

***Morinda citrifolia* Linn Leaf Extract Possesses Antioxidant Activities and Reduces Nociceptive Behavior and Leukocyte Migration**

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ABSTRACT Herbal drugs have been used since ancient times to treat a wide range of diseases. *Morinda citrifolia* Linn (popularly known as “Noni”) has been used in folk medicine by Polynesians for over 2,000 years. It is reported to have a broad range of therapeutic effects, including effects against headache, fever, arthritis, gingivitis, respiratory disorders, infections, tuberculosis, and diabetes. The aim of this study was to investigate the antioxidant, anti-inflammatory, antinociceptive, and antibacterial properties of the aqueous extract from *M. citrifolia* leaves (AEMC). Antioxidant activity was observed against lipid peroxidation, nitric oxide, and hydroxyl radicals. The antinociceptive effect of AEMC was observed in the acetic acid-induced writhing test at the higher dose. Moreover, AEMC significantly reduced the leukocyte migration in doses of 200 and 400 mg/kg and showed mild antibacterial activity. Together, the results suggest that properties of *M. citrifolia* leaf extract should be explored further in order to achieve newer tools for managing painful and inflammation conditions, including those related to oxidant states.

KEY WORDS: • anti-inflammatory activity • antinociceptive activity • antioxidant activity • *Morinda citrifolia*

INTRODUCTION

MORINDA CITRIFOLIA LINN, POPULARLY known as “noni,” has been used in traditional Polynesian medicine for over 2,000 years. *M. citrifolia* (Rubiaceae) is native from southeast Asia to Australia and is cultivated in Polynesia, India, the Caribbean region, and central and northern South America.^{1,2} Cultures native to these regions favored using *M. citrifolia* for treating major diseases and used it for nourishment in times of famine. Noni fruit has been recognized by cultures as an excellent source of nutrition.³ Noni leaves have been consumed as a vegetable by many 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.⁴

About 160 phytochemical compounds in the noni plant have already been identified, and the major secondary metabolites are phenolic compounds, organic acids, and

alkaloids.⁵ Phenolic compounds are examples of herbal compounds with widely reported antioxidant and anti-inflammatory properties.

Whereas noni juice and fruit have been well characterized chemically and pharmacologically,^{1–3} few data are available regarding the properties of *M. citrifolia* leaves. Despite the lack of experimental data on potential therapeutic properties, the use of noni for different purposes is still widespread in many parts of the world. However, infusions prepared with the aerial parts (stems and leaves) of noni are used in folk medicine in northeast Brazil to treat painful conditions and as a sedative. Thus, the aim of the present study was to evaluate the antioxidant, antibacterial, antinociceptive, and anti-inflammatory properties of aqueous extracts from *M. citrifolia* leaves (AEMC).

MATERIAL AND METHODS

Samples

M. citrifolia leaves were collected in São Cristóvão, 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

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University of Sergipe. The AEMC was prepared by boiling in distilled water (7.5%; w/v) for 15 minutes; the solvent evaporated under reduced pressure and lyophilized.

Phytochemical screening

To detect the presence of various chemical constituents in AEMC, phytochemical screening was performed according to the method described by Matos.⁶ The extract was qualitatively analyzed for the presence of phenols, condensed tannins, alkaloids, volatile coumarins, flavonoids, saponins, and triterpenes. This analysis was conducted by observing colorimetric variation after the addition of specific reagents.

Total phenolic content

The total phenolic content was assayed by using the Folin–Ciocalteu reagent, following a slightly modified version of Singleton and colleagues' method.⁷ The AEMC (100 mg) was diluted to 100 mL of water. An aliquot (100 μ L) of AEMC was added to 6 mL of deionized water and 500 μ L of the Folin–Ciocalteu reagent. The mixture was shaken for 1 minute. After addition of 2 mL of 15% Na₂CO₃ solution, the mixture was shaken for 0.5 minute. The solution was then diluted with deionized water to a final volume of 10 mL. After incubation for 120 minutes at 23°C, the total phenolic content was determined by using a spectrophotometer at 750 nm. Gallic acid was used as the standard, and total phenolic content was expressed as gallic acid equivalents (g/kg of AEMC) through the calibration curve with gallic acid. The calibration curve range was 50–500 mg/mL ($R^2 = 0.998$).

