

Thymoquinone counteracts oxidative and inflammatory machinery in carrageenan-induced murine paw edema model

Hayfa Hussin Ali Hijazy

Umm Al-Qura University

Naief Dahran

University of Jeddah

Hussam A. Althagafi

Al Baha University

Fahad Alharthi

Taif University

Ola A. Habotta

Mansoura University

Atif Abdulwahab A. Oyouni

University of Tabuk

Mohammad Algahtani

Security Forces Hospital Program Makkah

Abdulrahman Theyab

University of Tabuk

Osama Al-Amer

University of Tabuk

Maha S. Lokman

Prince Sattam bin Abdulaziz University

Khalaf F. Alsharif

Taif University

Ashraf Albrakati

Taif University

Hatem K. Amin

Galala University

Shauq Mumtaz Dawood

Osmania University

Rami Kassab (✉ rami.kassap@yahoo.com)

Helwan University Faculty of Science <https://orcid.org/0000-0002-1520-1601>

Rania A. Ellethy

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Abstract

Thymoquinone (TQ) is an active constituent in *Nigella sativa*, and is extensively reported for its distinguished antioxidant, and anti-inflammatory bioactivities. Despite the local protective response of the acute inflammation, it contributes to the development of various disease conditions such as cell death, organ damage, or genesis of tumors. Hence, in this study, the effects of orally administered TQ (50 and 100 mg/kg) for 14 days against edema development, oxidative stress, and inflammation were investigated in paw edema induced by carrageenan in mice. Indomethacin (10 mg/kg) was used as a reference drug. The results revealed that TQ reduced the paw edema volume in a time-dependent manner, attenuated acetic acid-provoked writhing movements, and reduced xylene-triggered ear edema. Hematological findings revealed marked normalization of altered counts of WBCs, and platelets. Furthermore, paw tissue levels of MDA and NO showed marked decreases together with increases Nrf2, GSH, SOD, CAT, GPx, and GR after TQ administration. Additionally, TQ decreased the levels of pro-inflammatory mediators, such as IL-1b, TNF- α , IL-6, MCP-1, CRP, MPO, and NF- κ B in the inflamed paw tissue. Moreover, appreciable decreases were recorded in COX-2 and its product (PGE-2) as well as the immune reaction of TNF- α in TQ-treated mice. Histopathological findings further validated the potential antioedematous, anti-inflammatory power of TQ in inflamed tissues. Conclusively, the obtained findings encourage the potent application of TQ to subside the acute inflammatory events because of its striking antioxidant and anti-inflammatory properties in the inflamed paw tissue.

Introduction

Inflammation is an ordered immune response to counteract tissue injury that can be triggered by noxious stimuli such as microbes, toxicants, irradiation, or even against the damaged cells (Alblihed 2020, Cordaro et al. 2020). The acute inflammatory reaction is accompanied by several microcirculatory events including changes in the vascular permeability, excess leukocytic infiltration, extra-production of pro-inflammatory mediators with subsequent redness, swelling, hyperthermia, and pain (Su et al. 2019, Zhang et al. 2020). These cellular and molecular sequences are considered as guarding mechanisms of tissues against the initial injury as well as restoring their homeostasis (Zhang et al. 2020). However, an uncontrolled inflammatory response may lead to chronic inflammation that contributes to the development of various chronic disorders such as cancers, neurodegeneration, autoimmune, cardiac, and aging disorders (Alblihed 2020, Pop et al. 2020).

During the inflammatory process, an excess amount of reactive oxygen species (ROS) are produced by the phagocytes (Mitrea et al. 2020). Owing to their instability, ROS tend to steal electrons from other cellular molecules that consequently trigger oxidative stress, depletion of the antioxidant system, and lipid peroxidation (Li et al. 2021, Pop et al. 2020). Further, ROS activate the transcription factor NF- κ B which control and maintain the inflammatory condition by stimulating the release of pro-inflammatory cytokines (El-Shitany & Eid 2019a, Yuan et al. 2020b). Cyclooxygenase-2 (COX-2) enzyme has a principal role in the production of prostaglandins in inflamed tissue (Abdel-Lateff et al. 2020). Based on the major

contribution of ROS in the initiation of inflammatory processes, their neutralization by antioxidants stand as a potent inhibitor for tissue inflammation and its consequences (Zhang et al. 2020).

Inflammation and edematous conditions are currently treated by synthetic anti-inflammatory agents that are unfortunately associated with various adverse effects (Abdel-Lateff et al. 2020, Akhtar &Shabbir 2019). For instance, nonsteroidal anti-inflammatory drugs like indomethacin, and ibuprofen are associated with GIT disturbances such as vomiting, bleeding, gastric ulcer, and diarrhea as well as increased risk of renal tubular necrosis and myocardial infarction (Alblihed 2020). Additionally, corticosteroids have established serious health conditions such as hypertension, compromised immunity, diabetes, and osteoporosis (Abdel-Lateff et al. 2020). In this regard, alternative anti-inflammatory therapy with maximum efficacy and minimum adverse effects is urgently needed (Zhang et al. 2020). Natural products have recently been accepted by the public and scientific community owing to their safety profile and abundant bioactive chemical ingredients (Majdalawieh &Fayyad 2015).

Nigella sativa (black cumin) is a member of *the Ranunculaceae* family and is used extensively to treat cardiovascular disorders, gastrointestinal diseases, respiratory diseases, hypertension, dyslipidemia, diabetes, and various types of cancer (Al Aboud et al. 2021, Pop et al. 2020). Its beneficial effects are explained by its bioactive components (Pop et al. 2020). Among them, thymoquinone (TQ) is an aromatic ketone derived from the seed oil extract of this plant (Al Aboud et al. 2021). TQ possesses various cytoprotective properties like antioxidant, anti-inflammatory, and anti-tumor activities (Lei et al. 2012). Several studies have shown that TQ effectively alleviated inflammatory conditions in experimental models of osteoarthritis (Wang et al. 2015), acute pancreatitis (Dur et al. 2016), otitis media (Gülmez et al. 2017b), corrosive oesophagitis (Karaca et al. 2017), and bronchial asthma (Su et al. 2016). Concerning the dermato-pharmacological effects, TQ was efficient against skin fungal and bacterial infections as well as skin tumors (Al Jabre 2005, Ivankovic et al. 2006, Kundu et al. 2013). Kundu et al. (Kundu et al. 2013) reported that TQ inhibits epidermal inflammation induced by phorbol ester via attenuation of NF- κ B and COX-2 in addition to enhancement of cytoprotective enzymes expression in mice skin. Moreover, topically applied TQ for four weeks significantly relieved the signs of allergy or hypersensitivity in hand eczema similarly to the effect of betamethasone (Yousefi et al. 2013).

As well-known, carrageenan is widely used for induction of acute inflammation by stimulation of cell infiltration, mainly neutrophils, and production of inflammatory mediators such as myeloperoxidase and cytokines (Zhang et al. 2020). Further, oxidative damage and lipid peroxidation are implicated in the progress of inflammatory responses. With this background in mind, we investigated the efficacy of TQ in carrageenan-induced paw edema in a mice model via examination of the oxidative stress markers, and inflammatory mediators in the inflamed skin.

Material And Methods

Chemicals and reagents

TQ (CAS number: 490-91-5), indomethacin (CAS number: 53-86-1), and carrageenan (CAS number: 9064-57-7) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other reagents were of high analytical grade.

Experimental animals and ethics statement

Male Swiss mice, 20–25 grams in weight and 8-10 weeks in age were obtained from the animal house of the VACSERA (Cairo, Egypt). They were housed in ordinary cages at 12 h light/dark cycle, $25 \pm 1^\circ\text{C}$ temperatures, and fed on rodent feed with free access to water. The animal care procedures are in agreement with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals 8th edition and the Institutional Animal Ethics Committee guidelines for Laboratory Animal Care at Zoology Department, Faculty of Science, Helwan University (Approval Number: HU2020/Z/RKA820-01).

