

Full Length Research Paper

Anti-inflammatory property and redox profile of the leaves extract from *Morinda citrifolia* L.

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Morinda citrifolia Linn (Rubiaceae), popularly known as noni, is widely used in folk medicine in the form of decoction and infusion, particularly as anti-inflammatory, depurative, anti-rheumatic and antiulcer remedy. The infusion of *M. citrifolia* L. leaves is used in popular medicine in Northeast of Brazil to treat inflammatory and painful diseases. The present study was designed to evaluate the antioxidant potential and the anti-inflammatory effect of the aqueous extract from the dried leaves from *M. citrifolia* L. (EAMC). The free radical scavenging activities were determined for different concentrations using *in vitro* models, and the inflammatory processes were evaluated by carrageenan-induced pleurisy. The study results indicated a significant dose-dependent antioxidant effect by noni extract as evaluated by total antioxidant potential (TRAP) and total antioxidant reactivity (TAR) assays. Noni extracts also exhibited modest catalase-like activity, and was able to inhibit the levels of proinflammatory cytokines. All experimental data indicate that the EAMC have not only remarkable antioxidant properties but also potential anti-inflammatory properties.

Key words: *Morinda citrifolia*, antioxidant, inflammation.

INTRODUCTION

Antioxidants are chemical substances that reduce or prevent cellular oxidation. These substances counteract the damaging effects of free radicals in tissues (Halliwell, 2008). An imbalance in the production and detoxification

of reactive species in cells leads to widespread damage to biomolecules, resulting in conditions such as cancer, heart disease, inflammation and several other diseases (Bandyopadhyay et al., 2007). By counteracting the

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deleterious action of free radicals, antioxidants are believed to play a preventive role in such diseases (Haliwell, 2008).

Plants are potential sources of natural antioxidants. They absorb the sun's radiation and generate high levels of oxygen as a secondary metabolite of photosynthesis. Oxygen is easily activated by ultraviolet (UV) radiation and heat from sunlight to produce, reactive oxygen species (ROS). Phytochemicals of plants consist of various antioxidant compounds to counteract these ROS in order to survive. Flavonoids and other polyphenols of foods and beverages have been associated with the decreased risk of age related diseases in several epidemiological studies (Esmaeili and Sonboli, 2010). Flavonoids have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of reactive oxygen, nitrogen, and chlorine species, such as superoxide $O_2^{\bullet-}$, hydroxyl radical $\bullet OH$, peroxy radicals RO_2^{\bullet} , hypochlorous acid (HOCl) and peroxyxynitrous acid (ONOOH) (Haliwell, 2008; Gasparetto et al., 2014).

Recent studies emphasize that recurrent or chronic inflammations associated with an oxidative stress have been implicated in various diseases such as cancer, diabetes, asthma and autoimmune diseases. The development of strategies for reducing inflammation and oxidation status could lead to effective treatments for these diseases. In this way, some natural products containing biological active molecules could participate to the prevention or the treatment of some of these diseases (Dussossoy et al., 2011). Herbal and natural products derived from plants have been used for centuries throughout the world in every culture. Recently, research has been intensified in *Morinda citrifolia* Linn (Rubiaceae) and its products as its benefits have become known (Zin et al., 2002). *M. citrifolia* L., popularly known as noni, has been used in traditional Polynesian medicine for over 2,000 years. *M. citrifolia* is native from Southeast Asia to Australia and is cultivated in Polynesia, India, the Caribbean region, and Central and Northern South America. The infusion of *M. citrifolia* L. leaves is used in popular medicine in Northeast of Brazil to treat inflammatory and painful diseases (Wang et al., 2002; Chan-Blanco et al., 2006; Serafini et al., 2011).

