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Mechanism involved in the anti-inflammatory effect of *Spiranthera odoratissima* (Manacá)

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Abstract: Acetic acid-induced writhing, hot-plate, carrageenan-induced pleurisy, formalin-induced pain, croton oil-induced ear edema, vascular permeability tests and phospholipase A_2 activity assay were used to study the analgesic and/or anti-inflammatory activity of the hydromethanolic fraction of ethanolic extract from *Spiranthera odoratissima* A. St.-Hil., Rutaceae, leaves (HMF) and its subfraction (sub-Fr_{10-28}). HMF and sub-Fr_{10-28} reduced the leukocyte migration on the carrageenan-induced pleurisy test; sub-Fr_{10-28} reduced the pain reaction time in the second phase of formalin-induced pain, as well as the ear edema and vascular permeability. Both HMF and sub-Fr_{10-28} inhibited the phospholipase A_2 activity. These results suggest that the analgesic effect of this plant could be, in part, due to an anti-inflammatory action produced by the inhibition of phospholipase A_2 activity.

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Introduction

Spiranthera odoratissima A. St.-Hil., Rutaceae, popularly known in Brazil as manacá, is found in the Brazilian Cerrado region and in folk medicine is used to treat different pathologies. The leaves and roots are used to treat rheumatism, abdominal pain, headache, muscle pain, stomach ache, kidneys and hepatic infections, urinary retention, as depurative and appetite stimulant (Galdino et al., 2007; Trevenzol et al., 2006; Ribeiro et al., 2005; Vila-Verde et al., 2003).

The evaluation of central nervous system (CNS) activity with the hexane fraction of ethanolic extract from manacá leaves indicates a depressant activity with this fraction (Matos et al., 2006). Matos et al. (2003) have shown the analgesic and anti-inflammatory activities of aqueous fraction of ethanolic extract from manacá leaves in the acetic acid-induced writhing, croton oil-induced ear and carrageenan-induced peritonitis, similarly, these results also were obtained with the ethanolic extract from manacá roots, and also a CNS depressant activity (Matos et al., 2004). These results could explain the popular use of this plant to treat pain and inflammatory process.

Phospholipases A_2 (PLA_{2s}) form a family of enzymes catalyzing the hydrolysis of membrane phospholipids into arachidonic acid, which is the major precursor of pro-inflammatory eicosanoids (Kini, 2003; Nevalainen et al., 2008; Burke & Dennis, 2009; Folmer et al., 2010). These enzymes are the main component of snake venom and this enzyme have been investigated because their similarity to mammalian phospholipases (Evangelista et al., 2008; Soares & Giglio,, 2003). Due to the role of PLA_{2s} in the inflammatory process, PLA_{2s} have been considered as potential targets in anti-inflammatory drug discovery, resulting in an interest in PLA_2 inhibitors.

The this work aimed to purify the hydromethanolic fraction of ethanolic extract from *S. odoratissima* leaves (HMF), isolate an active sub-fraction responsible for the anti-inflammatory effect, and also assays the effect this sub-fraction upon the PLA_2 activity as the possible anti-inflammatory mechanism.

Material and Methods

Plant material

The leaves from *Spiranthera odoratissima* A. St.-Hil., Rutaceae, were collected in a region of Cerrado, in Senador Canedo City, Goiás, Brazil (762 m, 16º45'45.2" S, 49º07'06.8" W). The samples were authenticated by Dr. José Realino de Paula (Pharmacy Faculty/UFG) and a voucher specimen has been deposited in the Federal

University of Goiás herbarium (UFG-30,275).

Crude extract and hydromethanolic fraction

The ethanolic extract from manacá leaves and fractions were obtained according to Matos et al. (2003).

Subfraction 10-28 (Sub-Fr_{10-28})

The HMF was fractionated using a column chromatography packed with Sephadex LH-20® eluted with methanol solution.

Phytochemical screening

The methods of Harborne (1984) and Ikhiri et al. (1992) were used to screen the HMF and sub-Fr_{10-28} for second metabolites. Only the second metabolites positive in HMF were screened in sub-Fr_{10-28}.

Animals

Male albino *Swiss* mice (25-30 g) provided by the Central Animal House of UFG were used in this study. The animals were maintained under controlled conditions of temperature and light (12 h dark/light), with water and food *ad libitum*. The experimental protocols were approved by Research Ethic Council of UFG (Protocol nº. 102/2008).

