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Anti-inflammatory and antinociceptive properties of the extracts from the leaves of *Porophyllum tagetoides* and *Annona reticulata*

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Abstract
The aqueous extracts of *Porophyllum tagetoides* and *Annona reticulata* were investigated for its anti-inflammatory and analgesic activities in animal models. The extracts at 200 and 300 mg/kg reduced significantly the formation of edema induced by carrageenan. In the acetic acid-induced writhing model, the extract showed a good analgesic effect characterized by a significant reduction in the number of writhes with two doses (100 and 200 mg/kg) used when compared to the untreated control group. Indomethacin at 10 mg/kg served as reference drug in all these tests. Moreover, *P. tagetoides* and *A. reticulata* also remarkably suppressed carrageenan-induced peritoneal leukocyte migration in rats. These results demonstrate that *P. tagetoides* and *A. reticulata* present remarkable anti-inflammatory activity, which supported its traditional use in the treatment of various diseases associated with inflammation.

Keywords: *Annona reticulata, Porophyllum tagetoides*, anti-inflammatory, paw edema, writhing

1. Introduction
*Porophyllum tagetoides (*Asteraceae) (Syn: *Porophyllum linaria)* is an annual warm-weather herb of green color that has an intense peculiar smell, it is known in Mexico as pepicha or pipicha. The tender, aromatic, pine-needle-like leaves can be eaten raw or used as a condiment. In México it is distributed in the States of Querétaro, Durango, Guanajuato, Hidalgo, Jalisco, Michoacán, México, Morelos, Puebla, Veracruz and Oaxaca. Its leaves are used in traditional medicine for the treatment of inflammatory processes and has showed antioxidant properties. It has volatile compounds, vitamin C, aldehydes, flavonoids, terpenes and polyphenolic content mainly, reason by which it is considered a powerful antioxidant remedy against many inflammatory and degenerative diseases [1-3].
Annona reticulata L., a native of tropical America, is completely naturalized and also cultivated in parts of India [4]. The bark of the plant is a powerful astringent and is given as a tonic. The plant has been used as an anti-inflammatory agent, in wound healing, and for its antianxiety, anti-stress, antimutagenic and spasmolytic effects [5]. Leaf and stem extracts show inotropic, positive chronotropic and spasmolytic activities [6,7]. In the West Indies and Central and South America, the fruit is used as an antidysenteric and anthelmintic [8]. The seed of the plant is reported to contain acetogenins, mainly cis- and trans-isomurisolenin, annoreticuin, bullatacin, squamocin and rolliniastatin [9]. The leaf and root contain mainly spathulenol, muurolene, copaene and eudesmol [10]. Reticullacinone, rolliniastatin-2 and molvizarin are reported to be present in the stem bark [11]. Dopamine, salsolinol, and coclaurine were isolated from leaves and stems [6,7]. In this study, were examined the effects antiedema, analgesic and inhibitory of cellular migration of aqueous extracts from *P. tagetoides* and *A. reticulata*.

2. Material and Methods
2.1. Animals
Adult male Wistar rats weighing between 250 and 300 g and Swiss albino mice (20-25 g) grown in our animal house, were used throughout the experiments. They were housed at 24 ± 0.5ºC with 12 h: 12 h light/dark cycle and free access to rat chow and water. Animals were randomly divided into groups of six animals each. All procedures were conducted in accordance to Institutional ethical guidelines, the Mexican Official Norm

(NOM-062-ZOO-1999) regarding technical Specifications for production, care and use of laboratory animals and the International Guide for Caring and Use of Laboratory Animals NRC 2002. The doses of the extracts and the fractions were chosen based on the yield obtained from them.

2.2 Plant Materials

The leaves of *P. tagetoides* and were obtained from Merced´s market, in Mexico City. *A. reticulata* was collected in Cerro Azul, Veracruz, México, in May of 2016. The plant´s botanical identity was verified at the Izta Herbarium of the Botanical Department of the Iztacala Faculty of Superior Studies of the National Autonomous University of Mexico (UNAM). The specimen deposited with the voucher number to *P. tagetoides* (26901) and *A. reticulata* (28914). was authenticated by Edith López Villafranco, biologist in charge of the Herbarium.

