



Schinus terebinthifolius Raddi (Brazilian pepper) leaves extract: in vitro and in vivo evidence of anti-inflammatory and antioxidant properties

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Abstract

The aim of this work was to evaluate the anti-inflammatory and antioxidant effects of ethyl acetate extract obtained from the leaves of Brazilian peppertree *Schinus terebinthifolius* Raddi (EAELSt). Total phenols and flavonoids, chemical constituents, in vitro antioxidant activity (DPPH and lipoperoxidation assays), and cytotoxicity in L929 fibroblasts were determined. In vivo anti-inflammatory and antioxidant properties were evaluated using TPA-induced ear inflammation model in mice. Phenol and flavonoid contents were 19.2 ± 0.4 and 93.8 ± 5.2 of gallic acid or quercetin equivalents/g, respectively. LC–MS analysis identified 43 compounds, of which myricetin-O-pentoside and quercetin-O-rhamnoside were major peaks of chromatogram. Incubation with EAELSt decreased the amount of DPPH radical (EC_{50} of 54.5 ± 2.4 $\mu\text{g/mL}$) and lipoperoxidation at 200–500 $\mu\text{g/mL}$. The incubation with EAELSt did not change fibroblast viability up to 100 $\mu\text{g/mL}$. Topical treatment with EAELSt significantly reduced edema and myeloperoxidase activity at 0.3, 1, and 3 mg/ear when compared to the vehicle-treated group. In addition, EAELSt decreased IL-6 and TNF- α levels and increased IL-10 levels. Besides, it modulated markers of oxidative stress (reduced total hydroperoxides and increased sulfhydryl contents and ferrum reduction potential) and increased the activity of catalase and superoxide dismutase, without altering GPx activity.

Keywords *Schinus terebinthifolius* · Oxidative stress · Phenolic compounds · Inflammation · Cytokines · Medicinal plant

Introduction

Inflammation is defined as immunological, biochemical, and cellular changes in response to molecular patterns associated to pathogens or cell, tissue damage (Rudrapal et al. 2022;

Upadhyay and Dixit 2015). The main clinical signs of this response are pain, heat, and redness, which are associated with the development of edema, vasodilation, and leukocyte migration to the injury site. If this process is not controlled, it leads to increased tissue damage, which worsens the loss

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of tissue function and drives to chronic inflammatory process (Herrero-Cervera et al. 2022).

Cells, such as macrophages and fibroblasts, are activated locally and systemically, inducing the release of mediators in the inflammatory condition (Upadhyay and Dixit 2015). Among these mediators, pro-inflammatory cytokines such as interleukins (IL-6) and the tumor necrosis factor- α (TNF- α) play a key role in the inflammatory response (Hirano 2020). In addition, several inflammatory stimuli, such as the excess of reactive oxygen (ROS) and nitrogen (RNS) species, contribute to the inflammatory process cascade (Tanabe et al. 2022).

During inflammation, there is a greater formation of ROS and RNS. The imbalance between the production of these species and the antioxidant defense mechanisms leads to oxidative stress, which plays an important role in inflammatory conditions (Doktorovova et al. 2014; Gutteridge and Halliwell 2018).

Several drugs are used to treat inflammation, especially, non-steroidal anti-inflammatory drugs and corticosteroids (Juthani et al. 2017). Despite the wide variety of anti-inflammatory drugs on the market, the adverse effects contribute to the continued need for more research to discover isolated molecules or mixtures of compounds such as those presented in medicinal plants that can serve as therapeutic alternatives (Souza et al. 2015).

Derivatives of herbal products are important sources for the discovery of new drugs (Amaral et al. 2020; Matsuo et al. 2011; Santos et al. 2021). For example, polyphenols are a group of metabolites found in parts of plants that have a series of biological activities, such as anti-inflammatory and antioxidant properties (Durazzo et al. 2019; Pimentel-Moral et al. 2018). There is great interest in the search and identification of secondary metabolites, such as polyphenols and other compounds from plant-based natural sources, since they can have valuable therapeutic potential.

One of the promising medicinal plants to treat inflammation is *S. terebinthifolius* Raddi (Anacardiaceae), known as Brazilian pepper tree. In folk medicine, the bark and leaves are used in as infusions and tinctures, to treat bacterial infections (Martínez et al. 1996), or to promote healing, anti-inflammatory and anti-ulcerogenic effect (Martorelli et al. 2011). The leaves can even be used for the green synthesis of silver nanoparticles (de Oliveira et al. 2021). Phytochemical studies of this species have resulted in the isolation of terpenes, monoterpenes, sesquiterpenes, and flavonoids (El-Massry et al. 2009; Matsuo et al. 2011).

A previous study showed that the acetate fraction of *S. terebinthifolius* leaves has anti-allergic activity when administered orally (Cavalher-Machado et al. 2008b). Although this represents consistent evidence of anti-inflammatory effect after oral administration of a *S. terebinthifolius* leaves fraction, there are no detailed reports

on the effect of preparations from *S. terebinthifolius* leaves in a model of skin inflammation after topical application. Thus, in this study, we prepared the ethyl acetate extract from the leaves of *S. terebinthifolius* (EAELSt) and tested the effect of this extract in selected in vitro models regarding its antioxidant effects and cytotoxicity activity, and in an in vivo model of skin inflammation and oxidative stress.

Materials and methods

Plant material

The leaves of *S. terebinthifolius* were collected in the municipality of São Cristóvão, State of Sergipe, at the coordinates (10° 55' 14.8" S, 37° 06' 11.9" O) with registration in the National Management System Genetic Heritage (SIGGEN) of number A6AC079. A specimen was identified and properly registered in the herbarium of the Federal University of Sergipe (UFS) with voucher 39,748. Leaves were placed in an oven (model MA-037) at 37 °C, with renewal and air circulation for 48 h until complete dehydration and reduced to powder. The powder from the leaves of *S. terebinthifolius* (3 kg) was subjected to extraction with ethyl acetate solvent by Soxhlet apparatus until complete exhaustion of the plant material. After this period, the material was filtered and concentrated on a rotary evaporator (Büchi® R-200, Merck KGaA, Darmstadt, Germany) under reduced pressure at a temperature of 40 °C, obtaining 295 g of ethyl acetate extract (EAE; 9.83% yield).

Quantification of total phenolics and flavonoids

Total phenolics content was quantified using the Folin–Ciocalteu method, as described by Sousa et al. (2007) with modifications. An aliquot of EAELSt (100 μ L, 1 mg/mL in methanol) was mixed with 6 mL of distilled water and 500 μ L of the Folin–Ciocalteu reagent (1 mol/L) and shaken for 1 min. After adding 2 mL of Na₂CO₃ (15%), the mixture was shaken for 30 s. The solution was diluted with distilled water to a final volume of 10 mL, incubated for 2 h at 23 °C, and the absorbance of the sample was measured by a UV–Vis spectrophotometer, model SP22, at 750 nm against a blank consisting of water and the other reagents. Total phenolics was determined by interpolating the absorbance of the samples against a calibration curve using the gallic acid standard (5–30 μ g/mL). The results were expressed in mg of gallic acid equivalents per g of extract (mg of GAE/g). All analyses were performed in triplicate.

Total flavonoids content was quantified using the colorimetric method according to Zhishen et al. (1999) with modifications. Aliquots of the EAELSt sample were mixed with 2 mL of distilled water and NaNO₂ solution

(5%, 0.15 mL). After 6 min, AlCl_3 solution (10%, 0.15 mL) was added and kept for 6 min. Then NaOH solution (4%, 2 mL) and 0.2 mL of distilled water were added until the volume of 5 mL was completed. Subsequently, the solution was kept at rest for 15 min. Total flavonoids was determined according to the quercetin standard curve, measured by spectrophotometer at 510 nm. The results were expressed in mg of quercetin equivalent/g of extract (mg of QE/g). All analyses were performed in triplicate.

