Cecropia pachystachya Improves Naproxen-induced Gastric Ulcers in Mice Through its Potent Anti-inflammatory, Antioxidant, and NO Production Activities

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Research Article

Keywords: Peptic ulcer, plants, antioxidants

Posted Date: May 16th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2895729/v1

Abstract

Peptic ulcer (PU) occurs as acute or chronic inflammation of the stomach and/or duodenum. It is characterized by oxidative stress, intense inflammation, and hemorrhage, which result from an imbalance between the defensive and harmful factors. Cecropia pachystachya (CP) contains flavonoids, terpenes, and polyphenols such as chlorogenic acid, which is known for its antioxidant properties. This study aimed to investigate the antiulcerogenic activities and properties of the CP leaf extract on naproxen (NPX)-induced gastric ulcers. MTT assay was used to evaluate CP cytotoxicity on intestinal epithelial cells (IEC-6). Gastric ulcer was induced in male Swiss mice by pretreating them with 0.5% carboxymethylcellulose (CMC, a vehicle control) or CP (3, 10, and 30 mg/kg reconstituted in 0.5% CMC) for 30 min, followed by administration of NPX (300 mg/kg) and then euthanization after 6 h. The stomach segments were collected for macroscopia, microscopia, glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO, a neutrophil infiltration marker), nitrite/nitrate, interleukin (IL)-6, and tumor necrosis factor (TNF)-α analysis. CP extract did not affect IEC viability. CP extract (1, 3, and 10 mg/kg) reduced ($p < 0.05$) NPX-induced macroscopic and microscopic gastric lesions. In addition, it (3.0 mg/kg) prevented the depletion of GSH levels and reduced MDA formation and MPO. TNF-α and IL-6 levels were also reduced in mice subjected to NPX-induced gastric ulcers, compared with the control group. Furthermore, the extract reversed the increase in nitrite/nitrate concentration induced by NPX in stomach tissues. Therefore, our results demonstrate that CP has anti-inflammatory and antioxidant activities against NPX-induced gastric ulcers.

1. Introduction

Peptic ulcer (PU), a nonmalignant ulcer that occurs in the stomach or duodenum, is one of the main gastrointestinal disorders with increasing worldwide incidence and prevalence rate [1]. It can lead to chronic inflammation and is mainly caused by Helicobacter pylori colonization; however, other factors such as naproxen and other non-steroidal anti-inflammatory drugs (NSAIDs), have been shown to cause PU with the same intensity [2]. The inhibition of gastric acid production and enhancement of gastric mucosal protection are the main approaches in clinical treatment [3]. It has been shown that increasing nitric oxide (NO) production in the stomach of patients with PU has gastroprotective effects as NO maintains gastric epithelium integrity and mucus barrier and inhibits the secretion of hydrochloric acid from parietal cells [4].

Several natural products with distinct action mechanisms such as antioxidant effects and cytoprotective or anti-secretory actions, have been used in PU treatment [5]. However, the effects of Cecropia pachystachya (CP) on PU have not been explored.

Previous studies have shown that CP has antioxidant and anti-inflammatory effects in croton oil-induced ear edema [8]. CP is a fast-growing tree mainly prevalent in Central and South America regions and is widely being cultivated in Brazil [6]. CP leaves have a high antioxidant effect, which is often attributed to its phenolic components [7].

In order to discover alternatives that favor or reverse the deleterious effects of oxidative and inflammatory processes, in the present study, we investigated the effect of the ethanolic extract of CP leaves on naproxen-induced damage in mice.

2. Materials and Methods

Animals

Swiss mice (25–30 g) were obtained from the Pharmacology Department of the Federal University of Ceará (UFC, Fortaleza, Ceará, Brazil). Each experimental group consisted of nine mice. All animal experiments in this study were approved by the Committee on the Ethical Treatment of Research Animals (Protocol No. 47/16) and conducted in accordance with the guidelines for animal experimentation at UFC.

CP and extraction

In March 2016, the CP leaves were harvested from the Francisco José de Abreu Matos Medicinal Plants Garden located at the FUC. A voucher specimen of the plant was deposited in the Herbarium Prisco Bezerra (#EAC 21709) at the FUC. Nearly 100 g of previously dried and ground leaves was extracted with ethanol (1 L) for 48 h. After that, the ethanolic solution was evaporated to yield a dark green solid (11%, w/w) that was used for further analysis.

Identification of secondary metabolites in CP extract from UPLC-ESI-QTOF (QTOF)

The chemical characterization of CP extract was performed using a Waters Acquity UPLC (Ultra Performance Liquid Chromatography) system coupled to two mass spectrometers (quadrupole and time of flight, QTOF) with an electrospray ionization interface (ESI). Separation was performed on a Waters Acquity BEH C18 column (150 mm × 2.1 mm, 1.7 µm). The mobile phase was composed of H2O (A) and acetonitrile (B) containing formic acid (0.1% v/v). The elution gradient varied from 2 to 95% at a flow rate of 500 µL min-1. The UPLC-ESI-QTOF analyses were recorded in positive (PI) and negative (NI) ionization modes, ranging from 100 to 1200 Da, with an acquisition time of 0.1 s, in the centroid mode. The ESI conditions were defined as follows: capillary voltage 2800 V, cone voltage 40 V, source temperature 120°C, temperature desolvation 330°C, gas flow of 20 L h -1, desolvation gas flow 600 L h-1, and microchannel plate voltage (MCP) at 1900 V. Prior to this analysis, the ethanolic extract was cleaned-up into a solid-phase extraction (SPE) cartridge (Phenomenex, C18). The fraction eluted with H2O/MeOH (20:80) was filtered through a 0.22 PTFE syringe filter (Allrom) and then injected.

Naproxen-induced gastric ulcer and experimental groups

Naproxen-induced gastric ulcer was established in mice as described by Santana et al. [10] with some modifications. The mice were pretreated with 0.5% carboxymethylcellulose (CMC, vehicle, via gavage) or ethanolic extract of CP leaves at concentrations of 1, 3, 10, and 30 mg/kg. After 30 min, the mice were administered NPX (300 mg/kg) or CMC (control group) via gavage and allowed for 6 h before euthanizing

with a lethal dose of ketamine (200 mg/kg) and xylazine hydrochloride (60 mg/kg). Then, the stomach was rapidly excised, opened along the greater curvature, and washed with 0.9% saline solution. Before collecting stomach segments for analysis, gastric mucosal lesions were evaluated.

Macroscopic analysis of gastric lesions

The glandular face images of the stomach were acquired using a photographic camera under the same angle for each animal's stomach. The ulcerated area and total gastric body area were determined using a computerized planimetry program (ImageJ®). The results are expressed as a percentage of the ulcerated gastric tissue (in relation to total gastric body area) [11].

Histopathological analysis

The stomach tissues were fixed in 10% formaldehyde solution for 24 h before transferring into a 70% alcohol solution. Then, they were embedded in paraffin and sectioned (4 μm). The sections were deparaffinized and stained with hematoxylin and eosin (H&E) for subsequent light microscopic examination (200x). The stained samples were evaluated according to a previously described modified histopathological score system (0–14) [12], which assessed the loss of cell architecture (0–3 scores), mucosal edema (0–4 scores), hemorrhage (scores of 0–4), and infiltration of inflammatory cells (scores of 0–3). An experienced pathologist, blinded to the experimental conditions, performed all histopathological evaluations.

Measurement of glutathione in gastric tissue

The levels of glutathione (GSH) in stomach tissue samples were measured as previously described [13]. Stomach tissue samples (50–100 mg) were homogenized in 1 mL of 0.02 M EDTA for each 100 mg of tissue. Then, 400 μL of the homogenate was mixed with 320 μL of distilled water and 80 μL of 50% trichloroacetic acid (TCA) for protein precipitation. The tubes were centrifuged for 15 min at 3000 rpm at 4°C, and then 400 μL of the supernatant was added to 800 μL of 0.4 M Tris buffer (pH 8.9) and 20 μL of 0.01 M dithio-nitrobenzoic acid DTNB (Sigma) and mixed for 3 min. The absorbance was read at 412 nm using a spectrophotometer, and the GSH levels were expressed in μg/g of tissue.

Malondialdehyde assay

The concentrations of malondialdehyde (MDA) were determined based on the thiobarbituric acid reaction, as previously described [14]. Stomach tissue samples were homogenized with 1.15% cold KCl to obtain a 10% homogenate. After that, 0.5 mL of the homogenate was added into a 10 mL tube and mixed with 3 mL of H3P4 (1%) and 1 mL of aqueous thiobarbituric acid (0.6%), followed by heating for 45 min at 100°C, cooling for 20 min on ice, and then addition of 4 mL of n-butanol. Thereafter, the contents were mixed for 40 s with a vortex mixer and centrifuged at 1200 rpm for 10 min. The absorbance of the first phase of the mixture (100 μL) was measured at 520 and 535 nm, and the results were expressed in mmol/g of tissue.

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined using a method previously described [15]. Briefly, stomach tissue samples (50–100 mg) were homogenized in 0.5% hexadecyltrimethylammonium buffer (1 mL for each 50 mg of tissue) and centrifuged (4000 rpm, 7 min, 4°C). The MPO activity in the resuspended pellet was analyzed by measuring the change in absorbance at 450 nm in the presence of o-dianisidine dihydrochloride and 1% hydrogen peroxide. The results were expressed in units of MPO per mg of tissue.