Xylene-induced ear edema test

For assessment of the anti-inflammatory action of TQ, a xylene-induced ear edema test was done following the method of Wang et al. (Wang et al. 2014). In brief, mice received the TQ and indomethacin prior to xylene application. After 1 hour, 20 μl of xylene was applied to the inner aspect of the right ear. One hour following the xylene administration, all mice were killed and both ears were sampled and weighed. The ear swelling was assessed depending on the difference in weight of both ears.

Antinociceptive activity of TQ

An acetic acid-induced writhing test was done to estimate the pain-relieving efficacy of TQ (Dey et al. 2010). One hour prior to acetic acid (0.5 ml of 0.6% aqueous solution) injection, all mice were treated with TQ and indomethacin. After that, mice were housed in separate cages. The writhing represents the contraction of the abdominal muscle for each mouse. After acetic acid injection for 20 minutes, these movements were counted for 10 minutes. Percent of inhibition was measured according to this equation:

$$\text{The percent of inhibition} = [(W_c - W_t) \times 100] / W_c$$

Where W_c = Number of writhes in control mice and W_t = Number of writhes in treated mice.

Induction of paw edema by carrageenan injection

After being adapted for two weeks under the lab conditions, mice were allocated into five groups as follows:

The first group (control): Mice were orally administered normal saline by oral gavage, simultaneously with the drug's administration to the other groups.

The second group (carrageenan): Mice were injected with carrageenan (100 μl of 1%) subcutaneously in the subplantar tissues of the left hind paw of each animal (Zhang et al. 2020).

The third and fourth groups were orally administered TQ at doses of 50 and 100 mg/kg body weight (Mansour et al. 2002) and the fifth group (indomethacin) was received 10 mg/kg/day of indomethacin as a reference drug according to Mondal et al. (Mondal et al. 2019).

Indomethacin and TQ were orally administered to the mice for seven repeated days prior to carrageenan injection. The paw volume was estimated by a Vernier caliper (LETICA Scientific Instruments, Barcelona, Spain) instantly prior to and after carrageenan injection at 2, 4, 6, and 8 hours. The obtained results were displayed as the difference in the paw volume (mL) in comparison with the right rear paw of the same animal. At 8 h, mice were sacrificed, and blood was collected from the retroorbital sinus. Paw skin samples were sampled and divided into two parts. The first part was immediately homogenized to obtain 50% (w/v) homogenate in an ice-cold medium containing 50 mM Tris-HCl (pH 7.4) and centrifuged at 500 × g for 10 min at 4°C. The supernatant was used for the biochemical analysis, whereas the second part was used for the histopathological examination.

Counting of white blood cells (WBCs) and platelets

Blood samples were collected in test tubes containing EDTA as an anticoagulant for counting WBCs using a hemocytometer according to the method described by Coles (Coles 1986).

Estimation of C-reactive protein (CRP) level

Serum level of CRP was estimated colorimetrically using DiaSys Diagnostic kit (Germany).

Evaluation of antioxidant enzymatic activities in paw tissue

Superoxide dismutase (SOD) and catalase (CAT) activities in paw tissue were estimated by the difference in the color intensity at 539 nm and 240 nm according to Fisher et al. (Fisher et al. 2003) and Aebi (Aebi 1984), correspondingly. Glutathione peroxidase (GPx) and glutathione reductase (GR) were measured according to the method explained by Paglia and Valentine (1967) and Factor et al. (1998), respectively. Further, glutathione (GSH) levels were determined by the reduction of Ellman's reagent to yield a yellow compound. The amount of reduced chromogen is indicative of GSH content and its absorbance was estimated at 405 nm (Ellman 1959). Malondialdehyde (MDA) in the paw tissue was estimated using thiobarbituric acid reactive substances at 535 nm (Ohkawa et al. 1979).

Cytokines and inflammatory mediators in paw tissue

ELISA kits (CUSABIO Life Sciences, Wuhan, China) were utilized for measurement of levels of interleukin-1 beta (IL-1 β ; cat. no: CSB-E04621m), tumor necrosis factor-alpha (TNF- α ; cat. no: CSB-E04741m), interleukin-6 (IL-6; cat. no: CSB-E04640r), cyclooxygenase-2 (COX-2; cat. no: CSB-E12910m), prostaglandin E2 (PGE2; cat. no: CSBPA040059), nuclear factor kappa beta (NF- κ B; cat. no: CSB-E13148r), and monocyte chemoattractant protein-1 (MCP-1; cat. no: CSB-E07430m) according to the manufacturer's information.

Myeloperoxidase (MPO) activity and nitric oxide (NO) levels in paw tissue

MPO activity was analyzed following the protocol of Bradley et al. (Bradley et al. 1982) with slight modifications. The paw tissue homogenate underwent three freeze-thawing cycles and centrifugation at $15,000 \times g$ for 10 min at 4°C. Next, MPO activity was evaluated by adding 200 μL of the paw supernatant to 2.8 mL of 50 mM phosphate buffer (pH 6.0) and 1 mL of 1.67 mM o-dianisidine hydrochloride containing 0.0005% (v/v) H_2O_2 . The MPO activity was calculated based on the change in the absorbance at 450 nm and presented as U/mg protein. Furthermore, levels of NO were measured as stated by Green et al. (Green et al. 1982) by adding the Griess reagent and sulfanilamide for 10 min in dark at 30°C, and the absorbance of the bright reddish-purple azo dye was assessed at 540 nm.

Hematoxylin and eosin staining

Paw tissue specimens were preserved in 10% neutral buffered formalin for 24 hours, dehydrated, and fixed in molten paraplast. The resulted blocks were cut into sections at 4–5 μm thickness and stained with hematoxylin and eosin. The microscopic examination was performed under a Nikon microscope (Eclipse E200-LED, Tokyo, Japan).

Immunohistochemistry procedures

Sections from paw tissues were incubated in 10% H_2O_2 for 30 min to eliminate endogenous peroxidase activity and blocked for one hour with 10% normal goat serum at room temperature. Sections were further incubated with primary anti-TNF- α antibodies (Abcam, Cambridge, MA; 1/1000) for 24 h at 4°C. Antibody detection was performed using the Histostain-Plus Bulk kit (Invitrogen) against rabbit IgG, and finally, 3,3'-diaminobenzidine was used for visualization. A Nikon microscope was used to capture photomicrographs at 400 \times magnification (Eclipse E200-LED, Tokyo, Japan). TNF- α expression was estimated by counting TNF- α (+) cells in random sections from each rat.

Statistical analysis

All data were expressed as the mean \pm standard error (SE) after being analyzed by one-way analysis of variance (ANOVA) and *post hoc* Duncan's multiple range test. Statistically significant differences were considered when *p* values were less than 0.05.

Results

The anti-inflammatory effect of TQ on ear edema in mice

The anti-inflammatory impact of TQ against xylene-induced ear edema is depicted in Fig. 1. The carrageenan-challenged group had significant ($P < 0.05$) inhibition in edema formation induced by xylene related to the control group. In contrast, TQ dose-dependently subsided xylene-induced edema in relation to the model group. Indomethacin at the dose of 10 mg/kg also caused a marked decrease in edema

formation ($P < 0.05$) related to control. These findings showed the anti-oedematous and anti-inflammatory effect of TQ against edema formation by xylene in mice.

Antinociceptive effect of TQ on writhing movements in mice

The effect of TQ on the pain sensation indicated by the number of writhes in mice is shown in Fig. 2. Notably, substantial increases ($P < 0.05$) were detected in the number of writhes after injection of carrageenan compared to those of the control group. However, TQ at doses of 50 and 100 mg/kg induced a noteworthy anti-nociceptive effect ($P < 0.05$) following carrageenan injection. The reduction was dose-dependent and the highest effect on the writhing count was observed at 100 mg/kg. Remarkably, the reference drug, indomethacin (10 mg/kg), declined the number of writhes noticeably related to the model group. These results revealed that the pre-treatment of TQ markedly relieved the pain as shown by the decreased number of writhes in mice.

