International Journal of Advanced Engineering Research and Science (IJAERS)
Peer-Reviewed Journal
ISSN: 2349-6495(P) | 2456-1908(O)
Vol-9, Issue-9; Sep, 2022
Journal Home Page Available: https://ijaers.com/
Article DOI: https://dx.doi.org/10.22161/ijaers.99.15

Antioxidant, anti-inflammatory and antimicrobial activities promoted by hydroalcoholic extract of *Laguncularia racemosa* (l) c.f. leaves Gaert

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Received: 05 Aug 2022,
Received in revised form: 02 Sep 2022,
Accepted: 08 Sep 2022,
Available online: 14 Sep 2022

***Keywords*— Combretaceae, Hydroalcoholic extract, biological activities, antimicrobials**

***Abstract*—** *This study aimed to evaluate the antioxidant, anti-inflammatory, antimicrobial and toxicity potential of the hydroalcoholic extract of the leaves of L. racemosa (EHALr) a mangrove plant. The results showed that the extract was able to promote moderate antioxidant activity. In acute toxicity assays, EHA-Lr showed low toxicity, with no significant changes in animal and organ weights and in biochemical and hematological parameters. In the evaluation of anti-inflammatory activity, the 200 mg/kg dose of EHA-Lr was most effective in reducing leukocyte migration in the paw edema model and in the LPS-induced acute lung inflammation model the 50 mg/kg dose was most effective in reducing plantar volume. The antimicrobial potential of the hydroalcoholic extract was observed against Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Pseudomonas aeruginosa, Serratia marcencens, Escherichia coli and Enterococcus faecalis strains, proving to be effective. However, the extract was most active against the Micrococcus luteus strain (0.93mg/mL). The hydroalcoholic extract of L. racemosa leaves showed anti-inflammatory and antimicrobial activities, with low toxicity, which makes it a strong promise to be applied or combined in clinical therapy.*

I. INTRODUCTION

Medicinal plants have been used to combat different diseases since the earliest records of human civilization. According to the World Health Organization (WHO), approximately 80% of the population in developing countries face difficulties in acquiring synthetic medicines and use traditional medicines, especially those of plant origin, to meet their basic health needs (SULTANA et al. 2015).

Through the development of research in the field of natural products, several biological activities have been systematically and widely studied using botanical material

as the main raw material, such as antimicrobial, anti-inflammatory, antioxidant, gastroprotective, anxiolytic and, more recently, antineoplastic (RTIBI et al. 2015; AL-ANSARI et at. 2019; SHARMA et al. 2017; GONULALAN et al. 2019; SIEW et al. 2019). In this intention, several botanical families have been studied seeking the determination of medicinal properties or aiming at the prospection of bioactive compounds. In Brazil, plants from practically all ecosystems are studied, from the Amazon, Atlantic Forest and Caatinga to the Mangrove, which is still little explored (LIPORACCI, 2014; BARTZ et al. 2015).

Four species occur in Brazilian mangroves (*Rhizophora mangle L.* - Rhizophoraceae, Avicennia schaeuriana Stapf & Leechm. ex Moldenke and Avicennia germinans (L.) Stearn - Acanthaceae, and *Laguncularia racemosa* (L.) C. F. Gaertn. - Combretaceae). The latter is popularly known as mangrove. It is of global range, with greater presence in North and South America, and overall, plays great importance for the ecosystem where it inhabits (SILVA, 2012; NETO et al. 2015). In this sense, *Laguncularia racemosa* (L) C. F. Gaertn has been the target of several studies involving its interaction in the mangrove ecosystem, in terms of pollution monitoring and in interaction studies with some microorganisms, however, pharmacological and phytochemical studies with this species are scarce (DE FREITAS GUEDES et al. 2018; REINERT et al. 2016; SODRÉ et al. 2013).

Some pharmacological studies indicate that *L. racemosa* has potential molluscicidal activity (MENDES et al. 2018), antioxidant activity (XUE et al, 2008) and inhibition of PLA2 and other pathways of the inflammation cascade (PINHO et al, 2014). And a phytochemical study revealed in this plant the presence of some compounds, such as tannins and flavonoids, with possible antioxidant activity and protein kinase inhibitors (PINHO, 2014).The literature shows that other species representing the Combretaceae family showed antimicrobial, antioxidant and anticancer activities, a fact that reveals a huge potential for further investigation of *L. racemosa*, considering that chemotaxonomic factors determine that species of the same family may possess similarities in phytochemical constitution and, consequently, biological activities (RAJABPOUR et al, 2019; SANTOS et al. 2018 SIMÕES et al. 2017; KATERERE et al. 2012).

In view of these possible biological activities and facing the high cost, therapeutic limitations and side effects evidenced with the drugs currently employed for the treatment of pathologies. The present study proposes an evaluation of the antioxidant, toxicity, anti-inflammatory and antimicrobial activities of the hydroalcoholic extract of *L. racemosa*.

II. MATERIAL AND METHODS

Reagents

Ethanol 100% (Vetec), methanol (Merck) heparin sodium 5.000 IU/mL (Cristalia), evans blue (Sigma), HEMSTAB EDTA 15 g/dL (Labtest), ketamine hydrochloride (Vetbrands), xylazine hydrochloride (Vetbrands); Griess reagent (Sigma), iodized alcohol, 70% alcohol, 9% saline, PBS (Phosphate Buffered Saline), carragenina e LPS (lipopolissacarídeo bacteriano) indometacina, NaCl Mueller-Hinton (HIMEDIA®) resazurina dexametasona Broth Heart Infusion (HIMEDIA®), Folin-Ciocalteu (Merck) carbonato de sódio (Merck), ácido ascórbico (Merck), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Merck), 2,2´- azinobis(3-etilbenzotiazolina-6-ácido sulfônico) (ABTS) (Merck), butylated hydroxytoluene (Merck), cloreto de alumínio(Merck), acetato de sódio (Merck).

