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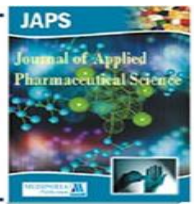

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Topical anti-inflammatory action of *Caryocar villosum* oil (Aubl) Pers.

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ABSTRACT

The piquia (*Caryocar villosum* (Aubl.) Pers.) has drawn the attention of the scientific community for its potential as an oilseed crop species. The aim of this study was to evaluate *in vivo* the topical anti-inflammatory activity of fixed oil from *Caryocar villosum* (Aubl.) Pers. – OCV. To evaluate anti-inflammatory activity, the following assays were used in rats: a) granuloma assay (cotton-pellet), b) carrageenan-induced paw edema and c) vascular permeability assay. Gas chromatography analysis from the OCV, transesterified with a BF3-methanol sample, showed the following fatty acid esters to be major compounds of this species: methyl hexadecanoate (32%), methyl octadecanoate (29%) and methyl (E)-octadecanoate (29%). The ED_{50} calculated from the granuloma assay was 531 mg/kg. In the granuloma assay, rat model of carrageenan-induced paw edema and vascular permeability of histamine test, groups treated with a topical dose of 531 mg/kg OCV showed significant differences ($p < 0.05$, analysis of variance (ANOVA) followed by a multiple comparison Student-Newman-Keuls test) when compared to the control groups treated with distilled water. Thus, a 531 mg/kg dose of OCV elicited a topical anti-inflammatory effect in rats, and the fatty acid esters identified in the fixed oil from *Caryocar villosum* participate in the detected topical anti-inflammatory activity because compounds with this characteristic are capable of modulating acute and chronic inflammatory responses.

Key words: *Caryocar villosum* (Aubl.) Pers., fixed oil, *in vivo* test, inflammation.

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INTRODUCTION

Some vegetal species have attracted the attention of researchers in several areas, such as pharmacology, biochemistry and phytochemistry. Because these species are characterized by the production of chemical compounds, such as fixed and essential oils, they are of great interest to the food, pharmaceutical, cosmetic and chemical industries (Alencar & Magalhães, 1979, Machado et al, 1995, & Max et al, 2004). Native plants, like the piquiá, have become important because of their potential to be an oilseed crop species. The oil is extracted from the fruit pulp, and is used for culinary purposes, by the cosmetic industry, in soap production, and as a medicinal product that helps prevent bronchitis, flu and colds, among others. The information supporting OCV as a drug is empirical. The fruit pulp has a high content of provitamin A, with an average of 200.000 UI, which would confer some beneficial effects on human health (Peixoto, 1973). The oil is traditionally used to relieve muscle pains and rheumatism (Clay et al, 2000). According to Galuppo et al.(2005), the presence of organic compounds, such as steroids, triterpenoids and others, characterize piquiá oil as a phytotherapeutic (medicinal compounds derived exclusively from plants) product. The high level of oleic acid enhances the fruit oil as an edible oil. The fruit is highly nutritive and is used to prepare juices and liquors. Europeans use the seeds under the name

of "souari", "sowari" or even "butternut". In addition, the seeds are used in the Guyanas for the manufacture of soaps. The wood is also resistant and durable, which permits is use in naval construction (boats) and civil construction (stakes and poles) (Clay et al, 2000).

The piquia tree is from primary forest and can reach 40 to 50 m in height, and it belongs to the Caryocaraceae family, highlighted by the *Caryocar villosum* (Aubl) Pers. (Piquiá) species (Clay et al, 2000). This plant is from the order Theales, which is composed of 25 species grouped in two genera, Caryocar and Anthodiscus. According to the study by Franco et al, 2004, the genus Caryocar has 16 species with 12 found in the Brazilian territory. Because of the wide spread use of piquiá tree oil (OCV), the aim of this work was to evaluate the topical anti-inflammatory action of OCV *in vivo*.

MATERIALS AND METHODS

Collecting botanical material

The botanical material was collected at the Tartarugal Grande, situated 17 km from the city of Tartarugalzinho, with coordinates N 01º26'06.6" (latitude) and 050º55'34.3" W (longitude), in Amapá state, Brazil.

