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Original article

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

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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.

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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 \times 0.25 mm d.i. \times 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 $\mu\text{g/ml}$) were added. The microplate was incubated at 37°C in 5% CO_2 for 72 h. Doxorubicin (DOX) (Sigma-Aldrich,

St. Louis, MO; 98-102%) was used as a positive control at a final concentration of 0.9 μ M (Zheng et al., 2012). Then, 10 μ l of MTT solution (5 mg/ml) diluted in DMEM was added, and the plate was incubated for 4 h. Finally, we added 150 μ l of isopropanol acidified with 0.04 M HCl and evaluated the samples with an ELISA reader (Thermoplate TP-Reader at 570 nm). The analyses were performed in triplicate, and the percentage of inhibition was calculated using the formula below: % inhibition = (absorbance of negative-control well) \times 100 negative control.

Determination of apoptosis by flow cytometry analysis

Apoptosis was assessed using an annexin V-FITC Kit (Sigma Aldrich, St. Louis, MO), according to the manufacturer's instructions. Briefly, MCF-7 cells were plated in a sterile 24-well plate at a concentration of 5×10^6 cells/ml. Then, the samples RES (10 μ l), and *P. heptaphyllum* fractions F12 and F14 (final concentration 40 μ g/ml) were added. A solution of hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) (final concentration of 10 μ M) was used as positive control. The plate was incubated at 37°C in 5% CO₂ for 72 h. After this period, the cells were colored with FITC-conjugated annexin V and propidium iodide for 15 min at room temperature, protected from light, and then analyzed by flow cytometry. The percentage of positive cells determined over 10,000 acquired events was analyzed by a FACSCalibur system equipped with a 488 nm argon laser and FCS Express 4 FlowCytometry software (De Novo Software, Los Angeles, CA).

Determination of caspase-3 for colorimetric assay

The caspase-3 activity was determined using a colorimetric method in which the presence of caspase-3 lysate produces p-nitroaniline (pNa), which generates a yellow color. MCF-7 cells were plated in a sterile 24-well plate at a concentration of 1×10^5 cells/ml. Then, samples of RES (10 μ l), and the *P. heptaphyllum* fractions F12 and F14 (final concentration of 40 μ g/ml) were added. As a positive control, we used DOX (final concentration of 0.9 μ M). The plate was incubated at 37°C in 5% CO₂ for 72 h. After this period, the determination of caspase-3 was performed according to the manufacturer's specification (Sigma-Aldrich, St. Louis, MO) using an ELISA reader (TP-Thermoplate Reader at 405 nm). The analyses were the average of eight replicates, and the results were expressed in Δ mOD_{405nm}/min (Posmantur et al., 1998).

Determination of TNF- α

To quantify the levels of TNF- α , MCF-7 tumor cells were plated in a sterile 24-well plate at a concentration of 1×10^6 cells/ml. Then, samples of RES (10 μ l), and the *P. heptaphyllum* fractions F12 and F14 (final concentration 40 μ g/ml) were added, and the plate was incubated at 37°C in 5% CO₂ for 72 h. As a positive control, we used DOX (final concentration of 0.9 μ M). After this period, we proceeded to read the plate in an ELISA reader (TP-Thermoplate Reader at 450 nm) using a commercial kit (Invitrogen, San Jose, USA) according to the manufacturer's instructions. Analyses were performed in replicates of 8.

ACE activity assay

Angiotensin Converting Enzyme (ACE) activity was determined by measuring the Gly-Gly (glycyl-glycine) cleavage product Hip-Gly-Gly (Hippuryl-glycyl-glycine) with ACE as described by Serra et al. (2005), with modifications. Briefly, the MCF-7 tumor cells were plated in a sterile 24 well plate at a concentration of 5×10^5 cells/ml, and 10 μ l samples of RES, and the *P. heptaphyllum* fractions F12 and F14 (final concentration of 40 μ g/ml) were added. DOX was used as a comparison known compound (final concentration of 0.9 μ M). The plate was incubated at 37°C in 5% CO₂ for 72 h. For the enzymatic reaction, an aliquot of supernatant was combined with the assay buffer and Gly-Gly substrate solution (100 mM, Sigma Aldrich, St. Louis, MO). After homogenization, the mixture was incubated for 35 min at 37°C. The reaction was stopped by the addition of solutions of sodium tungstate (300 mM) and sulfuric acid (0.33 mM). The system was mixed with 2,4,6-trinitrobenzene sulfonic acid (TNBS) staining reagent (Sigma Aldrich, St. Louis, MO). After 20 min under light, the plate absorbance was read in an ELISA reader (TP-Reader Thermoplate at 405 nm). The blank solution was prepared similarly except the sodium tungstate and sulfuric acid solutions were added before the enzyme solution. The assay was also carried out in the presence of 10 μ l of a solution of captopril (final concentration of 64 nM). The assays were performed in replicates of 8. The ACE activity was calculated according to the equation: % activity = (A \times 100) / B, where A is the measured absorbance at 405 nm of the well of cells, and B is the absorbance of rabbit lung.