High-performance liquid chromatography–photodiode-array detection analysis

The high-performance liquid chromatography (HPLC) analyses were performed on a liquid chromatograph (Prominence, Shimadzu, Kyoto, Japan) that was equipped with a vacuum degasser (DGU-20A3), autosampler (SIL-10A), 2 high-pressure pumps (LC-6A), and a photodiode-array detector (DAD) system (SIL-10A) coupled with a CBM 20A interface. Data were collected by using LC Solution software (Shimadzu). Analysis was performed on the analytical C18 Luna column (250 mm \times 4.6 mm interior diameter; 5- μ m particle diameter; Phenomenex, Torrance, California, USA) under the following conditions: flow rate 1.0 mL/minute and injection volume of 25 μ L, with mobile phase consisting of water and acetonitrile. The gradient elution for samples was as follows: 5%–50% (acetonitrile) in 30 minutes, 50%–100% (acetonitrile) in 5 minutes, 100% (acetonitrile) in 3 minutes. The DAD was set at 254 nm for acquiring chromatograms, and ultraviolet spectra were recorded between 190 and 800 nm. Identification was based on co-injections of the reference compounds and comparisons of absorption spectra.

In vitro experiments: antioxidant activity

Thiobarbituric acid test. We used the thiobarbituric acid reactive species (TBARS) assay to quantify lipid peroxidation and an adapted TBARS method to measure the

antioxidant capacity of AEMC, with egg yolk homogenate used as the lipid-rich substrate.^{8–10} Briefly, egg yolk was homogenized (1% w/v) in 20 mM phosphate buffer (pH, 7.4), and 1 mL of homogenate was sonicated. Lipid peroxidation was induced by addition of 0.1 mL of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) solution (0.12 M). Reactions were carried out for 30 minutes at 37°C. After cooling, AEMC (0.5 mL) was centrifuged with 0.5 mL of trichloroacetic acid (15%) at 1200 g for 10 minutes. An aliquot of 0.5 mL from supernatant was mixed with 0.5 mL thiobarbituric acid (0.67%) and was heated at 95°C for 30 minutes. After cooling, the absorbance of the samples was measured by using a spectrophotometer at 532 nm. The results are expressed as percentage of TBARS formed by AAPH alone (induced control).

Nitric oxide–scavenging assay. Nitric oxide was generated from sodium nitroprusside and measured by using the Griess reaction. Sodium nitroprusside in aqueous solution at physiologic pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by using the Griess reagent. Scavengers of nitric oxide compete with oxygen, thereby reducing production of nitric ions. Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with 3.0 mL of different concentrations (1, 10, 100 μ g/mL and 1 mg/mL) of AEMC and incubated at 25°C for 60 minutes. The samples were added to Griess reagent (1% sulfanilamide, 2% H₃PO₄, and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was measured at 546 nm.¹¹

Hydroxyl radical–scavenging assay. Hydroxyl radicals were generated by a Fenton system (FeSO₂-H₂O₂). When exposed to hydroxyl radicals, the sugar deoxyribose is degraded to malonaldehyde, which generates a pink chromogen on heating with thiobarbituric acid at low pH. The method for determining the scavenging on hydroxyl radicals was performed according to a previously described procedure.¹²

In vitro experiments: antibacterial activity

Microdilution assays. Microorganisms were obtained from the American Type Culture Collection and were available in the Laboratory of Mycological Research at the Federal University of Santa Maria, Brazil.

