND1 and IND2: indomethacin (25 mg/kg), TQ40: thymoquinone (40 mg/kg). The numbers following TQ are the doses in mg/kg body weight. Error bars indicate±standard deviation.

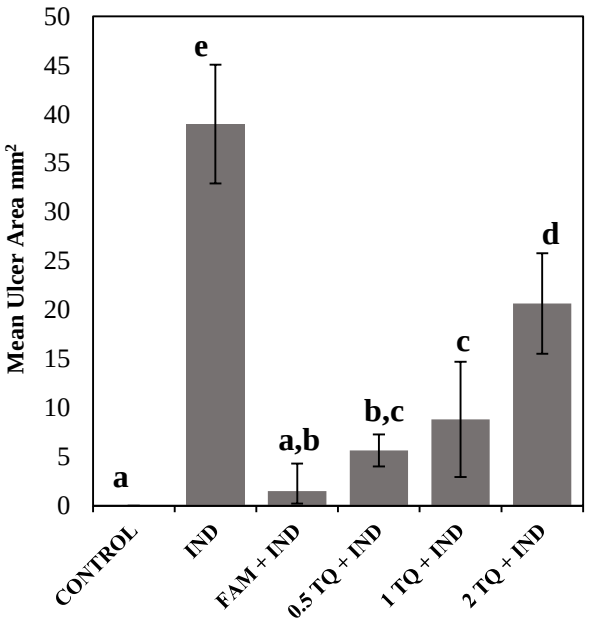

Figure 2: Results of EXP3. Effects of TQ and famotidine on indometacin induced gastric damage in rats. TQ, thymoquinone; FAM, famotidine; IND, indomethacin. Error bars indicate±Standard Deviation. Means in the same column with the same letter do not differ significantly (oneway ANOVA followed by Duncan test ($p<0.05$)).

4. Discussion

In the present study, the indomethacin-induced gastric ulcer model in rats was used for the first time to investigate the gastroprotective effect of TQ. Additionally, the antioxidant effect of TQ was examined in ulcerative gastric tissues.

Induction of gastric ulcers in rats by indomethacin is an established model of NSAID-induced ulcers in humans (23). NSAID usage deteriorates stomach mucosal integrity, which results in erosions, ulcers, hemorrhages, and perforations in gastric mucosa. This effect on gastric mucosa reduces the production of bicarbonate, mucus, and cytoprotective prostaglandins. It also causes the formation of free radicals (ROS) and lipid peroxidation (24). Though several factors are involved in gastric mucosal damage, oxygen-derived free radicals play a notable role in the pathogenesis of NSAID damage. ROS are continuously generated during typical physiologic incidents and removed by antioxidant defense mechanisms. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in the cellular membrane or intracellular molecules (25).

We started by testing 20 and 40 mg/kg body weight doses of TQ in indomethacin-induced gastric ulcers. We were unable to dissolve TQ in water and had to prepare a homogenous suspension of it in water. This observation is in accordance with recent findings that aqueous solubility of thymoquinone is in the range of 549-669 μg/mL (26). Unexpectedly, pretreatment with TQ did not produce any improvement. Moreover, the application of 40 mg/kg TQ alone resulted in hemorrhage in rat stomachs (Figure 3, C and D), and ulcer area of the TQ40+IND1 group increased when compared with the IND1 group. 20 mg/kg TQ exhibited no improvement in terms of ulcer formation (Figure 1, light bars).

