Antinociceptive and anti-inflammatory effects of *Caryocar coriaceum* Wittm fruit pulp fixed ethyl acetate extract on zymosan-induced arthritis in rats

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**Abstract**

The ethyl acetate extract from the fruit pulp of *Caryocar coriaceum* Wittm (Caryocaraceae), popularly known as pequi, has wide applications in popular medicine. Preclinical tests have demonstrated the therapeutic properties of the oil. We investigated the antinociceptive and anti-inflammatory effects of Pequi *C. coriaceum* Wittm ethyl acetate extract (PCCO) on zymosan-induced arthritis in rat knee joint. The animals were pretreated with PCCO for 7 consecutive days or with a single dose. Paw elevation time (PET), leukocyte infiltration, myeloperoxidase activity (MPO) and cytokine levels were assessed 4 h after zymosan injection. Synovial tissue was harvested for immunohistochemical analysis, edema and vascular permeability. We observed a significant decrease in PET with PCCO pretreatment. PCCO showed a significant reduction of leukocyte migration and a decrease in MPO. Decreases were observed in cytokine release in the synovial fluid and TNF-α and cyclooxygenase-1 immunostaining in synovial tissue. Edema was inhibited by treatment with all doses of PCCO. The data suggest that PCCO exerts antinociceptive and anti-inflammatory effects on arthritis in rats.

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1. Introduction

Arthritis is an inflammatory disease that affects the synovial joints. It promotes social effects in terms of financial cost, disability, and lost productivity. Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), are important mediators of the perpetuation of the disease (Feldmann et al., 1996). In addition to conventional treatment with non-steroidal anti-inflammatory drugs, disease-modifying drugs and glucocorticoids, new drugs that have fewer side-effects are desirable and continually researched. The fact is, the long-term use of these drugs has been shown to result in liver and gastrointestinal disorders. An alternative to treating the disease is the inclusion of medicinal plants. These are used to treat a wide variety of clinical diseases. Herbal formulas have been widely used for the treatment
of arthritis throughout history in China, Japan, and other Asian countries (Wang et al., 2013).

*Caryocar coriaceum* Wittm (*Caryocaraceae*) grows in dry plain areas in the northeastern region of Brazil, such as in the Araripе region in Ceará State (Matos, 2007; Oliveira et al., 2010). Fruit pulp oil extracted from pequi *C. coriaceum* Wittm (PCCO) is widely used in folk medicine for the treatment of colds and flu, rheumatism, external ulcers, muscle pain, and inflammation (Agra et al., 2007). However, few studies have demonstrated the specific effects of PCCO. Medical application for many years and popular acceptance of the efficacy of a natural product are sufficient for it to be relevant as an important natural therapy (Matos, 2007). However, studies on the possible pharmacological potential of PCCO are incipient. Few reports have been found in the literature on its anti-inflammatory effect. The PCCO literature has referred to its anti-inflammatory (Saraiva et al., 2011; Oliveira et al., 2010) and gastrotrophic (Quirino et al., 2009) effects, topical wound healing properties (Quirino et al., 2009; Oliveira et al., 2010; Batista et al., 2010), and antimicrobial activity (Costa et al., 2011; Saraiva et al., 2011). The use and observations of natural products in folk medicine for the treatment of inflammatory diseases suggest that these substances have important active constituents that can be used to develop new anti-inflammatory and analgesic drugs. Thus, we investigated the antinociceptive and anti-inflammatory effects of the fruit pulp oil extracted from pequi in a model of acute arthritis in rats. We used zymosan, a polysaccharide from the cell wall of *Saccharomyces cerevisiae* that produces acute and severe inflammation, leukocyte and lymphocyte proliferation, and antibody production (Frasnelli et al., 2005).

2. Material and methods

2.1. Botanical material

The plant material used for this study was the fruit pulp from Pequi (*C. coriaceum* Wittm). The fruit was collected in the cerrado area of the Araripе region (Barreiro Grande, Crato, CE, Brazil; 7°21′ 53.1′S, 39°28′42.6′W; 892 m altitude) in January 2009 by local residents. A voucher specimen of *C. coriaceum* Wittm was deposited in the Prisco Bezerra Herbarium of the Federal University of Ceará (no. 44523) and identified by Prof. Dra. Ligia Queiroz Matias.

2.2. Obtaining fixed ethyl acetate extract of pequi (*C. coriaceum Wittm*)

The extraction of fixed ethyl acetate extract was performed by Prof. Dr. Irwin Rose Alencar de Menezes in the Department of Biological Chemistry, Laboratory of Molecular Pharmacology and Chemistry, Regional University Cariri (URCA). The pulp of the Pequi fruit, in natura, was removed manually with the aid of a knife, separating the inner mesocarp endocarp that is the resistant portion of the fruit (thorns and almond). The pulp (104.14 g) was placed in a Soxhlet extractor in contact with ethyl acetate solvent for 3 h using a hot extraction method. At the end of the process, an oil + ethyl acetate solution was obtained. Subsequently, the solution was subjected to rotatory evaporation (Fisatom rota-evaporator), followed by heating in a water bath with controlled temperature (70 ± 2 °C) to remove residual ethyl acetate and avoid decomposition of the thermosensitive ethyl acetate extract substances. The ethyl acetate extract had a 5.17% yield.

