

Protocatechuic Acid Counteracts Oxidative Stress And Inflammation In Carrageenan- Induced Paw Edema in Mice

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Research Article

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Abstract

PCA (protocatechuic acid), a phenolic compound found in teas, fruits and vegetables, is widely recognized with its antioxidant and anti-inflammatory activities. Here we verified the protective role of PCA on carrageenan-induced paw edema in mice. PCA (25 mg/kg and 50 mg/kg) administration was applied for five consecutive days prior to the carrageenan injection. Diclofenac sodium (20 mg/kg) was used as a reference drug. PCA pretreatment notably decreased the volume of the developed edema and alleviated the histopathological alterations induced by carrageenan. Additionally, PCA administration enhanced the cellular antioxidant capacity as demonstrated by the increased levels of catalase, superoxide dismutase and reduced glutathione, in addition to the decreased malondialdehyde level in the edematous tissue. Interestingly, PCA administration was able to suppress the developed inflammatory response upon carrageenan injection as indicated by the decreased levels and expression of pro-inflammatory cytokines and mediators including tumor necrosis factor alpha, interleukin-1 beta, interleukin-6, inducible nitric oxide synthase, nitric oxide, cyclooxygenase-II, prostaglandin E2, monocyte chemoattractant protein-1, myeloperoxidase and nuclear factor kappa B. These results collectively confirm the protective effect of PCA against carrageenan-induced paw edema owing to its antioxidant and anti-inflammatory characteristics.

1. Introduction

Inflammation is an innate immunity response of the body toward tissue injury, involving complicated series of reactions that can be triggered by a variety of agents such as toxins and bacteria (Adegbaju et al. 2020). Acute inflammation is mainly associated with fluid exudation, neutrophil infiltration and vasodilation coupled with pain and tissue damage (Medzhitov 2008). During acute inflammation, several immune cells are recruited at the site of injury. In order to encounter tissue damage, these activated cells overproduce free radicals and inflammatory molecules (Zhang et al. 2020). Additionally, imbalance between pro-oxidants and antioxidants, lipid peroxidation, nitric oxide over release are essential events during the inflammatory state (Ahmad et al. 2012).

Inflammation is mainly treated by non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (Abbate et al. 2016). Although these anti-inflammatory medications can treat a variety of inflammatory diseases, their application exhibit negative side effects. Hepatorenal complications and gastrointestinal impairments are widely recorded with NSAIDs, while glucocorticoids cause cardiotoxicity, neuronal deficits, dermal impairments osteoporosis, and obesity (Harirforoosh et al. 2013, Oray et al. 2016). Therefore, finding anti-inflammatory drugs with minimum adverse reactions and more efficiency are required. Carrageenan-mediated paw edema in experimental animals is widely used as acute inflammatory model to examine the potential anti-inflammatory property of novel agents.

Due to their wide medicinal applications, naturally occurring phenolic compounds have received a great attention (Alsharif et al. 2020). PCA (protocatechuic acid) is a phenolic compound found in vegetables (onion, *Allium cepa* L.), fruits (grapes, *Vitis vinifera*) and other plants and spices such as rosemary

(*Rosmarinus officinalis* L.) (Kakkar & Bais 2014). Previous reports demonstrated that PCA has several biological and pharmacological properties. Bhattacharjee et al. (Bhattacharjee et al. 2017) demonstrated that PCA isolated from *Sansevieria roxburghiana* showed hypoglycemic effect and inhibited cardiac oxidative damage and inflammation associated with the development of animal diabetic model. Moreover, PCA administration restored the activity of neuronal antioxidative proteins and suppressed neuronal inflammation upon cadmium intoxication (Al Olayan et al. 2020). Additionally, PCA extracted from leaves of *Trianthema portulacastrum* was found to attenuate dermal wounds via inhibiting oxidative and inflammatory responses (Yadav et al. 2017). Furthermore, PCA showed hepatoprotective activity following cisplatin exposure through enhancing antioxidant molecules and downregulating pro-inflammatory cytokines (Habib et al. 2021).

Hence, the current study was designed to examine the anti-edematous effect of PCA administration through evaluating the redox and inflammatory status along with histopathological changes following carrageenan injection in mice.

2. Materials And Methods

2.1. Chemicals

Protocatechuic acid (CAS Number: 99-50-3, purity $\geq 97.0\%$) and carrageenan (CAS number: 11114-20-8) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Whereas, diclofenac sodium (Declophen) was obtained from Pharco Pharmaceuticals, Alexandria, Egypt. The used reagents were of high analytical grade.

2.2. Animals and experimental design

Forty-five adult male Swiss albino mice aged between 10 and 12 weeks, weighing 20-25 g were obtained from the King Fahd for Medical Research animal house at King Abdul Aziz University in Jeddah, Saudi Arabia. Animals were adapted for one week before being included in the study under standard laboratory conditions (12 hours of light, 12 hours of darkness) (humidity: $50 \pm 10\%$) in a temperature-controlled room ($22 \pm 2^\circ\text{C}$). Mice were arbitrarily categorized into the following 5 groups (nine mice in each group):

Control group: mice received normal saline solution for 5 successive days.

Carrageenan (CGN) injected group: mice were injected into the left hind paw's sub-plantar tissues with 1% w/v carrageenan dissolved in normal saline (0.1 ml) (Zhang et al. 2020).

Protocatechuic acid+carrageenan (PCA-L+CGN): mice orally treated with PCA (25 mg/kg) for 5 days prior to CGN injection.

Protocatechuic acid+carrageenan (PCA-H+CGN): mice orally treated with PCA (50 mg/kg) for 5 days prior to CGN injection.

diclofenac sodium+carrageenan (DIC+CGN): mice orally treated with DIC (20 mg/kg) according to (Zhang et al. 2020) before the CGN injection.

The applied doses of PCA was selected based on preliminary experiment using 12.5, 25, 50 mg/kg. The higher doses (25 and 50 mg/kg) were found to more effective in the treatment of paw edema induced by carrageenan.

Protocatechuic acid and diclofenac sodium (an anti-inflammatory reference drug) were dissolved in normal saline. The paw tissue thickness was assessed before and after carrageenan injection (at 2, 4, 6, and 8 hours). The measurements were estimated as the difference in paw volume (ml) and were compared to the same animal's right hind paw. Mice were sacrificed after 8 hours, and paw tissue samples were collected and divided into two parts. One part was homogenized in ice-cold medium containing 50 mM Tris-HCl (pH 7.4) and centrifuged at 500 x g for 10 minutes at 4°C to produce a 50% (w/v) homogenate. The biochemical measurements were performed on the paw supernatant, while the histopathological changes and molecular studies were performed on the second sample part.