The antioedematous activity of TQ in carrageenan-injected mice

As displayed in Fig. 3, subplanter injection of carrageenan developed marked edema in the rear paw skin after 1 hour that reached its maximum after 8 h. However, mice treated with either TQ or indomethacin for five days before carrageenan injection showed a notable decrease in ($P < 0.05$) the volume of paw edema at a time-dependent manner after 2, 4, and 8 h when compared to the model group. Remarkably, treatment of the carrageenan-injected group with the higher dose of TQ (100 mg/kg) evoked the maximum inhibition after 8 h that achieved a comparable antioedematous activity with that of 10 mg/kg/day of indomethacin. The results reflect the antioedematous action of TQ on the developed edema by attenuating the vascular variations following carrageenan injection.

Effect of TQ on CRP levels, WBCs, and platelets count in carrageenan-injected mice

Significant ($P < 0.05$) increases were recorded in levels of CRP, WBCs, and platelet count after carrageenan injection compared to the control group. Adversely, the level of CRP, WBCs, and platelet counts of indomethacin and TQ-treated mice recorded a significant decrease from those of the model group. as displayed in Fig. 4.

Effect of TQ on oxidative stress biomarkers in paw tissue in carrageenan-injected mice

Considering the implication of oxidant free radicals in the pathogenesis of inflammatory reactions and the ability of dietary antioxidants in scavenging or neutralizing free radicals, the oxidative stress biomarkers were examined in paw tissue (Fig. 5). Significant depletion ($P < 0.05$) was detected in the content of GSH associated with notable inhibitions ($P < 0.05$) of the enzymatic activities of SOD, CAT, GPx, and GR in the carrageenan group in comparison with the control group. Also, carrageenan injection-induced noteworthy increments in the levels of MDA in paw tissue in relation to the control group. In contrast, pre-treatment of TQ or indomethacin before carrageenan injection significantly boosted ($P < 0.05$) the activities of antioxidant enzymes and GSH levels ($P < 0.05$) if compared with the model group. It is noteworthy that the pre-treatment of carrageenan injected mice with TQ at the dose of 100 mg/kg

efficiently restored the levels of GSH and antioxidant enzymes in paw tissue to be near the normal control values. Also, the MDA levels in paw tissue of mice that were treated with the high dose of TQ evoked non-significant change in the control group.

In order to better understand the antioxidant potency of TQ against carrageenan-induced oxidative injury in paw tissue, the levels of nuclear and cytoplasmic Nrf2 were assessed in all tested groups (Fig. 6). In comparison with the controls, carrageenan injection provoked significant increases ($P < 0.05$) in the cytoplasmic Nrf2 together with decreases ($P < 0.05$) in its nuclear levels in the paw tissue of treated mice. In contrast, the pre-treatment of the model group with TQ reversed notably the carrageenan-induced alterations in both cytoplasmic and nuclear Nrf2 ($P < 0.05$) in relation to the model group. Further, administration of indomethacin-induced marked increase ($P < 0.05$) in the nuclear Nrf2 without any significant changes in the cytoplasmic one when compared to the model group.

Effect of TQ on pro-inflammatory cytokines and NF- κ B in paw tissue of carrageenan-injected mice

Modulating effect of TQ on the levels of inflammatory mediators in the inflamed paw tissues after carrageenan injection is shown in Fig 7. In comparison with the control group, tissue levels of IL-1 β , TNF- α and IL-6 were meaningfully augmented ($P < 0.05$) in the model group. Meanwhile, the administration of indomethacin or TQ at 50 and 100 mg/kg before carrageenan injection notably subsided ($P < 0.05$) the inflammation via lessening these indices in comparison with the carrageenan-injected group. In addition, immunohistochemical examination showed that carrageenan injection increased the expression of TNF- α as compared to its expression in the control group, while the administration of either indomethacin or TQ lessened its expression in the paw tissue as compared to the model group (Figure 8).

To elucidate the implicated mechanisms in the anti-inflammatory activity of TQ, levels of NF- κ B were assessed following carrageenan injection to mice. As known, NF- κ B has a vital role in the regulation and control of the expression of inflammatory mediators. Our results showed a marked increase ($P < 0.05$) in the level of NF- κ B in the inflamed paw tissue in the model group relative to the control mice. Remarkably, TQ and indomethacin notably lessened ($P < 0.05$) the level of this critical transcriptional factor upon carrageenan application in mice. Moreover, TQ at doses of 100 mg/kg was able to reduce the levels of NF- κ B in paw tissues close to those in the control group reflecting its ability to suppress the development of acute inflammatory reactions following carrageenan application.

Effect of TQ on the levels of NO, COX-2, and PGE-2 in paw tissue following carrageenan injection in mice

COX-2 has an important role during inflammatory events by modulating the production of PGE2 from the arachidonic acid cascade. Compared to the control group, our results demonstrate noteworthy increases ($P < 0.05$) in the levels of NO, COX-2, and PGE2 after the injection of carrageenan. Adversely, pre-treatment with indomethacin and TQ at doses of 50 and 100 mg/kg lessened ($P < 0.05$) the levels of these indices in paw tissues when compared with the model group. These results indicate that TQ induced remarkable deactivation to COX-2 with a subsequent decrease in its product, PGE2, in the inflamed paw tissue related to the carrageenan-treated group (Fig. 9).

Effect of TQ on the levels of MCP-1 and MPO in paw tissues after carrageenan injection in mice

For assessment of leukocytic migration and infiltration in the inflamed paw tissue, levels of MCP-1 and MPO were measured (Fig. 10). Compared to the normal mice, intraplantar injection of carrageenan triggered the activity ($P < 0.05$) of MPO and increased ($P < 0.05$) the level of MCP-1 when compared with the control group. Interestingly, pre-treatment of carrageenan-challenged mice with TQ or indomethacin-induced significant reduction ($P < 0.05$) in MPO activity and MCP-1 content in paw tissue compared to the model group. These findings signify the anti-inflammatory effect of TQ that is mediated by inhibition of neutrophil migration and infiltration at the inflamed foci.

Histological changes in inflamed paw tissue in response to TQ treatment

As presented in Fig. 11, histopathological examination of paw tissue of carrageenan-exposed mice exhibited epithelial hyperplasia, infiltration of inflammatory cells, and subepidermal edema. These inflammatory signs were markedly decreased following both doses of TQ.

Discussion

Inflammation studies are currently one of the principal hubs of scientific research worldwide. Looking for novel substances for alleviating inflammatory ailments is still a subject of intense interest. Considering the associated adverse effects with the presently used anti-inflammatory medications, improvement of drugs from natural compounds is the product strategy for the treatment of inflammatory response (Cordaro et al. 2020, Zhang et al. 2020).

For a preliminary screening and understanding of the anti-oedematous and anti-inflammatory effects of TQ, the model of xylene-induced ear edema and inflammation was utilized. In agreement with previous reports (Akhtar & Shabbir 2019, Cui et al. 2020, Liu et al. 2020), the application of xylene on the ear edema in our study resulted in excess release of inflammatory mediators, inflammatory cell infiltration, local vasodilatation, capillary permeability, and edema. In contrast, pre-treated mice with TQ displayed a significant decrease in ear edema that indicates the suppressive effect of TQ on capillary permeability, edema development, and inflammation in mice ears. Demirel et al. (Demirel et al. 2018) reported that topically applied TQ resulted in a marked decrease in tissue edema in acute otitis externa in rats.

Pain is an unpleasant sensation that is associated with inflammation and generated by PGE₂, histamine, serotonin, bradykinin, and inflammatory cytokines like TNF- α , IL-1 β , IL-6, and IL-8 in the peritoneal fluid (Liu et al. 2020). For assessment of the peripheral antinociceptive effect of TQ, acetic acid-triggered abdominal writhing was established. Injection of acetic acid-induced tissue damage and release of inflammatory mediators such as histamine, bradykinin, and PG that in turn arouse nociceptors (Karim et al. 2019, Paradee et al. 2021). This process involves the activation of COX that catalyzes the conversion of arachidonic acid to PGE₂ at the peritoneal receptors (Moharram et al. 2021). However, TQ antagonized acetic acid-induced-shriving in the abdominal muscles that advocates its peripheral anti-nociceptive effect via the inhibition of inflammatory mediators and the PG pathway. It was formerly stated that

administration of TQ relieved notably the formalin-induced pain in rats and the antinociception was mediated through the NO/cGMP/K_{ATP} channel pathway (Parvardeh et al. 2018). Also, *N. sativa* fixed oil, including TQ possesses appreciable anti-inflammatory and analgesic effects (Mahboubi et al. 2018). Amin et al. (Amin et al. 2014) found that TQ alleviated markedly the neuropathic pain resulting from chronic constriction of the sciatic nerve in rats.