Collecting plant material and obtaining extract from the leaves *Laguncularia racemosa*

The leaves of the species *L. racemosa* were collected in the mangrove of Tamandaré (8° 44′ 54″ South, 35° 6′ 14″ West), southern coast of the State of Pernambuco, identified and deposited in the Herbarium UFP- Geraldo Mariz, located in the center of Biocências of the Federal University of Pernambuco, with the accession number *Laguncularia racemosa* (Combretaceae); Soares, C. (01) UFP 83.203. Then, the plant material was dried in an oven (Tecnal, TE-393/1) at 45 ºC for 48 hours, then ground in a knife mill (FRITSCH- Pulverisette 14) and sieved in a 80 mesh particle size range according to the methodology proposed by Melo et al. (2022).

To obtain the hydroalcoholic extract, 50 g of the plant material was used for 500 mL of ethanol/water solution 70% (v/v), in which it was macerated at room temperature for 48 hours, kept in amber glasses, under the shelter of sunlight and with occasional agitation. After that, this extractive material was filtered, concentrated in a rotary evaporator, under reduced pressure, at a temperature of 40-50°C and then lyophilized to obtain the hydroalcoholic extract of Laguncularia racemosa dry leaves. The yield of the extract was determined by Equation 1.

$$\text{Yield (\%)} = \left(\frac{\text{Mass of the extract obtained (g)}}{\text{Mass of leaves (g)}}\right) * 100\% \quad (1)$$

Partial characterization of the hydroalcoholic extract by UV/Visible spectroscopy

Determination of total phenolic content

Total phenolic content was determined according to Nerys et al. (2022) and Royani et al. (2022) with modifications.

For this, 1 mL of extract at the concentration of 1000 µg/mL was added to 1 mL of Folin-Ciocalteu reagent (1/10 v/v) and allowed to react for 1 minute. Subsequently, 2 mL of sodium carbonate (2% w/v) was added to the system (extract + reagent), homogenized in vortex and incubated for 2 hours in the absence of light under ambient conditions (25°C). The samples were analyzed at a wavelength of 765 nm in a UV-vis spectrophotometer (Hewlett-Packard, model 8453).

The equipment blank was formed under the same conditions as the sample, using distilled water instead of the extract. After incubation, the total phenolic content of the extract was calculated using a curve prepared with standard gallic acid at different concentrations (3.12 -500 µg/mL). The total phenolic content was expressed as mg GAE (gallic acid equivalent) per g extract. The tests were performed in triplicate.

Determination of total flavonoid content

The assay for determination of total flavonoid content was performed according to the methodology proposed by Nerys et al. (2022) and Royani et al. (2022) with few modifications. The extract was diluted in 70% ethanol at a concentration of 1000 µg/mL. In a 10 mL volumetric flask, 1.0 mL of the extract solution, 1.0 mL of the 2% ethanol-aluminum chloride reagent was added and the volume was made up with ethanol. The absorbances were determined after 30 min at 425 nm in a spectrophotometer (Hewlett-Packard, model 8453). The equipment blank was formed under the same conditions as the sample, using water instead of the extract. After incubation, the total flavonoid content present in the extract was calculated using a curve with the quercetin standard at different concentrations (3.12 -500 µg/mL). The total flavonoid content was expressed as mg QE (quercetin equivalent) per g extract. The tests were performed in triplicate.

Determination of total flavonols content

The content of total flavonols in the hydroalcoholic extract was previously determined by Nerys et al. (2022) with modifications. The extract was diluted in 70% ethanol at a concentration of 1000 µg/mL. The assay consisted of 2 mL of extract, 2 mL of AlCl 3 (2%)/ethanol, and 3 mL of sodium acetate (50 g/L). The mixture was stirred and incubated for 2.5 h at 20°C. After this period, the absorbances were determined at 440 nm in a spectrophotometer (Hewlett-Packard, model 8453). The equipment blank was formed in the same proportions as the sample, using water instead of the extract. After incubation, the total flavonol content present in the extract was calculated using a quercetin curve at different concentrations (3.12 -500 µg/mL). The total flavonol content was expressed as mg QE (quercetin equivalent) per g extract. The tests were performed in triplicate.

Determination of total tannin content

The total tannin content in the hydroalcoholic extract was previously determined by Nerys et al. (2022) and Maobe et al. (2022) with modifications. The extract was diluted in 70% ethanol at a concentration of 1000 µg/mL. The assay consisted of 2 mL extract, 3 mL distilled water, 0.5 mL Folin-ciocalteu reagent. The system was reacted for 3 min. After this period, 1.5 mL of 17% sodium carbonate (Na_2CO_3) and 3 mL of distilled water were added, totaling 10 mL. The samples were homogenized and incubated in the dark for 2 hours. After this period, the absorbance of the samples was determined by a UV/Vis spectrophotometer (Hewlett-Packard, model 8453) at a wavelength of 725 nm. The equipment blank was formed in the same proportions as the sample, using water instead of the extract. After incubation, the total tannin. The content present in the extract was calculated using a curve using standard tannic acid prepared with standard quercetin at different concentrations (3.12 -500 µg/mL). The tannin content was expressed as mg TAE (tannic acid equivalent) per g extract. The tests were performed in triplicate.

In vitro antioxidant activity

The DPPH free radical scavenging activity was determined by the method described by Ita et al. (2022) and Nerys et al. (2022) with minor modifications. For the DPPH assay, 0.70 mL of sample or standard (ascorbic acid or BHT) extract with varying concentrations (0 to 1000µg/mL) were added to the same volume of DPPH methanolic solution (100 µM). The mixtures were shaken vigorously and left to incubate for 20 minutes in the dark at room temperature. A decrease in absorbance was measured at 515 nm against a methanol blank without DPPH using a Hewlett-Packard spectrophotometer, model 8453. The absorbance measured for the control solution was in the range of 0.75 ± 0.01. The percentage of DPPH discoloration inhibition was calculated using Equation 2.

$$[\text{DPPH}](\%) = \left(\frac{\text{ABS control-ABS sample}}{\text{ABS control}}\right) \text{x} 100 \qquad (2)$$

Control ABS: is the absorbance of the control; sample ABS: absorbance of the samples at different concentrations.

