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ARTICLES

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Full Length Research Paper

Ethnobotanical study of *Arruda quilombo* community in the State of Ceará, Brazil

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The aim of the present study was to investigate the use and storage of medicinal plants in the *Arruda quilombo* community in the state of Ceará, Brazil. Semi-structured interviews were conducted for the determination of traditional knowledge. The qualitative-quantitative collective subject discourse method was used for the data analysis, focusing the discussion on six categories. The Chi-square test with a 5% level of significance was used to analyze the relationship between the age of the interviewee and the postharvest care of medicinal plants. The majority of the *quilombo* community (76.19%) cultivates the medicinal plants used. Among this total, 57.14% of the interviewees store the plants in plastic bags and do not establish an expiration date. On health risks, 97.62% of the community members reported medicinal plants are natural and therefore cause no harm. Thus, the population is unaware of the care required for storing medicinal plants or the administration of a safe dose. The statistical analysis revealed that the storage of medicinal plants and ingestion of home-remedy phytotherapeutic medications by pregnant women is not associated with age group.

Key words: Safe use, storage, toxicity, medicinal plants.

INTRODUCTION

Traditional communities, such as the *quilombo* communities in Brazil, make up part of the immense cultural diversity of humanity. The collective knowledge of these communities is passed down from generation to generation and constitutes a source of immeasurable cultural wealth. Such traditional knowledge results from many years of experience and constitutes a connection between the community and the natural environment, from which community members gather food and medicinal plants as well as practice rituals and other cultural activities (Davies and Kassler, 2015).

The *Arruda quilombo* community in the state of Ceará, Brazil, has no healthcare center, which leads to the search for folk medicine solutions based on medicinal

plants, such as the use of herbal brews, shamans and healers as well as other folk practices (Marques, 2010). However, the use of such products does not exempt the user from the toxic effects such medicines can cause. Thus, an investigation was performed to identify how this community uses medicinal plants. The authors analyzed the planting, harvesting, modes of preparation, doses and storage of medicinal plants, all phases of which could be subject to contamination. The situation was more serious when the plants were collected from roadsides or near farms on which pesticides are used, which could lead to the accumulation of pollutants and toxic substances. This possibility is accentuated at more vulnerly time, such as during pregnancy, when the consumption of medicinal plants should be avoided (Araujo et al., 2014).

The aim of the present study was to investigate the use of medicinal plants in the *Arruda quilombo* community in the municipality of Araripe, state of Ceará, Brazil. The authors analyzed the storage of medicinal plants, doses ingested and the relationship between the method of storage and age as well as the restrictions to the consumption of plant-based "medicines" as a function of age.

MATERIALS AND METHODS

Study area

The *Arruda quilombo* is located in a rural area of the municipality of Araripe (7° 11' 44.37''S; 40° 15' 23.76''W) in the state of Ceará, Brazil (Figure 1), approximately 17 km from the center of the city. The *quilombo* families previously occupied the *Coqueiro* site during the time of slavery in the 18th century. In 1980, the families migrated to a location denominated *Bolandeira dos Estevãos,* which is currently called *Arruda*. The land was acquired through a purchase and in 2010, the members of the community requested the permission from the federal government to increase the acreage due to the fact that the land was insufficient for the sustenance of the group. The community is formed by 39 residences made up of the remainder of the *quilombos* that practice subsistence farming. The community relies on water from cement cisterns, electricity and a school for children (Marques, 2010).

Ethnobotantical survey

After clarifications regarding the project and the obtainment of the community's authorization through the signing of the statement of informed consent, the project was submitted to the Human Research Ethics Committee of *Universidade Regional do Cariri* (URCA) in compliance with Resolution 510/2016 of the Brazilian National Board of Health, which determines the ethical guidelines for research involving human subjects. Approval was obtained from the committee (process number: 51480515.5.0000.5055). Due to the fact that the traditional knowledge investigated was in a *quilombo* community, the project was sent for authorization by the National Artistic and Historical Heritage Institute [*Instituto do Patrimônio Artístico e Histórico Nacional (IPHAN)*] and subsequently directed to the Genetic Heritage Management Committee [*Conselho de Gestão do Patrimônio Genético (CGEN)*], which is linked to the Secretary of the Environment. Semi-structured interviews with appropriate, accessible language were conducted with the *quilombo* community (Albuquerque et al. 2010) between April and June 2016. A representative of each family was interviewed, totaling 42 informants.

Data analysis

The socioeconomic data of the interviewees were analyzed using descriptive statistics (percentage and frequency data). The questionnaires were analyzed using the qualitative-quantitative collective subject discourse (CSD) method. The *Quali Quanti Soft* program, with licensing linked to *URCA* (Lefevre and Lefevre, 2005) was used for this step. The collective discourses were identified numerically from 1 to 6 and groups into six discussion categories according to the question. The chi-square test of independence was used to analyze the proportion of the safe use of plant resources. The level of significance was set to 5%.

RESULTS AND DISCUSSION

In the remaining *Arruda quilombo* community, the female gender accounted for 83.33% of the interviewees, which is similar to findings described in previous ethnobotanical studies (Arnous et al., 2005; Freitas et al., 2012). With regard to schooling, none of the community members had a university education, 33.33% were illiterate and 66.67% had attended school, but did not continue their studies. Among the latter group, 54.77% had an elementary school education and 11.9% had an incomplete high school education. The difficulty in attending school in the *Arruda quilombo* community is associated with the absence of schools in the location to continue one's studies through to the end of high school. With regard to marital status, 83.33% of the interviewees were married and 16.67% were single. This demonstrates the importance of marriage, which is a way for outsiders to become legal members of *quilombo* community (Marques, 2010). A total 35.71% of the interviewees were older than 50 years of age.

During the interview, the source of knowledge was identified. For this first discourse, the following question was posed: From whom did you learn to use medicinal plants? The answer corresponded to *CSD* 1:

"*I learnt from the older ones here– my mother, grandmother, mother-in-law, great grandmother, aunt, my husband's grandmother, my father and older people in Coqueiro. My grandmother was a blesser; blessed people here and in Coqueiro. My aunt prayed here and my mother is a blesser here and a healer for the children here.*"