The noni fruits have been used as a folk medicine for the treatment of many diseases including diabetes, high blood pressure, inflammation and cancer (Chan-Blanco et al., 2006). Noni leaves have been consumed as a vegetable food by multiple cultural groups. For this reason, it is included in the World Health Organization's and Food and Agriculture Organization's, food composition tables for East Asia and the Islands of the Pacific (West et al., 2007). It has been shown that noni leaves contain several phytochemicals including phenolic compounds (Serafini et al., 2011) such as flavonoids (Sang et al., 2001; Takashima et al., 2007; Deng et al., 2008). Regarding its biological activity, the noni juice and fruit demonstrated an antibacterial, anti-cancer, anti-

antioxidant, antinociceptive and anti-inflammatory activity (Wang and Su, 2001; Wang et al., 2002; Su et al., 2005; Dussossoy et al., 2011; Brown, 2012; Zin et al., 2012; Gupta and Patel, 2013). Other in-vivo studies include the reduction of blood glucose levels in mice with streptozotocin-induced diabetes, hypotensive effects in dogs by the intravenous injection of the water-soluble extract from the roots and an analgesic effect of the aqueous extract injected intraperitoneally in mice (Youngken et al., 1960; Younos et al., 1990; Yamaguchi et al., 2002; Nayak et al., 2007).

Whereas, noni juice and fruit have been well characterized pharmacologically, few data are available regarding the properties of *M. citrifolia* leaves. In this regard, this study aims at investigating the antioxidant and the anti-inflammatory effects of the aqueous extract from *M. citrifolia* leaves.

MATERIAL AND METHODS

Plant material and preparation of the extract

M. citrifolia leaves were collected from São Cristóvão in March 2013, Sergipe, Brazil (10°18'20.7"(S); 36°39'7.2"(W)). Herbarium voucher specimens (registry number 13503) were prepared, and deposited at the Department of Biology of the Federal University of Sergipe. To prepare the *M. citrifolia* leaves aqueous extract from *M. citrifolia* (EAMC), fresh leaves were dried in a ventilated oven (45°C). Previously powdered dried leaves were prepared by decoction in distilled water (7.5% (w/v) in 15 min; the solvent evaporated under reduced pressure and lyophilized.

Drugs and reagents

AAPH (2,2'-Azobis(2-methylpropionamide) dihydrochloride), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 2-deoxyribose, glycine, Griess reagent, SNP (sodium nitroprusside), H_2O_2 (hydrogen peroxide), catalase and SOD (superoxide dismutase) were purchased from Sigma (USA). Indomethacine and Trolox were purchased from Sigma (Brazil).

Animals

Adult male albino Swiss mice (25 to 35 g) bred in animal house were used. Animals were housed at controlled temperature (22±2°C) with a 12 h- light/dark cycle, standard lab chow and tap water *ad libitum*. Animals were habituated to the experimental room for at least 2 h before the experiments and used only once. All protocols employed have been approved by the Local Ethic's Committee (process number: CEP/UFMS # 27/09) and are in accordance with the US guidelines for the care and use of Laboratory animals (NIH publication #85 to 23, revised in 1985). The number of animals used was the minimum necessary to demonstrate the consistent effects of the drug treatments.

Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

The total reactive antioxidant potential (TRAP) is employed to estimate the nonenzymatic antioxidant capacity of samples *in vitro*. This method is based on the quenching of luminol-enhanced

chemiluminescence (CL) derived from the thermolysis of AAPH as the free radical source (Lissi et al., 1992; Gasparotto et al., 2014). The background CL was measured by adding 4 ml of AAPH (10 mM) dissolved in glycine buffer (0.1 M, pH 8.6) to a glass scintillation vial. Then 10 μ L of luminol (4 mM) was added to each vial and the CL was measured until constant light intensity. After this stabilization time, 10 μ L of Trolox solution (water-soluble vitamin E analogue) or 10 μ L of sample was added, and the CL was measured in a liquid scintillator counter working in the out of coincidence mode. The last count before the addition of Trolox or samples was considered as 100%. The count time was 10 s, and the CL emission was monitored for 3000 s after the addition of Trolox or samples. The luminescence emission was recorded in a Micro Beta luminescence counter (Perkin Elmer, Waltham, LA). Graphs were obtained by plotting percentage of counts per minute (% cpm) versus time (s). The Area Under the Curve (AUC) was calculated using GraphPad Prism software. The total antioxidant reactivity (TAR) was also analyzed in the same samples used for TRAP readings. The TAR results were calculated as the ratio of light intensity in absence of samples (I₀)/light intensity right after sample addition. Although TAR and TRAP evaluations are obtained in the same experiment, they represent different observations, since the TAR is more related to the antioxidant quality (reactivity, the scavenging capacity in a short-term period) and TRAP is more related to the antioxidant amount and kinetic behavior.