The microdilution method was used for antibacterial activity tests, as recommended by the National Committee for Clinical Laboratory Standards.¹³ Media were placed into each of 96 wells of the microplates. AEMC solutions at 256 and 512 mg/mL were added into the first rows of the microplates, and 2-fold dilutions of the compounds (256–0.06 mg/mL) were made by dispensing the solutions to the remaining wells. Ten-microliter culture suspensions were inoculated into all the wells. The sealed microplates were incubated at 35°C for 24 hours in a humid chamber. The

lowest concentration of the extracts that completely inhibit macroscopic growth was determined, and minimum inhibitory concentrations (MICs) were reported. Antimicrobial activity of the AEMC was tested against gram-positive and gram-negative bacteria.

To classify the activity of the extract, the following criteria were observed: MIC up to 100 $\mu\text{g}/\text{mL}$ is considered to indicate good antimicrobial activity; MIC from 100 to 500 $\mu\text{g}/\text{mL}$ is considered to represent moderate activity; and MIC greater than 1,000 $\mu\text{g}/\text{mL}$ is considered to show inactivity.^{14–16}

In vivo experiments

Animals. Male Swiss mice (25–30 g) were kept in a controlled-temperature room (mean temperature \pm standard deviation, $21^\circ\text{C} \pm 2^\circ\text{C}$) with light/dark cycles of 12 hours each and were allowed free access to food (Purina chow) and water. Experimental protocols and procedures were approved by the Federal University of Sergipe Animal Care and Use Committee (no. 27/09).

Pharmacologic experiments. All nociception and anti-inflammatory tests were carried out by the same observer, and experiments were conducted in a quiet room at constant temperature of 23°C . Drugs were administered orally at a dose/volume of 0.1 mL/10 g.

Antinociceptive activity: acetic acid-induced writhing. This test was performed according to the methods of Koster *et al.*¹⁷ and Broadbear *et al.*¹⁸ Abdominal contractions were induced by intraperitoneal injection of a 0.9% solution of acetic acid (10 mL/kg) in mice ($n=6$ per group). The animals in different groups were treated with AEMC (100, 200, and 400 mg/kg orally), the reference drug (morphine, 3 mg/kg, or acetylsalicylic acid, 200 mg/kg), and vehicle (saline)

60 minutes before the acetic acid administration. The number of muscular contractions was counted for 15 minutes after injection.

Anti-inflammatory activity: leukocyte migration to the peritoneal cavity. Leukocyte migration was induced by intraperitoneal injection of carrageenan (1%, 0.25 mL) into the peritoneal cavity of mice 60 minutes after administration of AEMC (100, 200, and 400 mg/kg orally), dexamethasone (2 mg/kg subcutaneously), or vehicle by modification of the technique previously described by Matos *et al.*¹⁹ The animals ($n=6$ per group) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and were euthanized by cervical dislocation 4 hours after carrageenan injection. Shortly after, saline containing EDTA (1 mM, 3 mL) was injected intraperitoneally. Immediately a brief massage was done for further fluid collection; the fluid was centrifuged ($1000 \times g$, 5 min) at room temperature. The supernatant was disposed and the precipitate was resuspended in saline. An aliquot of 10 μL from this suspension was dissolved in 200 μL of Turk solution, and the total cells were counted in a Neubauer chamber under optic microscopy. The results were expressed as the number of neutrophils/mL. The percentage of the leukocyte inhibition = $(1 - T/C) \times 100$, where T represents the treated groups' leukocyte counts and C represents the control group's leukocyte counts.

Statistical analysis

The data were evaluated by 1-way analysis of variance followed by the Tukey test. The results are expressed as the means \pm standard error to show variation in groups. Data were analyzed by using Prism software, version 5.0 (GraphPad, La Jolla, CA, USA), and differences were considered significant when the P value was less than .05.