2.3. Assessment of oxidative stress markers in the paw tissue

Malondialdehyde (MDA), a lipid peroxidation marker was quantified in the paw supernatant based on the method described by Ohkawa et al. (1979). The reduced glutathione (GSH) level was determined according to the method of Ellman (1959). The superoxide dismutase (SOD) in addition to catalase (CAT) activities was measured according to the methods of Nishikimi et al. (Nishikimi et al. 1972) and Aebi (Aebi 1984), respectively.

2.4. Assessment of inflammatory mediators in the paw tissue

Tumor necrosis factor alpha (TNF- α , Cat. No: CSB-E04741m), interleukin-1 beta (IL-1 β , Cat. No: CSB-E04621m), interleukin-6 (IL-6, Cat. No: CSB-E04627m), interleukin-8 (IL-8, Cat. No:), cyclooxygenase-2 (COX-II, Cat. No: CSB-E12910m), prostaglandin 2 (PGE2, Cat. No: CSB-PA040059), and monocyte chemoattractant protein-1 (MCP-1, Cat. No: CSB-E07430m), nuclear factor kappa B (NF- κ B P65,, Cat. No:) concentrations were assessed in paw tissue supernatant by ELISA kits obtained from CUSABIO Life Sciences, Wuhan, China according to the manufacturer's instructions. Nitric oxide (NO) level in paw supernatant was assessed in accordance with the protocol defined by Green et al. (Green et al. 1982). Myeloperoxidase (MPO) activity in the inflamed tissue was quantified using Bradley et al. (Bradley et al. 1982) method with slight modifications. Homogenates were frozen and thawed three times before being centrifugation for 10 min at 10,000 \times g, 4°C. 200 μ l of the supernatant was added to 2.8 mL of 0.05 M (pH= 6) phosphate buffer and 1 ml of 1.6 mM o-dianisidine hydrochloride containing H₂O₂, 0.0005% (v/v). The absorbance change was measured at 460 nm and MPO activity was expressed as U/mg protein.

2.5. Genes expression

The total RNA from the paw tissues was extracted and then reverse-transcribed into cDNA in accordance with Abdel Moneim protocol (Abdel Moneim 2016). qRT-PCR was used to determine the mRNA levels of *iNOS* (inducible nitric oxide synthase) and *Ccl1* (C-C motif chemokine ligand 1) using the QuantiTect SYBR Green PCR kit (Qiagen, Germany) and the Applied Biosystems 7500 Real-Time PCR device. The housekeeping gene *GAPDH* was used for data normalization. Table 1 shows the primers sequences used for *GAPDH*, *iNOS* and *Ccl1*. The comparative delta CT ($2^{-\Delta\Delta CT}$) method was used to assess the relative expression levels of genes.

2.6. Histopathological examination

Paw tissue samples were collected and fixed in 4% neutral formaldehyde for 24 h. Tissue was then paraffinized and sliced into 4 μm -thick sections. To determine the histological alterations in the different groups, the sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

2.7. Statistical analysis

The results were depicted as mean \pm SD (standard deviation). SPSS version 17 was used to perform the data analysis. To determine the differences between the groups, a one-way analysis of variance (ANOVA) was used, followed by a Post Hoc Duncan's test. The level of significance was set at less than 0.05 ($p < 0.05$).

3. Results

3.1. Effect of PCA pre-treatment on carrageenan-induced paw edema

The increase in paw volume in a time-dependent manner was one of the first signs appeared after carrageenan intraplantar injection. This increase was significant relative to the control ($p < 0.05$). PCA pretreatment at doses of 25 and 50 mg/kg caused a significant reduction in the left hind paw volume compared to the carrageenan group ($p < 0.05$). The reference drug, diclofenac sodium (20 mg/kg), also reduced post-carrageenan edema significantly, and its effect was very similar to that recorded following PCA administration at the higher dose (50 mg/kg) (Fig. 1).

3.2. Effect of PCA pre-treatment on carrageenan-induced histological alterations

As shown in Fig. 2, carrageenan caused subepidermal edema, neutrophil infiltration and disruption of tissue structure. However, PCA pretreatment notably attenuated these alterations. Similarly, diclofenac sodium reduced the histological injury in paw tissues of carrageenan injected mice.

3.3. Effect of PCA pre-treatment on oxidative stress parameters of edematous tissue in carrageenan -induced paw edema

To assess the impact of PCA on the antioxidant capacity in carrageenan injected mice, the MDA and GSH contents in addition to the SOD and CAT activities were determined in the edematous tissue. Carrageenan injection markedly reduced the levels of CAT, SOD and GSH and increased the MDA level as compared to the control mice (Fig. 3). On the contrary, PCA and diclofenac sodium pretreatment significantly increased the levels of CAT, SOD and GSH as well as decreased the MDA level in the edematous tissue of carrageenan injected mice. Interestingly, the higher dose of PCA (50 mg/kg) showed a better improvement in the antioxidant capacity as compared to the reference drug (diclofenac sodium 20 mg/kg).

3.4. Effect of PCA pre-treatment on inflammatory mediators in carrageenan -induced paw edema

In comparison to control mice, a significant increase in TNF- α , IL-1 β , IL-6 and IL-8 levels was observed as well as ccl1 expression level in the inflamed tissue following carrageenan injection. PCA pretreatment at doses of 25 mg/kg and 50 mg/kg reduced these elevated cytokine levels significantly. The reference drug, diclofenac sodium (20 mg/kg), decreased these cytokines levels substantially as well, and its effect was superior to that of the two PCA doses (Fig. 4).

As shown in Fig. 5, carrageenan significantly increased the COX-II activity and PGE2 level ($p < 0.05$) in the carrageenan-injected mice. PCA pretreatment extensively attenuated these elevations in a dose-dependent manner. Similarly these PCA doses (25 mg/kg and 50 mg/kg) decreased the expression of iNOS and NO level in comparison with CGN group. As expected, diclofenac sodium at a dose 20 mg/kg significantly reduced COX-II, PGE2, iNOS, and NO levels in the inflamed tissue.

Figure 6 illustrated that, the CGN injection had substantially elevated MPO activity and MCP1 level as compared to the control group; reflecting neutrophil infiltration at the inflamed skin. Interestingly, both doses of PCA in addition to diclofenac sodium significantly reduced these inflammatory indices.

