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Full Length Research Paper

Pharmacological effects of two polar fractions from *Annona coriacea* Mart in animal models

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In the current investigation, the antinociceptive and anti-inflammatory effects of the ethyl acetate and butanol fractions from *Annona coriacea* leaves were evaluated using standard experimental test models. The antinociceptive activity of *A. coriacea* was studied by acetic acid-induced writhing, formalin and hot-plate tests, and the anti-inflammatory activity was determined by carrageenan-induced rat paw edema and carrageenan-induced pleurisy. Inhibition of the acetic acid-induced abdominal contortions was observed at the doses of 50 and 100 mg/kg. After injection of formalin, the dose of 100 mg/kg inhibited the time spent paw licking in the first phase, while the second phase was inhibited at the doses of 10, 50 or 100 mg/kg. In the hot-plate assay, the reaction time was increased at 90 min in animals that received 100 mg/kg. Doses of 50 and 100 mg/kg inhibited carrageenan-induced paw edema, the exudate volume, and leucocyte migration. The present results demonstrate that fractions of *A. coriacea* have antinociceptive and anti-inflammatory effects, supporting the use of this plant in folk medicine.

Key words: Annonaceae, *Annona coriacea*, antinociceptive activity, anti-inflammatory activity.

INTRODUCTION

Documentation of the indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources (Muthu et al., 2006). Plants which are used in different parts of the world for the treatment of similar diseases may be deemed to be effective in pharmacological terms (Cakilcioglu and Turkoglu, 2010). Therefore, establishment of the local names and indigenous uses of plants has significant potential societal benefits (Bağcı, 2000).

One of the most important sources of active substances has been sustained in medicinal plants research (Balunas and Kinghorn, 2005; Rout et al., 2009). Nowadays, medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, inflammation and pain (Balunas and Kinghorn, 2005). Thus, new drugs have been isolated from plants and their therapeutic properties have been proven to scientific validation (Younes et al., 2007; Itokawa et al., 2008; Bero et al., 2009; Agnihotri et al., 2010).

A. coriacea Mart. (Annonaceae), popularly known as araticum or araticum-do-campo, is found in the different regions of Brazil, being cultivated in the Brazilian Cerrado because of its delicious fruit (Meira and Saporetti, 2002; Pontes et al., 2004). Considering folk medicine reports, in the state of Ceará, Brazil, the leaves of this plant are used as carminative, stomachic, and anti-helminthic, in the treatment of stomatitis, neuralgia, rheumatism headaches, furuncles and ulcers (Sousa et al., 2007).

Phytochemical studies of the roots of *A. coriacea* have identified diterpenoids with clerodane skeleton (Ferrari and Pellizoni, 1971). Acetogenins, such as coriedienin,

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coriacin, 4-deoxicoriacin, gigantetronenin and gigantecin were also described and these compounds showed cytotoxic activity against tumour cells (Yu et al., 1994; Silva et al., 1995, 1996). Other important description was the characterization of an *A. coriacea* lectin (ACLEC) isolated of the seeds from this plant (Coelho et al., 2003), that increased the migration of neutrophils in the abdominal cavity in mice (Coelho et al., 2006). Furthermore, the extract of the seeds produced genotoxic effect (Fagundes et al., 2005) and showed to be active as insecticide (Souza et al., 2007). In addition, the methanol extract of the *A. coriacea* leaves revealed analgesic and anti-inflammatory properties (Sousa et al., 2007).

In order to contribute with the pharmacological basis for the better understanding of the use of *A. coriacea* in folk medicine, the present study was designed to investigate the antinociceptive and anti-inflammatory effects of two polar fractions obtained by partition of the methanol extract using animal models.

MATERIALS AND METHODS

Plant material

The plant material used in this study was collected in Camocim, Ceará, northeast region of Brazil, in February 2008. The identification of the species was made by Dr. Rita de Cássia Almeida Lafetá, and a voucher specimen (nº 195596) was deposited in the Herbarium of the Universidade Federal do Rio de Janeiro, Brazil.