2.3. Chemical composition analysis of pequi ethyl acetate extract

The fatty acid components were obtained as their methyl esters. A sample of 0.2 g of the ethyl acetate extract was subjected to reflux (30 min) with methanolic potassium hydroxide. The ethyl acetate extract was then converted to methyl esters by methanolysis with acid catalyst. Isolation and etherification of free and total fatty acids were performed according to well-known reported procedures (Hartman and Lago, 1973). The methyl esters of fatty acids were analyzed by gas chromatography on a Hewlett-Packard Model 5971, equipped with capillary column and FID detector. The operating conditions were oven temperature program start at 35–180 °C at a rate of 4 °C/min, then heated at a rate of 10 °C/min to 250 °C and held isothermally; injector and detector temperature of 250 and 200 °C, respectively, and H2 as a carrier gas flowing at 0.8 ml/min. The identification was carried out by co-injection of authentic compounds and retention times. Percentage area values were obtained electronically from the GC-FID response. The ethyl acetate extract was then characterized by a high content, 64.9% of unsaturated fatty acids. The two major components identified were oleic acid (55.79%) and palmitic acid (34.18%). Other constituents found were palmitoleic acid (0.27%), stearic acid (1.73%), linoleic acid (1.80%), heptadecenoic acid (5.86%) and 11-eicosenoic acid (0.37%) (Costa et al., 2011; Saraiva et al., 2011).

2.4. Animals

The experiments were performed in male Wistar rats, weighing 180–200 g (Central Animal Facility, Federal University of Ceará), with six animals per experimental group. The rats were housed in cages in a special silent room under a controlled temperature and light/dark cycle, with food and water available ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Federal University of Ceará (protocol no. 83/11) and performed in accordance with EU Directive 2010/63/EU for animal experiments. Efforts were made to reduce the number of animals and their stress throughout the experiments.

2.5. Experimental protocols

The ethyl acetate extract of the fruit pulp of pequi (PCCO) was diluted in 2% Tween 80 at concentrations and administered by oral gavage at doses of 100, 200 and 400 mg/kg, either 45 min before zymosan-induced arthritis or for 7 consecutive days, always at the same time each day. The zymosan injection was done 24 h after the last administration of PCCO.

The negative control received orally 500 μl of vehicle (saline + 2% Tween 80). These doses were chosen based on Quirino et al. (2009), in which no death and no other signs of toxicity were observed in animals treated with 2000 mg/kg PCCO. The 100% survival of the animals treated with 2000 mg/kg indicates that this extract can be considered as “no defined toxicity” according to the classification of the European Union, and its oral LD50 would be greater than this dose (Quirino et al., 2009). The rats were divided into six groups, with six animals per group. Each experimental group was subjected to the induction of knee joint inflammation (with the exception of the naïve group). The control group was given water. In three groups, PCCO was administered 45 min before zymosan-induced arthritis or for 7 consecutive days at doses of 100, 200, and 400 mg/kg. Another group was treated with the standard drug (dexamethasone) in each experimental test at a single dose 2 h before arthritis induction. The experimental groups were divided into subgroups to assess articular incapacitation and joint edema. The animals were then euthanized under general anesthesia to collect the fluid and synovial tissue to evaluate leukocyte recruitment, myeloperoxidase (MPO) activity, and cytokine release and perform immunohistochemistry. Changes in vascular permeability were evaluated by Evans Blue dye extravasation in joint tissue 6 h after zymosan injection.
2.6. Zymosan-induced arthritis in rat knee joint

The rats received an intra-articular (i.a.) injection of zymosan (1 mg/joint; 50 μl total volume), dissolved in sterile saline, into the right knee joint. The control group received only saline. Four hours after the zymosan or saline injections, the animals were anesthetized with a combination of ketamine (240 mg/kg) and xylazine (60 mg/kg) and euthanized.

2.7. Rat knee joint incapacitation test

The evaluation of articular incapacitation was performed using the articular incapacitation test according to Tonussi and Ferreira (1992), with modifications (Rocha et al., 1999). The rats were placed on a rotary drum (aluminum cylinder, 30 cm diameter × 50 cm width) that was covered with an aluminum screen of the same dimensions. The drum had a capacity of three animals and rotated at 3 rotations per minute (rpm) for 1 min. The hind paws were shod with specially designed metal gaiters that were connected to the data port of a computer with a data acquisition program (incapacitation registration system v1.0). When the rat touched the gaiter with the metal floor, a circuit closed. At the end of 1 min, the computer recorded the right paw elevation time (PET; i.e., the time the animals remained with the right paw raised without touching the floor). The PET was measured before zymosan injection (basal time) and each hour after zymosan injection until the 4th hour. Thus, an increase in the PET was interpreted as being proportional to the nociception felt by the animal. Twenty-four hours before the experiment, the animals were trained on the rotary drum and allowed a period of adaptation to the environment.