Catalase-Like activity

The capacity of EAMC to degrade hydrogen peroxide (H₂O₂) added in the incubation medium ("catalase-like" or "CAT-like" activity) was measured as described earlier (Aebi, 1984, Quintans-Junior et al., 2013). Catalase-like activity was monitored based on the rate decomposition of H₂O₂. Data were expressed as percentage of the rate decomposition of H₂O₂.

Superoxide-dependent adrenaline autooxidation ("SOD-Like" Activity)

The ability of EAMC to degrade hydrogen peroxide (H₂O₂) added in the incubation medium ("superoxide dismutase-like" activity or "SOD-like" activity) was measured as previously described (Bannister and Calabrese, 1987, Quintans-Junior et al., 2013). Superoxide production was determined by measuring spectrophotometrically by the inhibition of adrenaline autooxidation at 480 nm.

Carrageenan-induced pleurisy

Pleurisy was induced in mice by intrapleural injection of 0.1 ml of a carrageenan suspension (300 μ g/cavity) diluted in sterile saline (NaCl 0.9%) as described by da Silva et al. (2014). Animals were pretreated with EAMC (100, 200 and 400 mg/kg orally), vehicle (saline) or indomethacin (10 mg/kg, i.p.) 60 min before the injection of phlogistic agent. Four hours after carrageenan injection, mice were killed by excess carbon dioxide and the pleural exudate was collected by pleural cavity lavage with 1 ml of PBS solution containing EDTA (10 mM). Several samples of the pleural fluid were collected for further determination TNF- α levels by ELISA or cells in a Neubauer chamber and cytocentrifuged.

Statistical analysis

Data are expressed as mean \pm S.E.M. The obtained data were evaluated by one-way analysis of variance (ANOVA) followed by

Tukey's test. Data analyses were performed using the GraphPad Prism 5.0 software. Differences were considered significant if $p < 0.05$.

RESULTS

Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

To further explore the redox profile of EAMC, the TRAP/TAR parameters were evaluated, which indicate the capacity of a given sample to act as a general antioxidant or prooxidant agent in a constant reactive species generating system. TRAP and TAR measurements showed a strong antioxidant capacity of the EAMC against the peroxy radicals generated by the AAPH system (Figure 1). The EAMC at 100 μ g/ml and 1 mg/mL had an antioxidant activity significantly stronger than Trolox (200 nM), which was used as a standard antioxidant reference compound.

Catalase-like activity

The study results showed a statistically significant increase in CAT-like activity at the highest concentration of EAMC tested (Figure 2). However, due to the small extent of this increase, the study was not able to state whether this H₂O₂-scavenger activity would be significant in a physiologic context.

Superoxide-dependent adrenaline autooxidation

The study observed a decrease in SOD-like activity by EAMC at the highest concentration (1 mg/mL) when compared to the superoxide-generating system (Figure 3).

Carrageenan-induced pleurisy

These results allowed the study to detect a marked inhibitory effect of EAMC on neutrophil, leukocytes cells migration and TNF- α level without altering mononuclear cells migration in the pleural exudates. As a reference drug, indomethacin (10 mg/kg; i.p.), intensely inhibited on neutrophil, leukocytes cells migration and TNF- α level without altering mononuclear cells migration in the pleural exudates (Figure 4).

DISCUSSION

Oxidative stress is the result of an unbalance in reactive species production and antioxidant defense, and is a main component in cancer, infectious diseases, cardio-