It is evident by *CSD* 1 that the responsibility of traditional knowledge regarding plants falls to the older people, who
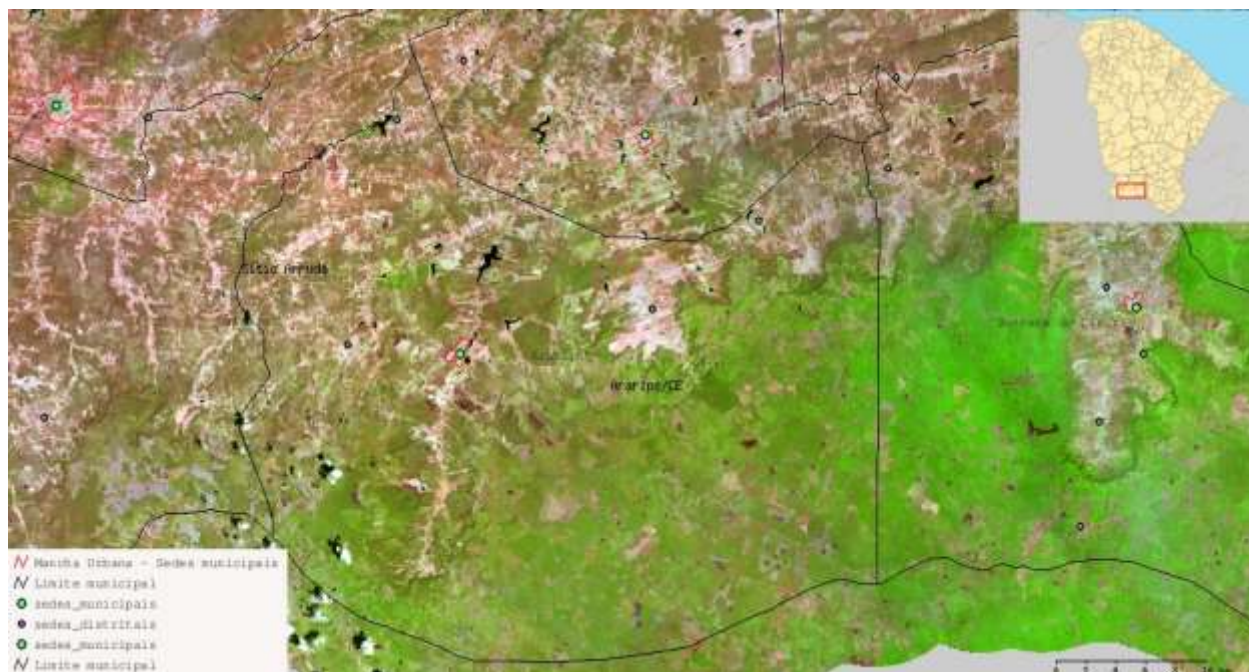
Figure 1. Location of municipality of Araripe in state of Ceará, Brazil, IPECE, 2014

pass this knowledge down to the younger individuals. Older individuals are known in their communities as local specialists due to their considerable knowledge on plants and/or animals in the region (Albuquerque et al., 2010). Thus, the blessers and healers of the *Arruda quilombo* are designated specialists in the community due to their knowledge regarding medicinal plants, herbs and potions. Their immemorial knowledge earns these individuals respect and admiration in the community and it is through these individuals that knowledge on the use of medicinal plants propagates. The origin of the medicinal plants used in the *quilombo* community is divided among cultivation in one's own yard (76.19%), gathering from nearby woods (19.05%) and, in some cases, purchasing (4.76%). *CSD* 2 presents the answer to the following question: Where did you find the plant mentioned?

CSD 2: "I plant it here in the yard. I plant it and take care of it so it does not die with the drought. When there is none here around the house, I look for it at my mother's house or a neighbor's house. I get cinnamon from the woods nearby and I get ipecac root from the field. I get catingueira [Caesalpinia pyramidalis] from the woods, but the drought kills everything. I also get "purging potato" [Operculina macrocarpa] and umburana-de-cheiro [Amburana cearensis] from the nearby woods. Others I buy at the market in Araripe. I buy mustard, others I plant in the yard. I plant everything at my house. We plant and have to take special care so that nothing dies."

CSD 2 demonstrates that cultivation favors the propagation of medicinal plants. Treatment aimed at curing or alleviating an illness with medicinal plants has increased in developing countries due to the difficult access to hospital facilities, exams and conventional pharmaceuticals as well as the ease of cultivating medicinal herbs (Junior et al., 2005). This is similar to what was observed in the community studied herein, in which members need to travel 3 km to a health unit in the district of Pajeú to treat a simple health problem or 17 km to the center of the city of Araripe for hospital care in more serious cases. Due to transportation difficulties, the distance constitutes an obstacle to the *quilombo* community with regard to treating health problems and the members therefore turn to treatment with medicinal herbs (Marques, 2010).

In *CSD 3,* the storage of medicinal plants in the *quilombo* community was investigated through the following question: Do you store part of the plant for when you will need it at a future date? How do you store it?

DCS 3:"When it dries in the sun, I pull up the plant, put it in a plastic bag and store it; peppermint, rue, dill weed, lemon grass, eucalyptus, Mexican mint, ipecac root– I store it all. Pull up the plant, dry it and store it. I get a bag, put it inside and hang it in the living room. It lasts a long time before running out. Some even get moldy. I dry rosemary in the sun and put it in a can and it lasts for a long time. I buy garlic and marcela [Achyrocline

satureioides]. *I put it in a plastic bottle and keep it for my whole life. I put others out to dry in the sun and store them for a very long time. I also store mallow, umburana (*Amburana cearensis*) in a bag for six months. I do not store other plants. I get them from the garden when I need them, because I always plant and have them in the garden or get them from the neighbor's yard. You just go to the plant, take the part you need and make the medicine."*

The drying and storage of medicinal plants is an issue of the utmost importance. If performed in an unduly manner, the active substance could be lost and pathogenic microorganisms could colonize the plant (Bochner et al., 2012). When drying and storage are performed in improper locations and/or in an inadequate fashion, fungi such as *Aspergilus flavus* and *Aspergilus parasiticus* can develop, which have toxic substances (aflatoxins), even small amounts of which can lead to the development of severe poisoning and liver cancer (Almeida, 2011). Fungi can contaminate vegetal tissue even before harvesting and subsequently develop due to a lack of quality with regard to cleaning and storage (Mendes, 1998). Therefore, care must be taken for the correct storage of herbs.

According to Mengue et al. (2001), medicinal plants should be stored in clean, dry places to avoid the proliferation of bacteria and fungi. Likewise, Jorge (2013) states that storage should only be for a short time to avoid the loss of active ingredients, which occurs through hydrolysis, metabolic degradation or degradation caused by light, enzymatic decomposition, degradation caused by heat, volatilization of essential oils and fungal contamination. Thus, medicinal plants should be dried well and stored in a dry, ventilated place in the absence of light and free of dust, rodents and insects. The author also states that medicinal plants should be stored in paper sacks or glass jars. Each species should have its packaging and a label with the name of the plant, date of collection, portion of the plant harvested and the name of the person who collected the material. If the species is aromatic, it should not be stored near other aromatic plants.

Packaging and storage means protecting the plant from infective agents and conserving its active substances, as medicinal plants should have adequate postharvest care to maintain the quality of the vegetal matter (Kffuri, 2011). The storage place should also be exclusive for this purpose or, if not, should at least be free of insects, odors, light, humidity, dust and radiation. Correct drying and storages allows a plant to last 10 to 12 months. Thus, one should chose a well-ventilated, shaded place for the drying process, which could be in flat layers that allows air circulation, such as on top of sieves, on top of cardboard boxes with holes or tied to a suspended rod

(Jorge, 2013). Members of the *Arruda quilombo* community do not take due care with regard to the safe storage of medicinal plants. Most interviewees (57.14%) store plants in plastic bags and other places in the home (Figure 2) and do not establish an expiration date.

