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Original article

Antinociceptive, anti-inflammatory and anxiolytic-like effects of the ethanolic extract, fractions and Hibalactone isolated from *Hydrocotyle umbellata* L. (Acariçoba) – Araliaceae

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ABSTRACT

Hydrocotyle umbellata Linn. (Araliaceae) is specie used in the treatment of inflammatory diseases. Crude extract (E-HU) was prepared from *H. umbellata* subterraneous parts and fractionated by liquid-liquid partition, resulting hexane fraction (HF-HU), dichloromethane fraction (DF-HU), ethyl acetate fraction (EAF-HU) and aqueous fraction (AF-HU). The hibalactone (HU-1) was isolated from the DF-HU and its structure was elucidated by ^{1}H NMR and ^{13}C NMR Spectroscopy, mass spectrometry and crystallographic x-ray analysis. The formalin-induced nociception was used to evaluate antinociceptive activity; carrageenan-induced edema and pleurisy tests to evaluate anti-inflammatory activity and light-dark box to evaluate anxiolytic-like activity in mice. The acute oral treatments with E-HU (1000 mg/kg), DF-HU (150 mg/kg), EAF-HU (400 mg/kg) and HU-1 (33 mg/kg) decreased the licking time in both phases of the formalin test. In the carrageenan-induced inflammation models, the treatment with the same doses of E-HU, DF-HU, EAF-HU and HU-1 reduced the paw edema formation and leukocytes account into pleural cavity. *In silico* findings suggest that hibalactone present anti-inflammatory activity by interacting with the enzymes 5-lipoxygenase and cyclooxygenase-2. In the light dark box, the treatments with DF-HU, EAF-HU and HU-1 revealed an anxiolytic like effect. Thus, the E-HU and fractions of *H. umbellata* showed antinociceptive, anti-inflammatory and anxiolytic like activities, as also hibalactone, a possible phytoconstituent responsible for the biological effects of this specie.

1. Introduction

The genus Hydrocotyle (Araliaceae) is constituted by aquatic or semiaquatic plants of temperate areas and tropical regions. Is represented by 17 species in Brazil with a great diversity of bioactive substances in terms of molecular patterns and metabolite variables [1,2]. *Hydrocotyle umbellata* Linn, popularly known as “acariçoba”, is native to the American continent and is traditionally used by communities in Argentina, Brazil and Cuba in the treatment of skin ulcers, eczema, dermatitis, psoriasis, erysipelas, rheumatism and others inflammatory processes [3]. The plant also has high relevance in phytotherapy and in the Ayurvedic medicine (Indian) because of its potential anxiolytic and memory stimulant effects [4].

The description of the phytochemical composition of *H. umbellata* indicates the presence of triterpenes, saponins, flavonoids, and polyacetylenes detectable in leaves and subterraneous parts, but in different

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amounts for each [5]. Studies revealed two new ergostane glycosides isolated from the leaves of *H. umbellata*, thereby contributing to the phytochemical knowledge of the specie and indicating a possible correlation with the biological activities attributed to the plant [6].

Rocha et al. [7] demonstrated the anxiolytic-like effects of the ethanol extract from *H. umbellata* subterraneous parts. Recently, Florentino et al. [8] showed that the ethanol extract of the acariçoba subterraneous parts presented analgesic and anti-inflammatory activities, and that a block of activity or reduction in the release of different mediators, such as histamine and serotonin, could be involved in these pharmacological effects. Despite the popular use of *H. umbellata*, the compounds and mechanisms of action involved in these anxiolytic-like, antinociceptive and anti-inflammatory effects still remain unclear.

Inflammation is a protective response to tissue injury caused by physical trauma, noxious stimuli by chemical agents, heat, and microbial effect. Currently, the non-steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of inflammation are the most frequently used class of drugs throughout the world, but their undesirable side effects on gastric mucosa, kidney, bronchus and cardiovascular system are well known, and have limited their use [9,10]. The natural products that can be a great source of new therapeutic compounds essential in treatment of disorders related to inflammation and pain [11,12].

This study aimed the fractionation of the ethanolic extract and isolation of phytochemical, besides to evaluate the antinociceptive, anti-inflammatory and anxiolytic-like effects of extract, fractions and hibalactone, a lignan isolated for the first time in *H. umbellata* L.

2. Materials and methods

2.1. Drugs and chemicals

The chemicals used were: carrageenan (Sigma, USA); dexamethasone (Decadron®, Ache, Brazil); Diazepam (Teuto, Brazil); dichloromethane (Merck, USA); ethyl acetate (Merck, USA); formaldehyde (Synth, Brazil); hexane (Merck, USA); indomethacin (Prodome, Brazil); methanol (Merck, USA); morphine sulphate (Dimorf®, Cristalia, Brazil); NaCl (Belga); Tween 80® (Vetec, Brazil); Türk liquid (Bioshop, Brazil); ethanolic extract (E-HU); hexane fraction (HF-HU); dichloromethane fraction (DF-HU); ethyl acetate fraction (EAF-HU); aqueous fraction (AF-HU) and isolated compound (HU-1). The E-HU, HF-HU, DF-HU, EAF-HU and AF-HU were dispersed in 2% Tween 80®. The HU-1 was solubilized in dimethylsulfoxide (10% DMSO). The indomethacin, morphine sulphate and diazepam were prepared in distilled water.

2.2. General experimental procedures

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra, HSQC and HMBC data were obtained on a Bruker Avance III spectrometer. Mass spectra (MS) were obtained with a MicroTOF (Brucker Daltonics, Bremen, Germany). The sample preparation for mass spectrometry analysis consisted of diluting 1 mg of each sample in 1 mL of methanol with 0.1% formic acid. The solution obtained was directly infused at a flow rate of 2 μL/min into the source of electrospray ionization, with nebulizer gas pressure of 0.5–1.0 bar, capillary voltage of 3.0 kV, and capillary transfer temperature of 250 °C.

X-ray diffraction data were collected using Mo-Kα radiation produced by an Incoatec IμS micro focus source. The diffracted beam intensities were measured with an APEX2 CCD area detector. The correction for the absorption effect was applied using the multi-scan method with SADABS and the structure was obtained with the Direct Methods algorithm method using SHELXTL. The final refinement of structural parameters was performed with F2 data, using the full matrix least squares method implemented in SHELXL-2014 [13]. The program SHELXL-2014 and the graphic production software ORTEP-3 were used inside the WinGX suite of programs [14]. Crystals of compound isolated suitable for x-ray diffraction were recrystallized from a DMSO solution at room temperature (25 °C). A single crystal was fixed with vacuum grease in an amorphous polymer micro loop and mounted on the goniometer head of the diffractometer Bruker-AXS Kappa Apex II Duo. The structural data were deposited in the Crystallographic Data Center Cambridge as Supplementary information (no. 1437908). Copies of the data can be obtained free of charge by contacting: CCDC, 12 Union Road, Cambridge CB2 1EZ, United Kingdom (Fax: (44) 1223336-033, e-mail: deposit@ccdc.cam.ac.uk or https://summary.ccdc.cam.ac.uk/structure-summary-form).