Detection of nitrite/nitrate levels

The nitrite/nitrate (NO_2^-/NO_3^-) levels, which were obtained as an indirect indicator of NO production in the gastric tissue samples, were measured by the Griess reaction [16]. Initially, 10% homogenate was prepared by adding 1.15% cold KCl to the stomach tissue samples (50 mg). Then, the homogenate was centrifuged for 15 min at 14,000 rpm, and NO_3^- (40 µL) was converted to NO_2^- by incubating overnight at room temperature with an enzyme solution (40 µL) containing nitrate reductase (1 U/50 µL, Sigma-Aldrich, USA), NADPH (5 mg/mL, Sigma-Aldrich), potassium phosphate buffer (pH 7.5), and ultrapure water in the ratio 1: 10: 20: 19, respectively. After that, a serial dilution of the NO_2^- reference standard curve was prepared and then 80 µL of Griess solution (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% phosphoric acid) was added to each well. The purple/magenta staining was measured on a plate reader with a 540 nm filter, and the tissue nitrite/nitrate levels were expressed as NOx (µM/mg of tissue).

Cytokine levels

Interleukin (IL)-6 and tumor necrosis factor (TNF)-α levels in the gastric tissues were quantified by ELISA using the DuoSet Kit (R&D Systems, USA). First, the samples were homogenized in PBS. Nunc-immunomicrowell 96-well solid plates (Sigma-Aldrich) were incubated overnight at room temperature with the capture antibody for IL-6 and TNF-α. Subsequently, the plates were washed three times with 200 µL of wash buffer (R&D Systems) and blocked with 200 µL of 1% BSA (R&D Systems) for 1 h. After blocking, 100 µL of samples was added to each well and incubated for 2 h at room temperature. Then, the plates were washed, as previously described, and incubated with IL-1β detection antibody at room temperature for 2 h. After washing, the plates were incubated with streptavidin (100 µL) at room temperature in the dark for 20 min. Then, the plates were washed and 100 µL of developing substrate solution (R&D Systems) was added to each well and incubated for 20 min at room temperature in the dark. The enzymatic reaction was stopped by adding 50 µL stop solution (H_2SO_4). Finally, the absorbance was measured at 450 nm and the result was expressed in pg/mL.

Statistical analysis

All quantitative results are expressed as mean ± standard error of the mean (SEM), and histopathological scores are expressed as the median. All statistical analyses were performed using GraphPad Prism 5.0

software. Differences between groups were analyzed with analysis of variance test (ANOVA), followed by the Bonferroni multiple comparisons test or Kruskal-Wallis test, and then the Dunn test. $P < 0.05$ was considered significant.

3. Results

Characterization of the *Cecropia pachystachya* leaf extract obtained in the UPLC-QTOF system

UPLC-ESI-QTOF analyses for CP were performed with the methanol:water fraction (80:20) from its CP ethanolic extract in order to concentrate the sample of secondary metabolites by eliminating inactive interfering compounds such as sugars, lipids, and chlorophyll. In this system, chemical characterization was performed using the molecular formula deduced from the exact mass (4 decimal places) and the isotopic ion pattern detected in the TOF-type mass spectrometer. Generally, the tolerance limit for the mass error is 5 ppm (difference between the theoretical and experimental masses of the ion divided by its experimental mass times 106). In addition, the ion fragmentation pattern as well as comparison with analytical standards and literature data reporting the occurrence of the compound in species of the genus/family reinforces the tentative identification. Considering that the chemical constituents have different physicochemical properties (polarity), it is advisable to analyze the samples in the two ionization modes generated by the electrospray source (ESI). In the positive (ESI+) mode, molecules appear protonated or as adducts with cations (e.g., Na+, K+), whereas in the negative mode (ESI-), molecules appear deprotonated or as adducts with anions (e.g., Cl-) [17]. Chromatograms of CP obtained in the positive and negative ionization modes are shown in Fig. 1.

In the CP leaf extract, 30 compounds were detected, among which 19 were tentatively identified, which included 10 flavonoids [(luteolin-C-hexoside-O-pentoside (4 and 6), isoorientin (5), orientin (7) apigenin-C-hexoside-O-pentoside (8), 6-C-galactosyl-6"-O-β-galactopyranosylapigenin (9 and 10), cinchonain 1a (11), rhamnetin 3-glucoside (13), and lupinifoline (14)], 3 quassinoid-type triterpenes bruceanol F and E (15, 16, and 17), chlorogenic acid (2), condensate tannin procyanidin C (3), taxane diterpene taxacustin (12), polyhydroxy fatty acid and thianshic acid (21 and 22), and alkylphenol cardanol (28) (Fig. 2, Table 1).

CP did not alter IEC viability

First, we investigated whether CP (0.625, 1.25, 2.5, 10, and 20 mg/mL) had any toxic effects on IECs using the MTT assay. We found that compared to the control (cells incubated with complete culture medium alone), CP was unable to alter IEC viability (Table 2) even after 72 h of exposure. These findings showed that CP is safe for use in animal models as it was not cytotoxic.

CP (1, 3, and 10 mg/kg) reduces gastric mucosal lesions induced by naproxen (NPX)

To investigate the possible gastroprotective role of CP in NPX-induced gastric ulcers, we used mice as a model system. We assessed whether CP could reduce NPX-induced gastric lesions. We found that mice receiving only NPX 300 mg/kg (2,43 ± 0,29) (Fig. 3B and 3G) showed an increase ($p < 0.05$) in

macroscopic gastric lesions when compared to the CMC group that showed no gastric lesions (Fig. 3A). However, pretreatment with CP (1, 3, or 10 mg/kg, Fig. 3C-E and 3G) reduced (0,76 ± 0,24; 0,77 ± 0,06, and 0,75 ± 0,29, respectively) ($p < 0.05$) the lesions induced by NPX in the gastric mucosa. However, CP at a dose of 30 mg/kg (2,36 ± 0,28) (Fig. 4) was not effective in decreasing gastric lesions induced by NPX (2,43 ± 0,29).

Photomicrographs of the gastric mucosa of mice treated with: Panel A: carboxymethylcellulose group (0.5% ml/kg, orally); Panel B: NPX group (300 mg/kg, v.o) arrows point to gastric injury induced by NPX; Panel C: Pretreatment group *Cecropia pachystachya* (1 mg/kg, v.o.) + NPX group (300 mg/kg, v.o); Panel D: Pretreatment group *Cecropia pachystachya* (3 mg/kg, v.o.) + NPX group (300 mg/kg, v.o); Panel E: Pretreatment group *Cecropia pachystachya* (10 mg/kg, v.o.) + NPX group (300 mg/kg, v.o); and Panel F: Pretreatment group *Cecropia pachystachya* (30 mg/kg, v.o.) + NPX group (300 mg/kg, v.o). Panels C, D, and E with pretreatment with *Cecropia pachystachya* showed a reduction in gastric injury induced by NPX.

Mice with or without gastric ulcer had their stomachs removed, opened, and washed with PBS solution 6 h after gastric ulcer induction. Then, the stomachs were stretched and photographed using a computerized planimetry program (image J®). Values are presented as mean ± SEM of gastric lesion levels expressed in mm2 of tissue. For statistical analysis, the one-way ANOVA test was used followed by the Bonferroni test, where $p < 0.05$ vs. CMC group and * $p < 0.05$ vs. NPX group.

CP (1, 3, and 10 mg/kg) decreases NPX-induced hemorrhage, edema, loss of gastric mucosa, and infiltration of inflammatory cells in the gastric mucosa

Next, we evaluated whether CP could prevent histological changes such as hemorrhage, edema, loss of cell architecture, and infiltration of inflammatory cells, caused by naproxen (300 mg/kg) in the gastric mucosa (Table 3). We observed that in comparison with the CMC group control, NPX (300 mg/kg) induced mild hemorrhagic lesions, extensive edema, loss of gastric mucosa architecture, and infiltration of intense inflammatory cells in gastric tissue samples (Fig. 5A and H-J). Pretreatment with CP (1, 3, or 10 mg/kg) decreased ($p < 0.05$) NPX-induced hemorrhage, edema, loss of gastric mucosa architecture, and infiltration of inflammatory cells (Fig. 5-C-E and H–J).

Photomicroscopy of the gastric mucosa in the 200x increase of mice treated with: Panel A: carboxymethylcellulose group (0.5% ml/kg, orally); Panel B: NPX group (300 mg/kg, v.o); Panel C: Pretreatment Group *Cecropia pachystachya* (1 mg/kg, v.o) + NPX (300 mg/kg, v.o); Panel D: Pretreatment group *Cecropia pachystachya* (3 mg/kg, vol) + NPX (300 mg/kg, vol); Panel E: Pretreatment group *Cecropia pachystachya* (10 mg/kg, v.o.) + NPX (300 mg/kg, v.o); and Panel F: Pretreatment group *Cecropia pachystachya* (30 mg/kg, v.o.) + NPX (300 mg/kg, v.o). Panel B group naproxen shows edema represented by the blue arrow, loss of cell architecture, and infiltrating inflammatory cells represented by the black arrow. Panels C, D, and E with CP reduced edema, loss of cell architecture, and infiltration of inflammatory cells.

