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Anti-inflammatory activity and modulate oxidative stress of *Bucida buceras* in lipopolysaccharide-stimulated RAW 264.7 macrophages and Carrageenan-induced acute paw edema in rats

Simon B. Iloki Assanga[1,2], Lidianys M. Lewis Luján[1*], Armida A. Gil-Salido[1], Claudia L. Lara Espinoza[1], Daniela Fernandez Angulo[1], Jose L. Rubio-Pino[1] and René Betancourt Riera[2]

[1]Rubio Pharma y Asociados S.A. de C.V., Blvd. García Morales, Km. 6.5 #330, El Llano, Hermosillo, Sonora, C.P. 83210, Mexico.
[2] Instituto Tecnológico de Hermosillo (ITH) Ave. Tecnológico y Periférico Poniente S/N, Col. Sahuaro, Hermosillo, Sonora, Mexico C.P. 83170.

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Since oxidative stress is an important mediator that lead or maintain inflammatory processes, the aim of this work is to evaluate the effects of *Bucida buceras* on inflammatory response in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and anti-inflammatory effect and redox biomarkers in carrageenan-induced paw edema in rats. *B. buceras* also known as "black-olive" belongs to the *Combretaceae* family and it is used as an ornamental evergreen tree in many city streets. This plant is widely distributed in tropical regions of Caribbean, Central America and northern South America. In a continuous effort to find more potent, non-toxic natural product inhibitors that suppress inflammation, the present study was carried out to analyzed the influence of aqueous extract on nitric oxide (NO), tumor necrosis factor (TNF-α), interleukin (IL)-6 and IL-1β in LPS-induced murine macrophages and paw thickness, NO, C-reactive protein (CPR), organoperoxide, oxidation protein and reducing power antioxidant in paw edema in rats. Results revealed that treatment with *B. buceras* aqueous extract inhibited not only the protein (albumin) denaturation but also, in LPS-induced inflammatory response, including increased secretion of pro-inflammatory cytokines (IL-6 and IL-1β) and NO were inhibited by aqueous extract in a concentration-dependent manner. Furthermore, *B. buceras* suppressed significantly edema in a dose-dependent fashion in inflamed rat paws; decrease the C-reactive protein, lipid peroxidation levels (OT) and oxidation protein product and exerted strong reducing antioxidant power. Thus, these results suggest that antioxidative properties of *B. buceras* attenuate inflammatory changes and support the application in complementary and alternative medicine.

Key words: Anti-inflammatory, macrophages, paw edema, nitric oxide, cytokines, oxidative stress biomarkers, C-reactive protein, anti-denaturation, *Bucida buceras*.

INTRODUCTION

Different cells and molecules form the immune system to distinguish and eliminate foreign agents (e.g. infections, toxins, allergens, burns, radiation or physical and chemical injuries) to protect the body. Although

inflammation is a first host immune response and a complex biological mechanism of the body to cell damage and vascularized tissue, inflammation that is uncontrolled and chronic becomes detrimental to tissues (Mueller et al., 2010; Pallarès et al., 2013; Yeh et al., 2014). During an inflammatory processes, some pro-inflammatory mediators are produced, among them interleukin (IL, tumor necrosis factor (TNF), interferon (INF-γ), pro-inflammatory enzymes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Mueller et al., 2010).

Dysregulated inflammation is a common mechanism leading to symptoms or pathology of many illnesses such as cancer, atherosclerosis, cardiovascular and pulmonary disorders, diabetes, allergies, asthma, Alzheimer's disease, osteoporosis, arthritis, periodontal disease and many forms of autoimmunity. Pathological inflammation is treated with small molecule drugs but this strategy only deletes the main symptoms of various illnesses. However anti-inflammatory drugs rarely cure these processes and long-term treatments are often limited by drug cost and toxicity. Many phytochemicals widely distributed in human diet and medicinal plants have anti-inflammatory properties (Arts and Hollman, 2005; Haines et al., 2010; Pallarès et al., 2013; Yeh et al., 2014).

Phytochemicals have been shown to inhibit pro-inflammatory signaling mechanisms such as redox reactions and the nuclear factor-kappaB (NF-κB) pathway, offering promise for the management of pathological inflammation. Several anti-inflammatory drugs have an antioxidant and/or radical scavenging activity. The mechanism of inflammation injury is attributed, in part, to oxidative stress induced by production of reactive oxygen species (ROS) from activated neutrophils and macrophages. This augmented production provoke to tissue injury by damaging macromolecules and lipid peroxidation of membranes (Pallarès et al., 2013; Lewis et al., 2015). Besides, ROS extend inflammation by promoting release of cytokines such as IL-1, TNF-α and INF-γ, which stimulate additional production of free radicals. As a result ROS are important mediators that lead or maintain inflammatory processes and, consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation (McCarty, 2004, 2011; Haines et al., 2010; Bian et al., 2012, 2013; Yeh et al., 2014).

Bucida buceras L. belongs to the family *Combretaceae* is native to Yucatan peninsula and along the coast of Mexico, Central America and northern South America to the Greater and Lesser Antilles. Plant amply cultivate for shade and ornamental use. Several scientific investigations about pharmacological active substances of this plant were found. *B. buceras* has demonstrated antimicrobial activity against different pathogens and cyto-toxicity against various human tumor cell lines (Mahlo et al., 2010, 2013).

Very recently, we have discovered antioxidant and oxygen free radical scavenging effects of crude extracts from *B. buceras* using different radical generating systems (superoxide, nitric oxide and diphenyl-picrylhydrazyl (DPPH) radical). The anti-inflammatory activity of many plants is related to the antioxidant property. Taking into account the former finding, we decided to evaluate the potential anti-inflammatory activity of *B. buceras* aqueous extract *in vitro* LPS-stimulated macrophages RAW 264 cell culture and *in vivo* model carrageenan-induced rat paw edema.

MATERIALS AND METHODS

Preparation of aqueous extract

B. buceras leaves collected in Hermosillo, Sonora, Mexico were dried and ground mechanically into fine powder. The powdered plant materials (50 g) were extracted with distilled water (350 mL) by boiling for 30 min. The solutions were filtered and the solvent was removed under vacuum at 40°C using a rotary evaporator. The dry aqueous extract was kept in a vacuum desiccator until use. Stock solution of 100 mg/mL (w/v) was obtained by dissolving aqueous extract in DMSO and was kept at -20°C until use.

Anti-denaturation activity

The reaction mixture (5 mL) consisted of 0.2 ml of egg albumin from fresh hen's egg (OVA), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations (0.76, 1.56, 3.12, 6.25, 12.5 µg/mL) of the test *B. buceras*. Similar volume of double-distilled water served as control reported by Dey et al. (2011). Thereafter the mixtures were incubated at 37±2°C in an incubator (Binder) for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm (Thermo Scientific Multiskan Spectrum). Acetylsalicylic acid (15 µg/mL) was used as reference drug. The percentage inhibition of protein denaturation was calculated by using the following formula:

% Inhibition of denaturation = (Abs of control - Abs of extract) / Abs of control

The extract concentration for 50% inhibition (IC_{50}) was determined form dose response curve by plotting percentage inhibition with respect to control against treatment concentration.

Propagation of the RAW 264.7 cell line

RAW 264.7 macrophage cells (ATCC) were maintained in Dulbecco´s modified eagle´s medium (DMEM from Sigma Aldrich) supplemented with 5% fetal bovine serum (FBS from Sigma

*Corresponding author. E-mail: lidianyslewis@rubiopharma.com, lidianys1@yahoo.es.

Aldrich), 2 mM L-glutamine (Sigma Aldrich) and 100 units mL^{-1} penicillin/streptomycin antibiotic mixture (Sigma Aldrich). Cells were incubated at 37°C in humidified air containing 5% CO_2. The cells were adherent and they reached confluence within 72 h. Confluent flasks were scraped with cell scraper.

Assessment of the viability of the cell line

The viability of the RAW 264.7 cell line after propagation was determined using the trypan blue exclusion technique. For this, cells were harvested using a cell scraper and they were centrifuged at 1800 rpm/10 min (Sigma) to a plug the resuspended in DMEM medium. Five microliters (5 µL) of the cell suspension was then mixed with forty-five microliters (45 µL) of trypan blue (5%). The total number of cell was counted using a hemocytometer and those that were stained dark blue (indicating dead cells) were identified for determination of viability.