The botanical material was collected in a fertile condition, with the presence of flowers or fruits, through standard botanical techniques. Once collected, the botanical material underwent a drying period of 48 hours in a wooden incubator. Subsequently, exsiccates were prepared and taken to the curator of the Amapá Herbarium (HAMAB) from the Institute of Scientific and Technological Research of the Amapá state (IEPA), Brazil, who received the following registration number: 7555, 5027, 14521, and 12702.

Acquisition of fixed oil from the fruits of *Caryocar villosum* (Aubl.) Pers.

The extraction of fixed oil from the fruits of *Caryocar villosum* was done by the solid-liquid extraction method using Soxhlet and hexane as an extraction solvent. Remaining extraction solvent was eliminated by boiling in a rotary evaporator.

Chromatographic analysis of OCV

To determine the components of OCV, gas chromatography coupled to mass spectrometry (GC-MS) was used, following the method described by Faria et al, 2011. The gas chromatographer (GC-MS), Thermo Scientific® model DSQ II®, was coupled to the Thermo Scientific® mass spectrometer equipped with a capillary column of fused silica (30 m x 0.25 mm i.d.) with a stationary phase DB5-ms (0.25 µm of film thickness), helium carrier gas adjusted to provide a linear velocity of 32 cm/s (measured at 100°C), an injector temperature at 240°C and a source temperature and other parts set at 200°C. The injector was of the following type: splitless flow, 0.1 µL of a solution of OCV transesterified with BF3-methanol; programmed temperature of 100°C-300°C with a gradient of 5°C/min. The quadrupole filter scanned from 39 to 550 daltons once every second. The ionization was acquired by the electron impact technique with an energy of 70 eV.

Each chemical component was identified by the comparison of its mass spectra (molecular mass and the fragmentation pattern) with existing spectra from the study by Adams and with spectra assessed by the equipment data bank (Wiley Registry of Mass Spectral Data - NIST).

Evaluation of anti-inflammatory activity

Animals

The initial project was submitted for approval by the Ethics in Research Committee from the Federal University of Amapá (Protocol 006A/2009), and all of the procedures were performed following the ethical guidelines from the International Council for Laboratory Animal Science and were in accordance with the national regulations on animal experimentation.

An albino strain of male Wistar rats with a body weight of 198 ± 20 g were obtained from the Multidisciplinary Center for Biological Research at the State University of Campinas. The rats underwent an adaptation period for a minimum of 7 days in polyethylene cages that were stacked in a ventilated rack system with controlled temperature (25°C ± 2°C), light/dark cycles of 12 h and *ad libitum* access to standardized food (Labina®) and water. One day prior to experimentation, the animals were deprived of food, but open access to water remained. At the end of the experiments, the animals were sacrificed in a CO_2 chamber.

Granuloma assay

The rats were separated into five treatment groups (5 rats/group). To initiate the granuloma assay implantation procedure, rats were maintained in aseptic conditions and were anesthetized by an intraperitoneal injection of sodium thiopental (45 mg/kg, Cristália Co., Brazil). A ventral longitudinal incision was then made on each animal, and the subcutaneous tissue was spread. Next, four hydrophilic 40 mg white dental cotton pellets (Johnson & Johnson) were implanted at equidistant points from the incision. Prior to implantation, the cotton pellets were sterilized in an autoclave in batches of four units, weighing 160 ± 3 mg, and they were immediately treated with a 5% aqueous solution of ampicillin before the implantation, according to method described by Meier (1950) and Niemegeers (1975).

The following drugs were administered 2 h following pellet implantation: topical OCV (100, 200, and 500 mg/kg), topical dexamethasone (positive control, 5 mg/kg) and orally administered distilled water (negative control group, 0.5 mL). The treatment was performed regularly each day for 6 days. On the seventh day, the animals were sacrificed in a CO_2 chamber. The granulomas were removed by dissection and dried in an oven for 24 hours at a constant temperature of 60°C. Next, the pellets were weighed in an analytical balance, and the results were calculated as the difference between the initial and final dry weight of each pellet.