CP (1, 3, and 10 mg/kg) decreases NPX-induced oxidative stress in gastric mucosa

To determine how CP significantly decreased the ulcer formation as well as the histological changes induced by NPX, we investigated whether its effects were mediated by its antioxidant activity by measuring GSH, an antioxidant, and MDA (an end product of lipid peroxidation product) levels. We found that NPX significantly decreased the levels of GSH (55,47 ± 2,67) in gastric tissue samples compared to the CMC group (108,4 ± 7,73) (Fig. 6). However, pretreatment with CP (3 mg/kg) (87,68 ± 3,45) prevented ($p < 0.05$) the depletion of glutathione concentrations induced by NPX. However, other CP concentrations (1, 10, or 30 mg/kg) failed to prevent NPX-induced depletion of glutathione concentrations [1 mg/kg (49,44 ± 1,42), 10 mg/kg (40,89 ± 8,81), and 30 mg/kg (38,97 ± 11,76)].

In relation to the end product of lipid peroxidation, NPX increased MDA concentrations (3489 ± 206,6) in gastric tissue compared with the CMC group (2233 ± 270,6). In contrast to the GSH levels, all CP concentrations (1, 3, and 10 mg/kg) decreased MDA levels [1 mg/kg (1892 ± 76,84), 3 mg/kg (1860 ± 93,10), and 10 mg/kg (1927 ± 85,27)] in the gastric tissue of mice subjected to NPX-induced gastric ulcer (3489 ± 206,6) (Fig. 7). Collectively, these data suggested that CP has potent antioxidant activity during NPX-induced gastric ulcer.

Stomach segments were collected to measure the MDA concentrations. Naproxen increased MDA concentration in the stomach when compared to the 0.5% CMC group. Cecropia pachystachya extract reduced stomach MDA concentration. Values are presented as mean ± SEM of MDA concentrations expressed in nmol/mg of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where $p < 0.05$ vs. CMC group 0.5% and * $p < 0.05$ vs. NPX group.

CP (1, 3, and 10 mg/kg) reverts NPX-induced inflammation in gastric mucosa

To further test how CP improved NPX-induced gastric ulcer, we evaluated its anti-inflammatory activity by measuring MPO activity, an indirect method for assessing neutrophil infiltration, and pro-inflammatory cytokine (IL-6 and TNF-α) levels. Mice subjected to NPX-induced gastric ulcer showed an increase ($p < 0.05$) in MPO activity (3,68 ± 0,68), suggesting that there was an increase in the infiltration of neutrophils in the stomach tissue compared to the CMC group (0,50 ± 0,07). However, mice pretreated with CP (1, 3, and 10 mg / kg) showed a notable reduction ($p < 0.05$) in MPO activity [1 mg/kg (0,50 ± 0,10), 3 mg/kg (0,15 ± 0,06), 10 mg/kg (0,08 ± 0,08), and 30 mg/kg (0,40 ± 0,06)] in the stomach of mice subjected to NPX-induced gastric ulcer (Fig. 8). Given that CP at 3 mg/kg was more effective in preventing NPX-induced oxidative stress and neutrophil infiltration, we only used that CP concentration for further analyses. Mice subjected to NPX-induced gastric ulcer demonstrated an increase ($p < 0.05$) in TNF-α (Fig. 10A) (158,7 ± 16,95) and IL-6 (145,0 ± 19,10) (Fig. 10B) levels in the stomach tissue compared to the TNF-α (78,14 ± 8,39) and IL-6 (83,95 ± 4,06) levels of the CMC group. Surprisingly, pretreatment with CP (3 mg/kg) reversed the NPX-induced increase ($p < 0.05$) in TNF-α (92,61 ± 2,34) (Fig. 10A) and IL-6 (98,49 ± 0,15) (Fig. 10B) levels (Fig. 10).

Segments of the stomach were collected to measure the concentrations of myeloperoxidase activity. Naproxen increased MPO activity compared to the 0.5% CMC group. Cecropia pachystachya extract reduced neutrophilic infiltration in the stomach. The values are presented as mean + SEM concentrations of MPO expressed in U/mg of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where $p < 0.05$ vs. CMC group 0.5% and * $p < 0.05$ vs. NPX group.

Stomach segments were collected to measure the concentrations of TNF-α and IL-6. Naproxen increased the stomach concentrations of TNF-α and IL-6 when compared to the vehicle group. Cecropia pachystachya in these animals reduced the concentration of TNF-α in the stomach. Values are presented as mean ± SEM concentrations of TNF-α and IL-6 expressed in pg/ml of tissue. For statistical analysis, the one-way ANOVA test was used followed by the Bonferroni test, where $p < 0.05$ vs. CMC group 0.5% and * $p < 0.05$ vs. NPX group.

CP (3 mg/kg) increased nitrite/nitrate level in gastric mucosa during NPX-induced gastric ulcer

NO is known to play an important role in promoting gastric mucosa formation, a protective essential component of the gastric epithelial barrier against injuries. Thus, we investigated whether CP was able to stimulate NO synthesis during NPX-induced gastric ulcer by measuring nitrite/nitrate levels using the Griess reaction. NPX reduced ($p < 0.05$) nitrite/nitrate concentrations (0,17 ± 0,09) in the stomach tissue compared to the CMC group (0,29 ± 0,04) ($p < 0.05$). However, pretreatment with CP (3 mg/kg) reversed the effect and increased the nitrite/nitrate concentration (0,27 ± 0,03) in gastric mucosa when compared with the naproxen group (0,17 ± 0,09) (Fig. 9).

Segments of the stomach were collected to measure the NO3/NO2 concentrations. Naproxen reduced the stomach nitrite and nitrate levels when compared to the 0.5% CMC group. Cecropia pachystachya (3.0 mg/kg) increased the nitrite/nitrate concentrations in the stomach of animals subjected to NPX-induced gastric ulcer. The values are presented as mean + SEM concentrations of nitrite/nitrate expressed in µmol/mg of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where $p < 0.05$ vs. CMC group 0.5% and * $p < 0.05$ vs. NPX group.

4. Discussion

The present study demonstrated that the ethanolic extract from CP leaves reduced NPX-induced injury in an experimental model of gastric ulcer in mice. The extract reduced edema, inflammatory cell infiltration, and alteration of the gastric mucosa architecture. For the first time, CP's gastroprotective effect was demonstrated as it decreased NPX-induced oxidative stress, prevented GSH depletion at a dose of 3 mg/kg, and reversed NPX-induced increase of MDA. Notably, the CP extract was not cytotoxic to epithelial cells of the digestive tract.

NSAID administration suppresses antioxidant defense, consequently increasing lipid peroxidation in gastric tissue, which results in gastric damage [22]. It has been previously demonstrated that NPX induces an increase in MDA levels [10, 23], which indicated increased oxidative stress. MDA is an

important target for free radical reactions in the presence of unsaturated bonds in membrane phospholipids. The consequence of this reaction is lipid peroxidation, which can result in loss of membrane fluidity, receptor alignment, and cellular lysis. Thus, damage induced by free radicals results in inactivation and reticular denaturation, which, together with interaction with nucleic acids, can induce DNA damage and result in mutations [24].

In the present study, 10 flavonoids and other compounds with antioxidant, anti-inflammatory, and antiplatelet biological activities [27] were identified in the CP extract. Studies have reported that flavonoids and other polyphenols can act as antioxidants because they can clear free radicals [19]. Structurally, phenolic compounds represent a wide variety of compounds having one or more aromatic rings attached to at least one hydroxyl group and/or other substituents. The biological activity is linked to the vast structural diversity of the compounds [26]. Other studies have shown that the antioxidant properties of phenolics orientin, isoorientin, and chlorogenic acid components are found in CP extract [6]. These constituents may explain the biological activities found in Cecropia species since they have been detected in C. pachystachya, which demonstrated anti-inflammatory, leishmanicidal, and hypoglycemic activities [47]. Thus, the effect of CP can be due to a major constituent or the synergy of these components.

In addition to flavonoids, the CP extract also contained cinchonain, which has antioxidant and antibacterial effects, and chlorogenic acid, which has a higher hydroxylation capacity, followed by gallic acid and caffeic acid. In biological systems, these acids have the capacity to interact in lipophilic and hydrophilic environments, therefore possessing an important biological capacity that includes minimizing the oxidative process [26]. Chlorogenic acid is considered a polyphenol with strong antioxidant action that is used to prevent diseases [42, 43]. The biological importance of the Cecropia was once again demonstrated in a review that shows how the active compounds orientin, isoorientin, isovitexin, vitexin as well as c-glycosylflavonoids present in the plant have anti-inflammatory and antimicrobial action [48]

Therefore, the extract demonstrated antioxidative activity in the stomach, which may partially explain its protective effect. These findings are in concordance with previous reports on the antioxidative activity of CP ethanolic extract in a mouse chronic stress model, which was analyzed for TBARS, CAT, GPX, and SOD activity [30].