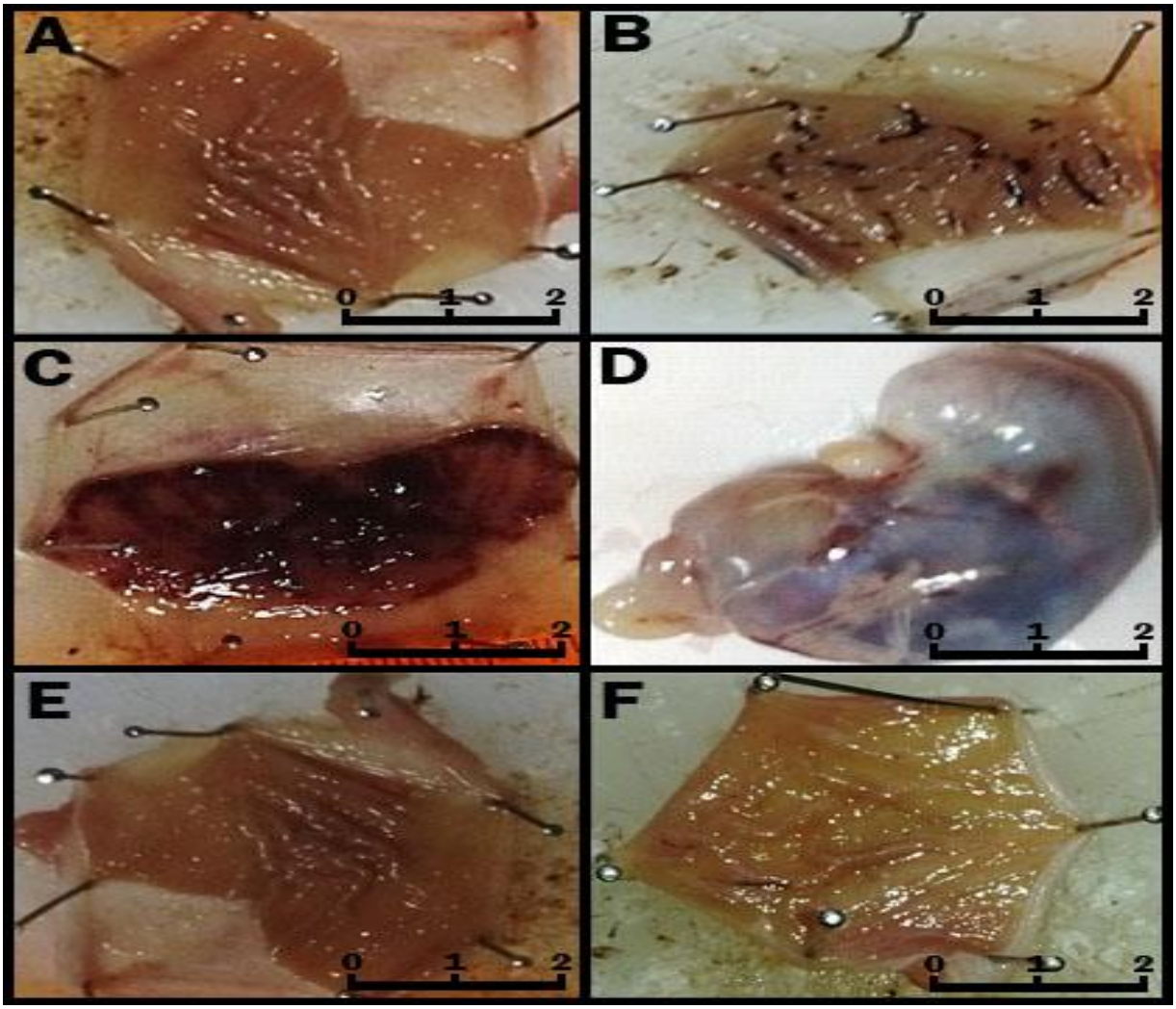

Figure 3: Ulcerous areas in the gastric tissues of indomethacin (IND)-induced ulcer model. Sections of the gastric tissues after IND administration were obtained from experimental groups. A: control group, B: IND, 25 mg/kg body wt., C: 40 mg/kg TQ alone, D: stomach before opening 40 mg/kg TQ, E: FAM 40 mg/kg, and F: 0.5 mg/kg TQ.