The leaves were dried under shade and then powdered with a mechanical grinder and stored in airtight container. Aqueous extract of the leaves was prepared with 100 g of powder in 1000 ml of distilled water, mixed for 30 min at 95ºC and then the aqueous extract was vacuum dried; each 100 g of dried leaves powder yielded 39 g of powder lyophilized. One part of the aqueous extract was adjusted at 60 mg/ml and adjusted to pH 7.4 for biological experiments. The other part of aqueous extract was extracted with 500 ml methanol in a Soxhlet apparatus for 8 h. Then the filtered extracts were taken to dryness at 40ºC using a rotary evaporator.

2.3. Acetic acid-induced writhing in mice

Abdominal constriction induced by intraperitoneal injection of acetic acid was carried out according to the procedures described previously by Collier *et al*. (1968) [12]. Briefly, acetic acid solution in saline (0.6% v/v, 10 ml/kg) was injected into the peritoneal cavities of mice, were placed in a large glass cylinder and the intensity of nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 20 min after acetic acid injection. Thirteen minutes before of acetic acid injection, was administered by orally, *A. reticulata* or *P. tagetoides* aqueous extract (300 mg/kg). The writhing response consists of a contraction of the abdominal muscle together with a stretching of the hind limbs. The antinociceptive activity was expressed as the writhing scores over 20 min.

2.4. Carrageenan-induced paw edema in rats

The anti-inflammatory activity of extracts was studied in rats (6 per group). Edema was induced according to the method described by Winter *et al*. (1962) [13]. Briefly, 0.1 ml of 1% carrageenan (type II, Sigma, St Louis, MO) in sterile saline solution was injected into the sub plantar tissue of the right hind paw and the left hid paw was injected 0.1 ml of saline (control). The paw volume was measured before injection of carrageenan or saline by the mercury displacement method, and the time course of edema formation was followed over 8 h. In separated groups of rats, either indomethacin (10 mg/kg) was administered as standard drug, saline solution and DMSO, and Tween 80 as control vehicles. Different doses of the extracts (200 and 300 mg/kg) from *A. reticulata* and *P. tagetoides* were administered orally 60 min before carrageenan injection. The volume increase of the inflamed paw was estimated by subtracting the volume of contralateral paw. The anti-inflammatory effect of the drugs was evaluated as the degree of edema inhibition.

2.5. Neutrophil migration into peritoneal cavity.

Experimental groups were orally treated with the aqueous extract from *A. reticulata* and *P. tagetoides* (300 mg/kg). The control groups were also given saline or DMSO by same route, and the standard reference groups were administered with dexamethasone (Merk-Sharp and Dohme; 1 mg/kg). One hour later, rat groups were injected with 3 ml of carrageenan (100 μg/ml) prepared in sterile saline solution into the peritoneal cavity; 4 h later, the rats were light ether anaesthetized and killed by cervical dislocation and the abdominal cavity was washed with phosphate buffered saline (pH 7.4) containing heparin (5 U/ml) (Sigma, St Louis, MO) and bovine serum albumin (0.1%). Briefly, 20 L peritoneal fluid was diluted 1:20 (v/v) in Turk solution for total cell counts in a Neubauer chamber. Samples of leukocytes from peritoneal lavage concentrated and resuspended in 5% bovine serum albumin. were applied to microscopy slides and the slides were then stained with Wright. Percentage of neutrophils in the samples was obtained by differential cell count [14]. The results were expressed as number cells /ml of collected fluid.