In carrageenan-injected mice, the level of NF- κ B p65, which regulates a number of inflammatory genes, increased. PCA and diclofenac sodium both reduced this level substantially, and the effect of the higher dose of PCA (50 mg/kg) was close to that of the reference drug (diclofenac sodium, 20 mg/kg) (Fig. 7).

4. Discussion

Inflammation is a normal body response toward any pathogens, injured tissue and toxins. Undesired adverse reactions are coupled with long term consumption of the available anti-inflammatory medications. Therefore, finding safe and more efficient alternative is currently mandatory. Carrageenan-

induced acute inflammatory response (edematous state) in experimental animals is used to examine the efficiency of the novel anti-inflammatory molecules. Here, the potential anti-edematous role of PCA (25 and 50 mg/kg) was investigated following carrageenan injection in mice. Carrageenan injection enhances excessive release of different pro-inflammatory mediators and potentiated vasodilatation, resulting in the development of paw and the increase in its size which is recorded after 8 hours. Additionally, microscopic examination revealed alterations in paw tissue architecture and the accumulation of infiltrating inflammatory cells and increased inter-fiber space were observed, these findings are in agreement with previous report (Zhang et al. 2020). Interestingly, PCA administration decreased significantly the volume of the developed paw edema after carrageenan injection and decreased the infiltrated immune cells at the site of injury; this effect may be attributed to its antioxidative characteristics.

Oxidative stress is a fundamental mechanism implicated in the pathogenesis of paw edema following carrageenan application. In the current study, a disturbance in the redox status was recorded as characterized by the elevated MDA and the declined GSH, SOD and CAT activities. Previous reports demonstrated the overproduction of ROS following carrageenan administration. These active molecules affect the plasma membrane integrity and enhance its lipoperoxidation (Zhang et al. 2020), which may explain the increased MDA (lipid peroxidation marker) in the present study. GSH, SOD and CAT are among the main quenching cellular antioxidants, their depletion indicate the development of oxidative stress and cellular impairments. It has been suggested that blocking lipoperoxidation and the enhancement of cellular antioxidants capacity may alleviate the oxidative challenge associated with the development of paw edema. Interestingly, PCA administration was able to suppress oxidative insults following carrageenan application through inhibiting lipoperoxidation and enhancing the depleted antioxidant proteins. The antioxidative activity of PCA was reported in earlier studies. Bhattacharjee et al. (Bhattacharjee et al. 2017) showed that PCA inhibited ROS production, lipoperoxidation, and protein carbonylation, and increased GSH along with antioxidant enzymes (SOD, CAT, GR, GPx) in the myocardial tissue of diabetic rats. PCA was also found to protect hepatic tissue against oxidative damage by decreasing MDA level and enhancing level of GSH and SOD in response to cisplatin exposure (Habib et al. 2021). Moreover, Al Olayan et al. (Al Olayan et al. 2020) stated that PCA provided neuroprotection against cadmium intoxication through enhancing cellular antioxidants gene expression and decreasing lipid peroxidation. Authors attributed these effects to its ability to upregulate nuclear-related factor 2 mRNA expression.

Carrageenan injection triggered excessive release of different inflammatory mediators in the paw skin as characterized by the increased COX-II activity and its product (PGE2) accompanied by upregulation of iNOS mRNA expression and its product (NO). The secretion of pro-inflammatory cytokines and the release of various inflammatory mediators such as COX-II and PGE2 are major events implicated during acute inflammatory responses. The elevated PGE2 reflects the overactivation of COX-II resulting in the progression of inflammation symptoms including redness, fever, swelling and pain [38]. iNOS is upregulated in response to overproduction of pro-inflammatory cytokines and its upregulation is coupled with NO formation which further interact with superoxide anions and produce highly reactive peroxynitrite

radicals resulting in oxidative and inflammatory responses (Alsharif et al. 2020). NO is also known to inflammatory responses such as vasodilation, increased vascular permeability, exudate formation, and prostaglandin synthase activation [39]. PCA administration was found to decrease levels and mRNA expression of the increased COX-II, PGE2, iNOS and NO in the inflamed tissue. The anti-inflammatory property of PCA has been discussed previously in different experimental models. Min et al. (Min et al. 2010) observed that PCA downregulated COX-II and iNOS, and diminished their products (PGE2 and NO) in RAW 264.7 cells treated with lipopolysaccharide. In addition, PCA showed anti-inflammatory activity following doxorubicin through downregulation of COX-II and iNOS in the renal tissue (Molehin et al. 2019).

Excessive amounts of inflammatory mediators including TNF- α , IL-1 β , IL-6 and IL-8 along with elevated level of NF- κ B were recorded following carrageenan injection. These results are in line with previous studies (Haddadi & Rashtiani 2020). Cross-talk between oxidative stress and inflammatory response has been confirmed in inflammatory conditions. Overproduction of ROS is strongly related to the development of inflammation upon carrageenan injection through activating immune cells and secretion of pro-inflammatory mediators. Additionally, higher lipid peroxidation product has been linked with oversecretion of pro-inflammatory cytokines (Ou et al. 2019). Macrophages and lymphocytes accumulate at the inflammation site and enhance releasing pro-inflammatory cytokines. The increased TNF- α and IL-1 β during inflammatory response cause hyperalgesia and inflammatory pain (Kadetoff et al. 2012). It has been reported that crosslink between the inflammatory mediators is demonstrated following carrageenan application. Carrageenan enhances phosphorylation of ERK1/2, JNK and p38 which trigger the secretion of TNF- α . Consequently, TNF- α enhance secretion of IL-1 β , IL-6, COX-II and PGE2 in addition to upregulation of iNOS expression (Ou et al. 2019). NF- κ B is transcriptional inflammatory mediator that regulates the activity and differentiation of immune cells in addition to the production of production of different inflammatory markers, which may explain the elevation in the examined inflammatory mediators following carrageenan injection (Almeer et al. 2019).