LC-MS/MS analysis

The EAELSt was analyzed by high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan), using an analytical chromatographic column C18 (Kromasil—250 m m \times 4.6 mm \times 5 μm), coupled to a mass spectrometer (Ion-TrapAmazonX, Bruker), with ionization by electrospray (ESI). The sample was solubilized in methanol (1 mg/mL), with subsequent filtration through polyvinylidene fluoride filters, with a 0.45 μm mesh. The developed chromatographic method used the solvents, methanol (solvent B) of chromatographic grade and ultrapure water type I (Milli-Q[®]), acidified with formic acid (0.1% v/v) (solvent A), with gradient analysis of concentration (5–100% B in 45 min). The injection volume was 10 μL and the flow rate was 0.6 mL/min. In the mass spectrometer, the samples were subjected to sequential fragmentation in MS3. The parameters used were: 4.5 kV capillary, 500 V final plate off set, nebulizer gas at 35 psi, dry gas (N_2) with flow rate of 8 mL/min, and temperature of 300 $^\circ\text{C}$. The sample was analyzed in the negative ionization mode and the identification of the compounds was based on the data (MS/MS) reported by the literature.

Antioxidant activity

DPPH free radical scavenging assay

The protocol used in this assay was adapted from Cheng et al. (2006), with modifications (Souto et al. 2020a, b). A stock solution of DPPH (2,2-diphenyl-1-picrilhidrazil, 0.208 mmol/L) was prepared in methanol. In triplicates, 100 μL of methanol (blank), gallic acid (standard curve: 1, 2, 3, 4 and 5 $\mu\text{g}/\text{mL}$), and samples were incubated with 100 μL of DPPH solution for 60 min. The absorbances were then read in a UV/Vis microplate spectrophotometer (SynergyMx[®], Biotek, Bad Friedrichshall, Germany) at 515 nm.

The effective antioxidant concentration required to decrease the initial DPPH radical concentration by 50% (EC_{50}) was calculated using % of DPPH reminiscent over 60 min, as opposed to the sample concentrations. The antioxidant concentration necessary to decrease the

initial DPPH concentration by 50% (EC_{50}) and antioxidant activity index (AAI) were also used for establishing the antioxidant potential of the samples (Scherer and Godoy 2009).

Lipoperoxidation assay

For the lipoperoxidation, the method of determining substances reactive to thiobarbituric acid was used (Ohkawa et al. 1979), with modifications (Souto et al. 2020a). The rat brain tissue was removed, and tissue homogenates were prepared in phosphate buffer solution (50 mmol/L; pH 7.0, 1:9 m/v. The homogenate was centrifuged at 800 $\times g$ in a Beckman centrifuge (4 $^\circ\text{C}$, 15 min) and the supernatant used in the assay. They were added in tubes (100 μL of rat brain homogenate in phosphate buffer 50 mmol/L, pH 7.4) incubated with 50 μL of different concentrations of EAELSt (200, 300, 400, and 500 $\mu\text{g}/\text{mL}$) at 37 $^\circ\text{C}$ during 30 min. Then 350 μL of acetic acid (20%, pH 3.5) and 600 μL of thiobarbituric acid (TBA, 0.36%) were added. Then they were incubated at a temperature of 85 $^\circ\text{C}$ for 1 h. Subsequently, they were cooled on ice and centrifuged at 500 $\times g$ for 15 min. The absorbance reading was performed at 532 nm. The results were expressed as a percentage of inhibition. Trolox (100 $\mu\text{g}/\text{mL}$) was used as a positive control. All analyses were performed in triplicate.

In vitro cell viability

This experiment was carried out in a culture of L929 fibroblasts exposed to different concentrations of the EAELSt, using the methylthiazolyl diphenyl tetrazolium bromide (MTT) technique, as previously described by us (Souto et al. 2020a, b). The cells were maintained in culture, seeded in 96-well culture plates (1×10^4 cells/well) and grown in culture medium (DMEM) containing NaHCO_3 (1.2 g/L), ampicillin (0.025 g/L), streptomycin (0.1 g/L) and 10% fetal bovine serum. Then, they were incubated with different concentrations of EAELSt (25, 50, 75 or 100 $\mu\text{g}/\text{mL}$), solubilized in dimethyl sulfoxide (DMSO, 0.1%) for 24 h at 37 $^\circ\text{C}$ and in an environment containing 5% CO_2 . Cell viability was assessed by adding an MTT solution (0.5 mg/mL in phosphate buffered saline) to the cells, which were then incubated at 37 $^\circ\text{C}$ for 3 h. After removing the MTT, DMSO was added to the plate for 10 min for the solubilization of the crystals of the tetrazolic salt and the absorbance was measured in a UV/Vis microplate spectrophotometer (SynergyMx[®], Biotek, Bad Friedrichshall, Germany) at 570 nm. The tests were carried out in triplicate in 3–4 independent experiments.

The results were expressed in percentage of cell viability based on normalized absorbance values.

Evaluation of the anti-inflammatory effect

Animals

Male Swiss mice (20–30 g) were obtained from the Animal Center of Federal University of Sergipe. Animals were kept at 21–23 °C with free access to feed and water under a 12-h light/dark cycle. All experiments were carried out according to the guidelines of the Brazilian College of Animal Experimentation and the National Institutes of Health and were approved by the Ethics Committee on Animal Use in Research of Federal University of Sergipe (Approval n° 06/2019).

Ear inflammation in mice

Ear inflammation was induced by 12-O-tetradecanoylforbol-acetate (TPA) in mice, according to a previous study (De Young et al. 1989), and adapted to our laboratory conditions (Cercato et al. 2021). Initially, the animals ($n = 5$ –6/group) were topically treated in the right ear with TPA (1 µg/ear). After 5 min, EAELSt (0.3, 1 or 3 mg/ear), dexamethasone (0.05 mg/ear; positive control) or vehicle (acetone, 20 µL/ear) were also applied to the ears. In the left ear of each animal, the equivalent volume of acetone was administered topically, and each animal served as its own control for the measurement of edema. Euthanasia was performed with inhaled isoflurane 6 h after induction. Then ear sites were cut out circularly with a punch (8 mm of diameter). The mass of the ear sites was measured. The edema values were expressed as the variation (Δ) of the mass (mg) by subtracting the left ear (non-inflamed) mass from the right ear (inflamed) mass.

Myeloperoxidase (MPO) activity assay

Ear samples were collected, weighed, cut into small pieces, and kept in test tubes in the presence of phosphate buffer (50 mmol/L, pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide). Then they were homogenized, and aliquots were centrifuged. The obtained supernatants were subjected to analysis of MPO activity.

In a 96-well plate, supernatants were added to the *o*-dianisidine dihydrochloride solution (0.167 mg/mL, prepared in 50 mmol/mL potassium phosphate buffer containing 0.005% of H₂O₂). Changes in absorbance

values at 460 nm for a period of 5 min and the results were expressed as units of MPO (UMPO/mg of tissue), considering 1 UMPO as the amount of enzyme that degrades 1 µmol of H₂O₂ at 25 °C, generating an absorbance variation of 0.0113 absorbance units, as previously described by Bradley et al. (1982).

Determination of cytokines concentration

The homogenates of the ears of the different experimental groups were used for cytokine quantification. For this purpose, 96-well microplates coated with monoclonal antibodies specific for IL-6, TNF- α or IL-10 were used. The determination was carried out according to the specifications of the manufacturers (Elisa Kits, ThermoFisher Scientific Inc., Waltham, Massachusetts, USA).