2.6. Acute toxicity of aqueous extracts.

The possible acute toxic effect of doses of 1.5, 3 and 5 g / kg of the aqueous extracts of *P. tagetoides* and *A. reticulata* was investigated by intraperitoneal administration to groups of 5 mice of strain CD-1. The mice were observed for one day to observe the appearance of signs of acute toxicity such as tremors, lack of mobility, seizures, ataxia, sedation or death [15].

2.7 Statistical Analysis

The results are presented as mean ± S.E.M. of experiments made on 6 animals per group. Differences between groups were evaluated by analysis of variance (one-way ANOVA) followed by the student´s t-test; P values < 0.05 were considered to be significant.

3. Results

Fig. 1 shows the change of rat paw volumes 4 h after of carrageenan intraplantar injection. Results indicated that indomethacin (10 mg/kg) ($p < 0.01$) inhibited the carrageenan induced edema.

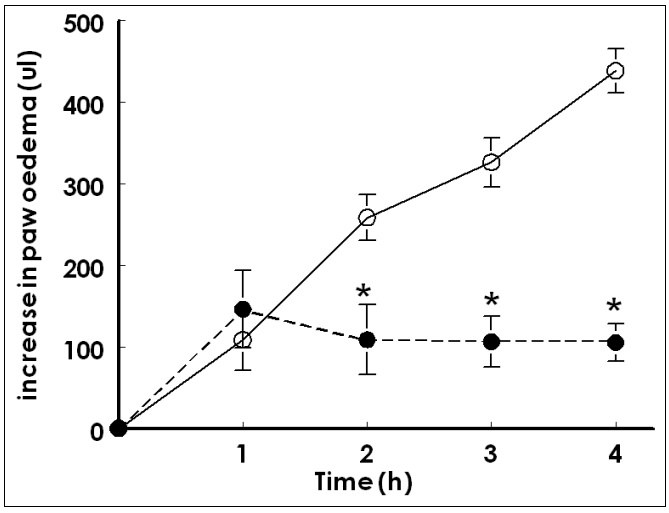

Fig 1: Effect of indomethacin (10 mg/kg) ● on paw edema volume induced by carrageenan. In all groups n = 5. * represents $p < 0.05$ compared to control (saline solution) ○.

Carrageenan-induced paw edema in the rat

The edema induced by the carrageenan injected subcutaneously into the hind paws in rats was followed for 4 h, the maximum effect was observed four hours after its injection (499 ± 55 µl) (Fig. 1). Aqueous extract of *P. tagetoides* (PTAE) (200 and 300 mg/kg) decreased volume of carrageenan - induced paw edema in the rats (383 ± 51 µl and 190 ± 63 µl), respectively) (Fig. 2). The extract aqueous of *A. reticulata* (ARAE) (200 mg/kg), also decreased the carrageenan - induced paw edema (296 ± 62 µL); indomethacin (10 mg/kg) showed better effect in decreased paw volume (87 ± 20 µl).