Effective dose 50 determinations (ED_{50})

The ED_{50} was determined by the granuloma assay. Groups of rats (5 rats/group) were treated with a topical administration of OCV 2 h following the implantation of inflammatory stimuli for a period of 6 days. Three doses of the test oil were administrated (100, 200 and 500 mg/kg), and the ED_{50} was determined by linear regression analysis with the Graph Pad InStat 3.0.

Carrageenan-induced paw edema in rats assay

Antiedematogenic action was evaluated by carrageenan-induced paw edema in rats, which is based on the volume variation of the back paw of the animals after the application of an edematogenic stimulus (carrageenan). The amount of edema was measured with a digital caliper as described by Favacho et al, 2011. Thus, the different experimental groups (5 rats/group) were treated topically with OCV (531 mg/kg), diclofenac potassium (positive control, 10 mg/kg) or orally administered distilled water (negative control, 0.5 mL). To induce edema, a 0.1-mL subplantar injection of 1% carrageenan (Carrageenan iota, Sigma) was applied to the posterior right paw of each rat thirty minutes after treatment with OCV, distilled water or diclofenac potassium. An equal volume of saline solution was injected to the posterior left paw, as a control. The volumes of each paw from the animals were measured before the application of the pro-inflammatory substance and hourly for up to 6 h after application of the edematogenic stimulus. A digital caliper (Zaas Precision) was used to measure the edema produced in each paw. The amount of edema was calculated by subtracting the measured volume of the paw injected with saline from the measured volume of the paw injected with carrageenan.

Vascular permeability assay

The method used to assess vascular permeability in this study was based upon the spectrophotometric quantification of the amount of Evans blue dye (Evans blue, 158-21 Nacalai Tec. Co, Kyoto, Japan) that is leaked into the interstitial space after treatment with an inflammatory mediator (histamine).

The various animal groups (5 rats/group) were shaved and topically treated with either OCV (531 mg/kg), distilled water (negative control, 0.5 mL) or diclofenac potassium (positive control, 10 mg/kg). After 1 hour, these animals received an intravenous injection of Evans blue dye (25 mg/kg), and 10 minutes later the histamine was intradermally administered (Histamine 50 µg, SIGMA H-7125). Each animal received four 0.1 ml injections in various, as previously described by Carvalho et al, 1999. Thirty minutes after the last injection, the animals were sacrificed in a CO_2 exposure, and the skin patches were removed from areas close to the injection sites by a punch biopsy 10 mm in diameter. Then, the skin patches were placed in test tubes that contained 3 mL of formamide (Merck 98%) and maintained in an oven with at a constant temperature of 37°C for 24 hours. After this incubation, the material was filtered and centrifuged (2500 rpm) for 10 minutes. The absorbance of Evans blue dye was measured with a digital spectrophotometer at a wavelength of 620 nm. The concentration of the dye was calculated by the optical density (OD) value multiplied by a factor that was obtained from a standard curve.

Statistical analysis

The InStat software version 3.0 was used for statistical analysis. The analysis of variance (ANOVA) test was used to calculate the statistically significant differences between groups, and was followed by the Student-Newman-Keuls multiple comparison test and linear regression analysis. Results obtaining a p-value of $p < 0.05$ were considered statistically significant.

RESULTS

Obtaining fixed oil from the fruits of *Caryocar villosum* (Aubl.) Pers.

Fixed oil (21.6 g) was obtained from 30.15 g of piquiá (*C. villosum*) fruit pulp through a liquid/solid extraction process by using soxhlet and hexane as an extractor liquid. The resulting yield was 72.06%. The fixed oil that was extracted from the pulp fruits showed a density corresponding to 1.42 g/L by the pycnometer method at a temperature of 25°C. The oil viscosity of the oil was 13.53 McP.