The results presented here demonstrated that NPX induced an increase in gastric tissue myeloperoxidase activity (MPO) [10, 36], whereas CP ethanolic extract reduced MPO activity in the stomach tissue of animals treated with NPX, indicating a reduction in neutrophilic infiltration. The infiltrated and activated neutrophils represent a source of reactive oxygen species, nitrogen species, and proinflammatory cytokines [33]. These data are consistent with those of previous studies that showed the antioxidant and anti-inflammatory properties of the methanol extract of CP leaves [6, 8]. In addition to decreasing NPX-induced hemorrhage, edema, loss of gastric mucosa, and infiltration of inflammatory cells in the gastric mucosa as seen by histopathological analysis, CP reversed the increased concentrations of TNF-α and IL-

6 in gastric tissue induced by NPX (Fig. 11). These data reinforce the anti-inflammatory activity of the CP extract and explain gastroprotection and reduction of ulcer size.

TNF-α and IL-6 are known to be expressed in the gastric mucosa and are important cytokines involved in ulcer pathogenesis. The transcriptional activation of the TNF-α gene depends on the transcription factor nuclear factor-kappa B (NF-kB). Thus, studies have suggested that inhibiting NF-kB activation reduces TNF-α production, which produces an anti-progression effect [37].

A few factors are involved in the pathogenesis of gastric lesions. It has been reported that gastric neutrophil accumulation, pro-inflammatory cytokine production, increased free radical production, reduced mucosal blood flow, and increased acid secretion are involved in pathogenesis; however, they have not been well elucidated [39]. While causing inflammation, NPX reduces nitrite/nitrate concentrations in the tissue, as demonstrated here. This finding is consistent with a previous report [41], which demonstrated that the NSAID-treated rats presented decreased concentrations of nitric oxide in the gastric mucosa when compared to the rats without gastric ulcer. On the contrary, the CP extract increased nitrite/nitrate concentrations, suggesting an increase in nitric oxide concentration in the tissue. Nitric oxide has a protective effect on the gastric mucosa because it induces vasodilation and increases blood flow necessary for the removal of mucosal-damaging factors such as free radicals [45, 46].

5. Conclusions

In conclusion, the CP ethanolic extract has an anti-inflammatory effect, an antiulcerogenic effect linked to an antioxidant, and the ability to increase nitric oxide concentrations in the gastric tissue. Hence, it may be valuable in the treatment of gastric ulcers.

Declarations

Author Contributions: D.S.M. designed and performed all experiments, analyzed the data, and wrote the manuscript. helped in the acquisition of data. assisted in analysis and interpretation, and helped revise the manuscript. wrote the discussion and helped revise the manuscript. We have helped revise the manuscript. is the principal investigator for the grant and helped with the experimental design, supervised the project, and helped to write the manuscript.

Funding: This work was supported by CAPES/DINTER (grant 23038044935/2009-12) and CAPES/Procad (Grant).

Acknowledgments: We acknowledge Maria do Socorro França Monte, Adalberto Júnior, and Flávia de Araújo Silva for technical assistance. We thank Prof Otilia Deusdênia Loiola Pessoa (Federal University of Ceará) for providing the plant material.

Conflicts of Interest: “The authors declare no conflict of interest.”

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Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures

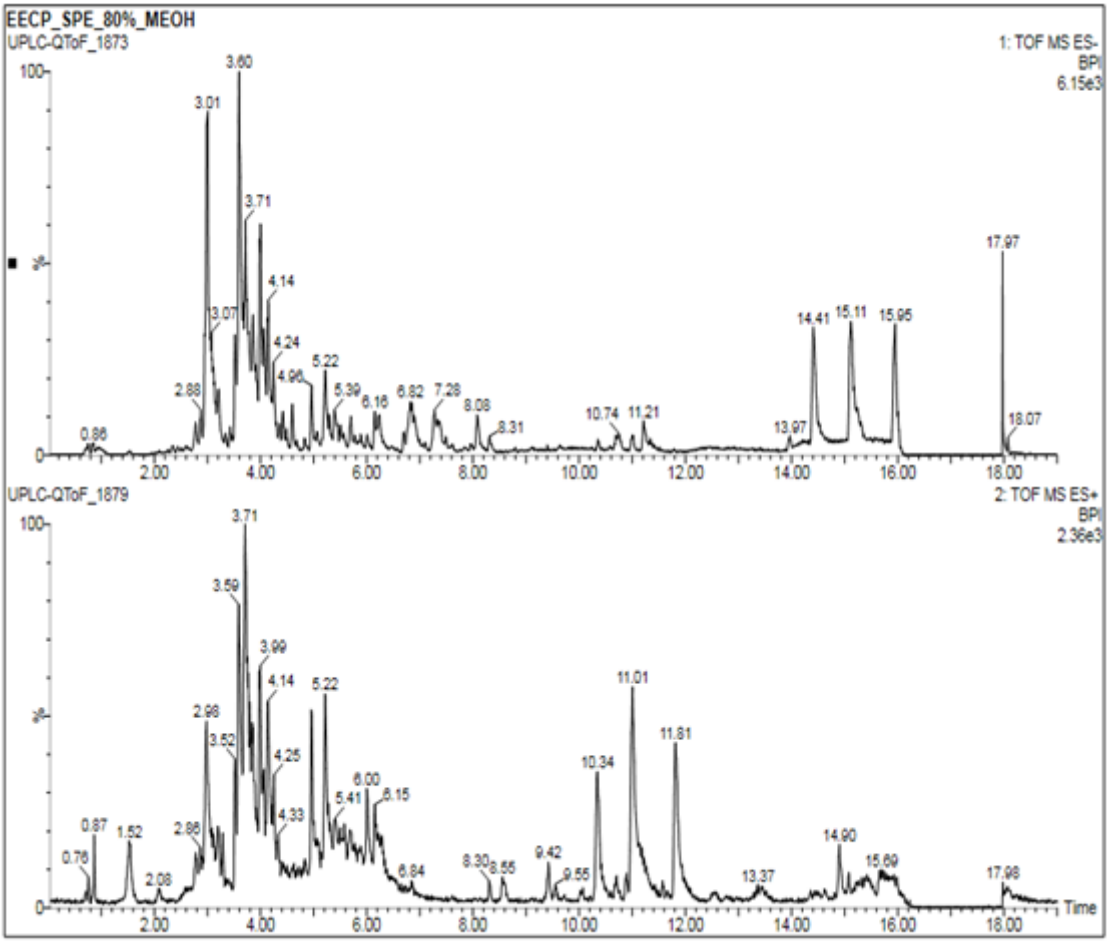

Figure 1

UPLC-QTOF Chromatograms of the ethanolic extract of CP leaves in the negative (A) and positive (B) ionization modes.

* represents the difference between the mass/charge of the ion observed in the mass spectrometer (experimental) and its theoretical mass/charge.