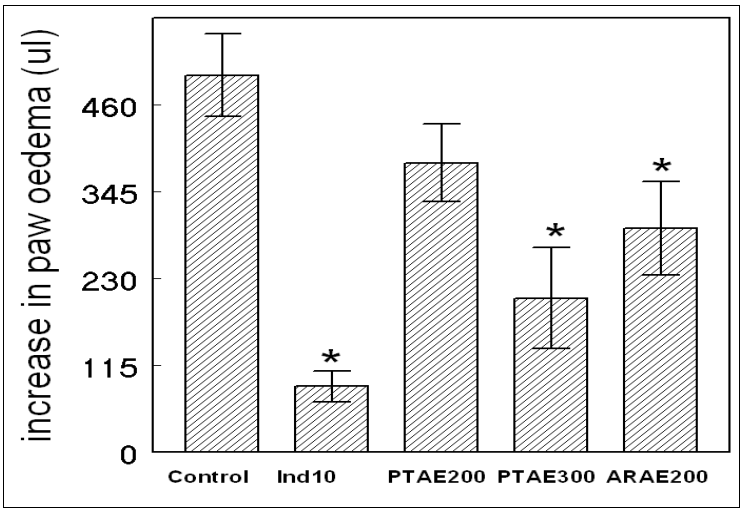

Fig 2: Effect of leaves aqueous extract of *P. tagetoides* (PTAE) and *A. reticulata* (ARAE) on carrageenan induced paw edema in rats, 4 h after intraplantar injection. Control carrageenan/saline; carrageenan/treatments: aqueous extract of *P. tagetoides* (200 and 300 mg/kg) and *A. reticulata* (200 mg/kg); indomethacin (Ind) (10 mg/kg). In all groups n = 6; * p < 0.05 compared with carrageenan/saline group.

Aqueous extract of *P. tagetoides* and *A. reticulata* was partitioned in methanol; soluble and insoluble fractions were assayed in carrageenan - induced paw edema in the rats. The edema induced by the carrageenan injected subcutaneously into the hind paws in rats was followed for 4 h, the maximum effect was observed 4 h after its injection (409 ± 38 µl) (Fig. 3). Methanol extract of *P. tagetoides* (PTME) and insoluble fraction in methanol of *A. reticulata* (ARR) (200 mg/kg) decreased paw volume (268 ± 31 µl and 297 ± 22 µl, respectively) (Fig. 3). The insoluble fraction in methanol of *P. tagetoides* and methanol extract of *A. reticulata* did not affect the carrageenan - induced inflammation.

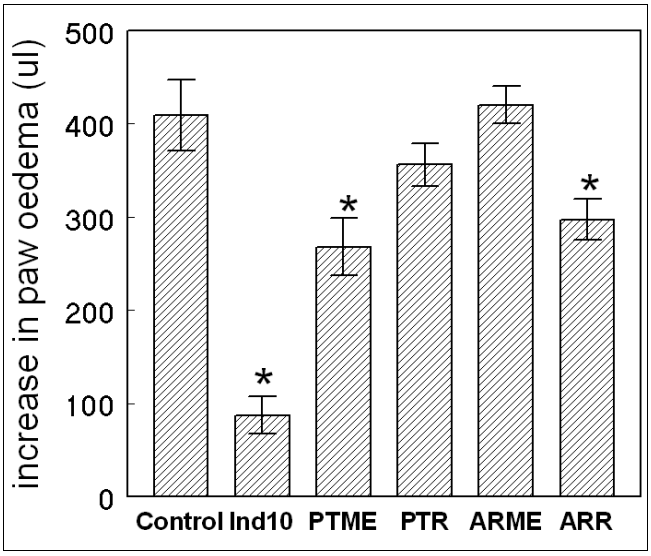

Fig 3: Effect of *P. tagetoides* methanol extract (PTME) (200 mg/kg), methanol insoluble fraction of *P. tagetoides* (PTR) (200 mg/kg), *A. reticulata* methanol extract (ARME) (200 mg/kg), and methanol insoluble fraction of *A. reticulata* (ARR) leaves (200 mg/kg) on carrageenan - induced paw edema in rats, 4 h after intraplantar injection of carrageenan. Control; standard treatment of indomethacin (10 mg/kg). In all groups n = 6. * represents p < 0.05.

***P. tagetoides* and *A. reticulata* aqueous extract inhibit neutrophil migration in the peritoneal cavity**

We evaluated the effect of *P. tagetoides* and *A. reticulata* aqueous extracts on carrageenan - induced neutrophil migration in the peritoneal cavity of the rats. It was found that administration of *P. tagetoides* and *A. reticulata* decreased the influx of neutrophils into the peritoneal cavity, compared to the carrageenan control group ($P < 0.05$); it was observed an inhibition of 40% for *P. tagetoides* and 54% for *A. reticulata* (300 mg/kg) (Fig. 4). Dexamethasone (1 mg/kg) inhibited the cellular migration in 67%.