Column chromatography (CC) was conducted on silica gel G60 (0.05–0.2 mm, Vetec, Brazil). Fractions were monitored by thin-layer chromatography (TLC) using silica gel G60 F254 (Vetec) and CC silica gel G60 0.05–0.2 mm (Vetec). To observe the chemical constituents in the TLC plates, UV light at 254 and 365 nm was used, which revealed vanillin–sulfuric acid solution followed by heating [15]. The phytochemical screening for compound isolated, possible bioactive marker, was realized by HPLC out using a Waters high performance liquid chromatograph (Massachusetts, USA) equipped with a quaternary pump, separator module e2695, diode array detector (PDA) in 2998 and data processing system Empower 2.0. and C18 (RP18) chromatography column 5 μm (4.6 × 250 mm) [16].

2.3. Plant material

Hydrocotyle umbellata L. (Araliaceae) was grown in the Garden of Medicinal Plants of the Pharmacy Faculty of the Federal University of Goiás (UFG), located at 16° 40′ 33″ S and 49° 14′ 39′ W at an altitude of 768 m above sea level. The plant material received botanic identification by Dr. Heleno Dias Ferreira, of the Institute of Biological Sciences, Federal University of Goiás (UFG). A voucher specimen of *Hydrocotyle umbellata* L. (Araliaceae), has been deposited at the Herbarium of Federal University of Goiás, Brazil, Conservation Unit PRPPG, under code number UFG-22394. The subterraneous parts were collected in the period from December 2013 to January 2014, and samples were washed with water, desiccated at 40 °C, and ground in a knife mill (Willye). The powder was refrigerated to −6 °C and stored in containers able to provide protection from moisture and light.

2.4. Extraction, fractionation and isolation

The ethanolic extract was obtained by cold maceration, employing 1000 g of vegetable drug *H. umbellata* and 95% ethanol as liquid extractor for three days, with occasional stirring. The ratio used corresponds to one part of pulverized material to five parts of ethanol. After maceration, filtration was performed with the aid of a funnel and filter paper, and the extract was concentrated on a rotary evaporator (MA-120, Tecnal) at 40° C. In order obtain an efficient extractive process, the maceration was carried out more twice, thereby obtaining the crude ethanolic extract of the subterraneous parts of *H. umbellata*, called E-HU (40 g), which were kept in lit, refrigerated to −6 °C conditions to prevent degradation by light and moisture [17]. Fractions were prepared from 30 g of the extract and dissolved in 250 mL of methanol–water solution (7:3). The mixture was subjected to liquid–liquid partition with increasingly polar solvents (*i.e.*, hexane, dichloromethane, and ethyl acetate) [17]. To this partition, the sample was solubilized in methanol–water solution and transferred to a separatory funnel. Added to 100 mL of hexane, stirred, and separated by phase, the hexane layer was collected in a clean, dry container. Two subsequent extractions with 100 mL of hexane were repeated, resulting in hexane fraction (HF-HU). This procedure was also performed for ethyl acetate and dichloromethane, resulting in the dichloromethane fraction (DF-HU) and ethyl acetate fraction (EAF-HU). The solvents of all fractions were evaporated under a rotary evaporator, and each fraction was desiccated to constant weight. Ultimately, the methanol was removed on a rotary evaporator, and the resulting aqueous fraction (AF-HU) was

lyophilized. The actual yield of the fractions: HF-HU (4.7 g), DF-HU (2.1 g), EAF-HU (5.8 g), and AF-HU (9.1 g).

Compund **1** was obtained from DF-HU (1.0 g). This fraction submitted on silica gel CC (1:40) and eluted with hexane–ethyl acetate (95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80), dichloromethane–ethyl acetate (50:50, 20:80, and 100%), methanol–ethyl acetate (1:1), and 100% methanol. Subsequently 5 mL aliquots were collected, which resulted in 105 fractions. After evaporation of the solvents, the fractions were monitored by TLC; the mobile phase was composed of mixtures of hexane and ethyl acetate (9:1, 8:2, 7:3), dichloromethane and ethyl acetate (1:1 and 4:6), and hexane, ethyl acetate, and methanol (10:10:1). Based on the retention factors (Rf) of the spots observed in UV light at 254/365 nm and developed with vanillin–sulfuric acid solution, fractions were pooled in 15 new fractions (DC-1 to DC-15). The DC-5 fraction showed the formation of a white crystal and was separated and identified as DC-5a; it was later evaluated by TLC using hexane and ethyl acetate (9:1), which resulted in 150 mg of pure substance **1** (HU-1).

Spectral data: Compound isolated 1, hibalactone. The compound appeared as a white crystal; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.04 (1H, s, H-2), 6.87 (1H, d, J = 8.0 Hz, H-5), 7.1 (1H, d, J = 8.12 Hz, H-6), 7.48 (1H, d, J = 1.9 Hz, H-7), 6.04 (-OCH_2O-, s, H-10), 6.69 (1H, s, H-2′), 6.56 (1H, d, J = 8.4 Hz, H-5′), 6.69 (1H, d, J = 8.4 Hz, H-6′), 2.98 (1H, dd, J = 12 and 4 Hz, H-7′), 2.6 (1H, dd, J = 12 and 4 Hz, H-7′), 4.26 (1H, m, H-9′), 3,7 (1H, m, H-8′), 5.91 (-OCH_2O-, d, J = 5.0 Hz, H-10′); ^{13}C −NMR (125 MHz, $CDCl_3$) δ: 128.13 (C-1), 108.65 (C2-H), 148.32 (C-3), 149.19 (C-4), 108.78 (C5-H), 125.77 (C6-H), 137.29 (C7-H), 126.09 (C-8), 172.64 (C-9), 101.74 (C10-2H), 131.46 (C-1′), 109.13 (C2′-H), 147.95 (C-3′), 146.54 (C-4′), 108.46 (C5′-H), 122.09 (C6′-H), 37.49 (C7′-2H), 39.86 (C8′-H), 69.48 (C9′-2H), 101.04 (C10′-2H). $[M+H]^+$ m/z 352.0660 of (error = 0.4 ppm). Crystallographic x-ray data, see S1 Table.

2.5. Experimental animals

All experiments were performed with adult male Swiss mice (*Mus musculus)* weighing 30–35 g obtained from the Central Animal House at UFG. The animals were maintained at constant room temperature (22 ± 2 °C) under a 12 h light–dark cycle with free access to standard food and water. The animals were acclimatized for seven days before the experiments began and randomly assigned to experimental groups (n = 9). The study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use. All experimental protocols were developed according to the principles of animal welfare designated by the Ethics Committee on Animal Experimentation and were approved by the Ethics Committee in Research at UFG (n°. 11/2014).

