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HEALTH SCIENCES

In vivo anti-inflammatory and antinociceptive effects, and *in vitro* antioxidant, antiglycant and anti-neuroinflammatory actions of *Syzygium malaccense*

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Abstract: *Syzygium malaccense* is popularly used to treat inflammation and pain-related ailments. The species was assessed regarding its antioxidant, antiglycant, anti-inflammatory, including anti-neuroinflammatory, and antinociceptive activities. Different models were employed to measure *S. malaccense* extract (ESM) antioxidant activity. The antiglycant activity was determined using the glucose-induced protein glycation model. LPS-induced neuroinflammation on murine BV-2 microglial cell line was used for anti-neuroinflammatory activity evaluation. The croton oil-induced ear edema test was accomplished to evaluate the *in vivo* anti-inflammatory activity. Acetic acid-induced writhing together with formalin-induced paw licking assays were performed to evaluate the antinociceptive potential. Finally, the chemical characterization was accomplished by a UHPLC-MS analysis. ESM presented relevant antioxidant and antiglycant activity. NO production by BV-2 cells was reduced, indicating the relevant neuroprotective activity. ESM significantly decreased the mice ear edema induced by croton oil and the nociceptive stimulus induced by acetic acid and formalin by central and peripheral mechanisms. The flavonoids myricitrin, myricetin and quercetin were identified and, as far as we know, the alkaloid reserpine was reported in the species for the first time. The antioxidant and antiglycant potential of ESM, may be related to the *in vivo* anti-inflammatory and antinociceptive effects, and to the *in vitro* neuroinflammation inhibition.

Key words: Antiglycant, antioxidant, inflammation, oxidative stress, pain.

INTRODUCTION

The inflammatory process is a severe response triggered by tissues to restore homeostasis after a harmful stimulus. Inflammation often causes noticeable symptoms, such as pain, which is one of the main reasons for medical sessions (Treed et al. 2019). The Global Burden of Diseases recognizes chronic pain as one of the leading causes of disability worldwide, which encourages national governments to consider pain management a public health priority and generate national strategies to challenge this issue (Fayaz et al. 2016). Pain reduces life quality and represents a significant risk for developing mental health disorders and suicidality, characterized by ideation, plans, and attempts to suicide (Campbell et al. 2015).

Furthermore, neurodegenerative disorders, including Alzheimer, Parkinson, Huntington, and multiple sclerosis, have emerged along with the increase of life expectancy worldwide (Zhang et al. 2010, Elmann et al. 2011). Those diseases are related to a neuroinflammatory process and

the excessive production of pro-inflammatory mediators by brain microglial cells (Elmann et al. 2011, Kim et al. 2018).

Oxidative stress, several chemical mediators and cellular components are involved in the inflammatory response, playing essential roles in the physiopathology of various high prevalence diseases, including rheumatoid arthritis, atherosclerosis, and asthma (Bhagavan et al. 2013). There is also evidence that oxidative stress is associated with neurotoxic mechanisms involved in the pathogenesis of neurodegenerative disorders (Neal & Richardson 2018). The brain oxidative stress may induce the formation of advanced glycation end products (AGEs), resulting from a non-enzymatic reaction that binds sugars to peptides or proteins. AGEs formation enhances the oxidative stress, inducing a positive feedback loop and favors the deposition of proteins in neurons, including amyloid-β, tau, α-synuclein, and prions, which are related to the neuronal cell death and, consequently, to the development of neurodegenerative diseases (Li et al. 2012).

In this context, several plants are used in traditional medicine for inflammation and pain-related ailments., including *Syzygium malaccense* (L.) Merr. & L. M. Perry (Myrtaceae), basionym *Eugenia malaccensis* L. This plant is native from Malaysia, Indonesia, Vietnam, and Thailand. However, it is commonly found in other tropical regions, as in the northern, northeastern, and southeastern regions of Brazil, where it is popularly known as "jambo" and "jambo vermelho" (Nunes et al. 2016, Fernandes & Rodrigues 2018). Different parts of this plant have been used in traditional medicine to treat various diseases, including dysentery, fever, bronchitis, skin diseases, and abdominal pain. It is also used as a diuretic, anti-inflammatory, antiviral and antifungal (Adebayo et al. 2015, Dustan 1997, Pedrollo et al. 2016, Whistler & Elevitch 2006).

The antioxidant and hypoglycemic potential were reported for the alcoholic extract obtained from the leaves of *S. malaccense* (Arumugam et al. 2016, Ramadhania et al. 2017). The ethanolic extract presented a significant topical inhibitory effect against the ethyl phenyl propiolate-induced rat ear edema challenge and a promising *in vitro* inhibition of the enzyme cyclooxygenase-1 (Dunstan 1997). Glycosylated flavonoids as myricetin derivatives (Arumugam et al. 2016), catechins, quercetin, and carotenoids were already identified in the leaves (Batista 2017).

The present study investigated the potential of the methanolic extract of *S. malaccense* leaves (ESM) in treating painful and inflammatory conditions, including neuroinflammation. In addition, its antioxidant and antiglycant activities were assessed, as oxidative stress and advanced glycation end products may be involved in the pathophysiology of brain inflammation. Besides, ESM chemical characterization was performed.

MATERIALS AND METHODS

Plant material

The leaves of *S. malaccense* were collected in Rio Novo, Minas Gerais State, Brazil (21°26'03.6"S 43°06'41.8"W). A voucher specimen (CESJ 46600) was deposited at Leopoldo Krieger Herbarium of Federal University of Juiz de Fora. The plant name has been checked with http://www.theplantlist.org, accessed in July 2020.

The plant material was dried at 40 °C, and the extraction process was performed by static maceration at room temperature with methanol until exhaustion. Finally, the extract was concentrated under reduced pressure using a rotary evaporator to obtain the methanolic extract (ESM), and stored in a refrigerator at 4 °C.

Chemicals

Indomethacin was purchased from Las Casas (Juiz de Fora, MG, Brazil). Morphine was obtained from Cristália (Itapira, SP, Brazil). Animal commercial chow was from Nuvital® (Colombo, PR, Brazil). Croton oil, Tween 80, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene, linoleic acid, bovine serum albumin (BSA), fetal bovine serum (FBS), *E. coli* serotype 111:B4 lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), aminoguanidine and fructose were from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate was from Labsynth (Diadema, SP, Brazil), and ammonium molybdate was from Vetec (Rio de Janeiro, RJ, Brazil). Dulbecco´s modified Eagle´s medium (DMEM) and antibiotic-antimycotic solution were acquired from Thermo-Fischer Scientific (Waltham, MA, USA). All other reagents were of the highest quality available.

