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Toxicological analysis and anti-inflammatory effects of essential oil from *Piper vicosanum* leaves

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ABSTRACT

This study assessed the anti-inflammatory effects of the essential oil from *Piper vicosanum* leaves (OPV) and evaluated the toxicological potential of this oil through acute toxicity, genotoxicity and mutagenicity tests. The acute toxicity of OPV was evaluated following oral administration to female rats at a single dose of 2 g/kg b.w. To evaluate the genotoxic and mutagenic potential, male mice were divided into five groups: I: negative control; II: positive control; III: 500 mg/kg of OPV; IV: 1000 mg/kg of OPV; V: 2000 mg/kg of OPV. The anti-inflammatory activity of OPV was evaluated in carrageenan-induced pleurisy and paw edema models in rats. No signs of acute toxicity were observed, indicating that the LD50 of this oil is greater than 2000 mg/kg. In the comet assay, OPV did not increase the frequency or rate of DNA damage in groups treated with any of the doses assessed compared to that in the negative control group. In the micronucleus test, the animals treated did not exhibit any cytotoxic or genotoxic changes in peripheral blood erythrocytes. OPV (100 and 300 mg/kg) significantly reduced edema formation and inhibited leukocyte migration analyzed in the carrageenan-induced edema and pleurisy models. These results show that OPV has anti-inflammatory potential without causing acute toxicity or genotoxicity.

1. Introduction

There is considerable interest in identifying new therapeutic agents obtained from plants used in popular medicine. One of the reasons of this interest is that drugs currently available have a number of adverse effects and new strategies are needed to improve treatment (Rates, 2001).

The genus *Piper* (Piperaceae family) comprises about 2000 species distributed in the tropical and subtropical regions. *Piper* species have been widely used in folk medicine for the treatment of various diseases such as bronchitis, intestinal pains, skin irritations and inflammation, and in the preparation of ceremonial drinks (Mesquita et al., 2006; Wang et al., 2014). Phytochemical analyses of this genus have demonstrated the occurrence of alkaloids, amides, propenylphenols, lignans, neolignans, steroids, pyrones, chalcones, dihydrochalcones, flavones and terpenoids (Sengupta and Ray, 1987; Parmar et al., 1997).

Although little information is available in the literature on the therapeutic or toxic effects of this plant, studies have shown that extracts obtained from some species of *Piper*, such as *Piper amalago*, *Piper nigrum*, *Piper longum*, *Piper abbreviatum*, *Piper umbellatum*,

Abbreviations: OPV, essential oil from *P. vicosanum* leaves; GC, gas chromatograph; GC–MS, mass spectrometer detector; OECD, Organisation for Economic Co-operation and Development; LD50, median lethal dose; PBS, phosphate buffer-saline; SEM, standard error of the mean; ANOVA, one-way analysis of variance.

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Piper officinarum present therapeutic properties, such as anti-inflammatory and antimicrobial activities, hepatoprotective effects, diuretic activity, among others (Rajeswary et al., 2011; Salleh et al., 2012; Novaes et al., 2014; Tasleem et al., 2014; Wan Salleh et al., 2014; Iwamoto et al., 2015). The especially well-studied species regarding their therapeutic activities, the fruits of *P. nigrum* L., possesses potent analgesic and anti-inflammatory activities, at doses of 10 and 15 mg/kg and these activities were attributed to piperine, its major alkaloid constituent (Tasleem et al., 2014).

Piper vicosanum Yuncker is a shrub that occurs in the Brazilian Atlantic Forest. The study carried out by Mesquita et al. (2006), revealed that this species possesses volatile oils with valuable biological activities, such as limonene (45.5%), α-pinene (6.1%), piperitone (3.4%), β-caryophyllene (1.4%), α-selinene (3.2%), D-cadinene (2.2%), spathulenol (0.3%), caryophyllene oxide (0.4%), β-pinene (0.2%), linalool and germacrene D traces, demonstrating the need for further studies about this species. Thus, due to the lack of studies in the literature using the *Piper* species, this study evaluated the anti-inflammatory effects of the essential oil extracted from *P. vicosanum* leaves (OPV) and determined the toxicological potential of this oil through acute toxicity, genotoxicity and mutagenicity tests.