Therefore, agents that are able to deactivate NF- κ B may be applied as anti-inflammatory drugs. Interestingly, PCA administration following carrageenan suppressed markedly the development of inflammation and its associated events. Former studies revealed the anti-inflammatory characteristics of PCA in different experimental protocols. PCA also was found to decrease the overproduced pro-inflammatory cytokines in the cortical tissue upon cadmium exposure (Al Olayan et al. 2020). Moreover, PCA suppressed the secreted TNF- α , IL-6 and IL-1 β . Authors attributed this effect to the deactivation of and NF- κ B signaling following LPS-mediated acute lung injury (Wei et al. 2012). Additionally, Crespo and collaborators (Crespo et al. 2017) demonstrated that PCA inhibited inflammatory response associated with colitis partially through decreasing pro-inflammatory cytokines and suppressing NF- κ B activity. PCA administration blockades the activities of COX-II, iNOS and NF- κ B in mouse epidermis following the exposure to 12-O-tetradecanoylphorbol-13-acetate (Cichocki et al. 2010). In another report, PCA downregulated the expression of COX-II and iNOS in the kidney tissue after the treatment with doxorubicin (Molehin et al. 2019). PCA was reported to attenuate production and expressions of TNF- α ,

IL-1 β , and iNOS, COX-II and PGE2 via the regulation of NF- κ B and MAPK activation in LPS- treated RAW 264.7 {Min, 2010 #2651}.

In the current study, carrageenan injection increased significantly MPO activity and MCP-1 level in the skin paw tissue. These results reflect the migration and infiltration of neutrophils and the degree of tissue damage at the inflamed tissue. These results are in agreement with previous reports (Abdel-Lateff et al. 2020, Almeer et al. 2019). Among other treatments (Zhang & de Mejia 2020), PCA was found to decrease the production of pro-inflammatory cytokines and MCP-1 level. This effect has been attributed to the attenuation of NF- κ B and JNK/MAPK-mediated inflammatory responses in adipocytes treated by LPS. Additionally, PCA protected against methotrexate-induced hepatorenal tissues through suppressing inflammatory mediators including MPO, NO, TNF- α and IL-1 β (Owumi et al. 2019). Moreover, PCA deactivated MPO and decreased the increased NO, TNF- α and IL-1 β in the hypothalamic, testicular and epididymal tissues following furan exposure.

Conclusion

The obtained findings clarified that PCA supplementation elicited a significant inhibitory effect against carrageenan injection-mediated paw edema. PCA markedly augmented the cellular antioxidant capacity, decreased pro-inflammatory cytokines production and improved the histopathological changes in the paw skin. The anti-inflammatory activity of PCA is potentiated mainly via deactivation iNOS, COX-II and NF- κ B. These results collectively point to the potential usage of PCA as an alternative anti-oxidative and anti-inflammatory agent.

Declarations

Author's contribution: The author has performed all the research items.

Funding: Self-funding

Data availability: Available upon request.

Conflict of interest: The author declares that there is no conflict of interest.

Ethical approval: All experimental protocols, including the use of animals, were approved by the Committee of Research Ethics for Laboratory Animal Care, Umm Al-Qura University (approval no. MATRR0200).

Consent to Participate: Not applicable.

Consent to Publish: Consented.

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Tables

Table 1 is not available with this version.

Figures

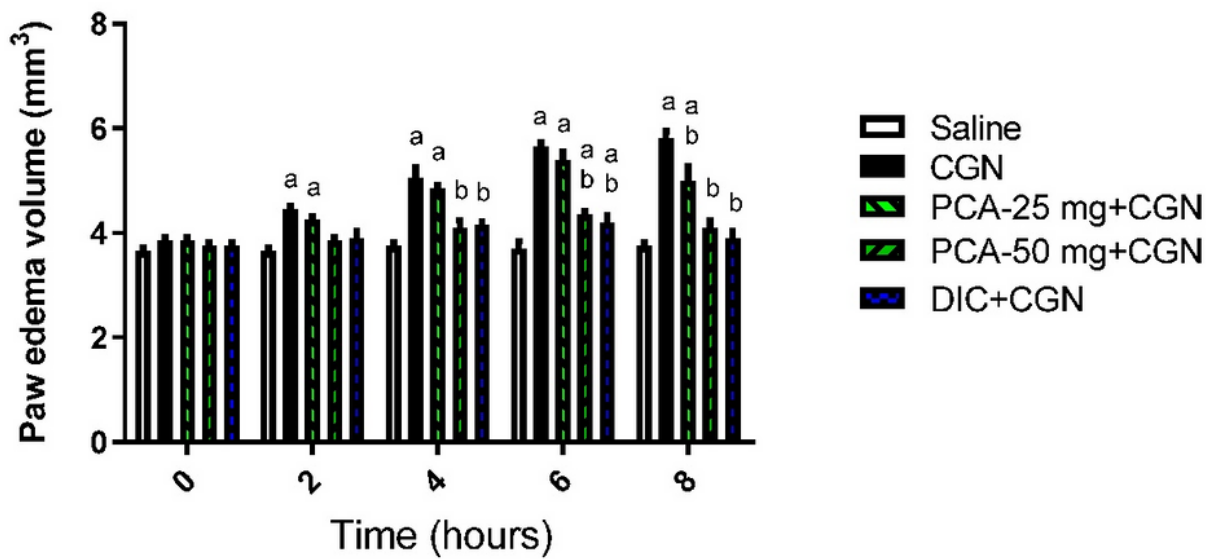
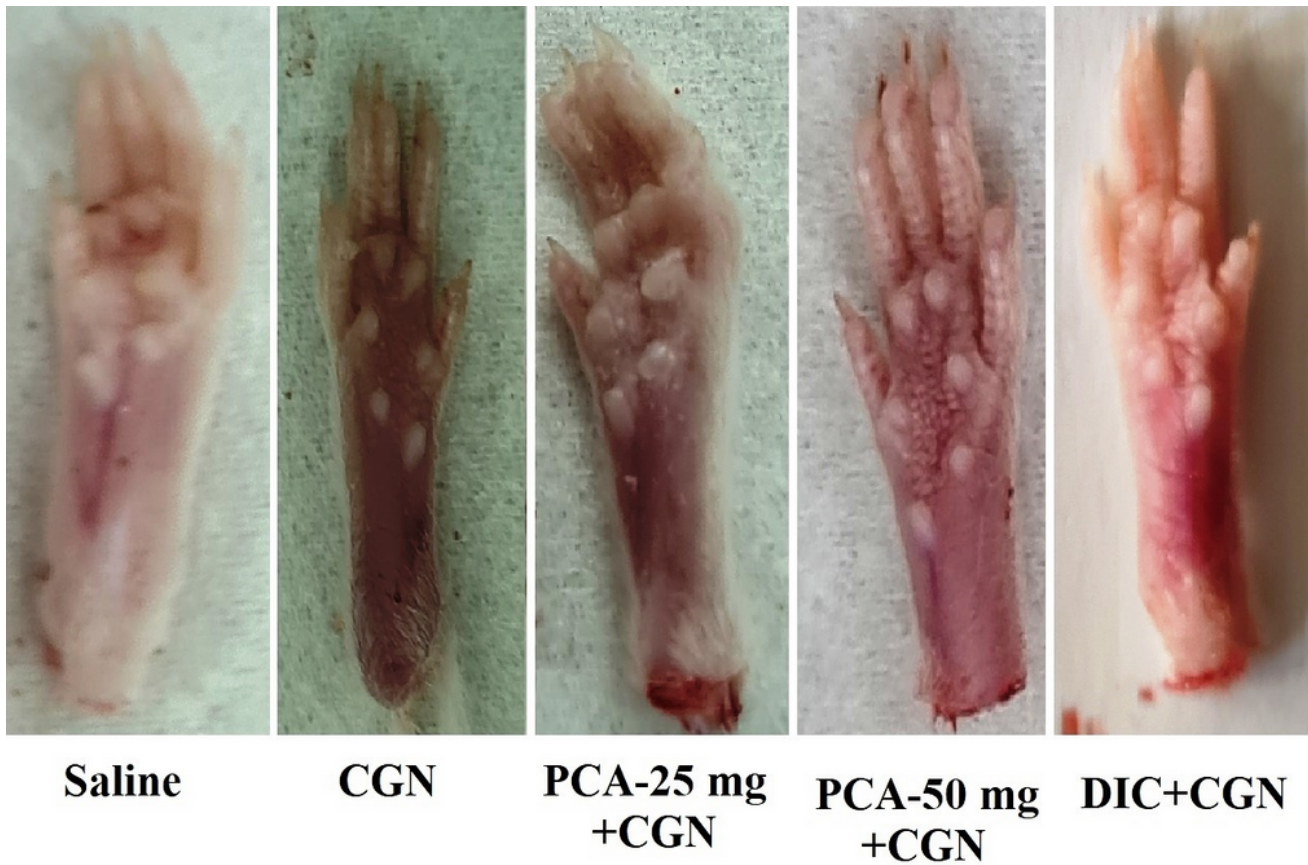


