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Original Article

Antitumor activity and toxicity of volatile oil from the leaves of *Annona leptopetala*

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ABSTRACT

Annona leptopetala (R.E.Fr.) H. Rainer, Annonaceae, is used in folk medicine like antitumor and anti-inflammatory. The aim of this study was to determine chemical composition, toxicity and antitumor activity of *A. leptopetala* leaves volatile oil. Fresh leaves were hydrodistilled and then the volatile oil chemical composition was assessed by gas chromatography and mass spectrometry. Toxicity was assessed using haemolysis, micronucleus and acute toxicity protocols. Antitumor effects were determined *in vitro* and *in vivo*, using sulforhodamine B assay and sarcoma 180 murine tumor model, respectively. Spathulenol was the major component identified (12.56%). The volatile oil showed *in vitro* antitumor activity mainly in leukemia cell line (K-562), with Total growth inhibit (TGI) (concentration producing TGI) of 0.64 μg/ml. In other hand, the volatile oil <250 μg/ml did not inhibit HaCat non-tumor cell line growth. The concentration that produced 50% haemolysis was 372.8 μg/ml. The 50% lethal dose in mice was approximately 447.2 mg/kg intraperitoneally. Sarcoma 180 tumor growth inhibition rates were 59.29% and 58.77% at 100 and 150 mg/kg intraperitoneally, respectively. The volatile oil presented moderate gastrointestinal toxicity and no genotoxicity was observed at 350 mg/kg. Thus, the volatile oil shows antitumor activity with moderate toxicity.

Introduction

Annona leptopetala (R.E.Fr) H. Rainer, Annonaceae, commonly known as "pinha-brava" is a tree or shrub endemic in Brazil used in folk medicine like antitumor and anti-inflammatory (Agra et al., 2007; David et al., 2007). Antioxidant and *in vivo* antitumor activities for extracts from *A. leptopetala* have been reported (David et al., 2007; Costa et al., 2012), in addition to antispasmodic effect in guinea pig ileum (Monteiro et al., 2008).

Different compounds has been obtained from *Annona* genus, such as flavonols (Júnior et al., 2016; Novaes et al., 2018), terpenes (Santana et al., 2017), tannins, saponins, cardiac glycosides, monosaccharides, aromatic and phenolic amino acids, steroids (Agu and Okolie, 2017), isoquinolonic and indolic alkaloids (Kuo et al., 2001), lignoids (Fevier et al., 1999) and acetogenins (Mangal et al., 2016). Regarding volatile oils from *Annona* species, monoterpenes and sesquiterpenes were isolated, including β-elemene (Kossouoh et al., 2007), bicyclogermacrene (Siqueira et al., 2011), α-copaene (Costa et al., 2013) and α-phellandrene (Meira et al., 2014). In addition, literature data showed antitumor activity for other components of *Annona* volatile oil, such as α-terpineol (Hassan et al., 2010), spathulenol (Bomfim et al., 2016), *trans*-caryophyllene (Hadri et al., 2010) and germacrene-D (Salvador et al., 2011),

Considering that the theory of synergistic action associated with the antitumor activity of volatile oils appears to be rather than its components separately (Bhalla et al., 2013), this study determined the chemical composition, antitumor activity and toxicity of the volatile oil from *A. leptopetala* leaves (ALO).

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Materials and methods

Drugs and reagents

RPMI 1640 culture medium, glutamine, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and fetal bovine serum (FBS) were obtained from GIBCO (Carlsbad, CA). 5-Fluorouracil (5-FU), Triton X-100, Tween-80, cyclophosphamide, Trizma base and sulforhodamine (SRB) were purchased from Sigma–Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Kits for biochemical analysis were purchased from LABTEST (Lagoa Santa, MG, Brazil). Sodium thiopental (Thiopentax®) was purchased from Cristália (Itapira, SP, Brazil) and heparin (Parinex®) from Hipolabor (Sabara, MG, Brazil). Doxorubicin (DOX) was from Tecnofarma International (Uruguay) and trichloroacetic acid (TCA) from Merck (Darnstadt, Germany).

Plant material

Annona leptopetala (R.E.Fr.) H. Rainer, Annonaceae, leaves were collected in August 2016 in Serra Branca, Paraíba State, Brazil. The plant material was identified by Dr. Maria de Fátima Agra. Voucher specimen number AGRA 3567 was deposited at the Herbarium Lauro Pires Xavier of Federal University of Paraíba (UFPB), Brazil.

Hydrodistillation of the volatile oil

Fresh leaves (1000 g) were collected over ice and hydro-distilled using Clevenger type apparatus for 4 h, at a temperature of 40 °C, yielding 400 mg of the volatile oil (yield of 0.04% relative to the weight of fresh material used). The resulting oil was dried with anhydrous sodium sulfate, stored in amber bottle and kept at 4 °C lower temperature. Thereafter, ALO was submitted for Gas Chromatography with Mass Spectrometry (GC-MS) analysis.