2. Materials and methods

2.1. Plant material, preparation and isolation of essential oil

Piper vicosanum leaves (5 kg) were collected at the Coqueiro Farm, Dourados, MS (latitude 22012′37, 7 ″south and longitude 54055′03, 2″west) in August 2014. A voucher specimen was identified by Dr. Elsie Franklin Guimarães and deposited (register: 4412) in the DDMS herbarium of the Federal University of Grande Dourados (UFGD).

The OPV was isolated from 400 g of fresh leaves by separate hydrodistillation using a Clevenger-type apparatus for 4 h. At the end of each distillation, the oils were collected, dried with anhydrous Na_2SO_4, measured, and transferred to glass flasks that were filled to the top and kept at a temperature of −18 °C for further analysis. The yield of the obtained oil was from fresh leaves 0.80%. The analyses were performed employing a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan), mass spectrometer detector (GC–MS 2010 Ultra) using a fused silica capillary column DB-5 (60 m length × 0 25 mm internal diameter × 0.25 mm thick film). The temperatures of the injector, detector and the transfer line were 250, 280 and 300 °C, respectively. The analysis conditions were: injection volume of 1 μL, 1:20 split injection, heating ramp of 50 °C initial temperature reached 280 °C at 3 °C min^{-1} and remained in the final temperature for 10 min. The mass spectrometer scan parameters included electron impact ionization voltage of 70 eV, the mass range of 45–650 *m*/*z* with 0.5 s sweep range. The identification was carried out using the calculated retention indexes with the same mixture of linear alkane (C_7–C_{40}) as external reference (Van den Dool and Kratz, 1963) associated to the index reported in the literature (Adams, 2001) and analysis of the mass spectra of the samples compared to the databases (NIST21 and WILEY229).

2.2. Animals

Adult male Wistar rats (90 days old, weighing approximately 340 g, n = 45) and females (60 days old, weighing approximately 300 g, n = 10) were used in the acute toxicity and inflammation study. Male Swiss mice (60 days old, weighing 30–40 g, n = 50) were used in genotoxicity and mutagenicity studies. All animals used in the experiments were provided by the UFGD. The animals were maintained under controlled temperature (23 °C), with a constant 12 h light–dark cycle and free access to food and water. The experimental procedures were in accordance with the Ethical Principles in Animal Research and approved by the Committee for Ethics in Animal Experimentation at the UFGD (Protocol number 024/2014).

2.3. Acute oral toxicity

Acute toxicity studies were carried out using the OECD (Organisation for Economic Co-operation and Development) - Guidelines 425 and ANVISA guidelines (Brazilian Health Surveillance Agency) (ANVISA, 2004; OECD, 2008).

OPV was administered by gavage, at a dose of 2000 mg/kg, to one female rat after 8 h of fasting. Sequentially, at intervals of 48 h, the same dose was administered to four female rats, totaling five treated animals. Under the same conditions, five females were treated with vehicle (sunflower oil) in order to establish a comparative negative control group (OECD, 2008).

The animals were observed periodically during the first 24 h after administering the essential oil and then once a day for 14 days. The five parameters of the Hippocratic screening (Malone and Robichaud, 1962) were analyzed: conscious state (general activity); activity and coordination of motor system and muscle toning (response to tail touch and grip, straightening, strength to grab); reflexes (corneal and headset); activities on the central nervous system (tremors, convulsions, straub, sedation, anesthesia and ataxia) and activities on the autonomic nervous system (lacrimation, cyanosis, ptosis, salivation and piloerection). The water and feed consumption and body weight were also recorded daily (OECD, 2008).

At the end of the observation period, all animals were anesthetized (Ketamine and xylazine, 25 and 10 mg/kg, respectively) and killed by decapitation. Organs (heart, lung, spleen, liver, and kidney) were removed, weighed and examined macroscopically.