Figure 1

Effect of protocatechuic acid (PCA, 25 and 50 mg/kg) and diclofenac sodium (DIC) on paw edema volume in carrageenan (CGN)-injected mice. Values are depicted as mean \pm SD (n = 9); ^a $P < 0.05$ against control mice; ^b $P < 0.05$ against carrageenan (CGN) treated mice.

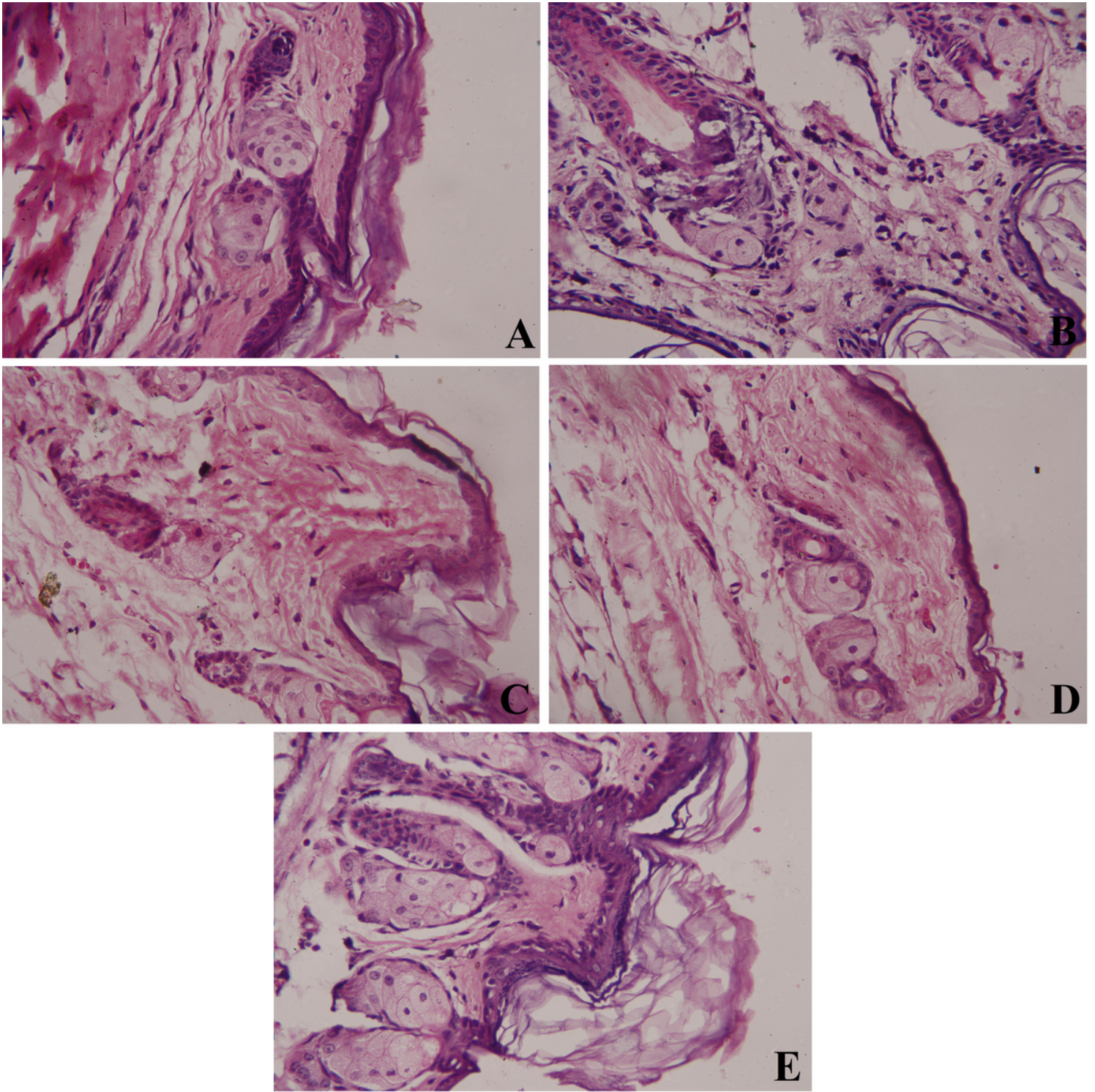


Figure 2

Effect of protocatechuic acid (PCA) and diclofenac sodium (DIC) histopathological alterations in the paw tissue following carrageenan injection.