Preparation of extracts

Dried and powdered leaves (600 g) were exhaustively extracted with methanol at room temperature by maceration, for a period of ten days. The obtained extract was filtered and evaporated under reduced pressure on a rotary evaporator. The residue was fractionated by liquid-liquid partition between ethanol: water (1:9) and organic solvents (hexane, dichloromethane, ethyl acetate and butanol) (Cechinel Filho and Yunes, 1998). After rota-evaporation, the fractions of ethyl acetate (EF) and butanol (BF) related with polar constituents were used to evaluate the pharmacological activities.

Preliminary phytochemical tests

The ethyl acetate and butanol fractions were subjected to preliminary screening for active phytochemical constituents such as alkaloids (Bertrand's, Bouchardat's, Dragendorf's and Mayer's tests), terpenes and steroids (Kedde's, Libermann-Buchard's and Baljet's tests), saponins (Foaming index), tannins (reactions with ferric chloride, lead acetate, copper salts, alkaloids and gelatin) and flavonoids (reactions with $AlCl_3$, H_3BO_3 and NaOH, and Shinoda test), following the conventional protocols suggested by Matos (1997).

Chemicals

Drugs and reagents used in this study (and their sources) were as follows: Acetic acid (Vetec Química Farm Ltd, Rio de Janeiro, Brazil), formaldehyde and acetylsalicylic acid (Reagen Quimibrás Ind. Química S.A., Rio de Janeiro, Brazil), morphine hydrochloride (Merck Inc., Whitehouse Station, USA), naloxone, indometacin and carrageenan (Sigma Chemical Co, St. Louis, USA).

Animals

Male Wistar rats (180 to 240 g) and male Swiss albino mice (25 to 30 g) were used in the experiments. These animals were provided by the central biotery of the Universidade Federal de Juiz de Fora (Minas Gerais state, Brazil), divided into groups, and kept in plastic cages at room temperature (25 ± 4ºC), with free access to Purina® rations and water. Animal care and the experimental protocol followed the principles and guidelines suggested by the Brazilian College of Animal Experimentation (COBEA) and were approved by the local Ethical Committee (Protocol number 054/2009).

Acetic acid-induced writhing response in mice

Antinociceptive activity was evaluated using the test of abdominal writhing induced by acetic acid in mice (Collier et al., 1968). Animals were divided into five groups of eight mice. Control group mice (sterile saline; 10 ml/kg) received an intraperitoneal (i.p.) injection of acetic acid 0.6% (0.25 ml) and 10 min later the writhes were counted over a period of 20 min. One group of mice received oral acetylsalicylic acid (200 mg/kg) as a reference compound, and the other three groups received oral doses of the fractions (10, 50 or 100 mg/kg), 1 h before the acetic acid injection.

Formalin-induced nociception in mice

Mice received subplantar injections of 20 µl 2.5% formalin (in 0.9% saline) and the duration of paw licking was determined over 0 to 5 min (first phase) and 15 to 30 min (second phase) after formalin injection (Hunskaar and Hole, 1987). Animals (n = 8) were pre-treated with fractions (10, 50 or 100 mg/kg; 0.1 ml per 10 g body weight, administered orally) or the reference compound, subcutaneous morphine (1 mg/kg), 1 h before administration of formalin. Control animals were treated with sterile saline (10 ml/kg).

Hot-plate latency assay in mice

Animals were placed on a hot-plate (Model LE 7406, Letica Scientific Instruments, Barcelona, Spain) heated at 55 ± 1ºC (Eddy and Leimbach, 1953). Three groups of mice (n = 8) were treated orally with fractions (10, 50 or 100 mg/kg; 0.1 ml per 10 g body weight) and the control group received sterile saline (10 ml/kg). Measurements were performed at time zero, 30, 60 and 90 min after drug administration, with a cut-off time of 40 s to avoid lesions to the animals' paws. The effect of pre-treatment with naloxone (1 mg/kg, subcutaneously) on the analgesia produced by the fractions (100 mg/kg) was determined in a separate group of animals. Morphine (1 mg/kg, subcutaneously) in the absence and presence of naloxone treatment was used as reference.