In vitro antioxidant activity

β-carotene/linoleic acid bleaching assay

Antioxidant activity was determined using the *β*-carotene bleaching assay (Ismail et al. 2004) with slight modifications. A stock solution of the *β*-carotene-linoleic acid mixture was prepared as follows: 50 µL *β*-carotene (10 mg/mL in chloroform), 20 µL linoleic acid, and 265 µL Tween 40 were dissolved in 1 mL of chloroform. The solvent was then entirely evaporated using nitrogen gas, and 40 mL of oxygen saturated water was added with vigorous shaking. After, 250 µL of this mixture were dispersed to a 96-well plate. Finally, 10 µL of blank, ESM, and quercetin (final concentration 1.20-38.5 µg/mL), used as the reference standard, were added. The emulsion system was incubated for 2 h at 45 °C, and the absorbance was read at 470 nm. The test was performed in triplicate. IC_{50} values were calculated and indicated the concentration of extract required to inhibit 50% of oxidation.

DPPH radical scavenging test

The radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was used to determine ESM free radical-scavenging activity (Blois 1958). One hundred microliters of ESM diluted in methanol were added to a 96-well microplate. Successive (1:2) dilution with methanol was then performed (250 - 0.98 µg/mL). Methanol solution of DPPH 20 µg/mL was added to each well. The microplate was incubated in the dark for 30 min, and the absorbance was measured at 517 nm. The experiment was carried out in triplicate. Quercetin and ascorbic acid were used as reference compounds. IC_{50} values were calculated and indicated the concentration of extract required to scavenge 50% of DPPH free radicals.

Phosphomolybdenum assay

This test was accomplished as described by Prieto et al. (1999). The phosphomolybdenum is produced after the reaction between Na_3PO_4 (28 mL, 0.1mol/L) with $(NH_4)_6Mo_7O_{24}.4\ H_2O$ (12 mL, 0.03 mol/L) and H_2SO_4 (20 mL, 3 mol/L) in water (sufficient quantity to 100 mL). Two milliliters of the reactive solution was added to 200 µL of ESM or rutin (200 µg/mL), used as the reference drug. The mixture was incubated in a water bath at 95 °C for 90 min. The absorbance was measured at 695 nm. The test was performed in triplicate. The total antioxidant capacity (TAC) was expressed as milligrams of ESM equivalent to 1 mg of ascorbic acid, using a seven-point standard calibration curve.

Ferric reducing antioxidant power assay (FRAP)

The ESM reducing power was determined by FRAP assay described by Oyaizu (1986) at different concentrations (serial dilution of 3.35-53.64 µg/

mL). Firstly, ESM was diluted in phosphate buffer pH 6.6 and potassium ferrocyanide [$K_3Fe(CN)_6$] 1% and incubated at 50 °C for 20 min. After that, trichloroacetic acid 10% was added, and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was diluted in 2.5 mL water and 0.5 mL $FeCl_3$ 0.1%, and the absorbance was measured using a spectrophotometer (700 nm). Ascorbic acid was used as the reference compound. This test was performed in triplicate, and the EC_{50} was calculated using the mean of five different concentrations.

Thiobarbituric acid reactive substances assay (TBARS)

The TBARS assay was used to verify ESM ability to inhibit lipid peroxidation in ground beef, as described by Uchiyama & Mihara (1978), with some modifications. Briefly, 100 g of ground beef and 67 mL of distilled and deionized water were mixed with 7.5, 15, or 30 mg of ESM dissolved in methanol. A solution containing beef, water, and methanol was used as the control, and butylated hydroxytoluene (BHT) 7.5, 15, or 30 mg diluted in methanol was used as the reference compound. These mixtures were blended until a smooth homogenate was formed and transferred to amber jars, stored at 5 °C for 5 days. The beef oxidation was then indirectly measured by a calibration curve prepared using the malonaldehyde (MDA) standard, reacting with the TBA/phosphoric acid solution. The measurement was accomplished using the absorbance spectrum of 535 nm. The results were expressed as µM of MDA formed by the reaction.

In vitro antiglycant activity

The *in vitro* antiglycant activity was determined using glucose-induced protein glycation models. The method was performed as previously described (Farsi et al. 2008) with some modifications. The ESM and aminoguanidine were evaluated at concentrations of 375-12.5 µg/mL in a medium containing BSA (2.5 mg/mL), glucose (0.2 M), sodium azide (0.6 g/L) and sodium phosphate buffer (pH 7.4). After the incubation time at 37 °C, the amount of fluorescent AGEs formed was determined using a fluorimeter after one, two, and ten days. The fluorescent intensity was measured at 330 nm (excitation) and 410 nm (emission). The antiglycant activity was expressed as the mean ± S.E.M. of AGE formation percentage compared to the vehicle.

In vitro anti-neuroinflammatory activity on murine BV-2 microglial cell line

Cell culture

Murine BV-2 microglial cell line was used to evaluate the anti-neuroinflammatory activity of ESM. The cells were maintained in DMEM enriched with 10% FBS and 1% of antibiotic solution (10.000 units/mL of penicillin, 10.000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B) at 37 °C, 95% humidity and 5% CO_2. The cultures were subcultivated in 25 and 75 cm^2 bottles, with 0.05% trypsin diluted in ethylenediamine tetraacetic acid (EDTA).

Cytotoxic activity on BV-2 cells

To evaluate whether ESM is toxic to BV-2 cells, the MTT assay (Mosmann 1983) was accomplished. Therefore, 4 x 10^4 cells/well were seeded in 96-well microplate and incubated overnight. Then, ESM 50 µg/mL was added, and the plate was incubated for 3 and 24 h at 37 °C, 100% humidity, and 5% CO_2. After the incubation time, the wells were aspirated, and 200 µL of MTT (0.5 mg/mL) diluted in DMEM were added to each well. Finally, after 2 h of the incubation period, the formazan crystals were dissolved

in dimethyl sulfoxide (DMSO)/ethanol (1:1). The control groups consisted of 0.3% DMSO or DMEM medium. The optical density was read at 570 nm. The test was performed in quintuplicate, and the results were expressed as mean ± S.E.M. of three independent experiments.