Histological analysis of the ears

In an independent experimental set, ears ($n = 5$) treated with the highest dose of EAE (3 mg/ear) or positive control (dexamethasone, 0.05 mg/ear) were used for histological analysis. Subsequently, the tissues were carefully removed, preserved in formaldehyde (10%), and submitted to routine techniques for histological analysis. Slices of 5 µm were stained with hematoxylin and eosin and were viewed and photographed under an optical microscope (Nikon, Tokyo, Japan) with a 20 \times magnification. The edema thickness was measured using the ImageJ[®] program. For this, four representative photographs of each ear were selected and the mean of five measurements of the thickness of the dermis was obtained (Chibli et al. 2014).

Determination of oxidative stress biomarkers

Total hydroperoxides were measured in mice ear as previously described by Jiang et al. (1992). The thiol levels were measured as described by Sedlak and Lindsay (1968). To assess the reducing capacity of samples, FRAP method was used (Oyaizu 1986), with minor modifications. The activity of antioxidant enzymes, namely, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), was determined as described by Doktorovova et al. (2014).

Statistical analysis

The data were expressed as mean \pm standard error of the mean (SEM) for the experimental number indicated in the legends of the figures and were evaluated for normality by the Shapiro–Wilk tests. As there was no impediment to parametric methods, the data were assessed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. In all these procedures, the statistical program

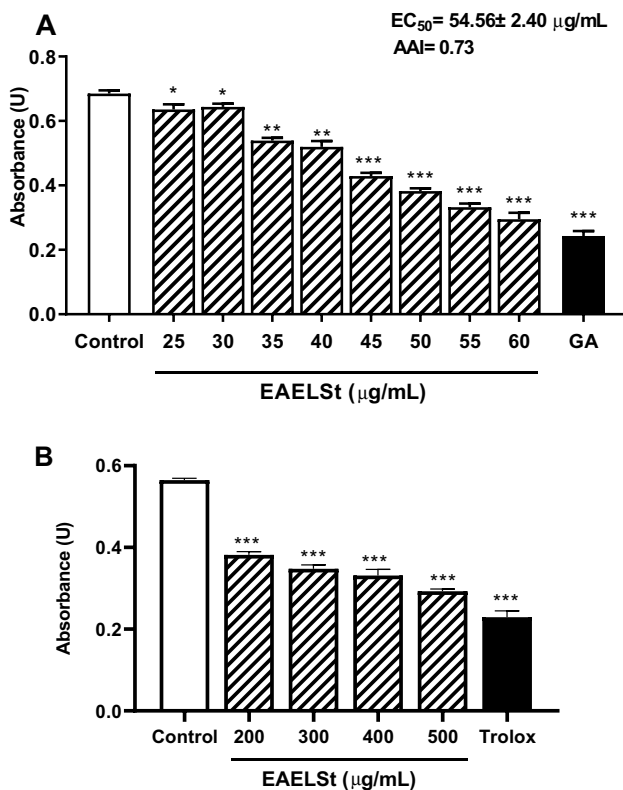


Fig. 1 The ethyl acetate extract from leaves of the *S. terebinthifolius* (EAELSt) reduces the amount of 2-diphenyl-1-picryl-hydrazil (DPPH) in vitro. EAELSt was tested against the DPPH radical (A) and lipoperoxidation (B) in vitro. The results represent men ± SEM of the absorbance values; n=3 experiments in triplicate. Gallic acid (AG; 1 µg/mL) or trolox (100 µg / mL) were used as controls. One-way ANOVA followed by Tukey’s test (*p < 0.05, **p < 0.01 or ***p < 0.001 vs control). EC₅₀ concentration that inhibits 50% of DPPH radical, AAI antioxidant activity index

GraphPad Prism (version 7.0) was used. Values with p < 0.05 were considered significant.

Results

Quantification of total phenol, total flavonoid, and antioxidant activity

The results show that the total phenol content in EAELSt was 19.21 ± 0.40 mg of GAE/g and the flavonoid content was 93.81 ± 5.17 mg of QE/g. It was also observed that EAELSt reduced the amount of the DPPH radical at concentrations of 25–60 µg/mL compared to the control group (Fig. 1A). Gallic acid (positive control; 1 µg/mL) also significantly reduced the DPPH radical compared to the control. The EC₅₀ calculated for the effect of EAELSt by the DPPH method was 54.56 ± 2.40 µg/mL and the IAA was 0.73, which is considered moderate. According to Scherer and Godoy (2009), a poor antioxidant has an AAI < 0.5, while a moderate one has an AAI between 0.5 and 1.0. The strong and very strong antioxidants are defined by AAI values between 1.0 and 2.0, and AAI > 2.0, respectively.

The data in Fig. 1B show that EAELSt significantly reduced spontaneous lipoperoxidation at concentrations of 200–500 µg/mL when compared to the control, which was also observed for the trolox (100 µg/mL).

LC–MS/MS analysis

Figure 2 represents the result of the analysis of EAELSt constituents by the LC–MS/MS spectroscopy. Spectroscopic

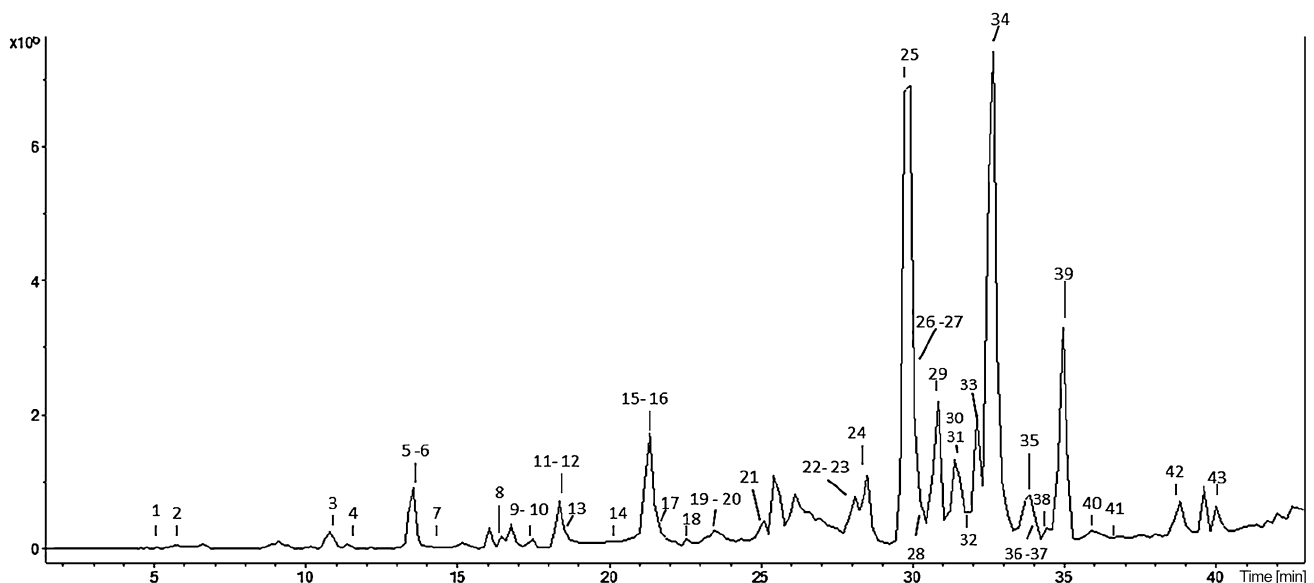


Fig. 2 Total chromatogram obtained for the ethyl acetate extract of the leaves of *S. terebinthifolius* by LC–MS/MS

Table 1 Identification of constituents of ethyl acetate extract from the leaves of *S. terebinthifolius* (EELSt)