2.6. Pharmacological evaluation

2.6.1. Formalin test

The formalin-induced nociception was performed as described by Hunskaar et al. [18]. Experimental groups of mice were treated with the CLT (10 mL/kg, p.o.), E-HU (1000 mg/kg, p.o.), HF-HU (300 mg/kg, p.o.), DF-HU (150 mg/kg, p.o.), EAF-HU (400 mg/kg, p.o.), AF-HU (600 mg/kg, p.o.), HU-1 (33 mg/kg, p.o.), indomethacin (10 mg/kg, p.o. − positive control for antinociceptive activity in the second phase) or morphine (5 mg/kg, s.c. − positive control for antinociceptive activity in the first and second phase). Either 60 min after oral treatment (p.o.) or 30 min after subcutaneous treatment (s.c.), 20 µL of 3% formalin was administered into the plantar surface of the right hind paw. Following injection of the phlogistic agent, the mice were placed into an acrylic box, under which a mirror was placed to enable the unhindered observation of the formalin-injected paw for 30 min. The pain reaction time (*i.e.*, licking time) was observed in two periods: 0–5 min (neurogenic pain) and 15–30 min (inflammatory pain). Results were expressed as the means ± SEM of licking time (s).

2.6.2. Carrageenan-induced paw edema

The paw edema test was performed as described by Winter et al. [19]. Experimental groups of mice were treated with CLT (10 mL/kg, p.o.), E-HU (250, 500 and 1000 mg/kg, p.o.), DF-HU (150 mg/kg, p.o.), EAF-HU (400 mg/kg, p.o.), HU-1 (33 mg/kg, p.o.) or indomethacin (10 mg/kg, p.o., positive control). One hour after treatment, each animal received an injection of 50 µL of 1% carrageenan in the right paw. The left paw, which received the same volume of 0.9% NaCl solution, was used as the control. The edema was measured by the difference in the volume between the paws using a plethysmometer (Ugo Basile, Italy) at 0, 1, 2, 3 and 4 h after injection with the phlogistic agent. Results were expressed as means ± SEM of the difference in the volume between the right and left paws (µL).

2.6.3. Carrageenan-induced pleurisy

The experimental groups of mice were treated with CLT (10 mL/kg, p.o.), E-HU (1000 mg/kg, p.o.), DF-HU (150 mg/kg, p.o.), EAF-HU (400 mg/kg, p.o.), HU-1 (33 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o., positive control). One hour after the treatments, animals received an injection of 100 µL of 1% carrageenan into pleural cavity. The pleural exudate was collected with 1 mL of heparinized PBS, 4 h after carrageenan administration. One aliquot was used to determine the total leukocyte content, using Türk liquid in a Neubauer's chamber. The final result was also adjusted through the volume unit used in the performed evaluation and expressed as means ± SEM of the number of leukocytes × 10^6/mL.

2.7. Molecular docking

Bioactivity prediction procedure with 2D hibalactone structure was performed with PASS tool [20]. The prediction result of compound is a list of potential biological activities with Pa and Pi values. Pa and Pi are estimates of probability for the compound to be active or inactive respectively for each type of activity from the activity spectrum. The activities presenting Pa > 0.7 were selected for the analysis.

Docking simulations with hibalactone and Human 5-lipoxygenase (PDB code: 3V99) and cyclooxygenase-2 (PDB code: 5IKR) were run with Dockthor server [21]. The calculations were performed inside a sphere of 7 Å radius centered at the CZ atom of the PHE177 residue for 5-lipoxygenase and CD1 atom of the LEU531 residue for cyclooxygenase-2. The advanced options were maintained as default. The most energetically favorable conformation was selected for the analysis. The docking protocol was validated by redocking the ligands of 5-LOX/arachidonic acid complex (PDB code: 3V99) and COX-2/mefenamic acid complex (PDB code: 5IRK). The 10 top-ranked redocked orientations were compared with the crystallographic orientations of arachidonic acid and mefenamic acid by calculation of heavy atoms RMSD (root-mean-square deviation) ≤ 2.0.

2.8. Light-dark box (LDB) test

The light dark was performed as described by Crawley and Goodwin [22] with the equipment purchased from Insight Scientific Equipments — Brazil (model EP 157C®). Acrylic apparatus consists of an illuminated compartment (40 cm × 30 cm) and a dark compartment (20 cm × 30 cm) demarcated by an opening (4 × 4 cm) that allowed the animals transition between the two compartments. Sixty minutes after oral treatments (CLT, 2.0% Tween 80, 10 mL/kg), DF-HU (150 mg/kg), EAF-HU (400 mg/kg) or HU-1 (33 mg/kg, p.o.), mice were placed individually at the center of the light compartment with their nose facing to the dark compartment. During 5 min were recorded the latency for dark compartment (s) and percentage of time spent in the light area.

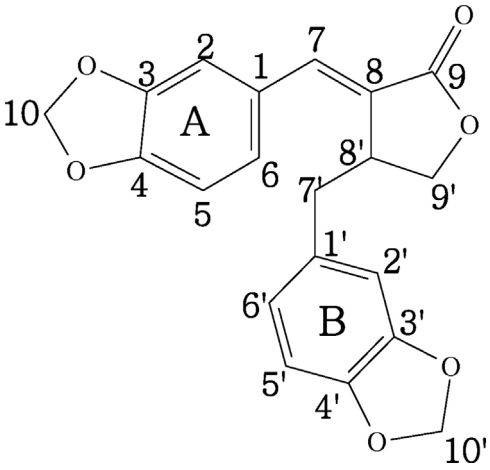

Fig. 1. Chemical structure of hibalactone isolated from the subterraneous parts of *Hydrocotyle umbellata* L.

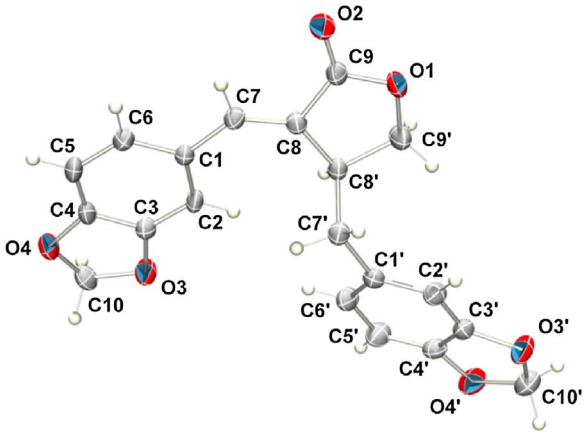

Fig. 2. ORTEP representation of compound 1, with the molecule A is shown, with atomic displacements indicated by ellipsoids with a probability density of 30%.

2.9. Statistical analysis

All results were expressed as means ± SEM. Data were analyzed statistically by one-way ANOVA followed by a *Student–Newman–Keuls* post hoc test or by two-way ANOVA followed by *Bonferroni's* post hoc test. Values of $p \leq 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism® version 5.0.

3. Results

3.1. Characterization of compound isolated

Hibalactone **(1)** was first time isolated in dichloromethane fraction (DF-HU) obtained from the ethanolic extract prepared with subterraneous parts of the *H. umbellate* L., by using a combination of different chromatographic methods.

