Original Article

Study of high dilutions of copaiba oil on inflammatory process

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ABSTRACT

The discovery of new drugs has led to a need to develop techniques to control the occurrence of toxic and collateral effects. This has enabled the advancement of homeopathic therapeutics as it presents major advantages against these effects. This study was designed to explore the effects of high dilutions of *Copaifera* (copaiba oil) on inflammation. This study considered the way the high dilutions were obtained (triturated form or mother-tincture-MT). The preparations were administered orally. The effects of the dilutions were tested using the rat paw edema induced by carrageenan; granuloumatous tissue induction and the edema induced by Croton oil. The high dilutions of copaiba oil obtained from both trituration and MT produced a statistically significant inhibitory effect of the carrageenan edematogenic process compared to control. The maximum effect was observed with dilution 30cH, with inhibition of edema by 73%, whereas indomethacin was 55%. Subcutaneous implantation of cotton pellets have induced a granulomatous tissue, evaluated 7 days after implantation. Daily treatment with dexamethasone produced 53% inhibition on the formation of granulomatous tissue. The 6cH dilution of copaiba oil inhibited in a statistically significant way the formation of granulomatous tissue compared to the control (18% and 16%, respectively). Edema in Croton-oil induced dermatitis was intense. Groups treated with dexamethasone and dilutions of copaiba oil presented similar responses, with inhibition by 57% and 48% respectively. Based on the results obtained in this study, it may be suggest that the Copaiba oil high dilutions possess an anti-inflammatory property supporting its use in the treatment of inflammatory disorders.

Keywords: *Copaifera langsdorffi*; Copaifera; Copaiba; Homeopathic dilutions; anti-inflammatory, dermatitis.

Introduction

The increasing discovery of new drugs and the introduction of new medications in the pharmaceutical market have led to a need to develop techniques to better control the occurrence of toxic and collateral effects of these products. This has enabled the advancement of homeopathic therapeutics as it presents major advantages against the occurrence of these effects, particularly when analyzed against specific therapeutic groups, as is the case with anti-inflammatories [1].

This study was designed to explore the possibility of testing, in a controlled way, the effects of high dilutions of *Copaifera* (copaiba oil) on three classical models of acute and chronic inflammation in rats. Thus, the study considered the way in which the high dilutions were obtained, from a triturated form and from a mother-solution. The preparations were administered by the oral route, following the methods recommended in the classical pharmacology.

Copaiba trees are native to the tropical region of Latin America, and also grow in West Africa. Copaiba oil was introduced to Europe as a drug against blennorrhagia in the 17th century. It has been listed in the London Pharmacopoeia of 1677, and in all editions of the United States Pharmacopoeia from 1820 to 1940, when it was

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admitted to the National Formulary. Nowadays, it is one of the medications most commonly used by the population of the Brazilian Amazon as an anti-inflammatory, anti-septic and cicatrizing agent, which has no access to pharmaceutical products or health services [1,2]. Among the medicinal properties of copaiba oil, the most studied are its anti-inflammatory effects [3, 4].

Other activities have been described for copaiba oil. Studies carried out by Brito et al., [5] with commercial copaiba oil in rats demonstrate the occurrence of diarrhea, weight loss and irritation in the rat behavior, at doses of 0.63 mL/kg.

Literature describes several pharmacological effects of substances detected or isolated from copaiba oils. Several sesquiterpenes compounds have been isolated or detected in copaiba oils. Among the sesquiterpenes, some properties such as gastric antiulcer [6], antiviral (including antirhinovirus) [7] activities have been described for α-curcumen and bisabolene. The latter has also been described as an abortive [8]. This compound has been described as an anticancer (cervical) [9] and caryophyllene and cadinene as anticariogenics [10]. The latter has been also described as a bactericide (MIC 800 ug/ml).

Among these, however, those which have been most studied and been proven active in a higher number of trials are caryophyllene and its oxide. Cariophyllene has been described in the literature as: anti-edematogenic [11], phago-repellent [12], antiinflammatory, antitumoral [13], bactericide, insectifuge [14] and antiallergic [15].

Carrageenan is a polysaccharide extracted from algae which induces a measurable local inflammatory response. It is the most commonly used paw edema model for evaluating the anti-inflammatory effect of drugs, generally quantified using a plethysmometer. It presents two inflammatory phases and a third uncharacteristic one. In the first hour after the injection of the carrageenan, there is an increase in vascular permeability mediated by histamine and serotonin. In the second hour, the increase in permeability is caused by kinines. In the third hour, the increase in vascular permeability occurs due to the action of the prostaglandins [16].

