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Additional Evidence for the Anti-inflammatory Properties of the Essential Oil of *Protium heptaphyllum* Resin in Mice and Rats.

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SUMMARY. **The objective of this study was to analyze the chemical composition and evaluates further the anti-inflammatory properties of essential oil of *Protium heptaphyllum* resin (EOP). The essential oil was analyzed by GC/MS. Fourteen constituents, accounting for 100% of the total oil, were identified. The main constituents of essential oil were limonene (49.96%), *trans*-β-ocimene (11.81%), eucalyptol (10.92%) and *p*-cymene (10.78%). EOP administered orally (100 and 200 mg/kg b.w.) significantly suppressed the development of carrageenan and egg albumin-paw edema and produced a significant inhibition of peritoneal vascular permeability induced by acetic acid. OEP also reduced peritoneal leukocytes migration, granuloma tissue formation and mast cell degranulation induced by compound 48/80 *(ex-vivo)*. These results appear to support the potential medicine use of EOP against inflammatory conditions.**

INTRODUCTION

Inflammation is the response of living tissue to mechanical injuries, burns, microbial infections, and other noxious stimuli that involve changes in blood flow, increased vascular permeability, activation and migration of leucocytes and the synthesis of local inflammatory mediators [1]. All the steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), despite their great number, cause undesired and serious side effects. Therefore, development of new and more powerful drugs is still needed. Medicinal plants have long been used worldwide in folk medicine as an alternative treatment of inflammatory processes of diverse origins [2].

Essential oils are volatile secondary metabolites and extensively used in pharmacy, medicine and aromatherapy. Their terpenoid components such as mono- and sesquiterpenes are recognized as potential anti-inflammatory agents. D-Limonene [*R*-(+)-isomer] is a monoterpene found in the essential oils of various plants, such as *Artemisia dracunculus* L. (Asteraceae), *Ziziphora taurica* subsp. cleonioides (Boiss) P.H. Davis (Lamiaceae) and other plants species as *Protium icicariba* (Burseraceae) [3-5]. Some studies with essential oils containing *R*-(+)-limonene as one of prevalent compounds or with pure limonene have demonstrated its anti-inflammatory activities and chemopreventive efficacy in preclinical model systems [6-8].

Protium heptaphyllum (Aubl.) March. (Burseraceae), is a medicinal plant widely encountered in the Amazon region and also in other parts of Brazil. The leaves and stem bark are used to treat gangrenous ulcers, inflammatory and to promote wound healing [9,10]. The resin collected from the trunk wood of *Protium heptaphyllum* is locally known as *almécega* or *breu branco* and is widely used for skin diseases, healing of ulcers, and as an analgesic [11]. Preliminary study has reported that essential oil of *Protium heptaphyllum* resin caused inhibition of the pleurisy induced by zymosan or lipopoly-

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saccharide in mice [12] and it was found to possess antinociceptive properties in mice models [13].

In the current study, the composition of essential oil of *Protium heptaphyllum* resin (EOP) was characterized by using GC-MS analysis and we have investigated therefore further the anti-inflammatory properties of the EOP in acute and chronic models of inflammation in rodents. In addition, we examined whether OEP could inhibit compound 48/80-induced degranulation of rat peritoneal mast cells.

MATERIALS AND METHODS

Plant material

The resin of *Protium heptaphyllum* (Aubl.) March. was collected on August 2, 2006, from Timon municipality, Maranhão State, Brazil, by scraping it from wound-stimulated trunk, after its identification by the Taxonomist Dr. Roseli Farias de Melo Barros. A voucher sample (TEPB 18247) has been deposited at the Herbarium Graziela Barroso of the Federal University of Piauí, Teresina, Brazil.

Extraction of plant material

The essential oil from the resin was extracted by hydrodistillation in a Clevenger-type apparatus for 3 h. The oil was dried over anhydrous Na_2SO_4 and was then sealed and stored at 4 °C in dark glass bottle until use.

Analysis of essential oil was performed on a Shimadzu, CG-17A under the following conditions: column: 5% phenylmethylpolysiloxane DB-5 fused silica capillary column (50 m x 0.25 mm, 0.25 ?m film thickness); carrier gas: helium (1.7 mL/min); injector temperature: 220 °C; detector temperature: 240 °C; column temperature: 60°-180 °C at 3 °C/min; mass spectra: electron impact, 70 eV. Individual components were identified using a computer search (Wiley 229 library) and by comparing their retention indices regarding the C_8-C_{30} series of n-alkanes [14].