2.8. Synovial fluid collection

The animals were anesthetized (240 mg/kg ketamine and 60 mg/kg xylazine; i.p.) and exsanguinated. The skin and right knee joint ligaments were removed, and the synovial cavity was washed with two injections of 200 μl of 10 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) followed by aspiration to collect the joint exudate. This joint lavage fluid was used for cell counting, leucocyte differential counting, myeloperoxidase activity and determination of cytokine levels.

2.9. Determination of leucocyte migration in synovial fluid

For the determination of leucocyte migration to synovial fluid it was performed a total leucocyte counting and differential counting of neutrophils in the joint lavage fluid collected as described above. The total number of leucocytes was counted in a Neubauer chamber using 20 μl of articular lavage solution diluted in 380 μl Turk (1:20 dilution). A 20 μl aliquot of this preparation (Turk solution + washed articular fluid) was placed in the Neubauer chamber, and the total number of cells was counted in the four quadrants of the chamber with the aid of an optical microscope (10 × magnification). The counts are expressed as the total number of leucocytes × 10⁶/ml. The researcher determined the number of cells and was blind to the treatments.

For differential neutrophil count, aliquots of articular lavage fluid were removed and centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was stored at −80 °C for later analysis (i.e., cytokine determination), and the cell pellet was resuspended in 200 μl of a solution of PBS plus EDTA. Slides for differential counts were prepared using an aliquot of the washed joint fluid (50 μl) subjected to cytocentrifugation at 1500 rpm for 10 min. The slides were then mounted, fixed for 4 min, and stained with eosin and hematoxylin. The slides were then washed in tap water and allowed to dry. Subsequently, differential cell counts were performed in slides under an optical microscope with a 40 × oil immersion objective. A hundred cells per slide were counted. The number of neutrophils present in the joint lavage was obtained by calculating the percentage of neutrophils (differential count) and total number of cells present in the joint lavage fluid. The results are expressed as the number of neutrophils × 10⁶/ml.

2.10. Assessment of myeloperoxidase (MPO) activity

The MPO activity assay has been described previously (Bradley et al., 1982). The collected joint lavage fluid was defrosted, diluted with phosphate buffer (pH 6.0) that contained hexadecyl trimethylammonium bromide (HTAB; Sigma), and centrifuged at 12,000 × g for 2 min. Myeloperoxidase activity was measured in the supernatants according to a method described previously (Bradley et al., 1982). The results are expressed as MPO units (U) per joint (1 U of MPO is defined as the amount of enzyme responsible for the degradation of 1 μmol of hydrogen peroxide/ min at 22 °C). Each group consisted of six animals.

2.11. Determination of cytokine levels

Cytokines (TNF-α, IL-1β, IL-6 and IL-10) were measured using the multiplex cytokine Bioplex test system (Bio-Rad Laboratories, Hercules, CA, USA). This system quantifies multiple biomarkers in each well of a plate (pg/ml). Antibodies are directed against the desired biomarker. They covalently couple the magnetic beads and react with the biomarker of interest. The data were analyzed using Bio-Plex Manager 3.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

2.12. Immunohistochemical analysis of TNF-α and COX-2

The animals were anesthetized (240 mg/kg ketamine and 60 mg/kg xylazine, i.p.) and euthanized. The skin and right knee joint ligaments were dissected, and the synovial tissue was excised and fixed in 10% neutral buffered formalin for 24 h. The samples were dehydrated and paraffin-embedded. Immunohistochemistry for TNF-α and COX-2 was performed using the streptavidin–biotin peroxidase method in formalin-fixed, paraffin-embedded tissue sections (5 μm thick) mounted on poly-l-lysine-coated microscope slides. The sections were deparaffinized and rehydrated using xylene and a graded series of alcohol. After antigen retrieval with citrate buffer (pH 6.0) at 95 °C (15 min), endogenous peroxidase was blocked twice (10 min) with 3% (v/v) hydrogen peroxide and washed in PBS. To block unspecific binding the slides were incubated with goat serum during 30 min. The sections were incubated overnight at 4 °C with primary goat anti-TNFα or goat anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS plus bovine serum albumin (BSA; 5%). The slides were then incubated with biotinylated donkey anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:400 in PBS–BSA 5%. After PBS washing, the slides were incubated with streptavidin horseradish peroxidase conjugate (StrepABC complex, Santa Cruz Biotechnology, Santa Cruz, USA) for 30 min according to the manufacturer’s protocol. Immunostaining was visualized with the chromogen 3,3′-diaminobenzidine (DAB; ABC staining system, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control sections were simultaneously processed as described above but with the primary antibody replaced with 5% PBS–BSA. None of the negative controls showed immunoreactivity to TNF-α or COX-2. The slides were counterstained with Mayer’s hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene, and coverslipped. Each group consisted of six animals. Positive labeling for TNF-α and COX-2 was determined by brown staining at the level of the cytoplasm in synovial cell layers and in...
inflammatory infiltrate using a Leica microscope (Leica, Wetzlar, Germany™) coupled with DFC232 camera at 400 × magnification. The program ImageJ (NIH) was used to quantify color saturation. The quantification of the area selected in the pictures was made differentiating higher color saturation selected areas (pixels) associated staining (brown). The procedure was based on color saturation associated to positive staining for a particular marker. The limits required for defining pixels selected and unselected were previously defined (Azevedo et al., 2013; Cavalcante et al., 2013).