The Chi-square test was conducted to determine the strength of the possible association between storage and age. The result of this test was $X^2 = 0.7$. Thus, no statistically significant association was found between how medicinal plants were stored and the age of the interviewee, which precludes the extrapolation of the result to the population.

CSD 4 addresses adverse reactions and contraindications regarding the use of plant-based medicines. For such, the following question was posed: Can the medicine you mentioned cause any harm to someone's health?

CSD 4- "It does good and it does not do any harm. Medicine from a plant is better than medicine from a drugstore. It makes us better. So, it does good and not harm. It does no harm at all."

This discourse demonstrates the notion that natural products are good. This concept was found among 97.62% of the interviewees, who stated that medicinal plants offer no health risks because they are natural. The concept of 'natural' employed by many individuals in relation to medicinal plants alludes to the belief that plants are healthy and do not release chemical substances (Mengue et al., 2001). Mistakenly, this concept has been disseminated in the population, leading to the risk of the occurrence of poisoning by medicinal plants that toxic chemical compounds as a defense against predators. The notion that a natural product is always healthy leads to the indiscriminate use and commercialization that is inadequately monitored by sanitation agencies (Araujo et al., 2014). The widespread notion that natural products cause no harm disregards the possibility of adverse reactions or toxicity and increases trust on the part of the population in religious beliefs despite the lack of proven efficacy (Rates, 2001). Medicinal plants can also act as xenobiotic agents that can activate the metabolism, with the effect manifested in a prolonged manner. Moreover, the non-identification of the toxic effect could lead to continual use, thereby compromising different systems of the body (Araujo et al., 2014). According to Almeida (2011), some species cause delayed harmful reactions in the organism due to the presence pyrrolizidine alkaloids, which, when ingested over a long period of time, cause liver and lung problems that can appear even years after use.

CSD 5 addresses interactions stemming from the use of both plant-based medicine and conventional pharmaceuticals. For such, the following question was

Figure 2. Storage of medicinal plants in *Arruda quilombo* community. A- Storage of seeds in plastic bottle with no label and with presence of insects; B- Storage in plastic bags with no label and exposed to humidity, light and dust; C- Improper storage on roof of interviewee's residence; D- Improper storage on roof of interviewee's residence (Source of photos: Gilmara Matias de Sousa).

posed: When you are sick, do you only take the plant medicine or do you take medicine from a drugstore?

CSD 5- "I first take the plant medicine. If it does not work, I go to the hospital. But I rarely go to the hospital because the doctor gives you the wrong medicine. But I also take medicine from the drugstore. You cannot mix the two medicines, because it causes harm to your health. I sometimes take a pain medication from the drugstore. Other times I take the plant medicine. What you cannot do is mix a plant medicine with medicine from the drugstore. Otherwise, it will poison you. You can only mix them as an anointment. One hour after you take the plant medicine, you can take the drugstore medicine. I do not go to the doctor. Drugstore medicine does not work. Plant medicine is what is good."

This discourse demonstrates that the members of the *Arruda quilombo* community used their own plant-based

medications and conventional pharmaceuticals. The majority of the interviewees (66.66%) took both types of medications, 30.95% preferred plant-based medications and 2.38% primarily took conventional pharmaceuticals. However, 73.81% of the interviewees recognized the danger in consuming the two types of medication together, which is a positive point in the culture. Paiva et al. (2007) stress the importance of warning the population regarding the use of medicinal plants as medication, as the ingestion of the active chemical substance is equivalent to medication sold in pharmacies and medicinal plants therefore need to be considered medications with possible side effects. Addressing drug interactions, Junior et al. (2005) warn that some medicinal plants potentiate the effect of a pharmaceutical drug, whereas others may inhibit the effect. It should be noted that some medications are produced from medicinal plants, metabolized through the same pathways in the organism and transformed into the same

substances. This was evidenced in *CSD 5*, when the community (71.43%) taking drugstore medication together with plant-based medicine had poisoning.

In CSD 6, the aim was to determine the administration of phytotherapeutic medications to risk groups. For such, the following question was posed: Can pregnant women, children and old people take plant medicine?

CSD 6: *"Yes, they can. Everyone can take it. There's no problem. Except pregnant women are not indicated because a pregnant woman is sensitive. Pregnant women do not take marcela, rue, "malva-sete-dor" [Plectranthus barbatus], aroeira [Myracrodruom urundeuva] orlosna [Artemisia absinthium] because they are bitter and they could lose the baby. Bitter remedies cause miscarriages, but others do not do any harm. Everyone can take other medicine. Children and old people take everything. Pregnant women can only take rosemary and peppermint. Children and old people take everything. There's no problem. Children take everything, all the medicines. It does not cause any harm."*

CSD 6 shows that the population in the *Arruda quilombo* is unaware of the danger of administering medication to children and elderly individuals. However, the discourse demonstrates concern for pregnant women, who are considered sensitive, and some active substances found in medicinal plants can cause miscarriage.

A total of 61.90% of the interviewees said that all age groups can take plant-based medicines without any restrictions. The danger in this thinking is the possibility of self-medication. In contrast, 38.10% of the interviewees reported concerns during pregnancy. Self-medication is dangerous for children and elderly individuals because these groups are more sensitive to poisoning (Samek, 2012). According to Kffuri (2011), infants and children require extra care and prudence should be exercised with regard to the ingestion of plant-based medication, which should preferably be prescribed by a specialist. Junior et al. (2005) stated that pregnant women and children are more sensitive to adverse reactions and therefore should not be submitted to treatment with herbal medicine. Regarding elderly individuals, Paula et al. (2012) reported that changes stemming from the aging process lead to greater sensitivity in the metabolism of drugs and poisoning in this age group can be lethal. In the statistical analysis of this variable, X^2 was larger than 0.05, indicating that the ingestion of phytotherapeutic medications by pregnant women in the community was not associated with the age of the interviewee.