In vitro LPS-induced neuroinflammation

This test was accomplished according to Wang-Yang et al. (2014). Therefore, 4 x 10^4 cells/well were seeded in 96-well microplate and incubated overnight. Then, the wells were aspirated, and 50 µg/mL of ESM, diluted in DMEM enriched with 2% FBS, were added. After 2 h, bacterial lipopolysaccharide (LPS) 1 µg/mL was also added, and the mixture was incubated for 24 h. The induction control consisted of DMEM with 2% FBS and LPS. The negative control, DMEM with 2% FBS, was used to evaluate the cell growth. After that, the supernatants were transferred to a sterile 96-well microplate for nitric oxide (NO) dosage. Then, 200 µL of MTT reagent (0.5 mg/mL) diluted in DMEM were added to each well, and the mixtures were incubated for 2 h. The insoluble formazan crystals were dissolved using 200 µL of DMSO/ethanol (1:1) solution per well. The optical density was evaluated at 570 nm. The NO level in cell cultures was measured using the Griess reaction (Schmidt & Kelm 1996). The test was performed in quintuplicate, and the results were expressed in the inhibition percentage compared to the induction control. The mean ± S.E.M. of three independent experiments was used.

In vivo anti-inflammatory and antinociceptive activities

Animals

Male Swiss mice (20-30 g, approx. 30 days old) were bred at the Center of Reproductive Biology of Federal University of Juiz de Fora. The animals were maintained in a room with 12 h/12 h dark/light cycle under standard temperature (22 °C) with water and food *ad libitum*; however, mice were fasted for 12 h before each experiment. Each group contained 8 animals. All experimental procedures followed the Ethical Principles of Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA – CEUA/UFJF Protocols n° 009/2009, 013/2013, 016/2013, 021/2013 and 028/2014)

Croton oil-induced ear edema test

The anti-inflammatory activity of ESM was evaluated as described by Schiantarelli and collabs (Schiantarelli et al. 1982). Firstly, animals were distributed in five groups and orally treated by gavage with ESM 50, 100, or 300 mg/kg, indomethacin 10 mg/kg (used as reference drug), or vehicle (12% Tween 80 diluted in NaCl 0.9%, v/v). All solutions were administered at 10 mL/kg. After 60 min, 20 µL of a fresh solution of 2.5% croton oil dissolved in acetone (v/v) was topically applied in the inner surface of the right ear, and the same volume of acetone (vehicle) was administered on the inner surface of the left ear of each mouse. Six hours after the beginning of the experiment, the animals were euthanized, and 6 mm diameter ear punch biopsies were obtained and individually weighed in an analytical balance. The weight difference between the right (inflamed) and the left (non-inflamed) ear biopsies was used to measure edema and, consequently, the inflammatory process.

Acetic acid-induced writhing test

The evaluation of the antinociceptive action of ESM was performed using the well-established acetic acid-induced writhing test (Koster et al. 1959). Five groups of animals were used. Mice were orally treated with ESM 50, 100, or

300 mg/kg, indomethacin 10 mg/kg, used as the reference drug, or vehicle (12% Tween 80 diluted in NaCl 0.9%, v/v). All treatments were administered at 10 mL/kg. After 60 min, mice received i.p. injection of acetic acid 0.6%, 10 mL/kg. The number of writhings (constriction of the abdominal wall combined with the extension of the hind paws) was counted for 30 min and indicated the level of mice nociceptive reaction.

Formalin-induced paw licking test

The formalin test was used to verify whether the ESM antinociceptive effect is related to a central or peripheral anti-inflammatory activity (Hunskaar et al. 1985). Firstly, animals were separated in six groups. Mice were orally pretreated with 10 mL/kg of ESM 50, 100 or 300 mg/kg, indomethacin 10 mg/kg, which was used as a reference drug that acts peripherally, or vehicle (12% Tween 80 diluted in NaCl 0.9%, v/v). Besides, morphine 7.5 mg/kg (10 mL/kg) was i.p. injected to be used as a reference antinociceptive drug with central mechanisms. After 60 min, 20 mL of 2% formalin prepared in saline was injected into each mouse's right hind paw's sup-plantar tissue. The licking paw time was timed during 5 min (first phase of the nociceptive stimulus) and between 15 and 30 min (second phase of the nociceptive stimulus) after the formalin injection.

Chemical characterization

UHPLC-MS analysis

The instrument consisted of an ultra-high pressure liquid chromatography (UHPLC) Shimadzu Nexera attached to a high-resolution mass spectrometer, a quadrupole time of flight (QTOF) Bruker Maxis, with an electrospray ionization operated in a positive mode. The chromatographic separation was achieved in a C18 Shim-pack XR-ODSIII (150 x 2.0 mm, 2.2 µm) at a controlled temperature of 40 °C, and the injection volume was set to 5 µL.

The mobile phase consisted of 0.1% formic acid in water as reservoir A and 0.1% formic acid in acetonitrile as reservoir B at a constant flow rate of 200 µL/min. The mobile phase was delivered according to the following elution program: 5.0% B (0.0 – 5.0 min); 5.0-100.0% B (5.0 – 45.0 min); 100% B (45.0 – 50.0); and one more minute to restore the column to the initial mobile phase condition. Samples were introduced into the interface through a heated nebulizer probe set at 200 °C. The optimized parameters for electrospray ionization (ESI) were a gas flow of 8 L/min, a nebulizer of 2.0 bar, a capillary voltage of 4.5 kV, and a gas temperature of 200 °C, with nitrogen used as the drying gas. Compass software version 1.5 was used to obtain data acquisition and quantification.

HPLC-UV analysis

This technique was used to confirm the presence of the alkaloid reserpine in ESM. Reserpine standard (0.15 mg/mL), ESM (2 mg/mL), and the mixture of both samples (Reserpine:ESM – 1:100) were injected at room temperature (25 °C) in the high-pressure liquid chromatography (HPLC) instrument attached to UV detector Agilent 1200 Series (Agilent, Santa Clara, CA). The mobile phase was a gradient consisted of water and acetonitrile 5.0% - 70% (0.0 – 10.0 min), then acetonitrile 70% - 80% (10.01 – 30.0 min). The volume injection was set to 20 µL. Agilent XDB-C18 column, the flow rate at 0.8 mL/min, and detection at 230 nm were used.

Statistical analysis

All antioxidant results were expressed as mean ± S.D. (standard deviation), except for TBARS assay. The software GraFit Data Analysis® 5.0 was used to calculate the IC_{50} in the DPPH scavenging assay

and β-carotene/linoleic acid bleaching method. Microsoft Excel® 2007 was used to analyze the FRAP assay. The comparison between groups was assessed by two-way ANOVA, followed by the Tukey test in TBARS assay (mean ± S.E.M - standard error of mean). Values were expressed as mean ± S.E.M. One-way ANOVA followed by the Tukey test was used for all other experiments. The software GraphPad Prism® 7.0 was used for the statistical analysis. p values < 0.05 were considered significant.