ABTS method

ABTS radical scavenging activity was determined according to the method described by Ita et al. (2022) and Nerys et al. (2022) with some modifications. The ABTS + stock solution was produced by reacting the aqueous ABTS solution (7 mM) with 2.45 mM potassium persulfate aqueous solution in equal amounts and allowed to react for 12-16 h at room temperature in the dark. Then, 1 mL of ABTS + solution was mixed with 0.50 mL of the extract at

different concentrations (0 to 1000µg/mL). The mixture was then incubated at room temperature for exactly 10 min in the dark. The control was prepared by mixing 1 mL of ABTS+solution with 0.50 mL of double-distilled water. The absorbance measured for the control solution was in the range of 0.38 ± 0.04. The percent elimination activity results were calculated as % inhibition using Equation 2. The experimental standards were ascorbic acid and BHT at the same concentrations as the extract. All experiments were performed in triplicate.

Determination of EC_{50}

The amount of antioxidant required to decrease the initial concentration of DPPH or ABTS by 50%, called EC_{50}, was determined from a non-linear fit, obtained graphically by plotting the concentration of the samples against the antioxidant capacity. Where, the higher the consumption of DPPH or ABTS by a sample, the lower its EC_{50} and the higher its antioxidant activity.

Experimental animals

For the biological activity studies, 40 adult male Swiss albino mice (*Mus musculus*) weighing between 25 and 35g - from the bioterium of the Antibiotics Department of the Federal University of Pernambuco - were used. In addition, 48 albino *Mus musculus* mice of the Balb/c strain were used (6 to 8 weeks old), which were bred and maintained in the Keizo Asami Laboratory of Immunopathology (LIKA), also located in the Federal University of Pernambuco.

The animals were housed in polyethylene cages with stainless steel bars. They had free access to water and balanced feed (Labina/Presence), and were kept in an environment with a temperature of 22 ± 2°C and controlled light providing a 12-hour light-dark cycle. All animals were submitted to fasting, with the withdrawal of the feed about 4 hours before the beginning of the experiment. However, during the experiment, the animals had free access to drinking water. The animals were kept according to the international guidelines of the Council for Experimental Laboratory Animals Council for Experimental Laboratory Animals (ICLAS).

Ethical Procedures

All experiments were performed according to the standards established by the Brazilian Society of Animal Science (SBCAL) and the standards established by the National Institute of Health Guide for the Care and Use of Laboratory Animals. This work was approved by the Ethics Committee on the Use of Animals of the Federal University of Pernambuco (CEUA-UFPE), under protocol number 23076.030374/2018-85. In addition, it is in accordance with current regulations in Brazil, especially Law 9.605 - art. 32 and decree 3.179 - art. 17, of 21/09/1999, which deals with the issue of animal use for scientific purposes.

Acute Toxicity Evaluation

The methodology recommended by the Organization for Economic Cooperation and Development Guideline 423 (OECD, 2001) was used for acute toxicology evaluation. Female mice (60 days old) were randomly assigned to two groups of three animals. A single dose was administered orally to the groups of test animals. The control group (n=3) received the vehicle water, the treated group (n=3) received the crude hydroalcoholic extract (EHA-Lr) at a dose of 2,000mg/kg.

The animals were observed for the first two hours and then every 24 hours daily for 14 days after administration of the extract. The evaluation was performed by the hypocratic screening method, in addition, weight, water and feed consumption were evaluated daily. On the 14th day, the animals were anesthetized with ketamine and xylazine (2:1; v/v) intraperitoneally to collect blood by cardiac puncture and perform hematological and biochemical tests. The liver, kidneys, spleen, brain, ovaries, lung, heart, and stomach were collected for micro and macroscopic analysis as well as to determine the relative weight of the organs.

Afterwards the whole experiment was repeated under the same initial conditions to confirm the results obtained. The results were analyzed and all parameters were evaluated in the same way and expressed as means between the groups. The relative weight of the organs was determined by Equation 3.

$$\text{Relative weight (\%)} = \left(\frac{\text{Organ weight (g)}}{\text{Animal weight (g)}}\right) * 100 \quad (3)$$

Anti-inflammatory activity

For the evaluation of the anti-inflammatory activity of the hydroalcoholic extract, the carrageenan-induced paw edema technique (Carrageenan-induced paw edema test) and LPS (bacterial lipopolysaccharide) described by Winter et al. (1962), Henriques et al, (1987) Hamad et al. (2019), Siraj et al. (2021) with modifications. Thus, the animals were divided into 5 groups containing one (n=8) animal per group, the groups generally received the substances orally, the groups treated with hydroalcoholic extract (EHA-Lr) at doses of 50, 100 and 200mg/kg. one. The standard used was indomethacin (non-steroidal anti-inflammatory drug) at a dose of 10 mg/kg. The negative control group received only water. One hour after treatment, inflammation was induced by an intra-plantar (i.pl.) injection of 50 µL of carrageenan (1%) in the right hind leg. The other leg of the animal was also analyzed for volume and compared to the paws that received the carrageenan. The paw volumes were measured

before induction by the flogging agent and after induction at 0.5, 1, 2, 3, 4, 5, and 6 hours after carrageenan. The volume of edema, in milliliters (mL), was recorded using a plethysmometer (Ugo Basile, Italy). The animal's hind paw was submerged up to the tibio-tarsal junction in the reading chamber of the device. The volume of fluid displacement was digitally recorded and corresponded to the volume of the paw. The results were expressed as the difference in volume (mL) between the foot that received carrageenan and the contralateral paw that did not receive carrageenan.

LPS-induced acute lung inflammation

Forty-eight female Balb/c mice were divided into six groups of (n=8) animals: control group with saline solution (NaCl 0.9%); LPS group; LPS + dexamethasone group (0.5 mg/kg); LPS + hydroalcoholic extract of L. racemosa groups (50, 100 and 200 mg/kg). The animals received saline, dexamethasone or the extract, by gavage, and after 1 hour were challenged with 25 µL of lipopolysaccharide (LPS 1 mg/mL saline) by intranasal instillation. Twenty-four hours after LPS challenge, the animals were euthanized by anesthetic overdose (ketamine 300mg/kg + xylazine 30mg/kg) and bronchoalveolar lavage was collected for leukocyte migration analysis. Following the protocols described by Lee et al. (2016), Daram et al. (2021) and Talwar et al. (2021) respectively.