Conclusion

The findings of the present study demonstrate that the

Arruda quilombo community in the city of Araripe, Brazil, has vast knowledge of medicinal plants. A total of 97.62% of the community members believe that medicinal plants are healthy and cause no harm, but are unaware of due care required with regard to drying, storage and the safe administration of medicinal plants. The findings also demonstrate that the ingestion of plant-based medications in the *quilombo* community studied and the storage of medicinal plants are independent of the age of the interviewee. As medicinal plants play a unique role in the treatment of health problems in this traditional *quilombo* community and constitute a therapeutic alternative as a way to circumvent the transportation difficulties of reaching local healthcare centers, it is necessary to warn the members of this community with regard to the postharvest storage and use of medicinal plants. Thus, the present study is of fundamental importance, as it offers a unique portrait of the use of medicinal plants by a traditional *quilombo* community and demonstrates the need to disseminate knowledge produced in the academic setting regarding safe postharvest methods and the administration of medicinal plants to these communities so that they may enjoy the benefits of biodiversity without harming their own health.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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Full Length Research Paper

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

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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

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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:

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

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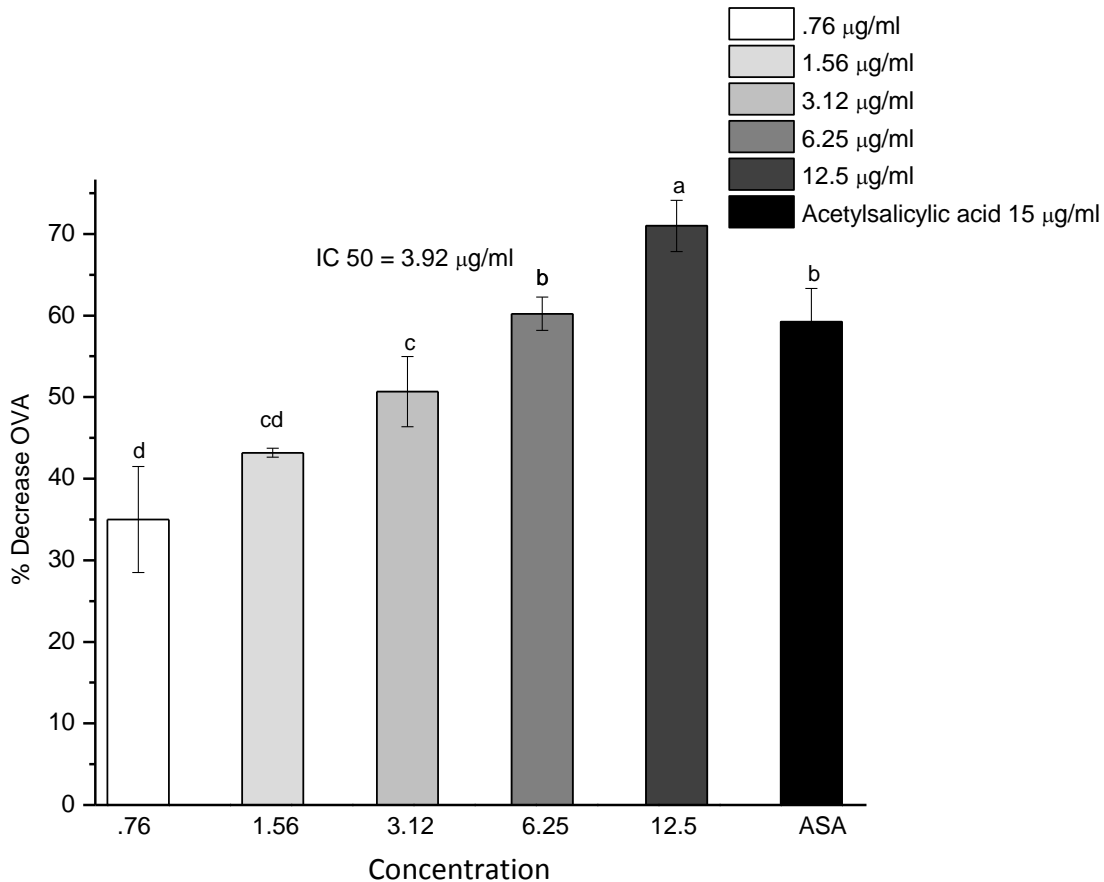
Figure 1. Effect of *Bucida buceras* on inhibition of ovalbumin denaturation (OVA). *Bucida buceras* (0.76 to 12.5 µg/mL Acetylsalicylic acid (ASA-15 µg/mL) was used as drug. Data are presented as mean ± S.D (n=5). Different letters indicate significant differences (p<0.05).