RESULTS

Antioxidant activity

The Table I summarizes the IC_{50} values and total antioxidant capacity obtained for ESM in DPPH scavenging, β-carotene/linoleic acid bleaching and FRAP assays. ESM inhibited lipid peroxidation on days 4 and 5 at all tested concentrations in TBAR assays; the results were statistically comparable to BHT at 15 and 30 mg, in contrast to BHT 7.5 mg, which showed no activity (Figure 1).

Antiglycant activity

ESM showed impressively antiglycant activity, considerably reducing the *in vitro* formation of the BSA-glucose complex. ESM and aminoguanidine activities were similar. However, ESM showed more significant inhibition of AGE formation when statistically compared to the reference drug at some tested concentrations and time points throughout the experiment (Figure 2).

In vitro evaluation of the anti-neuroinflammatory activity of ESM in BV-2 microglial cell line

Firstly, it was verified if ESM interfered with BV-2 cell viability. ESM did not show cytotoxicity up to 100 µg/mL (data not shown). LPS was used to induce the inflammatory process on BV-2 cells at noncytotoxic levels (Figure 3), so that the cells induced and treated with ESM remained viable (as the control, C-). As shown in Figure 4, ESM significantly reduced NO production induced by LPS.

Table I. Antioxidant activity of *Syzygium malaccense* methanolic extract (ESM) evaluated by β-carotene/linoleic acid, DPPH, phosphomolybdenum, and FRAP assays.

Samples	β-carotene / linoleic acid bleaching (IC_{50} - µg/mL[a])	DPPH scavenging activity (IC_{50} - µg/mL[a])	TAC (mg/mg sample[a, b])	FRAP (EC_{50} - µg/mL[a])
ESM	17.43 ± 2.05	11.87 ± 0.66	0.46 ± 0.03	20.28 ± 0.02
Ascorbic acid	nd	0.41 ± 0.27	nd	4.27 ± 0.06
Rutin	nd	nd	0.85 ± 0.02	nd
Quercetin	1.18 ± 0.62	1.02 ± 0.04	nd	nd

nd, not determined. [a]Values expressed by mean ± S.D.[b] TAC means total antioxidant capacity expressed as mg of ascorbic acid equivalent to mg of sample.

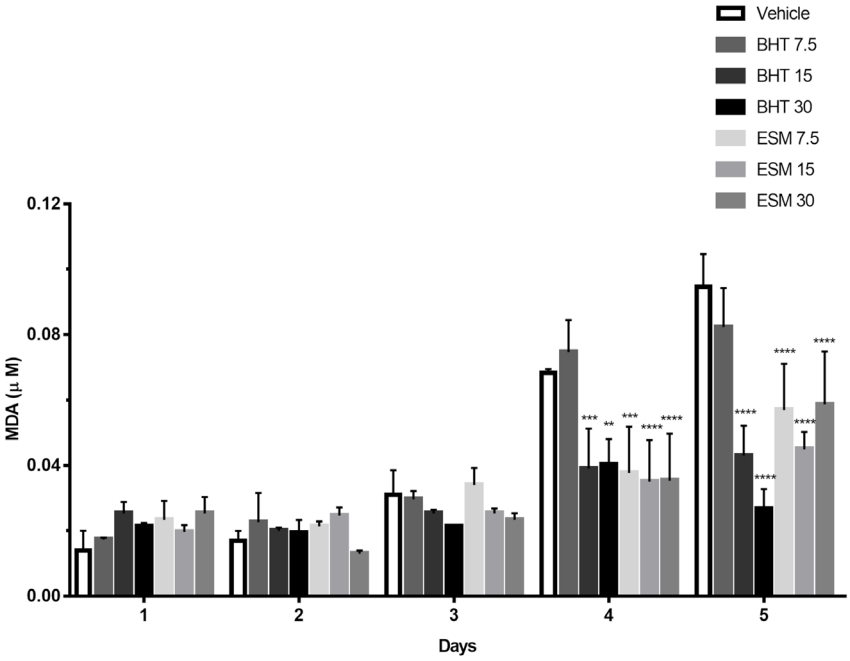

Figure 1. ESM antioxidant activity in the TBARS assay. The ground beef oxidation was indirectly measured by a calibration curve prepared using the malonaldehyde (MDA) standard. The measurement was accomplished using the absorbance spectrum at 535 nm. The results were expressed as μM of MDA formed by the reaction. The comparison between groups was assessed by two-way ANOVA, followed by the Tukey test (mean ± S.E.M). Significant values: ***p* <0.01; ****p* <0.001; *****p* <0.0001 compared to the vehicle.

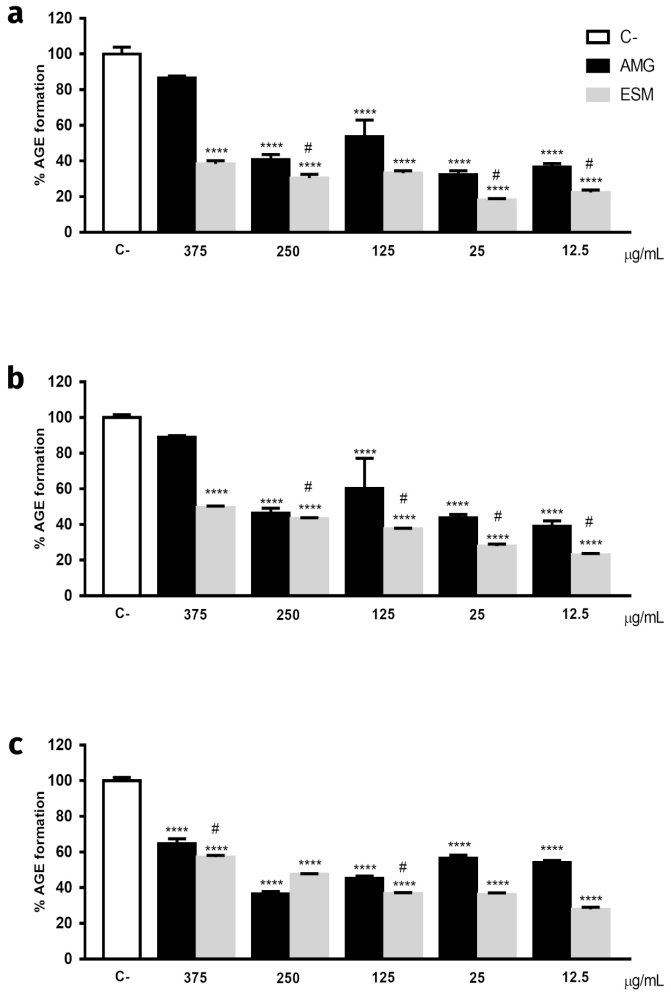

Figure 2. Effect of ESM at different concentrations (12.5-375μg/mL) on AGE formation. Fluorescent intensity was measured after (a) one, (b) two, and (c) ten days at 330 nm (excitation) and 410 nm (emission). The antiglycant activity was expressed as the mean ± S.E.M. of AGE formation percentage compared to the vehicle (100%). Significant values:*****p* <0.0001 compared to the vehicle; # means statistically equal to the reference drug (AMG-aminoguanidine) at the same concentration.