Isolated compound 1 (Fig. 1) was presented as crystalline, white, and soluble in chloroform. The ^{1}H and ^{13}C NMR data of these compounds were in full agreement with data reported in the literature [23,24]. This data showed characteristic signals of lignans. ^{1}NMR spectrum indicates the presence of two methylenedioxy groups attached to the aromatic rings A and B, which absorb in δ = 5.91 and 5.88. Signals for unhydrogenated carbon were δc = 172.64, typical for a lactone five-membered and δc = 69.48, characteristic of carbon oxymethylene, suggesting the existence of a dibenzylbutyrolactone.

S1 Fig shows the ESI(−)-Q-TOF mass spectrum of compound 1. Basically, compound 1 was detected in protonated form $[M+H]^+$ of *m/z* 353.0660 with an error of 0.4 ppm. In all cases, the high-resolution mass spectrum of ESI-Q-TOF-MS analysis enabled the detection of compound 1 with strong accuracy in terms of mass.

X-ray diffraction data regarding showed the isolated molecule of compound **1** crystallizes in a non-centrosymmetric space group $P2_1$ with two independent molecules per asymmetric unit. In the superposition, molecules A and B have the same chirality and different torsion angles regarding benzodioxole groups around the σ bonds C1–C7 and C1′–C7′ (S2 Fig). The ORTEP-3 representation of 1 illustrates the molecular structure of the compound (Fig. 2). From the results obtained, it is observed a chiral center in the C8′ atom, a double bond in C7 = C8 and carbonyl group C9 = O, which corroborate the identification of the molecule as the lignan hibalactone, whose mass and molecular formula are 352.337 Da and $C_{20}H_{16}O_6$, respectively.

3.2. Formalin induced-nociception

Oral administration of E-HU in a dose of 1000 mg/kg significantly reduced the pain reactivity in both phases of the formalin test compared to activity in the control group. In the first phase (0–5 min), animals in the control group showed a licking time (s) of 64.78 ± 2.37, which treatment with E-HU or morphine (5 mg/kg s.c., positive control) reduced by 47.1% ($p < 0.001$) and 85.6% ($p < 0.001$), respectively (Fig. 3). The group treated with indomethacin (10 mg/kg, p.o., anti-inflammatory positive control) significantly decreased its licking time (s) only during the second phase. In the second phase (15–30 min), treatments with E-HU, indomethacin, and morphine reduced licking time by 56.8%, 50.9% and 99.8% ($p < 0.001$), respectively, compared to that in the control group (161.6 ± 19.16), (Fig. 3).

Evaluation of the antinociceptive activity of the fractions and HU-1 was also performed in this test. However, only DF-HU (150 mg/kg) and EAF-HU (400 mg/kg) significantly reduced pain reactivity time in the first phase, by 36.9% ($p < 0.05$) and 37.6% ($p < 0.05$), respectively, when compared to control group (61.88 ± 5.86). In the second phase, when compared to the control group (203.0 ± 20.51), these fractions reduced by 41.9% ($p < 0.01$) and 42.2% ($p < 0.01$), respectively. The hexane fraction (HF-HU 400 mg/kg) and the aqueous fraction (AF-HU 600 mg/kg) did not affect the formalin-induced nociception. The isolated compound **1** (HU-1 33 mg/kg) reduced licking time in both phases, in the first phase, licking time was reduced by 46.81% ($p < 0.001$) compared to the control group (62.0 ± 4.31). In the second phase, licking time was reduced by 49.5% ($p < 0.001$) compared to the control group (187.8 ± 13.48), as showed in (Fig. 3).

3.3. Carrageenan-induced paw edema

The treatment of the animals with E-HU inhibited edema formation, in a dose-dependent manner, until the third hour. In the first hour, E-HU (250, 500 and 1000 mg/kg) reduced by 29.56% ($p < 0.01$), 36.91% ($p < 0.01$) and 58.76% ($p < 0.001$), respectively. In the second hour, E-HU (250, 500 and 1000 mg/kg) reduced by 33.90% ($p < 0.01$), 40.47% ($p < 0.001$) and 48.18% ($p < 0.001$), respectively. In the third hour, E-HU (250, 500 and 1000 mg/kg) reduced by 15.85%, 20.89% ($p < 0.05$), 27.05% ($p < 0.01$) respectively when compared to the control groups (Table 1).

The treatment of the animals with DF-HU, EAF-HU and HU-1 inhibited edema formation until third hour. In the first hour, the compounds decreased by 36.52% ($p < 0.001$), 30.43% ($p < 0.05$) and 27.83% ($p < 0.05$), respectively, while in the second hour, by 38.77% ($p < 0.001$), 31.09% ($p < 0.001$) and 26.05% ($p < 0.01$), also respectively. In the third hour, only the treatment with DF-HU and HU-1 significantly reduced edema formation by 22.21% ($p < 0.01$) and 16.46% ($p < 0.05$), respectively. In the fourth hour, any treatments significantly reduced the formation. However, the group treated with indomethacin, an anti-inflammatory positive control, showed decreased paw edema at 2 until 4 h after carrageenan-induced edema (Table 1).

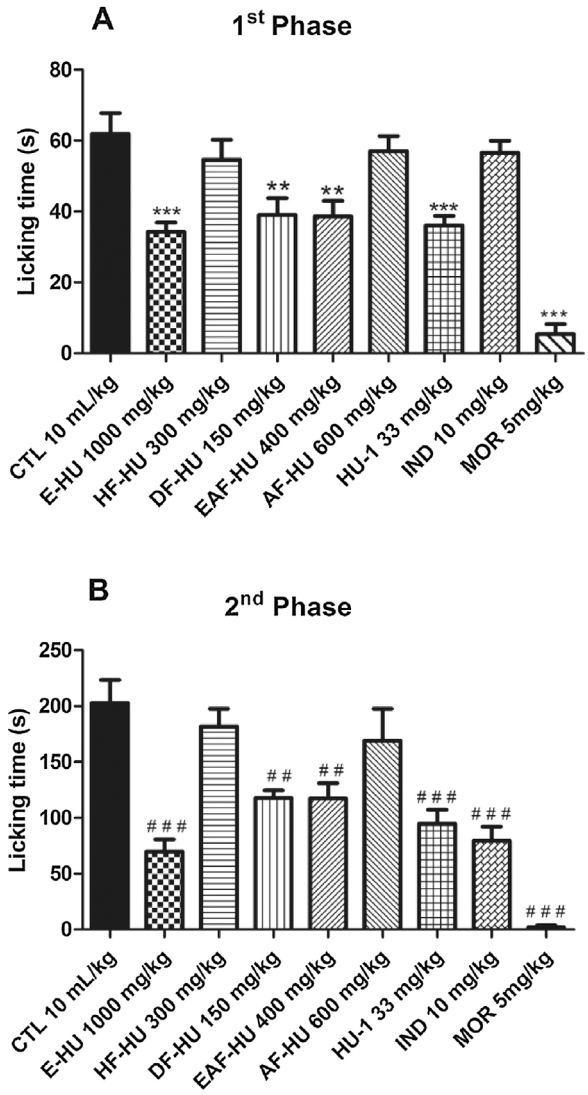

Fig. 3. Effect of the extract, fractions and isolated compound from *H. umbellata* on mice during the formalin test (a) in the first phase (0–5 min) and (b) second phase (15–30 min), with CTL (control − 2% Tween 80®, 10 mL/kg, p.o.), ethanolic extract (E-HU, 1.000 mg/kg, p.o.), hexane fraction (HF-HU, 400 mg/kg), dichloromethane fraction (DF-HU, 150 mg/kg), ethyl acetate fraction (EAF-HU, 400 mg/kg), aqueous fraction (AF-HU, 600 mg/kg), HU-1 (33 mg/kg p.o.), indomethacin (IND, 10 mg/kg, p.o.) and morphine (MOR, 5 mg/kg s.c.). Vertical bars represent Mean ± SEM of pain reaction time (s); * $p \leq 0.05$ and ** $p \leq 0.01$ (compared to first phase control group) and ## $p \leq 0.01$ and ### $p \leq 0.001$ (compared to second phase control group) according to ANOVA followed by a *Student–Newman–Keuls* post hoc test.