Peak	RT	[M-H]	MS ⁿ m/z	Compound
1	5,1	341,0	MS ² [341,0]: 178,7; 160,9; 142,8 MS ³ [341,0 → 178,7]: 160,8; 112,8; 100,7; 88,9	Disaccharide
2	6,6	172,9	MS ² [172,9]: 154,8; 110,8; 92,8; 82,9; 72,9	Shikimic acid
3	10,8	330,9	MS ² [330,9]: 270,9; 240,8; 210,8; 192,9; 168,8 MS ³ [330,9 → 168,8]: 124,8	Galloylhexoid
4	12,2	331,0	MS ² [331,0]: 270,8; 240,8; 168,9 MS ³ [331,0 → 270,8]: 210,8; 168,8 MS ³ [331,0 → 168,9]: 124,6	Galloylhexoid
5	13,2	331,0	MS ² [331,0]: 270,8; 240,8; 210,8; 168,8 MS ³ [331,0 → 168,8]: 124,8	Galloylhexoid
6	13,5	168,8	MS ² [168,8]: 124,8; 96,8; 80,9; 68,9	Gallic acid
7	13,9	298,9	MS ² [298,9]: 136,8 MS ³ [298,9 → 136,8]: 92,9	Hydroxybenzoic acid-O-hexoside
8	16,5	325,0	MS ² [325,0]: 281,0; 168,8; 154,8; 136,8; 124,8 MS ³ [325,0 → 168,8]: 124,8	Galloyl-chiquimic acid
9	17,2	483,0	MS ² [483,0]: 331,0 MS ³ [483,0 → 331,0]: 270,8; 210,9; 168,8; 124,9	Digalloyl glucose
10	17,5	325,0	MS ² [325,0]: 281,0; 168,8; 124,8 MS ³ [325,0 → 168,8]: 124,8	Galloyl-chiquimic acid
11	18,0	359,0	MS ² [359,0]: 196,9; 181,8; 152,9	Siringenic acid -O-hexoside
12	18,4	320,9	MS ² [320,9]: 168,9; 124,8	Digallic acid
13	18,5	152,8	MS ² [152,8]: 108,8	Protocatechuic acid
14	20,1	289,0	MS ² [289,0]: 244,9; 204,8; 178,8 MS ³ [289,0 → 244,9]: 226,9; 202,8; 186,8; 160,7	Catechin
15	21,4	320,9	MS ² [320,9]: 168,9; 124,8	Digallic acid
16	21,5	182,8	MS ² [182,8]: 167,8; 123,8	Methyl gallate
17	21,9	477,0	MS ² [477,0]: 324,9 MS ³ [477,0 → 324,9]: 168,8; 124,8	Digalloylchiquimic acid
18	22,5	473,0	MS ² [473,0]: 320,9; 168,8 MS ³ [473,0 → 320,9]: 168,9; 124,8	Trigallic acid
19	23,4	335,0	MS ² [335,0]: 182,8 MS ³ [335,0 → 182,8]: 167,7; 123,8	Galloylmethyl gallate
20	23,5	472,9	MS ² [472,9]: 320,8; 168,7 MS ³ [472,9 → 320,8]: 168,7; 124,7	Trigallic acid
21	25,0	441,0	MS ² [441,0]: 288,9 MS ³ [441,0 → 288,9]: 244,9; 204,8; 202,9; 178,8; 124,9	Epicatechin-O-gallate
22	25,1	473,0	MS ² [473,0]: 320,9 MS ³ [473,0 → 320,9]: 168,8; 124,7	Trigallic acid
23	28,1	334,9	MS ² [334,9]: 182,8 MS ³ [334,9 → 182,8]: 167,8; 123,8	Galloylmethyl gallate
24	28,5	479,0	MS ² [479,0]: 315,9; 316,9; 270,8; 178,9	Myricetin-O-hexoside
25	28,7	449,0	MS ² [449,0]: 315,8; 270,8 MS ³ [449,0 → 315,8]: 270,8; 178,8	Myricetin-O-pentoside
26	29,0	615,0	MS ² [615,0]: 462,9; 300,8 MS ³ [615,0 → 300,8]: 270,9; 178,8; 150,9	Quercetin-O-galloyl-hexoside
27	29,7	463,0	MS ² [463,0]: 315,9; 270,8; 178,9 MS ³ [463,0 → 315,9]: 286,9; 270,8; 178,8; 150,8; 136,7	Myricetin-O-rhamnoside
28	30,3	615,0	MS ² [615,0]: 462,9; 300,9 MS ³ [615,0 → 300,9]: 178,8; 150,8	Quercetin-O-galloyl-hexoside
29	30,8	463,0	MS ² [463,0]: 300,9 MS ³ [463,0 → 300,9]: 270,8; 254,8; 178,8; 150,8; 120,8	Quercetin-O-hexoside
30	31,4	433,0	MS ² [433,0]: 300,8 MS ³ [433,0 → 300,8]: 270,8; 254,8; 178,8; 150,8; 120,7	Quercetin-O-pentoside

Table 1 (continued)

Peak	RT	[M-H]	MS ⁿ m/z	Compound
31	31,4	615,0	MS ² [615,0]: 462,9; 316,9 MS ³ [615,0 → 316,9]: 270,9; 178,8; 150,8; 136,6	Myricetin-O-(O-galloyl)-deoxyhexoside
32	31,6	585,0	MS ² [585,0]: 300,9 MS ³ [585,0 → 300,9]: 178,8; 150,8; 120,8	Quercetin Gallo Pentose
33	32,1	599,0	MS ² [599,0]: 312,9; 284,5 MS ³ [599,0 → 312,9]: 210,7; 168,9; 124,8	Kaempferol-O- (gallo) hexoside
34	32,7	447,0	MS ² [447,0]: 300,9 MS ³ [447,01 → 300,9]: 270,8; 254,9; 178,8; 150,9	Quercetin-O-rhamnoside
35	33,7	417,0	MS ² [417,0]: 283,9; 254,9; 226,8 MS ³ [417,0 → 283,9]: 254,9; 240,9; 226,8	Kaempferol-O-pentoside
36	33,9	599,0	MS ² [599,0]: 447,0; 300,9 MS ³ [599,0 → 300,9]: 178,8; 150,8	Galoilquercetin-O-rhamnoside
37	34,1	585,0	MS ² [585,0]: 300,9 MS ³ [585,0 → 300,9]: 178,7; 150,7	Quercetin Gallo Pentose
38	34,6	417,0	MS ² [417,0]: 284,9; 254,8; 226,8 MS ³ [417,0 → 284,9]: 254,8; 241,0; 226,8	Kaempferol-O-pentoside
39	35,0	431,0	MS ² [431,0]: 284,9; 254,9; 226,9	Kaempferol-O-rhamnoside
40	36,1	300,9	MS ² [300,9]: 273,0; 178,8; 150,8; 120,9	Quercetin
41	36,5	477,0	MS ² [477,0]: 314,9; 299,8; 271,8; 242,9	Isorhamnetin-O-hexoside
42	38,8	537,0	MS ² [537,0]: 442,9; 416,9; 399,0; 374,9; 330,9	Amentoflavone
43	40,0	327,1	MS ² [327,1]: 291,0; 229,0; 210,9; 209,0; 170,8; 164,9	Oxo-dihydroxy-octadecenoic acid

Table 2 Effect of the incubation with ethyl acetate extract of the leaves of *S. terebinthifolius* (EAELSt) on L929 fibroblasts viability

Stimulus	Mean of absorbance ± SEM
Control	0.282 ± 0.029
EAELSt (25 µg/mL)	0.267 ± 0.049
EAELSt (50 µg/mL)	0.264 ± 0.012
EAELSt (75 µg/mL)	0.244 ± 0.017
EAELSt (100 µg/mL)	0.250 ± 0.011

Data are shown as mean of absorbance detected at the end of the test. One-way ANOVA followed by Tukey's test, *n* = 3, experiments performed in triplicate)

data allowed the identification of 43 substances, for which the identity is detailed in Table 1. The phytochemical profile showed that the EAE is rich in polyphenolic compounds, mostly derived from gallic and ellagic acids. In this table, it is possible to observe that the peaks 25 and 34 have the largest area, which refer to myricetin-O-pentoside and quercetin-O-rhamnoside.