Carrageenan mediated-paw edema is a well-known experimental model for the acute inflammatory response in addition to screening the anti-inflammatory potential of natural compounds and synthetic chemicals (Liu et al. 2020). In this model, carrageenan injection in the mice's rear paws resulted in intense inflammatory events with a biphasic phenomenon (Mehrzadi et al. 2021). The early phase (0 to 1 h post-injection) is characterized by over secretion of serotonin, bradykinin, and histamine. These mediators initiate tissue edema through the encouragement of the local blood flow and an increase in the capillary permeability (Zahra et al. 2020). The delayed phase (after 1 h and peaked after 8 h) is associated with leukocytic migration and prostaglandin (Zhang et al. 2020). As previously reported (Mehrzadi et al. 2021, Zahra et al. 2020), our study revealed a visible increase in the paw volume after intraplantar injection of carrageenan as compared to that of the control untreated paws. However, pre-treatment of mice with TQ in doses of 50 and 100 mg/kg decreased significantly the edema volume of the paws injected with carrageenan. Likewise, indomethacin exhibited a significant anti-edema effect and reduced paw volume of the induced paws. These results imply the potent anti-inflammatory effect of TQ that is probably because of the inhibition of mediators of inflammation.

In accordance with former studies, our results revealed notable increases in WBCs count and platelet levels in carrageenan-injected mice in comparison with the control (Akhtar &Shabbir 2019, Shabbir et al. 2018). These results indicated the activation of the immune system against tissue injury caused by foreign agents (Akhtar &Shabbir 2019). Treatment with TQ normalized the levels of WBCs and platelets, which reflects its potential immunomodulatory effect. Our results are in harmony with earlier reports (Gülmez et al. 2017a, Nemmar et al. 2011), which revealed the normalization of disordered hematological markers by TQ in experimental models of otitis media and arthritis in rats.

CRP, a sensitive acute-phase protein, is a key inflammatory mediator that is secreted by hepatic tissue after being stimulated with cytokines such as IL-1, IL-6, and TNF- α (Hamsa &Kuttan 2011). It indicates the extent of tissue inflammation and its increase is a predictive risk factor for the occurrence of organ damage (Jisha et al. 2019). In this study, carrageenan injection increased the CRP level in paw tissue that indicating a strong inflammatory response of the injured tissue and this agrees with former findings (Jisha et al. 2019, Zhang et al. 2020). On the contrary, the level of CRP displayed a notable decline in TQ-treated groups which explains its powerful anti-inflammatory property. Treatment of arthritic rats with TQ decreased significantly the levels of CRP in an experimental model of rheumatoid arthritis (Arjumand et al. 2019).

In inflammatory conditions, excess ROS is the culprit of cellular and organ damage (El-Shitany &Eid 2019a, Mehrzadi et al. 2021). In agreement with former authors (Alblihed 2020, Mehrzadi et al. 2021),

marked increments were noticed in the levels of MDA accompanied by notable decreases in GSH levels and SOD, CAT, GR, and GPx activities in the paw tissues of carrageenan-injected mice. In addition, carrageenan injection resulted in notable increases in the levels of Nrf2 in the cytoplasm with decreases in the nucleus in the paw tissue of the model group. RT-PCR analysis that was performed by El-shitany and Eid revealed that carrageenan injection significantly decreased the Nrf2 expression of mRNA (El-Shitany & Eid 2019b). Remarkably, the treatment of mice with both TQ and indomethacin before carrageenan counteracted ROS overproduction and lessened the oxidative deleterious effects of carrageenan in mice paw tissue. These results are in accordance with former reports (Al Aboud et al. 2021, Dera et al. 2020, Dur et al. 2016, Kundu et al. 2013). Because of its potent radical scavenging ability, TQ alleviates the cellular oxidative stress and lessens the consequent lipid peroxidation (Al Aboud et al. 2021). Supporting former studies (Dera et al. 2020), the antioxidant efficacy of TQ may refer to the elevation of Nrf2 levels and its nuclear translocation along with blockage of Keap1 and induction of HO-1 expression. Our finding revealed that TQ at both tested doses decreased the cytoplasmic Nrf2 and increased its nuclear levels in the paw tissue. Normally, Nrf2 is attached to Keap1 in the cytosol, but under stress conditions of excess ROS production, it moves to the nucleus and binds to the antioxidant response element (ARE), and initiates the activation of cytoprotective enzymes (Albarakati et al. 2020, El-Khadragy et al. 2021, Yuan et al. 2020a). It was reported that TQ treatment abrogated the lung inflammation in a rat model of lung fibrosis through enhancement of the Nrf2/HO-1 signaling pathway (Ahmad et al. 2020). Modulation of Nrf2 signalling by TQ administration was also reported previously in different animal models (Amin et al. 2021, Hamdan et al. 2019, Sabir et al. 2022).

Furthermore, excessive ROS generation is strongly related to the progression of inflammation following carrageenan injection by activating immune cells and secretion of pro-inflammatory mediators (Alblihed 2020). In our study, carrageenan injected mice displayed noteworthy elevations in levels of pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) in paw tissue in comparison with control mice and this agrees with former studies (Alblihed 2020, Cui et al. 2020, Mehrzadi et al. 2021). These inflammatory cytokines are produced by macrophages and monocytes, fibroblasts, and endothelial cells in situations of inflammatory reactions and cellular stress (Zhang et al. 2020). TNF- α , a pro-inflammatory cytokine, is involved in the regulation of the secretion of other inflammatory mediators (Liu et al. 2020, Mehrzadi et al. 2021). Also, IL-1 β is a pro-inflammatory cytokine, that enhances both local and systemic immune reactions (Mehrzadi et al. 2021). Furthermore, IL-1 β and TNF- α trigger the expression of various enzymes, such as COX-2 with subsequent production of prostaglandins as PGE-2 (Alblihed 2020, Zhang et al. 2020). COX-2/PGE-2 signaling pathway plays an important role in various inflammatory diseases (Alblihed 2020). In the present study, intraplantar injection of carrageenan-induced the production of inflammatory mediators such as COX-2 and PGE-2, as reported in former studies (El-Shitany & Eid 2019a, Mitrea et al. 2020, Zhang et al. 2020). In contrast, TQ treatment attenuated notably all these inflammatory mediators in paw tissue, which indicates its potent anti-inflammatory effect and this is in harmony with previous authors (Kundu et al. 2013, Su et al. 2016, Wang et al. 2015). TQ was reported to decrease the protein and gene expression of COX-2 in mice skin exposed to phorbol ester (Kundu et al. 2013). The

reduction of these pro-inflammatory cytokines represents a key target to control and regulate the development of acute and chronic inflammation (Alblihed 2020).

It has been reported that both IL-1 β and TNF- α trigger the production of inducible nitric oxide synthase (iNOS), resulting in the development of inflammatory responses via the production of NO (Alblihed 2020, Mehrzadi et al. 2021). Our results revealed high NO levels in paw tissue injected with carrageenan which is similar to other reports (El-Shitany & Eid 2019a, Zhang et al. 2020). NO is an intercellular molecule that maintains the tissue homeostasis of numerous physiological functions (Alblihed 2020). However, high NO levels under pathological circumstances liberate peroxynitrite radicals that result in severe cellular damage such as oxidation of DNA and peroxidation of lipid molecules with subsequent tissue oxidative injury and inflammatory events (Zhang et al. 2020). Besides, earlier reports stated that high NO enhances the liberation of arachidonic acid from the cellular membrane which accordingly activates COX-2 and PGE-2 formation (Alblihed 2020, Zhang et al. 2020). Further, elevated NO levels increase the COX-2 half-life via the production of free radicals and inhibition of COX-2 auto inactivation (Alblihed 2020). Similar to previous reports (Saghir et al. 2019, Wang et al. 2015), TQ pre-treatment decreased the NO levels in inflamed tissue which is assumed by the regulation of inflammatory cytokines and the iNOS signalling pathway.

