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Original article

Triterpenes from the *Protium heptaphyllum* resin – chemical composition and cytotoxicity

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ABSTRACT

Protium heptaphyllum (Aubl) Marchand, Burseraceae, is popularly used as an analgesic and anti-inflammatory agent. However, the cellular mechanism of action remains unknown. This study aims to evaluate the chemical composition of *P. heptaphyllum* resin and cytotoxicity on a breast cancer cell line (MCF-7). The chemical composition of the resin was determined by Gas Chromatography coupled to a Mass Spectrometer. The cytotoxicity was evaluated using an MTT assay. Annexin V-FITC, caspase-3, Angiotensin Converting Enzyme activity and Tumor Necrosis Factor alpha (TNF- α) assays were performed to evaluate apoptosis and inflammatory events. The resin consisted of triterpenes, such as α- and β-amyrin. Cytotoxicity was only observed in fractions enriched with α- and β-amyrin. The resin and fractions elicited antiproliferative activity, increased activity of caspase-3 and ACE, and a decrease in the TNF-α level. Altogether, the resin and fractions enriched with α- and β-amyrin promoted cytotoxicity and apoptosis.

Introduction

Protium heptaphyllum (Aubl.) Marchand, Burseraceae, known as "almécega", "breu", and "almíscar", originated in South America, exudes an oily resin composed of a mixture of triterpenes from the α-amyrin (ursane) and β-amyrin (oleane) series, and an essential oil rich in mono- and sesquiterpenes (Siani et al., 1999a; Maia et al., 2000). Ethnopharmacological studies report the use of this type of resin for ulcer treatment; there are also reports of its use as an analgesic and anti-inflammatory agent (Correa, 1978; Brandão et al., 2008).

The anti-inflammatory effect of the mixture of α-and β-amyrin, the essential oil, and the crude resin of this plant has been previously reported (Siani et al., 1999b; Aragão et al., 2007). Research aimed at minimizing the effects of breast cancer and the inhibition, reversal or delay of its appearance has received wide interest, and natural products have been used for these purposes (Newman and Cragg, 2012). The role of inflammation in the pathogenesis of human cancers is well established (Allavena et al., 2008). Compounds including inflammatory eicosanoids, reactive oxygen species (ROS) and cytokines are involved in this process, and their levels are endogenously regulated (Basu et al., 2013).

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Antitumor activity is possibly a result of the inhibition of inflammation produced by the tumor, the suppression of the expression of certain oncogenes, the activation of a suppressor, and the modulation of defense systems, including antioxidant and immune functions (Liu et al., 1994). The participation of the rennin angiotensin system (RAS) as a pro-apoptotic and anti-apoptotic agent in tumor cells is not well understood (George et al., 2010).

Considering the anti-inflammatory activity of *P. heptaphyllum* resin, the present study aimed to evaluate the chemical composition, cytotoxicity, and pro-apoptotic nature of the crude resin and other fractions on a mammary adenocarcinoma cell line (MCF-7). The studied fractions were enriched with the isomers α- and β-amyrin from *P. heptaphyllum*. The participation of ACE and TNF-α in these events was also evaluated.

Materials and methods

Plant material

The resin of the stem of the species of *Protium heptaphyllum* (Aubl.) Marchand, Burseraceae, was collected in May 2009 on the Ilha de Guriri, Espírito Santo. A specimen was deposited at the herbarium of the University of Vila Velha (UVV/ES 1802) and identified by botanist Solange Zanotti Schneider.

Fractionation of the resin

An aliquot of the resin obtained from the stem of *P. heptaphyllum* (90 g) was dissolved in dichloromethane to eliminate impurities, such as sand and pieces of wood. The clear resin (78.1 g) was subjected to chromatographic separation in a silica gel column (162 g) using a pentane: CH_2Cl_2 gradient (100:00 - 00:100), followed by a CH_2Cl_2:EtOAc gradient (10:00 - 00:10). Fractions of 20 ml, eluted with *n*-hexane: EtOAc (4:1), were collected and then analyzed by thin layer chromatography (TLC) (silica gel 60 F254), yielding 38 fractions which were combined into fifteen primary fractions (FR1-FR15) based on the TLC patterns.