2.13. Analysis of knee joint swelling

Swelling of the knee joint was assessed by measuring the transverse diameter of the right knee that was injected with zymosan. A digital caliper (Digimatic Caliper, Mitutoyo Corporation, Kanagawa, Japan) was used to measure edema. The measurement was performed before zymosan administration (time zero) and every hour for 5 h thereafter. To measure the joints, the animal was carefully immobilized by the researcher, and the diameter of the knee was determined by a second researcher. Edema is expressed in millimeters. The data are expressed as the mean difference between the articulate diameters measured every hour after the zymosan injection and the diameter measured immediately before the zymosan injection (articular range diameter in mm).

2.14. Assessment of vascular permeability in the joint

The animals received an intravenous injection of Evans blue dye (25 mg/kg) dissolved in saline, 30 min before sacrifice (6 h after zymosan-induced arthritis). The knee joint and surrounding tissues from six animals per group were dissected, removed, and weighed. Evans blue dye that was present in the tissue was extracted by immersing the tissue overnight in 2 ml of formamide PA solution. The amount of dye present in the formamide solution was estimated by measuring spectrophotometric absorbance at 418 nm.

![Fig. 1. Anitnociceptive effect of PCCO pretreatment in zymosan-induced articular incapacitation. All of the groups were injected with zymosan (1 mg, 50 μl, i.a.) to induce arthritis and water (control [C], 1 ml, p.o., 45 min prior to zymosan), PCCO (100, 200, and 400 mg/kg, p.o., 45 min prior to zymosan in a single dose or 100, 200, and 400 mg/kg, p.o., once per day for 7 days prior to zymosan treatment); or dexamethasone (Dexa; 4 mg/kg, s.c., 2 h prior to zymosan). Joint disability was assessed before the zymosan injection and every hour thereafter until the 4th hour after zymosan administration. The paw elevation time (PET) was recorded in seconds. The data are expressed as the mean ± SEM of the PET in six animals per group. (A) Kinetics of articular incapacitation in a single dose of PCCO. (B) Peak of articular incapacitation in a single dose of PCCO (i.e., the highest value between the 3rd and 4th hours after the injection of zymosan); (C) Kinetics of articular incapacitation in repeated doses of PCCO. (D) Peak of articular incapacitation in repeated doses of PCCO. *p < 0.05, compared with control group (ANOVA followed by Newman–Keuls test).]
Fig. 2. Effect of PCCO pretreatment on leukocyte influx in the knee and on myeloperoxidase (MPO) activity in joint fluid. All of the groups were injected with zymosan to induce arthritis, and water; PCCO (100, 200, and 400 mg/kg, p.o., in a single dose or 100, 200, and 400 mg/kg, repeated doses), or dexamethasone. The naive group included animals that received no zymosan injection and no other treatment. The total number of leukocytes was measured 4 h after joint lavage. Myeloperoxidase activity was assessed in the supernatant of synovial fluid. The results are expressed as units of MPO/μL lavage (mean ± SEM) in six animals per group. The data are expressed as the mean ± SEM cell number in six animals per group. (2A) Total leukocytes in a single dose of PCCO. (2B) Total polymorphonuclear neutrophils in a single dose of PCCO. (2C) Total leukocytes in repeated doses of PCCO. (2D) Total polymorphonuclear neutrophils in repeated doses of PCCO. (2E) Myeloperoxidase activity in repeated doses of PCCO. *p < 0.05, compared with control group (ANOVA followed by Newman–Keuls test). #Significant difference between the control group and naive group. †Significant difference between dexamethasone group and naive group.
620 nm. The concentration was determined by comparison with a standard curve of known amounts of Evans blue dye in the extraction solution, which was assessed using the same assay. The amount of Evans blue dye (μg) was then calculated per milligrams of joint tissue (Kwan et al., 1996).