Drugs e solvents

Dexamethasone, Decadron (Ache); indomethacin, Indoci (Merck Sharp & Dohme); morphine, Dimorf® (Cristália); acetone (Vetec); acetic acid (Vetec); hydrochloric acid (Vetec); sulfuric acid (Vetec); carrageenan (Sigma); aluminum chloride (Vetec); calcium chloride (Isofar); ferric chloride (Baker analyzed); sodium chloride (Belga); chloroform (Proquímicos); ethanol (Vetec); hexane (Proquímico); sodium hydroxide (Merck); methanol (F.Maia); croton oil (Sigma); quercetin (Sigma); egg yolk suspension (Newprox); Tris (hydroxymethyl) aminomethane (Merck); vanillin (Sigma); *Caudisona durissa collilineata* venom (Center of Biological Studies and Research-PUCGO).

Pharmacological tests

Acetic acid-induced abdominal writhing test

Groups of nine mice were treated *p.o.* with vehicle (10 mL/kg), HMF (50, 150 and 500 mg/kg), or indomethacin (10 mg/kg) (Hendershot & Forsaith, 1959; Koster et al., 1959).

Hot plate test

Groups of seven mice were treated with vehicle (10 mL/kg, *p.o.*), HMF (500 mg/kg, *p.o.*) or morphine (10 mg/kg, *s.c.*) (Woolfe & MacDonald, 1944). The time to pain reaction was recorded at the times of -60, -30, 0, 30, 60, 90, 120, 150 and 180 min in the hot plate (at 55±0.5 ºC).

Carrageenan-induced pleurisy test

Groups of eight mice were treated *p.o.* with vehicle (10 mL/kg), HMF (50, 150 and 500 mg/kg), sub-Fr_{10-28} (10, 20 and 40 mg/kg) or dexamethasone (2.0 mg/kg) (Vinegar et al., 1973). The total number of leukocytes in the exudates was determined using a Neubauer chamber.

Formalin-induced pain test

Groups of ten mice were treated with vehicle (10 mL/kg, *p.o.*), sub-Fr_{10-28} (20 mg/kg, *p.o.*), indomethacin (10 mg/kg, *p.o.*) or morphine (10 mg/kg, *s.c.*) (Hunskar & Hole, 1987).

Croton oil-induced ear edema test

Groups of nine mice were treated *p.o.* with vehicle (10 mL/kg), sub-Fr_{10-28} (20 mg/kg) or dexamethasone (2.0 mg/kg) (Zanini et al., 1992).

Vascular permeability test

Each animal received 200 µL of 2.5% Evans blue dye solution, intravenously, then were submitted to the protocol of carrageenan-induced pleurisy test (Vinegar et al., 1973). Groups of nine mice were treated *p.o.* with vehicle (10 mL/kg), sub-Fr_{10-28} (20 mg/kg) or dexamethasone (2.0 mg/kg). The exudates were centrifuged (1200 x *g*, 10 min) and the absorbance of the collected solution was measured at 600 nm. The amount of dye leakage was calculated from the absorbance measurements.

Gel plate assay of phospholipase A_2 activity

The inhibitory effect of HMF (375, 750 and 1,500 µg/mL) and sub-Fr_{10-28} (150, 300 and 600 µg/mL) on PLA_2 activity was assayed plates containing agarose-egg yolk gels as substrate (Habermann & Hardt, 1972). The *Caudisona durissa collilineata* poison was used as PLA_2 source. Eight replicates were done for each concentration. The results were expressed as hydrolyses halos area.

Statistical analysis

The data were expressed as mean±SEM of absolute value or percentage of control group. The results were analyzed using ANOVA and Student-Newman-Keuls test as post-test, or using only Student's t-test. P values less than 0.05 ($p<0.05$) were considered significant (Sokal & Rohlf, 1981).

Results

Extractive process and Phytochemical screening

The ethanolic extract yielded 35.7%, compared to the powder, and the yields of the hexanic (HF), chloroformic (CF), and hydromethanolic (HMF) fractions were 0.5, 3.5 and 35% (w/w), respectively, compared to ethanolic extract. Phytochemical screening showed the presence of anthraquinones, flavonoids, coumarins and tannins in HMF and only the presence of tannins in sub-Fr_{10-28}.

Fractionation of hydromethanolic fraction

The aliquots (10 to 28) were grouped according to its Rf on TLC (ethanol:acetic acid solution (80:20, v/v) as moving phase and sulfuric vanillin 1% was revelator. The obtained fraction was named sub-Fr_{10-28}, with yield of 13.1% compared to HFM.