Bacterial strains and inoculum preparation

The strains tested included the species *Staphylococcus aureus* (UFPEDA 02), *Micrococcus luteus* (UFPEDA 100), *Bacillus subtilis* (UFPEDA 86), *Pseudomonas aeruginosa* (UFPEDA 416), *Serratia marcencens* (UFPEDA 352), *Escherichia coli* (UFPEDA 224) and *Enterococcus faecalis* (UFPEDA 138) from the microorganism collection of the Antibiotic Department of the Federal University of Pernambuco. To prepare the inoculum, the bacteria were cultured in Broth Heart Infusion (HIMEDIA®) plus 5% defibrinated sheep blood (Sigma) at 35°C for 24-48 hours. After incubation, the obtained colonies were suspended in sterile 0.9% NaCl solution and adjusted according to McFarland 0.5, corresponding to a concentration of 1.5 x 108 colony forming units CFU/mL (Stoppa et al. 2009).

In vitro antimicrobial activity

The antibacterial activity of the hydroalcoholic extract of *L. racemosa* was quantitatively evaluated by the broth microdilution technique (CLSI, 2021). All samples were tested in triplicate and Erythromycin was used as an evaluation parameter, as a positive control and dimethylsulfoxide (DMSO) 5%, as a negative control. In the technique used, 100 µL of Mueller-Hinton broth (HIMEDIA®) was pipetted into each well of the microtiter plate. Then, 100 µL of the stock solution of the hydroalcoholic extract was added to the first well, with subsequent homogenization, followed by serial dilutions to obtain the 8 final concentrations for the hydroalcoholic extract (0.23 to 30 mg/mL). Then, the plates were incubated for 24 hours to determine the Minimum Inhibitory Concentration (MIC). After incubation, the plates were read, using the bacterial growth developer resazurin (0.01 mg/mL) was added to all wells, followed by incubation for 1 hour and subsequent reading of the plates (Cortinhas et al. 2013). The minimum inhibitory concentrations were considered those capable of inhibiting the growth of the microorganism in 90% of the bacterial isolates. The experiments were performed in triplicate. The standard drug used was streptomycin, under the same conditions as the hydroalcoholic extract of *L. racemosa*.

Statistical analysis

The carrageenan-induced paw edema experiments were statistically evaluated statistically evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni test with 95% confidence interval, using Graph Pad prism 5.0 software. Values of "p" less than 0.05 ($p<0.05$) were considered as indicative of significance. The others experiments were statistically evaluated by one-way analysis of variance (ANOVA), followed by the Newman-Keuls test with a 99% confidence interval, using Graph Pad Prism 7.0 software. P-values less than 0.01 ($p<0.01$) were considered indicative of significance as indicative of significance. In parallel, the antimicrobial activity (MIC) data obtained were plotted using Microsoft Excel® version 2010.

III. RESULTS AND DISCUSSION

Yield of obtaining the hydroalcoholic extract and partial characterization by UV/Visible spectroscopy

The yield of the hydroalcoholic extract obtained from the leaves of *L. racemosa* was 12.7% a value close to that obtained by Soares (2018) who evaluating the same extract obtained a yield of 10.47%. Knowing the constituents of the hydroalcoholic extract of *L. racemosa* is an important step for possible applications. In table 1 presents the total contents of phenols, flavonoids, flavonols, and tannins respectively.

Table 1. Content of phenolics, flavonoids , flavonols and tannic acid determined by UV/Vis spectroscopy present in the hydroalcoholic extract.

Constituents	Hydroalcoholic Extract
Total Phenolic Content (mg EGA/g extract)	445,4 ± 0,1
Total Flavonoid Content (mg EQ/g extract)	247,2 ± 0,9
Total Flavonols content (mg EQ/g extract)	155,7 ± 1,9
Tannin content (mg ETA/g extract)	34,7 ± 0,8

Average ± standard deviation. EAG: gallic acid equivalent . EQ: quercetin equivalent . ETA: tannic acid equivalent.

Few works are reported in the literature performing the characterization of the hydroalcoholic extract of *L. racemosa*. Among these are those performed by Soares (2018) who verified the presence of total phenols, tannins, flavonoids and coumarins and quantitatively presented total phenols and tannins content of 128.24 ± 2.6 and 113 ± 0.43 mg EAT/g, respectively, of flavonoids 85.75 ± 7.19 mg ER/g and coumarins 226.36 ± 8.7 mgEC/g. In addition, Rodrigues et al. (2015), Mendes et al. (2018) and Costa et al. (2021) identified different phenolic compounds present in *L. racemosa* extract.

***In vitro* antioxidant activity**

Extracts of different plants are described in the literature as good antioxidant agents. In many cases this activity is directly related to the presence phenolic constituents present (Wang et al. 2021; Xu et al. 2021). Phenolic compounds act as radical scavengers and sometimes as metal chelators, acting at both the initiation and propagation stages of the oxidative process Wang et al. 2021). Figure 1 shows the antioxidant activity curves promoted by hydroalcoholic extract of *L. racemosa* at different concentrations for DPPH (A) and ABTS (B) assays respectively.