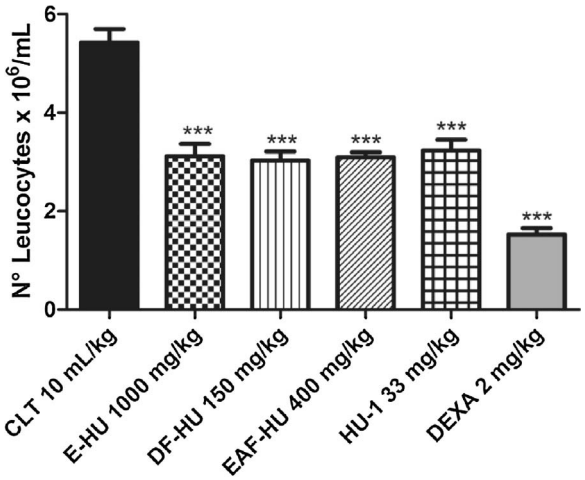

Fig. 4. Effect of the extract, fractions and isolated compound from *H. umbellata* L. on the carrageenan-induced pleurisy test, in mice. CTL (control − 2% Tween 80®, 10 mL/kg, p.o.), ethanolic extract (E-HU, 1.000 mg/kg, p.o.), dichloromethane fraction (DF-HU, 150 mg/kg), ethyl acetate fraction (EAF-HU, 400 mg/kg), HU-1 (33 mg/kg p.o.), and DEXA (Dexamethasone − 2 mg/kg p.o., positive control). Vertical bars represent Mean ± SEM of the number of leukocytes into pleural cavity; ***p ≤ 0.001 (compared with control group) according to ANOVA followed by a *Student–Newman–Keuls* post hoc test.

3.4. Carrageenan-induced pleurisy

When compared with the control group (5.430 ± 0.273 × 10^6/mL) the treatment with E-HU (1000 mg/kg), DF-HU (150 mg/kg), EAF-HU (400 mg/kg), HU-1 (33 mg/kg) and dexamethasone (2 mg/kg) reduced the number of leukocytes migrated by 42.5% ($p < 0.001$), 44.2% ($p < 0.001$), 43.0% ($p < 0.001$), 40.48% ($p < 0.001$) and 71.9% ($p < 0.001$), respectively. As showed in Fig. 4.

3.5. Molecular docking

The bioactivity prediction data of compound 1performed with PASS tool revealed potential activities including anti-inflammatory, anti-neoplastic and antidepressant propensity (Table 2). These suggested activities presented Pa > 0.7, which indicates that hibalactone is very likely to exhibit the activity experimentally, thus supporting our experimental results regarding anti-inflammatory activity.

Moreover, since the PASS tool compares chemical structures of compounds to predict activities, it indicates that hibalactone present molecular characteristics consistent with anti-inflammatory molecules. Considering that most of the drugs approved for anti-inflammatory diseases interfere in the arachidonic acid (AA) pathway, the key enzymes 5-LOX and COX-2 were selected for molecular docking

Table 1
Effect of the ethanolic extract from *Hydrocotyle umbellata* (E-HU − 250, 500 and 1000 mg/kg p.o.), dichloromethane fraction (DF-HU − 150 mg/kg p.o.), ethyl acetate fraction (EAF-HU − 400 mg/kg p.o.) and isolated compound **1** (HU-1 − 33 mg/kg) on the paw edema test in mice. CTL (Control − 2% Tween 80®, 10 mL/kg p.o.) and indomethacin (IND − 10 mg/kg p.o., positive control). Values are expressed as mean ± SEM of the difference between the volumes of the paws; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group, according to two-way ANOVA followed by *Bonferroni's* post-hoc test.

Treatment (p.o.)	Paw edema (μL) and inhibition percentage				
	Basal	1st h	2nd h	3rd h	4th h
CTL	3.00 ± 1.52	115.0 ± 8.97	119.0 ± 5.26	97.14 ± 2.86	83.75 ± 2.63
E-HU 250 mg/kg	4.23 ± 1.89	81.0 ± 6.29** (29.56%)	78.66 ± 5.08** (33.90%)	81.74 ± 5.31 (15.85%)	82.32 ± 4.75 (1.70%)
E-HU 500 mg/kg	3.45 ± 1.24	72.55 ± 7.2*** (36.91%)	70.84 ± 10.14*** (40.47%)	76.84 ± 5.43* (20.89%)	74.28 ± 8.73 (11.31%)
E-HU 1000 mg/kg	5.14 ± 1.51	47.42 ± 7.98*** (58.76%)	61.66 ± 9.98 *** (48.18%)	70.86 ± 5.39** (27.05%)	72.40 ± 5.95 (13.55%)
DF-HU 150 mg/kg	6.00 ± 2.21	73.00 ± 3.00*** (36.52%)	72.86 ± 2.86 *** (38.77%)	75.56 ± 3.38** (22.21%)	75.00 ± 2.68 (10.45%)
EAF-HU 400 mg/kg	3.75 ± 1.83	80.00 ± 4.47* (30.43%)	82.00 ± 9.69*** (31.09%)	83.00 ± 4.22 (14.56%)	80.00 ± 6.83 (4.48%)
HU-1 33 mg/kg	5.71 ± 2.02	83.00 ± 5.97** (27.83%)	88.00 ± 6.39** (26.05%)	81.15 ± 5.80* (16.46%)	82.75 ± 6.41 (1.83%)
IND 10 mg/kg	3.04 ± 1.50	105.58 ± 5.10 (8.20%)	81.70 ± 5.01*** (31.93%)	62.04 ± 4.40*** (36.13%)	54.5 ± 2.70*** (34.92%)

Table 2
Top-ranked bioactivity predicition data of hibalactone using PASS tool, being Pa the estimate of probability for the compound to be active and Pi the estimate of probability for the compound to be inactive).

*Pa	*Pi	Activity
0,944	0,004	Membrane integrity agonist
0,940	0,004	Apoptosis agonist
0,872	0,003	Tubulin antagonist
0,861	0,005	Anti-inflammatory
0,751	0,006	Neurotransmitter uptake inhibitor

simulations.