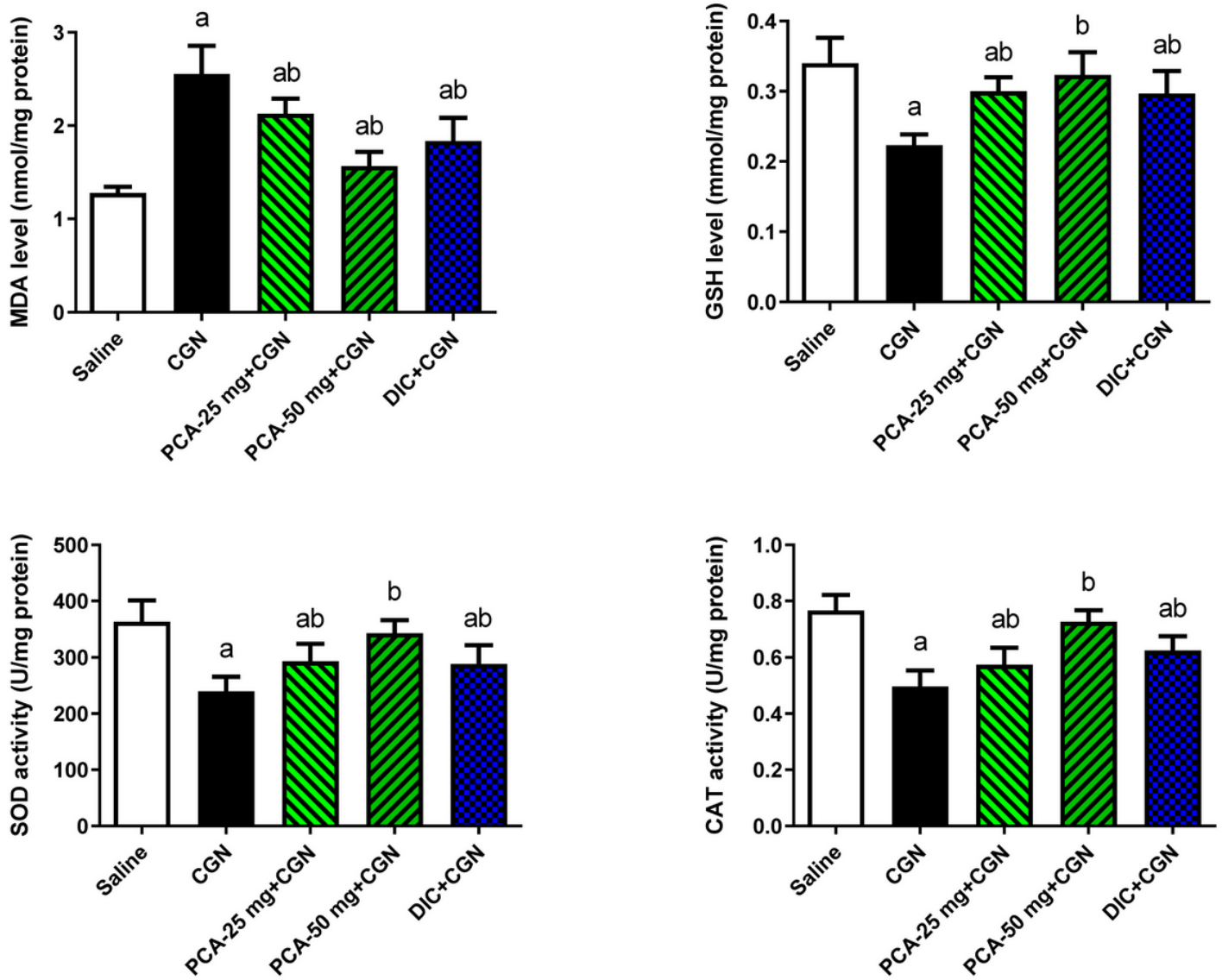


Figure 3

Effect of protocatechuic acid (PCA, 25 and 50 mg/kg) and diclofenac sodium (DIC) on level of MDA, GSH, SOD and CAT activities following carrageenan (CGN)-induced paw edema. Results are depicted as mean \pm SD (n = 9); ^a $P < 0.05$ against control mice; ^b $P < 0.05$ against carrageenan (CGN) treated mice.