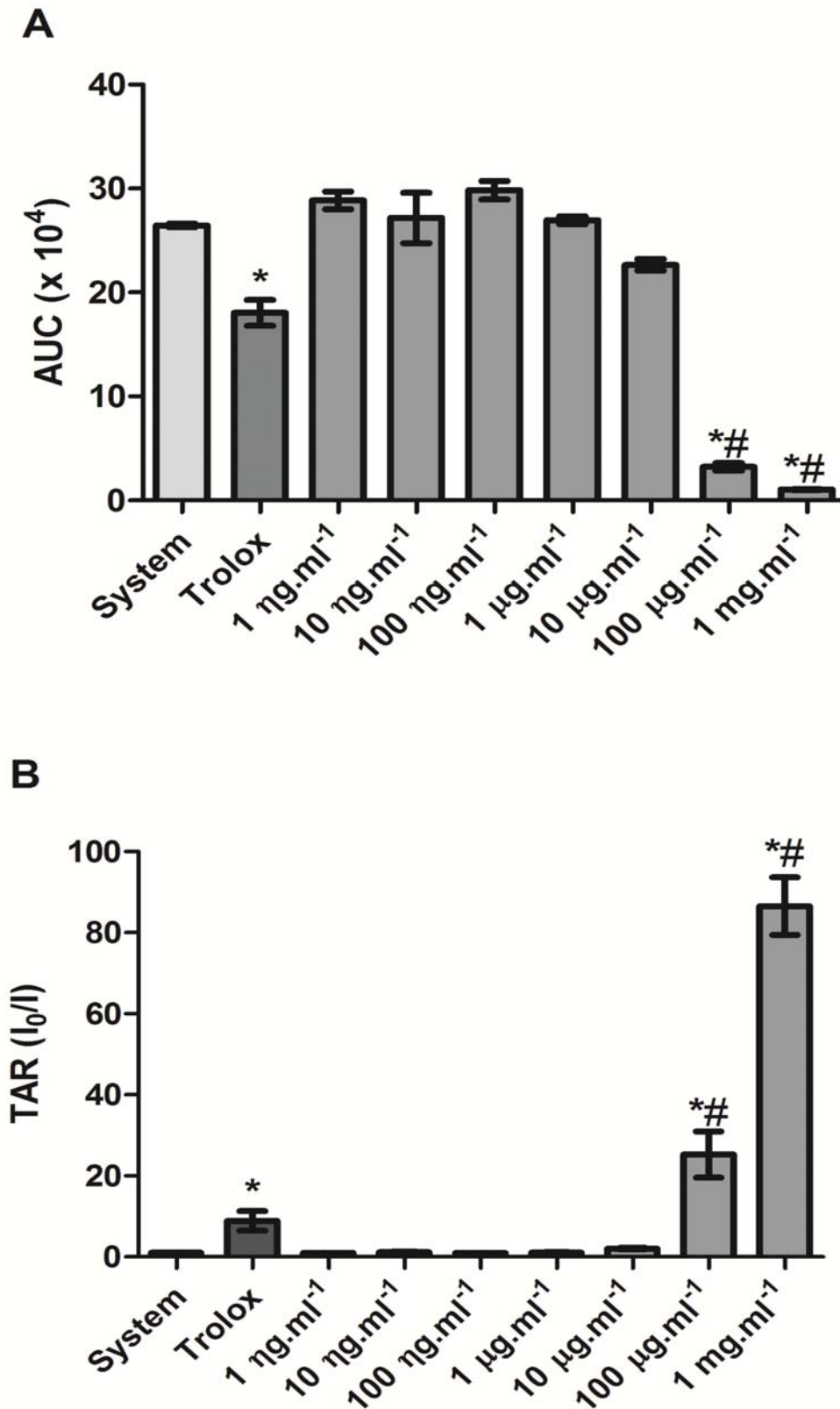


Figure 1. (A) Total radical-trapping antioxidant parameter (TRAP) at different concentrations. (B) The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in absence of samples expressed as percent of inhibition (I_0/I). Values represent mean \pm S.E.D., experiments in triplicate, * $p < 0.001$ different from system; # $p < 0.001$ different from Trolox (ANOVA followed by Tukey).