Chromatographic analysis of OCV

The gas chromatographic analysis from the samples of OCV transesterified with BF3-methanol, was composed of the following compounds: methyl hexadecanoate (32%), methyl octadecanoate (29%) and methyl (E)-octadecanoate (29%) (Figure 1 and Table 1).

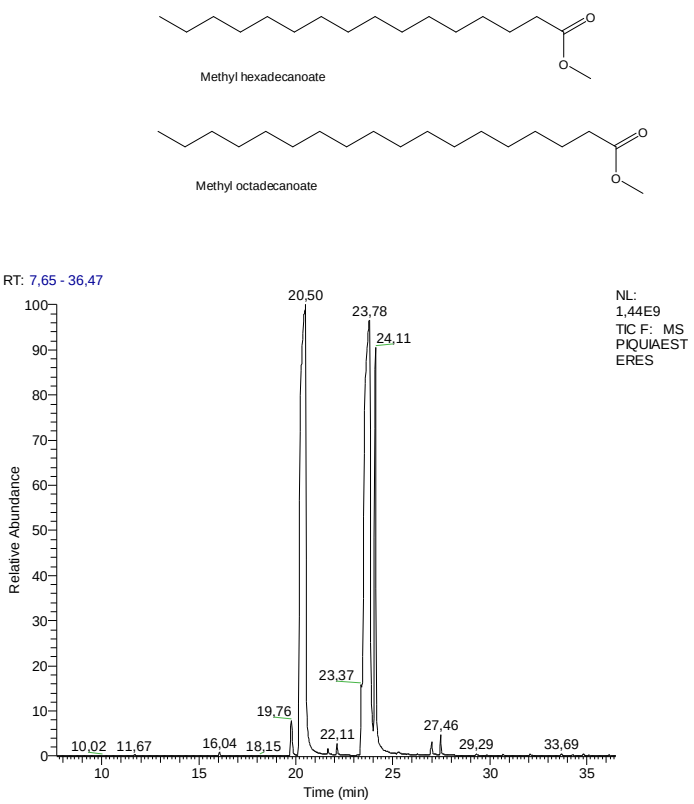

Fig 1. Chromatogram from the fixed oil of *Caryocar villosum* transesterified with BF3-Methanol, obtained by gas chromatography coupled to mass spectrometry.

Table 1. Compounds identified in the fixed oil of *Caryocar villosum* transesterified with BF3-Methanol, obtained by gas chromatography coupled to mass spectrometry

Retention Time (RT)	Fatty acid esters	%
11.67	Methyl dodecanoate	0.08
16.04	Methyl tetradecanoate	0.3
19.76	Methyl (*Z*)-9-hexadecenoate	2.53
20.50	Methyl hexadecanoate	32.17
22.11	Methyl heptadecanoate	0.83
23.37	Methyl (*Z,Z*)-9,12-octadecadienoate (methyl linoleate)	2.0
23.78	Methyl (*E*)-octadecenoate	29.03
24.11	Methyl octadecanoate	29.13
27.00	Methyl 11-eicosenoate	0.95
27.46	Methyl eicosanoate	1.47
29.29	Heptacosane	0.11
30.68	Methyl decosanoate	0.11
32.06	Octacosane	0.11
33.69	Methyl tetraconsanoate	0.15

Granuloma assay

Pellet implants in the subcutaneous region of animals led to the production of granulomatous tissue in all groups by the end of the seventh day with the control group (distilled water treated) being the most severe. The topical administration of OCV in different groups of rats ranging from 100, 200 and 500 mg/kg for 6 days led to a dose dependent inhibition of granulomatous tissue formation. This inhibition reached 43% in rats treated with OCV at 500 mg/kg and 55% with 0.5 mg/kg dexamethasone (Figure 2).