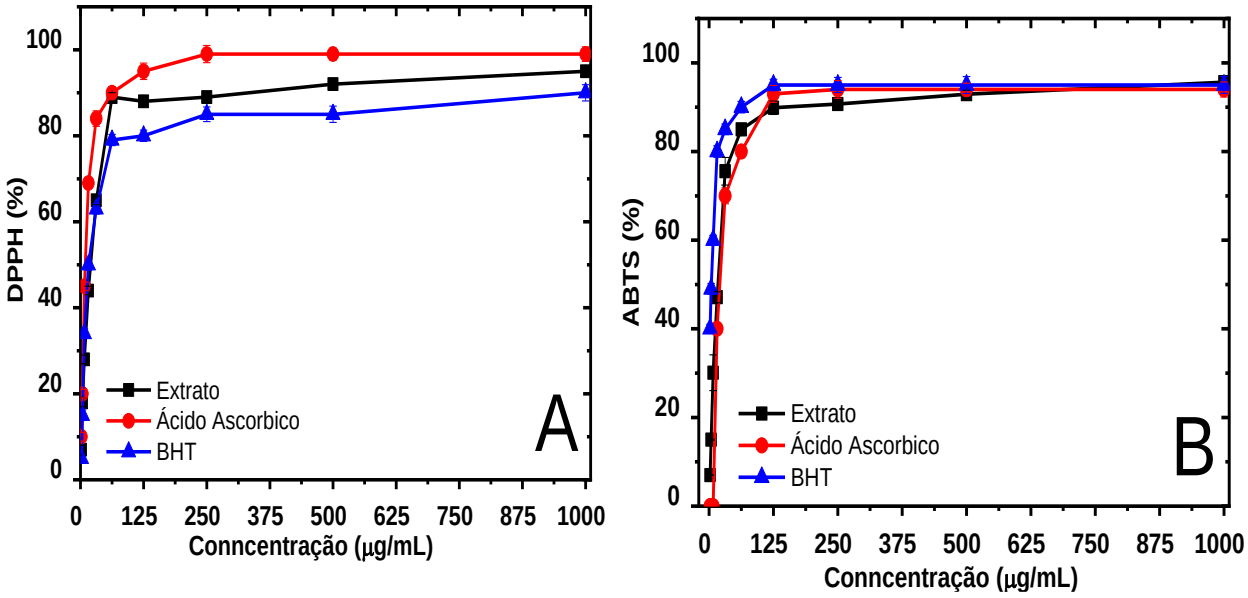

Fig.1. Antioxidant activity promoted by hydroalcoholic extract of L. racemosa by in vitro DPPH (A) and ABTS (B) methods at different concentrations compared to ascorbic acid and butylated hydroxytoluene (BHT) standards, respectively.

The results presented in Figure 1 show that the extract promotes increased antioxidant activity with increasing concentration for both activity assays. Similar profile were obtained by Ahmed et al. (2014), Kaneria et al. (2018) and Bui et al. (2021) evaluating different extracts of plants belonging to the Combretaceae family. Through the obtained curves it was possible to determine the EC50 values (concentration capable of capturing radicals by 50%) for the extract for each of the assays. Table 2 shows the EC50 values for the extract and for the standards ascorbic acid and butylated hydroxytoluene (BHT).

Table 2. EC50 results for L. racemosa hydroalcoholic extract and for ascorbic acid and butylated hydroxytoluene (BHT) standards against DPPH and ABTS assays

Samples	DPPH EC_{50} (µg/mL)	ABTS EC_{50} (µg/mL)
L. racemosa extract	41,90 ± 3,15	21,78 ± 0,5
Ascorbic Acid	18,97± 0,4	5,24 ± 0,1
Butylated Hydroxytoluene (BHT)	7,75 ± 0,1	13,37± 0,01

Average ± Standard Deviation

The results presented in Table 2 show that the hydroalcoholic extract presented lower results when compared to the evaluated standards. This fact indicates that the extract presents moderate antioxidant activity in vitro. Moreover, it showed better results for the ABTS radical scavenging assays when compared to the DPPH assay.

This better result may be related to the versatility of the ABTS radical scavenging method when compared to the DPPH radical scavenging assay, since the latter shows better results when the molecules evaluated are polar, while the ABTS assay evaluates polar and apolar compounds at the same time (Floegel et al. 2011; Schaich et al. 2015).Similar data to those expressed in Table 2 were found by Ibrahim and collaborators (2022), of which their DPPH showed IC 50 of 50.3 µg/ml for mangrove ethyl acetate extract (MEE).

Similar results were obtained by Ahmed et al. (2014) obtained mean EC 50 values of the extracts for DPPH and ABTS antioxidant assays ranging from 0.21 - 12 µg/mL (*Combretum padoides*), 0.25 - 16 µg/mL (*Combretum vendae*), 0.33-9.41 µg/mL (*Combretum woodii*) and 4, 97-85 µg/mL (*Combretum bracteosum*), respectively, while the mean EC 50 values for the positive controls ascorbic acid and trolox were 1.28 - 1.51 and 1.02-1.19 µg/mL, respectively. All crude extracts inhibited lipid peroxidation of linoleic acid by more than 80% at the concentration of 64 µg/mL.

Awotunde et al. (2017) evaluating the Hydroethanol and Saponin fractions from Terminalia schimperiana root found that the polar hydroethanol fraction showed potent DPPH antioxidant activity with EC50 value of 19.36± 0, 436 µg/mL and ABTS elimination activity with EC50 value of 0.9420 ± 0.011 µg/mL, while the Saponin fraction has moderate DPPH elimination activity with EC50 of 59.33 ± 0.417 µg/mL and moderate ABTS elimination activity with EC50 value of 2.273 ± 0 µg/mL.

Sousa et al. (2021) performing different antioxidant assays, found that the intermediate fraction of the stem bark (28.5 ± 0.60 µg/mL) and the ethyl acetate fraction of the leaves (40 ± 0, 56 µg/mL) showed higher % inhibition of DPPH free radical activity, while the intermediate stem bark fraction (27.5 ± 0.9 µg/mL) and the hydromethanol fraction of the leaves (81 ± 1.4 µg/mL) demonstrated inhibition of the ABTS free radical. These results show that the hydroalcoholic extract of *L. racemosa* can be used as a promising antioxidant agent.

Acute Toxicity Evaluation

The results of the toxicity assays showed that the hydroalcoholic extract of *L. racemosa* was not able to promote signs of toxicity and mortality within a period of 14 days after administration of the extract. The lethal dose, 50% (LD50) of this extract was estimated to be above 2000 mg/kg. Furthermore, the results indicated no statistically significant differences between control and extract treated mice in the amount of food, weight, water consumed and the relative weight of different organs (lung, ovary, brain, heart, kidney, liver, spleen and stomach) as presented in Table 3.