2.4. Comet assay and micronucleus test with peripheral blood

Mice were divided into five groups (n = 10/group). Three groups received OPV, at doses of 500 (group III), 1000 (group IV), or 2000 (group V) mg/kg body weight by oral route (gavage). The negative control animals (group I) were exposed to sunflower oil (vehicle) by gavage and to saline (0.1 mL/10 g body weight, i.p). The positive control animals (group II) were treated intraperitoneally with cyclophosphamide (Fosfaseron, Filaxis) at a dose of 100 mg/kg (Navarro et al., 2014). The OPV dosages were based on our determination of the LD_{50} (median lethal dose), which was higher than 2000 mg/kg. The treatment was performed once, and OPV was dissolved in sunflower oil and cyclophosphamide was dissolved in saline before administration. Cyclophosphamide was used to induce DNA damage, which has already demonstrated *in vivo* and *in vitro* genotoxicity activity in mouse bone marrow cells and peripheral blood (Fenech et al., 1999).

For the Comet assay, 24 h after treatment with OPV, 20 μL of blood was collected from each animal of each group. Slides were pre-coated with 5% agarose, and 20 μL of blood of each individual with 120 μL of agarose LPM (1.5%) at 37 °C were placed on the slide.

The coverslips were removed and the slides immersed in freshly prepared lysis solution with 89 mL of lysis stock solution (2.5 M NaCl, 100 mM EDTA, 10 mMTris, pH 10 adjusted with NaOH, 89 mL of distilled water and 1% of sodium lauroylsarcosine), 1 mL of Triton X − 100 and 10 mL of DMSO, for 1 h at 4 °C in the dark. The slides were placed on an electrophoresis chamber filled with buffer pH > 13 (300 mMNaOH and 1 mM EDTA, prepared with a stock

solution of 10 N NaOH and EDTA 200 mM, pH 10) at 4 °C for 20 min for DNA denaturation. Electrophoresis was performed at 25 V and 300 mA (1.25 V/cm). The slides were then neutralized with buffer pH 7.5 (0.4 M Tris–HCl) for three 5-min cycles, air dried, fixed with 100% ethanol for 10 min. The slides were stained with 100 μL of ethidium bromide (20 μg/mL) and covered with a coverslip. The material was examined with a fluorescence microscope, equipped with an excitation filter of 515–560 nm and a barrier filter of 520 nm at 40× magnification.

Each slide was identified and analyzed in a blind test. The cells (200 per animal; 100 for each time-point) were analyzed randomly. The comet findings were classified as follows: class 0 (no damage); class 1 (comet tail shorter than the diameter of the nucleoid); class 2 (comet tail once or twice the diameter of the nucleoid); and class 3 (comet tail more than twice the size of the nucleoid). Comets with a turbid aspect or a head that was too small were excluded from the analyses, as they could represent dead cells (da Silva et al., 2010). A total damage score was determined by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing all resulting values.

The micronucleus test with pheripheral blood was conducted according to Hayashi et al. (1990). Blood was collected from the tail of animals from all groups (n = 10 animals/group) at 24, 48, and 72 h after treatment. Slides were previously prepared with 20 μL of acridine orange, and 20 μL of peripheral blood was added in the center of the prepared slides. Slides were slip covered and stored in a freezer. The slides were examined using blue light fluorescence microscope (488 nm), an orange barrier filter, and magnification of 100×. Two thousand cells per animal (two slides per animal/treatment) were examined and micronucleated cells were counted. The micronuclei frequency was examined at 24 h, 48 h, and 72 h after treatment.

2.5. Carrageenan-induced rat pleurisy

Rats (n = 5 animals/group) were orally treated with OPV (100 and 300 mg/kg) or vehicle (0.9% saline – control or naïve group). The positive control group received a dose of 1 mg/kg of dexamethasone subcutaneously. Pleurisy was induced in experimental groups by intrapleural injection of 100 μL of 1% carrageenan diluted in saline, as previously described (Kassuya et al., 2009). The naïve group received 100 μL of sterile saline by intrapleural injection. After 4 h, the animals were euthanized and the pleural cavity was rinsed with 1 mL phosphate buffer-saline (PBS). An aliquot of 20 μL of lavage (exudate) was collected from the pleural cavity, and diluted with Turck (1:20) and used for total leukocyte count in a Neubauer chamber (Kassuya et al., 2009; Santos et al., 2012).