Statistical analysis

The results were expressed as the mean, plus or minus the standard error of the mean (SEM) and \pm the standard deviation (SD). They were analyzed by one-way ANOVA. The significance of the difference between the means was determined using the *post-hoc* Tukey method, adjusted for multiple comparisons with significance above 5% ($p < 0.05$).

Results and discussion

Identification of the fractions of the *P. heptaphyllum*

The resin of *Protium heptaphyllum* (Aubl.) Marchand, Burseraceae, was dissolved in dichloromethane and subjected to a series of chromatographic techniques, producing fractions enriched in triterpenes. Isolation purification was not possible due to the presence of isomers. The fractions F12 and F14, evaluated in the bioassays, were analyzed by GC-MS; the major components identified were the isomers α - and β -amyrin in various proportions. The compound identification was performed by comparing the spectra with the NIST library of MS software, AMDIS version 2.1. The ratio of α - and β -amyrin was 3:1 in fraction F12 and 2:1 in fraction F14 (Fig. 1).

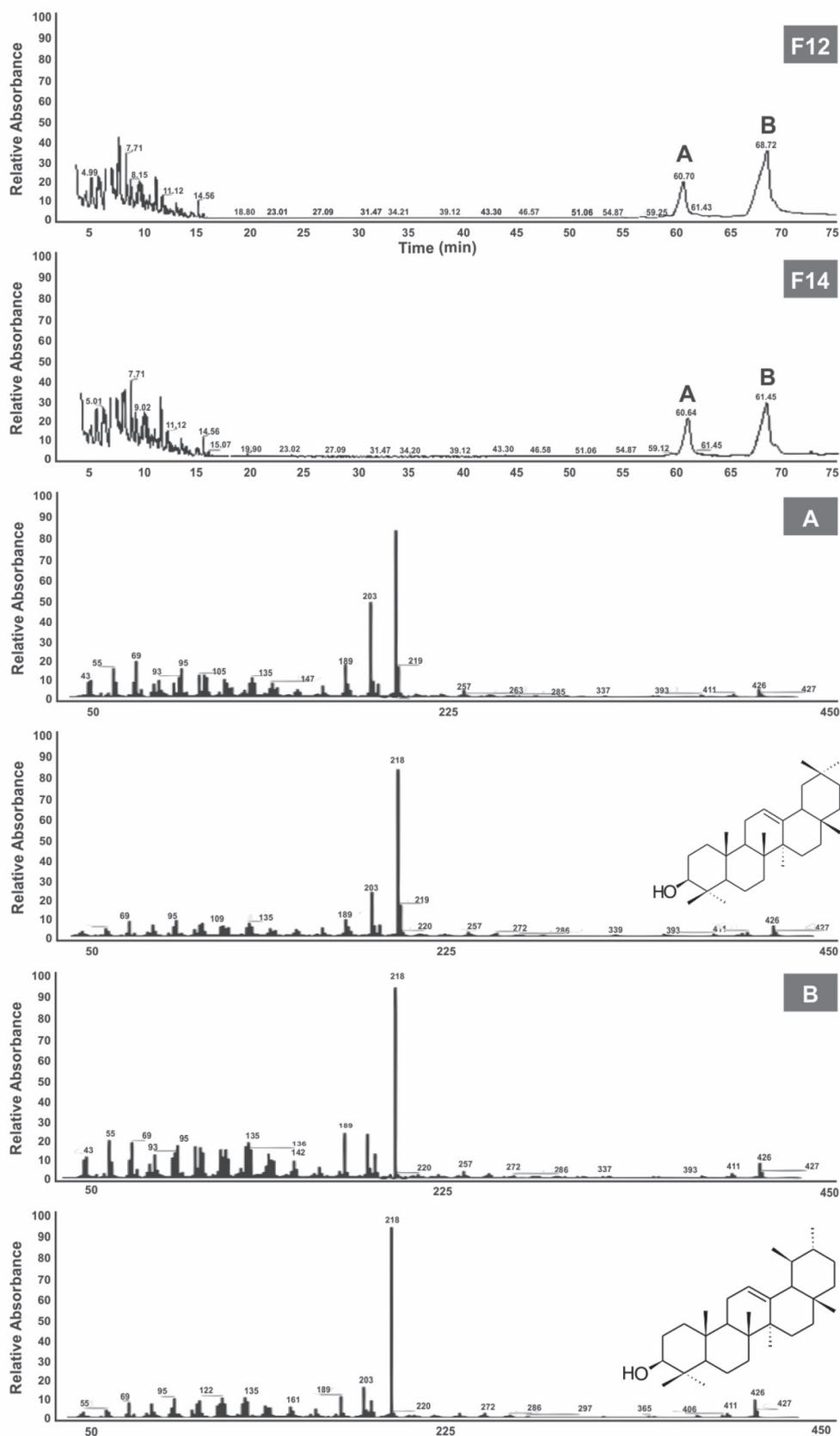


Figure 1 – Profiles of fractions F12 and F14 of *Protium heptaphyllum* with the GC-MS attributions of the components detected and the MS spectra of β -amyrin (A) and α -amyrin (B).