Regulation of inflammatory reaction is a complex process related to several pathways such as the NF- κ B signalling pathway that controls the expression of various pro-inflammatory mediators (El-Shitany & Eid 2019a). In accordance with former studies (El-Shitany & Eid 2019a, Liu et al. 2020, Mitrea et al. 2020), this study found a significant increment in paw NF- κ B levels in the carrageenan group compared to the control. During pathological conditions, NF- κ B translocates into the nucleus to control the release of different inflammatory mediators (Cui et al. 2020). Accordingly, the elevations in levels of inflammatory cytokines such as TNF- α , IL-1 β , IL-6, NO, COX-2, and PGE2 are attributed to the activated NF- κ B in the inflamed paw tissue following carrageenan injection (Alblihed 2020, Cui et al. 2020, Zhang et al. 2020). This in turn explains the anti-inflammatory ability of TQ as evidenced by low levels of NF- κ B in TQ-treated mice. Former authors stated the ability of TQ to subside tissue inflammatory response by deactivation of NF- κ B and MAPKs signaling (Arjumand et al. 2019, Dera et al. 2020, Wang et al. 2015). Also, TQ was reported to lessen the nuclear translocation and the DNA binding of NF- κ B by hindering the phosphorylation and consequent degradation of I κ B- α in mice skin exposed to phorbol ester (Kundu et al. 2013).

Acute inflammation is characterized by adhesion and infiltration of white blood cells, particularly neutrophils. MPO is a protein secreted from neutrophils and is a reliable index to assess the degree of neutrophil infiltration (Abdel-Lateff et al. 2020). Its reaction with H₂O₂ and halides results in hypochlorous acid that possesses a strong oxidant with microbicidal activities (Alblihed 2020). However, a high level of MPO is indicative of tissue injury in acute inflammatory response (Cui et al. 2020). In harmony with former reports (Abdel-Lateff et al. 2020, Cui et al. 2020, Zhang et al. 2020), this study revealed marked increases in MPO activity and MCP-1 levels in paw edema tissues. This suggests that carrageenan resulted in infiltration of neutrophils in inflammatory sites as confirmed by histological alterations. MCP-1

is a vital chemokine that controls the immigration and infiltration of monocytes/macrophages at the inflamed foci (Zhang et al. 2020). Elevated MCP-1 levels were recorded following carrageenan injection in a model of acute pleurisy; demonstrating the infiltration of leukocytes in the inflamed tissues (Lansley et al. 2017). In this study, mice that received TQ displayed low levels of MPO and MCP-1 in inflamed paw tissues and this is similar to former reports (Al Aboud et al. 2021, Karaca et al. 2017). TQ administration decreased the levels of hepatic MCP-1 in sepsis injury in BALB/c mice and MCP-1 levels of murine microglia cells treated with lipopolysaccharide (Taka et al. 2015, Wang et al. 2019). These findings further demonstrate the TQ protection against inflammation through blocking the migration and infiltration of immune cells in inflamed tissue as validated by the inhibition of MPO and downregulation of proinflammatory cytokines including MCP-1.

Conclusion

The current study collectively validated that TQ administration exerted remarkable inhibitory activity on the carrageenan-induced paw edema and consequently associated pain in mice. TQ was able to significantly enhance the cellular antioxidant activity, and lessen pro-inflammatory cytokines secretion. The anti-inflammatory characteristic of TQ is mediated by inhibition of COX-2 and NF- κ B. Moreover, pre-treatment with TQ also provoked marked attenuation of ear edema which further confirms its potency in acute inflammatory models. Taken together, our results demonstrated that TQ possesses interesting anti-inflammatory, anti-oxidative, and analgesic activities that will be of interest for further investigation.

Declarations

Author contributions

Conceptualization and supervision: R.B.K. Animal treatments, molecular and biochemical investigations: O.A.H., R.B.K., A.A.A.O. A.T., and M.A. Histological methodology and investigation: O.A. and M.S.L. Data analysis, software, data curation, and visualization: K.F.A., A.A., H.K.A., S.M.D. and R.A.E. Writing-reviewing and editing manuscript: R.A.E., H.H.A.H., and N.D., Resources H.A.A., F.A. H.H.A.H., and N.D.

All authors approved the final version of the manuscript.

Data availability: All relevant data are within the paper.

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Ethics approval

The animal care procedures are in agreement with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals 8th edition and the Institutional Animal Ethics Committee

guidelines for Laboratory Animal Care at Zoology Department, Faculty of Science, Helwan University (Approval Number: HU2020/Z/RKA820-01).

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Figures

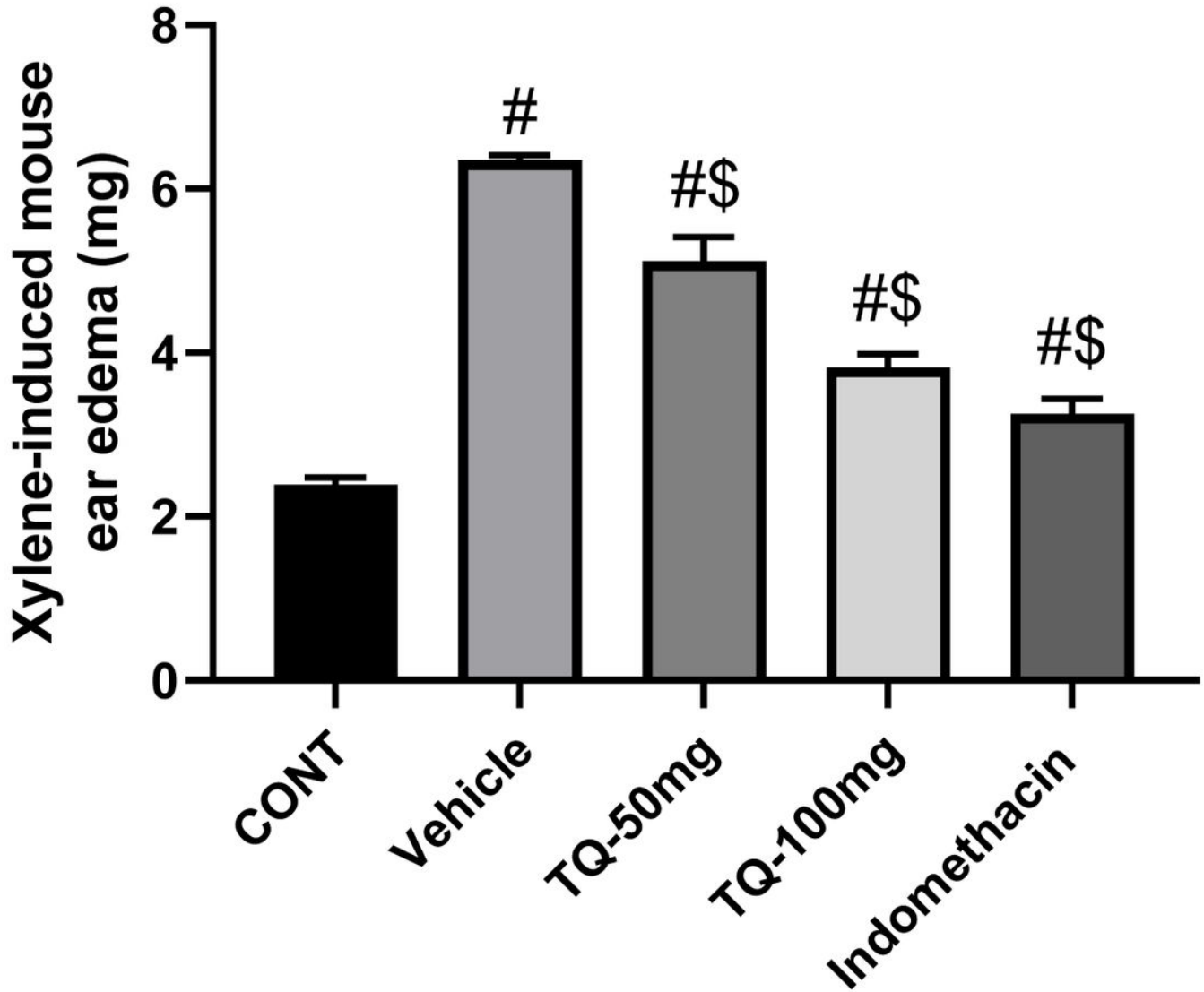


Figure 1

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on ear edema volume in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