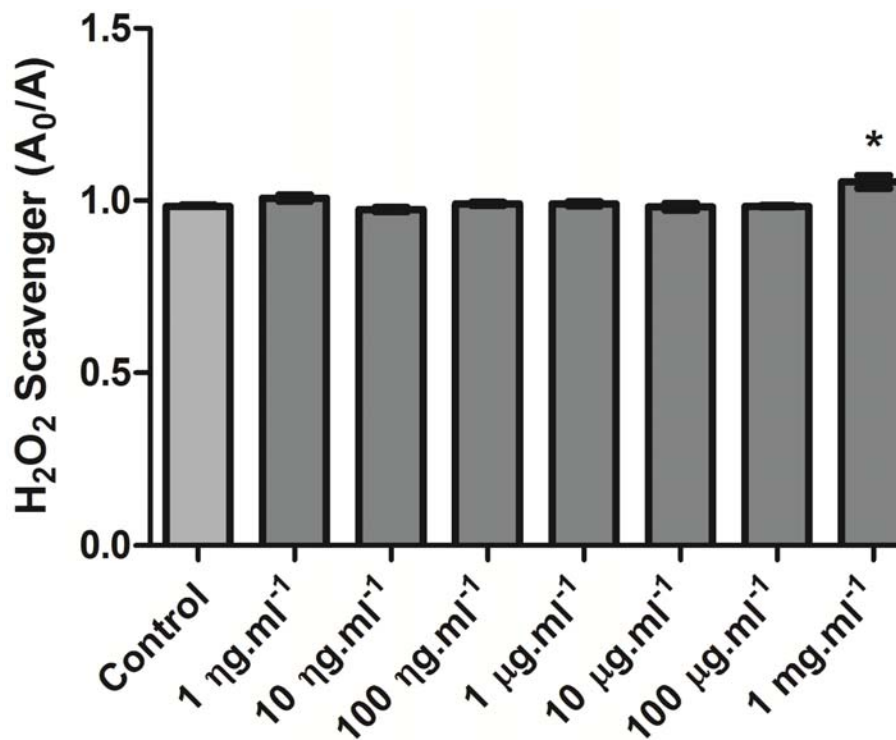


Figure 2. Catalase-like (CAT-like) activity. CAT-like activity was measured in a catalase reaction buffer with H₂O₂. Values represent mean \pm S.E.D., experiments in triplicate. *** $p < 0.001$ different from control + NPS (ANOVA followed by Tukey).

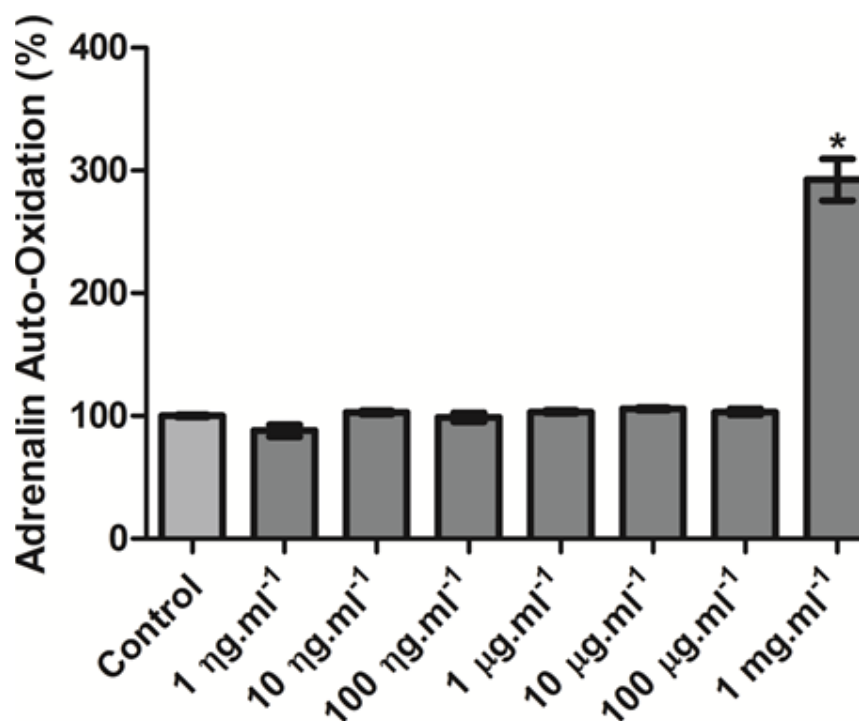


Figure 3. Superoxide dismutase-like (SOD-like) activities. SOD-like activity was determined by following formation of adrenochrome in a SOD reaction buffer containing native purified catalase and adrenaline. Values represent mean \pm S.E.D., experiments in triplicate. *** $p < 0.001$ different from control + NPS (ANOVA followed by Tukey).