Carrageenan-induced edema in rats

Anti-inflammatory activity was assessed on the basis of inhibition of paw edema induced by the injection of 0.1 ml 2% carrageenan (an edematogenic agent) into the subplantar region of the right hind paw of the rat (Winter et al., 1962). Male Wistar rats were divided into groups of six animals which received oral doses of fractions

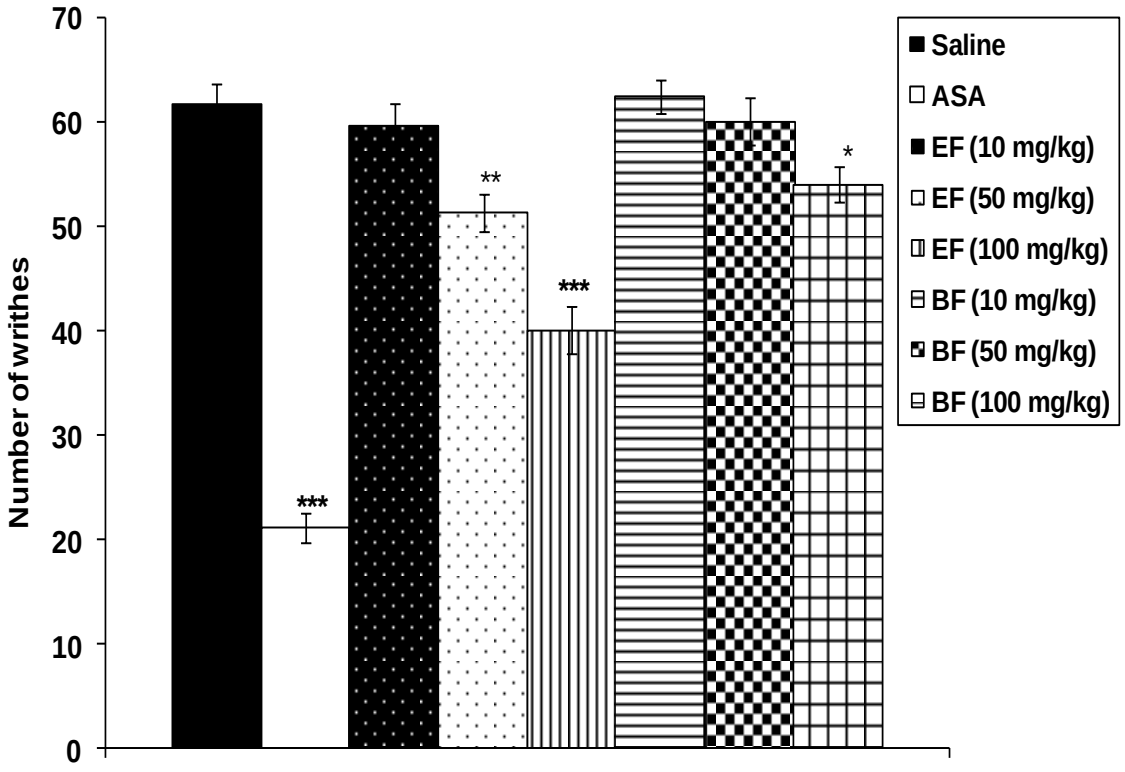

Figure 1. Effects of the polar fractions from *A. coriacea* leaves on acetic acid-induced writhing in mice. ASA, Acetylsalicylic acid; EF, ethyl acetate fraction; BF, butanol fraction. Data are mean ± S.E.M. of eight mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *vs.* control group (saline).

(10, 50 or 100 mg/kg; 0.1 ml per 10 g body weight), sterile saline (10 ml/kg) and indomethacin (10 mg/kg) 1 h before the injection of carrageenan. Paw volume was measured after 4 h after administration of carrageenan using a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain).

Carrageenan-induced pleurisy in rats

Pleurisy was induced in male Wistar rats by intrapleural administration of 0.5 ml 2% carrageenan suspension in saline solution between the third and fifth ribs on the right side of the mediastinum (Vinegar et al., 1973). Fractions (10, 50 or 100 mg/kg), sterile saline (10 ml/kg) or indomethacin (10 mg/kg) per oral (p.o.) were given 60 min before injection of the irritant. Animals were killed 4 h after carrageenan injection, and the skin and pectoral muscles were retracted. A longitudinal incision was made between the third and fifth ribs on each side of the mediastinum. The exudate was collected and transferred to a 15 ml conical centrifuge tube and the total volume was determined. A 20 µl aliquot of the exudate was used to establish the total leucocyte count in Neubauer chambers.