Animals used

Male Albino Swiss mice (20–25 g) and male Wistar rats (150–200g) were used. The animals were maintained under a controlled temperature of 24 ± 2 °C and 12 h light/12 h dark cycle with water and food *ad libitum*. The experimental protocols used in this study were approved by the Committee for Animal Ethics of the Federal University of Piauí (UFPI), Teresina-PI, Brazil (02/08-CEP/UFPI).

Carrageenan-induced paw edema

Paw edema was induced by subplantar injection of 0.1 ml of 1% (w/v) carrageenan in saline into the right hind paw of each rat. The volume of the injected paw was measured after 0, 1, 2, 3, 4 and 5 h of carrageenan administration using a digital plethysmometer (model 7159 Ugo Basile®). The EOP at 50, 100 and 200 mg/kg, the reference compound indomethacin (10 mg/kg) and vehicle (2% Tween 80) in a volume of 10 ml/kg were given orally one-hour prior to carrageenan treatment [15].

Egg albumin induced paw edema

The same protocol as described above was used. One hour after treatment edema was induced by injection of fresh egg albumin (0.1 ml, 1% w/v in saline). The volume of the injected paw was measured after 0, 1, 2 and 3 h of induction of edema. Chlorpheniramine, 60 mg/kg p.o., was used as a reference [15,16].

Acetic acid-induced peritoneal capillary permeability

Groups of mice were treated orally with the EOP (50, 100 e 200 and mg/kg), acetylsalicylic acid (250 mg/kg) or vehicle (2% Tween 80) in a volume of 10 ml/kg. One hour after these treatments, the mice were given 10 ml/kg of 1% solution of Evans Blue solution in normal saline intravenously. Five minutes later, each mouse received intraperitoneally 10 ml/kg of 1% acetic acid solution. Thirty minutes after acetic acid injection, the animals were killed, the peritoneal exudates were collected after being washed with 5 ml of normal saline and the concentration of Evans Blue was measured by absorbance at 610 nm in a spectrophotometer [17]. The dye extravasation was quantified from a standard curve and expressed in µg.

Carrageenan-induced peritonitis

The method described by Carvalho *et al.* [18] was used. Groups of 6 animals were treated orally with EOP (50, 100 and 200 mg/kg), vehicle (2% Tween 80) and dexamethasone (1 mg/kg, s.c.), followed by 1% carrageenan (0.2 mL, i.p.) 1 h later. Four hours after the intraperitoneal injection, rats were killed and 2 mL of modified PBS (heparin, 10 IU/mL) were injected into the peritoneal cavity. Total cell counts were performed using a Neubaüer chamber.

Cotton pellet-induced granuloma formation

This was carried out using the method of Swingle & Shideman [19]. Two 10±1 mg sterilized pellets of cotton were implanted subcutaneously, one on each side of the animal's abdomen, under anesthesia and with a sterile technique. They were treated orally with EOP (50, 100 and 200 mg/kg), ibuprofen (300 mg/kg), the reference compound or the vehicle (2% Tween 80), once daily for 7 days. On Day 8, the mice were sacrificed and the pellets with the surrounding granulation tissues were collected, and their wet and dry weights were measured. The granulomas were dried at 60 °C for 24 h to obtain the dry weights.

Peritoneal mast cell degranulation

Four groups of rats were included for the ex vivo study that aimed to demonstrate the effects of EOP and ketotifen on the peritoneal mast cell degranulation. The first and second groups of animals served as normal and vehicle-treated controls and received orally, normal saline or 3% Tween 80 (vehicle for EOP), respectively, in a volume of 10 ml/kg, whereas the third and fourth groups were treated orally with EOP- (200 mg/kg) or ketotifen (1 mg/kg), respectively. Two hours later, the animals were killed by cervical dislocation and half a centimeter pieces of mesenteric vascular plexus were collected from the respective groups into each of the glass tubes containing Ringer Locks fluid (10 ml). Mast cell degranulation was induced by incubation of tubes containing mesenteric vascular tissue collected from EOP, ketotifen, or vehicle-treated groups with compound 48/80 (final concentration, 0.4 µg/ml) for 30 min. The concentration of 0.4 µg/ml of compound 48/80 was chosen for this study based on our preliminary studies, which caused mast cell disruption to the extent of 90%. The same volume of distilled water (instead of compound 48/80 solution) was added to tubes containing mesenteric tissue obtained from the normal control rats that received only the vehicle. After 30 min incubation, the mesenteric tissue was mounted on glass slides, allowed to dry at room temperature and stained with toluidine blue (0.1%) for the observation of mast cells by light microscopy. At least five optical fields were chosen for each tissue sample (five samples per group) and the number of total mast cells present, and the percentage of cells disrupted/degranulated were noted [20].

Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M). One-way analysis of variance (ANOVA), followed by Student Newman Keul's test, was used to analyze the significance of differences between groups. Values were considered statistically significant at $P < 0.05$.

RESULTS

Essential Oil Analysis

The hydrodistillation of *Protium heptaphyllum* resin produced oil with a 2.11% (w/w) yield, based on the dry weight of the plant. The constituents of the essential oil obtained are presented in Table 1. The essential oil analysis identified 14 compounds, which represented 100% of the compounds present in EOP. The major constituents of the oil were the monoterpenes limonene (49.96%), *trans*-β-ocimene (11.81%), eucalyptol (10.92%), *p*-cymene (10.78%), α-Phellandrene (9.976%) and α-Terpineol (1.82%).

Constituent	RI [a] (DB-5)	Relative abundance (%)
α-Phellandrene	1004	9.97
Sabinene	975	0.49
α-Pinene	936	0.50
Cis-β-ocimene	1008	trb
β-Phellandrene	1041	trb
β-Pinene	1045	0.20
γ-Terpinene	1069	trb
Trans-β-ocimene	1043	11.81
p-Cymene	1025	10.78
Limonene	1031	49,96
Eucalyptol	1064	10.92
Terpinolene	1190	trb
α-Terpineol	1193	1.82
Linalool	1100	0,26
ND	-	2,32
Total		100

Table 1. Chemical composition of the *P. heptaphyllum* resin essential oil. RI, retention indices relative to C8—C30 *n*-alkanes. [b] tr, trace (<0.1%). ND: not determined.

Effect on carrageenan-induced paw edema

Interplantar injection of carrageenan in rats caused a local inflammatory response; this increase was observed at 1 h and peaked 4 h after the application of the phlogistic agent. Administration of EOP (100 and 200 mg/kg; p.o.) 1 h before injection carrageenan caused a significant ($P < 0.01$) and dose dependent inhibition of increase in paw edema, by 34.7 and 41.1% respec-

tively 2 h after carrageenan administration. Indomethacin (10 mg/kg, p.o.) produced an inhibition of 51.2% (Table 2).

Effect on egg albumin induced paw edema

The effects of EOP on egg-albumin induced inflammation in rats are as shown in Table 3. There was significant suppression of the inflammatory process at doses of 100 and 200 mg/kg when compared to the control. Peak inhibitory effect was recorded with a dose of 200 mg/kg at 1, 2 and 3 h post-egg albumin injection (77.0, 62.0 and 82.0%, respectively). This effect was comparable to that of Chlorpheniramine (60 mg/kg; 77.0, 79.0 and 79.0%, respectively).

Effect on acetic acid-induced vascular permeability

EOP at all doses (50, 100 and 200 mg/kg, p.o.) and acetylsalicylic acid (250 mg/kg, p.o.) produced significant inhibitions by 49.17, 56.23, 71.41 and 84.61%, respectively, on the vascular permeability increase induced by intra-peritoneal acetic acid in mice (Table 4).

Effect on carrageenan-induced peritonitis

The results show that EOP (50, 100 and 200 mg/kg, p.o.) also inhibited peritoneal leukocyte migration at the rate of 23.1, 58.3 and 67.4 %, respectively. The highest dose of EOP inhibited leukocytes migration comparable to that induced by dexamethasone (1 mg/kg, s.c.) which produced 71.0 % of inhibition as shown in Table 5.

Effect on cotton pellet induced granuloma formation

Treatment with EOP (100 and 200 mg/kg, p.o.) and ibuprofen (300 mg/kg, p.o.) caused significant inhibition of granuloma tissue formation by 37.7 and 40% respectively, when compared to the vehicle treated mice (Table 6).