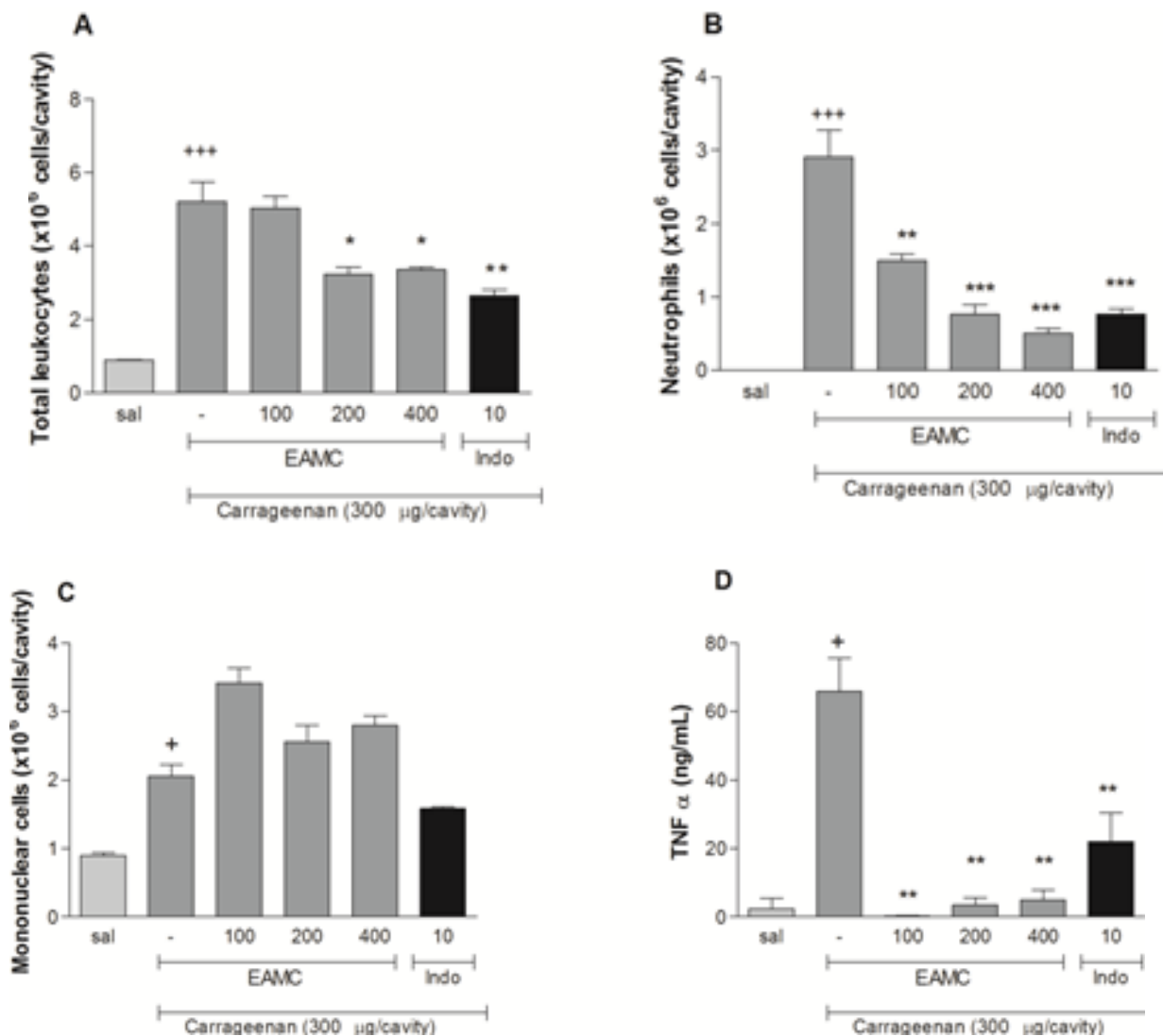


Figure 4. Effect of EAMC (100, 200 or 400 mg/kg, i.p.) or indomethacin (INDO, 10 mg/kg; i.p.) on the inflammation by carrageenan in mouse pleurisy. The analyses were performed 4 h after carrageenan injection (300 μ g/cavity) to evaluate the recruitment of total leukocytes (A), neutrophils (B), mononuclear cells (C), and to assess tumor necrosis factor alpha (TNF- α) levels (D). Data were expressed as mean \pm SEM, for a minimum of 5 animals. ⁺⁺⁺ $p < 0.001$ or ⁺ $p < 0.05$ compared with the saline-injected mice; ^{*} $p < 0.05$, ^{**} $p < 0.01$ and ⁺⁺⁺ $p < 0.001$ compared with the control group (vehicle) (ANOVA followed by Tukey test).

vascular disorders, and neurodegenerative conditions (Gelain et al., 2009). Pharmacological agents showing therapeutic efficiency against some diseases may exert antioxidant properties in target tissues, which may be related to their mechanism of action. *M. citrifolia* L. (Rubiaceae), the noni, has been used in traditional medicine in northeast Brazil to treat painful conditions. In the present work, a screening of redox activities and anti-inflammatory actions of the EAMC was performed.