In the granuloma test, Repetto and Lesuy [17] demonstrated the existence of three phases after the implantation of the pellets. The first phase, which covers the first three hours after implantation, has been known as the transudative phase; the second, from three to 72 hours, is the exudative phase; and the third, from 72 hours to the sixth day, the proliferative phase. Chronic inflammation appears when the inflammatory agent is not overcome, and is characterized by the presence of proliferative elements in the inflamed area, such as lymphocytes, plasmocytes, mast cells, monocytes and histiocytes. In addition, fibroblasts, blood capillaries and other repair tissues also proliferate at the site [18].

The granuloma test represents a chronic inflammatory model, in which the development process may be inhibited by anti-inflammatory steroid drugs. The most commonly used test to evaluate the topical anti-inflammatory activity of new drugs is the Croton oil-induced rat ear erythema test. This oil is a vascular irritant, causing leukocyte infiltration of polymorphonuclears, as well as intracellular edema and topical dermatitis. In the initial phase, there is degranulation of the mast cells; in rodents, these granules contain inflammatory mediators with a predominance of serotonin. Zibetti et al., [19] demonstrate the role of serotonin in analgesia. There is an increase in vascular permeability and leukocyte chemotaxis.

This study was designed to explore, in a controlled way, the effects of homeopathic high dilutions of copaiba oil on three known experimental models of acute and chronic inflammation in rats. The different forms of obtaining the final product were therefore considered in this study, according to the techniques of homeopathic practice. The aim of this study was to verify the anti-inflammatory effect of copaiba oil in different high dilutions.

Materials and methods

The oil of *Copaifera langsdorfii* Desf. used in this study was acquired from Brasmazon Ltd. High dilutions of this oil were prepared by Laboratório Industrial Almeida Prado Ltd., using two distinct processes. The fisrt process used the mother tincture method and the second the trituration method, according to guidelines indicated in Farmacopéia Homeopática Brasileira [20]. The high dilutions (6cH, 12cH and 30cH) were prepared by trituration with lactose, mother-tincture and using 30% hydro alcoholic solution as a control, both of them by the Hahnemannian method.

Male Wistar rats weighing between 180 – 200g and Swiss albino mice were acquired at the Biotery at University of Alfenas (UNIFENAS). The animals were kept in boxes hosting 5 animals each, placed in acclimatized cages (27°C), with light-dark periods of 12 hours each and automatically controlled humidity conditions for 24 hours prior to the experiments, with free access to water. Rats were randomly assigned to 5 experimental groups: Group 1: Copaiba oil 6cH; Group 2: Copaiba oil 12cH; Group 3: Copaiba oil 30cH; Group 4: indomethacin (10 mg/kg, p.o.); Group 5: Control (0.9% saline solution. 0.5 ml, p.o.).

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In each experiment, the tested substances were administered by oral route, at different time intervals, before the application of the inflammatory stimulus. High dilutions of copaiba oil, *Copaifera* 6cH, 12cH and 30cH were administered 3 days before induction of inflammation, at a dose of 0.5mL and 2 hours after granuloma test (insertion of cotton pellets). Treatment was maintained for 6 days 0.5 mL in one daily dose. Indomethacin was administered 10 mg/kg *per os*, just as the control which received 0.5 mL of 0.9% saline solution *per os*. The experiment was conducted double-blind, and was approved by the Research Ethics Committee of the University of Alfenas, #23A/2005.

Plantar edema was induced using 1.000 µg/paw of carrageenan (0.1 mL, Iota - carrageenan - Fluka - Biochemika) injected into the sole of the right hind paw of rats. A same volume of 0.9% saline solution was injected into the animals' left paw. Edema was measured 2 and a half hour after application, as it corresponds to its peak. [21]

Induction of granulomatous tissue followed the method described by Meier et al. [22] and Niemegeers et al. [23]. The following drugs were administered daily, by oral route: 0.5 mL of 30% hydroalcoholic solution (control group, n = 8), 0.2 mg/kg of dexamethasone (n = 8, MSD Co.), and high dilutions, *Copaifera* 6cH, 12cH and 30cH (0,5 mL/animal), starting the treatment two hours after the implantation of the pellets, up to the sixth day. On the seventh day, the animals were sacrificed. The granulomas were removed for dissection, and submitted to drying for 24 hours at a temperature of 60°C. Next, they were weighed using analytical scale (Explorer Ohaus – Marte). The weight of the granuloma was calculated by the difference between the initial dry weight and the final weight.