2.15. Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Values of p < 0.05 were considered statistically significant. Prism 5.0 software (GraphPad, San Diego, CA, USA) was used to perform the statistical analyses.

3. Results

3.1. Antinociceptive effect of PCCO pretreatment in zymosan-induced articular incapacitation

Pretreatment with PCCO (400 mg/kg) 45 min before the zymosan injection significantly decreased the PET at the 3rd and 4th hour (p < 0.05 and 0.001, respectively; Fig. 1A). Paw elevation time peak, corresponding to the higher value at the 3rd or 4th hour, was significantly different in the groups treated with 200 and 400 mg/kg PCCO (p < 0.05 and 0.001, respectively) compared with the control group. The group treated with dexamethasone (4 mg/kg) also exhibited a significant decrease in PET (p < 0.001) at the 3rd and 4th hours (Fig. 1B).

3.2. Antinociceptive effect of repeated PCCO pretreatment in zymosan-induced articular incapacitation

The animals were treated with PCCO (100, 200, and 400 mg/kg) for 7 consecutive days prior to the induction of knee arthritis by zymosan. The PET significantly decreased at the 4th hour (p < 0.01, 0.01, and 0.001, respectively). Dexamethasone administration (4 mg/kg, s.c.) 2 h before zymosan-induced arthritis significantly inhibited (p < 0.001) joint incapacitation at the 4th hour (Fig. 1C). PCCO treatment reduced articular incapacitation at the peak of articular incapacitation (i.e., 4 h after zymosan-induced arthritis; Fig. 1D).

3.3. Effect of PCCO pretreatment (single dose) on leukocyte influx in the knee

Leukocyte infiltration in the joint cavity was evaluated 4 h after zymosan administration. PCCO treatment (100, 200, and 400 mg/kg) 45 min before zymosan treatment significantly inhibited (p < 0.05) leukocyte influx compared with the control group (Fig. 2A). Dexamethasone (4 mg/kg, s.c.) 2 h before zymosan administration significantly inhibited (p < 0.05) leukocyte influx measured 4 h after zymosan treatment (Fig. 2A) compared with the control group. The differential cell count showed a significant decrease (p < 0.05) in polymorphonuclear cells with PCCO pretreatment (100, 200, and 400 mg/kg) and dexamethasone pretreatment (4 mg/kg, s.c.) 2 h before zymosan administration compared with the control group (Fig. 2B).

3.4. Effect of PCCO (repeated administration) on leukocyte influx in the knee

PCCO treatment (100, 200, and 400 mg/kg) for seven consecutive days prior to the induction of arthritis by zymosan significantly inhibited (p < 0.05) leukocyte influx at the 4th hour of arthritis compared with the control group (Fig. 2C). Dexamethasone administration (4 mg/kg, s.c.) 2 h before the induction of inflammation significantly inhibited (p < 0.05) leukocyte influx at the 4th hour of arthritis (Fig. 2C) compared with the control group. The differential cell count revealed a significant decrease (p < 0.05) in polymorphonuclear cells induced by PCCO treatment (100, 200, and 400 mg/kg) for seven consecutive days prior to the induction of arthritis by zymosan compared with the control group (Fig. 2D). Dexamethasone administration (4 mg/kg, s.c.) 2 h before the induction of inflammation significantly inhibited (p < 0.05) the influx of cells compared with the control group.

3.5. Effect of PCCO on myeloperoxidase (MPO) activity in joint fluid

Treatment with PCCO (100, 200, and 400 mg/kg) for seven consecutive days prior to the induction of arthritis by zymosan significantly inhibited (p < 0.05) MPO activity at the 4th hour of arthritis compared with the control group. Dexamethasone administration (4 mg/kg, s.c.) 2 h before the induction of arthritis by zymosan significantly inhibited (p < 0.05) MPO activity at the 4th hour of arthritis compared with the control group. No difference was observed between the groups treated with different doses of PCCO (Fig. 2E).

3.6. Effect of PCCO pretreatment on joint swelling

Knee joint swelling was evaluated in the injected knee joint by measuring the transverse diameter of each joint. PCCO (100 mg/kg) was administered 45 min before the induction of arthritis by zymosan. A significant reduction (p < 0.05) was observed from the 2nd hour compared with the control group. The 200 and 400 mg/kg doses of PCCO significantly reduced edema only at the 3rd hour compared with the control group. Dexamethasone administration (4 mg/kg, s.c.) 2 h before zymosan-induced arthritis significantly inhibited (p < 0.05) edema at the 2nd hour (Fig. 3A).

PCCO administered at doses of 200 and 400 mg/kg for seven consecutive days before zymosan-induced arthritis significantly reduced (p < 0.05) edema at the 2nd hour compared with the control group. PCCO at a dose of 100 mg/kg significantly reduced (p < 0.05) edema at the 3rd hour compared with the control group. Dexamethasone administration (4 mg/kg, s.c.) 2 h before the induction of inflammation significantly inhibited (p < 0.05) edema at the 2nd hour with the control group (Fig. 3B).

