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Original Article

Schinus terebinthifolius: phenolic constituents and *in vitro* antioxidant, antiproliferative and *in vivo* anti-inflammatory activities

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ABSTRACT

Schinus terebinthifolius Raddi, Anacardiaceae, native to Brazil, is referred to as "pimento-rosa" and is used to treat inflammatory disease in folk medicine. Studies have reported important pharmacological properties, but these effects have still not been fully exploited. This study reports that the crude extract and isolated compounds of *S. terebinthifolius* (leaves) have *in vitro* antioxidant, antiproliferative, and *in vivo* anti-inflammatory activities. The samples were evaluated for antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl, β-carotene/linoleic acid and 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulphonic acid reagents. The anti-inflammatory effects were assayed against a carrageenan-induced paw oedema model in mice to test doses of 100 and 300 mg/kg at different time points in addition to myeloperoxidase activity analysis. The antiproliferative activity was evaluated using ten human tumour cell lines. Two derivatives of gallic acid and four flavonoids were isolated and exhibited considerable antioxidant activity. The extract and its compounds showed selectivity towards ovarian cancer cells, with GI_{50} values ranging from 1.9 to 6.5 μg/ml. Sample extracts and methyl gallate significantly inhibited carrageenan-induced oedema in the mice paw oedema experimental model. The calculated topological polar surface area for methyl gallate (86.98 Å^2) showed good intestinal absorption. The effects reported herein are be related to the presence of flavonoids and the galloyl phenolic derivative content.

Introduction

Schinus terebinthifolius Raddi, Anacardiaceae, is an evergreen shrub that grows in South and Central America. In Brazil, it is popularly known as "pimenta-rosa", "aroeira-vermelha", "aroeira-pimenteira", "aroeira-da-praia", "aroeira-negra" and/or "aroeira-de-minas" and used in folk medicine for treatment of several health disorders as well anti-inflammatory processes (Morton, 1978; Gazzaneo et al., 2005). Its biological applications have been described since the first edition of the Brazilian Pharmacopoeia, published in 1926. A Brazilian gel-based aqueous bark extract of *S. terebinthifolius* has been marketed since 1999 for the treatment of vaginitis and cervical vaginitis (Leite et al., 2011). The Brazilian Pharmacopoeia recommends the decoction of *S. terebinthifolius* for use as a natural anti-inflammatory agent (Santos and Amorim, 2002). Pharmacological studies with extracts obtained from leaves have reported antioxidant, anti-allergic, antimicrobial, anti-inflammatory, antiulcer and antiadherent properties as well as wound-healing properties (Castelo Branco Neto de et al., 2006; Carvalher-Machado et al., 2008; Gomes et al., 2010; Johann et al., 2010; Carvalho et al., 2013; Barbieri et al., 2014; Uliana et al., 2016). Chemical studies showed that polyphenolic and flavonoid are major constituents of the extracts of *S. terebinthifolius* leaves (Farag, 2008; El-Massry et al., 2009; Santana et al., 2012).

Investigations by our research group show that the essential oil of *S. terebinthifolius* fruits contains a predominance of monoterpenes, with β-pinene as the major constituent. This oil was effective against persistent inflammation caused by Complete Freund

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Adjuvant (CFA) or acute inflammation induced by carrageenan in the paw or in pouches (Formagio et al., 2011). In another study, the extract of the leaves has anti-inflammatory, immunomodulatory, chemopreventive, antigenotoxic and antimutagenic effects owing to phenol and flavonoid compounds (Fedel-Miyasato et al., 2014a,b).

The oxidative damage induced in cells and tissues is related to the aetiology of various diseases including inflammatory and cancer diseases. Thus, the natural products are candidates for these tests because the insertion of food compounds or phytopharmaceuticals may be an important alternative to treat inflammation and the prevention of cancer. Although cancer is a specific disease, it has been only slightly defined in terms of traditional medicine. Recent contributions to the armament of chemotherapeutic agents, in alliance with natural products approved as drugs in this 30-year time frame, include paclitaxel (Taxol®), isolated from *Taxus brevifolia*; the alkaloids vincristine and vinblastine from *Catharanthus roseus*; camptothecin and derivatives from *Camptotheca acuminata*; combretastatin from *Combretum caffrum* (Newman et al., 2005; Newman and Gragg, 2012); and curcumin from the rhizome of *Curcuma longa* (Aggarwal and Bharti, 2003) in addition to synthetic derivatives or combinations of agents such as flavopiridol and roscovitine (Newman et al., 2002; Dancey and Sausville, 2003), justifying the importance of the search for cancer therapy. Even if *S. terebinthifolius* has been proposed as a folk remedy in the treatment of inflammation, more studies must be reported. Therefore, we evaluated the *in vitro* antioxidant, antiproliferative and *in vivo* anti-inflammatory activities of methanolic extracts and compounds isolated from *S. terebinthifolius* (leaves). As a complement, a computational study for predicting the ADME properties of compounds was performed by determining the lipophilicity, topological polar surface area (TPSA), absorption (% ABS) and simple molecular descriptors using Lipinski's rule.