Groups	Dose (mg/kg, p.o.)	Edema value (ml) and inhibition rate (%)				
		1 h	2 h	3 h	4 h	5 h
Control	10 ml/kg	0.434 ± 0.062	0.574± 0.06	0.660 ± 0.04	0.88 ± 0.04	0.648 ± 0.06
EOP	50	0.385 ± 0.05	0.440 ± 0.04	0.562 ± 0.03	0.773 ± 0.05	0.523 ± 0.03
EOP	100	0.224 ± 0.04* (34.1)	0.375 ± 0.04* (34.7)	0.580 ± 0.02	0.754 ± 0.05	0.602 ± 0.01
EOP	200	0.230 ± 0.02* (32.3)	0.338 ± 0.02** (41.1)	0.442 ± 0.04* (33.0)	0.715 ± 0.03* (18.7)	0.522 ± 0.04
Indomethacin	10	0.180 ± 0.03** (58.5)	0.280 ± 0.03** (51.2)	0.382 ± 0.03** (42.1)	0.505± 0.06** (43.1)	0.418 ± 0.02** (35.5)

Table 2. Effect of the *Protium heptaphyllum* resin essential oil (EOP) on carrageenan-induced paw edema in male Wistar rats (n=8 for each group). Each point represents the mean volume of the paw ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* control groups (ANOVA followed by Student–Newman–Keuls test) Values in parentheses indicate the percentage inhibition rate.

Groups	Dose (mg/kg, p.o.)	Edema value (ml) and inhibition rate (%)		
		1h	2h	3h
Control	10 ml/kg	1.152 ± 0.076	0.698 ± 0.126	0.564 ± 0.192
EOP	50	0.905 ± 0.078* (21.4)	0.415 ± 0.046** (40.5)	0.487 ± 0.293
EOP	100	0.830 ± 0.056* (28.0)	0.334 ± 0.094** (53.0)	0.278 ± 0.192** (51.0)
EOP	200	0.265 ± 0.106** (77.0)	0.268 ± 0.069** (62.0)	0.045 ± 0.061** (82.0)
Chlorpheniramine	60	0.274 ± 0.108** (77.0)	0.146 ± 0.078** (79.0)	0.117 ± 0.127** (79.0)

Table 3. Effect of the *Protium heptaphyllum* resin essential oil (EOP) on egg albumin-induced paw edema in male Wistar rats (n=8 for each group). Each point represents the mean volume of the paw ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* control groups (ANOVA followed by Student–Newman–Keuls test) Values in parentheses indicate the percentage inhibition rate.

Groups	Dose (mg/kg, p.o.)	Dye leakage (µg)	Inhibition (%)
Control	10 ml/kg	0.066 ± 0.01	
Control acetic acid	–	4.403 ± 0.70	
EOP	50	3.869 ± 0.33	12
	100	3.133 ± 0.33*	28.8
	200	1.415 ± 0.33**	67.8
Acetyl salicilic acid	250	0.928 ± 0.23**	78.9

Table 4. Effect of the *Protium heptaphyllum* resin essential oil (EOP) on acetic acid-induced vascular permeability in male Swiss mice (n=8 for each group). Each point represents the mean volume of the paw ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* control groups (ANOVA followed by Student–Newman–Keuls test).

Groups	Dose (mg/kg, p.o.)	Number of total leukocytes (x 10^7)	Inhibition (%)
Control	10 ml/kg	8.912 ± 0.50	
EOP	50	6.850 ± 0.46*	23.1
	100	3.710 ± 0.63**	58.3
	200	2.900 ± 0.57**	67.4
Dexamethasone	1 (s.c.)	2.580 ± 0.69**	71.0

Table 5. Effect of the *Protium heptaphyllum* resin essential oil (EOP) on carrageenan-induced peritonitis in male Wistar rats (n=6 for each group). Each point represents the mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* control groups (ANOVA followed by Student–Newman–Keuls test).

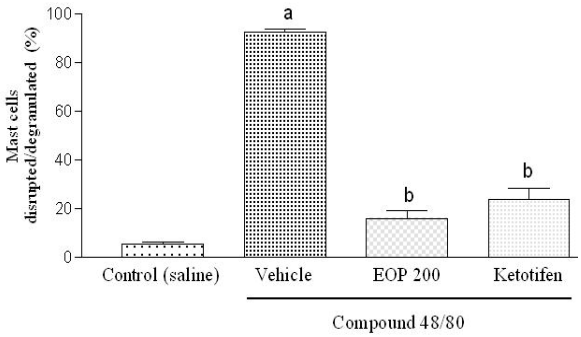

Figure 1. The effect of oral pretreatments with OEP (200 mg/kg), ketotifen (1 mg/kg) or vehicle (3% Tween 80; 10 mL/kg) or normal saline (10 mL/kg) on Compound 48/80 (0.4 µg/mL)-induced degranulation of rat peritoneal mast cells *ex vivo*. Each column represents mean ± S.E.M. (n=4). **a** $^{**}P < 0.01$ *vs.* saline-treated control; **b** $^{**}P < 0.01$ *vs.* vehicle-treated control (ANOVA and Bonferroni test).