3.7. Effect of PCCO on vascular permeability in the knee

Cell influx in synovial fluid was accompanied by increased vascular permeability in the joint, evaluated by Evans Blue dye extravasation. The maximum effect was observed at the 6th hour after zymosan-induced arthritis (p < 0.05) compared with the control group. PCCO (100, 200, and 400 mg/kg) was administered for seven consecutive days before the zymosan injection. A significant decrease (p < 0.05) in vascular permeability was observed with the different doses of PCCO. Dexamethasone (4 mg/kg, s.c., 2 h before zymosan injection), used as a control treatment, significantly inhibited (p < 0.05) the increase in vascular permeability at the 6th hour after arthritis induction (Fig. 3C).

3.8. Effect of PCCO on cytokine release in the joint fluid

PCCO treatment (100, 200, and 400 mg/kg) once per day for seven consecutive days or dexamethasone (4 mg/kg, s.c.) 2 h before the zymosan-induced arthritis significantly reduced (p < 0.05) TNF-α and IL-1β levels in synovial fluid in the arthritic knee, as compared to the zymosan group (Fig. 4A and B, respectively). No significant difference in the levels of TNF-α and IL-1β...
was observed between the treated groups of PCCO and naive group. No significant difference in the levels of IL-6 (Fig. 4C) and IL-10 (Fig. 4D) was observed among groups.

3.9. Effect of PCCO on TNF-α and COX-2 immunoreactivity in synovial tissue

The immunohistochemical analysis of the synovium showed distinct immunological profiles between groups. The synovium in the naive group had a normal structure, with low TNF-α (Fig. 5C and D) and COX-2 (Fig. 6C and D) immunostaining. Synovial tissue in the control group (zymosan-induced arthritis) showed intense cellular infiltrate and synovial layer cells (synovial membrane) with TNF-α (Fig. 5E and F) and COX-2 (Fig. 6E and F) immunostaining. Fig. 5G and H and Fig. 6G and H show the synovial membranes in articular knees in rats treated for seven consecutive days with PCCO (200 mg/kg). In this group, we observed a reduction of cellular infiltrate with a consequent decrease in TNF-α and COX-2 immunostaining.

The immunohistochemical analysis of synovial tissue in the rats treated with dexamethasone (4 mg/kg, s.c.) 2 h before zymosan administration showed a decrease in TNF-α (Fig. 5I and J) and COX-2 (Fig. 6I and J) immunostaining.

4. Discussion

The current study showed the antinociceptive and anti-inflammatory effects of the PCCO on the arthritis induced by zymosan. Zymosan produces severe erosive synovitis and is associated with increases in vascular permeability, edema, leukocyte migration and hypernociception (Rocha et al., 1999). Originally, the zymosan-induced arthritis is used extensively in experimental studies as a phlogistic agent, causing acute or subacute inflammation in various experimental models (Yamada et al., 2013; Gondim et al., 2012; Chaves et al., 2011; Vale et al., 2006). Articular incapacitation in zymosan-induced arthritis in the rat knee is also associated with the activation of nociceptors located in periarticular structures and not in the synovium (Rocha et al., 1999).

The "C. coriaceum Wittm" is a very versatile plant with regard to their utilities (Silva Filho, 1992). However, the use of its fruit has a major influence on rural farmers' income residents because of their food, nutritional and therapeutic properties (Almeida and Silva, 1994). The extraction of “pequi pulp oil” is popularly held by cooking the kernels in water and the oil separated by decantation. Several studies have shown a significant change in the chemical composition of cooked oil compared with oil in natura. Ramos et al. (2001) noted the loss of carotenoids derived from conventional baking pequi pulp (Caryocar brasiliense Camb) averaged 30 25%, corresponding to the average loss of 12.11% on vitamin A. In this regard, the sale of ethyl acetate extract from the fruit pulp of C. coriaceum Wittm (Caryocaraceae) has advantages as it can be stored and sold in the period between harvests, having a greater demand because of its medicinal properties against inflammation, bronchitis, colds and flu.

To evaluate the antinociceptive effect of PCCO in zymosan-induced arthritis, we tested knee articular incapacitation in rats. This test was developed to study articular incapacitation, defined as the inability of the animal with experimentally induced arthritis to walk normally, measured by the time of paw suspension (Tonussi and Ferreira, 1992). In the present study, we assumed that joint incapacitation reflects hypernociception following inflammatory insult in the joint. For an additional evaluation of the antinociceptive effect of PCCO we also verified its effect upon paw mechanical hypernociception induced by carrageenan.