In vitro cell viability

Table 2 shows that EAE did not alter the viability of L929 fibroblasts at concentrations between 25 and 100 µg/mL when compared to control.

Ear inflammation in mice

The topical application of TPA (1 µg/ear) induced an increase in the mass of mice right ear sites by 20.4 ± 1.0 mg in relation to the left ear, which confirmed the formation of edema (Fig. 3A). This figure also shows that in the ears with administration of EAELSt after TPA, lower ear edema was observed at the doses of 0.3 (*p* < 0.05), 1 (*p* < 0.01), and 3.0 mg/ear of extract (*p* < 0.001) compared with the TPA group. As a positive control, in the ears in which TPA and dexamethasone (0.05 mg/ear) were administered, there was also less edema compared to the TPA plus vehicle group (*p* < 0.001).

Topical application of TPA also increased MPO activity in the animals' right ear compared to acetone (control) group (*p* < 0.001; Fig. 3B). The activity of this enzyme was lower in the ear of animals submitted to the administration of EAELSt at doses of 0.3 (*p* < 0.05), 1 (*p* < 0.01), and 3.0 mg/ear (*p* < 0.05) compared to the TPA group. Dexamethasone also decreases the MPO activity compared to the TPA plus vehicle group (*p* < 0.01).

Cytokine concentration in mice ears

Topical administration of TPA produced a higher concentration of IL-6 and TNF-α in comparison to the acetone group (*p* < 0.001; Fig. 4A, B). The concentration of IL-6 was lower in the ear of animals submitted to administration of

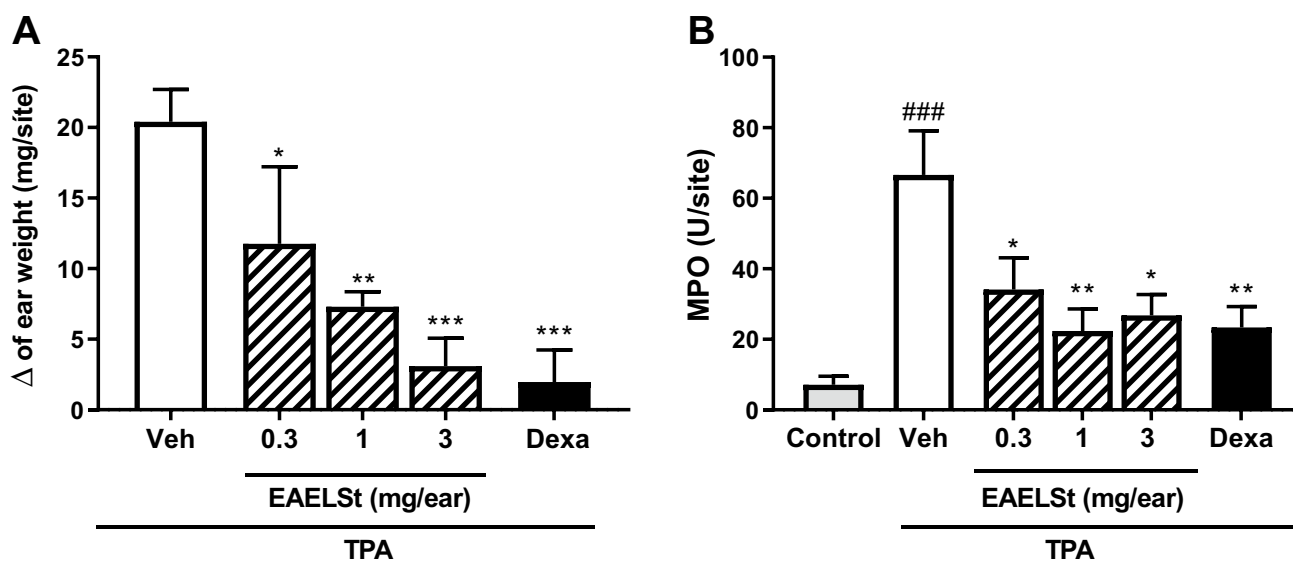


Fig. 3 Effect of treatment with ethyl acetate extract from the leaves of *S. terebinthifolius* (EAELSt) on ear edema (A) and myeloperoxidase (MPO) activity (B) in mice ear. Animals were submitted to concomitant topical administration of 12-O-tetradecanoilforbol-13-acetate (TPA; 1 μ g/ear) and EAELSt or dexamethasone (Dexa). Data

are shown as mean \pm SEM of the variation of ear weight (right–left ear site) and MPO activity (U/ear site) for $n=5$ animals. One-way ANOVA followed by the Tukey test; ### $p<0.001$ vs. control group (right ear site), * $p<0.05$, ** $p<0.01$ or *** $p<0.001$ vs. TPA + vehicle)

EAELSt at doses of 0.3 ($p<0.01$), 1 ($p<0.001$), and 3.0 mg/ear ($p<0.001$) in comparison to TPA group. Only the treatment with EAELSt at 3.0 mg/ear ($p<0.001$) reduced TNF- α , in comparison to TPA group.

Figure 4C shows that animals submitted to topical administration of TPA presented lower concentration of IL-10 compared to the acetone group ($p<0.001$). Interestingly, the doses of 0.3 and 1.0 mg/ear reversed the effect of TPA on the IL-10 concentrations ($p<0.001$ compared to TPA group), leading to values similar to the control group. However, in the group treated with 3.0 mg of EAELSt/ear or dexamethasone (0.05 mg/ear), the concentration of IL-10 was not different from the TPA plus vehicle group.

Histological analysis

Given the effects observed for the inflammatory parameters, the dose of 3 mg/ear was chosen for histological analysis. Representative images from the light microscopy of mice ears are shown in Fig. 5. We observed that the application of TPA (Fig. 5B) increased the ear thickness, with characteristics mainly of edema, that differs from the animal that receives only acetone (control, Fig. 5A). Figure. 5C shows that in the ear submitted to the application of TPA and to the

treatment with EAELSt at 3 mg/ear, it was possible to observe the preservation of the tissue through the reduction of edema. The same occurred for the treatment with dexamethasone (0.05 mg/ear, Fig. 5D).

Determination of oxidative stress and antioxidant enzymes activity

Administration of TPA increased total hydroperoxides in mice ear ($p<0.01$) and this effect was reduced by the treatment with EAELSt at 3 mg/ear ($p<0.01$), but not at 0.3 or 1 mg/ear, when compared to TPA plus vehicle group (Table 3).

When assessing the concentration of SH groups, the animals in the TPA group showed a lower content of these groups when compared to animals in the control group ($p<0.01$). In animals submitted to administration of EAELSt at 0.3 and 3 mg/ear ($p<0.05$), but not 1 mg/ear, the concentration of sulfhydryl groups was higher than in TPA plus vehicle group. Besides, in animals administered with TPA, FRAP was decreased when compared to control group ($p<0.001$). This effect was partially reverted by the administration of EAELSt at 3 mg/ear, but not 0.3 or 1 mg/ear, when compared to TPA plus vehicle group (Table 3).