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
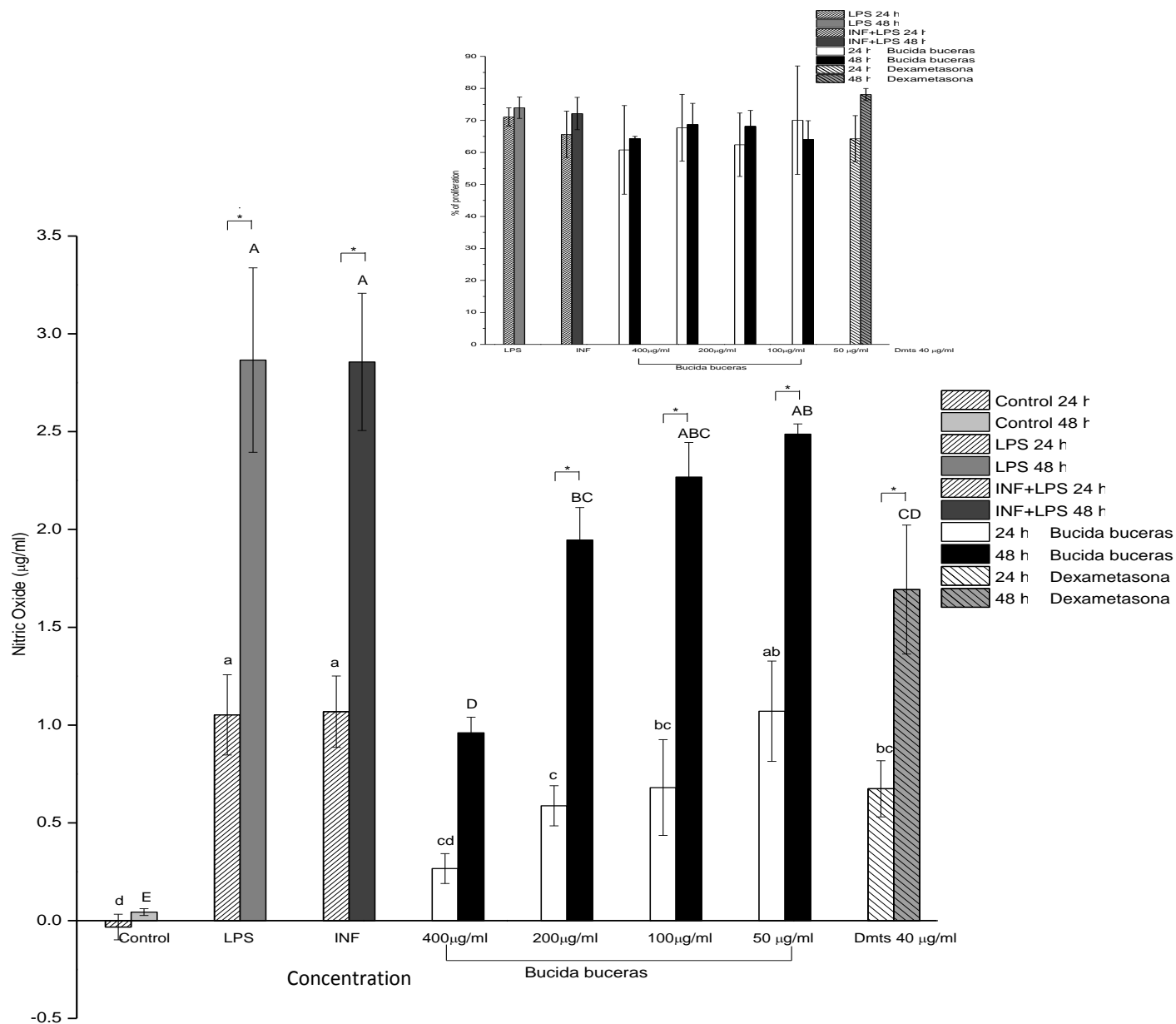
Figure 2. Influence of *Bucida buceras* on NO production by RAW 264.7 macrophages exposed to LPS at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (0.1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone drug (40 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
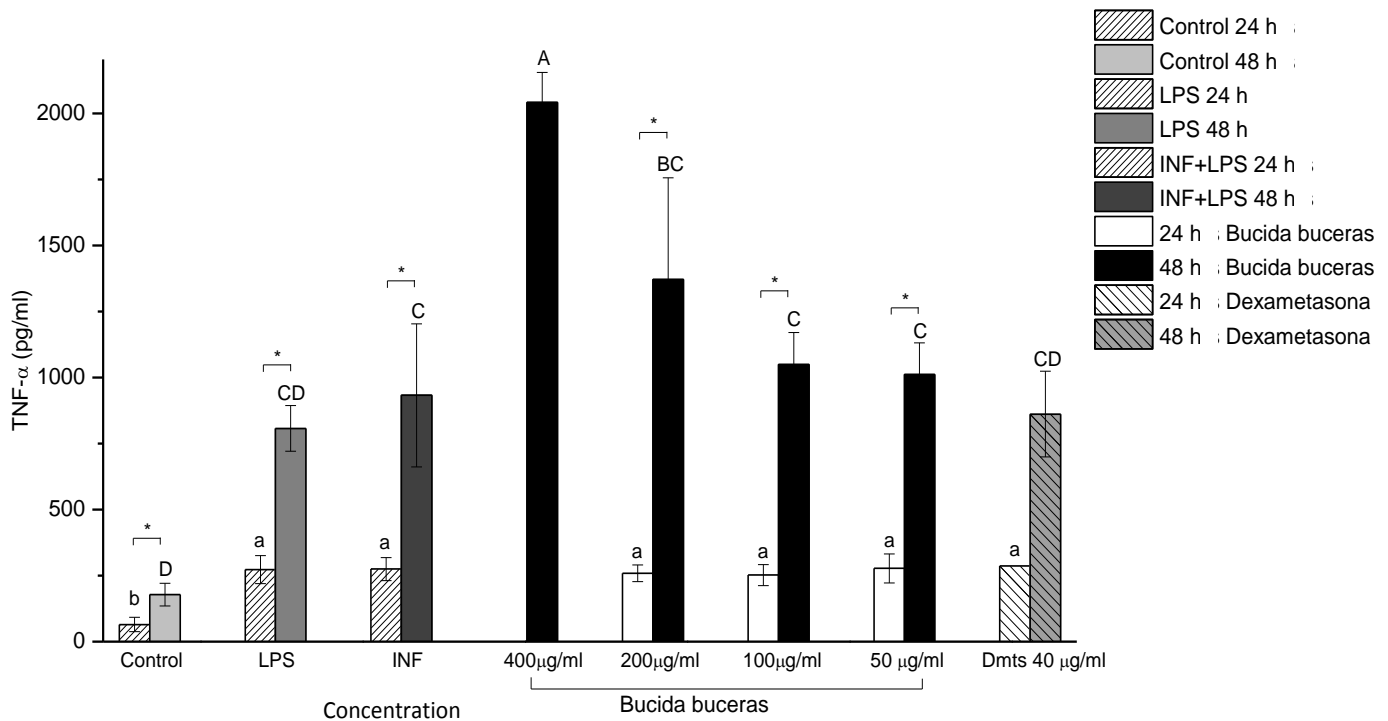
Figure 3. Effect of *Bucida buceras* on tumor necrosis factor (TNF-α) by RAW 264.7 macrophages exposed to LPS and LPS+INF-γ at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and 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*
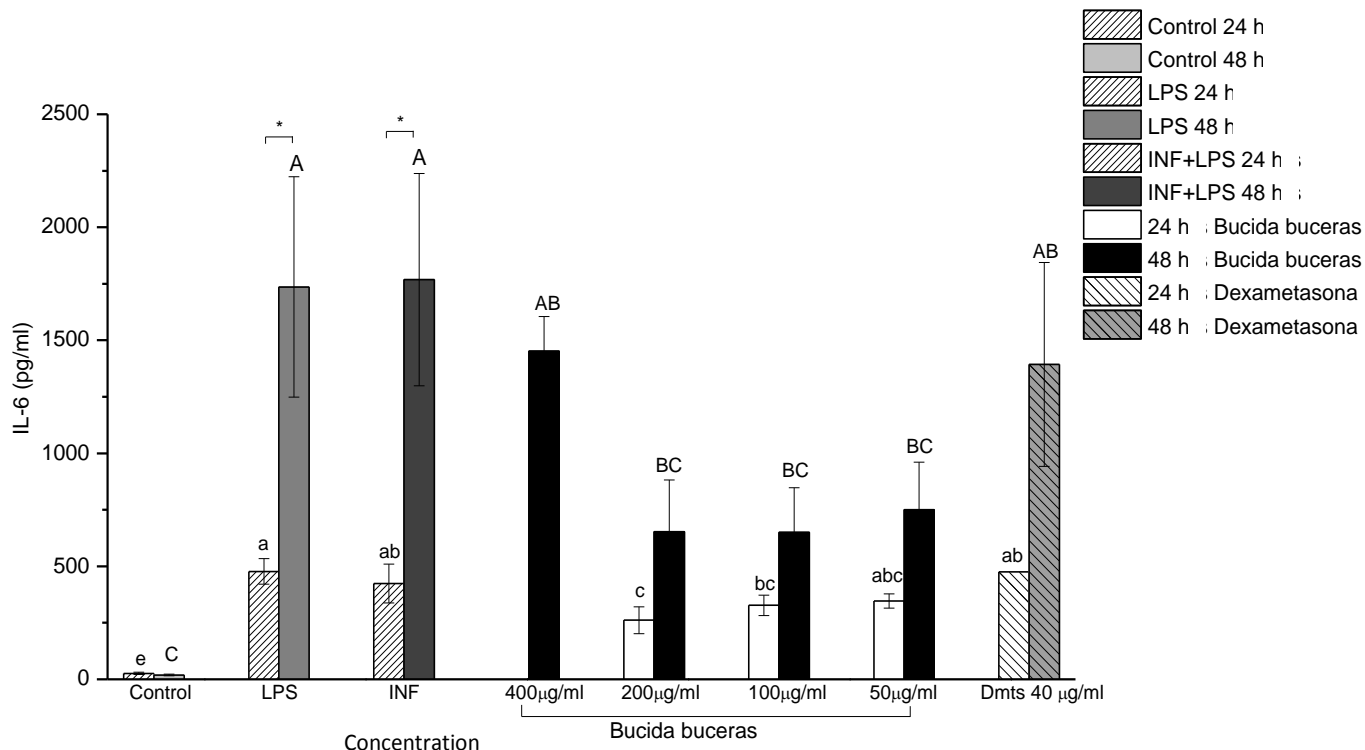
Figure 4. Effect of *Bucida buceras* on interleukin-6 (IL-6) y RAW 264.7 macrophages exposed to LPS and LPS+INF-γ at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone 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.
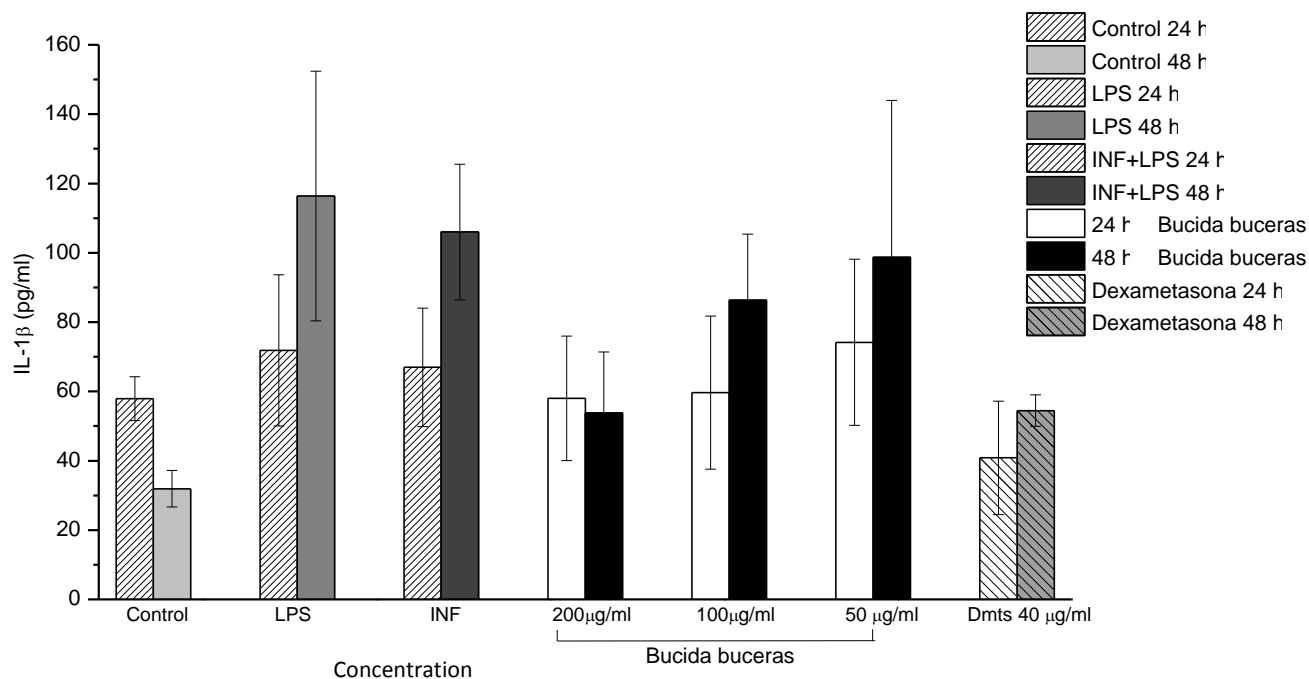

