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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