Determination of anti-inflammatory activity

To examine the effect of aqueous *B. buceras* extract on inflammation, we use macrophages stimulated with lipopolysaccharide (LPS: Escherichia coli, serotype 011:B4). RAW 264.7 cells were seeded at a density of 5×10^4 cell per well in 12 well plates and incubated for 24 h at 37°C. On the following day LPS and interferon-γ (INF-γ) were added at final concentration of 1 µg/mL and 0.004 µg/mL, respectively. At the moment, test substances (*B. buceras*) in < 0.1% DMSO solution in DMEM were added to final concentrations of 50, 100, 200, 400 µg/mL. The cells were then incubated for a further 24 h at 37°C. On the third day (48 h), the media was removed and centrifuged at 1800 rpm to remove cells; the supernatant was aliquoted and use in immunoassay for IL-6, IL-1β and TNF-α and nitrite oxide (NO) production. Cells, which were not treated with LPS/ INF-γ, served as a negative control and cells incubated with DMSO and LPS/ INF-γ served as control, dexamethasone at 40 µg/mL served as positive control.

Measurement of cytokine concentrations

The production of IL-6, IL-1β and TNF-α in 100 µL of cell supernatant each were determinate by a commercially available ELISA kit assay according to the manufacturer´s protocol (R&D Systems). All incubation steps were performed at room temperature. The optical density at 450 nm was measured with microplate spectrophotometer reader Thermo Scientific. Standard curves were calculated within each ELISA assay in order to determine the concentration of the secreted cytokines.

Colorimetric assay for nitric oxide (NO) production

Nitric oxide amounts were measured in the cell culture supernatants obtained after activation with LPS/ INF-γ in the absence and presence of extract and plasma of rats carrageenan-induced. NO production was measured using Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide (in 60% of acetic acid) and 0.1% naphthylethylenediamine dihydrochloridein], and the optical density was measured using microplate spectrophotometer reader Thermo Scientific at 550 nm. A standard curve of sodium nitrite (µg/mL) was used to find the concentrations of nitrites produced (Green, 1982).

MTT assay

The colorimetric assay using methyl thiazollyl tetrazolium (MTT) was performed simultaneous with the ELISA assay. The viability of LPS/ INF-γ-stimulated cells after treatment with *B. buceras* extract was based on the mitochondrial-dependent reduction of MTT to formazan. After removing the supernatant for ELISA analysis, 10 µL of MTT solution (5 mg/mL) were added to the cells, and the cells were incubated for 4 h at 37°C. Formed formazan crystals were dissolved with 0.05 N HCl in isopropanol (100 µL), and the absorbance was read at 570 nm on a microplate reader (Thermo Scientific Multiskan Spectrum), using a reference wavelength of 655 nm (Mosmann, 1983).

Percent viability = OD of *B. buceras* extract treated sample/OD of LPS/ INF-γ-stimulated cells x 100, where OD is optical density.

Animals

A total of 45 adult male Sprage-Dawley rats (125-200) g were obtained from Laboratory Animals Unit, University of Sonora, Mexico. Animals were housed in controlled conditions of temperature (22 ± 2°C), humidity (40 ± 2%) and light (12 h. light/dark cycle) and allowed free access to diet LabDiet chow and water *ad libitum*. Experiments were performed during the light phases of the cycle. The Animal Ethics Committee of the University of Sonora, Mexico, approved all of the experimental protocols used (No. 853).

For the anti-inflammatory activity and oxidative stress indicators animals were divided into six groups with five animals in each group: Group 1 (extract 1) received dose of 250 mg/kg of body weight, p.i, of *B. buceras*; Group 2 (extract 2) dose of 125 mg/kg, p.i; Group 3 (extract 3) dose of 62.5 mg/kg, p.i, while Group 4 (Carrageenan control) at 1%; Group 5 (PBS control) and Group 6 (standard drug) received the nonsteroidal anti-inflammatory drug (NSAID) Nimesulide at dose of 4 mg/kg, p.i.

Carrageenan-induced paw edema in rats

Paw edema induced by subcutaneous injection of λ-carrageenan into the right hind paw of rats, as was described previously by Hussein et al. (2012). The anti-inflammatory effect of aqueous *B. buceras* extract was evaluated by decrease in percentage of inhibition of the paw thickness. In this study, six groups (n=5 rats for each groups) were employed. Three groups were pretreated with different doses 62.5, 125 and 250 mg/kg, p.i. of the *Bucida* extract for 3 days consecutively. The negative control (not induced with inflammation by carrageenan) received an equivalent volume of vehicle (Buffer phosphate saline-PBS), and the positive control group received the drug nimesulide-suspension (UL-Flam-ULTRA) at dose of 4 mg/kg, p.i.

After pretreatment with *B. buceras* aqueous extract, vehicle or nimesulide, the rats were injected subcutaneously with (0.1 mL/paw) 1% carrageenan in PBS. After the carrageenan injection, the paw thickness was measured at several time points (0, 3, 5 24 h) using a Dial Caliper (0-150 mm/0.02 mm). The paw thickness was determined at 0 h (C_0): left paw thickness without Carrageenan injection and 3, 5, 24 h after Carrageenan injection (C_t). Measure of paw edema was expressed as the mean paw thickness ± S.D. The percentages of inhibition compared to negative controls (Carrageenan-inflammation) were calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{(C_t - C_o)_{(\text{Carrageenan})} - (C_t - C_o)_{\text{Treated group}}}{(C_t - C_o)_{(\text{Carrageenan})}} \times 100$$

Preparation of blood serum samples

Five hours after carrageenan injection, the rats were anesthetized with sodium Pentobarbital 40 mg/kg p.i and the blood were collected by cardiac puncture in heparinized tubes. The blood was centrifuged at 1800 rpm for 10 min (4°C), the plasma was aliquoted and store at -20°C until use.

Qualitative determination of C-Reactive protein (CRP)

At 50 µL of samples to be tested were added 50 µL of the CRP-latex reagent. The mixture was extended over the entire surface of the circle with a stirrer on a mechanical rotator 80-100 r.p.m for 2 min (PCR-Turbilatex, SPINREACT). The presence or absence of visible agglutination immediately after removing the slide from the rotator is defined as a positive result. Positive and negative controls are used as a comparative pattern for a better result interpretation.

Measurement of biochemical test to evaluate oxidative stress

Organic peroxide and hydrogen peroxide concentration was measured using oxidation of ferrous ion to ferric ions by hydroperoxides under acid conditions. The ferric ions binds with the indicator dye xylenol orange (3,3´-bis (N,N-di(carboxymethyl)-aminomethyl)-0-cresolsulfone-phatein, sodium salt) to form a stable coloured complex, which can be measured at 560 nm (Gil et al., 2003). Briefly, at 50 µL of serum were added 500 µL of reagent solution [100:1 mixture (v/v) of 125 µM xylenol orange and 25 mM ferrous ammonium sulfate (in 20% of sulfuric acid) and incubated for 30 minutes to room temperature. The total organoperoxide were determined by comparisons to the hydrogen peroxide standard curve, and the results were expressed as (µM).

Advanced oxidized protein product (AOPP) concentration was determined in serum based on spectrophotometric detection in an adapted microassay system using chloramine-T as a standard and measure optical density at 340 nm. The results were expressed as µM chloramine T equivalents (Witko-Sarsat et al., 1998).

The total antioxidant was estimated in serum using the Ferric Reducing Antioxidant Power (FRAP) microassay based on the reduction of a ferric-2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) (Benzie and Strain, 1996). The FRAP reagent was prepared in acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily. 5 µL of samples test diluted with 20 µL of distilled water were added to 150 µL of FRAP reagent. The absorbance of the mixture was measured using microplate spectrophotometer reader Thermo Scientific at 595 nm after 4 min. The standard curve was prepared by iron (II) sulfate solution, and the results were expressed as µM Fe (II).

Statistical analysis

Experimental results are expressed as the means ± SD from experiments carried out in at five experiments independent in triplicate. A multifactorial analysis of the variance (ANOVA), statistical significance and Pearson's correlation coefficient were performed in NCSS, 2007. P values <0.05 were regarded as significant by Tukey multiple-range test. Graphs were generated using Origin 8 software and the error bars represent the SD.

RESULTS

In vitro anti-denaturation of oval-albumin (OVA) was used to evaluate the anti-inflammatory effect of *B. buceras* in this research. The result shown a concentration dependent inhibition of protein (albumin) denaturation by the aqueous extract (0.76 to 12.5 µg/mL), an IC_{50} value of 3.92 µg/mL for reduction of albumin denaturation was found (Figure 1). Acetylsalicylic acid at the concentration of 15 µg/mL used as positive control (drug) showed inhibition of protein denaturation, however, the effect of acetylsalicylic acid was less effective concentration compared with *B. buceras*.