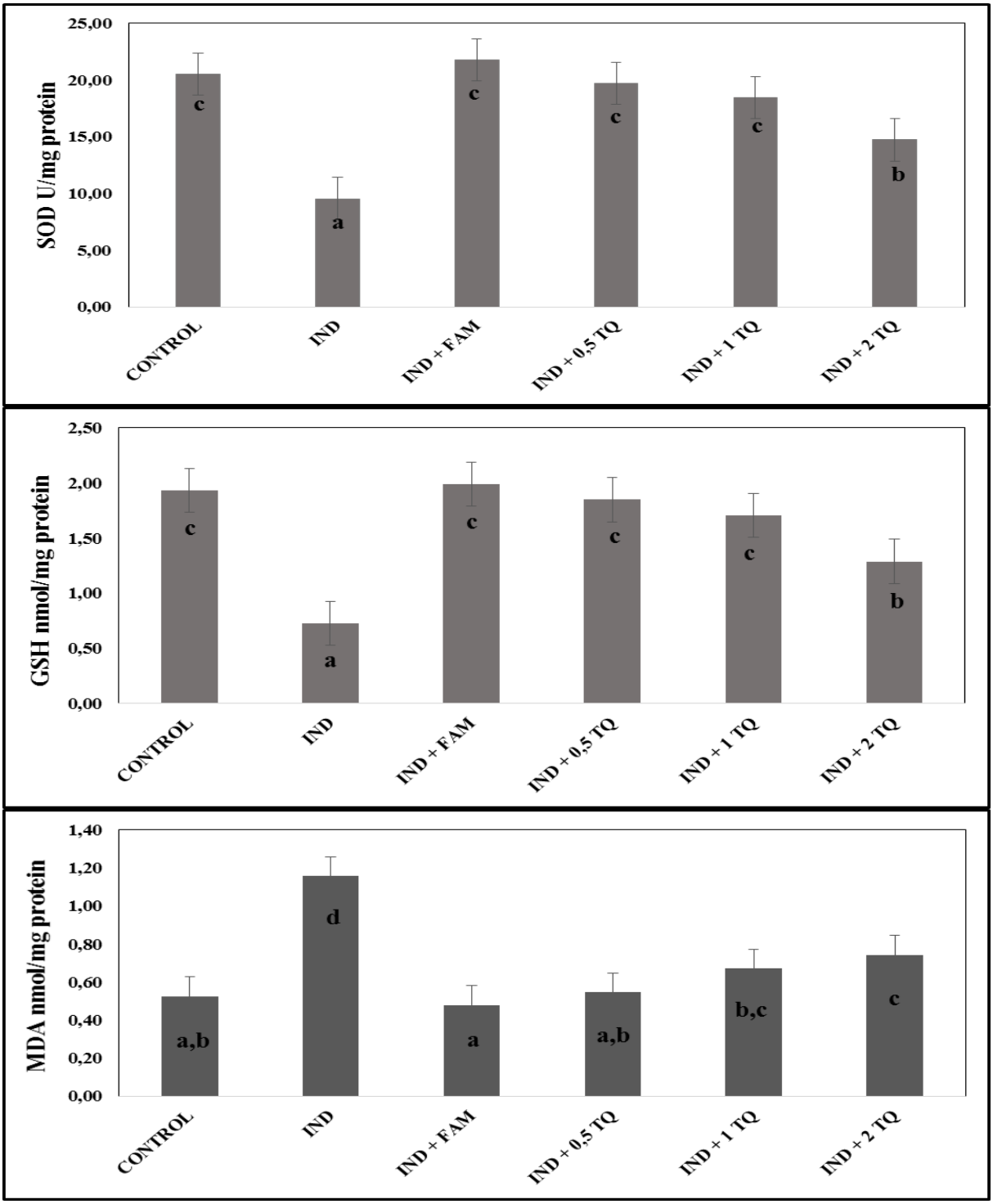

Figure 4: Effects of thymoquinone and famotidine pretreatment on changes in SOD activity GSH and MDA levels induced by indomethacin for EXP 3. TQ, thymoquinone; FAM, famotidine; SOD, superoxide dismutase; GSH, glutathione; MDA, lipid peroxidation. Error bars indicate±Standard Deviation Means in the same column with the same letter do not differ significantly (Oneway ANOVA followed by Duncan test ($p<0.05$).

We tested pretreatment with 10, 5, and 3 mg/kg TQ in EXP2. Although the mean ulcer area exhibited a steady decrease with the decreased amounts of TQ (Figure 1, dark bars), the final result was far from our expectations and reported results (13, 25). Therefore, we decided to use even lower doses (2, 1, and 0.5 mg/kg) of TQ in EXP3.

Results of EXP3 (Figure 2) reveals that TQ has a potent influence on the development of indomethacin-induced gastric ulcers in rats. 0.5 mg/kg TQ decreased mean ulcer area by 85.48% while 1 and 2 mg/kg TQ decreases mean ulcer area by 77.35 and 47.17%, respectively. 40 mg/kg famotidine, as expected, decreased ulcer area by 96.15%.

These data put forward a new argument that pretreatment with low-dose TQ inhibits indomethacin-induced gastric ulcers in rats while high doses are ineffective or toxic.

Previous studies claimed that much higher doses of TQ (up to 60 mg/kg) had antiulcer effects (11, 12). It is safe to assume that the TQ amount will be significantly lower in Nigella sativa seeds. Therefore, it may be reasonable and efficient to use high doses of Nigella sativa raw products such as extracts and oils. High doses of TQ or TQ-enriched fractions, however, are irrational when compared with our results.

The mechanisms underlying this phenomenon may be the fact that TQ inhibits COX-1 derived PGE_2 production in vitro, with an IC_{50} value of 2.6 μM, and COX-2 derived PGE2 production with an IC_{50} value of 0.3 μM (27). Also, TQ was declared to dose-dependently induce oxidative damage in mitochondria (16). Zubair, Khan (17) demonstrated that TQ was antioxidant in low doses but pro-oxidant in high doses. Putting it all together, we can speculate that high doses of TQ act as a pro-oxidant and have the potential to inhibit COX-1 and COX-2 significantly. This inhibition can reduce the production of PGE_2, leaving gastric tissue open to acid damage. Also worth mentioning is the severe hemorrhage caused by 40 mg/kg TQ in intact rats, which suggests that TQ may be directly acting on gastric tissue and damaging the integrity of it. We cannot speculate anything about the mechanism of this suggestion yet, due to lack of data. However, new studies may shed light on the subject.