Table 3. Ponderal evolution, water and feed consumption, and relative weight of the organs of mice in the control group and the group treated with hydroalcoholic extract of L. racemosa leaves at a dose of 2,000 mg/kg.

Parameters	**Control**	**EHA-L.r**
Initial weight (g)	25,1 ± 1,58	24,7 ±1,81
Final weight (g)	36,0 ± 1,08	34,4 ± 2,67
Water consumption per animal (mL)	10,67 ± 0,95	11,20*± 0,74*
Feed consumption per animal (g)	5,69 ± 0,41	6,49*± 0,18*
Relative weight of organs (%)	**Control**	**EHA-L.r**
Lung	9,70 ± 0,46	9,04 ± 0, 37
Ovary	0,04 ± 0,01	0,03 ± 0,01
Brain	4,17± 0,46	4,09 ± 0,33
Heart	1,82 ± 0,26	1,58 ± 0,42
Kidney	6,00 ± 064	6,27 ± 0,69
Liver	56,00 ± 064	53,15 ± 5, 86
Spleen	6,27 ± 0,69	7,30 ± 0,85
Stomach	10,27 ± 0,69	10,01 ± 0,47

Mean ± Standard Deviation; *p<0.05. Significant after two-way analysis of variance (ANOVA) followed by Bonferroni test with 95% confidence interval when compared to the control group.

In addition to weight, water consumption, food consumption, and relative organ weight, the effects of the hydroalcoholic extract of L. racemosa on biochemical and hematological parameters were analyzed. These results are presented in Table 4.

Table 4. Biochemical and hematological parameters of the control and treated groups with hydroalcoholic extract of Laguncularia racemosa in acute toxicity at a dose of 2,000 mg/kg.

Biochemical parameters	**Control**	**EHA-L.r**
ng/dL)	: 9,73	: 7,33
ine (mg/dL)	0,03	: 0,06
J/L)	± 63,3	± 24,6
J/L)	: 9,50	: 23,6
e Phosphatase (U/L)	± 36,7	± 30,1
Total (g/dL)	: 1,12	: 0,52
n (g/dL)	: 0,26	: 0,21
e (mg/dL)	± 10,8	: 9,29
Hematological Parameters	**Control**	**EHA-L.r**
RBC (mm3)	: 0,33	: 0,41
Hemoglobin (g/dL-1)	± 0,03	: 0,05
Hematocrit (%)	,31	,46
MCV (fL)	: 0,05	: 0,07

MCHC (pg)	± 0,07	± 0,01
MCHC (%)	± 0,32	± 0,28
Platelets (mm3)	3 ± 0,26	3 ± 0,21
Total Leukocytes (mm3)	: 0,48	: 0,29
Segmented neutrophils (%)	40	45
Eosinophils (%)	01	04
Lymphocytes (%)	57	50
Monocytes (%)	02	01

Mean ± Standard Deviation; *p<0.05. Significant after two-way analysis of variance (ANOVA) followed by Bonferroni test with 95% confidence interval when compared to the control group.

Biochemical and hematological parameters remained in the normal range, with no statistically significant difference between the mice in the EHA-L.r treated and untreated groups (Table 3).Non-significant levels of toxicity of other hydroalcoholic extracts obtained by different species of the Combretaceae family in animal models have been described in the literature.

Kpemissi et al. (2020) evaluating the toxicity of the hydroalcoholic extract of *Combretum micranthum* found that during the acute toxicity assay, no mortality or adverse effects were observed at the dose of 5,000 mg/kg. Daram et al. (2021) evaluating the ethanolic extract of *Terminalia catappa*, found that during the 14-day observation period, no adverse symptoms were found without any mortality for the maximum dose administered during the study (4,000 mg/kg). Similar results were obtained by VIlegas et al. (2018) who evaluating the hydroethanolic extract of *Terminalia argentea* Mart. Leaves found that the doses of 1000 and 2000 mg/kg did not alter the weight gain of mice over the 14-day period nor did it alter the relative weight of the organs analyzed. No macroscopic changes were observed in the organs analyzed.

Jayesh et al. (2017), demonstrated low toxicity for *Terminalia bellirica* (Gaertn.) extract. Meanwhile, a review on the toxicity of *Terminalia sericea* Burch. ex DC. evidenced that some compounds and extracts of this plant had moderate toxicity (Mongalo et al. 2016).

Moreover, the cited studies show that the evaluated extracts were not able to promote changes in biochemical and hematological parameters of different animals. These findings thus reinforce the importance of pharmacological research on representatives of this family.

Carrageenan-induced paw edema test

The results of the carrageenin-induced paw edema test were presented in Figure 2. The animals that received the intraplantar injection of carrageenan demonstrated the induction of edema, which was evidenced by the increase in volume of the paws that received the stimulus being analyzed at different time intervals. The group of animals pre-treated only with the vehicle (water) showed extremely significant edema when compared to indomethacin and to all doses of the extract EHA-Lr at all corresponding times. The extract EHA-Lr in all doses tested proved to be efficient in reducing the edema provoked by carrageenan at all observed times.

In this mouse model of paw edema, the extract EHA-Lr significantly reduced edema volume and showed superior inhibition compared to indomethacin. The paw edema induced by carrageenan in rats is associated with the synthesis of mediators involved in the inflammatory process (Farça et al. 2001; Hamad et al. 2019; Sharma et al. 2020). Among these we can highlight some of the most relevant ones such as: histamine, serotonin, bradykinin, nitric oxide and prostaglandins, whose release is closely related to the chemotaxis process of cells in particular leukocytes to the inflammatory sites (Farça et al. 2001; Hajare et al. 2001).

Although the carrageenan-induced paw edema model is more resistant in mice, this logistic agent is also capable of inducing edema formation. Studies have shown that albumin in mice demonstrates extravasation for more than 24 hours after its stimulation (Henriques et al.1987; Farça et al. 2001; Hajare et al. 2001; Hamad et al. 2019; Siraj et al. 2021). The paw edema performed in mice can be described through two very distinct phases characterized as follows: the 1st phase corresponding to (0 - 24 hours) after the inflammatory stimulus, and the 2nd phase (after 24 hours). In the initial period corresponding to the 1st phase a predominance of neutrophils occurs, they in turn have the ability to release inflammatory agents such as histamine, bradykinin, serotonin, and prostaglandins (Henriques et al.1987; Farça et al. 2001; Hajare et al. 2001; Hamad et al. 2019; Siraj et al. 2021).