The effect of treatment with various concentrations of *B. buceras* (50-400 µg/mL) on lipopolysaccharide-induced nitric oxide production was studied in RAW 264.7 macrophages. After incubation, macrophages without LPS produced low level of nitrite in medium (Figure 2). After stimulation with LPS for 24 and 48 h, nitrite concentration in medium increased remarkable, reaching values of 1.05 ± 0.20 and 2.86 ± 0.47 (µg/mL) respectively. Nitric oxide levels can increase rapidly within minutes to hours in response to a potent exogenous inducer (LPS) of the inducible nitric oxide synthase (iNOS) during inflammatory stimuli as well as by endogenous inducers such as cytokines TNF-α and IL-1β.

The cell viability in the presence of 50 - 400 µg/mL of extract and/or LPS+INF-γ for 24 and 48 h was evaluated by MTT assay (Figure 3 inset). *B. buceras* at the concentrations of ≤ 400 µg/mL did not influence the cell viability and the cytotoxic effect (≈ 20%) was at level of LPS. When the cells were treated with different concentration (50-400 µg/mL) of *B. buceras* significantly inhibited the nitrite production a dose-dependently in both times treatment (24 and 48 h), but the inhibition effect was greater at 24 h. The IC_{50} values determined for the reduction of NO production were 222.76 and 298.61 µg/mL at 24 and 48 h, respectively. The IC_{50} values of the *B. buceras* within the nontoxic concentration range suggest that the inhibition action seems to be mediated interaction of extract with NO and not cytotoxic property of extract.

As a positive control, cells were incubated with the steroidal anti-inflammatory drug dexamethasone. A slight reduction of NO level was observed when incubating cells with 40 µg/mL of drug despite dexamethasone inhibit the expression of iNOS. Notably *B. buceras* at 400 µg/mL significantly reduced the NO content by more efficient than Dexamethasone drug.

Since the results indicated that test extract inhibited NO production we also evaluated the cytokine levels in response to treatment with *B. buceras* (Figure 4). In addition to NO, proinflammatory cytokines are important mediators of inflammatory responses. This study indicates that after LPS and/or LPS+ IFN-γ stimulus, TNF-α increased significantly relative to the basal control levels and this raise was more marked at 48 h. On the other hand, TNF-α in LPS-stimulated cells and treated with *B. buceras* extract at 24 h not reveled inhibition but

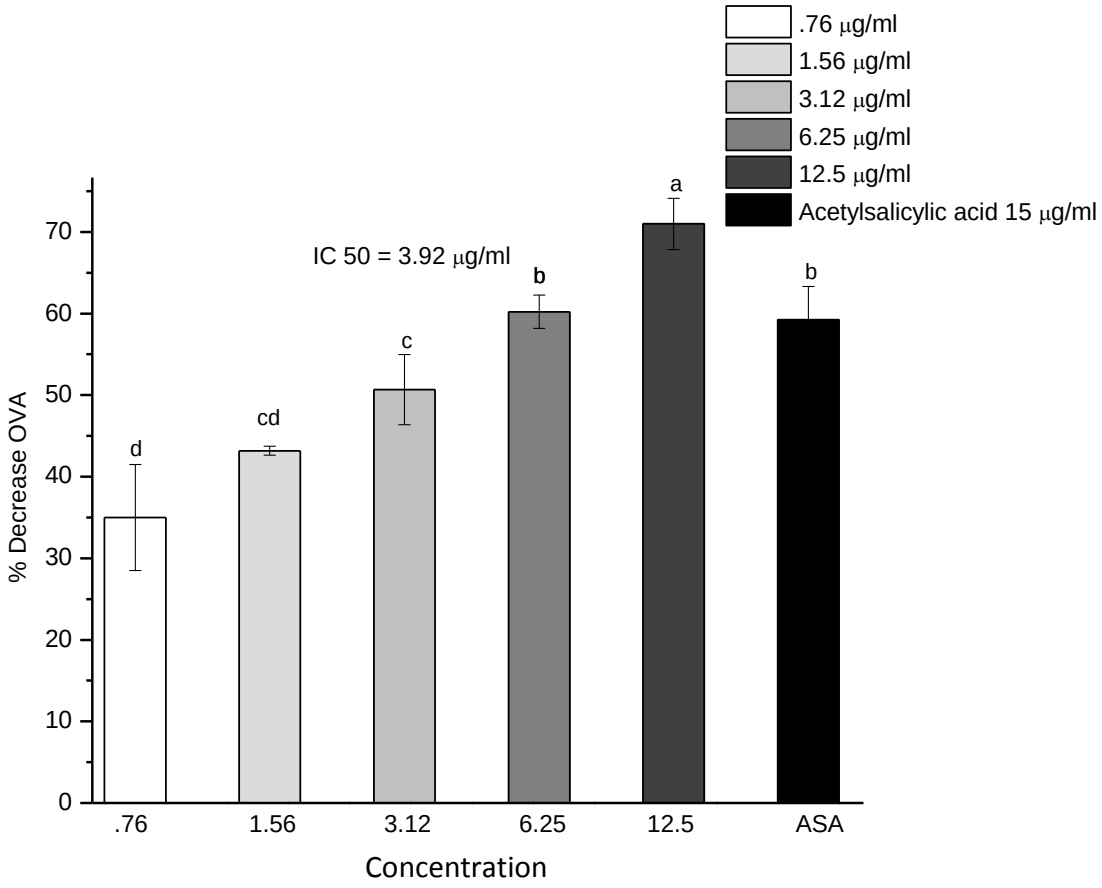

Figure 1. Effect of *Bucida buceras* on inhibition of ovalbumin denaturation (OVA). *Bucida buceras* (0.76 to 12.5 µg/mL Acetylsalicylic acid (ASA-15 µg/mL) was used as drug. Data are presented as mean ± S.D (n=5). Different letters indicate significant differences ($p<0.05$).

surprisingly increased in a concentration-dependent manner compared with the LPS control cells at 48 h. Dexamethasone drug control (40 µg/mL) did not inhibit TNF-α level.

The treatment of RAW 264.7 cells with endotoxin LPS caused a significant increase in the production of Interleukin 6 (IL-6) at 24 and 48 h reaching values of 477.50 and 1735.77 pg/mL respectively. IL-6 production was significantly and dose-dependently reduced by *B. buceras* at 24 h. An IC_{50} (242.95 µg/mL) could only be determined for the reduction of IL-6 production at 24 h. Nevertheless, as observed in Figure 5 *B. buceras* in 400 µg/mL did not show complete inhibition of IL-6 at 48 h compared to inhibition (>50%) in concentrations 50-200 µg/mL.

The mouse macrophage-like cell line Raw 264.7 was used for investigated the influence of LPS and LPS+ IFN-γ stimulus and inhibiting effect of *B. buceras* on IL-1β production. When macrophages were stimulated with the combination of LPS (1 µg/mL) and INF-γ (4 ng/mL), IL-1β level was similar to LPS alone at 24 and 48 h. In the presence of *B. buceras* concentration of IL-1β was decreased in dose-dependent manner at 48 h compared with that of LPS-treated RAW 264.7 cells. The incubation of stimulated macrophages with 200 µg/mL of *Bucida* extract markedly reduces the IL-1β production by almost 50% at 48 h similar to dexamethasone drug.

Carrageenan-induced acute paw edema in rats

The anti-inflammation activity of *B. buceras* in acute-phase inflammation (carrageenan edema) was conducted. The time-dependent curve shows that the paw size increased till 5 h (7.00 ±0.82 mm) after carrageenan injection and remained elevated thereafter 24 h (Figure 6). In control group rats, which received injection of PBS induced in paw thickness of 3.69 ± 0.24 mm over 5 h (data not shown).