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical significance was analysed by the one-way analysis of variance followed by the Student Newman-Keuls test. p Values below 0.05 were considered significant.

RESULTS

Preliminary phytochemical tests

The phytochemical screening indicated that the ethyl acetate and butanol fractions obtained from leaves of *A. coriacea* contain flavonoids, tannins and alkaloids.

Writhing response induced by acetic acid in mice

The fractions of *A. coriacea* reduced significantly the abdominal contortions (Figure 1): EF (50 mg/kg = 51.37 ± 1.82, $p < 0.01$; 100 mg/kg = 40.12 ± 2.23, $p < 0.001$) and BF (100 mg/kg = 54.12 ± 1.71, $p < 0.05$). At the dose of 200 mg/kg, the acetylsalicylic acid decreased 65.80% when compared with the control group.

Effects on formalin-induced nociception in mice

The subplantar injection of formalin produced a biphasic characteristic response (Figure 2). At dose of 100 mg/kg, the first phase of paw licking was reduced ($p < 0.001$) in 13.83% (EF) and 13.98% (BF) when compared with the

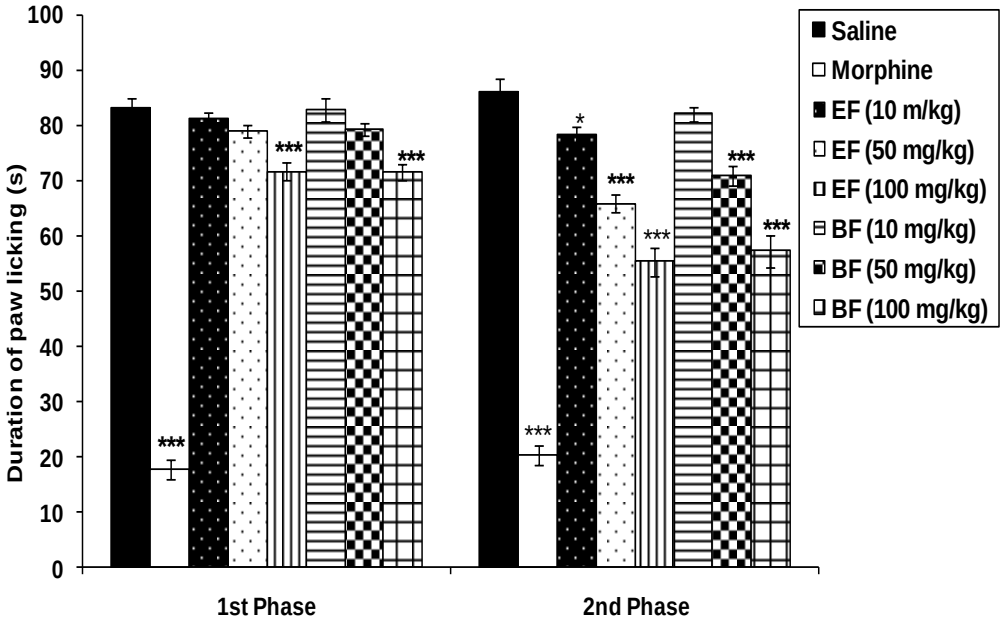

Figure 2. Effects of the fractions from *A. coriacea* leaves on formalin-induced nociception in mice. EF, Ethyl acetate fraction; BF; butanol fraction. Data are mean ± S.E.M. of eight mice. *, $p < 0.05$, ***, $p < 0.001$ *vs* control group (saline).

control group. The ethyl acetate fraction inhibited the second phase at doses of 10 (9.00%), 50 (23.65%) and 100 mg/kg (35.84%). The second phase of paw licking was also reduced by butanol fraction in 17.85 and 33.52% to 50 and 100 mg/kg, respectively. As expected, morphine (1 mg/kg) was active in both phases of formalin-induced paw licking.