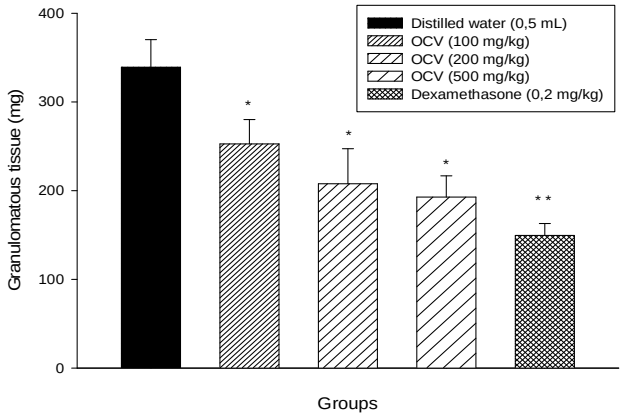

Fig 2. The effect of the topical administration of *Caryocar villosum* fixed oil (OCV 100, 200 and 500 mg/kg), distilled water (0.5 mL) and dexamethasone (0.2 mg/kg) for six days on granulomatous tissue formation. The bars show the mean ± SE of 5 animals/group, *p < 0.05, **p < 0.01, (Student-Newman-Keuls multiple comparison test).

Effective dose 50 (ED_{50})

There was a dose dependent anti-inflammatory effect of OCV administration (100, 200 and 500 mg/kg) in the rat granuloma assay with a Pierce correlation coefficient of r = 0.8409 and an ED_{50} of 531 mg/kg (Figure 3).

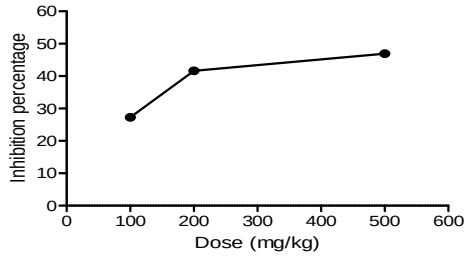

Fig 3. The effect of topical administration of *Caryocar villosum* (100, 200 and 500 mg/kg) fixed oil for six days on granulomatous tissue formation in rats. The points express the mean of n = 5 animals/group. R = 0.8409, Y = - 49.66 + 8.88X. ED_{50} = 531 mg/kg.

Carrageenan-induced paw edema in rat

The administration of carrageenan (0.1 mL at 1%) produced a visible edema in the paw of the rats that was measurable one hour after induction with maximal peak of after four hours. OCV topical treatment (531 mg/kg) 30 minutes prior to the application of the edematogenic stimulus (carrageenan) antagonized the formation of edema during the 6 hour experiment and reached a maximum peak of edema after four hours at which the inhibition was 30% (p < 0.001). The group treated with topical diclofenac potassium at 10 mg/kg had a 46% inhibition of the maximal peak of edema (p < 0.001). Both results were significantly different from the control (orally administered distilled water, 0.5 mL) (p < 0.05)(Figure 4).

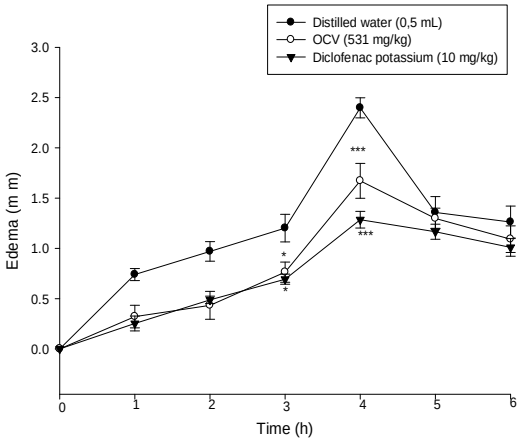

Fig 4. Effect of the topical administration of *Caryocar villosum* fixed oil (OCV 531 mg/kg), diclofenac potassium (10 mg/kg) and distilled water (0.5 mL) on rat paw edema that was induced by intraplantar injection of carrageenan (1000 µg/paw). The points express the mean ± SE of 5 animals (*p < 0.05, ***p < 0.001, Student-Newman-Keuls multiple comparison test).