TQ has become popular because of its proven therapeutic properties, and it is a common substance of not only *Nigella sativa* but also lots of other medical plants (28). Studies have shown that compounds found in seeds of *Nigella sativa* have anticancer (29), antitumor (30), anti-inflammatory and analgesic (31), antioxidant (32) and immuno-enhancing (33) effects. Studies about the gastro-protective effect of TQ are also available (11-13, 15, 25, 34). These studies were about ethanol-induced gastric damage and gastric ischemia/reperfusion damage.

Among TQ doses, 0.5 mg/kg TQ had the highest efficiency in all tests. Additionally, when compared to the FAM group (positive control group), the gastro-protective effect of 0.5 TQ group was statistically the same. There is a linear relationship between the dose increase in TQ and damage increase in gastric tissues. In a previous study, the gastroprotective activity of Nigella sativa oil and its constituent, thymoquinone, was studied against gastric mucosal injury induced by ischemia/ reperfusion in rats. In this study, similar to our results, it was shown that when the dose increased, the damage increased (13).

One of the most significant markers in ulcer physio-pathogenesis is the increase in free oxygen radicals induced by oxidative stress. The role of free oxygen radicals and oxidative stress was defined in IND induced gastric ulcers (35-38). Numerous researchers investigated TQ and other *Nigella sativa* products in oxidative stress-linked diseases, such as ulcers, in clinical and experimental studies, because of their antioxidant properties (28). TQ was tested routinely in numerous experiments (25) and its radical scavenging properties emerged as partly the source of its protective effects. Quinone structure in TQ shows excellent redox property, can penetrate easily from the cell membrane, and scavenge free oxygen radicals (39). In another metabolic pathway, TQ decreased oxidative stress by inducing cellular glutathione (40) and acted as an antioxidant (41).

Enzymatic (CAT, SOD, GPx) and non-enzymatic (vitamins, GSH) antioxidant defense systems are available to prevent oxidative damage (42). The cells' natural protection system against the destructive actions of free radicals contains the protection enzyme SOD and the antioxidant molecule, GSH. SOD, GSH, and MDA are all established indicators of the antioxidant capacity of the body (the protection against the damage caused by oxidative stress). An increase in mucosal levels of MDA and a decrease in SOD and GSH levels accompanied the gastric lesions observed in this study. Cell membrane damage caused by indomethacin may contribute to elevated MDA levels. The decreased SOD and GSH levels and the increased MDA levels in our study were in accordance with previous research studying indomethacin-induced nephrotoxicity (43-45). These results suggest that low doses of TQ reduce oxidative stress in stomach. We can say that TQ prevented the depletion of antioxidant enzymes, including GSH, in the present study. This suggestion is supported by macroscopic analysis findings, indicating that the administration of the TQ significantly prevented ulceration damage. Previous studies suggesting potential antioxidant capacity of TQ support our findings (46, 47). Also, TQ supplementation reverses lead-induced oxidative stress (14, 39).

5. Conclusions

The cytoprotective role of antioxidants in the prevention and treatment of gastric lesions has been investigated comprehensively in several studies. As far as the authors know, this is the first study to demonstrate that TQ might be a potential inhibitor of indomethacin-induced gastric ulcers. Biochemical analysis and macroscopic investigations revealed that low-dose TQ has protective effects. Pretreatment with 0.5 and 1 mg/kg doses of TQ can decrease the ulcer index and boost the recovery of gastric lesions induced by indomethacin in rats. The fact that the administration of low-dose TQ enhances SOD, GSH and MDA parameters in indomethacin-induced gastric ulcer supports this conclusion. The strong redox potential of quinone in the TQ structure may have scavenged the free radicals caused by IND, leading to a decrease in oxidative stress and protection of gastric tissue from ulceration.

In contrast, pretreatment with high-dose TQ was ineffective in protecting gastric tissue from indomethacin-induced damage. Moreover, high-dose TQ can disintegrate gastric tissue and cause hemorrhage. This may be attributed to TQ's ability to inhibit COX-1 and COX-2, and act as pro-oxidant in high doses.

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Conflict of interest statement
None of the authors has a commercial interest, financial interest, and/or other relationship with manufacturers of pharmaceuticals, laboratory supplies, and/or medical devices or with commercial providers of medically related services. All the listed authors have read and approved the submitted manuscript.

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Author Contributions
Turan C: Managing the in-vitro and in-vivo process of experiments. Bayir Y: Constructing an idea or hypothesis for manuscript Karagoz Y: Taking responsibility in the construction of the whole or body of the manuscript Albayrak A, Erkayman B: Managing the in-vivo process of experiments Duysak L: Managing the in-vitro process of experiments.

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