In zymosan-induced articular incapacitation, only the dose of 400 mg/kg of PCCO (acute treatment) decreased the PET. We also pretreated rats with PCCO at daily doses for seven consecutive days. Our group observed that single oral doses did not inhibit paw edema induced by carrageenan, but seven days of PCCO administration was
assumed to be effective. With daily injections of PCCO, the PET was decreased by all the doses of PCCO. The plantar hypernociception induced by carrageenan was also inhibited by the daily pretreatment with PCCO, showing that antinociceptive effect develops in other model [Supplementary material]. Comparisons of the single and repeated dose treatments suggest that daily doses of PCCO may lead to an accumulation of substances in the body that prevent hypernociception. When treated daily with different doses of PCCO, the rats exhibited a decrease in articular incapacitation. When treated with a single dose, only the higher doses of PCCO prevented hypernociception induced by zymosan.

The antinociceptive effect of PCCO may reflect anti-inflammatory activity through the action of fatty acids that are present in PCCO. Sales et al. (2009) reported that the Mediterranean diet reduces pain and stiffness in patients with arthritis. The Mediterranean diet is based on olive oil, which is rich in oleic acid. PCCO also consists mainly of oleic acid, and this may inhibit the hypernociception caused by zymosan in this experimental model of arthritis. Our data are consistent with the literature, which has shown that foods rich in fatty acids with the presence of palmitic acid, which is the second major constituent of PCCO, have anti-inflammatory and antinociceptive activity (Shah and Alagawadi, 2011).

Deciga-Campos et al. (2007) showed that palmitic acid analog represents the first selective inhibitors of “N-palmitoyl ethanolamine hydrolase”. These compounds are devoid of affinity for CB1 and CB2 receptors and characterized by high percentages of inhibition of N-palmitoyl ethanolamine-selective acid amidase (Vandevoorde et al., 2003). This enzyme is essential points of control in inflammation and pain. The inhibition attenuates inflammation and tissue damage (Solorzano et al., 2009).

Saraiva et al. (2011) reported that the topical use of PCCO has anti-inflammatory effects in models of ear edema induced by different irritants (e.g., croton oil, capsaicin, arachidonic acid, phenol, and histamine), indicating possible properties of PCCO in the therapeutic treatment of acute cutaneous inflammation, such as absorption and possible local action at inflammatory focus. These data corroborate the present results, and PCCO may also have systemic anti-inflammatory activity.

Articular incapacitation is directly associated with the migration of neutrophils into the joint cavity (Guerrero et al., 2008). Since the migration of leukocytes from the blood circulation to the affected tissue is critical step in the inflammatory process and neutrophils play a critical role in hyperalgesia, drugs that inhibit the migration of neutrophils to inflammatory foci may be an alternative for pain control. These substances may also be able to modify the fundamental process for certain inflammatory diseases that lead to tissue injury. Cunha et al. (2003) showed that such drugs inhibit the synthesis of or antagonize leukotriene B4 (LTB4) receptors effectively inhibit neutrophil migration, and prevent the development of hyperalgesia in inflammatory processes that are characterized by adaptive immunity and in some experimental models of arthritis. In the present study, single treatments with all of the doses of PCCO inhibited the influx of leukocytes, thus

**Fig. 4.** Effect of PCCO on cytokine release in the joint fluid. All of the groups were injected with zymosan (1 mg, 50 μl, i.a.) to induce arthritis and water (control [C], 1 ml, p.o., once per day for 7 days prior to zymosan), PCCO (100, 200, and 400 mg/kg, p.o., once per day for 7 days prior to zymosan), or dexamethasone (Dexa; 4 mg/kg, s.c., 2 h prior to zymosan). The naive group included animals that received no zymosan injection and no other treatment. The results are expressed as the mean ± SEM concentration of (A) TNF-α, (B) IL-1β, (C) IL-6, and (D) IL-10 in six animals per group. *p < 0.05, compared with control group (ANOVA followed by Newman–Keuls test). †Significant difference between the control group and naive group. **
Fig. 5. Effect of PCCO on TNF-α immunoreactivity in synovial tissue. (A, B) Negative control group (no primary antibody). (C, D) Naive group. (E, F) Control group. (G, H) The group treated with 200 mg/kg PCCO. (I, J) The group treated with 4 mg/kg dexamethasone. (K) The data are expressed as the percentage ± SEM of the labeled area in six animals per group. *p < 0.05, compared with control group (ANOVA followed by Newman–Keuls test). #Significant difference between the control group and naive group.
Fig. 6. Effect of PCCO on COX-2 immunoreactivity in synovial tissue. (A, B) Negative control group. (C, D) Naive group. (E, F) Control group. (G, H) The group treated with 200 mg/kg PCCO. (I, J) The group treated with 4 mg/kg dexamethasone. (K) The data are expressed as the percentage ± SEM of the labeled area in six animals per group. *p < 0.05, compared with control group (ANOVA followed by Newman–Keuls test). #Significant difference between the control group and naive group.
indicating an inhibitory effect of PCCO on cell migration to the inflammatory foci. The inhibition of leucocytes and neutrophils by daily doses of PCCO was more evident compared with a single administration of PCCO.