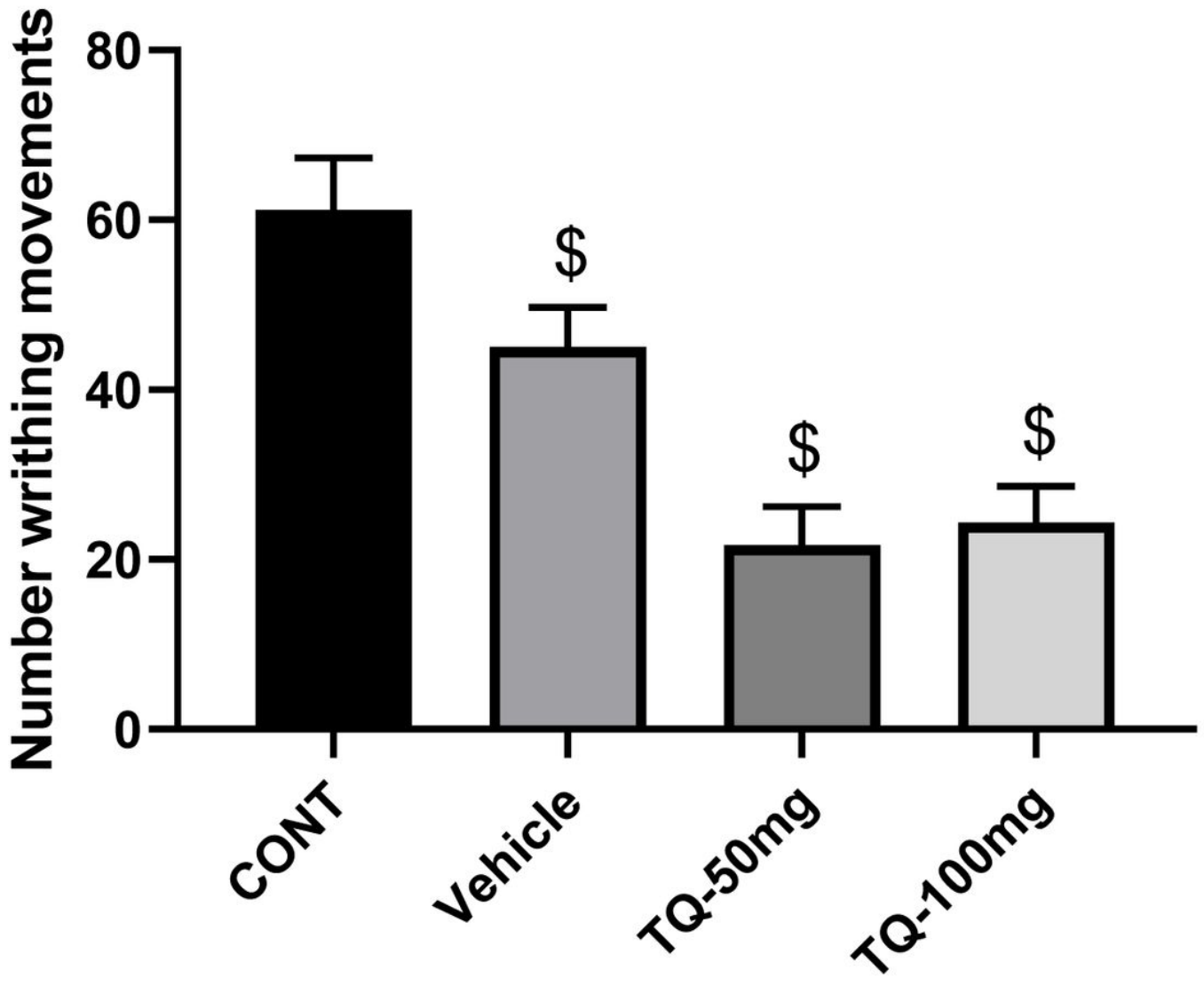


Figure 2

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on writhing movements in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0:05 indicates a significant change versus the control group; \$p < 0:05 indicates a significant change versus the carrageenan-injected group.

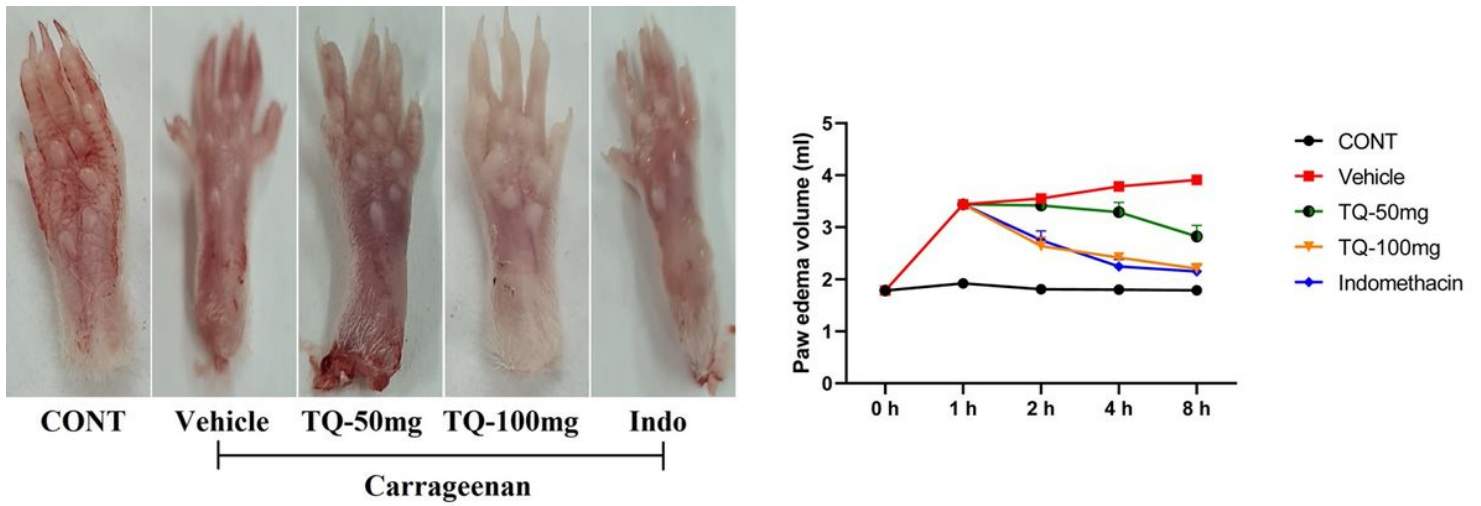


Figure 3

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on paw edema volume in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

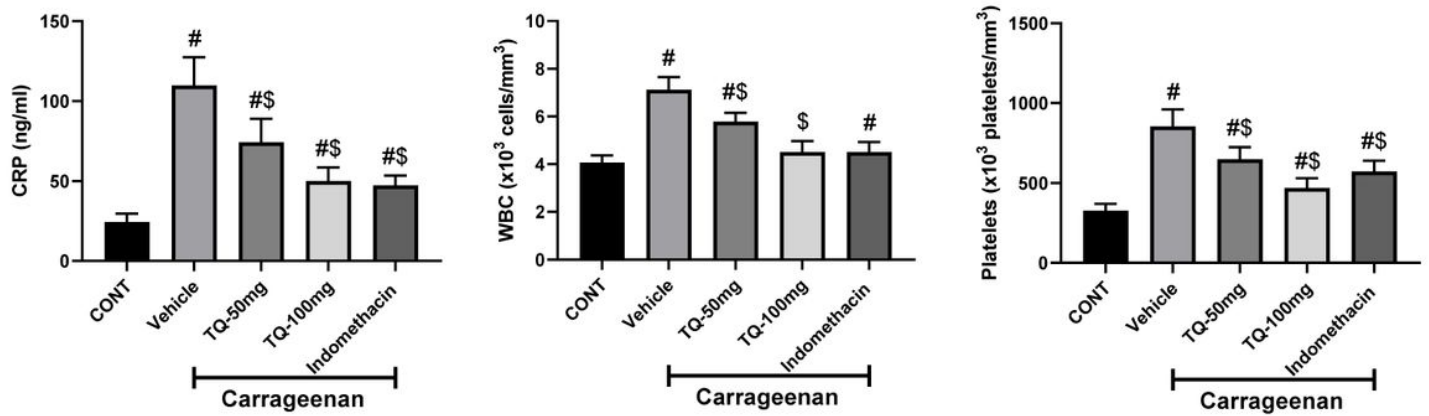


Figure 4

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on CRP levels, WBCs, and platelet count in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