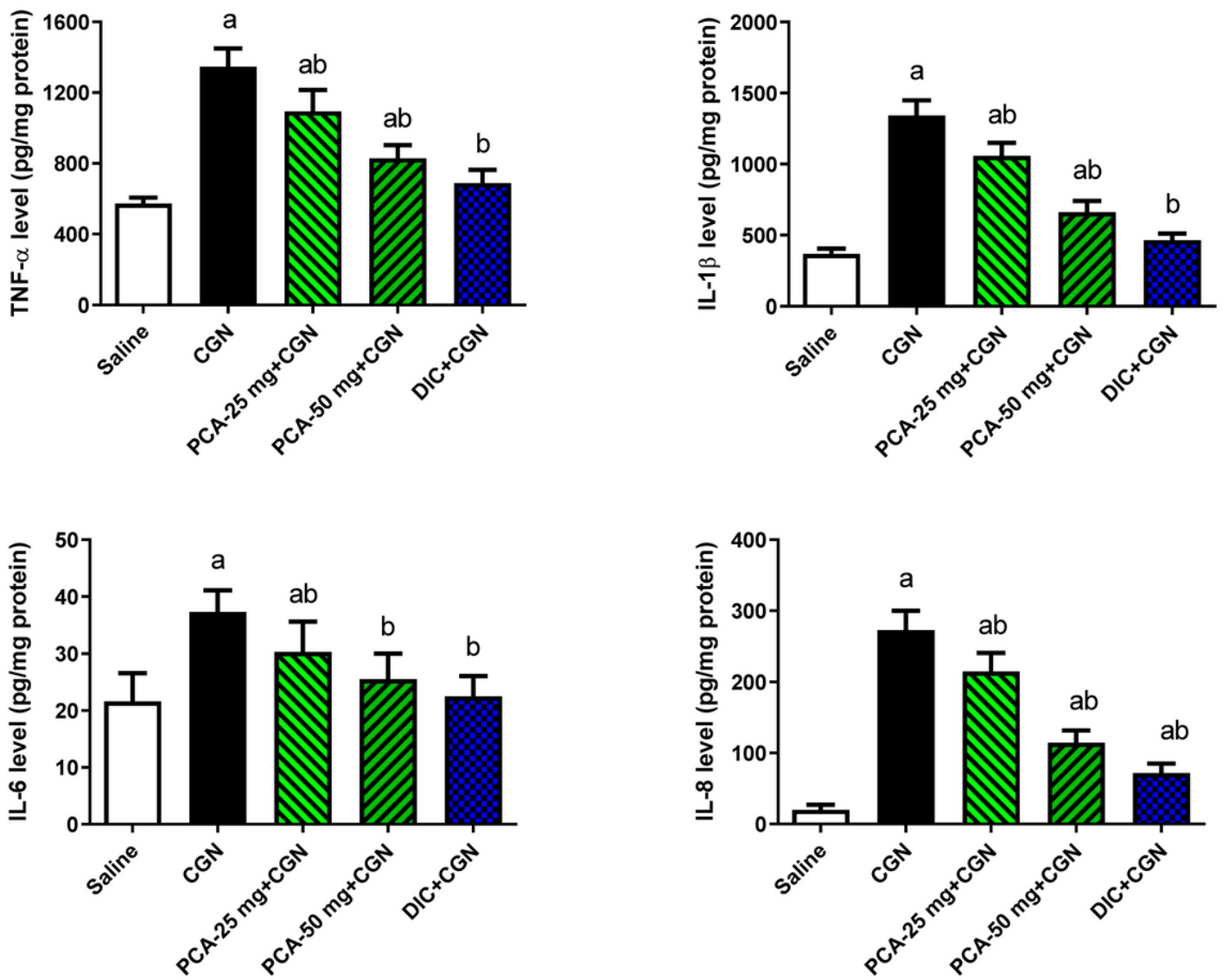


Figure 4

Effect of protocatechuic acid (PCA, 25 and 50 mg/kg) and diclofenac sodium (DIC) on TNF- α , IL-1 β , IL-6 and IL-8 levels following carrageenan (CGN)-induced paw edema. Results are depicted as mean \pm SD (n = 9); ^a $P < 0.05$ against control mice; ^b $P < 0.05$ against carrageenan (CGN) treated mice.

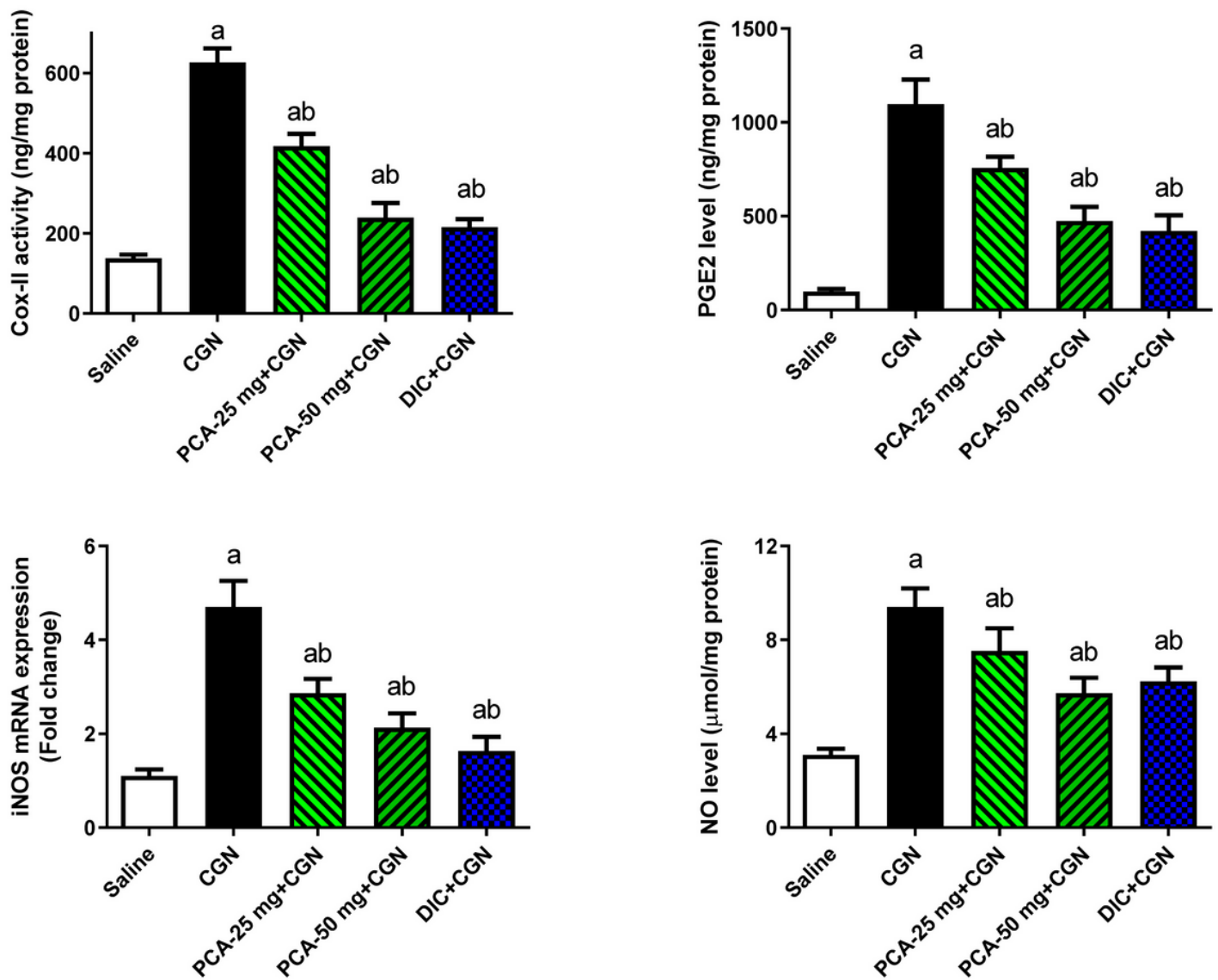


Figure 5

Effect of protocatechuic acid (PCA, 25 and 50 mg/kg) and diclofenac sodium (DIC) on COX-II activity and PGE2 along with mRNA expression of *iNOS* and NO level in mice following carrageenan (CGN)-induced paw edema. Results are depicted as mean \pm SD (n = 9); ^a $P < 0.05$ against control mice; ^b $P < 0.05$ against carrageenan (CGN) treated mice.