The docking protocol was validated using the complex 5-LOX/arachidonic acid (PDB code: 3V99) and the complex COX-2/mefenamic acid (PDB code: 5IRK). The redocking procedure with arachidonic acid revealed that 5 of the 10-top ranked orientations presented heavy atoms RMSD < 2 when compared with the crystallographic conformation. In relation to mefenamic acid, all the 10 top-scored had RMSD < 2. The docking protocol was thus able to reproduce the conformation and the binding mode of arachidonic acid and mefenamic acid (Figs. S3 and S4, respectively), validating simulations with hibalactone.

Docking simulations within 5-LOX revealed that hibalactone was capable of accommodating in the end of the active site cavity and performing electrostatic and hydrophobic interactions (Fig. 5). The hibalactone butyrolactone moiety was able to interact with His-600 residue, which is involved in the appropriate positioning of the substrate for catalysis [25]. Moreover, the two hibalactone benzodioxole moieties interact with Ala-603 residue, which has been considered necessary during the substrate catalytic cycle [25].

Other interesting interaction occurs between the hibalactone benzodioxole moiety and Phe-177 residue, which is required for 5-LOX to process AA 5-hydroperoxide and its metabolites [25]. The binding mode of hibalactone within 5-LOX revealed interactions with the key residues for the substrate recognition, which may generate a molecular competition between hibalactone and AA for binding to the active site, contributing to reduce the production of the proinflammatory leukotrienes.

On the other hand, the possible interference in the COX-2 pathway was also investigated and docking simulations revealed that hibalactone interact in the entrance of the active site cavity, performing electrostatic and hydrophobic interactions (Fig. 6). The main interaction occurs between the hibalactone benzodioxole moiety and Tyr-385 and Ser-530 residues. It has been described that most of the NSAIDs exercise its inhibitory activity mainly through interacting with these residues [26].

The interaction with Tyr-385 may also interfere in the substrate catalysis, since it promotes the hydrogen abstraction from C-14 of AA, which is the first step in the AA oxygenation [27]. The binding mode of hibalactone within COX-2 revealed that it was able to accommodate in the same NSAIDs binding cleft and interact with key residue for substrate catalysis, thus preventing the production of prostaglandins.

3.6. Light-dark box (LDB) test

In the light dark box test, the treatments with dichloromethane fraction, isolated compound and diazepam revealed an increased in the latency to dark compartment when compared to control group (Fig. 7a). The control group showed a latency time of 6.7 ± 1.5 s while treated groups with DF-HU, HU-1 and diazepam increased the latency to 24, 20 and 15 s. Furthermore, the treatments of the animals with DF-HU, EAF-HU, HU-1 and diazepam increased the time spent in the light area in 32, 38, 45 and 47%, respectively as shown in Fig. 7b.

4. Discussion

Hibalactone (HU-1) belongs to the lignan class, organic molecules that show promise in the development of new drugs due to their anti-inflammatory, antimicrobial, antineoplastic, antiviral, antifungal, leishmanicide, antiangiogenic, antioxidant, anti-ulcer, antiplatelet, hepatoprotective, and antirheumatic activities [28].

The lignan dibenzylbutyrolactone, such as, cubebin isolated from *Piper cubeba* Linn. (Piperaceae) [29] and arctigenin isolated from *Forsythia koreana Nakai* (Oleaceae) [30] were described with pronounced anti-inflammatory activities. Others lignan such as fargesin, isolated from *Magnolia fargesii* (Magnoliaceae) [31] also been reported previously with antinociceptive and anti-inflammatory effects.

The present study demonstrating that E-HU of the subterraneous parts of *H. umbellata*, some fractions and HU-1, compound isolated from *H. umbellata*, have significant antinociceptive (formalin induced-nociception test), anti-inflammatory (carrageenan induced paw edema and pleurisy tests) and anxiolytic-like (light-dark box test) effects.

The formalin test is a model described by Hunskaar et al. (1985) used to evaluate nociception due the formalin administration causing a biphasic response. Neurogenic pain (first phase) occurs during the first 5 min after formalin injection and this pain is caused by direct activation of nociceptors. However inflammatory pain (second phase), which occurs from the 15–30 min after formalin injection and is caused by tissue injury and the subsequent release of inflammatory mediators, such as, histamine, serotonin and prostaglandins. In this test the administration of classical NSAIDS inhibited just late phase, whereas opioids inhibit both phases [32,33].

As presented in Fig. 3, the E-HU (1.000 mg/kg) blocks both the early and late phases of formalin-induced nociception, while indomethacin suppressed only later phase. The results of this test suggest that the extract, could act both centrally and peripherally to reduce the pain, as descript by Florentino et al. [8]. In an attempt to verify the compound(s) responsible for the antinociceptive activity demonstrated by E-HU, the same tests were performed with the fractions (HF-HU, DF-HU, EAF-HU and AF-HU) and the compound isolated HU-1.

The result with the fractionation of the ethanolic extract of *H. umbellata* showed that DF-HU and EAF-HU retained the analgesic effect observed in E-HU and presented similar activity in both phases of the formalin test, whereas HF-HU and AF-HU did not exhibit antinociceptive activity. Additionally, we also observed that HU-1 showed similar effect as the DF-HU and EAF-HU fractions. The dose of HU-1 (33 mg/kg) used were based on the relative amount in the fraction isolated DF-HU. These results suggest that the compound **1** is a phytochemical responsible, at least in part, for the antinociceptive effect observed with DF-HU and E-HU in both phases of the formalin test. Possibly this lignan collaborates with the activities attributed the plant and may be used as a bioactive marker for *H. umbellata* L.

The results inhibiting both phases of the formalin test does not allow the separation of independent anti-inflammatory effects of central analgesic effects, making it necessary to evaluate the extract, fractions and isolated compound **1** in specific models of inflammation. Thus, to confirm the hypothetical anti-inflammatory activity of E-HU, DF-HU, EAF-HU and isolated compound **1** was used carrageenan-induced edema and pleurisy tests.

The carrageenan-induced paw edema is a model of acute inflammation widely used to study new anti-inflammatory drugs. Edema evolution involves a gradual and complex response, marked by the release of inflammatory mediators [19]. The development of edema is an event that involves the release of inflammatory mediators such as histamine, serotonin, bradykinins and prostaglandins [34].