Pretreatment with *B. buceras* aqueous extract (62.5, 125 and 250 mg/kg, p.i.) for 3-day and 5h after carrageenan significantly ($P < 0.05$) inhibited the paw edema in a dose-dependent manner, as shown in Figure 7. *B. buceras* treated at 250 mg/kg resulted in a

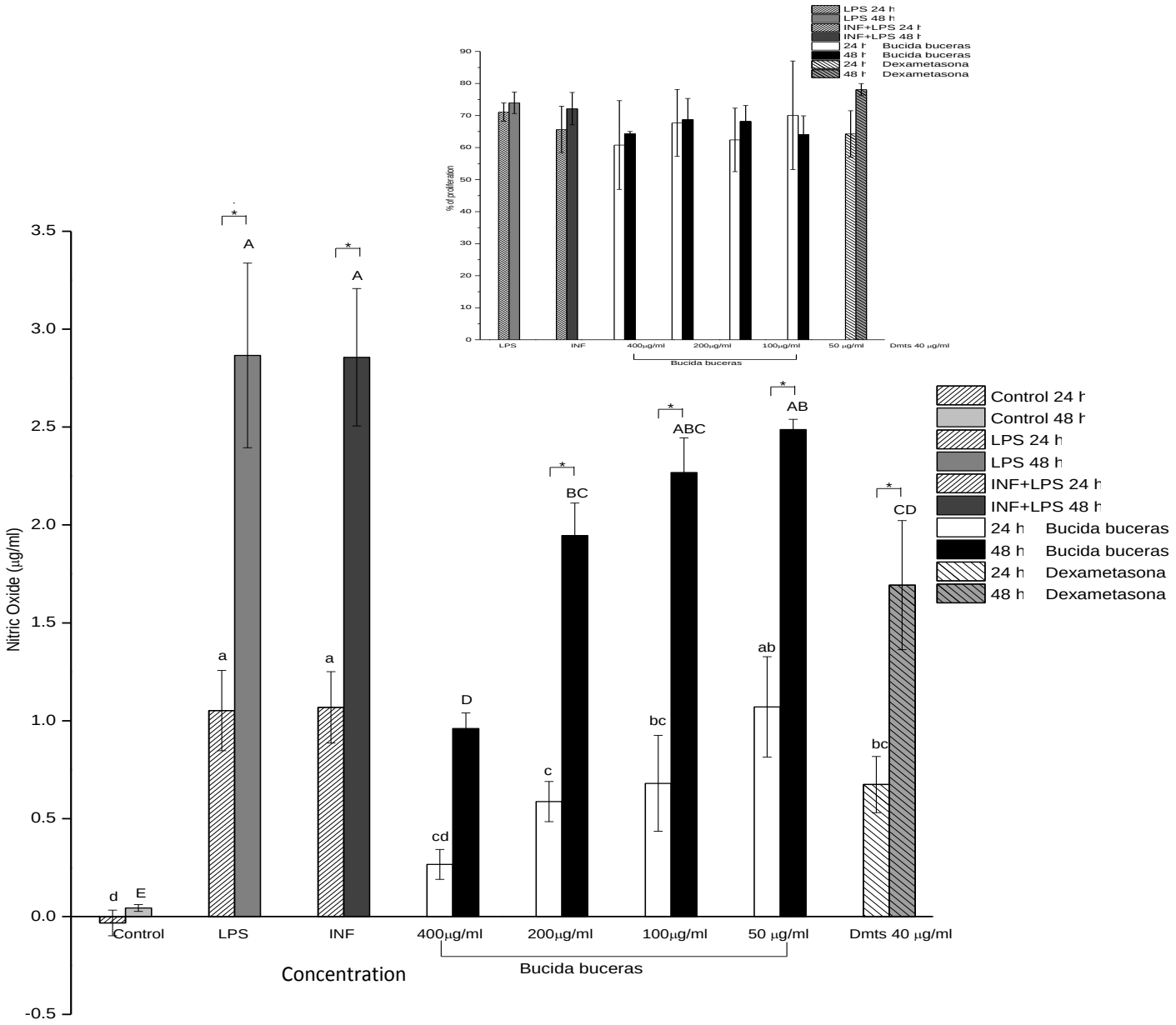

Figure 2. Influence of *Bucida buceras* on NO production by RAW 264.7 macrophages exposed to LPS at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (0.1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone drug (40 µg/mL). NO production was measured by the Griess reaction assay. The inset shows the cytotoxic effects of LPS, LPS/ IFN-γ, *B. buceras* extracts and dexamethasone drug control (40 µg/mL). Cell viability was measured by MTT assay. Data are presented as the mean ± S.D (n=5). Different lower case and capital letters indicate significant differences ($p<0.05$) at 24 and 48 h respectively. * Asterisk indicates significant differences ($P< 0.05$) between 24 and 48 h.

spectacular significative ($P< 0.05$) inhibition of the edema (91.77±0.91 %) when compared to the standard drug nimesulide (51.63±7.21 %). Nimesulide (4 mg/kg, p.i.) also inhibited the increase in paw thickness but much less than *Bucida*-250 mg/kg. The effects of 62.5 and 125 mg/kg of *B. buceras* were almost similar (were not significantly different) to the NSAID Nimesulide with percentages of inhibition of 40.82±10.11 and 49.35±11.35% respectively. Total inhibition of inflammation processes induced with carrageenan was found in *B. buceras* at 250 mg/kg.

The inhibitory activity of *B. buceras* in rats was examined by measuring the C-reactive protein (CRP), nitric oxide and oxidative stress biomarkers. After post-carrageenan injection was produced an increase in CRP production (80% positive result) compared with normal control (0% positive result). Increase amount of CRP is nonspecific but sensitive markers of the acute

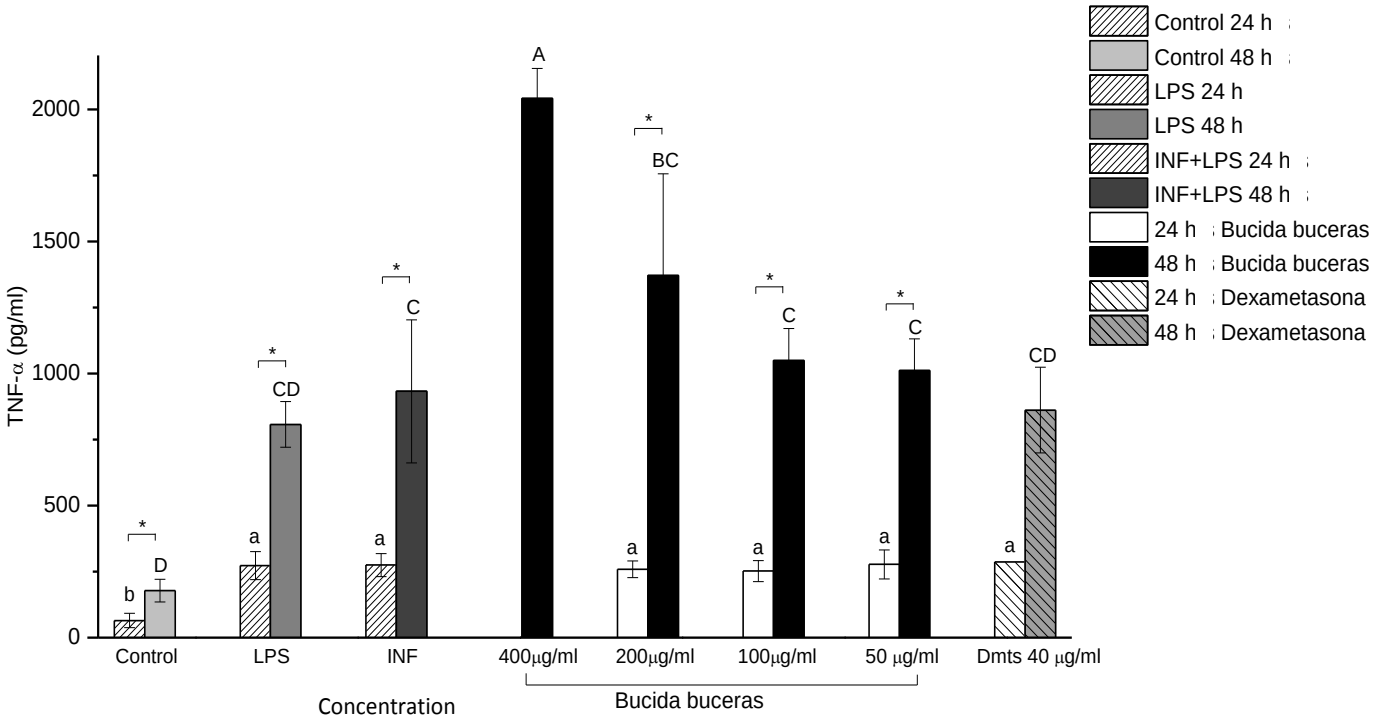

Figure 3. Effect of *Bucida buceras* on tumor necrosis factor (TNF-α) by RAW 264.7 macrophages exposed to LPS and LPS+INF-γ at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone drug (40 µg/mL). Data are presented as the mean ± S.D (n=5). Different lower case and capital letters indicate significant differences ($p<0.05$) at 24 and 48 h respectively. * Asterisk indicates significant differences ($P< 0.05$) between 24 and 48 h.

inflammatory response. *B. buceras* extract decrease the percentages of positive result of CRP dose-dependently (Table 1). Pretreatment of 250 mg/kg of *B. buceras* has a greater effect on reducing CRP production. This result corroborates the almost completely suppressing the edema by carrageenan for this dose of aqueous extract.