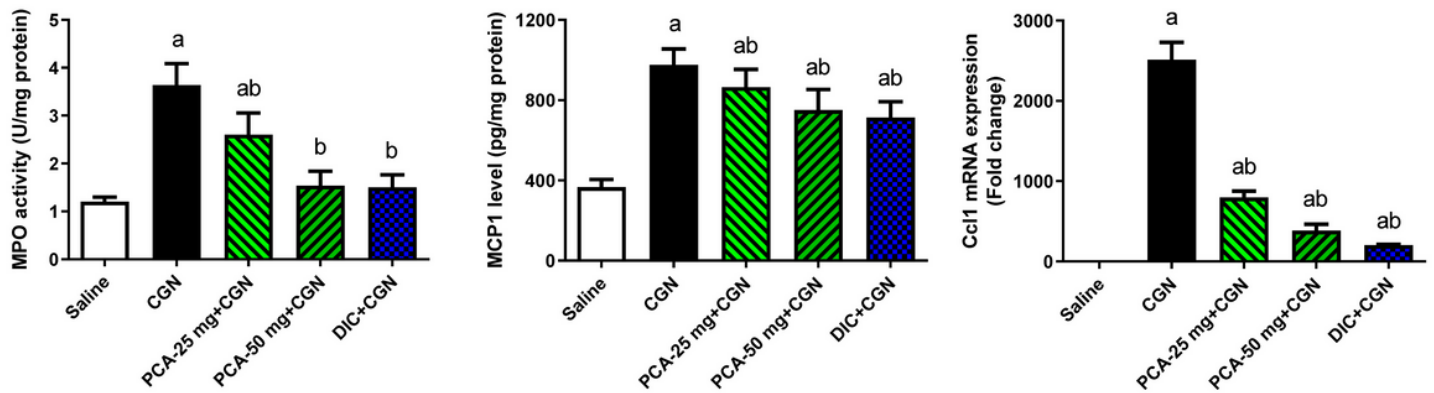


Figure 6

Effect of protocatechuic acid (PCA, 25 and 50 mg/kg) and diclofenac sodium (DIC) on MPO activity and MCP1 level along with *cc1* mRNA expression in mice following carrageenan (CGN)-induced paw edema. Results are depicted as mean \pm SD (n = 9); ^a $P < 0.05$ against control mice; ^b $P < 0.05$ against carrageenan (CGN) treated mice.

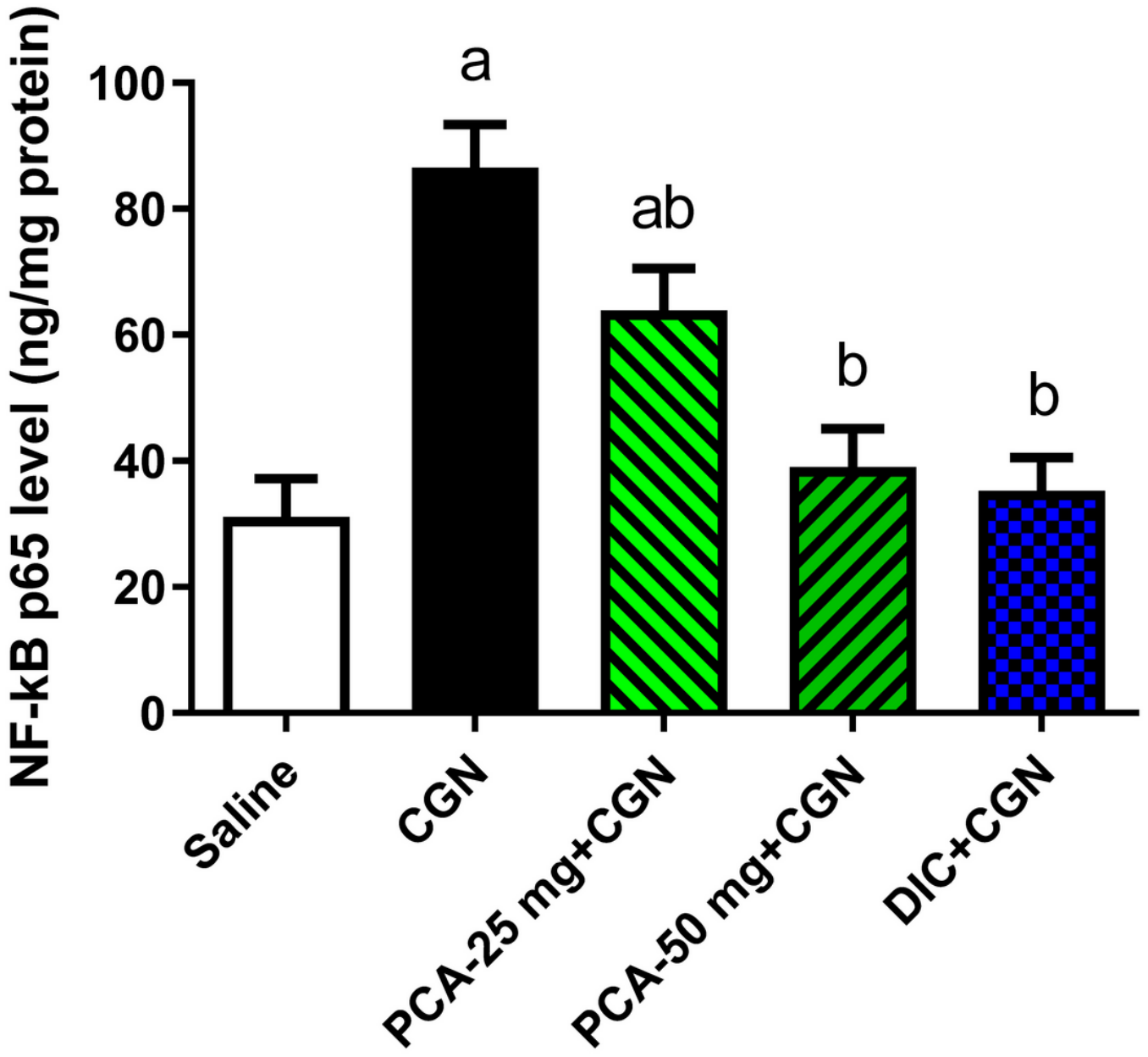


Figure 7

Effect of protocatechuic acid (PCA, 25 and 50 mg/kg) and diclofenac sodium (DIC) on NF-κB p65 level in mice with carrageenan (CGN)-induced paw edema. Results are depicted as mean ± SD (n = 9); ^a *P*<0.05 against control mice; ^b *P*<0.05 against carrageenan (CGN) treated mice.