Effects on hot-plate latency assay in mice

The effect of the fractions in the hot-plate assay varied according the doses and the observation time (Table 1). No significant antinociceptive effects were observed at time zero, 30 and 60 min. The reaction time was increased significantly ($p < 0.05$ or $p < 0.01$)) only at 90 min in animals that received EF or BF at dose of 100 mg/kg. Hot-plate testing was also performed in the presence of naloxone, an opioid antagonist. Naloxone reduced the morphine and the fractions-induced antinociceptive effect (Table 1).

Effects on carrageenan-induced edema in rats

To confirm the anti-inflammatory effect suggested by the formalin test, the two polar fractions were evaluated using the carrageenan-induced paw edema model (Figure 3). The edema inhibition was observed for 3 h after carrageenan application of doses of 50 mg/kg ($p < 0.05$) with 27.42% (EF) and 24.19% (BF), and 100 mg/kg ($p < 0.05$) with 30.64% (EF) and 27.42% (BF) in relation to the control group. 4 h after carrageenan injections, the doses of 50 mg/kg ($p < 0.05$) reduced the edema in 28.33% (EF) and 25.00% (BF) and 100 mg/kg ($p < 0.01$) with 36.67% (EF) and 33.33% (BF). The indomethacin reduced the paw edema in 32.26% ($p < 0.05$) and 41.67% ($p < 0.01$) at 3 and 4 h, respectively.

Effects on carrageenan-induced pleurisy in rats

The pleurisy effects demonstrated that doses of 50 ($p < 0.01$) and 100 mg/kg ($p < 0.001$) of the fractions significantly reduced the exudate volume (Table 2). The number of total leucocytes was inhibited at the doses of 50 ($p < 0.05$ or $p < 0.01$) and 100 mg/kg ($p < 0.001$) (Table 2). EF reduced the exudate volume in 21.35% (50 mg/kg) and 36.52% (100 mg/kg), while BF decreased by 19.66 and 42.69% at doses of 50 and 100 mg/kg when compared to the respective control. Leucocyte migration inhibition also occurred from doses of 50 (EF= 13.23 ± 0.34 × 10^3 cells/mm^3; BF= 13.57±0.27 × 10^3 cells/mm^3) and 100 mg/kg (EF= 11.08 ± 0.23 × 10^3 cells/mm^3; BF= 11.70 ± 0.29). Indomethacin reduced the exudate volume and the leucocyte migration as expected.

DISCUSSION

In the present study, preliminary phytochemical tests were performed to detect the active constituents present

Table 1. Effects of the fractions from *A. coriacea* leaves on the latency time of mice exposed to the hot plate test.

Group	Dose (mg/kg)	Time after drug administration (s)			
		0 min	30 min	60 min	90 min
Control	Saline	6.25±0.75	6.50±0.53	6.50±0.71	6.50±0.57
	10	6.12±0.48	6.62±0.62	6.50±0.57	7.25±0.53
EF	50	6.62±0.37	6.87±0.58	6.75±0.72	7.50±0.53
	100	6.87±0.67	7.00±0.78	7.75±0.82	8.75±0.49**
	10	6.50±0.57	6.12±0.51	6.37±0.73	6.62±0.68
BF	50	6.12±0.48	6.25±0.59	6.62±0.59	7.37±0.37
	100	6.62±0.56	6.37±0.65	7.50±0.87	8.37±0.46*
Morphine	1	6.75±0.45	10.37±0.53***	14.25±0.84***	17.50±0.84***
Naloxone+morphine	1+1	6.50±0.46	7.25±0.77	7.37±0.80	7.87±0.61
Naloxone+EF	1+100	6.12±0.48	6.62±0.82	7.62±0.68	8.12±0.55
Naloxone+BF	1+100	6.37±0.53	6.75±0.80	7.25±0.75	7.75±0.53

EF, Ethyl acetate fraction; BF, butanol fraction. Data are mean ± S.E.M. of six rats. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ *vs.* control group.