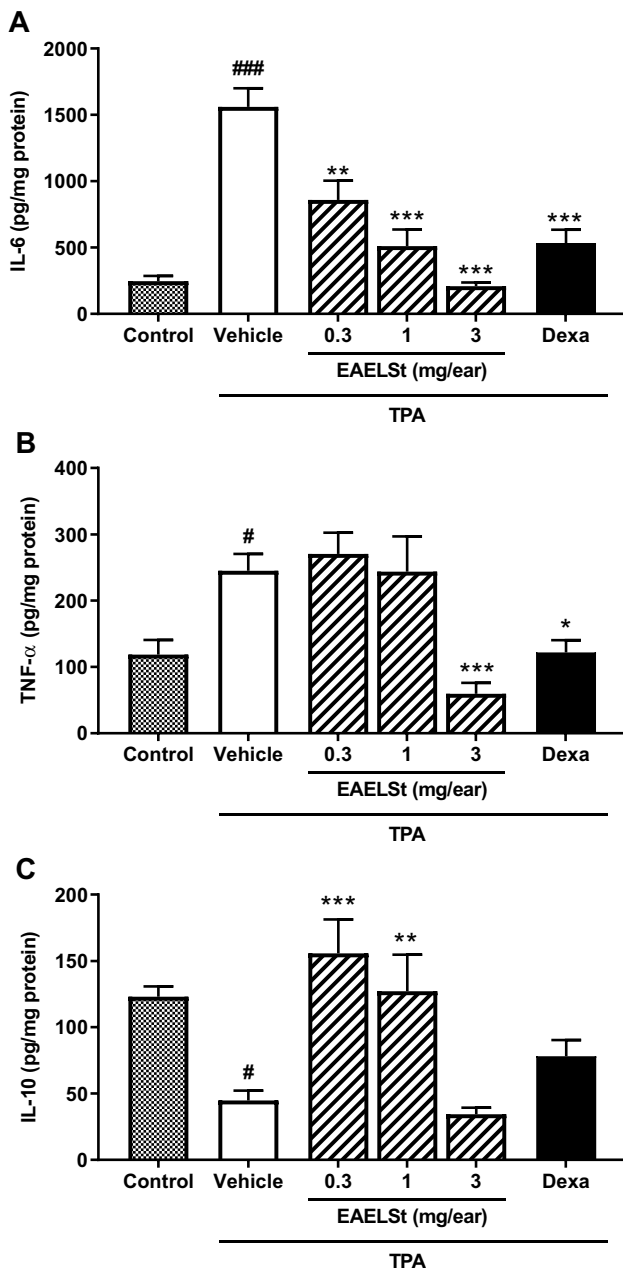


Fig. 4 Effect of ethyl acetate extract from the leaves of *S. terebinthifolius* (EAELSt) on cytokines concentration in mice ear. Animals were submitted to concomitant topical administration of 12-O-tetradecanoilforbol-13-acetate (TPA; 1 µg/ear) and EAELSt or dexamethasone (Dexa). IL-6 (A), TNF-α (B) or IL-10 concentrations are expressed as mean ± S.E.M. (n=4). One-way followed by Tukey test. #p < 0.05 or ###p < 0.001 vs. control group (right ear) and *p < 0.05, **p < 0.01 or ***p < 0.001 vs. TPA + vehicle group

The activities of SOD, CAT, and GPx were also investigated and are shown in Table 3.

Administration of TPA reduced both CAT (p < 0.01) and SOD activities (p < 0.001), in comparison to control group.

In animals treated with 0.3 and 1 mg of EAELSt/ear, we observed that the activity of CAT was higher in comparison to the TPA + vehicle group (p < 0.01 for 0.3 mg/ear and p < 0.001 for 1 mg/ear), but this effect did not occur in animals administered with 3.0 mg of extract/ear.

On the other hand, animals that received treatment with EAELSt at 3 mg/ear showed greater SOD activity (p < 0.001) when compared to the TPA plus vehicle group. However, this difference was not observed in animals that received 0.3 and 1 mg/ear of EAELSt. GPx activity did not differ among the experimental groups (p = 0.7496).

Discussion

In the present study, we show results about the in vitro antioxidant effect and in vivo anti-inflammatory and antioxidant effect of EAELSt in a model of skin inflammation, which seems to correlate with the composition of the extract.

The chemical characterization of the components presented in the EAELSt showed a high concentration of phenolic compounds and total flavonoids, which may have a greater correlation with pharmacological effects. Similarly, El-Massry et al. (2009) observed the presence of a high concentration of phenolic compounds in the ethanolic extract of the leaves of *S. terebinthifolius*, however, using the maceration technique for extraction.

The analysis of the chemical composition of the EAE by LC-MS/MS confirmed the presence of phenolic compounds and their derivatives with a total of 43 compounds identified. Among them, the major peaks area in the chromatogram were for myricetin-O-pentoside, quercetin-O-rhamnoside, and kaempferol-O-rhamnoside. Rosas et al. (2015), using the hydroalcoholic extract of the leaves of *S. terebinthifolius*, identified the presence of polyphenols such as gallic acid, methyl gallate, and penta-galloyl glucose. These data partially corroborate our findings, since these compounds were also identified in EAE, but to a lesser extent. In a study by Uliana et al. (2016), ferulic and caffeic acids, and quercetin were the major components identified by mass spectroscopy in the extracts.

In this study, it was possible to verify an antioxidant potential by reducing the free radical DPPH. The fact that EAE reduced the amount of this radical in all concentrations tested suggests that the chemical constituents of EAE may act as donors of H⁺ which indicates a mechanism for reducing the DPPH free radical (Floegel et al. 2011; Shahidi and Zhong 2015). In the study by El-Massry et al. (2009), a greater antioxidant activity was observed in the ethanolic extract than in the methanolic or dichloromethane extracts from the leaves of *S. terebinthifolius*. These data corroborate

Fig. 5 Light microscopic images representative of histological sections of mice ears. Animals were submitted to administration of acetone in the right ear (Control, **A**), 12-O-tetradecanoilforbol-13-acetate (TPA; 1 µg/ear) in the presence of acetone (**B**) or concomitant topical administration of TPA and EAELSt (3 mg/ear) (**C**) or dexamethasone (Dexa, **D**). Scale bars represent 1 mm

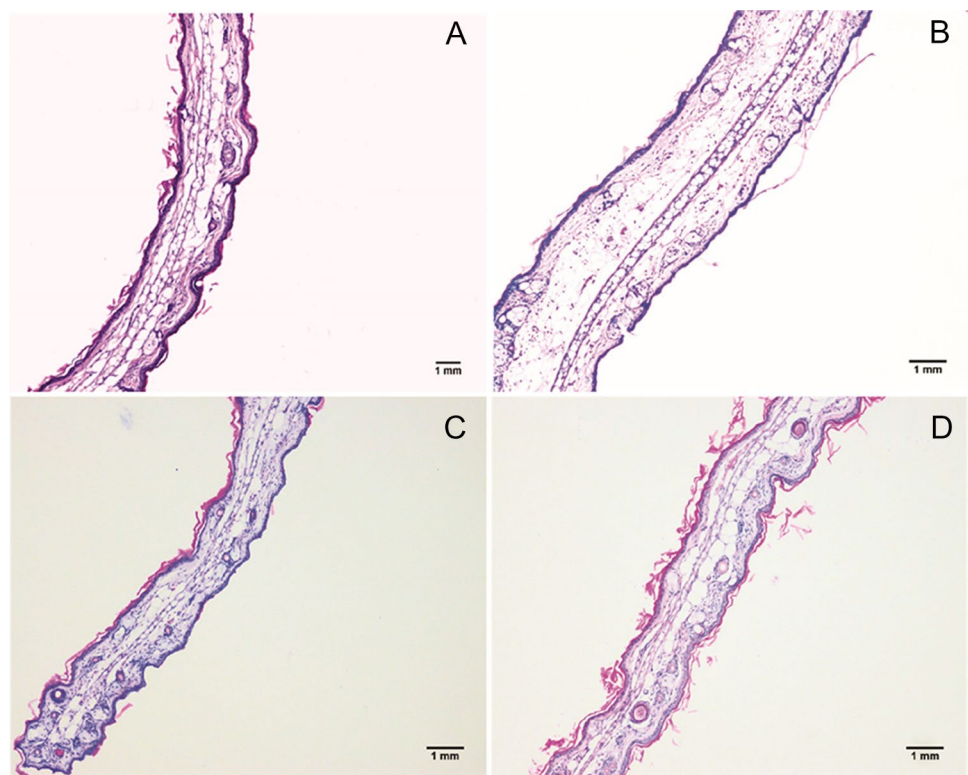


Table 3 Effect of the treatment with ethyl acetate extract from leaves of *S. terebinthifolius* (EAELSt) on oxidative stress biomarkers and antioxidant enzyme activity in mice submitted to ear inflammation model