In order to evaluate the antioxidant activity of a natural product, it is crucial to work with different assays, taking into consideration the various oxidation aspects in the systems under scrutiny (Esmaili and Sonboli, 2010). In

this context, the antioxidant activity of the EAMC were analyzed for the levels/activities of non-enzymatic antioxidants and compared with the activity of the well-known antioxidants. Due to this, the study examined the total antioxidant capacity of the EAMC on TRAP/TAR methods. The TRAP assay is widely used to determine the non-enzymatic antioxidant capacity in plant extracts, which is mostly dependent on the content on secondary metabolites with redox activity (Dresch et al., 2009).

The total antioxidant reactivity (TAR) index indicates the instantaneous decrease luminescence associated with the sample addition into peroxy-generating system. While TRAP indicates the quantity of antioxidants pre-

sents in the plant extracts, the TAR indicates their antioxidant effectiveness (Gasparotto et al., 2014). The study results on TRAP/TAR assays showed that the EAMC had a significant antioxidant capacity can be attributed to the total phenolic content.

To confirm the antioxidant activity of the EAMC the study verified its ability to decompose hydrogen peroxide and oxygen. The primary scavenger of ROS is the enzyme superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide and oxygen (Slesak and Miszalski, 2003). It has been proposed that SOD may play a vital role in the supplying H_2O_2 to peroxidase and preventing peroxidase inactivation by superoxide anion radicals (Zielinski et al., 2006).

The study observed a decrease in SOD-like activity by EAMC at the highest concentration when compared to the superoxide-generating system. Differences in the relative antioxidant potential of model compounds were observed when one compound is strongly antioxidant with one method and pro-oxidant with another (Moure et al., 2001). For such reason, the antioxidant activity of a compound must always be evaluated with different tests, in order to identify different mechanisms (Melo et al., 2011). To confirm the antioxidant activity of the EAMC the study evaluated the catalase-like activity. CAT constitutes the most efficient and elaborates system available in both plants and animals to control H_2O_2 concentrations. CAT catalyzes the dismutation of H_2O_2 to one molecule of H_2O and a half molecule of O_2 . H_2O_2 is a normal product of mitochondrial electron transport, oxidation of fatty acids and photorespiration. The first reaction responsible for the generation of H_2O_2 is the transfer of electrons to molecular oxygen, which produces the superoxide radical (Montavon et al., 2007). Plant extracts exhibiting H_2O_2 -scavenger activity may prevent a variety of deleterious effects derived from oxidative damage. The study results showed an increase in CAT-like activity at the highest concentration of EAMC. However, due to the small extent of this increase the study was not able to state whether this H_2O_2 -scavenger activity would be significant in a physiologic context.

The results of this study, are in accordance with a study showing that the root methanol extract and the ethyl acetate partitions of all parts of the *M. citrifolia* tested had antioxidant activity, similar to the positive controls, using the ferric thiocyanate method and thiobarbituric acid test (Zin et al., 2002). Moreover, other study demonstrated antioxidant activity of the fruit juice from *M. citrifolia* in both lipid hydroperoxide and tetrazolium nitroblue assays (Wang and Su, 2001). Natural antioxidants present in medicinal plants are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Natural products contain free radical scavengers such as polyphenols, flavonoids, and phenolic compounds. A number of scientific reports indicate that terpenoids, steroids, and phenolic compounds such as tannins, coumarins and flavonoids exert protective effects

due to their antioxidant properties (Chandrasekhar et al., 2006; Sreelatha and Padma, 2010). Several studies have shown that the antioxidant activity associated with medicinal plants is attributed to the total content of phenolic compounds.