Acetic acid-induced abdominal writhing test

The treatment with HMF (150 and 500 mg/kg) reduced in a dose-dependent manner the abdominal writhes from 84.3±4.48 (control group) to 69.8±7.9 and 43.4±5.6%, respectively (Figure 1).

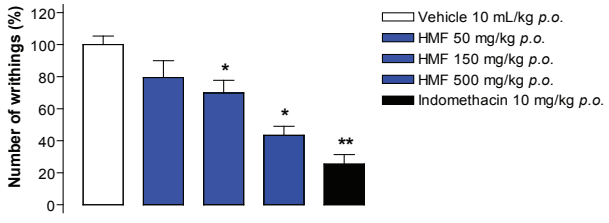

Figure 1. Effect of hydromethanolic fraction from *Spiranthera odoratissima* leaves ethanolic extract (HMF 50, 150 and 500 mg/kg, *p.o.*) in the number of acetic acid-induced writhing in mice. Indomethacin (10 mg/kg, *p.o.*) was used as positive control. Vertical bars represent mean±SEM in percentage of control group of accumulated writhes in 30 min for each experimental group. *$p<0.05$; **$p<0.01$ when compared to control group.

Hot plate test

The treatment with HMF did not alter the latency to pain reaction. Morphine increased the latency at the times 30 and 60 min after the treatment (9.1±0.8 and 10.4±2.1 s, respectively) when compared to control group (4.8±0.7 and 4.3±0.7, respectively) (Figure 2).

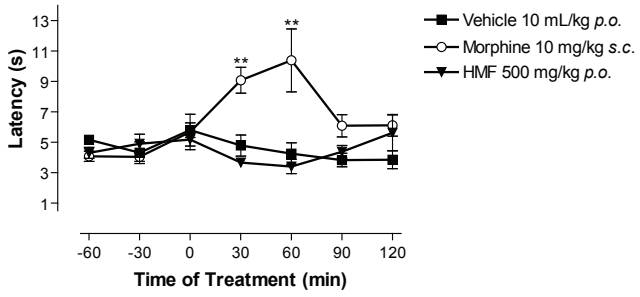

Figure 2. Effect of hydromethanolic fraction from *Spiranthera odoratissima* leaves ethanolic extract (HMF 500 mg/kg, *p.o.*) in the latency, in seconds, to reaction in mice. Morphine (10 mg/kg, *s.c.*) was used as positive control. Vertical bars represent mean±SEM for each experimental group. **$p<0.01$ when compared to control group.

Carrageenan-induced pleurisy test

The treatment with HMF (50, 150 and 500 mg/kg) and sub-Fr_{10-28} (20 and 40 mg/kg), decreased the leukocyte migration to pleural cavity from control value of 6.03±0.61 x 10^6 leukocytes/mL to 3.63±0.355; 3.542±0.321; 2.97±0.67; 3.74±0.307; 2.79±0.484 x 106 leukocytes/mL, respectively (Figure 3).

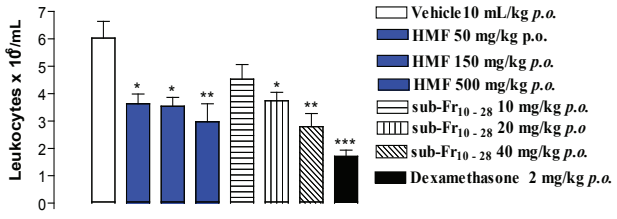

Figure 3. Effect of hydromethanolic fraction from *Spiranthera odoratissima* leaves ethanolic extract (HMF 500 mg/kg, *p.o.*) in carrageenan-induced pleurisy in mice. Dexamethasone (2.0 mg/kg, *p.o.*) was used as positive control. Vertical bars represent mean±SEM of total number of migrated leukocytes/mL. *$p<0.05$; **$p<0.01$; ***$p<0.001$ when compared to control group.

Formalin-induced pain test

The treatment with sub-Fr_{10-28} (20 mg/kg) did not reduced the licking time (time licking the paw after

intraplantar injection of formalin) at the neurogenic phase, but reduced the licking time at the inflammatory phase from 163.2±10.90 s (control group) to 116.7±8.88 s. Morphine reduced the licking time at both phases and indomethacin reduced the licking time only at the second phase (Figure 4).