Croton oil-induced dermatitis

The method described by Tubaro et al. [24] was used. A cutaneous inflammation was induced by the application of 0.1 mL (1 mg/ear) of a Croton oil solution (Sigma Co.) in acetone, on the surface of the mice right ear. The same volume of acetone was applied to the left ear. Thirty minutes after application of this stimulus, the topical treatment was applied. One group (n = 8) was treated with the high dilutions, *Copaifera* 6cH, 12cH and 30cH (0.1 ml/animal), another with dexamethasone (n = 8) (2.5 µg/ear), and the control group (n = 8) received only 30% hydroalcoholic solution (0.5 ml). The anti-inflammatory response was evaluated 6h after application of the stimulus, when the rats were sacrificed and an ear sample of 8mm in diameter was removed to establish the difference in weigh between the sample of control ear (left) and the stimulated ear (right). The results obtained were expressed in weigh (mg).

Statistical analysis

Statistical analysis of results was carried out by variance analysis (ANOVA) followed by Tukey–Kramer multiple comparison test; to compare measurements, Student T test was used. Levels of significance $p < 0.05$ were considered statistically significant [25].

Results

Inhibition of plantar edema

Injection of carrageenan into the rats' paws produced edema which peaked 2.5h after application (Figure 1).

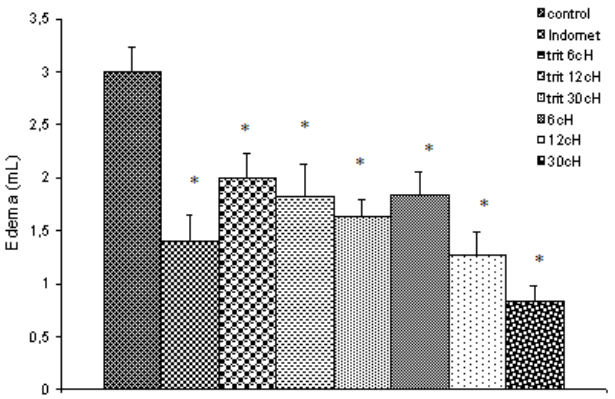

Figure 1. Effect of the administration of high dilutions of copaiba oil (p.o.), obtained by trituration (trit. 6cH, trit. 12cH, trit. 30cH), solution (6cH, 12cH, and 30cH), indomethacin (10 mg/kg) and control (EtOH 30%, 0.5mL) on carrageenan-induced rat paw edema (1000 µg/paw). The bars represent the Mean ± Standard Error of the Mean. *p < 0.05, Student t test.

Distinct treatments with high dilutions of copaiba oil obtained from both trituration and MT produced a statistically significant inhibitory effect of the edematogenic process by comparison to control. In both groups treated with high dilutions (from trituration and from MT), effects were dilution-dependent, with correlation coefficients r = 0.9983 and r = 0.9992, respectively (Figure 1). Therefore, maximum effect was observed with dilution 30cH, where inhibition of edema was 73%, whereas with indomethacin was 55%. The results did not show statistical significant differences among the treatments, but all groups were statistically significant different from the control group (Figure 1).

Induction of granulomatous tissue

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Subcutaneous implantation of cotton pellets led to the formation of granulomatous tissue, evaluated 7 days after implantation. Daily treatment with dexamethasone produced 53% inhibition on the formation of granulomatous tissue, while from high dilutions of copaiba oil, only dilution 6cH obtained by both methods - trituration and solution from MT - were able to inhibit in a statistically significant way the formation of granulomatous tissue compared to the control (18% and 16%, respectively). The treatment with dexamethasone was the most effective, differing statistically from all treatments (Figure 2).

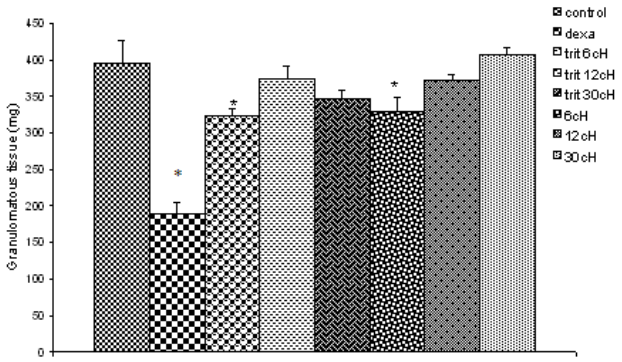

Figure 2. Effect of the administration of high dilutions of copaiba oil (p.o.), obtained by trituration (trit. 6cH, trit. 12cH, trit.. 30cH), and solution (6cH, 12cH, and 30cH), dexamethasone (0.2 mg/kg) and control (EtOH 30%, 0.5mL) for 6 days on the formation of the granulomatous tissue. The bars represent the Mean ± Standard error of the Mean. *p < 0.05, Student t test.