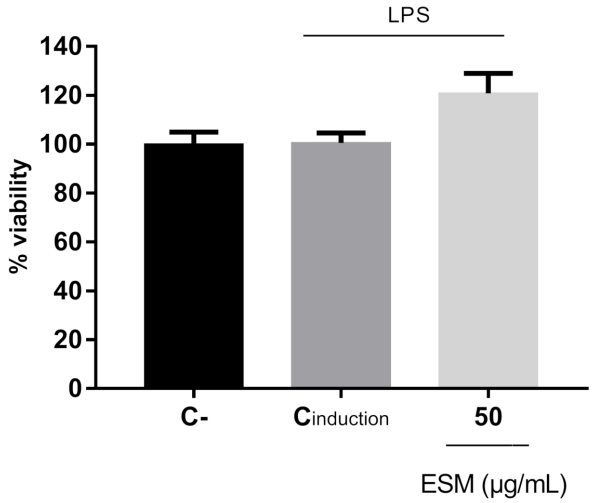

Figure 3. Effect of ESM 50 µg/mL on murine BV-2 cells microglial cells viability. Induction control ($C_{induction}$ - DMEM with 2% FBS and LPS) and negative control (C- - DMEM with 2% FBS) were used. The test was performed in five independent experiments, each in triplicate. Results were expressed in inhibition percentage compared to the induction control. The mean ± S.E.M. of was used.

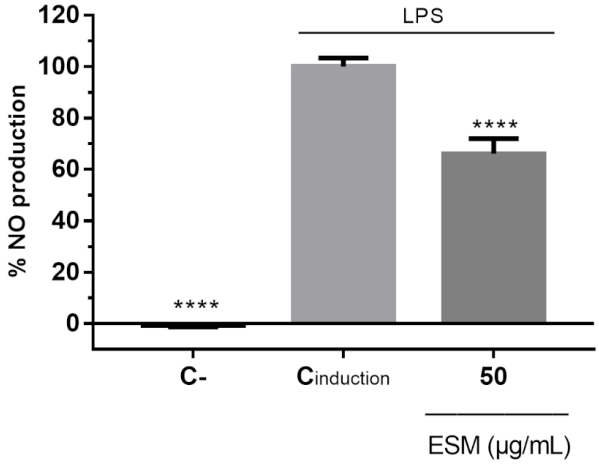

Figure 4. Effect of ESM 50 µg/mL on NO production in an *in vitro* model of LPS-induced neuroinflammation. Murine BV-2 cells microglial cell line was used. Induction control ($C_{induction}$ - DMEM with 2% FBS and LPS) and negative control (C- - DMEM with 2% FBS) were used. The test was performed in five independent experiments, each in triplicate. Results were expressed in inhibition percentage compared to the induction control ($C_{induction}$). The mean ± S.E.M. was used. Bars with ** are extremely different (p <0.0001).**

In vivo anti-inflammatory and antinociceptive activities

As shown in Figure 5, ESM significantly reduced the mice ear inflammatory edema induced by croton oil at all tested doses. No statistical difference was found when compared to indomethacin, an NSAID often used in clinical practice (Rang et al. 2016). This result is quite interesting as it reinforces the ethnopharmacological uses of *S. malaccense*.

As shown in Figure 6, ESM reduced the nociceptive stimulus induced by acetic acid in an inverse manner to the dose administered. In addition, Figure 7(a-b) reports that ESM reduced the licking paw time in both formalin test phases. Only the doses of 100 and 300 mg/kg were useful in the neurogenic phase. ESM at all tested doses significantly reduced the nociception in the inflammatory phase; however, the effectiveness of ESM 300 mg/kg was particularly notable.

Chemical characterization of ESM by UHPLC-MS

The public repository of mass spectra for small chemical compounds MassBank (Horai et al. 2010) was used as the database to identify the compounds present in ESM. According to the exact mass and the fragments (MS1 and MS2) obtained, three flavonoids: myricitrin (*m/z* 465.1022) and its aglycone myricetin (*m/z* 319.0450), quercetin (*m/z* 303.0504), and the alkaloid reserpine (*m/z* 609.2713) (Figure 8a-d) were identified.

The flavonols aglycones presented predominantly the MS2 fragment *m/z* 153.0199 and, in accordance to the mass spectrometry fragmentation pathways described (Bindu et al. 2014), reserpine presented the following MS2 fragments: *m/z* 448.1910, *m/z* 397.2070, *m/z* 365.1790, *m/z* 195.0630 and *m/z* 174.0890.

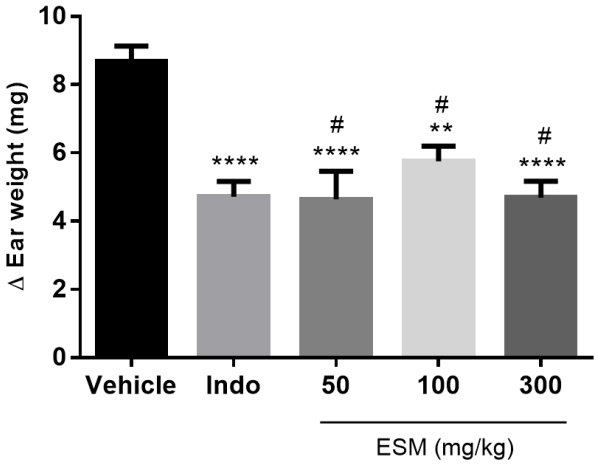

Figure 5. Topical effect of different doses of ESM on croton-oil-induced ear edema in mice. The vehicle, indomethacin 10 mg/kg, and ESM 50, 100, and 300 mg/kg were administered orally 60 min before topical application of 2.5% croton oil diluted in acetone. The weight difference between the right (inflamed) and the left (non-inflamed) ear biopsies was used to measure edema and, consequently, the inflammatory process. Each column's values represent the mean±S.E.M. ANOVA followed by the Tukey test, used as *post hoc*. Significant values:**p < 0.01 and ****p <0.0001 compared to the vehicle group; # means statistically equal to the reference drug (indomethacin).