The results showed that E-HU (250–1.000 mg/kg) significantly inhibited the paw edema in dose-dependent manner, as was seen by Florentino et al. [8]. Similar effect was observed with DF-HU, EAF-HU and HU-1, indicating that the extract, fraction and the isolated compound may decrease the edema formation in a similar pathway as

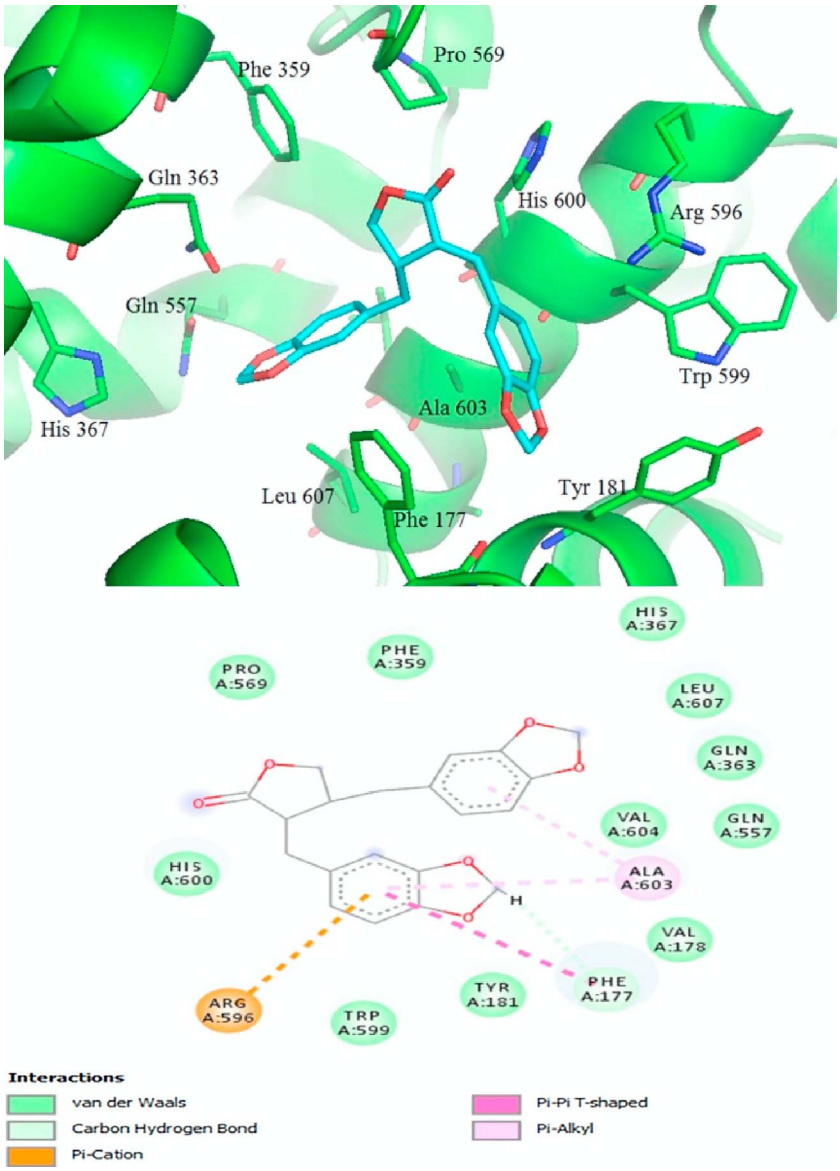

Fig. 5. (A) Docking 3D model of hibalactone (carbon atoms in blue) within 5-LOX active site, highlighting the main intermolecular interactions with protein residues. (B) 2D interaction diagram between the hibalactone and 5-LOX protein residues.

indomethacin. The HF-HU and AF-HU fractions have not been tested in this model, because they did not show antinociceptive activity in formalin test.

Pleurisy test is a well-characterized experimental model of inflammation used to evaluate cell migration and other inflammatory parameters [35]. As presented in Fig. 4, the treatment with E-HU, DF-HU, EAF-HU and HU-1 reduced number of migrated leucocytes to pleural exudate confirming the anti-inflammatory effect seen in the previous experiments of formalin and paw edema. Taken together, these data suggest that anti-inflammatory activity of extract, fractions and hibalactone might be attributed to the reduced of general inflammatory mediators.

Some lignans have anti-inflammatory activity due to inhibition of lipoxygenase and cyclooxygenase [36]. Recently, a study was carried out to evaluate *in vivo* and *in silico* anti-inflammatory mechanism of action of the semisynthetic cubebin derivatives, hinokinin and obenzylcubebin (lignans dibenzylbutyrolactone). The results indicated all compounds were active in the paw edema test. Additionally, docking analysis showed that lignans displayed stable ligand-receptors relevant for cyclooxygenase-2, indicated that this class of compounds may act by inhibition of the cyclooxygenase activity [37]. In another study showed that phylligenin (lignan) isolated from the fruits of *Forsythia koreana* have anti-inflammatory activity. In addition, this study indicated that phylligenin exhibited this effect by inhibit COX-2-mediated prostanoid synthesis by inhibiting COX-2 [38].

Docking simulations within COX-2, reveled electrostatic and hydrophobic interactions with Tyr-385 and Ser-530 residues. These interactions of hibalactone to the actives sites in 5-LOX or COX-2 residues might cause the anchoring of the molecule on the active site resulting in activity against the enzyme. To docking within 5-LOX: His-600 residue, Ala-603 residue, Phe-177 residue, contributes to reduce the production of the pro-inflammatory leukotrienes.

In silico findings suggest the hypothesis of hibalactone presenting anti-inflammatory activity by interfering in the arachidonic acid pathway. Hibalactone present molecular structure viable for interacting with the key enzymes 5-LOX and COX-2, performing intermolecular interactions in the enzymes active sites and thus modulating this dual target. Recently, dual COX-2/5-LOX enzyme inhibitors are considered significant in managing the molecular mechanisms during inflammation [39]. Thus this results may justify the hibalactone effects on the edema formation and cellular migration, however further studies are needed to comprehend this possible mechanism of action and other possible target of hibalactone.