Group	TH (mol/L)	SH (µmol/mg of protein)	FRAP (Fe ³⁺ /Fe ²⁺)	CAT (ΔE/min/mg of protein)	SOD (U/mg of protein)	GPx (mUA/mg of protein)
Control	2.76 ± 0.51	2.93 ± 0.30	0.63 ± 0.02	0.073 ± 0.007	7.75 ± 0.41	0.87 ± 0.04
TPA + vehicle	5.79 ± 0.42 ^{###}	1.03 ± 0.21 ^{###}	0.34 ± 0.01 ^{###}	0.026 ± 0.002 ^{###}	1.89 ± 0.15 ^{###}	0.73 ± 0.06
TPA + EAELSt (0.3 mg/ear)	4.89 ± 0.42	2.45 ± 0.45*	0.40 ± 0.01	0.085 ± 0.005 ^{***}	2.34 ± 0.38	0.88 ± 0.21
TPA + EAELSt (1 mg/ear)	6.31 ± 0.60	1.91 ± 0.16	0.38 ± 0.01	0.074 ± 0.014 ^{**}	2.15 ± 0.31	0.97 ± 0.17
TPA + EAELSt (3 mg/ear)	2.64 ± 0.12 ^{**}	2.48 ± 0.38*	0.47 ± 0.02 ^{***}	0.039 ± 0.009	4.33 ± 0.35 ^{***}	0.94 ± 0.09

Mice ear submitted to topical administration of 12-O-tetradecanoilforbol-13-acetate (TPA; 1 µg/ear) and concomitant treatment with EAELSt. Data are expressed as mean ± S.E.M. for the quantification of total hydroperoxides (TH), sulfhydryl groups (SH), ferrum reducing potential (FRAP), and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. One-way ANOVA followed by Tukey test (^{###}*p* < 0.01 or ^{###}*p* < 0.001 vs. acetone group; **p* < 0.05; ***p* < 0.01 or ^{***}*p* < 0.001 vs. TPA + acetone)

our study, considering that EAE presented a high concentration of total phenols and flavonoids, associated with antioxidant capacity. Flavonoids can act directly or indirectly as antioxidants (Jucá et al. 2020), so that the antioxidant activity is related to the amount of hydroxyl group in its structure (Havsteen 2002).

To complement the evaluation of antioxidant activity *in vitro*, the evaluation method by inhibiting lipoperoxidation in a biological matrix consisting of rat brain homogenate was used. The results obtained indicate that there was a protective effect for the formation of MDA for all evaluated

EAELSt concentrations. MDA is formed during oxidative degeneration as one of the products of free radicals and serves as a marker of lipoperoxidation (Alam et al. 2013). Based on the study by Lesjak et al. (2018), it is possible to suggest that EAELSt effect is related to the presence of phenolic compounds, such as quercetin and its derivatives, which has already been shown to have inhibitory effects on MDA (Lu et al. 2018; Tian et al. 2021).

Before the study in a model of skin inflammation *in vivo*, a cytotoxicity test with L929 fibroblasts was carried out, to verify whether EAELSt presented any cellular toxicity.

Using the MTT test, we showed that EAELSt did not have a cytotoxic effect until the concentration of 100 µg/mL in this cell line. There is no information on the toxicity of *S. terebinthifolius* leaves in in vitro studies. However, data from other authors showed that the ethanolic extract of the bark of this plant did not produce acute or subacute toxicity (45 days of administration) in Wistar rats of both sex, indicating that the oral pretreatment does not cause cytotoxic effect (Lima et al. 2009).

Despite the ethnobotanical suggestions of this species having an anti-inflammatory effect, few studies have investigated its chemical composition and its association with anti-inflammatory and antioxidant activity in vivo. For this purpose, the TPA-induced ear inflammation model was used to evaluate the topical anti-inflammatory effect of EAELSt in vivo. The time point of 6 h was chosen because it is the peak time of edema formation, and it was previously reported to show infiltration of neutrophils, according to reference study we used to perform this assay (De Young et al. 1989).

In the present study, it was shown that the topical application of EAELSt reduced the edema caused by TPA in the range doses of 0.3–3 mg/ear. These data indicate that EAE influences the vascular component of the inflammatory response that contributes to edema formation, suggesting a topical anti-inflammatory effect. Accordingly, histological analysis reinforced that treatment with EAELSt at the high dose used reduced edema.

The mechanism for the formation of edema induced by TPA is not completely understood. However, evidence indicates that treatment with TPA activates protein kinase C, promotes release of eicosanoid mediators, such as prostaglandins and leukotrienes, increased expression of cyclooxygenase-2, migration of leukocytes, and increased concentrations of cytokines IL-1 β and TNF- α (Carlson et al. 1985; Oliveira et al. 2017), which suggests the involvement of these pathways in the anti-inflammatory effect of EAELSt. In agreement with our data, Fedel-Miyasato et al. (2014) showed that oral treatment with the methanolic extract of the leaves of *S. terebinthifolius* reduced the edema induced by Croton oil in mice ear, similar to that observed in the present study, but these authors did not report any other inflammatory markers in their model.

In addition to the anti-edematogenic effect, a similar effect was observed for doses of EAELSt on MPO activity, which reflects the inhibition of neutrophil migration to the inflamed site. Neutrophil infiltration, characteristic of acute inflammation, was assessed indirectly through the activity of MPO, an enzyme located in neutrophil azurophil granules (Jorch and Kubes 2017). The recruitment of neutrophils occurs through the stimulation of cytokines and chemokines, which, in turn, initiates a series of interactions

between different types of leukocytes and endothelial cells (Timmerman et al. 2016).

The study by Rosas et al. (2015) corroborates the reduction in neutrophil migration observed in our study. These authors showed that the oral pretreatment with the hydroethanolic extract obtained from the leaves of *S. terebinthifolius* inhibited the migration of neutrophils in a model of pleurisy induced by zymosan. In the same study, using the zymosan-induced arthritis model, there was also a reduction in joint edema and inhibition of neutrophil migration to the joint.

Our data raised the possibility that components of the EAE may act to reduce the migration of neutrophils. In the study by Rosas et al. (2015), gallic acid, another component found in EAE, reduced the in vitro migration of isolated human neutrophils stimulated with N-formylmethionyl-leucyl-phenylalanine. These data reinforce that the phenolic compounds identified in EAELSt may be responsible for the anti-inflammatory effects shown in the present study.

Increased concentration of IL-6 and TNF- α is associated with cutaneous inflammatory response, as well as with other pro-inflammatory cytokines (Murakawa et al. 2006; Scheller et al. 2011). Accordingly, in the study by Blaser et al. (2016), it was shown that the use of a TNF- α antagonist inhibited both edema and TPA-induced concentrations of TNF- α . We chose to measure TNF- α and IL-6 because these are cytokines involved since the initial stage of the inflammatory response. Many other inflammatory mediators might also be reduced by the administration of the extract. However, as we worked with the crude extract, the exact mechanism of action cannot be fully disclosed, provided that a variety of compounds that can contribute to the beneficial effects have been identified.

We showed a reduction of IL-6 and TNF- α in the ears treated with EAELSt, which corroborates our data on the reduction of edema and MPO. It is interesting that all doses of EAELSt reduced the concentration of IL-6, but only the highest dose of this extract decreased the concentration of TNF- α , which suggests differential modulation between these cytokines in the evaluated time point.