2.6. Carrageenan-induced rat paw edema

Rats (n = 5 animals/group) were orally treated with OPV (100 and 300 mg/kg) or vehicle. Another group was treated subcutaneously with dexamethasone (1 mg/kg). After 1 h, the animals received a solution of 50 μL carrageenan injection (300 μg/paw) in the right paw. The other paw received the same volume of sterile 0.9% saline. A plethysmometer was used to measure the paw edema after 1; 2; 3 and 4 h of carrageenan injection. Results were expressed as μL and the difference between right and left paws were quantified as edema (Kassuya et al., 2005).

2.7. Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). Difference among groups was evaluated by analyses of variance (one-way ANOVA) followed by Tukey–Kramer or Newman–Keuls tests. Statistical differences were considered to be significant at $p < 0.05$. Graphs were performed using GraphPad Prism Software (San Diego, CA, U.S.A).

3. Results

The gas chromatography/mass spectrometry (GC–MS) analysis identified 60 compounds in the OPV. Most of the compounds were ϒ-elemene (14.16%), α-alaskene (13.44%) and limonene (9.10%) (Table 1).

After the acute toxicity test, the dose of 2000 mg/kg (limit test – OECD, 2008) of OPV did not cause the death of any animal. The female rats exposed presented no behavioral changes during the treatment period, as well as no changes in water and food consumption and ponderal evolution were observed, related to the control group (data not shown). No abnormality was found in the organs at necropsy.

Table 2 presents the overall frequency of damaged cells, the division among the classes of damage and score ± SEM for the comet assay. These results showed that the groups treated with different doses of OPV did not exhibit increase in the frequency of genotoxic damage, when compared to the negative control group. Conversely, these parameters were significantly different from those observed in the positive control group.

Regarding the micronuclei frequency (Table 3), the results indicated that groups treated with OPV exhibited averages similar to those observed in the negative control group (at all time-points), and that these differed from those observed in the positive control group. These data showed that exposure to OPV did not alter the number of micronucleated polychromatic erythrocytes.

One hour after the carrageenan-induced inflammation, the control group continued to show edema, whereas the groups treated with OPV at doses of 100 and 300 mg/kg showed a significant decrease in edema compared to the control group (Fig. 1A) and this reduction continued after the second, third and fourth hour of observation (Fig. 1B, C and D). The inhibitions were 78 ± 2% and 75 ± 3% after 2 h and 80 ± 2% and 86 ± 3% after 4 h, respectively. The animals treated with dexamethasone, the positive control, showed a significant reduction at all time points, with inhibitions of 93 ± 7% after 2 h and 85 ± 5% after 4 h. Additionally, the oral administration of OPV significantly inhibited the leukocyte migration at all doses tested (100 and 300 mg/kg), with inhibitions of 70 ± 3% and 85 ± 2%, respectively, and higher inhibition at a dose of 300 mg/kg (Fig. 2). For the positive control the inhibition was 91 ± 2%.

4. Discussion

Species belonging to the Piperaceae family are well known due to their therapeutic properties in folk medicine (Alves et al., 2008), however, evidenced-based information is limited. To our knowledge, the present study may represent the first research that demonstrates the anti-inflammatory activity of the essential oil of *P. vicosanum* leaves without causing toxic effects.

In this study, the results indicate that acute administration of OPV has low oral toxicity in rats, since no mortality or clinical signs were observed. Thus, the oil tested falls in Class 5 (a substance with oral lethal dose (LD_{50}) higher than 2000 mg/kg), hence considered of low toxicity (OECD, 2008). Similar results were found by Rodrigues Silva et al. (2008) and da Silva et al. (2014), that after the acute toxicity analysis of other species of *Piper* (*Piper ovatum and P. umbellatum)* did not observe toxicity signs at doses of 5000 mg/kg or 2000 mg/kg, respectively.