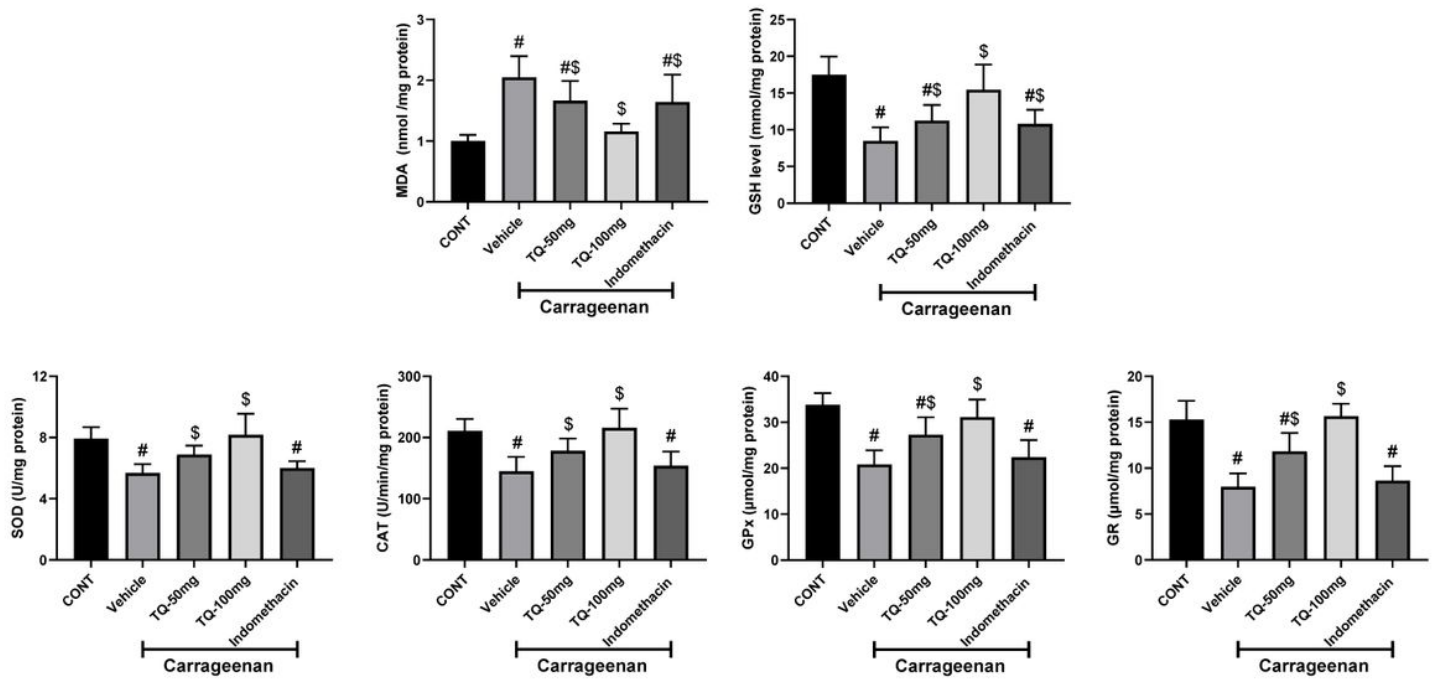


Figure 5

Effect of thymoquinone ((TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on oxidative stress markers in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

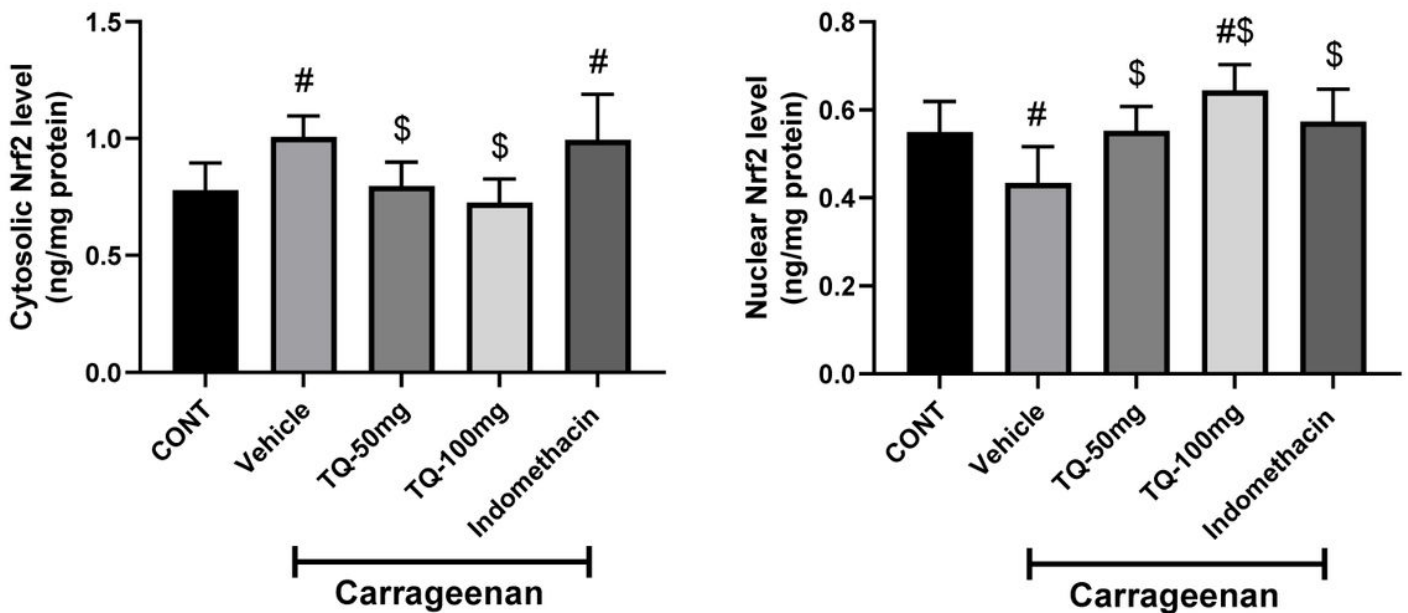


Figure 6

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on the cytoplasmic and nuclear levels of Nrf-2 in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