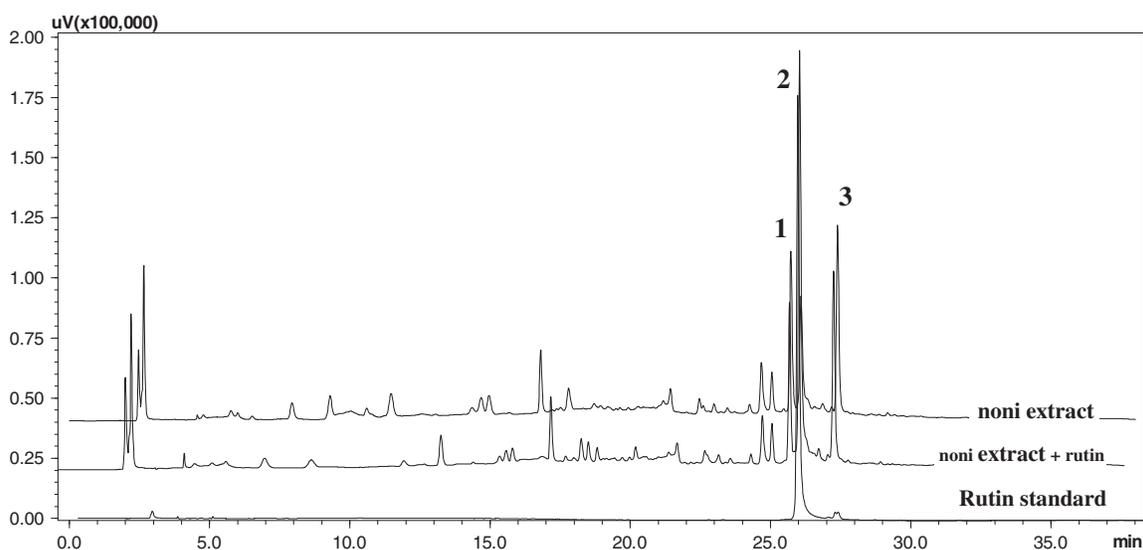


FIG. 1. High-performance liquid chromatograms at 254 nm of aqueous extract from *Morinda citrifolia* leaves and spiked with rutin. Kaempferol glycosides (peaks 1 and 3) and rutin (peak 2).

RESULTS AND DISCUSSION

The AEMC was chemically analyzed (qualitative method). The phytochemical screening of AEMC showed the presence of alkaloids, coumarins, flavonoids, tannins, saponins, steroids, and triterpenoids. Total phenolic content of the extract was 196.8 mg of phenolic equivalents (gallic acid) per gram of extract.

Qualitative analysis of AEMC was also performed by HPLC. Figure 1 shows the chromatographic profiles of AEMC and standards; good separation and resolution of peaks are visible. The phenolic compounds were characterized by retention times relative to external standards, peak spiking, and comparison of their ultraviolet spectra with the literature. HPLC-DAD analysis of the aqueous extract from leaves of noni revealed the presence of 3 major compounds (peaks 1–3), all showing ultraviolet spectra typical of flavonols.²⁰ The flavonols identified in noni are derivatives of quercetin and kaempferol.^{21,22} According to the results, flavonoids are present in the AEMC analyzed by HPLC-DAD, and, on the basis of the use of commercial standards, peak 2 was identified as quercetin-3-*O*-rutinoside (rutin). According to the published data on the ultraviolet spectra (Fig. 2) and the elution sequence in HPLC-DAD, peaks 1–3 were suggested as kaempferol glycosides.²³ According to Deng *et al.*,²³ flavonol glycosides, mainly rutin, are the major compounds in noni leaves.

It has been proposed that phenolic compounds are antioxidants and anti-inflammatory agents. Furthermore, much attention has been given to the relationship between the antioxidant and anti-inflammatory properties of phenolic compounds *in vitro* and *in vivo*.²⁴ Another anti-inflammatory property of phenolic compounds (*e.g.*, flavonoids) is their ability to inhibit neutrophil degranulation.²⁵ Indeed, studies have shown that certain flavonoids downregulate nitric oxide production in response to inflammatory stimuli.²⁶