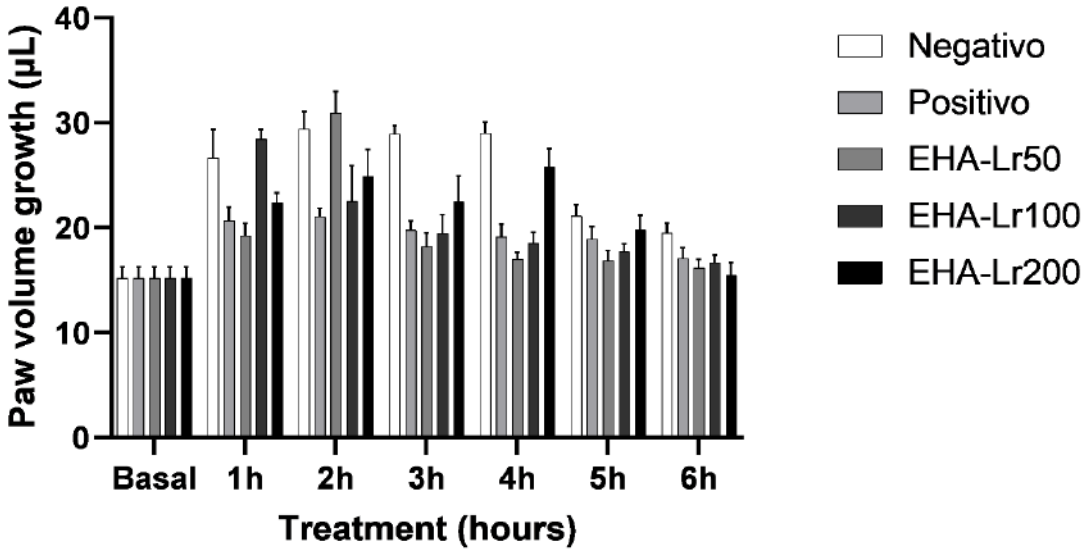

*Fig.2.Effect of EHA-Lr extract (50, 100 and 200mg/kg, v.o) and indomethacin (10mg/kg, v.o) on paw edema formation in mice. Mean ± Standard Deviation; *p<0.05. Significant after two-way analysis of variance (ANOVA) followed by Bonferroni test with 95% confidence interval when compared to the control group.*

The mechanism by which the inhibition of edema formation occurs is directly linked to the synthesis of PGs, and is compatible with the action generated by drugs classified as nonsteroidal anti-inflammatory drugs (NSAIDs), where the cyclooxygenases pathway is involved.

Hydroalcoholic extracts of different species belonging to Combretaceae family. Sharma et al. (2020) found that using the ethanolic extract of the stem bark of Anogeissus latifolia prevented paw volume increase in both in vivo models with percentage of inhibition of 44.40 and 46.21, respectively at 5 hours. To this end, Hamad and colleagues (2019) found that the ethanolic extract of Combretum aculeatum Vent at a concentration of 400 mg/kg decreased paw edema (only 32 ± 1.9% increase in paw weight after 4 hours) compared to indomethacin (28.6 ± 2.5%). Similar results to the current study (Figure 2), were obtained by Siraj et al. (2021) evaluating the anti-inflammatory effect of Terminalia myriocarpa ethanolic extract in paw edema trials found that doses of 250 and 500 mg/kg were able to reduce edema and showed promising results when compared to carrageenan. These results show that the extracts obtained from different species belonging to the Combretaceae family are able to reduce paw edema in different animals.

LPS-induced acute lung inflammation

Figure 3 presents the results of the effect of EHA-Lr extract (50, 100 and 200mg/kg, v.o) and dexamethasone (0.5mg/kg, v.o) on leukocyte migration in acute lung inflammation.

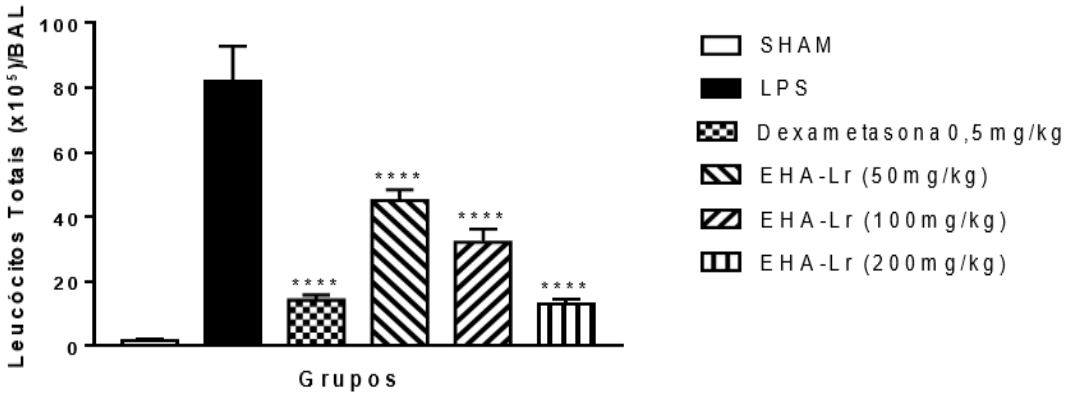

Fig.3. Effect of EHA-Lr extract (50, 100 and 200mg/kg, v.o) and dexamethasone (0.5mg/kg, v.o) on leukocyte migration in acute lung inflammation.

EHA-Lr extract has been shown to significantly reduce the migration of polymorphic nucleated leukocytes in the brocoalveolar lavage of animals subjected to LPS-induced acute lung inflammation. According to Liang et al. (2018) LPS is the main activator of inflammatory cells, being further responsible for the induction of neutrophils and active participation in the production and release of inflammatory cytokines, stating that the reduction of these inflammatory cytokines exerts proven therapeutic effects.