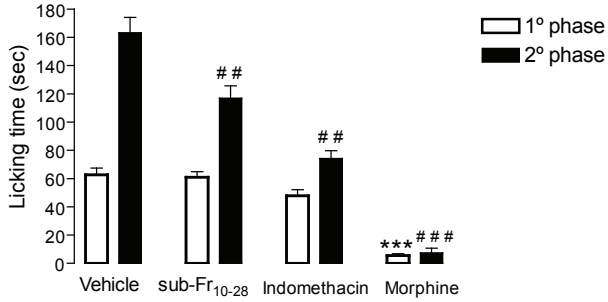

Figure 4. Effect of sub-Fr_{10-28} (20 mg/kg, *p.o.*) in formalin-induced pain in mice on first phase (0-5 min) and second phase (15-30 min). Morphine (10 mg/kg, *s.c.*) and indomethacin (10 mg/kg, *p.o.*) were used as positive control. Vertical bars represent mean±SEM of licking time, in seconds. ***p<0.001 compared with the control group of the first phase. $^{\#\#}p$<0.01; $^{\#\#\#}p$<0.001 compared with the control group of the second phase.

Croton oil–induced ear edema and vascular permeability tests

The treatment with sub-Fr_{10-28} (20 mg/kg) reduced the ear edema formation as well as the Evan's blue dye concentration in pleural exudates (Table 1).

Table 1. Anti-inflammatory effect of sub-Fr_{10-28} in croton oil-induced ear edema and vascular permeability tests.

Test Group	Oedema (mg)	Inhibition (%)	Evan's blue (µg/mL)	Inhibition (%)
Vehicle (10 mL/kg)	17.8±0.77	_____	4.10±0.49	____
Sub-Fr_{10-28} (20 mg/kg)	14.0 0.42**	21.35	2.07±0.50*	49.51
Dexamethasone (2 mg/kg)	4.0±1.26***	77.53	1.70±0.42***	58.54

Dexamethasone (2.0 mg/kg) was used as positive control. *p<0.05; **p<0.01; ***p<0.001 when compared to control group.

Gel plate assay of phospholipase A_2 activity

HMF (375, 750 and 1500 µg/mL) reduced the halo formatted, in a concentration-dependent manner, to 76.8±6.0; 54.7±4.3; 26.1±1.2 % of control group, respectively. Sub-Fr_{10-28} (150, 300 and 600 µg/mL) also reduced the halo formatted to 80.1±7.1; 70.9±4.9; 66.9±3.0 % of control group, respectively (Figure 5).

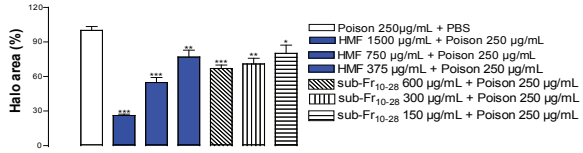

Figure 5. Effect of hydromethanolic fraction from *Spiranthera odoratissima* leaves ethanolic extract (HMF 1500, 750 and 375 µg/mL) and sub-Fr_{10-28} (600, 300 and 150 µg/mL) in gel plate assay of phospholipase A_2 activity. Vertical bars represent mean±SEM in percentage of halo area of control group. *p<0.05; **p<0.01; ***p<0.001 when compared to control group.

Discussion

Medicinal plants shown a large range of secondary metabolites that may show biological activities, among these plants is *Spiranthera odoratissim*a A. St.-Hil., Rutaceae, a plant from Brazilian Cerrado with analgesic and anti-inflammatory activities (Matos et al., 2003).

With the acetic acid-induced abdominal writhing test, a high sensibility but low specificity model (Koster et al., 1959), was demonstrated that HMF posses antinociceptive activity, this results is in accordance with Matos et al. (2003), that demonstrated the same effect with this fraction, and also with Matos et al. (2004), that demonstrated the antinociceptive effect with the ethanolic extract from manacá roots in this test.

In order to evaluate the involvement of central mechanisms in the antinociceptive effect of HMF, we realized the hot plate test, which is frequently used to evaluate centrally mediated antinociceptive activity and utilize thermal stimulus to induce pain (Rinaldi et al., 2009), in this test HMF did not reduce the latency to pain reaction which suggests no supra-spinal analgesic activity.