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.²⁵

Thiobarbituric acid test

Lipid peroxidation has been defined as the biological damage caused by free radicals that are formed under oxidative stress.²⁷ Several plant extracts have been shown to inhibit lipid peroxidation, as measured by TBARS results. The lipids in membrane are continuously subjected to oxidant challenges. Oxidant-induced abstraction of a hydrogen atom from an unsaturated fatty-acid chain of membrane lipids initiates the process of lipid peroxidation, which propagates as a chain reaction. In the process, cyclic peroxides, lipid peroxides, and cyclic end peroxides are generated, which ultimately are fragmented into aldehydes such as malondialdehyde.²⁸

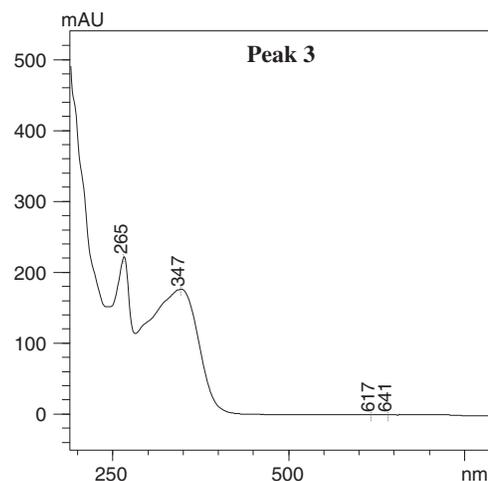
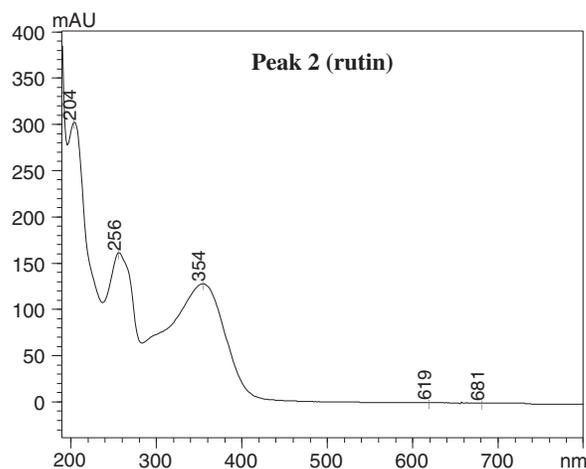
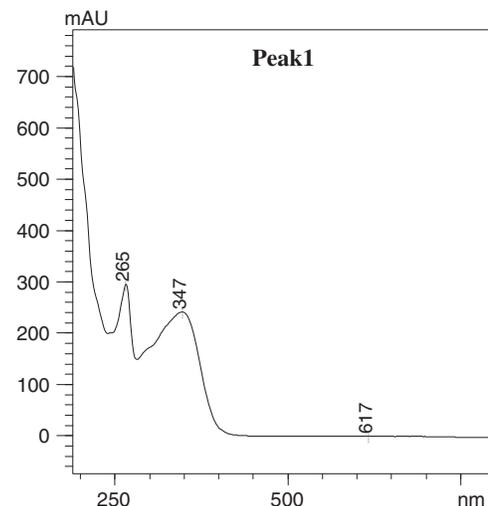


FIG. 2. Photodiode array ultraviolet spectra of flavonol glycosides (peaks 1–3) of aqueous extract from *Morinda citrifolia* leaves. AU, arbitrary units.

AEMC (all doses) inhibited the amount of TBARS generated by AAPH (Fig. 3), indicating a protective effect against lipid peroxidation. This result suggests that the AEMC may exert antioxidant protection on biomolecules such as phospholipids, triacylglycerols, and polyunsaturated