The reduction in the amount of leukocytes in the inflammatory brocoalveolar exudate when compared to the values found in the vehicle group and the group that received the reference drug (dexamethasone 0.5mg/kg, v.o.). It was also observed that the reduction of leukocyte migration in the brocoalveolar lavage in the model of acute lung inflammation was closely related to the dose used of EHA-Lr extract because the higher the dose used the greater was the reduction in the number of cells observed.

For Gao et al. (2020), pleurisy is associated with pleural inflammation and oxidative stress. The injury produced in the lungs and the inflammatory induction by carrageenan increases the levels of inflammatory cell exudates, protein leakage, and the pro-inflammatory mediators themselves. Similar results to those presented in figure 3 were obtained by Moreira et al. (2020), who found that treatment with *T. argentea* extract was able to reduce total protein production after stimulation with LPS, especially when the 300 mg/kg concentration was used, when compared to the other concentrations (5 and 60 mg /kg) or vehicle. Furthermore, according to the reduction of total proteins in the pleurisy process ascertained, the plant extract at the highest concentration (300 mg/kg), reduced the number of neutrophils and mononuclear cells at the same site. To a lesser extent, a reduction in the number of neutrophils was observed in mice treated with 5 and 60 mg/kg of the plant extract, when compared to those that received vehicle alone.

Other studies using the same pleurisy model, such as that of Silva-Balin et al. (2018), also demonstrated anti-inflammatory activity at 300 mg/kg, however, the authors used an ethanolic extract obtained from the fruit of *Bromelia balansae*. According to Shorkry and colleagues (2022), the induction of acute inflammation in vivo by LPS provides significant data for evaluating the pharmacological activity of extracts. The same highlights that Ivy ethanolic extract at 200mg/kg especially decreased LPS-induced pro-inflammatory mediators and oxidative stress. Thus, corroborating with the findings of this study (Figure 3), considering that the EHA-Lr extract at 200mg/kg reduced the number of neutrophils as much as its treatment with dexamethasone.

In another study, Daram et al. (2021) evaluating the ethanolic extract of Terminalia catappa found that at the concentration of 500 mg/kg it was able to promote the decrease in total leukocyte count, lymphocyte count and myeloperoxidase enzyme activity. To this end, the granulocyte count was increased in almost all three treatments and the percentage data of total leukocyte, granulocyte, and MPO activity. Thus, the anti-inflammatory potential without causing acute toxicity of extracts from medicinal plants, such as those reported in this study, may contribute to new pharmaceutical applications.

Thus, murine models provide suitable frameworks for studying the anti-inflammatory activity of medicinal plants, mainly by counting cell migration during a stimulation of an inflammation-inducing agent. In this context, Talwar et al. (2021) found that the aqueous extract of *Terminalia paniculata* (400 mg/kg) also reduced carrageenan-induced leukocyte migration (50.92 ± ± ± 5.24%) in exudates found due to stimulation with LPS. These results show that the hydroalcoholic extract of *Laguncularia racemosa* has promising anti-inflammatory activity for the treatment of lung inflammation.

In vitro antibacterial activity

The results of antibacterial activity showed that the extract EHA-Lr proved to be very effective in inhibiting bacterial growth with considerably satisfactory values, being able to inhibit the growth of Micrococcus luteus strain (UFPEDA 100). The other strains showed moderate to low results when compared to the erythromycin control (Table 5). The mechanisms of antimicrobial activity promoted by different extracts are not yet well elucidated, however, it is known that phenolic compounds are responsible for the in vitro activity (Puupponen-Pimiä et al. 2001; Gomes et al. 2022).

Table 5. Determination of the minimum inhibitory concentration of EHA-Lr against the tested bacterial strains

Nd: Not determined of the evaluated concentrations 0.23 to 30mg/mL

Microorganisms	Erythromycin (mg/mL)	EHA-L.r (mg/mL)
Staphylococcus aureus (UFPEDA 02)	0,6	7,5
Micrococcus luteus (UFPEDA 100)	0,6	0,93
Bacillus subtilis (UFPEDA 86)	1,2	7,5
Pseudomonas aeruginosa (UFPEDA 416)	Nd	3,75
Serratia marcencens (UFPEDA 352)	0,6	3,75
Escherichia coli (UFPEDA 224)	1,2	3,75
Enterococcus faecalis (UFPEDA 138)	0,3	3,75

Philomène-Kokora (2013) found that the ethanolic and aqueous extracts of *Terminalia mantaly* H. Perrier (Combretaceae) show inhibitory activity on all tested strains (*Escherichia coli* and *Staphylococcus aureus*). The inhibitory diameters ranged from 7.66 mm to 35 mm. The Minimum Inhibitory Concentrations (MIC) of the extracts ranged from 0.78 mg/mL and 2.5 mg/mL. The ethanolic extracts inhibit the growth of bacteria at lower concentrations than the aqueous extracts.

Kuet et al. (2010) when evaluating the antimycobacterial, antimicrobial and antifungal activities of a species from the same family found that individuals belonging to the combretaceae family have excellent minimum inhibitory concentration values for bacterial strains such as: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. Anokwuru et al. (2021) evaluated the broad-spectrum antibacterial potential of methanolic extracts of species representing four genera of Combretaceae (*Combretum*, *Pteleopsis*, *Quisqualis*, *Terminalia*). They found that these were more active against *Bacillus cereus* and *Salmonella typhimurium* strains, with mean MIC values of 0.70 mg/mL, 0.52 mg/mL and 0.45 mg/mL, respectively. These findings show that the hydroalcoholic extract of *Laguncularia racemosa* has promising anti-inflammatory activity for the treatment of bacterial inflammation.

IV. CONCLUSION

The hydroalcoholic extract of Laguncularia racemosa showed moderate antioxidant and antibacterial activity in vitro. Regarding anti-inflammatory activity, it was able to reduce paw edema in mice and prevent acute lung inflammation. The results obtained in this study provide supportive data for the use of Laguncularia racemosa for therapeutic purposes.

V. CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

ACKNOWLEDGMENTS

The authors thank CNPQ and CAPES for the structural and financial support to this work.

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