Despite the therapeutic properties, several medicinal plants have genotoxic or mutagenic effects (Hong and Lyu, 2011; de

Table 1
Chemical composition of the essential oil of *P. vicosanum*.

Retention time (min)	Compound	RI_a	RI_b	%
7.675	α-Pinene	934	932	1.11
8.165	Camphene	948	946	0.04
9.225	β-Pinene	979	974	1.74
11.033	Limonene	1026	1024	9.10
11.293	Sylvestrene	1032	1030	3.18
11.625	(E)-β- Ocimene	1040	1044	1.22
24.815	Silphinene	1341	1345	0.58
25.349	α-Longipinene	1354	1350	0.53
26.144	Longicyelene	1372	1371	0.81
26.321	α-Copaene	1376	1374	0.26
26.607	2-epi-α-Funebrene	1383	1380	2.98
26.906	β-Bourbonene	1390	1387	0.54
27.294	α-Chamipinene	1399	1396	3.35
27.893	Caryophyllene	1413	1417	2.51
28.724	ϒ-Elemene	1434	1434	14.16
28.899	α-Guaiene	1438	1437	1.33
29.021	6,9-Guaiadiene	1441	1442	0.47
29.319	cis-Muurola-3,5-diene	1448	1448	2.14
29.399	α-Himachalene	1450	1449	0.10
28.899	α-Guaiene	1438	1437	1.33
29.021	6,9-Guaiadiene	1441	1442	0.47
29.319	cis-Muurola-3,5-diene	1448	1448	2.14
29.399	α-Himachalene	1450	1449	0.10
29.876	α-Acoradiene	1463	1464	1.85
30.141	β-Acoradiene	1268	1469	1.03
31.055	cis-β-Guaiene	1491	1492	0.84
31.233	cis-Cadine-1,4-diene	1495	1495	1.94
31.362	α-Selinene	1498	1498	1.50
31.870	α-Alaskene	1511	1512	13.44
32.129	ϒ-cadineno	1518	1513	1.73
32.432	δ-cadineno	1524	1522	2.10
32.825	α-Cadinene	1536	1537	4.12
33.052	ϒ-Cuprenene	1542	1542	0.23
33.233	Hedycaryol	1546	1546	0.28
33.444	β-vetivenene	1553	1554	0.25
34.038	Palustrol	1567	1567	1.18
34.357	Spathulenol	1575	1577	2.90
34.961	Caryophyllene oxide	1586	1582	1.51
35.164	Viridiflorol	1596	1592	1.66
35.431	Ledol	1603	1602	1.19
36.357	1-epi-Cubenol	1628	1627	0.14
36.490	ϒ-Eudesmol	1631	1630	0.31
36.696	cis-Cadin-4-en-7-ol	1636	1935	0.14
36.842	epi-α-Muurolol	1641	1640	1.74
37.272	α-Cadinol	1653	1652	0.43
37.413	Valerionol	1656	1656	0.69
37.583	Allohimachalol	1661	1661	2.13
37.803	Intermedol	1666	1665	0.40
38.321	5-neo-Cedranol	1682	1684	0.11
38.854	Z-Apritone	1690	1689	0.17
39.376	1,4-hydroxy-4,5-dihydro-Caryophyllene	1710	1706	0.53
42.448	(Z)-α-trans-Bergamotolacetate	1798	1794	0.19
42.913	Vetivenicacid	1812	1811	0.38
43.607	Cyclopentadecanolide	1832	1832	0.36
43.844	Eudesm-7(11)-en-4-olacetate	1839	1839	0.61
44.246	o-methylβ-Pipitzol	1851	1853	0.21
44.752	(E)-β- Santalolacetate	1866	1867	0.15
46.022	Evodione	1904	1904	0.35
46.614	Totarene	1922	1922	0.33
47.514	Columellarin	1951	1952	0.36
48.409	Bifloratriene	1979	1977	0.31
50.124	(Z)-Falcarinol	2034	2035	0.14
51.231	(Z)Isoeugenylbenzylether	2071	2072	0.67
53.529	Abienol	2148	2149	1.21

RI, retention index.