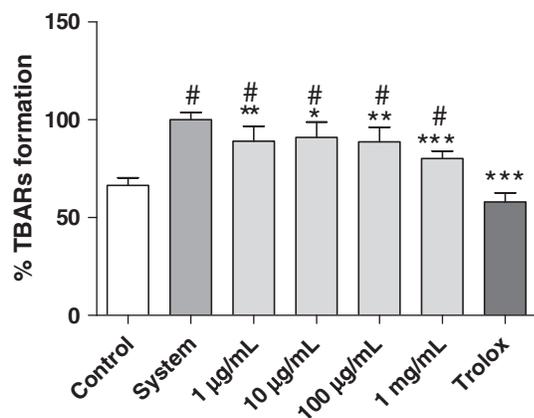


FIG. 3. Ability of different concentrations of aqueous extract from *Morinda citrifolia* leaves to prevent thiobarbituric acid reactive species formation was analyzed. Values represent mean \pm standard error; experiments were performed in triplicate. * $P < .05$, ** $P < .01$, *** $P < .001$ versus system. # $P < .05$ versus Trolox (analysis of variance followed by Tukey test). TBARS, thiobarbituric acid reactive species.

fatty acids *in vivo*, which are lipids essential to cellular membrane function and paracrine signaling.

Hydroxyl radical-scavenging assay

The hydroxyl radical is an extremely reactive species formed in biological systems and has been implicated as a highly damaging species in free radical pathology. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity.^{11,29,30} The hydroxyl radical-scavenging capacity of an extract is directly related to its antioxidant activity. The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids, and proteins.¹¹ We observed that the AEMC reduced deoxyribose oxidative damage at all concentrations (Fig. 4). The extract was highly effective in inhibiting hydroxyl damage. All doses tested (1, 10, and 100 $\mu\text{g}/\text{mL}$ and 1 mg/mL) exhibited a

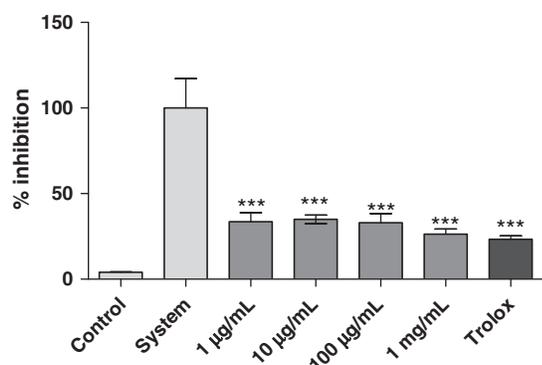


FIG. 4. Hydroxyl radical-scavenging activity of aqueous extract from *Morinda citrifolia* leaves. Values represent mean \pm standard error; experiments were performed in triplicate. *** $P < .001$ versus system (analysis of variance followed by Tukey test).

significant ($P < .05$) antioxidant activity, which was similar to Trolox® (Hoffman-LaRoche, Basel, Switzerland), a synthetic analogue of α -tocopherol used as an antioxidant standard.

Nitric oxide-scavenging assay

Nitric oxide plays an important role in various types of inflammatory processes. It is produced by macrophages in the course of the inflammatory response. It is mutagenic and can interfere with DNA repair processes.³¹ In this study, the AEMC was checked for its inhibitory effect on nitric oxide production.

To determine the ability of AEMC as a reactive nitrogen species scavenger, we evaluated the nitric oxide-scavenging activity by incubating AEMC with sodium nitroprusside, a chemical inducer of nitric oxide production. Figure 5 shows that AEMC at doses of 100 $\mu\text{g}/\text{mL}$ and 1 mg/mL significantly decreased nitric oxide production (11% and 19% of inhibition, respectively).

Microdilution assays

The AEMC showed no activity against the gram-positive and gram-negative bacteria tested except for *Aeromonas hydrophila* (Table 1). This finding could be related to the absence of anthraquinones in the extract because Chan-Blanco *et al.*² and Wang *et al.*¹ demonstrated the antimicrobial activity of this compound.