Effect of *Bucida buceras* on nitric oxide production in carrageenan induce paw edema

The carrageenan treatment increased significantly the production of NO serum compared with those treated with PBS control, measure as nitrate over 5 h in our results (Figure 8). In contrast NO production was significantly and dose-dependently reduced by *B. buceras* respect to the carrageenan-induced paw edema. The Nimesulide drug suppressed this production almost completely (39.5% respect to carrageenan) similar to control while *B. buceras* at dose of 250 mg/kg reached an inhibition of 24.44%, being less effective than nimesulide.

In parallel with the anti-inflammatory assays, we performed biochemical markers to evaluate oxidative stress given that oxygen radical generation play an important role in the maintenance of carrageenan paw edema. Rats with carrageenan paw edema had higher serum levels of organic peroxide total (OT) and advanced oxidized protein (AOP) product concentrations than the control group. Lipid hydroperoxide total concentrations in carrageenan group showed almost 3-fold increase compared to the control (Figure 9A), while the level of oxidized serum protein was increased by 6-fold respect to the normal control group (Figure 9B).

Accumulated evidence supports the crucial role of the reactive oxygen and nitrogen species during inflammation. Our results revealed a significant increase of OT levels due to the carrageenan intervention in serum and indicate a rise of the lipid peroxidation levels in the inflammation of rats. After *B. buceras* treatment, OT biomarker was effectively reduced in a dose-dependent fashion and dose of 250 mg/kg was significantly recovered comparable to the control group. This result demonstrates that the reducing capacity of the *B. buceras* is protective to lipid peroxidation in rats subjected to oxidative stress by carrageenan-induced. The antiinflammatory response of Nimesulide was less efficient than 125 and 250 mg/kg of extracts, which reached levels of inhibition of 42.7 and 74.6% in relation to carrageenan.

Advanced oxidation protein product is not only a marker of oxidative stress, but also acts as an inflammatory mediator generated by activated neutrophils. *B. buceras*

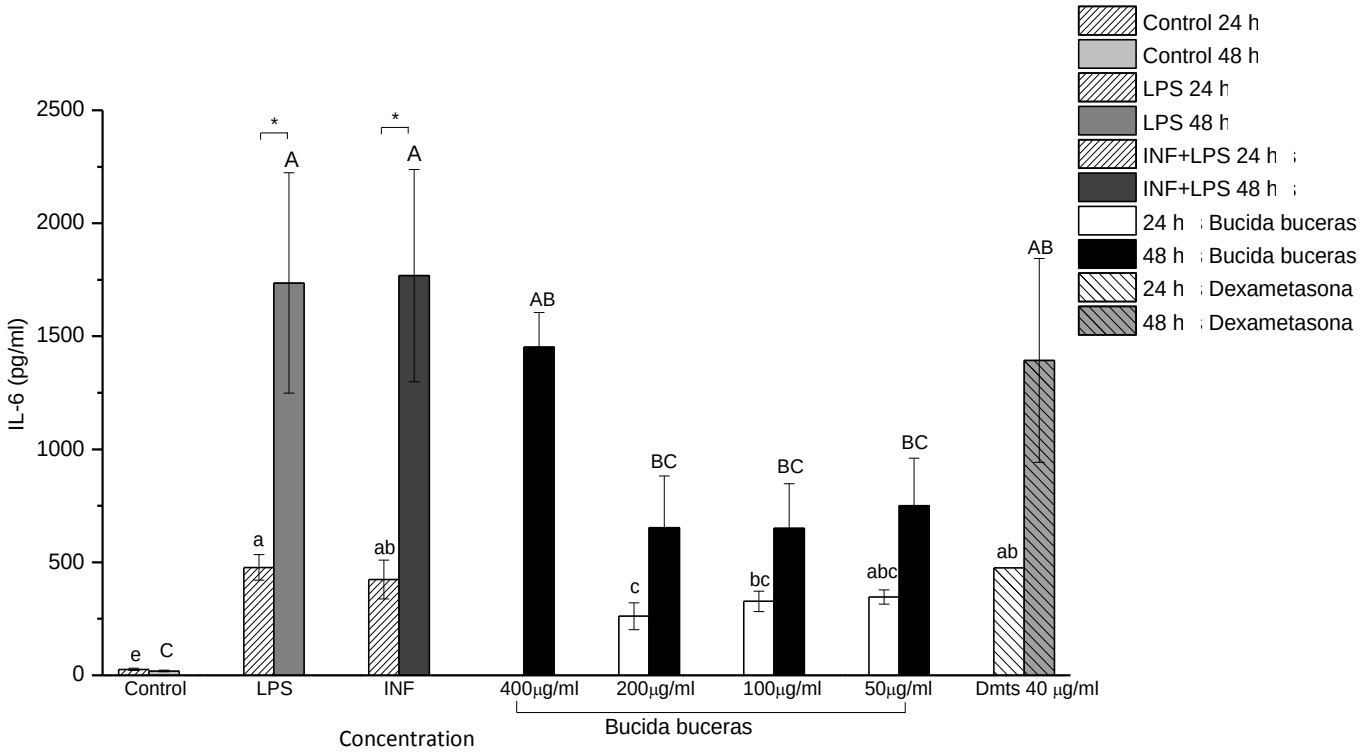

Figure 4. Effect of *Bucida buceras* on interleukin-6 (IL-6) y RAW 264.7 macrophages exposed to LPS and LPS+INF-γ at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone drug (40 µg/mL). Data are presented as the mean ± S.D (n=5). Different lower case and capital letters indicate significant differences ($p<0.05$) at 24 and 48 h respectively. * Asterisk indicates significant differences ($P<0.05$) between 24 and 48 h.

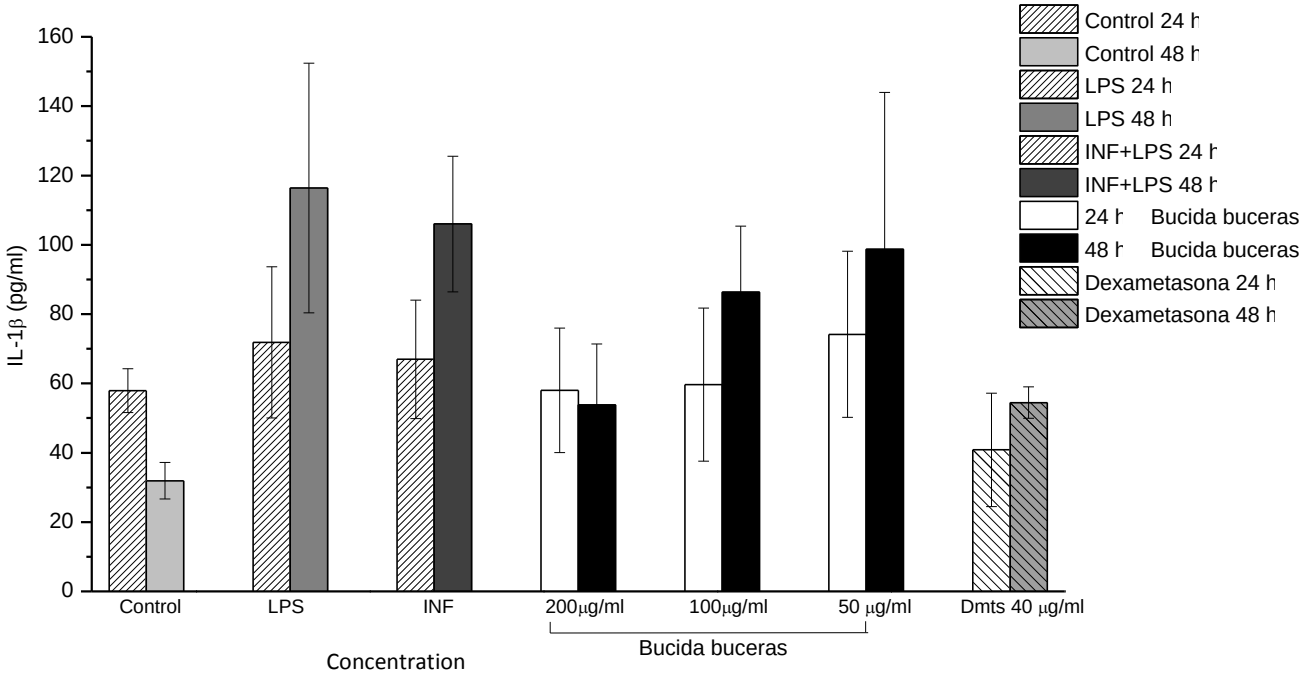

Figure 5. Effect of *Bucida buceras* on interleukin-1β (IL-1β) by RAW 264.7 macrophages exposed to LPS and LPS+INF-γ at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone drug (40 µg/mL). Data are presented as the mean ± S.D (n=5).