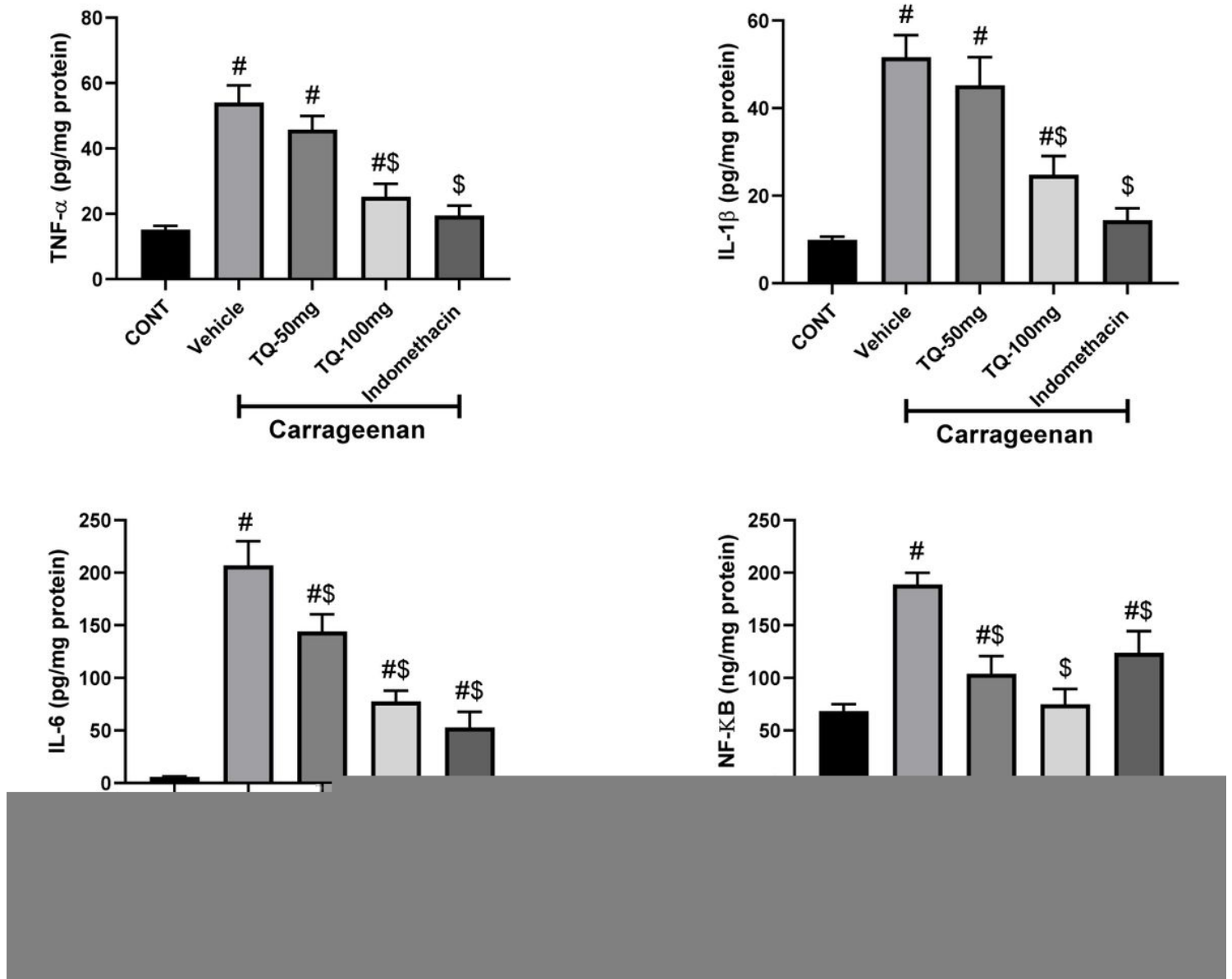


Figure 7

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on inflammatory cytokines and NF- κ B in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

Figure 8

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on the TNF- α immune expression in paw skin upon carrageenan exposure, Scale bar = 80, 400x.

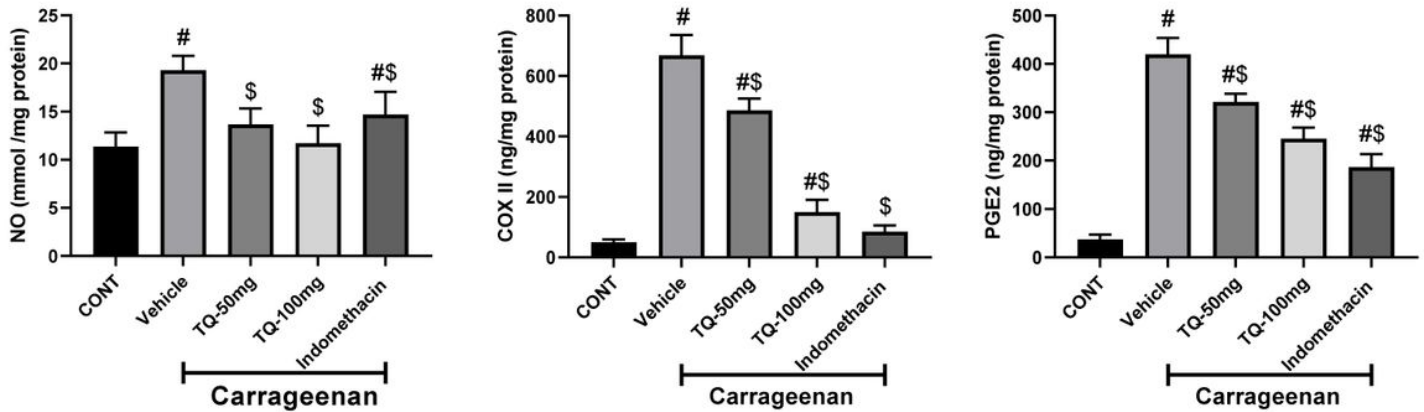


Figure 9

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on the levels of NO, COX-2, and PGE-2 in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

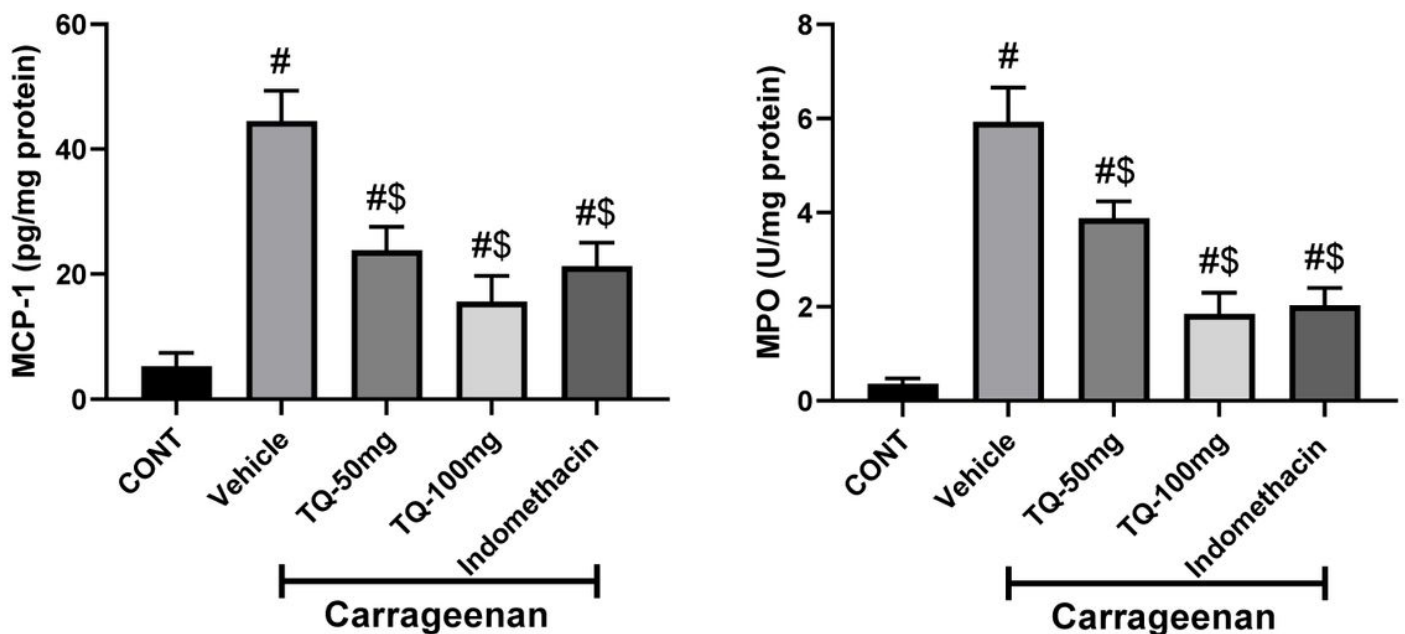


Figure 10

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on the levels of MCP-1 and MPO in carrageenan-injected mice. Data are represented as mean \pm SE (n = 7); #p < 0.05 indicates a significant change versus the control group; \$p < 0.05 indicates a significant change versus the carrageenan-injected group.

Figure 11

Effect of thymoquinone (TQ, 50 and 100 mg/kg) or indomethacin (10 mg/kg) on histopathological alterations in paw skin upon carrageenan exposure, white star inflammatory cells infiltration, red star subcutaneous edema, Scale bar =80, 400x.