The reduction in the cell migration and chemotaxis are important activities shown by different anti-inflammatory drugs (Catão-Dias & Sinhorini, 1999; Cummings, 1999; Servant et al., 2000). The carrageenan-induced pleurisy is an acute inflammation model that allows the quantification of leukocytes attracted to pleural cavity under the action of chemotactic agents, such as leukotrienes and interleukins, this cell migration is sensitive to steroidal and non-steroidal anti-inflammatory drugs (Vinegar et al., 1973; Higgs et al., 1980; Middleton & Kandaswami, 1992; Brooks & Day, 1991). The HMF and sub-Fr_{10-28} reductions in leukocyte migration suggests an anti-inflammatory activity that can be associated with the inhibition of late phase of the inflammatory process, where the chemotactic process and the cell migration happen (Di Vaio & Freitas, 2001; Contran et al., 1999), and this anti-inflammatory activity could explain the antinociceptive effects already observed.

The formalin-induced pain test is divided in two phases: the neurogenic phase (first 5 min after formaline intraplantar injection) that involves a direct activation of sensory fibers C by formaldehyde as well as participation of peripheral mediators stimulating directly the nociceptors; and inflammatory phase (15 to 30 min after the injection) that involves the release and production of inflammatory mediators (Rocha et al., 2007). The sub-Fr_{10-28} showed an antinociceptive activity only in the second phase, confirming that the observed effects with the treatment with sub-Fr_{10-28} are not due to central analgesic effect and possibly due to an anti-inflammatory action, such as inhibition in synthesis or release of inflammation mediators (prostaglandins, thromboxanes, leukotrienes, among others).

The sub-Fr_{10-28} anti-inflammatory activity was evaluated in the croton oil–induced ear edema and vascular permeability tests. The croton oil is a vascular irritant agent and causes leukocyte migration to the region, edema and topic dermatitis, in the croton oil-induced ear edema the edema intensity is considered as an inflammatory parameter that can be measured (Zanini et al., 1992; Montello, 2002). In this test, sub-Fr_{10-28} inhibited the edema formation, and considering that sub-Fr_{10-28} was able to inhibit the licking time only in the second phase of formalin test (inflammatory phase), it is very likely to considerate that the ear edema inhibition be due to the anti-inflammatory molecules present in this fraction. Corroborating with the presence of anti-inflammatory compound in sub-Fr_{10-28}, we observed a reduction in vascular permeability, an effect produce by anti-inflammatory drugs (Gehlen et al., 2004).

The PLA_2 catalyzes the hydrolysis of membrane phospholipids to produces archidonic acid, which is involved in the synthesis of eicosanoids, this forms the first step in inflammatory pathway, and in addition to inflammation this enzyme has been implicated in the etiology of atherosclerosis and cancer (Sato et al., 2009; 2008; Murakami et al., 2005). The identification of a specific PLA_2 inhibitor is an important step in the study of news potential anti-inflammatory agents (Yu et al., 1998).

Several snakes venom, including *Caudisona durissa collilineata* posses among the main constituents the enzyme PLA_2, with has high structural homology with human PLA_2 (Ponce-Soto et al., 2002). The used of snakes PLA_2 enzyme in the investigation of PLA_2 specific inhibitors has been described in some studies (Borges et al., 2005; Cotrim et al., 2011; da Silva et al., 2008). In our study, HMF and its sub-fraction (sub-Fr_{10-28}) were able to inhibit the PLA_2 activity, indicating that the anti-inflammatory activity of HMF and sub-Fr_{10-28} evolves, at least in part, the inhibition in PLA_2 activity.

The phytochemical screening showed the presence of tannins, anthraquinones, flavonoids, and coumarins in HMF, and only the presence of tannins in sub-Fr_{10-28}. The ability of tannins to inhibit phospholipase A_2 is already established for ellagic acid and derivates (da Silva et al., 2008; Glaser et al., 1995; Chandra et al., 2007), a potent anti-inflammatory found in several plants, for example in *Lafoensia pacari*, a plant with anti-inflammatory activity (Galdino et al., 2009; Rogerio et al., 2006). *S. odoratissima* tannins could presented a similar activity. The others secondary metabolites, presents in HMF, have been report as antinociceptive and anti-inflammatory compounds (Corrêa et al., 2008; Coutinho et al., 2009; Melo et al., 2009; Seo et al., 2009), this fact could explain the better effect of HMF in PLA2 activity assay.

In conclusion, the present study demonstrated the inhibition of PLA_2 activity as one mechanism involved in the anti-inflammatory effect of sub-fraction from hydromethanolic fraction of ethanolic extract from *Spiranthera odoratissima* leaves. Through identification of active molecule will be possibly study others mechanisms involved in inflammation process, like cytokines level and other enzymatic activities.

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