Materials and methods

Plant material

The leaves of *S. terebinthifolius* Raddi, Anacardiaceae, were collected at the Medicinal Plants Garden of Federal University of Grande Dourados (22°11′43.7″S, 54°56′08.5″W and 430 m) in November 2014. A voucher specimen was deposited in the Herbarium of the UFGD under the number DDMS 4600 and was identified by Dr. Maria do Carmo Vieira. Authorization for accessing and studying samples from the Brazilian genetic heritage site was obtained from the Brazilian government through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq authorization no. 010220/2015-1 – CNPq/CGEN/MMA).

Extraction, fractionation and identification procedures

Leaves (860 g) were dried and extracted by maceration with methanol, filtered, concentrated under reduced pressure and lyophilized to yield the methanolic extract (MEST) (42.7 g). The MEST (30 g) was partitioned with hexane, chloroform and ethyl acetate. The chloroform fraction (1.8 g) was submitted to column chromatography (CC) silica gel, yielding sitosterol-3-*O*-β-glucopyranoside (64 mg). Fractionation of part of the ethyl acetate fraction (6.2 g) by CC in silica gel was performed using a mixture of hexane/EtOAc and EtOAc/MeOH, in increasing polarity, to afford compounds **1** (25.4 mg), **2** (19.4 mg) and **3** (12.2 mg). The hydromethanolic fraction (13 g) was purified by successive CC on Sephadex LH-20 using H_2O, H_2O/MeOH 7:3–3:7, and MeOH as eluents, yielding compounds **4** (22.8 mg), **5** (17.8 mg) and **6** (26 mg). The ^{1}H and ^{13}CNMR spectra were collected using a Varian Mercury Plus BB spectrometer operating at 300 MHz and 75.5 MHz using CD_3OD as the solvent and tetramethylsilane (TMS) as the internal standard.

HPLC analysis

The LC data with MEST include caffeic acid (Rt = 14.78 min), *p*-coumaric acid (Rt = 25.82 min), luteolin (Rt = 62.08 min), quercetin (Rt = 64.62 min) and apigenin data (Rt = 66.79 min). These data were described in a previous report by our research group (Fedel-Miyasato et al., 2014a).

In vivo *anti-inflammatory activity*

Animals

Male *Swiss* mice (25–35 g) were used for *in vivo* anti-inflammatory evaluation and were provided by the Universidade Federal da Grande Dourados. The mice were kept under a 12 h light-dark cycle with controlled humidity (60–80%) and temperature (22 ± 1 °C). Two hours before the experiments, the animals were placed in the laboratory and were used only once for experiments (n = 5/group). All experimental procedures were performed in accordance with the U.S. National Institute of Health and were approved by the ethics committee for research on laboratory animals of the UFGD (Nbr. 005/2010).

Carrageenan-induced paw oedema

Five groups of Swiss mice were orally treated with MEST (100 and 300 mg/kg) and **2** (100 and 300 mg/kg) as well as a vehicle. Two groups were treated intraplantarly with **2** (10 and 100 mg/kg). One group of mice was treated subcutaneously with an anti-inflammatory positive control drug dexamethasone (1 mg/kg). After 1 h, the animals received an intraplantar injection (50 μl) of a solution of carrageenan (300 μg/paw, diluted in sterile 0.9% saline) into the right hind paw. The contralateral paw received only saline and was used as a control.

The oedema was the difference in thickness of both paws using a digital micrometre (DIGIMESS 110-284) at several time points (0.5, 1, 2, and 4 h) after carrageenan injection. The results were expressed in μm (Kassuya et al., 2009).

Myeloperoxidase (MPO) activity

MPO activity was measured in the paw after 6 h to evaluate indirect neutrophil migration to this tissue (De Young et al., 1989). The paw tissue was homogenized in 5% (w/v) 80 mM phosphate buffer at pH 5.4 containing 0.5% of hexadecyltrimethylammonium bromide. The homogenate was centrifuged at 3200 × *g* and 4 °C for 20 min. Thirty microliters of each supernatant was mixed with 100 μl of 80 mM phosphate buffer, 85 μl of 0.22 M phosphate buffer and 15 μl of 0.017% H_2O_2 in a 96-well plate. The reaction was initiated with 20 μl of 3,3,3-tetramethylbenzidine (dissolved in N,N-dimethylformamide). The plate was maintained at 37 °C for 3 min, after which the reaction was interrupted by adding 30 μl of 1.46 M sodium acetate (pH 3.0). The enzymatic activity was determined by measuring the optical density at 630 nm and was expressed as mOD/mg of protein.

In vitro *antiproliferative activity*

MEST and other compounds were assessed in the following ten human tumour cell lines from various tissues, kindly provided by the National Cancer Institute (Frederick, MA, USA): U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (ovarian expressing the multiple drug resistance phenotype), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon),

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