N.I: not identified

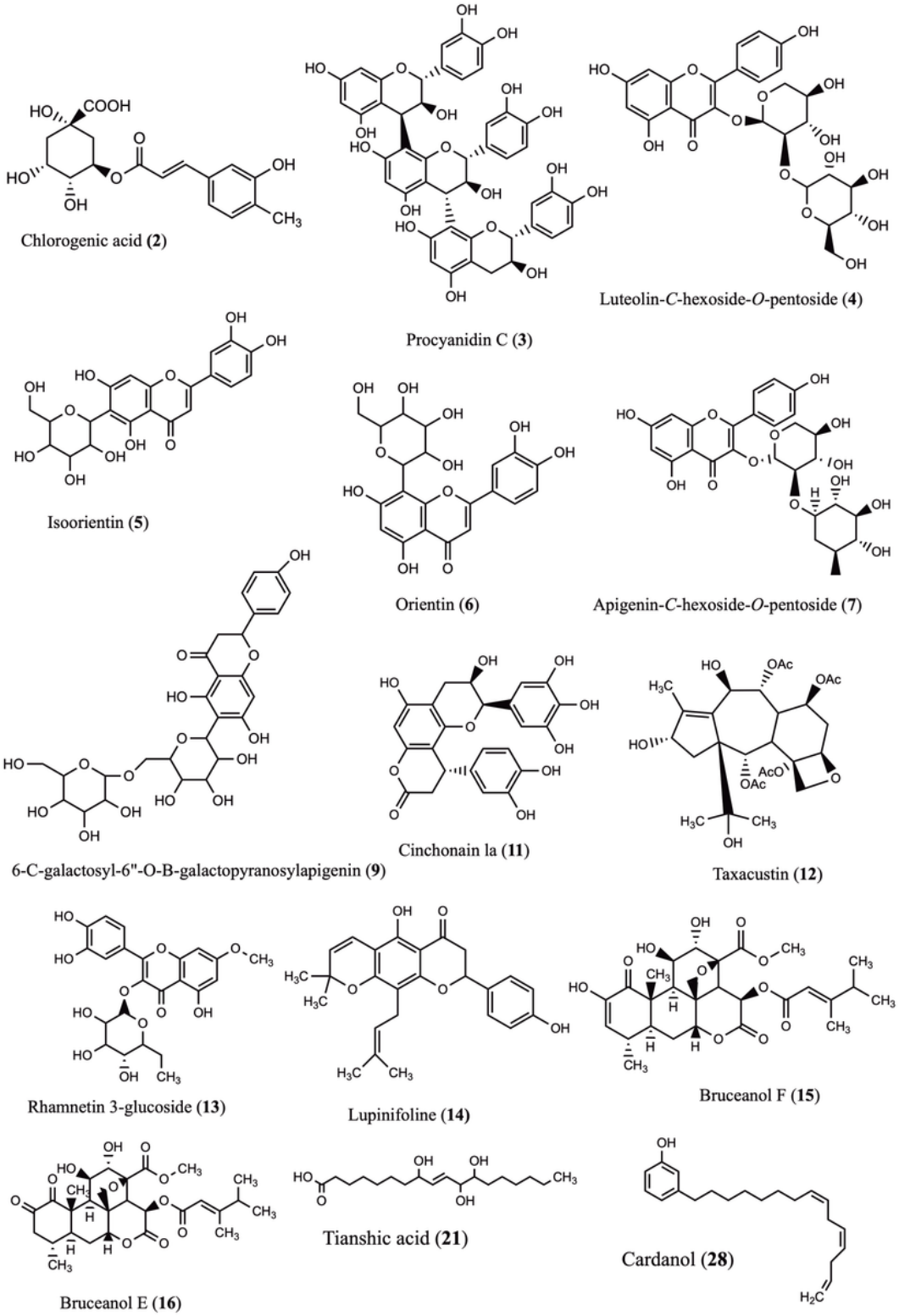

Figure 2

Structures of Cecropia pachystachya

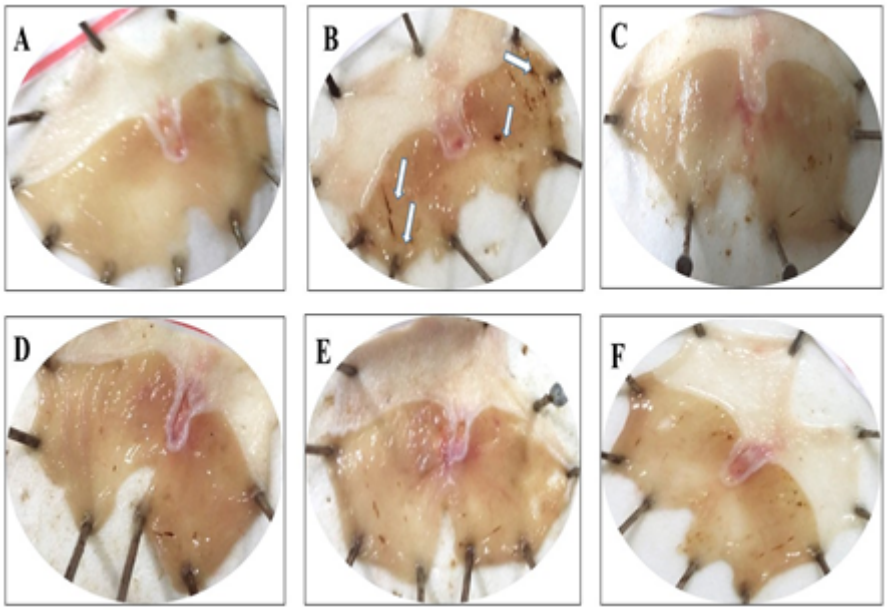

Figure 3

Photographs of the gastric mucosa of mice treated with CMC (panel A) or CP (panels C, D, E, and F) in the NPX (panel B) gastric lesion model.

Photomicrographs of the gastric mucosa of mice treated with: Panel A: carboxymethylcellulose group (0.5% ml/kg, orally); Panel B: NPX group (300 mg/kg, v.o) arrows point to gastric injury induced by NPX; Panel C: Pretreatment group Cecropia pachystachya (1 mg/kg, v.o.) + NPX group (300 mg/kg, v.o); Panel D: Pretreatment group Cecropia pachystachya (3 mg/kg, v.o.) + NPX group (300 mg/kg, v.o); Panel E: Pretreatment group Cecropia pachystachya (10 mg/kg, v.o.) + NPX group (300 mg/kg, v.o); and Panel F: Pretreatment group Cecropia pachystachya (30 mg/kg, v.o.) + NPX group (300 mg/kg, v.o). Panels C, D, and E with pretreatment with Cecropia pachystachya showed a reduction in gastric injury induced by NPX.

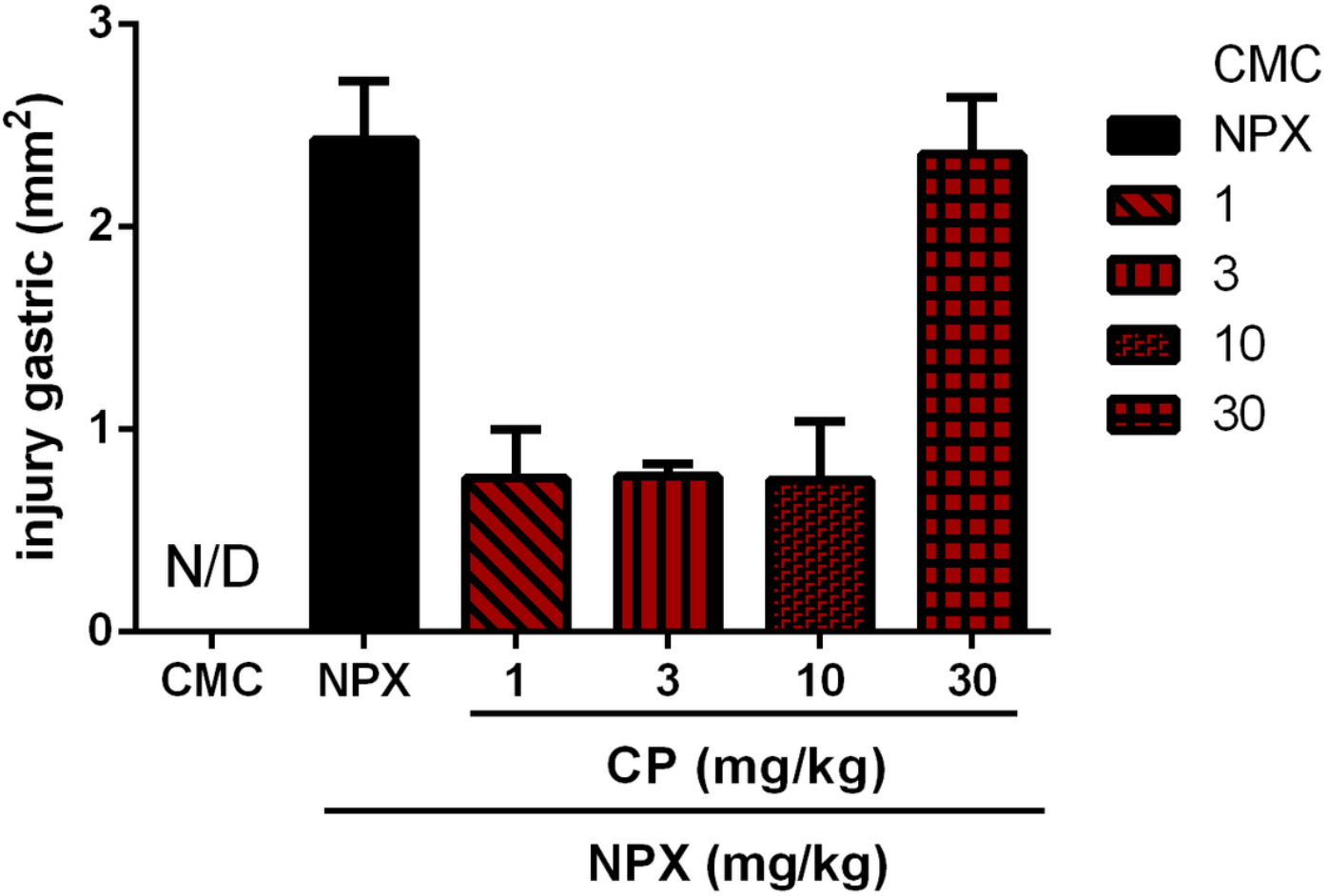

Figure 4

Macroscopic evaluation of gastric mucosal lesions.

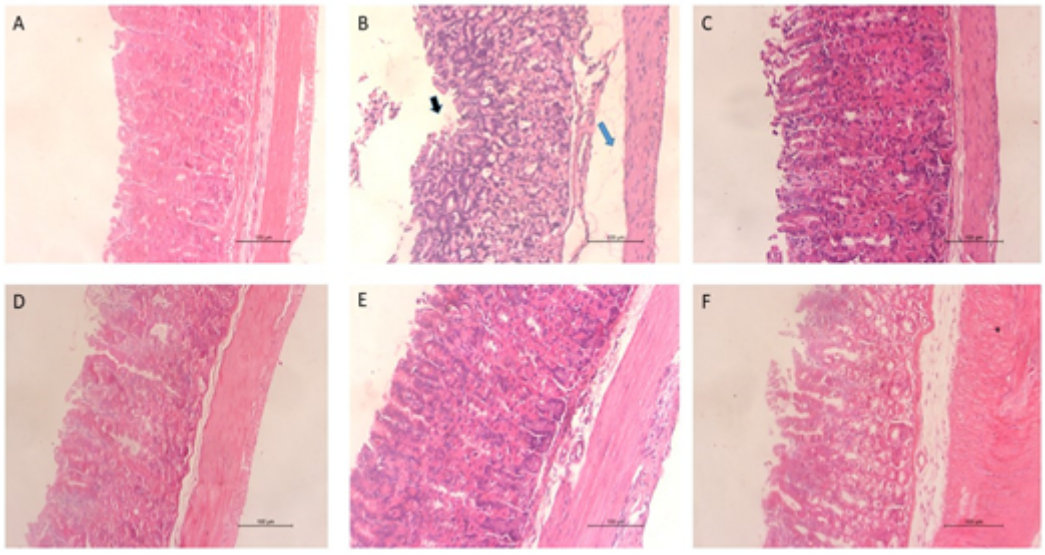

Figure 5

Photomicrographs of the gastric mucosa of CMC (panel A) or CP (panels C, D, E, and F) treated mice in the NPX (panel B) gastric lesion model.