Acetic acid-induced writhing

Acetic acid-induced writhing is a visceral pain model widely used to evaluate peripheral antinociceptive activity. Although this test is a nonspecific model (*e.g.*, anticholinergic, tricyclic antidepressants, and antihistaminic and other agents

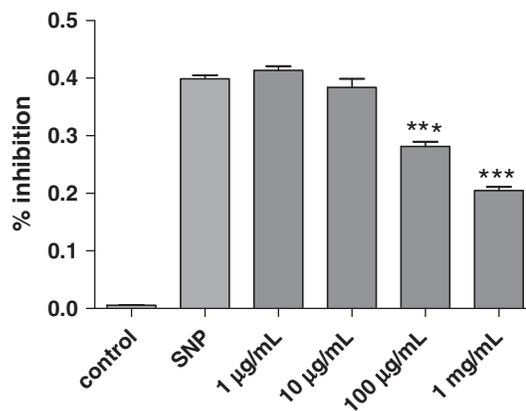


FIG. 5. Nitric oxide radical-scavenging activity of aqueous extract from *Morinda citrifolia* leaves. Values represent mean \pm standard error; experiments were performed in triplicate. Control group refers to Griess absorbance in the absence of nitric oxide inductor, and SNP group is the nitric oxide inductor sodium nitroprusside (5 mM). Different concentrations of the extracts were incubated along with sodium nitroprusside. *** $P < .001$ versus sodium nitroprusside (analysis of variance followed by Tukey test).

TABLE 1. INHIBITORY CONCENTRATION OF AQUEOUS EXTRACT FROM *MORINDA CITRIFOLIA* LEAVES AGAINST BACTERIA

Microorganisms	ATCC No.	Gram stain result	MIC (mg/mL)
<i>Salmonella typhi</i>	8739	Gram-negative	>10
<i>Escherichia coli</i>	9120	Gram-negative	10
<i>Klebsiella pneumoniae</i>	9341	Gram-negative	5
<i>Proteus mirabilis</i>	10031	Gram-negative	5
<i>Pseudomonas aeruginosa</i>	25923	Gram-negative	1.25
<i>Aeromonas hydrophila</i>	29212	Gram-negative	0.625
<i>Micrococcus species</i>	14153	Gram-positive	2.5
<i>Staphylococcus aureus</i>	27853	Gram-positive	2.5
<i>Enterococcus faecalis</i>	7966	Gram-positive	5

ATCC, American Type Culture Collection.

show activity in this test), it is widely used for analgesic screening and involves local peritoneal receptors (cholinergic and histaminic receptors) and the mediators acetylcholine and histamine.^{24,32,33} These peripheral nociceptive fibers are sensitive to both narcotic analgesic (morphine) and nonsteroid anti-inflammatory drugs, such as aspirin.³⁴

The results depicted in Figure 6 showed that the AEMC (400 mg/kg), given 60 minutes before, produced a significant ($P < .001$) reduction in abdominal constrictions induced by acetic acid in mice. This result suggests that AEMC may also help inhibit prostaglandin synthesis because nociceptive mechanisms involve the processing or release of arachidonic acid metabolites via cyclooxygenase and prostaglandin biosynthesis.³⁵ Because cyclooxygenase may be activated by nitric oxide,^{36,37} a possible link between the antioxidant and antinociceptive activities exerted by AEMC might be its nitric oxide-scavenging ability.

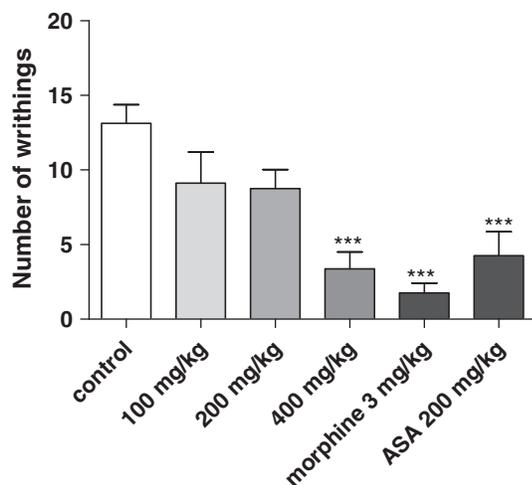


FIG. 6. Effects of aqueous extract from *Morinda citrifolia* leaves on the acetic acid-induced writhing test. Vehicle (control), aqueous extract (100, 200, and 400 mg/kg), morphine, or acetylsalicylic acid were administered orally 0.5 hour before acetic acid injection. Each column represents mean \pm standard error ($n=6$). *** $P < .001$ versus control (analysis of variance followed by Tukey test). ASA, acetylsalicylic acid.