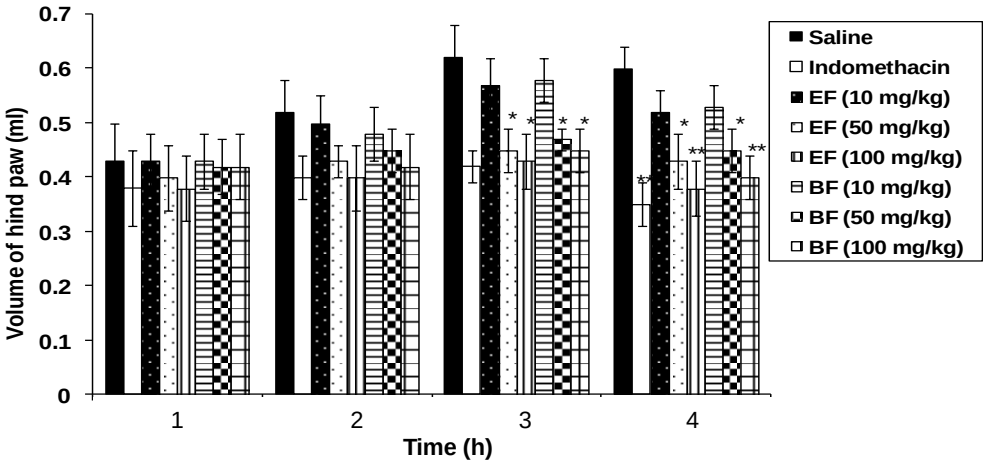

Figure 3. Effects of the polar fractions from *A. coriacea* leaves on carrageenan-induced paw edema in rats. EF: ethyl acetate fraction, BF: butanol fraction. Data are mean ± S.E.M. of six rats. *$p < 0.05$, ***$p < 0.01$ *vs.* control group (saline).

Table 2. Effects of the polar fractions from *A. coriacea* leaves on pleural exudation and number of leucocytes in carrageenan-induced pleurisy in rats.

Group	Doses (mg/kg)	Exudate volume (ml)	Inhibition (%)	Nº Leucocytes (× 10^3 cells/mm^3)	Inhibition (%)
Control	Saline	1.78±0.09	-	14.70±0.28	-
	10	1.75±0.08	1.68	14.63±0.31	-
EF	50	1.40±0.06**	21.35	13.23±0.34**	10.00
	100	1.13±0.08***	36.52	11.08±0.23***	24.62
	10	1.77±0.08	-	14.65±0.27	-
BF	50	1.43±0.07**	19.66	13.57±0.27*	7.69
	100	1.02±0.05***	42.70	11.70±0.29***	20.41
Indomethacin	10	0.62±0.06***	65.17	9.57±0.25***	34.90

EF, Ethyl acetate fraction; BF, butanol fraction. Data are mean ± S.E.M. of six rats. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs control group.

in the two polar fractions investigated. Flavonoids, tannins and alkaloids were revealed in *A. coriacea* leaves and these substances have been identified into other species of the Annonaceae family (Santos and Salatino, 2000; Hu and Wu, 2007; Leboeuf et al., 1981, 1982; Silva et al., 2007; Bako et al., 2005).

Regarding the writhing test, the intraperitoneal injection of acetic acid produces high levels of prostaglandins PGE2α and PGF2α (Deraedt et al., 1980) and releases sympathetic nervous system mediators (Duarte et al., 1988; Hokanson, 1978), promoting pain and inflammation. Our results supported that *A. coriacea* fractions (Figure 1) presented antinociceptive property by reducing abdominal writhing, suggesting inhibition of prostaglandins synthesis (Duarte et al., 1992). Previously, the same observation was reported by our research group using methanol extract (Sousa et al., 2007).

The two polar fractions also produced significant inhibition in the phases of formalin-induced pain. The formalin test is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. This test produces a distinct biphasic response that is characterized by two phases (first and second phases) (Hunskaar and Hole, 1987). Centrally acting drugs such as opioids inhibit both phases equally, while peripherally acting drugs such as aspirin, indomethacin and dexamethasone only inhibit the second phase (Shibata et al., 1989). The second phase is suggested as an inflammatory response that can be inhibited by anti-inflammatory drugs (Hunskaar and Hole, 1987; Rosland et al., 1990). According to the Figure 2, the effect observed with the fractions suggests that antinociceptive activity may be resulting from central and peripheral actions, confirming the writhing test.

The central action was proven in the hot-plate test (100 mg/kg), showing maximal effect after 90 min (Table 1). Our results indicate that the analgesia induced by the fractions could be dependent on the opioid system, since previous treatment with naloxone reversed the effect (Table 1). It is possible that this effect was due to polar constituents, which together providing the action in a synergistic way.