The study results show an antioxidant and anti-inflammatory potential exhibited by the extracts in a dose-dependent manner. It has also been found in other studies that the EAMC contains polyphenols (Rasal et al., 2008; Yang et al., 2007; Dussossoy et al., 2011) and therefore, the antioxidant and anti-inflammatory effects of this extract may depend on its phenolic components. Also, flavonol glycosides, lipid glycosides, triterpenoids, polysaccharides, iridoids, alkaloids, lignans, trisaccharide, fatty acid esters, anthraquinones, scopoletin, vitamin C, minerals, octoanoic acid, potassium, sitosterol, β -carotene, vitamin A, and linoleic acid have been isolated from noni fruits, roots and leaves (Chan-Hong et al., 2006; Rasal et al., 2008; Yang et al., 2007). Recently, the total phenolic content of the EAMC was reported to be 196.8 mg of phenolic equivalents (gallic acid) per gram of extract (Serafini et al., 2011). In addition to these, high-performance liquid chromatography (HPLC) fingerprint of the EAMC demonstrated pharmacologically important phyto-chemical, namely, the flavonoid rutin (Serafini et al., 2011).

It has been proposed that phenolic compounds are antioxidants and anti-inflammatory agents. Nevertheless, there is a relationship between the antioxidant and anti-inflammatory properties of phenolic compounds. Therefore, the biological effects of the leaf extract may depend on its phenolic components (Serafini et al., 2011). The anti-inflammatory property of phenolic compounds is associated with their ability to inhibit neutrophil degranulation. Indeed, studies have shown that certain flavonoids down regulate nitric oxide production in response to inflammatory stimuli. In addition, specific flavonoids are known to chelate iron, thereby removing a causal factor for the development of free radicals. Direct inhibition of lipid peroxidation is another protective measure. Selected flavonoids can reduce system complement activation, thereby decreasing the adhesion of inflammatory cells to the endothelium and in general resulting in a diminished inflammatory response.

Lipid mediators such as prostaglandins and leucotrienes and cytokines such as TNF- α , IL-1, IL-6 and IL-8 are involved in the carrageenan-induced pleurisy. These mediators promote accumulation of neutrophils and mononuclear cells from blood vessels and activate endothelial cells (Fröde and Medeiros, 2001). In addition, these mediators promote the plasmatic extravasation that contributes to the formation of pleural exudates and migration of leukocytes, which peaked at 4 h after administration of the phlogistic agent (Fröde et al., 2001; Menegazzi et al., 2008). The investigation of anti-inflammatory property of EAMC was evaluated in the

carrageenan-induced pleurisy. The study was able to detect a marked inhibitory effect of different doses of EAMC on neutrophil, leukocytes cells migration and TNF- α level without altering mononuclear cells migration in the pleural exudates. A possible explanation for these findings may be the fact that EAMC, through activation of PPAR α and γ , inhibits the COX-2 expression and prostaglandin synthesis that is involved in the exudates formation in the inflammatory process (Hotta et al., 2010).

Dussosoy et al. (2011), also observed that several polyphenols belonging to the coumarin, flavonoids and phenolics acid groups, and two iridoid present in Noni juice have demonstrated reduced carrageenan-induced paw edema, directly inhibited cyclooxygenase COX-1 and COX-2 activities and inhibited the production of nitric oxide (NO) and prostaglandins E(2) (PGE(2)) in activated J774 cells, in a dose dependent manner. This study showed that Noni's biological effects include anti-inflammatory action through NO and PGE pathways that might also be strengthened by anti-oxidant effects. Together, the results suggested that properties of *M. citrifolia* extract should be explored further in order to achieve newer tools for managing painful and inflammation conditions, including those related to oxidant states.

Also, may be a source of new therapeutic candidates with a spectrum of activity similar to the current anti-inflammatory non-steroids such as indomethacine. Further studies are underway to investigate which compounds in the extract are responsible for the anti-inflammatory activity and the precise mechanism and site of action.

CONCLUSION

The data reported in this study show that *M. citrifolia* has important antioxidant and anti-inflammatory properties. The inhibition of proinflammatory cytokine TNF- α production seems to be a key event to these effects, as does the antioxidant effect, but further studies can clarify the exact mechanisms underlying the effects of *M. citrifolia*. Altogether, the results obtained in this study, along with previously reported data on *M. citrifolia* (Serafini et al., 2011), suggest that this plant may be an interesting candidate for the development of new therapeutic options for the treatment of inflammatory disorders.

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Conflict of interest

The authors declare no conflict of interest.

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