The F2 fraction obtained from the pentane: CH_2Cl_2 (3:2) gradient was re-chromatographed with pentane and CH_2Cl_2 (10:00 - 00:10) elution gradient on silica gel (9.9 g), yielding 165 fractions to provide five grouped (FR2-1 to FR2-5) fractions according to their chemical profile by TLC. The F2-3 fraction (7g) obtained from the elution with a pentane: CH_2Cl_2 (3:2) gradient was re-chromatographed on a column of silica gel (106.3 g) using isocratic elution with CH_2Cl_2:EtOAc:MeOH (1.8:5.0:0.1), resulting in 44 fractions, combined into eight fractions (FR2-3-1 to FR2-3-8). In an attempt to isolate the isomeric constituent, re-fractionation of the FR2-3-4 fraction (4.1 g) was performed on silica gel, using an elution gradient of pentane:CH_2Cl_2, CH_2Cl_2:$CHCl_3$, $CHCl_3$, and $CHCl_3$:MeOH, yielding nineteen fractions (F1 to F19). However, even after subsequent attempts to isolate the isomers, they remained a mixture, as analyzed by GC-MS. The F12 and F14 fractions resulting from the elution of $CHCl_3$ and $CHCl_3$:MeOH, respectively, were mixtures with majority of two triterpenes (Fig. 1) and were utilized in the bioassays.

Gas chromatography coupled to Mass spectrometry (GC/MS)

The analysis of the chemical constituents of the resin was performed using a gas chromatograph (Trace Ultra, ThermoScientific®) coupled to a mass spectrometer (DSQII, ThermoScientific®). The volatile substances were separated on a DB-5 capillary column (30 m × 0.25 mm d.i. × 0.25 μm, J&W Scientific®, Folson, California, USA). The initial temperature was 70°C for 5 min, and the temperature was then increased to 250°C using a temperature ramp of 3°C/min before reaching and keeping the final temperature for 5 min. We used helium gas with a constant flow of 1 ml/min. The injector temperature was maintained at 220°C, and the temperature of the GC/MS interface was maintained at 250°C. The mass detector was operated by ionization with electron impact (+70 eV) using the scan mode, held at 35-450 MHz. The samples were diluted with hexane (1 mg/ml) and injected into the GC/MS in duplicate; 1.0 μl was injected with the injector in *splitless* mode. The identification of the substances contained in the resin was performed by comparing the similarity of the obtained mass spectra obtained with those in the literature (Adams, 2001; NIST/EPA/NIH, 2005) (Fig. 1). The relative percentages of these compounds were calculated from the mean areas of the chromatograms.

Cell line

A mammary adenocarcinoma (MCF-7, ATCC-HTB22) cell line was used and maintained in Dulbecco's Modified Eagle's Medium (DMEM) culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with a 10 ml solution of penicillin G, streptomycin and L-glutamine (Sigma-Aldrich, St. Louis, MO) and 20% fetal bovine serum (Gibco, Invitrogen Corporation, Grand Island, NY).

Preparation of samples for assays

The test samples used in the biological assays were fractions containing the isomers α- and β-amyrin (F12 and F14) and the crude resin (RES). These were dissolved in PBS, dimethylsulfoxide (DMSO) (0.09%) and propylene glycol (1%). The final concentration of dichloromethane in the assay was less than 0.003%.

Cellular cytotoxicity assay with colorimetric method of the MTT

Cytotoxicity was determined using the colorimetric MTT (3-bromide-[4.5-dimethyl-thiazol-2-yl]-2.5-diphenyl-tetrazolium) method (Sigma-Aldrich, St. Louis, MO), in which the tetrazolium salt is converted into the formazan salt by living cells, turning the culture blue (Mosmann, 1983). MCF-7 tumor cells were plated in sterile 96 well plates at a concentration of 5×10^4 cells/ml. Then, 10 μl samples of RES, and *P. heptaphyllum* fractions F12 and F14 (final concentration of 1.71 to 40 μg/ml) were added. The microplate was incubated at 37°C in 5% CO_2 for 72 h. Doxorubicin (DOX) (Sigma-Aldrich,