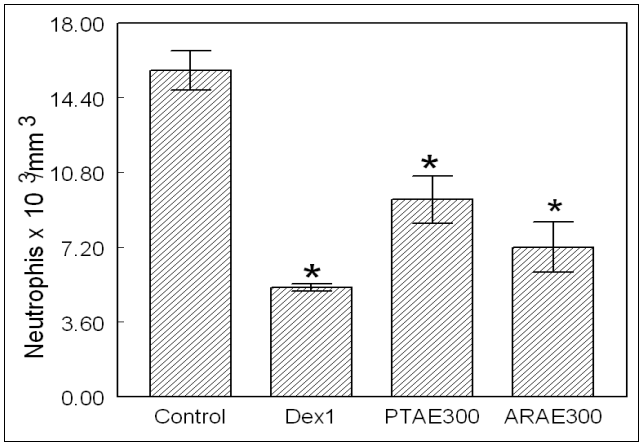

Fig 4: Effect of the leaves aqueous extract of *P. tagetoides* (PTAE) (300 mg/kg), *A. reticulata* (ARAE) (300 mg/kg) and dexamethasone (Dex) (1 mg/kg) on neutrophil migration into the peritoneal cavity induced by carrageenan. Neutrophil migration was determined 4 h after the injection of carrageenan 300 μg/3ml cavity. Mice previously treated with vehicle (saline and carrageenan). The data are expressed as mean ± SEM, $n = 6$. Symbols indicate statistical difference ($P < 0.05$, Tukey test). *compared to the saline/carrageenan group.

Acetic acid-induced writhing response in mice

Aqueous extract of leaves of *P. tagetoides* (300 mg/kg) and *A. reticulata* (200 mg/kg) inhibited the number of abdominal

writhes (50 %, and 63%, respectively), indomethacin (10 mg/kg) also inhibited the writhes in 80% (Figure 5).

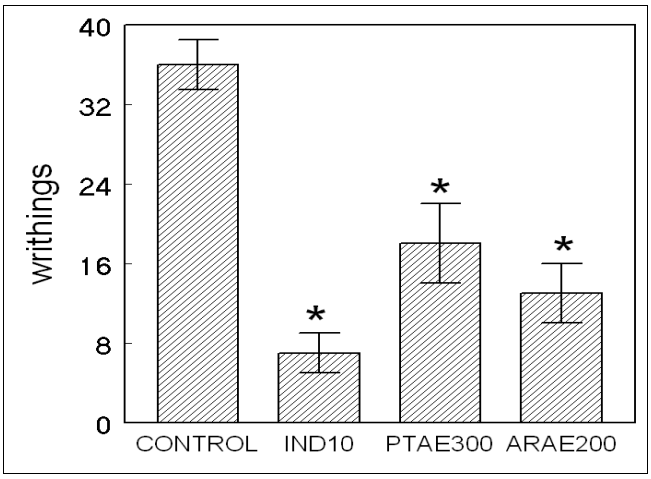

Fig 5: Effect of the leaves aqueous extract of *P. tagetoides* (PTAE) (300 mg/kg) and *A. reticulata* (ARAE) (300 mg/kg) on acetic acid-induced writhing reflex in mice. The data are expressed as mean ± SEM, $n = 6$. Symbols indicate statistical difference ($p < 0.05$, Tukey test). *compared to the saline/acetic acid group.

Acute toxicity assay

Aqueous extract of *A. reticulata* did not cause deaths in doses of 3 and 5 g/kg; however, the aqueous extract of *P. tagetoides* was lethal from the dose of 1.5 g/kg.

A. reticulata aqueous extract
3 mg/kg immobility, no death
5 mg/kg hyperventilation, no death

P. tagetoides aqueous extract
1.5 g/kg intense contortions, ataxia, death
3.0 g/kg seizures, death
5.0 g/kg seizures, death

4. Discussion

Current pharmacological evaluation of aqueous and methanolic extracts of leaves *of P. tagetoides* and *A. reticulata* was carried out to assess its anti-inflammatory and anti-nociceptive potential and to justify its use in the traditional medicine. Aqueous and methanolic extracts of leaves of *P. tagetoides* and *A. reticulata* (200 and 300 mg/kg) significantly reduced the carrageenan - induced paw edema in rats; inhibited neutrophil migration into the peritoneal cavity induced by carrageenan; caused a nearly 60% reduction in the number of acetic acid induced abdominal writhes in mice as compared to the untreated control group, showing its anti-inflammatory and analgesic effects.