GC-MS analysis

Analysis of the oil was carried out on a Shimadzu GC-MS instrument under the following conditions: DB-5 ms (30 m × 0.25 mm internal diameter, film thickness 0.25 μm), fused-silica capillary column, programmed temperature of 60–240 °C (3 °C/min), injector temperature at 220 °C, helium carrier gas adjusted to a linear velocity of 32 cm/s (measured at 100 °C), splitless injection (2 μl of hexane solution 1:1000), split flow adjusted to yield a 20:1 ratio, septum sweep constant at 10 ml/min, Electron Ionization Mass Spectrometry (EIMS) electron energy of 70 eV, ion source and connections at 200 °C. The quantitative data for the volatile constituents were obtained by peak-area normalization using a Focus Gas Chromatography with Flame Ionization Detector (GC/FID), operated under GC-MS similar conditions except for the carrier gas, which was nitrogen. The retention index was calculated for all the volatile constituents by volatile oil co-injection using an *n*-alkane (C8-C20, Sigma–Aldrich) homologous series applying the equation of Van den Dool and Kratz (1963). Individual components were identified by comparison of both mass spectrum and Gas Chromatography (GC) retention data with previously analyzed authentic compounds stored in our private library, as well as with the aid of commercial libraries containing mass spectra, and retention indices of volatile compounds commonly found in volatile oils (Adams, 2001).

Cell lines

The tumor cell lines used were: U251 – glioma, MCF-7 – breast, NCI/ADR-RES - multidrug-resistant ovarian, 786-0 – kidney, NCI-H460 – non-small cell lung cancer, PC-3 – prostate, OVCAR – ovarian, HT29 – colon and K562 – leukemia, and HaCaT human keratinocytes served as the normal cell line. The cells lines were cultivated in RPMI-1640 supplemented with FBS 10%, glutamine 2 mM, penicillin 100 U/ml, streptomycin 100 μg/ml and HEPES 2 mM, at 37 °C with CO_2 5%, in the Chemical, Biological and Agricultural Pluridisciplinary Research Center, State University of Campinas, Campinas, Brazil. Sarcoma 180 tumor cells were maintained in the peritoneal cavity of Swiss mice.

Animals

Swiss albino mice (*Mus musculus*), females (36–42 g), were obtained from the Dr. Thomas George Bioterium of Research Institute in Drugs and Medicines of Federal University of Paraíba, Brazil. The animals were randomly housed in cages containing six animals with free access to food and water. All animals were kept on a 12 h/12 h off light-dark cycle (lights on at 6 am). All procedures were previously approved by the Animal Studies Committee (CEUA) of UFPB, n°. 0912/10.

Haemolysis assay

The haemolytic activity of ALO was tested using mouse erythrocytes according to Kang et al. (2009). Erythrocytes from fresh blood samples were suspended in phosphate buffered saline (PBS) to make a 1% (v/v) solution. Red blood cell suspension was incubated with various concentrations (0–750 μg/ml) of ALO dissolved in DMSO (5%, v/v, in PBS) in plates on a shaker for 60 min and then centrifuged. The absorbance of the supernatants was read at 540 nm using a UV–vis spectrophotometer (UV-1650PC Shimadzu) to measure the extent of red blood cell (RBC) lysis, and the concentration producing 50% haemolysis (HC_{50}) was determined. Positive controls (100% haemolysis) and negative controls (0% haemolysis) were also determined by incubating erythrocytes using Triton X-100 1% in PBS and DMSO 5% in PBS, respectively. The haemolysis assay was performed in quadruplicate and repeated three times.

In vitro *antitumor activity*

The sulforhodamine B assay (SRB) was performed as described by Monks et al. (1991). This assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by TCA. SRB is a bright-pink aminoxanthene dye with two sulphonic groups that bind to basic amino acid residues under acidic conditions and dissociate under basic conditions.

Cells in 96-well plates (100 μl cells/well) were exposed to different concentrations of ALO (0.25, 2.5, 25 and 250 μg/ml) in DMSO/RPMI/FBS 5% at 37 °C and CO_2 5%, for 48 h. Final DMSO concentration did not affect cell viability. Cells were then fixed with TCA solution (50%, v/v), and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content. DOX (0.025–25 μg/ml) was used as the positive control. Three measurements were obtained: at the beginning of incubation (T0) and 48 h post-incubation for compound-free (C) and exposed (T) cells. Cell proliferation was determined according to the equation: cell proliferation = $100 \times [(T - T0)/C - T0]$. The cytostatic effect was observed when $T0 \leq T < C$, while cytocidal effect occurred when $T < T0$. The experiments were done in triplicate to calculate the total growth inhibition (TGI) (concentration that produces TGI).

Acute preclinical toxicity

The evaluation of acute preclinical toxicity of ALO was performed according to the Guide for the conduct of non-clinical toxicology studies and safety pharmacology necessary for the

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