Vascular permeability assay in rats

The administration of histamine in the control group (orally administered distilled water 0.5 mL) led to an increase in vascular permeability, detected by the leaking of Evans blue dye. The topical treatment with OCV (531 mg/kg) one hour prior to the administration of inflammatory mediators, was able to antagonize the response to histamine by 40% (p < 0.001). The group treated with diclofenac potassium at 10 mg/kg was able to inhibit histamine response by 57% (p < 0.001). Both responses were

statistically significant when compared to the control group ($p < 0.05$, Student-Newman-Keuls multiple comparison test) (Figure 5).

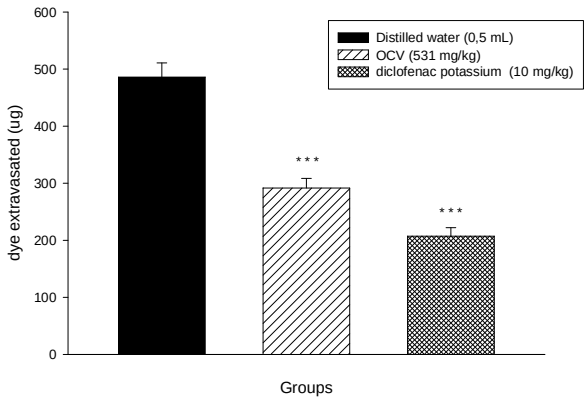

Fig 5. Effect of the topical administration of *Caryocar villosum* fixed oil (OCV 531 mg/kg), diclofenac potassium (10 mg/kg) and distilled water (0.5 mL) on vascular permeability induced by histamine. The bars show the mean ± SE of 5 animals/group (***$p < 0.001$, Student-Newman-Keuls multiple comparison test).

DISCUSSION

It is known that naturally derived drugs account for 30% of all drugs that are available for therapy. Recent results from pharmaceutical companies have shown in some complex diseases that these natural products represent an extremely valuable source for the production of new chemicals because they are composed of specialized chemical structures that have been selected for by millions of years of evolution (Calixto, 2005). Among these rich sources of new chemicals, is the family Caryocaraceae. Numerous members compose this family from the vegetal kingdom, and they have a broad range of activity, including the genus *Caryocar*. Many C14 to C24 carboxylic acids were identified in the *Caryocar villosum* fixed oil transesterified with BF3-methanol. Among these, the following compounds of the highest abundance were identified: methyl hexadecanoate (32%), methyl octadecanoate (29%) and methyl (*E*)-octadecenoate (29%). Several models of inflammation from different animal species have been used to evaluate the potential anti-inflammatory activity of various compounds and their mechanisms of action. The diversity of these models is due to differences in the etiology and clinical manifestations of inflammatory reactions. Thus, there has been a great need to generate models that reproduce the basic characteristics of each type of inflammation (Lenz, 2009). The model of granulomatous tissue induction described by Swingle & Shideman suggests that there are three phases after pellet implantation. In this study, the action was due to the proliferative phase beginning at 72 hours post-implantation and lasting until the sixth day (Swingle & Shideman, 1972). The rat granuloma assay is a commonly used and reliable method for determining the anti-inflammatory action of different agents because the implanted cotton pellets interfere with the proliferative components of the inflammatory process (Bailey et al, 1982).

The common steps of granuloma formation at the site of pellet implantation initially involve fluid accumulation and protein material as well as neutrophil infiltration. After six days, the granuloma is characterized by the formation of a vascularized fibrous capsule that contains fibroblasts and infiltrating mononuclear cells (Bailey et al, 1982). According to Dalmora and Oliveira (1999), the smaller the fibrous capsule the more intense the anti-inflammatory effect of the drug. Indeed, this is what happened in granulomas taken from rats treated with OCV and diclofenac potassium. The results from the granuloma assay in this study showed a 43% ($p < 0.01$) and a 55% reduction of inflammation compared to negative controls (distilled water) when rats were treated with 531 mg/kg OCV and dexamethasone, respectively. Dexamethasone was used in this assay because it is a corticosteroid that controls the rate of protein synthesis. The main effect of this drug is a profound change in the lymphocyte immune response that is due to anti-inflammatory and immunosuppressant action, which can prevent or suppress several types of inflammatory processes (Bailey et al, 1982). The inhibition of granulomatous tissue formation suggests that the active ingredients in OCV produce an action that is similar dexamethasone and may be an alternative anti-inflammatory medication. According to Bjorkman (1996) and Wallace (1997), there is no ideal anti-inflammatory therapeutic that has a robust effect on inflammatory disorders with minimal side effects. Furthermore, topical treatment with OCV (531 mg/kg) 30 minutes prior to the application of edematogenic stimulus (carrageenan), significantly inhibited ($p < 0.001$) the formation of edema during the six hour experiment with a maximum peak of inhibition at 30% after four hours, which was similar to the 46% inhibition in the group treated with diclofenac potassium (10 mg/kg).