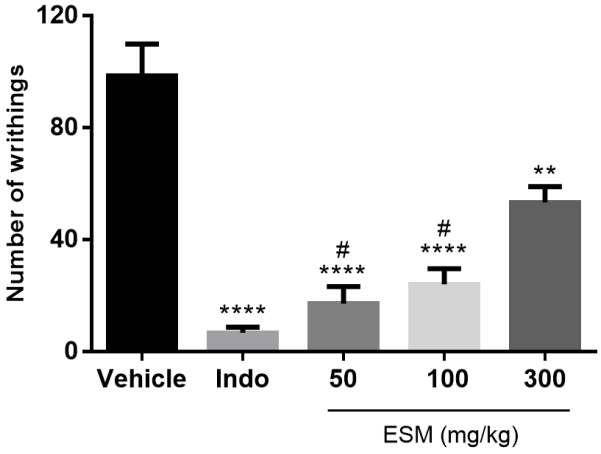

Figure 6. Effect of different doses of ESM on the number of writhings in acetic acid-induced in mice. The vehicle, indomethacin 10 mg/kg, and ESM 50, 100, and 300 mg/kg were administered orally 60 min before acetic acid 0.6% injection. The number of writhings was counted for 30 min. The values of each column represent the mean±S.E.M. ANOVA followed by the Tukey test, used as *post hoc*. Significant values:**p < 0.01 and ****p <0.0001 compared to the vehicle group; # means statistically equal to the reference drug (indomethacin).

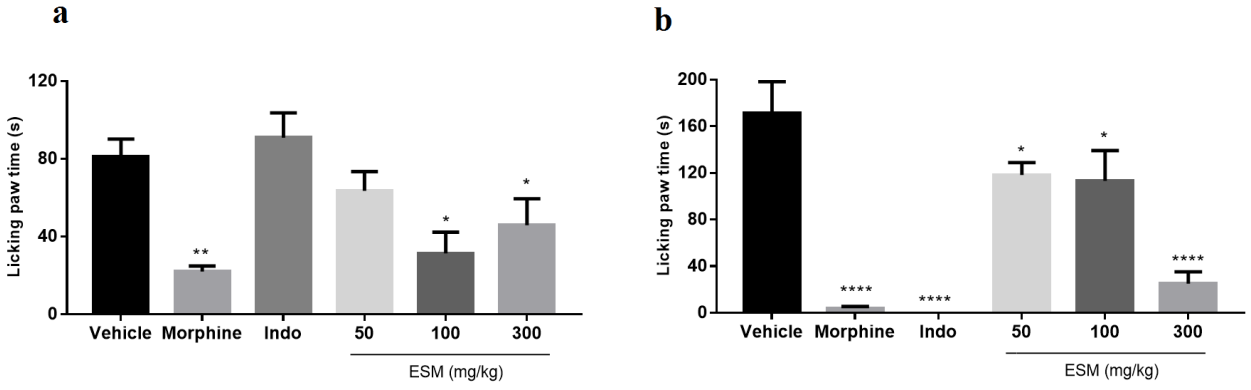

Figure 7. Effect of different doses of ESM on licking paw time in **the first (a) and second (b) phases** of the formalin test in mice. Vehicle, indomethacin 10 mg/kg and ESM 50, 100, and 300 mg/kg were administered orally, and morphine 7.5 mg/kg was administered intraperitoneally 60 and 30 min before formalin injection, respectively. The licking paw time was counted for 5 min (first phase) and during 15-30 min (second phase) after formalin injection. Each column's values represent the mean±S.E.M. ANOVA followed by the Tukey test, used as post hoc. Significant values: *p < 0.05 and ****p <0.0001 compared to the vehicle group.