Photomicroscopy of the gastric mucosa in the 200x increase of mice treated with: Panel A: carboxymethylcellulose group (0.5% ml/kg, orally); Panel B: NPX group (300 mg/kg, v.o); Panel C: Pretreatment Group Cecropia pachystachya (1 mg/kg, v.o) + NPX (300 mg/kg, v.o); Panel D: Pretreatment group Cecropia pachystachya (3 mg/kg, vol) + NPX (300 mg/kg, vol); Panel E: Pretreatment group Cecropia pachystachya (10 mg/kg, v.o.) + NPX (300 mg/kg, v.o); and Panel F: Pretreatment group Cecropia pachystachya (30 mg/kg, v.o.) + NPX (300 mg/kg, v.o). Panel B group naproxen shows edema represented by the blue arrow, loss of cell architecture, and infiltrating inflammatory cells represented by the black arrow. Panels C, D, and E with CP reduced edema, loss of cell architecture, and infiltration of inflammatory cells.

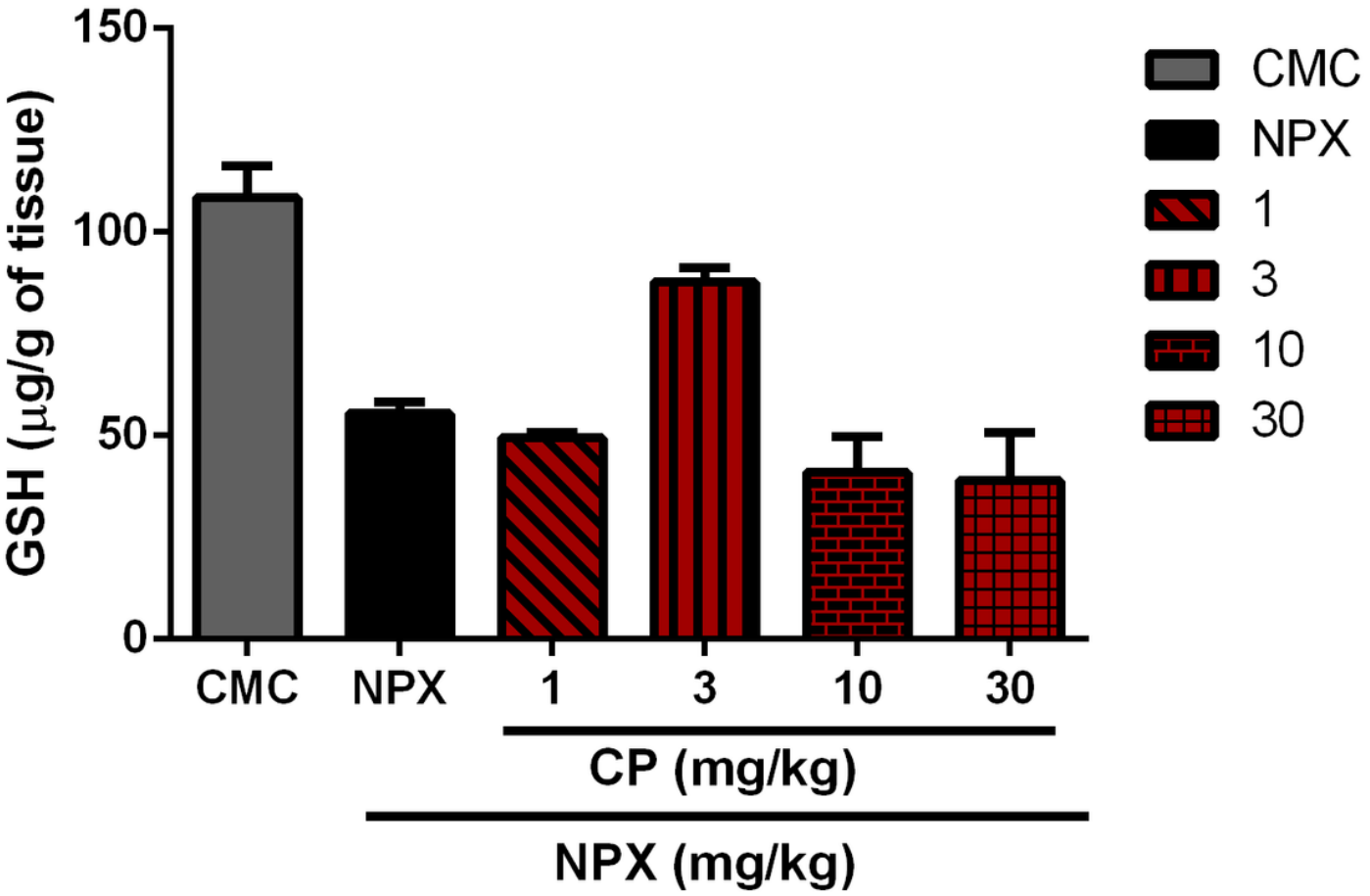

Figure 6

Effect of Cecropia pachystachya on glutathione concentration in the gastric mucosa of mice subjected to NPX-induced gastric ulcer. Stomach segments were collected to measure the GSH concentrations. It was observed that naproxen decreased the glutathione concentrations in the stomach when compared to the 0.5% CMC group. Administration of Cecropia pachystachya (3 mg/kg) in these animals prevented the

depletion of GSH concentrations in the stomach. Values are presented as mean ± SEM of GSH concentrations expressed in µg/g of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where p <0.05 vs. CMC group 0.5% and * p <0.05 vs. NPX group.

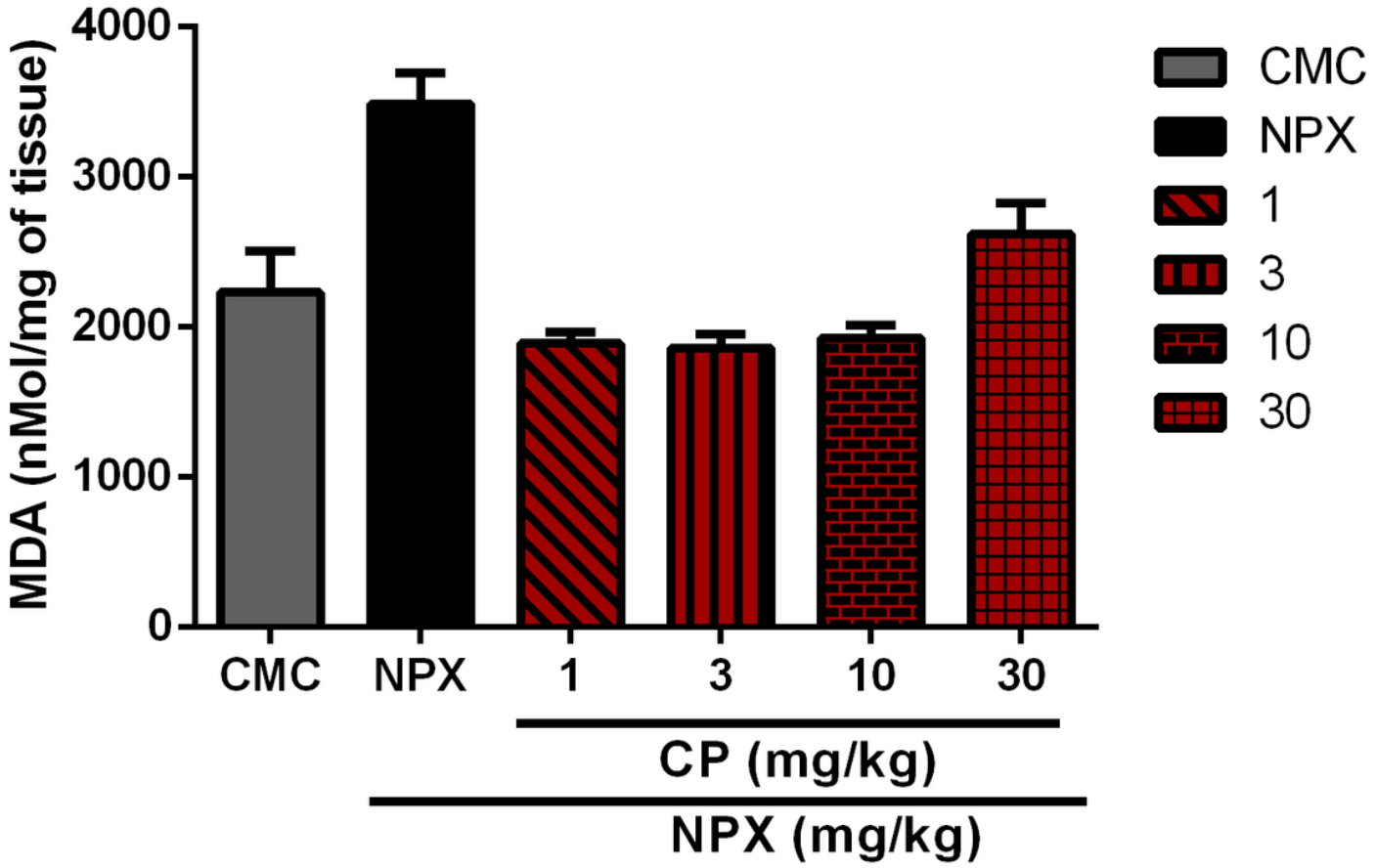

Figure 7

Effect of Cecropia pachystachya on malondialdehyde concentrations in the gastric mucosa of NPX-treated mice

Stomach segments were collected to measure the MDA concentrations. Naproxen increased MDA concentration in the stomach when compared to the 0.5% CMC group. Cecropia pachystachya extract reduced stomach MDA concentration. Values are presented as mean ± SEM of MDA concentrations expressed in nmol/mg of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where p <0.05 vs. CMC group 0.5% and * p <0.05 vs. NPX group.