Peritoneal mast cell degranulation

The percent numbers of degranulated mast cells in saline treated normal rats, in vehicle-treated rats and in rats treated with EOP (200 mg/kg) and ketotifen (1 mg/kg) were in the order of 5.50, 92.50, 21.90 and 20.20%, respectively (Figs. 1 and 2). Compared with vehicle-treated controls, treatment with EOP and ketotifen significantly reduced the compound 48/80-induced degranulation by the extent of 76.32 and 78.16%, respectively.

DISCUSSION

The present study showed that essential oil of *Protium heptaphyllum* resin exhibited a significant anti-inflammatory activity against animal models rats and in mice induced by various stimuli, representing different phases of inflammation.

Acute inflammation is characterized by vasodilatation, the exudation of protein-rich fluid (plasma) and leukocytes infiltration (cell migration) into the site of injury [21]. The carrageenan model is characterized by the production of a biphasic edema. In the relatively rapid early phase (1 to 2 h), edema formation is mediated by histamine and serotonin and the late phase (3 to 4h), kinins and prostaglandins contribute to the edema [22]. The egg albumin-induced edema is useful in detecting activity in acute inflammation. Fresh egg albumin-induced hind paw edema results from the release of histamine and serotonin [23]. The results showed that EOP (100 and 200 mg/kg) administered 1h before injection of carrageenan or egg albumin caused a significant and doses dependent inhibition of increase in paw edema. Maximum inhibitory effect was recorded with a dose of 200 mg/kg at 2 and 3 h post-carrageenan and egg albumin injection, respectively. These results suggest that the EOP may effectively suppress the exudative phase of acute inflammation.

To further evaluate anti-inflammatory effects suggested by the paw edema experiments, we tested EOP in acetic acid-induced vascular permeability and carrageenan-induced peritonitis models. The vascular permeability induced by

Groups	Dose (mg/kg, p.o.)	Granuloma wet weight (mg)	Inhibition (%)	Granuloma dry weight (mg)	Inhibition (%)
Control	10 ml/kg	131 ± 0.13		45 ± 0.06	
EOP	50	132 ± 0.22		35 ± 0.06	
EOP	100	83 ± 0.07**	36.6	28 ± 0.02**	37.7
EOP	200	79 ± 0.06**	39.7	27 ± 0,02**	40.0
Ibuprofen	300	68 ± 0.03**	48.0	20 ± 0.01**	55.5

Table 6. Effect of the *Protium heptaphyllum* resin essential oil (EOP) on cotton pellet granuloma in male Swiss mice (n=8 for each group). Each point represents the mean ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* control groups (ANOVA followed by Student–Newman–Keuls test).

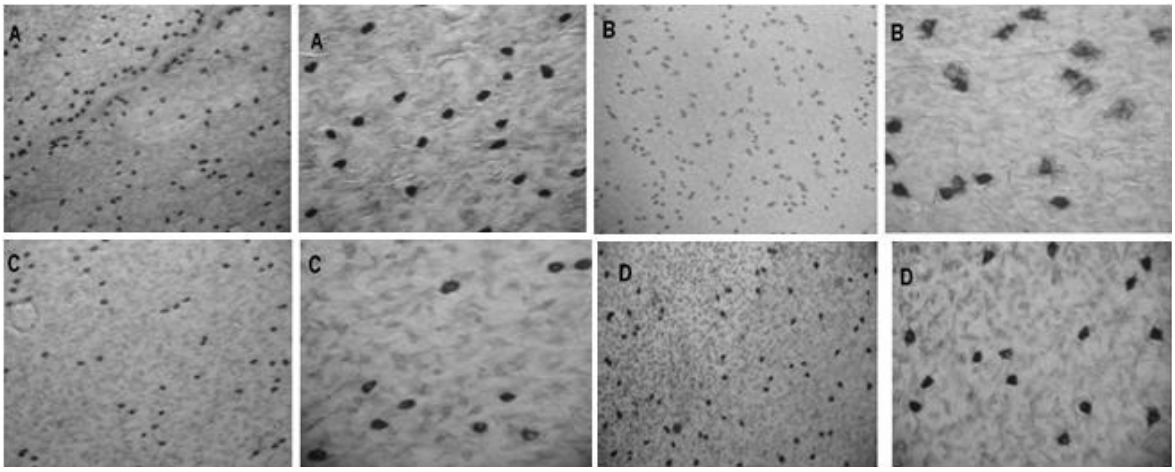

Figure 2. The representative microphotograph of toluidine blue (100 X; 400 X) stained rat peritoneal mast cells subjected or not to compound 48/80 (0.4 µg/ml)-induced degranulation ex vivo. (**A**) Mast cells from saline-treated rats; (**B**) Mast cells from vehicle (2% Tween 80; 10 ml/kg)-treated rats incubated with compound 48/80, showing extensive mast cell degranulation; (**C**) Mast cells from EOP (200 mg/kg); (**D**) Ketotifen (1 mg/kg)-treated rats incubated with compound 48/80, showing inhibition of degranulation.