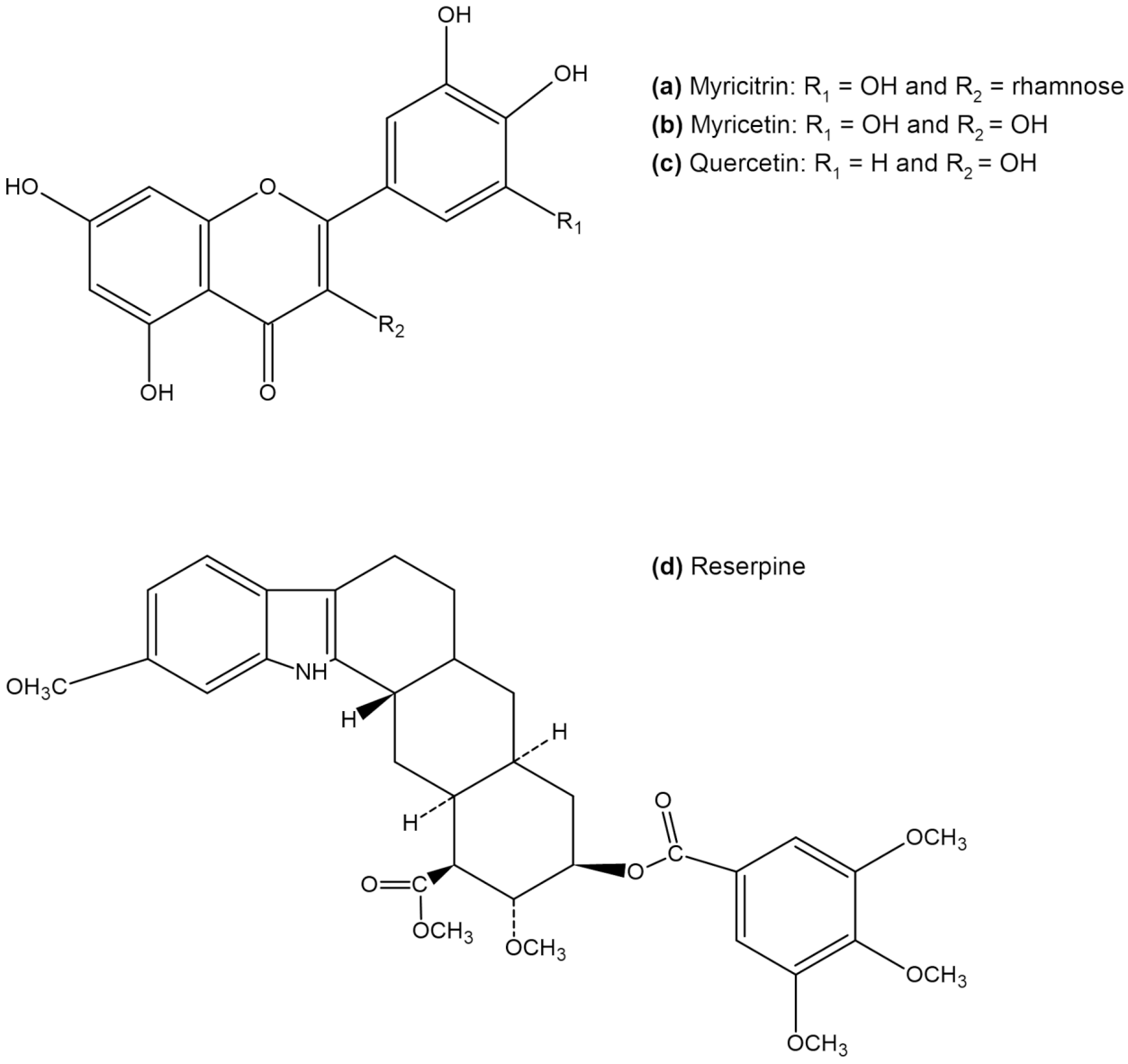

Figure 8. Chemical structure of myricitrin (a), myricetin (b), quercetin (c), and reserpine (d).

DISCUSSION

Antioxidant activity

Antioxidant effectiveness is related to various distinct aspects, including sample scavenging capacity, concentration, solubility, metal chelating activity, reducing potential, and lipid peroxidation inhibition. Thus, different assays were accomplished (Alam et al. 2013, Scio et al. 2012). DPPH assay was performed as it is quite simple, reproducible, and appropriate to evaluate free radical scavenging activity of compounds with different polarities (Scio et al. 2012). In contrast, β-carotene/linoleic acid bleaching assay is useful to verify the sample ability to inhibit lipid peroxidation, mainly due to its high correlation with the *in vivo* impairment associated with oxidative stress (Ismail et al. 2004). Also, phosphomolybdenum assay is convenient to verify the total antioxidant capacity of polar and non-polar compounds

(Prieto et al. 1999). The FRAP test is suitable for reducing power assessment (Oyaizu 1986).

In summary, the results suggested that ESM presents relevant antioxidant activity (Table I), as IC_{50} or EC_{50} values below 100 µg/mL are considered significant for plant extracts, which are endowed with several active and non-active chemical constituents at low concentrations (Cos et al. 2006).

Malonaldehyde (MDA) produced by lipid peroxidation in the TBARs assay is generated in ground beef throughout the experiment (Embuscado 2015). Thus, MDA quantification provides valuable data to predict the *in vivo* antioxidant potential of ESM.

Antiglycant activity

Glycation is a non-enzymatic reaction in which a reducing sugar binds to a circulating or structural protein, including collagen, neural proteins, hemoglobin, and albumin (Ma et al. 2016). Advanced Glycation End-products (AGEs) are irreversible heterogeneous compounds produced after subsequent dehydration, oxidation, and cyclization reactions (Grzegorczyk-Karolak et al. 2016). AGEs interact with specific receptors (RAGEs) in a variety of cells, which favors the synthesis of reactive oxygen species due to NADPH oxidase activation, and stimulates the NF-κB pathway, inducing the release of several cytokines, including IL-1, IL-6, TNF-α, endothelin-1, and tissue factor, generating an inflammatory process, which becomes the molecular basis of several pathologies, such as diabetes, nephropathies and cardiovascular and neurodegenerative diseases (Chuah et al. 2013, Park et al. 2012, Sun et al. 2016). It is well known that cerebrospinal fluid from patients with Alzheimer's disease and the frontal cortex of patients with Parkinson present high levels of AGEs (Chen et al. 2012, Schinkovitz et al. 2018).

The antiglycant effect of ESM may be attributed, at least in part, to its antioxidant capacity, as reactive oxygen species are generated during all the steps of glycation reaction (Liu et al. 2018). The antioxidant and antiglycation effectiveness of ESM encouraged evaluating its activity after LPS-induced inflammation in microglial cells. This assay is mediated by nitric oxide free radical and may be useful to predict ESM anti-neuroinflammatory potential.

In vitro evaluation of the anti-neuroinflammatory activity of ESM in BV-2 microglial cell line

Neuroinflammation plays an important role in the pathogenesis of several neurodegenerative diseases. Microglia cells are brain-resident phagocytes that exert various functions related to the host defense when activated (Bozic et al. 2015). Microglia activation is characterized by the release of different pro-inflammatory mediators, such as interleukins, tumor necrosis factor-alpha (TNF-α), and reactive oxygen species (ROS), including nitric oxide (NO). These mechanisms may be induced by exposure to toxins, including LPS, and are strictly related to neuroinflammation and brain injuries (Gan et al. 2015).

Although NO is an important biologically active molecule which fulfills several functions in the body, its high levels, produced by activated microglial cells, may induce the formation of reactive oxygen species, which generates brain oxidative stress and contributes to the neuronal cell death and the oligodendrocyte degeneration process associated with demyelinating diseases (Eguchi et al. 2011).

Several different mechanisms can inhibit the increase of NO levels by LPS exposure; however, ESM might reduce them by the neutralization of the NO· radical itself. More importantly, those results, together with the

inhibition of AGEs formation, suggested that ESM presented neuroprotective potential, which is quite relevant due to the current emerging of neurodegenerative diseases (Elmann et al. 2011, Kim et al. 2018, Zhang et al. 2010).

In vivo anti-inflammatory and antinociceptive activities

Croton oil-induced ear edema test

Croton oil is a natural product endowed with phorbol esters, which stimulate the release of several inflammatory mediators, including transcription factors, cytokines, and enzymes, such as cyclooxygenase, 5-lipoxygenase, and phospholipase-A_2 (Pascual & Glass 2006, Saraiva et al. 2011). For this reason, different inflammatory pathways are activated, so that croton oil-induced ear edema test is entirely appropriated to verify a possible anti-inflammatory activity of a tested drug independent of its mechanism of action (Pinto et al. 2015).

Although the phlogistic agent was topically applied in this test, this screening assay was used to verify the action of anti-inflammatory chemical compounds administered both topically and orally. This test is also entirely appropriated to evaluate natural compounds, as it requires small amounts, and it is rapidly accomplished with reproducible results (Gábor 2003).

Although many mediators are involved in the inflammatory processes induced by croton oil (Pascual & Glass 2006, Saraiva et al. 2011), it was not possible to predict ESM mechanism of action. However, its antioxidant capacity may contribute, at least in part, to reduce the mice ear edema. It is well known that oxidative stress is associated with inflammation development (Bhagavan et al. 2013).