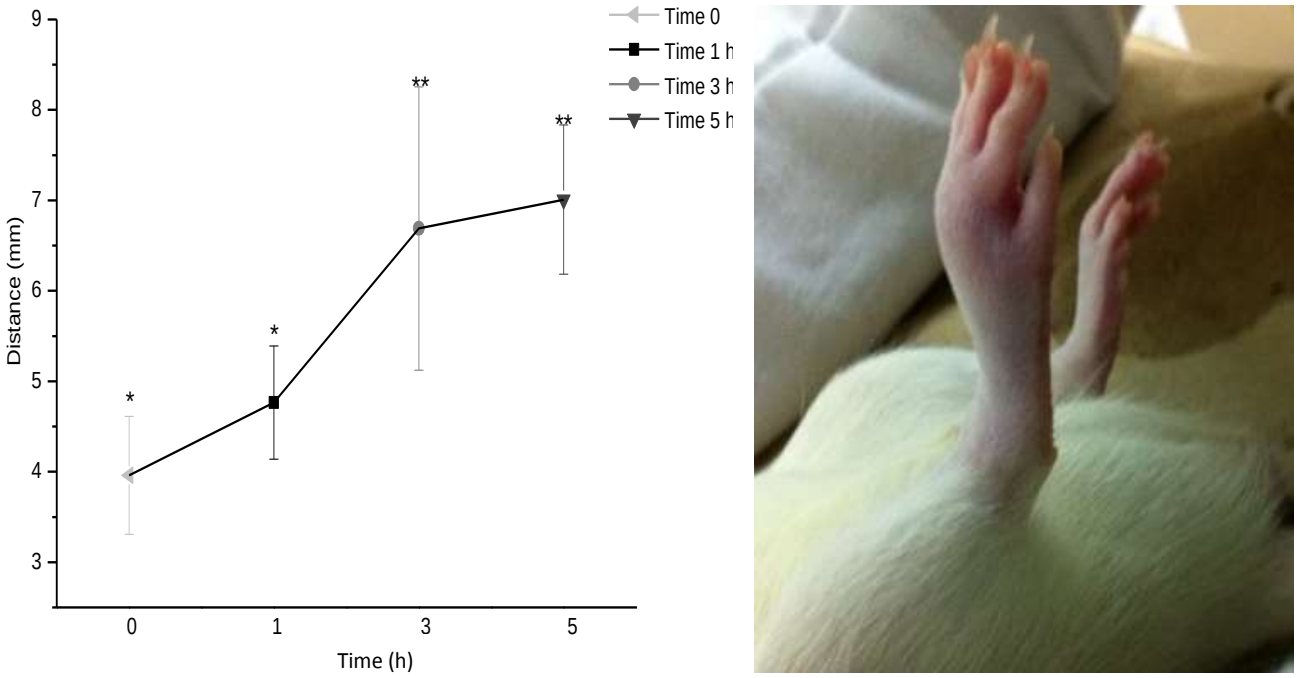

Figure 6. Paw size in acute edema in rats after carrageenan treatment at different time of subplantar injection (0, 1, 3 and 5 h). Paw thickness was measured using a caliper placed at the border of the phalanges and metatarsals. Data are expressed as the mean paw thickness ± S.D. * Asterisks indicate significant differences (P<0.05) at various times.

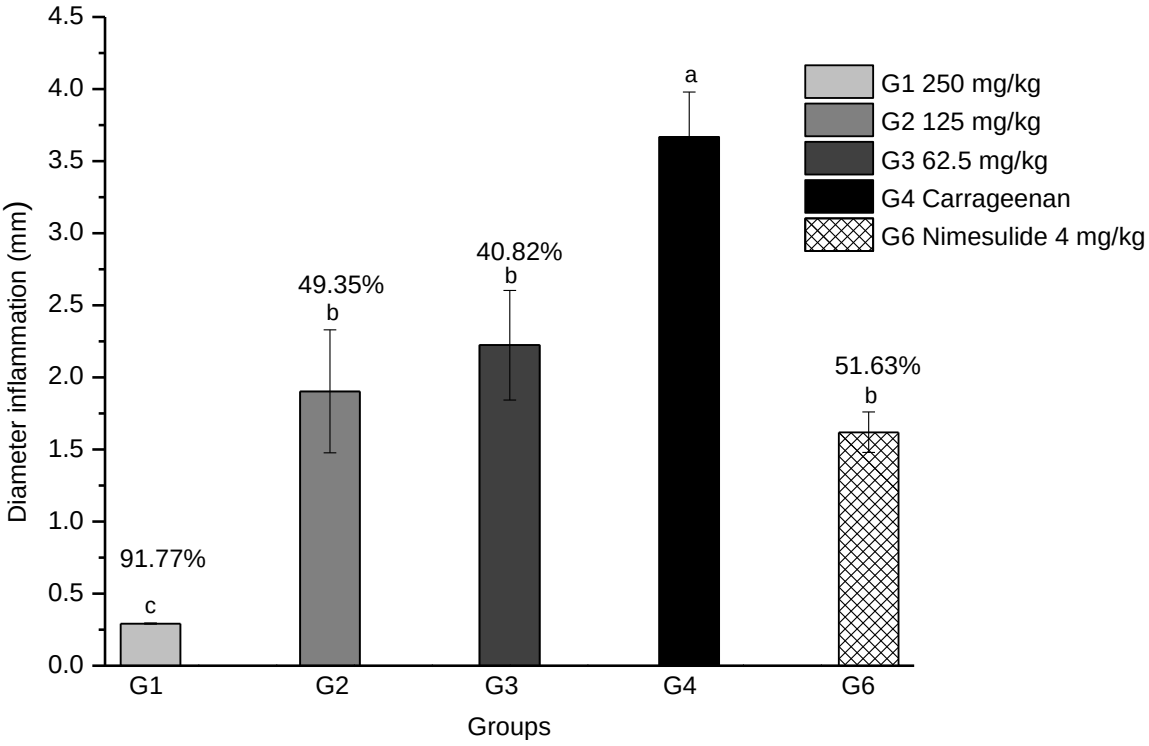

Figure 7. Effect of *Bucida buceras* on the footpad edema post-carrageenan injection in rats. Animals were pretreated with aqueous extract of *B. buceras* (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G7 (Nimesulide-drug control). Data are presented as the mean ± S.D (n=3). Different letters indicate significant differences (p<0.05). Numbers above bars indicate percentage of inhibitions of paw edema in each group compared with carrageenan.

Table 1. Effect of *Bucida buceras* aqueous extract in the C-reactive protein positive result on carrageenan paw edema.

Groups	*Bucida buceras*			Carrageenan	Nimesulide	PBS	Normal control
	250 mg/kg	125 mg/kg	62.5 mg/kg				
CRP (%+)[1]	25	40	60	80	60	20	0

[1]positive result (visible agglutination) in PCR-Turbilatex, SPINREACT.