Croton oil-induced dermatitis

Edema in Croton-oil induced dermatitis was intense, reaching 10mg in the control group. Groups treated with dexamethasone and dilutions of copaiba oil presented similar responses, with inhibition of 57% and 48% respectively, which was not statistically significant. There was no statistically significant difference among all groups treated (Figure 3).

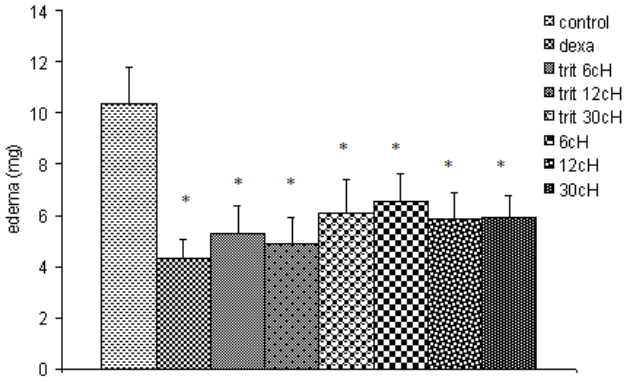

Figure 3. Effect of the administration of high dilutions of copaiba oil (p.o.), obtained by trituration (trit.. 6cH, trit. 12cH, trit. 30cH), and solution (6cH, 12cH, and 30cH), dexamethasone (2.5 µg/ear) and control (EtOH 30%, 0.5mL) on croton oil-induced rat paw edema (50 µg/paw). The bars represent the Mean ± Standard Error of the Mean. *p < 0.05, Student t test.

Discussion

The development of therapeutics able to modulate the inflammation process without suppressing the desirable effects of physiological aspects of inflammation could be an interesting alternative to obtain better efficacy of tissue response against exogenous aggression. Although inflammation is very important to homeostasis and tissue integrity, the action of proteolytic enzymes, chemical mediators and free radicals may aggravate the situation, in some cases [26].

Carrageenin-induced oedema is an experimental model widely used for the evaluation of anti-inflammatory activity of new medications. This model is used for acute responses, because the participation of mediators such as prostaglandins and kinins is intense at the third hour after the stimulus [27]. Administration of carrageenan (0.1 ml at 1%) into the rats' paws induced edema gradually, measurable from the first hour after induction and reaching its peak 2.5 hours after application. Treatment with high dilutions of copaiba oil prepared from both trituration and MT produced a statistically significant inhibitory effect of edema formation when compared to the control group. Inhibition was maximum with dilution 30cH (73%), higher significantly than indomethacine (53%). This suggests that the mechanism might involve interference on the activity of cyclooxygenase [21].

Many studies indicate that the inhibition of prostaglandin synthesis is the principal mechanism of the therapeutic actions of NSAIDS (nonsteroidal anti-inflammatory drugs). Indomethacin represents a remarkable exception,

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because it is more effective on anti-inflammatory tests than in enzyme inhibition tests. [2]

The test of erythema in mouse ears induced by croton oil is commonly used for the evaluation of new anti-inflammatory drugs [28, 29] Dermatitis induced by croton oil represents a model of acute inflammatory response. The oedema is mediated by cycloxygenase metabolism of arachidonic acid [30, 31, 32]. Regarding the Croton-oil induced dermatitis, groups treated with high dilutions of copaiba oil showed statistically significant results when compared to control ($p < 0.05$, Student "t" test), which were not statistically different from the effect of dexamethasone, a steroidal anti-inflammatory drug, obstructing the generation of these mediators.

The experimental models used in this study explore what is conventionally denoted pharmacological activity, as expressed by the presence of various signs and symptoms, such as edema. While conventional anti-inflammatory drugs are designed to overcome the enzyme mechanism involved in the inflammation process (for example the production of prostaglandins), it is proposed that homeopathic treatment regulates the pathological excess of inflammation.

Based on the results obtained in this study, we can suggest that the Copaiba oil high dilutions possess an anti-inflammatory property supporting its use in the treatment of inflammatory disorders.

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