The model of inflammation response induced by the injection of carrageenan in the paw of rats was introduced in 1962 (Winter et al, 1962). Since then, this model has been widely used to screen for compounds that have anti-inflammatory potential (Arya & Kumar, 2005, Crunkhorn & Meacock, 1971, Posadas et al, 2004, Vinegar et al, 1969). Research with experimental models inducing inflammation in rodents, such as the model of carrageenan-Induced paw edema, allows for an acute determination of the dose-response of a particular drug. In addition, *in vivo* models of non-steroidal anti-inflammatories allow for the determination of the activity profile of anti-inflammatory drugs (Giraidel et al, 2005). This model allows for the identification of drugs with anti-inflammatory activity. For example, the study of Carvalho et al (1999). observed a 77% inhibition of paw edema 3-4 hours following the administration of the phlogiston agent (carrageenan). The treatment of rats with topical OCV (531 mg/kg) led to a significantly antagonized ($p < 0.001$) response to histamine of 40%, which was comparable to the 57% inhibition in the group treated with diclofenac potassium (10 mg/kg).

Binny et al (2010) claimed that increased vascular permeability is due to the shrinkage of endothelial cells that results in the appearance of inter-endothelial spaces, which then allows protein macromolecules to leak into the injured interstice. Thus, most models of vascular permeability in rats rely on the measurement of extravasated proteins. Several studies have used this model to evaluate drugs with anti-inflammatory activity. For

example, Faria et al (2011) demonstrated that *Rosmarinus officinalis*, which is able to antagonize the inflammatory response to histamine, was able to reduce vascular permeability by 50%. To assess dye leakage, intradermal injections of histamine were used, which is important for the initiation of the primary events of acute inflammation. This method ensures the measurement of the monophasic response of increased vascular permeability. In order to have an effect, histamine must bind to the H_1 receptors that are present in endothelial cells. The binding of histamine to these receptors results in endothelial cell contraction that produces intercellular spaces from which the plasma proteins can cross into the interstitial space (Binny et al, 2010). Wallace observed that topically administrated non-steroidal anti-inflammatories have been used for decades to relieve the pain of musculoskeletal tissues (Wallace, 1997). This administration route probably reduces adverse effects by maximizing the local effect and minimizing systemic toxicity. The major problem has been the penetration into the target-tissue and, therefore, the clinical efficacy. The identification of methyl hexadecanoate (32%), methyl octadecanoate (29%) and methyl (*E*)-octadecanoate (29%) fatty acid esters, in the fixed oil from *Caryocar villosum* transesterified with BF3-methanol, may explain the topical anti-inflammatory effects found several of the experiments. The antinociceptive and anti-inflammatory activities of these compounds were studied by Lima et al (2005) using standard in vivo preclinical tests in mice and rats. The study by Carvalho (2011) reported that compounds resembling arachidonic acid metabolites, mainly prostaglandin E_2, are important to the physiological maintenance of the cutaneous system because this prostaglandin is involved in the homeostasis of epidermal and dermal cells. Thus, these compounds would be able to modulate the acute and chronic inflammatory response.

CONCLUSIONS

The fatty acid esters identified in the fixed oil from *Caryocar villosum* are related to the detected topical anti-inflammatory activity because compounds with this characteristic are capable of modulating acute and chronic inflammatory responses.

ACKNOWLEDGE

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