The anti-inflammatory activity was demonstrated by the inhibition of formation of paw edema in rats induced by the injection of carrageenan that is a suitable test for evaluating anti-inflammatory drugs, which has frequently been used to assess the anti-edematous effect of natural products [16]. The experimental model exhibits a high degree of reproducibility. In rats, the inflammatory response induced by carrageenan is characterized by a biphasic response; marked edema formation, the initial phase, is attributed to the release of histamine and serotonin. The second phase of edema is due to the release of prostaglandins, protease and lysosome [17-19]. The second phase is sensitive to most clinically effective anti-inflammatory drugs [17, 20, 21]. Therefore, it is suggested that the mechanism of anti-inflammatory action of the aqueous and methanol extracts of *P. tagetoides* may be related to inhibition of prostaglandin and other autacoids, however the effect was lower for aqueous extract of *A. reticulata*.

Cell recruitment during inflammation depends on the orchestrated release of local mediators which is responsible for local vascular and tissue changes as well as for the recruitment of host defense cells. The inflammation induced by carrageenan involves cell migration, plasma exudation and production of mediators, such as nitric oxide, prostaglandin E_2, IL-1β, IL-6 and TNF-α [22, 23]. These mediators are able to recruit leukocytes, such as neutrophils, in several experimental models. The aqueous extract of leaves of *P. tagetoides* and *A. reticulata* inhibited leukocyte migration induced by i.p. injection of carrageenan (in peritonitis model). A putative mechanism associated with this activity may be inhibition of the synthesis of many inflammatory mediators whose involvement in the cell migration is well-established.

The acetic acid-induced writhing test is the most useful model for preliminary studies of anti-nociceptive activity, is a well recommended model in evaluating medicinal agents for their analgesic property) [24, 25]. This pain paradigm is widely used for the assessment of peripheral analgesic activity due to its sensitivity and response to the compounds at a dose which is not effective in other methods. The local peritoneal receptor could be the cause of abdominal writhings [26]. Pain sensation in acetic acid induced writhing is elicited by producing localized inflammatory response due to release of free arachidonic acid from tissue phospholipids via cyclooxygenase (COX), and producing prostaglandin specifically PGE_2 and $PGF_{2\alpha}$, the level of lipoxygenase products may also increase in peritoneal fluids [27, 28]. These prostaglandin and lipoxygenase products cause inflammation and pain by increasing capillary permeability. The substance inhibiting the writhing will have analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition [27]. *P. tagetoides* and *A. reticulata* also demonstrated analgesic activity as shown by inhibition of pain sensation in the mice. Response to pain, observed as abdominal writhing movement, was significantly reduced indicating that *P. tagetoides* and *A. reticulata* possesses analgesic activity. The exact mechanism of analgesic activity of *P. tagetoides* and *A. reticulata* cannot be ascertained in this study because the model of analgesic used is only a strong indicator of the presence of analgesic activity in a compound.

Guillet *et al*. (1998) [29] found that *P. gracile* and *P. ruderale* contain the insecticide alpha-terthienyl [30], which would explain the acute toxicity observed in the aqueous extract of *P. tagetoides*.

Therefore, it is concluded that aqueous extract of leaves of *Porophyllum tagetoides* and *Annona reticulata* extracts are capable of inhibiting inflammatory reactions as well as pain. The results provided experimental evidence for its traditional use in treating various diseases associated with inflammation and pain. The mechanism involved is not determined and elucidated in the present study and is therefore the likely focus of subsequent research.

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