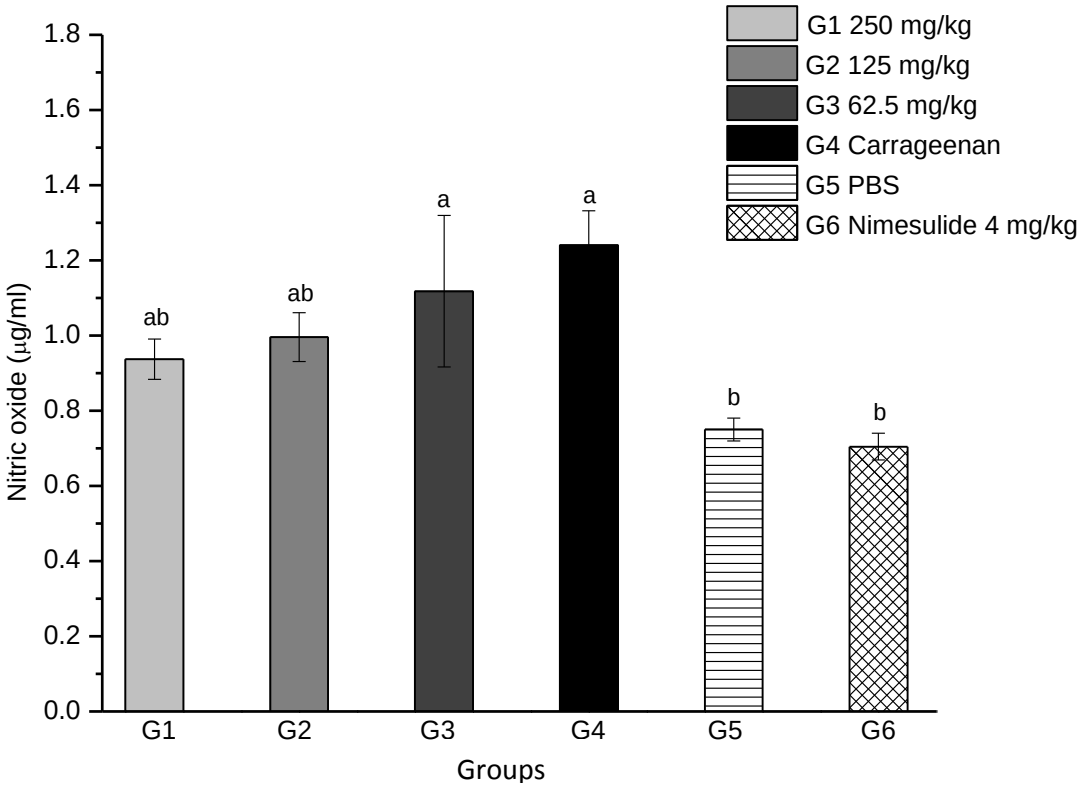

Figure 8. Inhibitory effect of aqueous extract of *Bucida buceras* on nitric oxide production in acute inflammation. Rats were pretreated with aqueous extract (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G5 PBS-control, G6 (Nimesulide-drug control). Data are presented as mean ± S.D (n=3). Different letters indicate significant differences ($p<0.05$) in different groups.

a slight reduction in oxidation protein and reached 17.8% of reduction at dose of 250 mg/kg respect to Carrageenan control (Figure 9B). These values did not differ significantly from that of the carrageenan group ($P>0.05$). The suppressed effect of NSAID nimesulide drug was very similar with that found for the extract.

On the other hands, the ferric reducing antioxidant power (FRAP) in *B. buceras* in 250 mg/kg group was significantly higher than low doses of extracts, as well as in the controls (Figure 10). FRAP concentrations in *Bucida* extracts groups showed 2-3.5-fold higher than the controls groups. In view of the fact that pretreatment with aqueous extract was able to reduce the paw thickness (inflammation) in relation to FRAP level. The present study revealed an irreversible highly significant correlation between paw thickness and FRAP ($r=-0.909$, $P<0.001$).

As expected, FRAP levels were significantly increased in animals pretreatment with *B. buceras*, correlations were observed between FRAP vs. OT ($r=-0.786$, $P<0.001$) and FRAP vs. NO ($r=-0.536$, $P<0.05$) but no correlation was observed between FRAP and AOPP ($r=-0.422$, $P>0.05$).

DISCUSSION

B. buceras is able to inhibit the denaturation of protein *in vitro.* Denaturation of tissue proteins is one of the well-

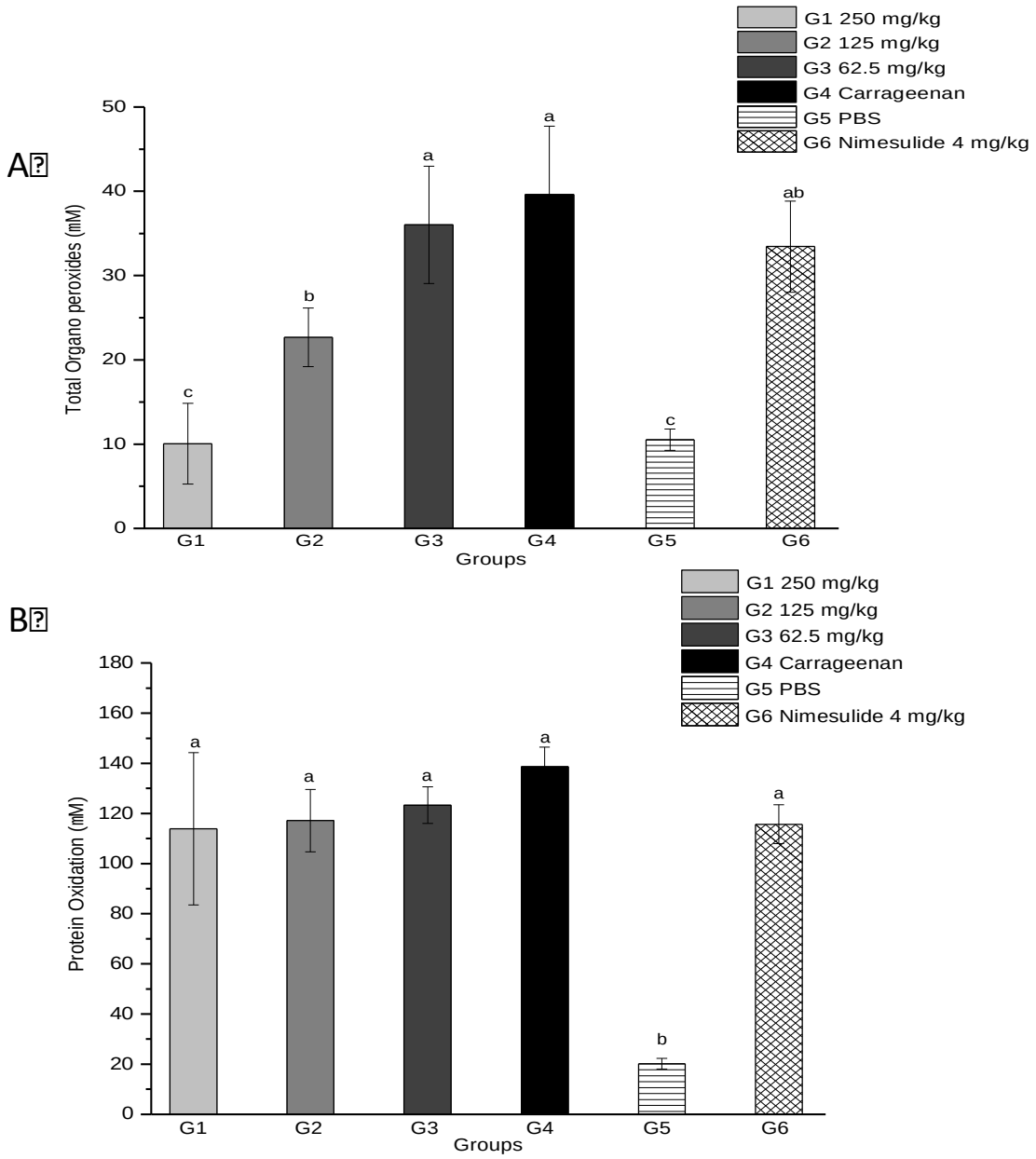

Figure 9. Inhibitory effects of aqueous extract of *Bucida buceras* on total organoperoxid lipid (A) and oxidation protein (B) production in acute inflammation. Rats were pretreated with aqueous extract (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G5 PBS-control, G6 (Nimesulide-drug control). Data are presented as the mean±S.D (n=3). Different letters indicate significant differences ($p<0.05$) in different groups.

documented causes of inflammatory and arthritic diseases. Production of auto-antigens in certain arthritic diseases may be due to denaturation of tissue proteins in vivo (Duganath et al., 2010; Dey et al., 2011; Tatti et al., 2012). In addition in mice, asthmatic were ameliorated by treatment with licorice extract via inhibition of ovalbumin-induced immediate airway constriction (Ram et al., 2006). Agents that can prevent protein denaturation therefore would be worthwhile for anti-inflammatory drug development.