Almeida et al., 2015), demonstrating the need to assess the genotoxic potential of herbal preparations (Kelber et al., 2014). In the present study, the genotoxic effects of OPV were evaluated using the comet assay, which detects DNA damage produced by chemical and physical agents (Gontijo and Tice, 2003). The OPV treatment did not increase the incidence of comets in all doses tested, indicating lack of genotoxicity of this oil. However, other genotoxicity tests must be performed to confirm this hypothesis.

In addition, the mutagenic effects of OPV were evaluated using the micronucleus test in peripheral blood, in which changes in DNA and/or damage to the spindle can be detected (Fenech, 2000). Moreover, in the present study OPV did not exhibit any mutagenic

Table 2
Frequency of damage, by the comet assay in mice treated orally with *P. vicosanum* (OPV).

Groups	Damaged cells	Classes of damage				Score
		0	1	2	3	
Negative control	16.70 ± 1.22[a]	83.30 ± 1.22	11.60 ± 1.76	5.10 ± 1.29	0.00 ± 0.00	21.90 ± 1.80[a]
Positive control	84.50 ± 1.32[b]	15.50 ± 1.32	42.20 ± 2.92	35.30 ± 3.22	7.00 ± 0.79	133.80 ± 3.75[b]
500 mg/kg OPV	15.20 ± 1.52[a]	84.80 ± 1.52	8.90 ± 0.72	5.60 ± 1.24	0.70 ± 0.33	22.20 ± 2.56[a]
1000 mg/kg OPV	17.20 ± 2.52[a]	82.80 ± 2.52	9.80 ± 1.70	5.60 ± 1.79	1.80 ± 0.74	26.40 ± 4.71[a]
2000 mg/kg OPV	18.30 ± 2.06[a]	81.70 ± 2.06	13.40 ± 1.62	3.80 ± 0.87	1.10 ± 0.52	24.30 ± 3.10[a]

[a] Values expressed as mean ± SEM, n = 10 animals/group. Different letters indicate statistically significant differences ($p < 0.05$; ANOVA/Tukey).
[b] Significantly different from negative control and OPV groups. Class 0 – no damage; class 1 – tail of comet shorter than the diameter of nucleoid; class 2 – tail of comet once or twice the diameter of nucleoid; class 3 – tail of comet more than twice the diameter of nucleoid.

Table 3
Frequency of micronuclei in mice treated orally with *P. vicosanum* (OPV).

Groups	Time (h)		
	24	48	72
Negative Control	5.20 ± 0.47[a]	5.50 ± 0.34[a]	5.10 ± 0.61[a]
Positive Control	35.20 ± 0.60[b]	27.90 ± 0.69[b]	27.40 ± 0.79[b]
500 mg/kg OPV	7.10 ± 0.58[a]	7.00 ± 0.85[a]	5.60 ± 0.54[a]
1000 mg/kg OPV	7.70 ± 0.79[a]	6.70 ± 0.63[a]	5.80 ± 0.44[a]
2000 mg/kg OPV	8.00 ± 0.85[a]	6.50 ± 0.62[a]	5.30 ± 0.50[a]

[a] Values expressed as mean ± SEM, n = 10 animals/group. Different letters indicate statistically significant differences. ($p < 0.05$; ANOVA/Tukey).
[b] Significantly different from negative control and OPV groups.

effects 24, 48 or 72 h after oral administration to mice.

The carrageenan-induced paw edema is a widely used method to evaluate nonsteroidal anti-inflammatory drugs. Anti-inflammatory drugs initially inhibit the cyclooxygenase enzyme, which is involved in prostaglandin synthesis (Anilkumar, 2010). Some species of the *Piper* genus have been tested for their anti-inflammatory activity, showing satisfactory results (de Queiroz et al., 2014; Tasleem, 2014; Iwamoto et al., 2015). The *P. nigrum* species presented anti-inflammatory activity through the paw edema method and this effect was attributed to presence of piperine (Tasleem, 2014). In our study, reduced carrageenan-induced paw edema was observed in both doses tested and this reduction after 2, 3 and 4 h of induction was dose-dependent.