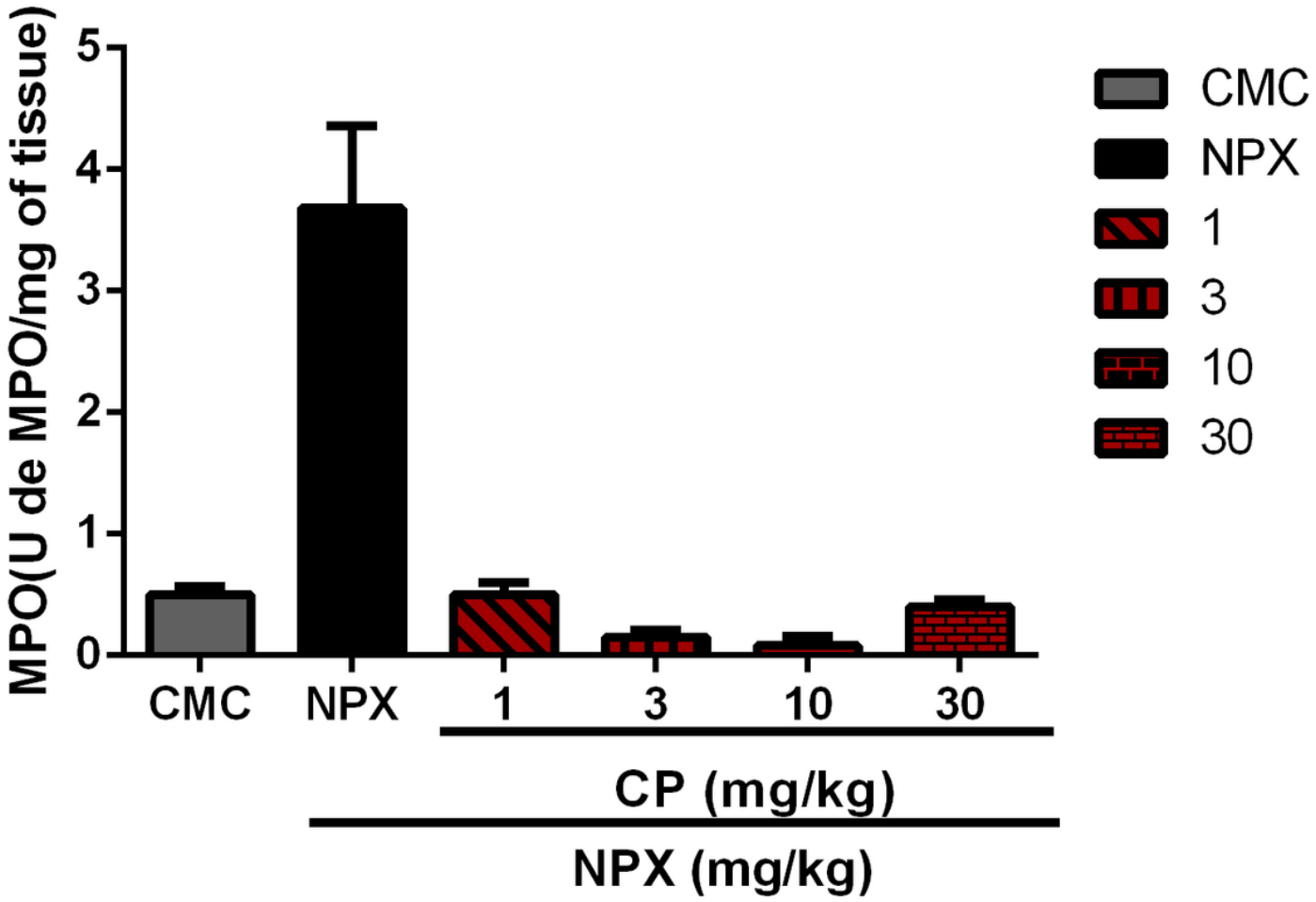

Figure 8

Effect of Cecropia pachystachya on myeloperoxidase concentration in the gastric mucosa of NPX-treated mice

Segments of the stomach were collected to measure the concentrations of myeloperoxidase activity. Naproxen increased MPO activity compared to the 0.5% CMC group. Cecropia pachystachya extract reduced neutrophilic infiltration in the stomach. The values are presented as mean + SEM concentrations of MPO expressed in U/mg of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where $p < 0.05$ vs. CMC group 0.5% and * $p < 0.05$ vs. NPX group.

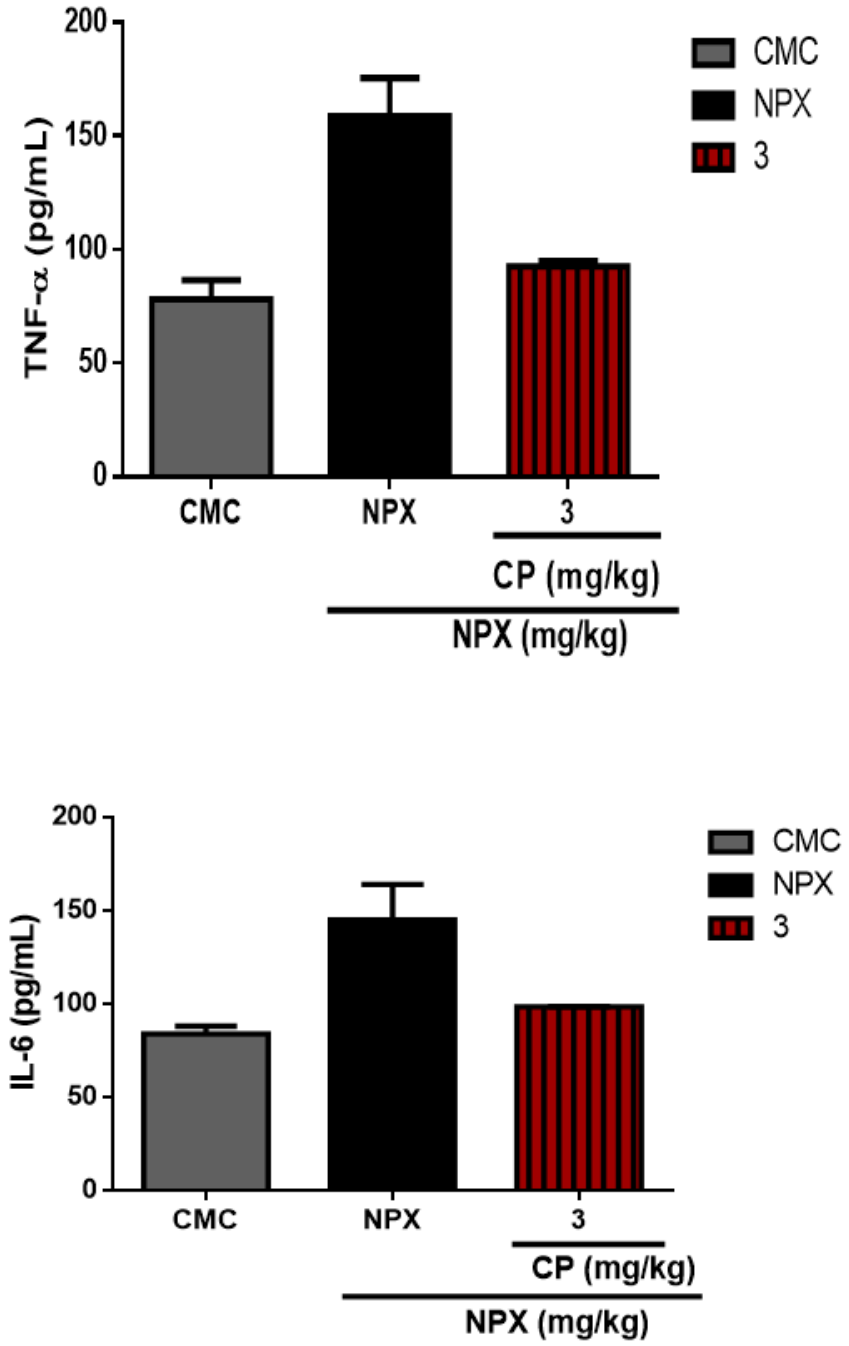

Figure 9

Cecropia pachystachya decreases the tissue concentrations of TNF-α (panel A) and IL-6 (panel B) in the stomach of mice subjected to NPX-induced gastric ulcer.