Acetic acid-induced writhing test

Both central analgesics and anti-inflammatory agents are suitable to respond to this test. The intraperitoneal injection of acetic acid in mouse induces the synthesis of eicosanoids and other inflammatory mediators, such as histamine and bradykinin, and the release of several cytokines, including TNF-α, IL-1, IL-6, and IL-8, which irritate the serous abdominal membranes and stimulate nociceptive neurons (Favero et al. 2014, Pinheiro et al. 2011). Mice respond to this test with stereotyped movements, characterized by abdominal writhing, dorsal abdominal muscle sprain and contraction of the whole body (Silva et al. 2013).

As shown in Figure 6, ESM reduced the nociceptive stimulus induced by acetic acid in an inverse manner to the dose administered. This find was not surprising, as similar dose-response curves of plant extracts in *in vivo* nociceptive models have been reported in the literature (Pinto et al. 2015, Huerta-Reyes et al. 2013). According to Williamson et al. (1996) it may be explained by the presence of several compounds in plant extracts, as a competitive or non-competitive antagonism may occur. An agonist's threshold dose may not be significantly high; however, in non-competitive antagonism, the maximum response may be decreased by an antagonist compound, which may be active even beyond the receptor, interfering in intracellular second messengers signaling pathways.

Formalin-induced paw licking test

This test helps distinguish whether a tested drug can inhibit nociception stimulus by central or peripheral mechanisms. The sub-plantar injection of formalin induces a biphasic behavioral response characterized by paw licking. In the first (neurogenic) phase, up to 5 min after the formalin injection, there is a direct sensitization of C and Aδ fibers

due to the release of neurogenic mediators, including excitatory amino acids, nitric oxide, and substance P. The second (inflammatory) phase, which occurs between 15 and 30 min after formalin injection, is related to the release of several inflammatory mediators, such as prostaglandins, histamine, bradykinin, and serotonin (Hunskaar et al. 1985, Saldanha et al. 2017, Silva et al. 2013). Thus, antinociceptive drugs that act centrally or in peripheral fibers, interfering in the central nociceptive pathways, are capable of inhibiting the licking paw time of both phases. Simultaneously, those that interfere in peripheral mechanisms can reduce the licking paw time only in the second phase (Monteiro et al. 2014). For those reasons, the opioid morphine and the NSAID indomethacin were both used as reference drugs.

Central non-opioid analgesics are active in both phases; however, they are more efficacious in the second phase (Valerio et al. 2009), explaining the mice's response towards ESM 300 mg/kg. It is noteworthy that ESM is a crude extract endowed with several chemical constituents acting separately as central and peripheral analgesics in synergism.

The chemical compounds produced during the oxidative stress, including hydrogen peroxide, peroxynitrite, and superoxide anion, are important mediators in inflammatory pain and may cause tissue injuries. Thus, antioxidant agents may reduce the nociceptive stimulus preventing the formation of free radicals in the site of inflammation or even centrally in the spinal cord (Valerio et al. 2009). As ESM showed remarkable antioxidant capacity, it is reasonable to speculate that the inhibition of free radicals may explain, at least in part, its antinociceptive activity.

Chemical compounds identified in ESM by UHPLC-MS

The anti-inflammatory and antioxidant activities of myricitrin are well known. Its ability to reduce the neuroinflammatory process was recently reported, which involves decreasing several chemical mediators, including NF-κB and MAPK signaling pathways, IL-1β, IL-6, TNF-α, MCP-1, and the enzymes COX-2 and iNOS (Yang et al. 2019). Also, Sobeh et al. (2019) demonstrated that myricitrin significantly modified ROS generation profile, glutathione levels and protein oxidation by interfering with mitogen-activated protein kinase (MAPK) signaling pathways, using the sodium arsenite-induced oxidative stress model on human keratinocytes. Besides, myricitrin presents relevant potential as a natural agent to treat neurodegenerative disorders, as this flavonoid can suppress the aggregation of various aberrant proteins, eliminate several abnormal proteins from cell environment and reduce the inclusions of misfolded proteins, which decreases the neurotoxicity of these anomalous biomolecules (Joshi et al. 2019). It is also noteworthy to mention that a previous study reported that four flavan-3-ol derivatives, including (+) – catechin, mearnsitrin, myricitrin, and quercitrin isolated from *S. malaccense* leaves exhibit inhibitory activity against COX-1 and COX-2 enzymes (Noreen et al. 1998). Besides, Arumugam et al. (2019) showed that a myricetin enriched fraction obtained from *S malaccense* inhibited the oxidative stress induced by hydrogen peroxide in pigmented epithelial cells of the human retina (ARPE-19).

The review carried out by Calis et al. (2020) pointed that a variety of flavonoids, especially those obtained from diet, including quercetin, have received great attention from the scientific community due to their potential to prevent neuroinflammation and neurodegenerative process. On the other hand, reserpine is

recognized as a classical antipsychotic and antihypertensive drug used in clinical practice. However, studies have reported that reserpine presents neurotoxicity in zebrafish (*Danio rerio*) assays (Wang et al. 2019) and may induce the development of depression and Parkinson's disease Khan et al. 2018, Rijntjes & Meyer 2019).

The flavonoids myricitrin, myricetin and quercetin, have been identified in fruits and leaves of *S. malaccense* (Arumugam et al. 2016, 2019, Batista 2017). However, no studies were reporting the identification of the alkaloid reserpine in this plant species as far as we know. To confirm this alkaloid's presence, the standard reserpine and ESM were eluted and coeluted at the same analytical conditions. The signals related to reserpine acquired after the standard, ESM, and co-elution injections, showed similar UV profile (UV max 217, 268 and 299 nm) and retention time (RT = 18.4 min), confirming the reserpine identification obtained by the mass spectrum analysis.

CONCLUSION

The present study corroborates to the ethnopharmacological uses of *S. malaccense*, as the methanolic extract obtained from the leaves showed relevant *in vivo* anti-inflammatory and antinociceptive effects. Besides, the anti-inflammatory action of *S. malaccense* also occurred *in vitro* on BV-2 microglial cells after LPS addition, which revealed the ESM potential to be used as a neuroprotective agent. The antioxidant and antiglycant activities may be related, at least in part, to the mechanism of the *in vitro* neuroinflammation inhibition promoted by ESM. This result is quite important due to the role of brain inflammation in the pathogenesis of neurodegenerative disorders. Although reserpine was identified in ESM, the flavonoids myricitrin, myricetin and quercetin, among other compounds, are more likely to be responsible for those effects.

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