Figure 5. Effect of *Bucida buceras* on interleukin-1β (IL-1β) by RAW 264.7 macrophages exposed to LPS and LPS+INF-γ at 24 and 48 h. Cells were incubated with aqueous extract of *B. buceras* (50, 100, 200 and 400 µg/mL, stimulated with LPS alone (1 µg/mL) and LPS (1 µg/mL) + IFN-γ (4 ng/mL) and dexamethasone drug (40 µg/mL). Data are presented as the mean ± S.D (n=5).
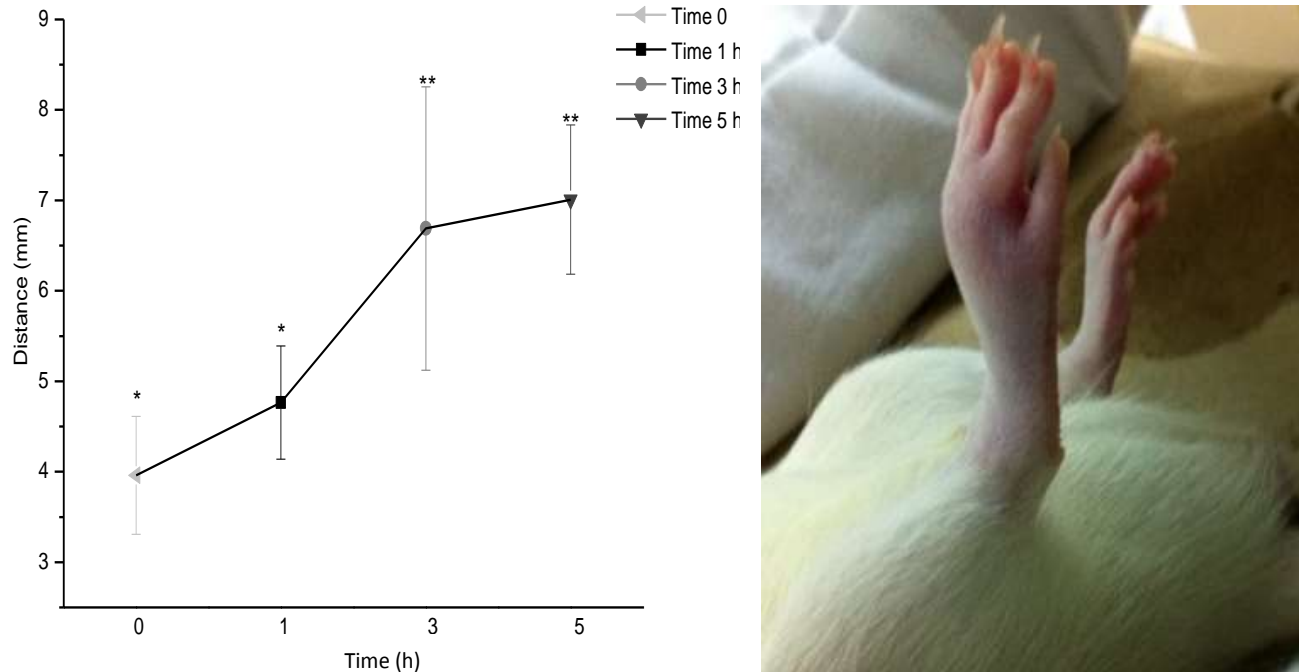
Figure 6. Paw size in acute edema in rats after carrageenan treatment at different time of subplantar injection (0, 1, 3 and 5 h). Paw thickness was measured using a caliper placed at the border of the phalanges and metatarsals. Data are expressed as the mean paw thickness ± S.D. * Asterisks indicate significant differences (P<0.05) at various times.
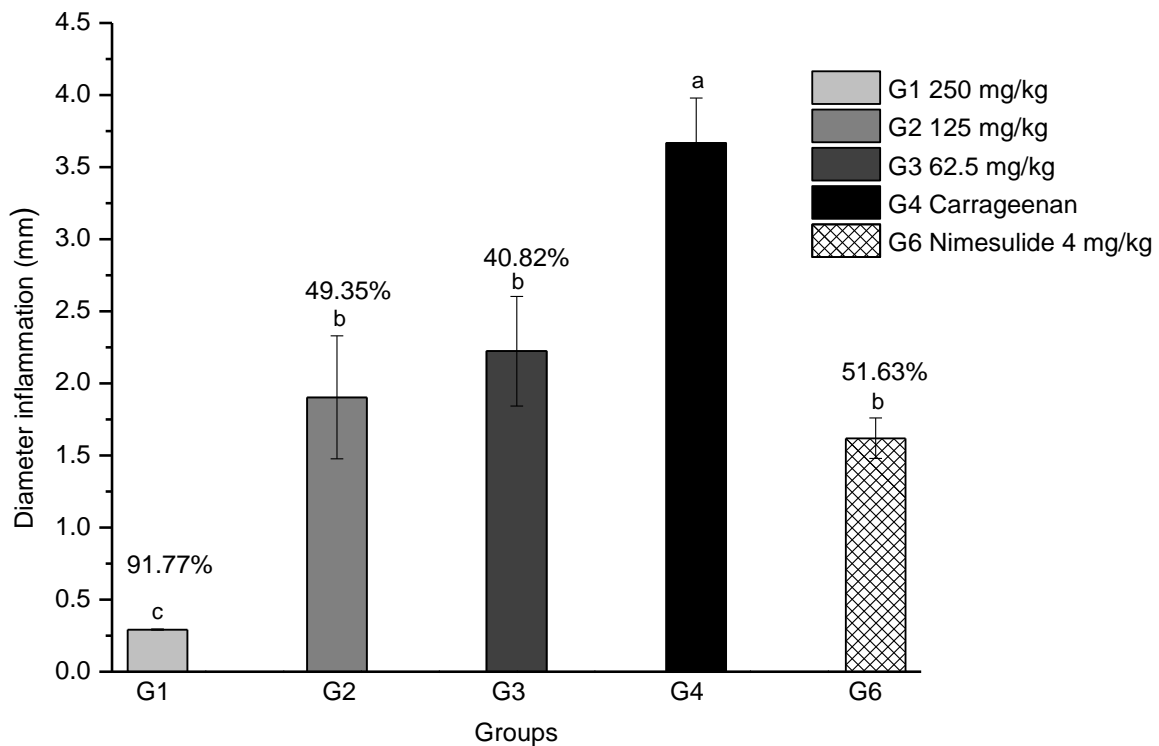