After demonstrating that the effect of the extract, fractions and

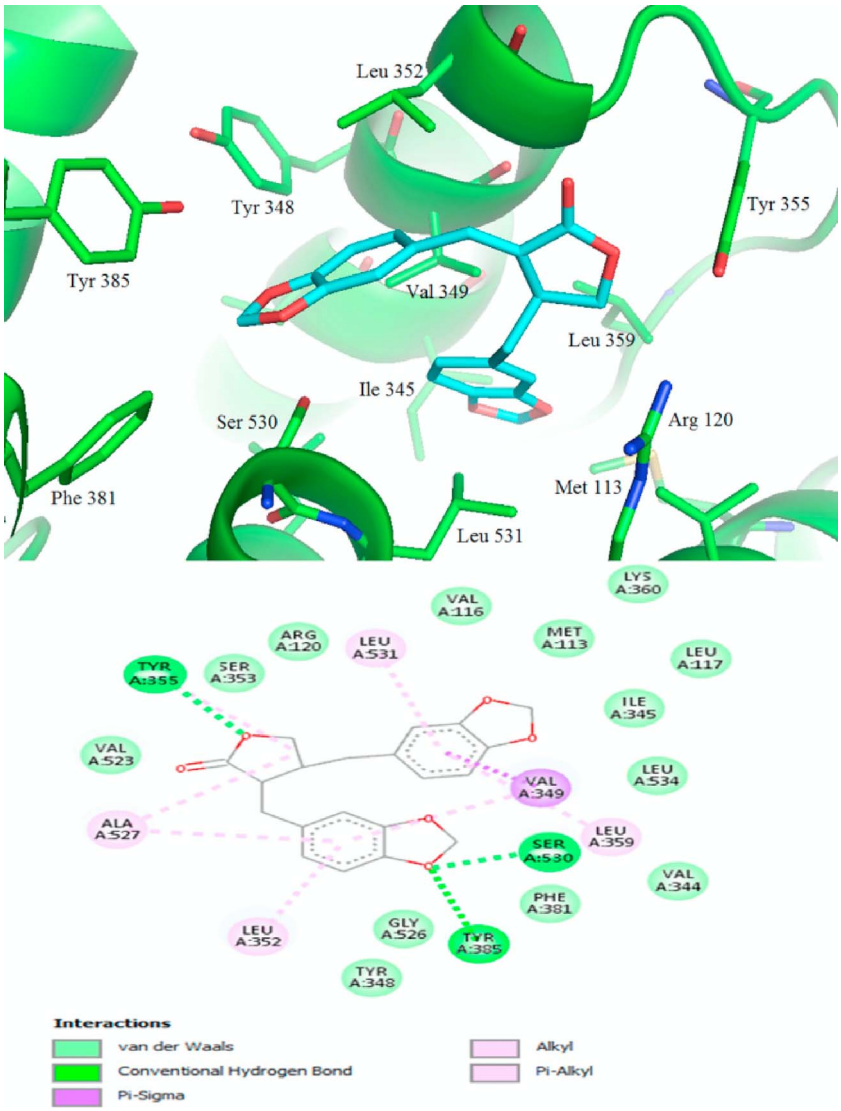

Fig. 6. (A) Docking 3D model of hibalactone (carbon atoms in blue) within COX-2 active site, highlighting the main intermolecular interactions with protein residues. (B) 2D interaction diagram between the hibalactone and COX-2 protein residues.

isolated compound in the second phase of the formalin test is due to an anti-inflammatory activity, we attempted to delineate if the effects seen in the first phase of this same test could be associated with some activity on the central nervous system (CNS). Previous literature data demonstrates that the ethanolic extract of subterraneous parts of *H. umbellata* possess mild sedative properties and this action is accompanied by an anxiolytic like effect in the elevated plus maze with any motor impairment as demonstrated in specific behavioral tests [7].

In the present study it was preceded with the investigation of the anxiolytic like effect of the fractions that were effective in the first phase of formalin test (DF-HU, EAF-HU) and isolated compound through the light dark box, a conventional test for assessment of anxiety-like behavior in the laboratory mice.

The underlining principle of LDB test is based on the aversion of

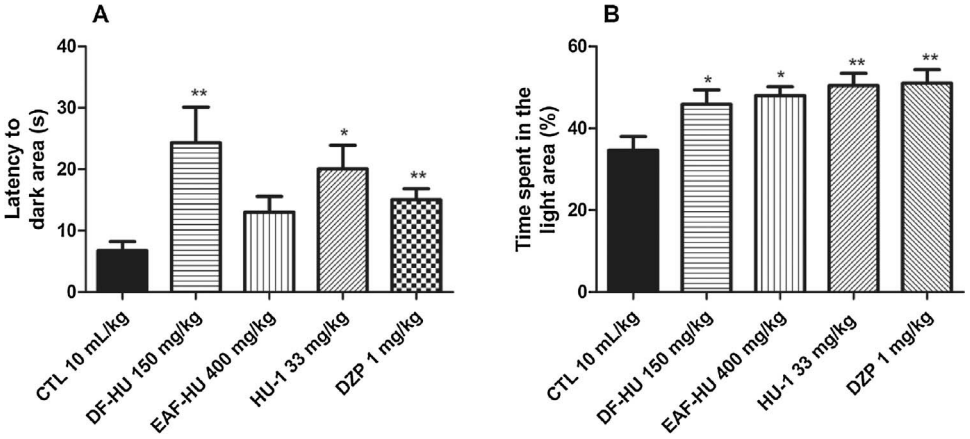

Fig. 7. Effects of oral administration of (CLT, 2.0% Tween 80, 10 mL/kg), DF-HU (150 mg/kg), EAF-HU (400 mg/kg), HU-1 (33 mg/kg) and Diazepam (1 mg/kg) on the (A) Latency to dark area (s) and (B) Time spent in the light area (%) in the light-dark box. Data represent the mean ± SEM. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared treated groups *x* control group using ANOVA followed by *Student-Newman-Keuls* as post-test or Student *t*-test when compared positive control *x* control group.

rodents to brightly illuminated areas, novel environment and light-induced mild stress [40]. When the animals spent more time in the light area corresponds to a predictive behavior of an anxiolytic like activity. The current results obtained in the LDB showed a decreased in the latency to dark compartment with the DF-HU and HU-1, but not with EAF-HU. Furthermore, it was verified an increased in the time spent in the light area providing evidences for the anxiolytic like effects of DF-HU, EAF-HU and hibalactone which may be one of the mechanisms responsible for reducing the reactivity time to pain seen in the first phase of the formalin test.

In the same way, diazepam, used as positive control in LDB test, also decrease the latency to dark compartment and increased the time spent in the light area. Literature survey also revealed that diazepam also reduces the reactivity to pain in both phases of formalin test and among the mechanisms for this action includes this sedative and anxiolytic property [41].

In summary, the present study demonstrated the antinociceptive, anti-inflammatory and anxiolytic like effects of the extract and fractions from *H. umbellata*. These findings are in accordance with the ethnopharmacological use of this plant to treat painful and inflammatory conditions, besides the CNS activities. Phytochemical screening indicated that these effects can be attributed, at least in part, of the presence of hibalactone, a lignan isolated for the first time in this plant. The results findings suggest the hypothesis of hibalactone present anti-inflammatory activity by interfering in the arachidonic acid pathway, by interacting with the enzymes 5-lipoxygenase and cyclooxygenase-2. In addition, it was found still exists the evidences for the anxiolytic like effects of DF-HU, EAF-HU and hibalactone which may be one of the mechanisms responsible for reducing the reactivity time to pain seen in the first phase of the formalin test. However, more pharmacological and chemical studies will be needed in order to better characterize the mechanism(s) responsible for biological effects and to identify other active secondary metabolites present in this specie.

5. Conclusions

The present study demonstrated the antinociceptive, anti-inflammatory and anxiolytic like effects of the extract and fractions from *H. umbellata*. These effects might be due to the presence of the hibalactone, a lignan isolated for the first time in this plant. The phytochemical screening demonstrated the presence this compound in E-HU, HF-HU and DF-HU. The antinociceptive and anti-inflammatory effects might be attributed to the reduced the general inflammatory mediators (histamine, bradykinin, serotonin and prostaglandins). *In silico* findings suggest the hypothesis of hibalactone present anti-inflammatory activity by interfering in the arachidonic acid pathway, by interacting with the enzymes 5-lipoxygenase and cyclooxygenase-2. It was found still exists the evidences for the anxiolytic like effects of E-HU, HF-HU, DF-HU and hibalactone which may be one of the mechanisms responsible for reducing the reactivity time to pain seen in the first phase of the formalin test. However, more pharmacological and chemical studies will be needed in order to better characterize the mechanism(s) responsible for biological effects and to identify other active secondary metabolites present in this specie.

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Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.08.140.

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