Other authors observed that the treatment with hydroethanolic extract of the leaves of *S. terebinthifolius* caused a reduction in the concentration of IL-6 and TNF- α in a model of arthritis induced by zymosan in mice (Rosas et al. 2015), which corroborates the effect observed in our study. It is also interesting that the treatment with the ethyl acetate fraction of the leaves of *S. terebinthifolius* decreased the concentrations of chemokines with a Th2 profile, namely eotaxin and CCL5/RANTES in ovalbumin-induced allergic pleurisy in rats (Cavalher-Machado et al. 2008a).

Considering the compounds presented in EAELSt, it is well described that flavonoids can reduce the formation of pro-inflammatory cytokines (Maleki et al. 2019). Thus, it is

possible that the anti-edematogenic effect and the reduction in the concentrations of IL-6 and TNF- α by EAELSt, in part, are attributed to the presence of phenolic compounds in this extract, such as quercetin, which can act solely or synergistically, most likely by modulating intracellular signaling pathways such as phosphatidylinositol-3-kinase or other tyrosine kinase proteins (Lolli et al. 2012; Yokoyama et al. 2015) or transcription factors like the nuclear factor κ B (Peng et al. 2018).

In the present study, the effect of EAELSt on IL-10 concentrations was also evaluated. The pretreatment with EAELSt only in the lowest doses (0.3 and 1.0 mg/ear) prevented the reduction of IL-10 levels produced by TPA. IL-10 is a cytokine that plays an important role in maintaining homeostasis and in responding to inflammatory stimuli by suppressing pro-inflammatory cytokines (Ouyang and O'Garra 2019). Considering this fact, it is possible to speculate that the concentrations of IL-10 in the ears would be linked to the concentrations of TNF- α . Thus, at the lowest doses of EAELSt, the increased concentrations of IL-10 would be compensating for the lack of reduction in TNF- α concentrations, which did not occur for the highest dose of the extract. Anyway, the results obtained indicate that the treatment with EAELSt modulated this anti-inflammatory cytokine, which confirms the action of this extract in the cutaneous inflammatory response induced by TPA.

These protective actions of EAELSt may be related to the compounds presented in this extract. These phenolic compounds, in addition to being able to modulate signaling pathways and transcription factors (Lolli et al. 2012; Peng et al. 2018; Yokoyama et al. 2015), are known for their antioxidant effects, which could contribute to the action on the inflammatory response. Thus, we also investigated whether EAELSt could alter the oxidative stress that accompanies the induction of skin inflammation induced by TPA.

In fact, treatment with EAELSt promoted modulation of the formation of hydroperoxides, sulfhydryl groups, and the potential to reduce iron. Our data indicate that there was an inhibitory effect on oxidative stress markers (by reducing total hydroperoxides and by increasing the sulfhydryl groups). The formation of hydroperoxides denotes initial stages of lipid peroxidation, since these species are primary products of lipoperoxidation (Esterbauer 1993). In turn, it is known that the sulfhydryl groups are present in the constitution of several proteins and oxidative stress causes oxidation in these groups, resulting in malfunction of the cellular structures (Santos et al. 2011). Thus, it is most likely that the phenolic compounds in EAELSt reduced the formation of hydroperoxides and preserved the sulfhydryl groups from possible changes induced by oxidative stress induced by TPA. Taken together, these parameters show the decrease in the lipid peroxidation, and increase in sulfhydryl

groups and in the Fe²⁺/Fe³⁺ rate, which strongly indicates the antioxidant effect of the extract in mice ears.

Additionally, it was observed that the highest dose of EAE increased the reduction potential indicating antioxidant effect through the FRAP method in vivo. It is known that, during oxidative stress, Fe³⁺ reacts with O₂⁻ becoming Fe²⁺. This occurs through the Fenton reaction, which leads to the formation of hydroxyl radical which is highly reactive (Shahidi and Zhong 2015). The data found suggest that the reducing potential of EAELSt possibly occurs by the action of polyphenolic compounds identified in this plant, as proven in other studies (Jeyadevi et al. 2013). In fact, the antioxidant activity of polyphenolic compounds such as quercetin identified in EAELSt is directly related to the amount of hydroxyl group, position, and glycosylation (Cai et al. 2006).

We also found that treatment with this extract increased the activity of SOD activity at 3.0 mg/ear. Since SOD is responsible for the conversion of \cdot O₂⁻ to H₂O₂ and water, this data shows that there was modulation of this enzyme to protect the tissue against oxidative stress. It has been documented in the literature that SOD can contribute to the resolution of inflammation through apoptosis of neutrophils, regulated by the H₂O₂ (Yasui and Baba 2006). For the CAT enzyme, treatment with 0.3 and 1 mg EAE/ear increased the activity of this antioxidant enzyme. CAT catalyzes the conversion of H₂O₂ into H₂O, which indicates that the increased activity of this enzyme results in the detoxification of free radicals.

Another interesting finding was related to the effect induced by the dose of 3 mg EAE/ear, which, despite not modulating CAT activity, reduced the formation of hydroperoxides, preserved the sulphidryl groups and increased SOD activity. One possibility would be that the GPx activity acts in a compensatory way at the different doses of the EAE; however, the GPx activity remained unchanged in all the doses evaluated. In this context, it can be suggested that, at the different doses of EAE, there was a compensatory effect between the activities of CAT and SOD that acted primarily in the detoxification process.

In the literature, studies involving oxidative stress in the model of ear edema used in the present study are still seldomly described. To our knowledge, this is the first study to demonstrate the effect of *S. terebinthifolius* on antioxidant markers and enzymes. It is important to highlight the involvement of oxidative stress in the inflammatory process, as tissue damage during this situation leads to an excess of oxygen and nitrogen reactive species (Hussain et al. 2016) and several transcription factors involved in inflammation, such as the nuclear factor- κ B, are activated by ROS (Li et al. 2002). Thus, it is plausible to suggest that the anti-inflammatory effect of EAELSt is associated, in part, with protection against oxidative damage. Besides, it is also possible to assume that

the anti-inflammatory effect is not solely due to the antioxidant activity, since the anti-inflammatory effect was detected even when using a lower dose, when the antioxidant effect was not fully achieved. Despite these facts, our data suggest that the EAELSt can be promising in the search for alternatives for the treatment of inflammatory conditions for topical use. Furthermore, this study showed that EAELSt is promising for the treatment of skin inflammation. Finally, this study can serve as a basis for future studies to better understand the pharmacological action and its possible mechanisms of action.

Conclusion

In this study, it was demonstrated that EAELSt promotes a topical anti-inflammatory effect in an animal model of acute TPA-induced skin inflammation. In addition, this extract showed antioxidant activity both in vitro and in vivo. From these data, it can be evidenced that the biological effect presented by EAE can bring perspectives to explore the therapeutic potential of this plant and enable the treatment of inflammatory conditions.

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Author contributions MSN, PHS, FFA, AYKVS, RGA, and LNA contributed to the conception and design of the study; JPRS, JFT, AMOS, CBC, MMM, SP, CP, EAC, and CSE contributed to sample prospection, data collection, data analysis, and interpretation of results; RGA, LNA, EBS, MISS, ASG, and JBS contributed to the validation of results, discussion, draft and final version of manuscript preparation. RGA, LNA, EBS, and CSE contributed to the management of the project. All authors approved the final version of the manuscript.

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Data availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest Authors report no conflicts of interests with respect to this research.

Ethical approval All experiments were carried out according to the guidelines of the Brazilian College of Animal Experimentation and the

National Institutes of Health and were approved by the Ethics Committee on Animal Use in Research of Federal University of Sergipe (Approval n° 06/2019).

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