Stomach segments were collected to measure the concentrations of TNF-α and IL-6. Naproxen increased the stomach concentrations of TNF-α and IL-6 when compared to the vehicle group. Cecropia

pachystachya in these animals reduced the concentration of TNF-α in the stomach. Values are presented as mean ± SEM concentrations of TNF-α and IL-6 expressed in pg/ml of tissue. For statistical analysis, the one-way ANOVA test was used followed by the Bonferroni test, where p <0.05 vs. CMC group 0.5% and * p <0.05 vs. NPX group.

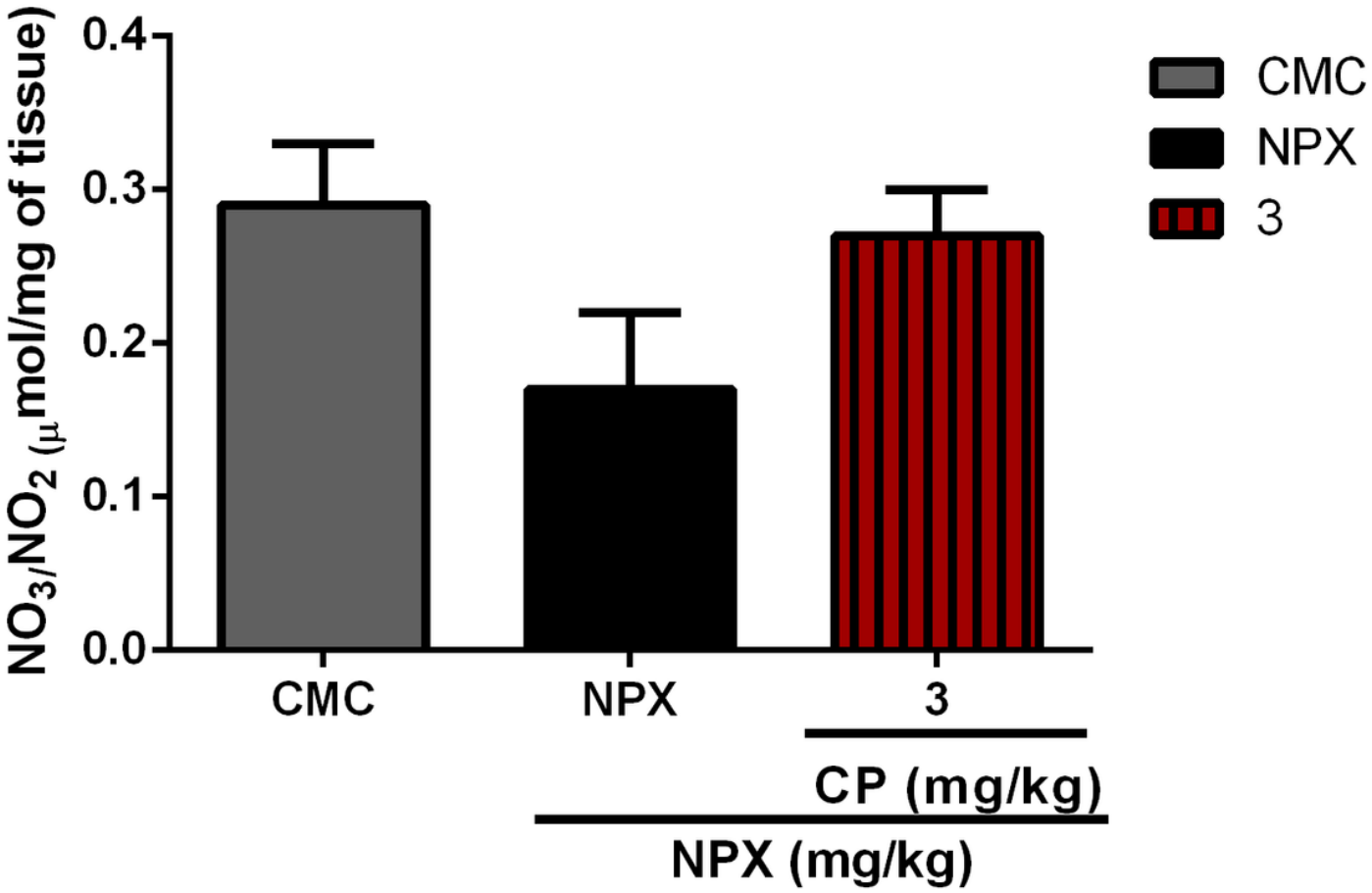

Figure 10

Cecropia pachystachya increases NO3/NO2 concentrations in the stomachs of mice subjected NPX-induced gastric ulcers.

Segments of the stomach were collected to measure the NO3/NO2 concentrations. Naproxen reduced the stomach nitrite and nitrate levels when compared to the 0.5% CMC group. Cecropia pachystachya (3.0 mg/kg) increased the nitrite/nitrate concentrations in the stomach of animals subjected to NPX-induced gastric ulcer. The values are presented as mean + SEM concentrations of nitrite/nitrate expressed in µmol/mg of tissue. For statistical analysis, one-way ANOVA followed by the Bonferroni test was used, where p <0.05 vs. CMC group 0.5% and * p <0.05 vs. NPX group.

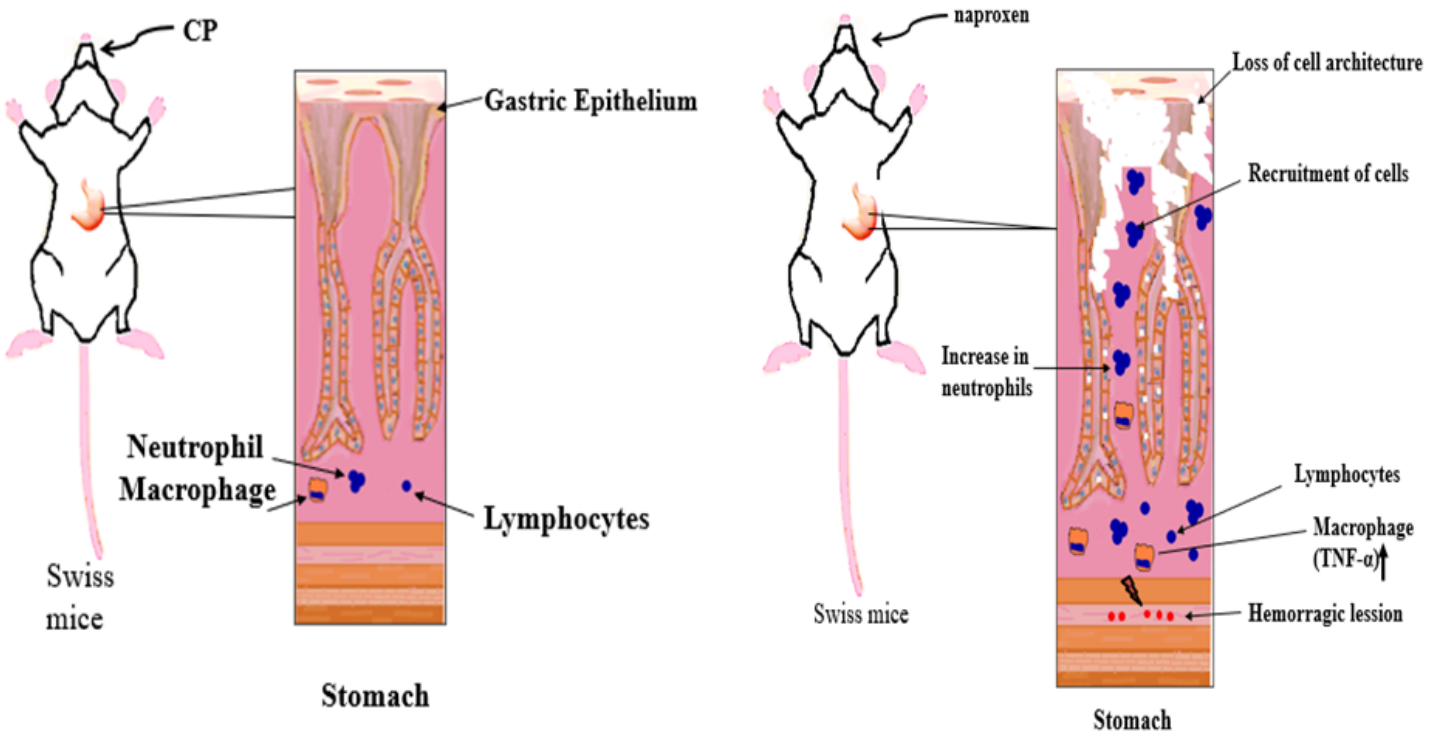

Figure 11

Representative scheme of the effect of NPX and the effect of CP in the epithelial gastric

Supplementary Files

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- Supplementary.png
- Table1.tif
- Table2.tif
- Table3.tif