In this study, LPS-stimulated macrophages as an acute inflammation model for used testing *B. buceras* extract for anti-inflammatory activity and modulates oxidative stress markers. Increased levels of NO, TNF, IL-1β and IL-6 from macrophages is a major feature of the pathophysiological manifestation of the inflammatory processes involved various diseases (Mueller et al., 2010; Haines et al., 2010; Pallarès et al., 2013). In the

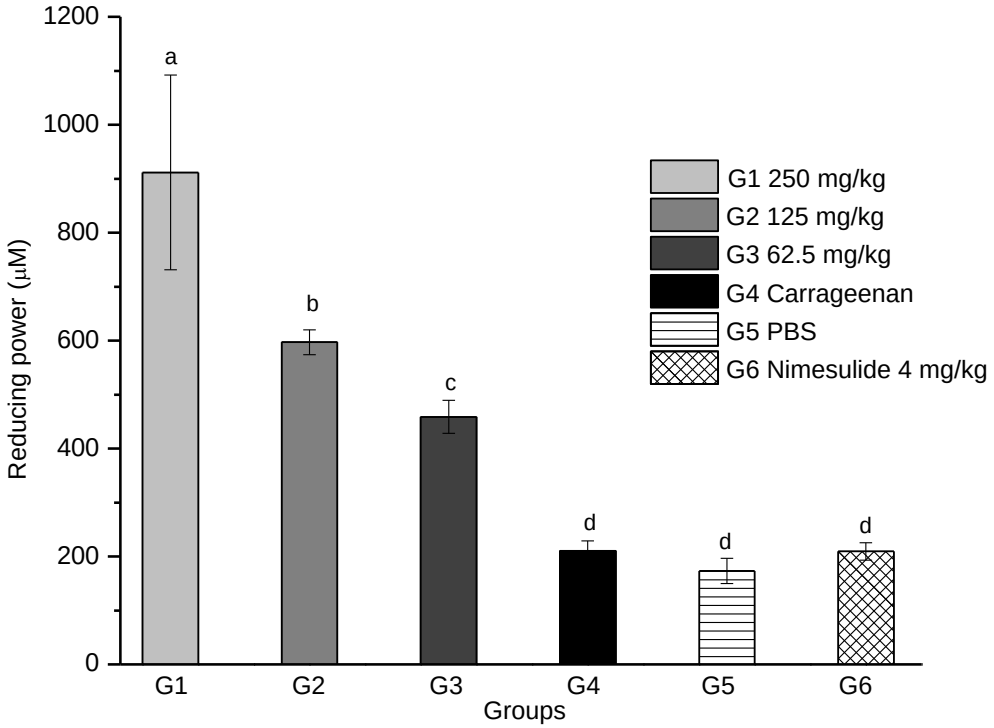

Figure 10. Effect of *Bucida buceras* on ferric reducing antioxidant power in carrageenan-induced paw edema in rats. Rats were pretreated with aqueous extract of *B. buceras* (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G7 (Nimesulide-drug control). Data are presented as the mean ± S.D (n=3). Different letters indicate significant differences ($p<0.05$) in different groups.

interaction with macrophages, LPS induces a variety of intracellular signaling cascades leading to the release of mediators pro-inflammatory (Salvemini et al., 1993). The amount of nitrite in medium of the cells stimulated with LPS+INF-γ was maintained at level similar to the LPS alone-stimulated samples. The presence of INF-γ did not lead to further iNOS activity, which can be explained by a finding from Xie et al. (1993), who found synergism of LPS and INF-γ in iNOS induction using LPS, concentrations of 1 up to 100 ng/ml. However, at 1 µg/mL of LPS, costimulation with 50 U/ml INF-γ did not result in a significant increase in iNOS promoter activity (Obermeier et al., 1999).

The ability of *Bucida* extract to enhance TNF-α production was not correlated with the reduction in NO level in the same concentrations. A possible regulatory role of NO on cytokine production by macrophages is partly controversial. Inhibition of NO lead to decreased TNF levels in RAW 264.7 after activation by LPS+ INF-γ (Chien et al., 2008; Levy and Simon, 2009). In contrast, Eigler et al. (1995) reported that TNF-α production was increased after inhibition of iNOS. Similar result was found in pomegranate extract where the TNF-α secretion and the IL-10 secretion increased simultaneously in RAW 264.7 cells stimulated with LPS, and thus it is not clear if it increased or reduced inflammation (Mueller et al., 2010). The results of various studies have shown that NO production and expression of iNOS activity is regulated by several mechanisms, including transcriptional control such as INF-γ, LPS, IL-6 and TNF-α. Although binding to these response elements generally confers positive regulation, negative regulation also has been reported (Austeanaa and Ross, 2001).

Oxidants such as superoxide, hydroxyl radicals, hydrogen peroxide and hypochlorous acid are formed at sites of inflammation, and appear to contribute to the tissue damage in some acute and chronic inflammatory diseases. *B. buceras* has powerful antioxidant properties. It was able to scavenge free radical (DPPH), NO and $O_2^{\cdot}$ radicals (IC_{50}= 7.82, 27.29 (NPS), 163.92 µg/mL respectively (Iloki et al., 2015). In this study would indicate that the inhibition of the paw edema could be due to its antioxidative and oxygen free radical scavenger properties. Edema is one of the fundamental actions of acute inflammation and is an essential parameter to be considered when evaluating compounds with potential anti-inflammatory activity (Hussein et al., 2012).

B. buceras leaves contain varying amount of polyphenols particularly flavonoids. We found that *Bucida* extract contains gallic acid, catechins, aesculetin, rutin and quercetin. The anti-inflammatory effect may be due to synergistic effect rather than a single constituent

Welton et al. (1986) demonstrated that quercetin and kaempferol inhibited cyclooxygenase-2 in rat peritoneal macrophages while catechin weakly inhibits cyclooxygenase-2 but a very high concentration (100 µM) (Noreen et al., 1998). Flavonols such as kaemperol, quercetin, morin and myricetin were found to better lipoxygenase inhibitors than flavones. Quercetin and catechins coupled their inhibitory action on TNF and IL-1β to an enhanced release of the anti-inflammatory cytokine IL-10 (Arts and Hollman, 2005; Santangelo et al., 2007).

Several doses of *Bucida* extract were used to ascertain if their effects could be dose-dependent and to assess which can be the most effective. Considering that the range intake of phenolic compounds in various human populations can be reach 25-1000 mg/day (Palláres et al., 2013), the dose of 62.5 mg/kg used in rats could be considered a low-moderate dose due to it is raw extract.

Changes in acute phase proteins reflect the presence and intensity of inflammation. In this study we found that *B. buceras* decreased the C reactive protein levels in serum in all doses, with the dose of 250 mg/kg reaching maximum activity. The lower dose of extracts decreased the CRP levels, similar to Nimesulide drug (4 mg/kg). IL-1, TNF-α, IL-6 modulates the production of most acute phase proteins in inflammatory reactions. Only, IL-6 stimulates the synthesis of C-reactive protein. *B. buceras* at different concentrations reduced the amount of CRP in serum, and the decrease suggests that *Bucida* extract has an anti-inflammatory activity by inhibiting the secretion of interleukin-6. This fact has great potential for recommending that natural *B. buceras* produce higher activity effect than NSAID nimesulide drug.

Recent papers suggest that AOP is not only a marker of oxidative stress, but also acts as an inflammatory mediator. Advanced oxidation protein products (AOPP) are markers of protein oxidation as a result of the action of free radicals generated by activated neutrophils and involved in inflammation (Alderman et al., 2002). Previous studies have showed that carrageenan-induced inflammation in rats increased the production of AOPP in serum. We have also shown that *B. buceras* slight decreased the levels.

Here we show that pretreatment of *B. buceras* results in an increase in the total antioxidant power. Phenolic compounds are a major contributor of antioxidant activity. Our previous studies in phytochemical analysis of different *Bucida* extracts showed the presence of carotene, triterpenoids, saponnin, phenols (tannin and flavonoids) that possess antioxidant and anti-inflammatory activities. The variability of active compounds present in *Bucida* extract is responsible for the marked increase in the reducing power. Terra et al., 2007 reported that the anti-inflammatory activity of grape seeds correlates positively with its radical activity and its total phenolic content. Other studies have shown that Bucida buceras extract is high in phenolic compounds and radical scavenging activity, and it is highly likely that phenolic compounds in Bucida buceras contribute to the anti-inflammatory activity.

Conclusions

This work demonstrated that *B. buceras* attenuated *in vitro* and *in vivo* anti-inflammatory effect in a LPS-stimulated macrophage and in an acute inflammation models. The mechanisms underlying this protective effect include (1) reduction of protein (albumin) denaturation; (2) decrease in nitric oxide and proinflammatory cytokines, such as IL-6 and IL-1β production; (3) inhibition of C reactive protein levels and the paw edema size; (4) diminution of lipid peroxidation and orgonoperoxide content and (5) elevation in reducing antioxidant power. Experimental findings support the potential use of *B. buceras* as a therapeutic agent for treating inflammatory diseases and support the application in complementary and alternative medicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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