The anti-inflammatory activity of OPV also was evaluated using the carrageenan-induced pleurisy model, which can confirm the results obtained by the paw edema test (Vinegar et al., 1973; Almeida et al., 1980). This is a classical test to assess this type of inflammation, forming an exudate in the pleural cavity characterized by infiltration of polymorphonuclear leukocytes, and the release of various important chemical mediators in the inflammatory process (Oliveira et al., 2012).

Anti-inflammatory drugs such as dexamethasone and indomethacin inhibit leukocyte migration between 3 and 6 h after

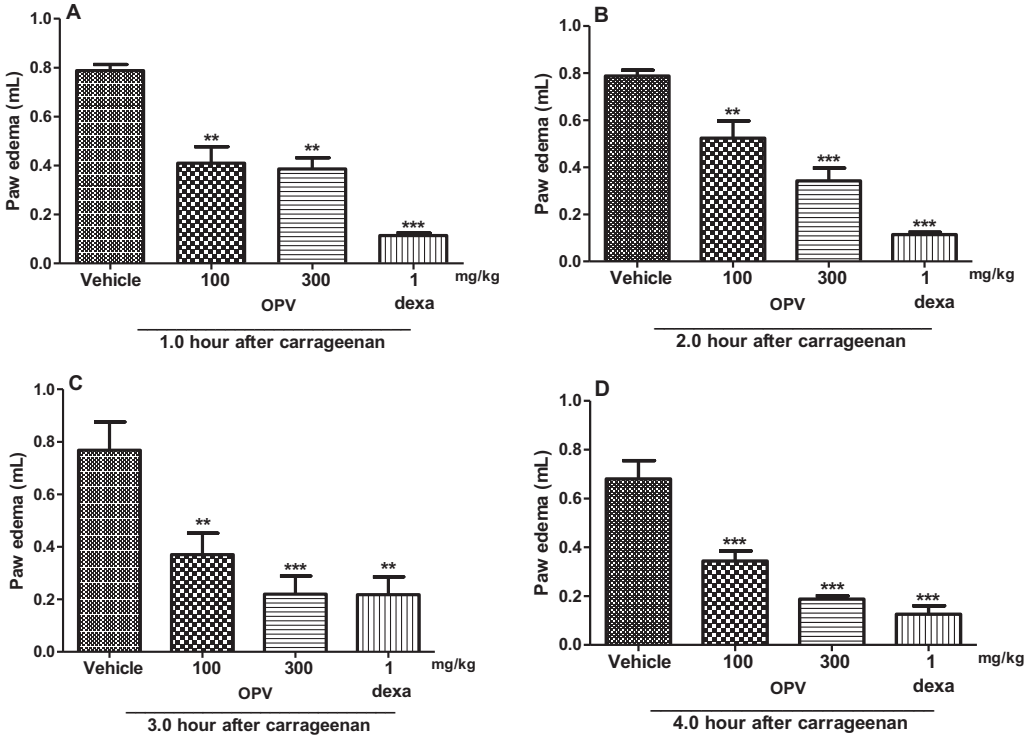

Fig. 1. Effect of oral administration of *P. vicosanum* (OPV) on carrageenan-induced paw oedema in rats. Animals received OPV (100 or 300 mg/kg, p.o.) or control (vehicle) or dexamethasone (DEXA, 1 mg/kg, s.c.) and after 1 h an intraplantar injection of carrageenan (300 μg/paw). Graphics (A), (B), (C), and (D) represent the evaluation of paw oedema after 1, 2, 3 and 4 h, respectively, after carrageenan injection. Each bar represents the mean ± SEM of 5 animals. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ when compared with the control group. One-way ANOVA followed by the Newman–Keuls test.