The anti-inflammatory activity of *A. coriacea* fractions suggested in the formalin test was confirmed by the carrageenan-induced paw edema model through the reduction on the displaced volume (Figure 3). This experiment is a suitable test for evaluating anti-inflammatory drugs, which has frequently been used to assess the anti-edematous effect of natural products (Panthong et al., 2003). Development of edema in the paw of the rat after injection of carrageenan is a biphasic event (Vinegar et al., 1969). The initial phase observed during the first hour is attributed to the release of histamine and serotonin (Crunkhon and Meacock, 1971), while the second phase is due to the release of bradykinin, prostaglandins, protease and lysosome (Crunkhon and Meacock, 1971; Vinegar et al., 1969; Di Rosa et al., 1971; Seibert et al., 1994; Nantel et al., 1999). The treatment with the fractions of *A. coriacea* reduced carrageenan-induced paw edema in rats showing that inflammatory mediators that participate of the second phase in the carrageenan response could be inhibited (Figure 3) (Stochla and Maslinski, 1982; Hwang et al., 1986; De Campos et al., 1996; Gilligan et al., 1994). The EF and BF fractions tested could be similar activity to non-steroid anti-inflammatory agents such as indometacin that inhibit the cyclo-oxygenase, reducing the biosynthesis of prostaglandin (Ueno et al., 2000).

Pleurisy produced by intrapleural injection of carrageenan leads to the formation of exudate in the pleural cavity (Ammendola et al., 1975; Almeida et al., 1980) and leucocyte migration (Almeida et al., 1980; Capasso et al., 1975). This method assesses the inflammatory infiltrate and confirms the obtained paw edema results. Non-steroidal anti-inflammatory drugs inhibit the accumulation of exudates and mobilization of leucocytes between 3 and 6 h after application of carrageenan (Almeida et al., 1980; Vinegar et al., 1973). By reducing the volume of exudate and the leucocyte migration, the *A. coriacea* fractions reinforce the anti-inflammatory effect observed in the paw edema test (Figure 3 and Table 2).

Antinociceptive and anti-inflammatory activities described in this study were also reported in the Annonaceae species, including *A. coriacea* (Shang-Hsin et al., 2005; Sousa et al., 2007, 2008, 2010; Oyemitan et al., 2008; Tanna et al., 2009). Probably, these properties are related with components detected by phytochemical screening of fractions from *A. coriacea* in this investigation.

Several mechanisms of action could speculate the pharmacological activities found in the present study. Flavonoids, for example, are potent inhibitors of nitric oxide synthase type 2 and protein tyrosine kinases that are enzymes involved in the nitric oxide/cyclic GMP pathway (Olszanecki et al., 2002). Flavonoids also can inhibit the pathways of the cyclooxygenase and/or lipoxygenase (Robak et al., 1998) and protein kinase C and/or L-arginine/nitric oxide (Meotti et al., 2005). These pathways have been implicated with molecular events related to the nociceptive (Machelska et al., 1997) and inflammatory (Kim et al., 2004) processes. In addition, flavonoids have shown ability to block phospholipase A2 and phospholipase C, which are key enzymes in inflammation (Middleton et al., 2000). The ability of flavonoids to inhibit the nuclear factor-kB (NF-kB) could clarify the anti-inflammatory activity of the fractions (Nam, 2006). Interestingly, compounds like alkaloids have been shown to possess antinociceptive and anti-inflammatory activities (Barbosa-Filho et al., 2006; Miguel et al., 2002; Gutser et al., 1998; Monsef et al., 2004; Pandurangan et al., 2010). All these observations could justify the action at the peripheral and central nervous system level.

However, additional studies are necessary to establish the possible correlation between activities and chemical composition of *A. coriacea* to ensure the appropriate medicinal use of this plant.

Therefore, the two fractions from *A. coriacea* leaves showed antinociceptive and anti-inflammatory effects as demonstrated by well established methods, suggesting a potential alternative for therapeutic purposes and supporting the use of this plant in folk medicine. However, further studies need to be conducted to ensure the safe use.

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