Figure 7. Effect of *Bucida buceras* on the footpad edema post-carrageenan injection in rats. Animals were pretreated with aqueous extract of *B. buceras* (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G7 (Nimesulide-drug control). Data are presented 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.
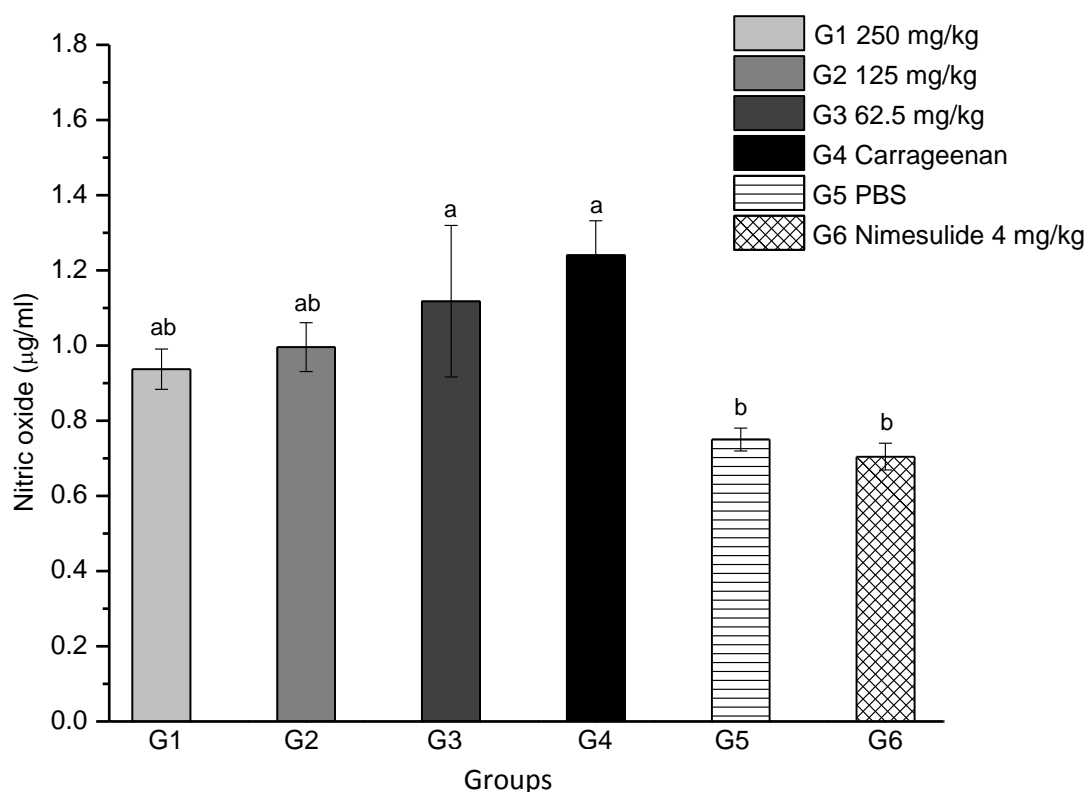