acetic acid causes an increase in peritoneal fluids of serotonin, histamine and prostaglandins. This leads to dilation of arterioles and venules and increased vascular permeability [17]. EOP (100 and 200 mg/kg) exhibited a dose-dependent and significant inhibition of degree of peritoneal increased vascular permeability produced by acetic acid in mice, indicating that the essential oil arises from its protection on the release of inflammatory mediators at the first phase. The carrageenan-induced peritonitis is known acute inflammatory model in which fluid extravasation and leukocyte migration involved in the inflammatory response can be easily detected. Various mediators are involved in the pathogenesis of carrageenan-induced peritonitis. In the early stage, endogenous amines and prostaglandins play an important role, while infiltrating polymorphonuclear leucocytes play a crucial role in the more delayed injury (3-4 h) [24,25]. EOP (100 and 200 mg/kg) significantly prevented recruitment of cells into the peritoneal cavity after injection of carrageenan. These results are consistent with previous study that has reported the inhibitory effects of essential oil of *Protium heptaphyllum* resin on protein extravasation and eosinophil migration induced by zymosan and lipopolysaccharide respectively [12].

The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of chronic inflammation. The wet weight of the cotton pellets correlates with the transudative, and the dry weight of the pellets correlates with the amount of the granulomatous tissue [26]. NSAIDs decrease the size of granuloma by inhibiting granulocyte infiltration and preventing an increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides which are natural proliferative events of granulation in tissue formation [27]. Administration of EOP (100 and 200 mg/kg) appear to be effective at inhibiting both wet and

dry weights of the cotton pellet when compared to the control. These results indicate that EOP is effective at inhibiting the proliferative phase of the inflammation process.

Mast cells are found resident in tissues throughout the body, virtually in all vascularized types of tissues [28]. Activated mast cell secrete a wide variety of preformed and neo-synthetised inflammatory mediators such as histamine, serotonin, leukotrienes, prostaglandins and many cytokines which are observed in settings of acute and/or chronic inflammation *e.g.,* vasodilatation, plasma extravasation and the recruitment and activation of granulocytes [29,30].

Mast cell degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells [31]. The most potent secretagogues include the synthetic compound 48/80. Compound 48/80 is a mixed polymer of phenethylamine crosslinked by formaldehyde and is known to stimulates only certain subtypes of mast cells, such as rat peritoneal mast cells, to induce the release of inflammatory mediators *via* phospholipase D and heterotrimeric GTP-binding proteins [32,33].

In this study carried out *ex vivo,* EOP was able to prevent mast cell degranulation, comparable to that caused by ketotifen (used as reference drug). Furthermore, EOP could inhibit the paw edema response, induced by compound 48/80 and dextran T40 in mice (unpublished observations). Taken together, these observations suggest that the protective effect of EOP against mast cell degranulation indicates that this action may be responsible for the observed anti-inflammatory effect of this plant. However the effect of EOP on other inflammatory mediators can not be excluded as mast cell is only one of the complex mechanisms in the pathogenesis of inflammation

The major constituent presented in EOP resin is limonene (49.96%), a monoterpenic compound present in nature and is the majority constituent of an essential oil series. Ozbek [34], showed that essential oil of *Foenicum vulgare* has anti-inflammatory effect and the anti-inflammatory activity shown by essential oil could be attributed in part to the two major components, limonene and α-pinene. For this reason, the anti-inflammatory effect showed by the EOP in this work can be explained, at least in part, by the presence of the limonene, not excluding the possibility of synergism between other constituents present in the oil.

CONCLUSION

The present study provided further *in vivo* e *ex vivo* experimental evidences indicating that the essential oil of *Protium heptaphyllum* resin showed significant anti-inflammatory activity in both acute and chronic inflammatory models when administered orally to animals. The biological activity could be partly explained by the presence of monoterpenes, such as d-limonene, the main constituent of EOP.

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