Leukocyte migration to the peritoneal cavity

To determine the probable anti-inflammatory activity of AEMC, the peritonitis-induced-by-carrageenan test was performed in mice. The carrageenan is known to be an excellent acute inflammatory agent in which fluid extravasation, leukocyte migration, and biochemical parameters in the exudate can be easily detected. Injection of carrageenan on the dorsal surface of mice initiates an inflammatory process.³⁸

Because chemotaxis is important in the initiation and exacerbation of an inflammatory response, our results confirm some of the signs of inflammation, such as a decrease in the leukocyte count, observed in the blood of mice that were treated by AEMC ingestion.

Figure 7 shows the inhibitory effect of AEMC (200 and 400 mg/kg) and dexamethasone (2 mg/kg subcutaneously) on carrageenan-induced responses. There was significantly ($P < .05$) decreased leukocyte migration (predominantly neutrophil migration).

The results of this test showed that the AEMC significantly reduced the number of recruited cells. These findings therefore suggest that AEMC contains active anti-inflammatory agents.

The role of nitric oxide in physiologic and pathologic processes such as inflammation is well-known. It is reported that nitric oxide itself expresses chemotactic activity.^{24,39} In addition, reactive oxygen species and reactive nitrogen species have also been reported to act as proinflammatory signals *in vivo* by stimulating the activation of tumor necrosis factor- α , interleukin-1 β , and interleukin-6 genes through the activation of the redox-sensitive transcription factor nuclear factor- κ B.⁴⁰ The antinociception and anti-inflammatory effects induced by AEMC could thus also be a

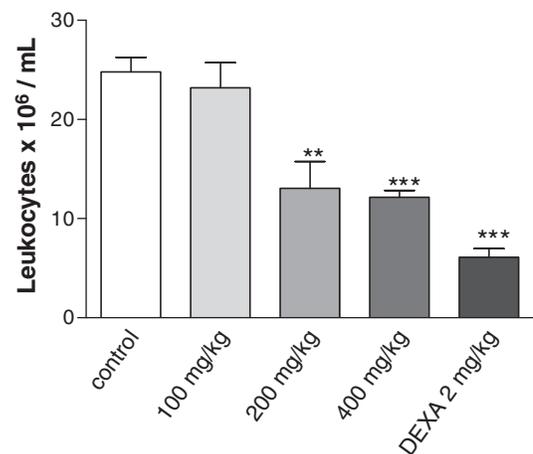


FIG. 7. Effect of aqueous extract from *Morinda citrifolia* leaves (100, 200, and 400 mg/kg) on leukocyte migration into the peritoneal cavity induced by carrageenan. Groups of mice were pretreated with vehicle or dexamethasone (2 mg/kg). Each value represents the mean \pm standard error. *** $P < .001$ and ** $P < 0.01$ versus control group (analysis of variance followed by Tukey test) ($n=6$). DEXA, dexamethasone.

consequence of its antioxidant ability, which may prevent the free radical-induced nuclear factor- κ B activation and consequent proinflammatory cytokine production, a cycle that perpetuates inflammatory processes.⁴¹

CONCLUSION

Our results indicate that AEMC has mild antibacterial activity and significant antioxidant, antinociceptive, and anti-inflammatory effects, which provide pharmacologic evidence for folk uses of *M. citrifolia* Linn. Other studies have reported that the *M. citrifolia* leaf extract contains polyphenols. Therefore, the biological effects of the leaf extract may depend on its phenolic components. However, the dosage used appears to be high, and additional studies are necessary to evaluate the potential toxicity of the extract and its possible safe and therapeutically efficient dosage in humans. In addition, further studies, isolation, and characterization will be undertaken to correlate the pharmacologic activities with the chemical constituents.

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AUTHOR DISCLOSURE STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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