Figure 8. Inhibitory effect of aqueous extract of *Bucida buceras* on nitric oxide production in acute inflammation. Rats were pretreated with aqueous extract (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G5 PBS-control, G6 (Nimesulide-drug control). 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-
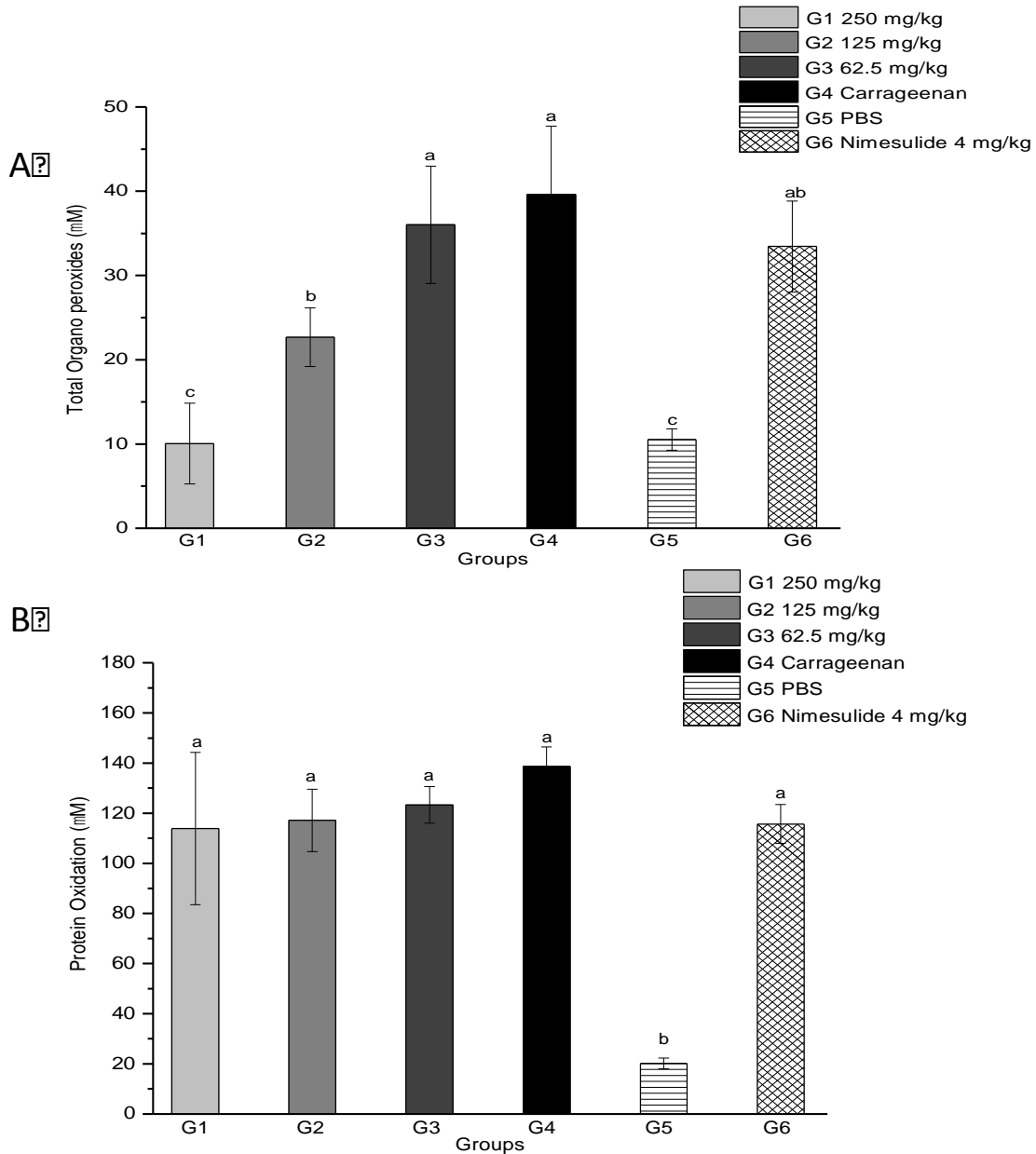
Figure 9. Inhibitory effects of aqueous extract of *Bucida buceras* on total organoperoxid lipid (A) and oxidation protein (B) production in acute inflammation. Rats were pretreated with aqueous extract (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, 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
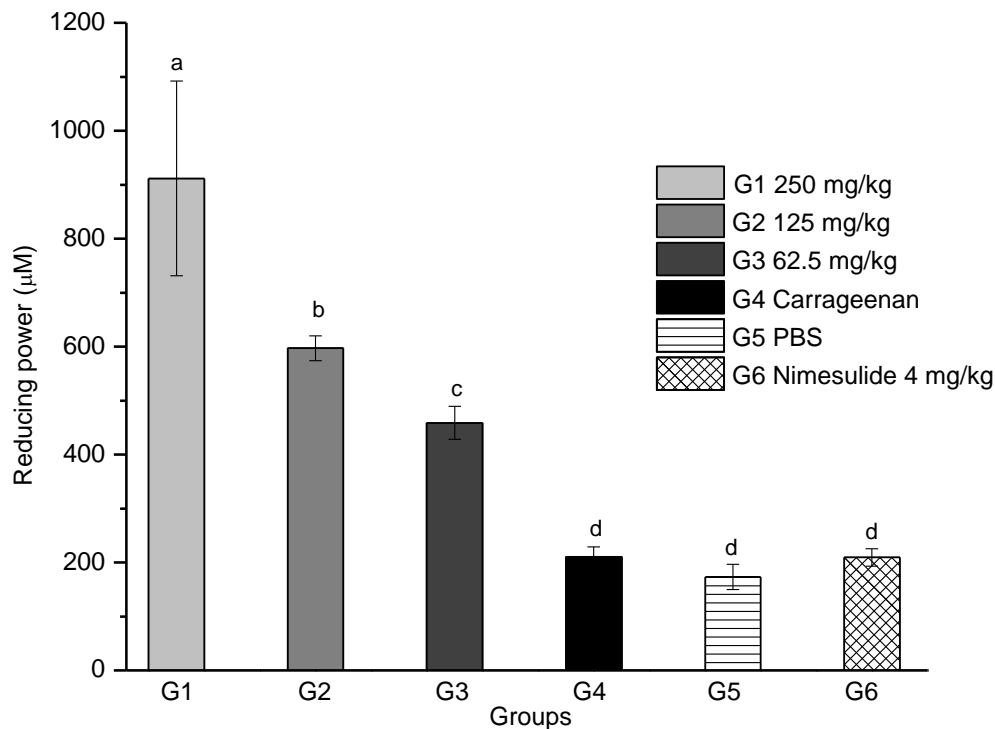
Figure 10. Effect of *Bucida buceras* on ferric reducing antioxidant power in carrageenan-induced paw edema in rats. Rats were pretreated with aqueous extract of *B. buceras* (250, 125 and 62.5 mg/kg p.c, p.i G1 to G3) for 3 days before carrageenan injection, G4 carrageenan, G7 (Nimesulide-drug control). 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 $O_2^{.-}$ 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.

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