Seven days of PCCO administration more effectively inhibited neutrophil migration and hypernociception compared with treatment with a single dose, and we decided to measure cytokine levels only in the groups that received repeated doses of PCCO. We measured the levels of cytokines in synovial fluid that may affect the inflammatory response triggered by rheumatoid arthritis. TNF-α and IL-1β play a key role in promoting rheumatoid cartilage degradation and activating endothelial cells (Bevaart et al., 2010). TNF-α plays a pivotal role in the genesis of inflammatory mechanical hypernociception in rats (Cunha et al., 1992) and the nociceptive response in mice (Ribeiro et al., 2000). Previous studies have shown that drugs that inhibit the production of this cytokine, such as thalidomide (Moreira et al., 1993) and pentoxifylline have effective analgesic action because of their ability to inhibit TNF-α and IL-1β production by synovial cells (Vale et al., 2006, 2004). In a review of infliximab, an antibody against TNF-α, Smolen (2009) reported that its use rapidly reduces signs and symptoms in the treatment of rheumatoid arthritis as well as Etanercept, a soluble TNF-α receptor antagonist (Lethaby et al., 2013).

IL-6 induces the production of IL-1β in inflammatory mechanical hypernociception in rats (Cunha et al., 1992). The inhibition of IL-6 by an antibody against IL-6 was shown to inhibit TNF-α, responsible hypernociception by mechanical planting (Cunha et al., 1992) but, in the present study, we did not observe significant differences in the levels of IL-6 in the joint between the naïve group, the groups with arthritis, and the treated groups.

IL-10 has a significant antiinociceptive effect in a model of articular incapacitation induced by zymosan. This effect may be attributable to inhibition of the release of the proinflammatory cytokines IL-1β and TNF-α (Vale et al., 2003). Despite this evidence, PCCO was ineffective in inducing the release of IL-10 in the present study, suggesting that the effect of PCCO is not attributable to the increased production of this cytokine in joint fluid.

Since palmitoylethanolamide (PEA), a palmitic acid derivative, has been proposed to be cannabinoid CB2 receptor agonist, we could suggest that this may have a relation with analgesic and anti-inflammatory effect. Actually, it is accepted that some of its pharmacological actions are reversed in the presence of a CB2 antagonist (Lambert and Di Marzo, 1999). Calignano et al. (1998) showed that PEA exhibits anti-inflammatory and analgesic effects, both suppressed by the administration of the CB2 cannabinoid receptor antagonist SR144528.

The immunohistochemical aspects of synovial tissue in the different groups showed distinct immunological profiles in the present study. Synovial membranes in the naïve group presented a normal structure, with the minimal presence of cellular infiltrate and low TNF-α and COX-2 immunoreactivity. Synovial membranes in the arthritic group presented intense cellular infiltrate, with marked TNF-α and COX-2 expression. Seven days of PCCO treatment (200 mg/kg) decreased cellular infiltrate, with a consequent decrease in TNF-α and COX-2 expression. Cyclooxygenase is an enzyme that performs the first step in prostaglandin synthesis from arachidonic acid, thus contributing to the inflammatory process. Prostaglandins are important proinflammatory mediators. During the initial phase of inflammation, prostaglandin E2 (PGF2α) is the main product of COX (Kapoor et al., 2005).

As we have found with PCCO, the anti-inflammatory effect of palmitic acid has been shown by the decrease in COX-2 expression and neutrophil influx, as well as decrease in mast cells activation and iNOS expression. In addition to its known anti-inflammatory activity, PEA also produces analgesia (Calignano et al., 1998; Calignano et al., 2001; Jaggar et al., 1998).

The present results demonstrated that an intra-articular injection of zymosan induced characteristic inflammation, with hypernociception and an increase in vascular permeability leading to edema, confirming previous studies (Rocha et al., 2003; Kanashiro et al., 2009, Chaves et al., 2011). We found that PCCO prevented joint edema as well as the increase in vascular permeability, similarly to dexamethasone. The PCCO treatment prevented edema and plasma extravasation. However, the inhibition of edema by PCCO in zymosan-induced arthritis was more pronounced with seven days of PCCO administration. The present data are consistent with the results reported by Saraiva et al. (2011), who used various inflammatory agents and found a significant anti-edematogenic effect of PCCO on mouse ear edema, indicating that topical application may be useful for the treatment of inflammatory skin diseases.

In conclusion, the present study found that PCCO had nociceptive and anti-inflammatory effects on arthritis induced by zymosan in rat knees, suggesting its possible application for the treatment of inflammatory joint diseases. The exact mechanism of its action is still under debate.

Acknowledgments

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing support for this research and Maria Silvandira França Pinheiro and Sorcoro Pinheiro França for technical assistance.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.08.017.

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