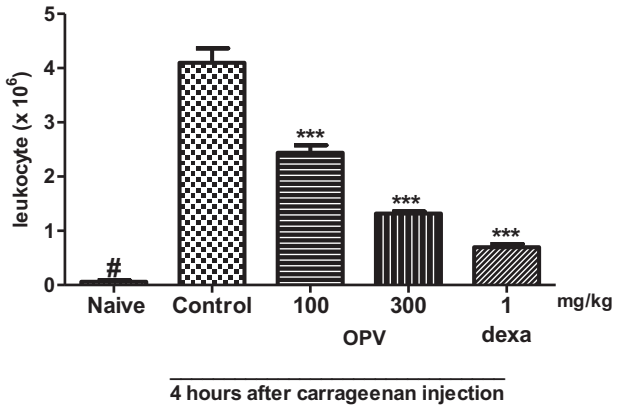

Fig. 2. Effect of oral administration of *P. vicosanum* (OPV) on the inhibition of the leukocyte migration at both doses tested on pleurisy test. Rats were treated 1 h before an intrapleural injection of carrageenan, with OPV (100 or 300 mg/kg, p.o.), dexamethasone (DEXA, 1 mg/kg, s.c.), or negative control (vehicle, saline solution). Naive group (# indicate statistic difference from vehicle group) received an intrapleural injection of sterile saline instead of carrageenan and also treated with saline solution. The bars express the mean ± SEM of 5 animals. $^{\#}p < 0.001$ when compared Naïve treated with negative control; $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$ when compared to negative control group. One-way ANOVA followed by the Newman–Keuls test.

carrageenan administration. Studies performed with the oil of *Piper aleyreanum* (1–100 mg/kg, p.o.) in rodents have shown that this oil decreased the total leukocytes count, neutrophils and mononuclear cells, through pleurisy tests, demonstrating the therapeutic potential of this plant (Lima et al., 2012). Similarly, *Piper marginatum* extract (0.5 and 1 g/kg) reduced the paw edema in rats by 80–90% in relation to the control group, but had a minor effect on the exudate volume and leukocytes migration in carrageenan-induced pleurisy (D'Angelo et al., 1997). However, in another study, Rodrigues Silva et al. (2008), found no anti-inflammatory activity in the hydroethanolic extract of *P. ovatum* leaves (500 mg/kg), since this extract did not reduce the pleural exudate volume.

In the present study, the treatment with OPV was able to significantly decrease the pleural exudate volume and the leukocyte recruitment in the pleural cavity at the two doses tested, and this effect was dose-dependent. These results, together with the paw edema test, reinforce the anti-inflammatory potential of this species.

Essential volatile oils, characteristic of most species of this genus, have also been object of several studies, revealing valuable biological activities and interesting compositions with monoterpenoids, sesquiterpenoids and seldom phenyl- and arylpropanoids (Mesquita et al., 2006). According to Mesquita et al. (2006), the composition of *P. vicosanum* leaves was dominated by monoterpenoids, with limonene and 1.8-cineole as major compounds. These data corroborate the results obtained in this study, in which the predominance of monoterpenoids (limonene) was identified in the OPV, in addition to sesquiterpenoids (ϒ-elemene and α-alaskene). It has been shown that D-limonene has immunomodulatory activity and inhibits the proinflammatory activities of CD4 + and CD8 + T lymphocytes (Lappas and Lappas, 2012) and exhibits antimicrobial effects, while β-elemene has potent anti-proliferation effects on some types of cancer cells (Lu et al., 2012). Thus, for these therapeutic properties, these compounds may be responsible for the effects found in this study.

The essential oil of *P. vicosanum* leaves showed anti-inflammatory activity which may be associated with the presence of most of the compounds identified in this study, such as limonene, ϒ-elemene and α-alaskene. Furthermore, OPV did not cause acute toxicity, genotoxicty or mutagenicity. More studies should be carried out to assess the exact mechanism of action responsible for the anti-inflammatory activity, as well as other aspects of toxicity.

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Transparency document

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Conflict of interest

The authors declare that there are no conflicts of interest.

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