Other studies have identified and isolated triterpenes from *P. heptaphyllum* resin, including the isomers α - and β -amyrin from the resin of this same species (Maia et al., 2000; Susunaga et al., 2001; Vieira Junior et al., 2005).

Cellular cytotoxicity by resin and fractions of the *P. heptaphyllum*

In the assay for cell viability using MTT, the *P. heptaphyllum* resin did not exhibit significant cytotoxicity against MCF-7 cancer cells at a concentration of 40 $\mu\text{g/ml}$. However, fractions F12 and F14 showed weak cytotoxicity, with IC_{50} values of 34.8 ± 2.9 and 38.2 ± 3.4 $\mu\text{g/ml}$, respectively. These values are in accordance with the concentrations recommended by the American National Cancer Institute (NCI) ($\text{IC}_{50} < 20$ $\mu\text{g/ml}$ for the extract and fractions) (Boyed, 1997). Furthermore, when those compounds were evaluated separately, each one, α -amyrin (IC_{50} 2.35 μg) and β -amyrin (IC_{50} 2.48 μg), showed a potent cytotoxic activity on breast cancer cell line, MCF-7 (El-Alfy et al., 2011). Nevertheless, the data El-Alfy et al. (2011) were not depicted as a concentration.

Activation of caspase-3 and apoptosis by resin and fractions of *P. heptaphyllum*

The inhibition of cell proliferation by activation of caspase-3 and the translocation of phosphatidylserine were observed in the fractions containing α - and β -amyrin (Figs. 2 and 3), suggesting that the induction of cell death by apoptosis is caspase-dependent. Also, increased levels of apoptosis have been associated with cell growth inhibition, indicating that the fractions are potentially oncogenic (Campisi, 2005).

The fraction with a higher percentage of α -amyrin (F12) showed greater apoptotic activity and greater induction of caspase-3. Curiously, at a concentration of 40 $\mu\text{g/ml}$, the resin showed no significant cytotoxicity, but induced the highest rate of apoptosis observed in all of the fractions, but minimally activated caspase-3 (Figs. 2 and 3).

This result may indicate that the resin promotes apoptosis by caspase-dependent and caspase-independent mechanisms simultaneously. Liang et al. (2001) concluded that MCF-7 cells may undergo apoptosis by sequential activation of effector

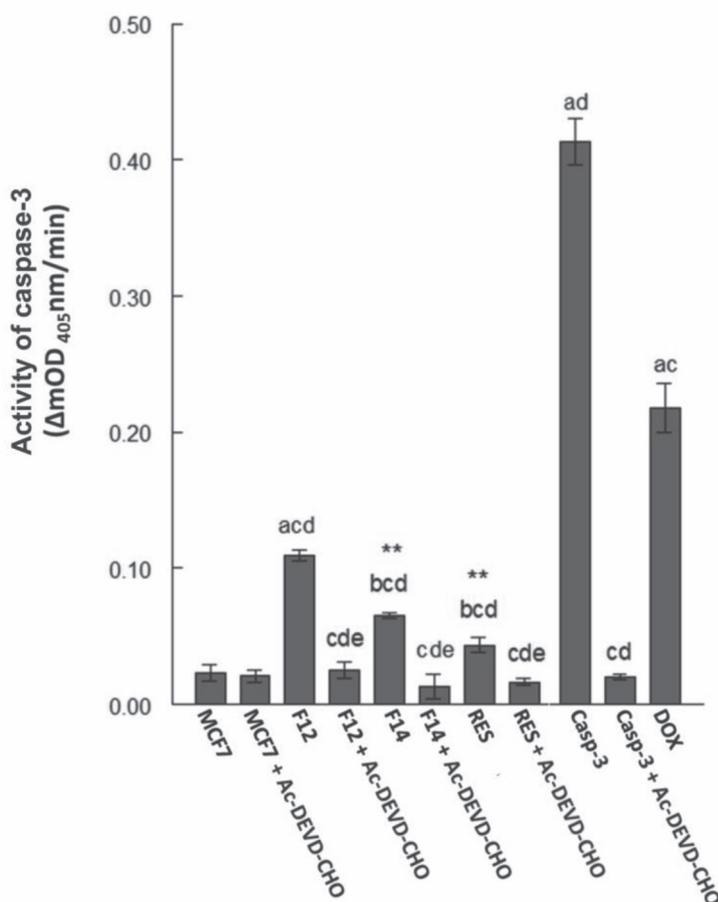


Figure 2 – Caspase-3 activity by ELISA in a breast cancer cell line (MCF-7). The cells were treated with essential *Protium heptaphyllum* crude resin (RES) and its fractions, enriched with α - and β -amyrin (F12 and F14). Ac-DEVD-CHO (caspase-3 inhibitor) and doxorubicin (DOX) were used at 40 $\mu\text{g/ml}$ for 72 h. The values represent the means \pm SEM; ^a $p < 0.01$ and ^b $p < 0.05$ compared to MCF-7. ^c $p < 0.01$ compared to CASP3. ^d $p < 0.01$ compared to DOX. ^e $p < 0.01$ compared to treatment of fractions with and without inhibition of CASP3. ^{**} $p < 0.01$ in relationship to fraction F12.

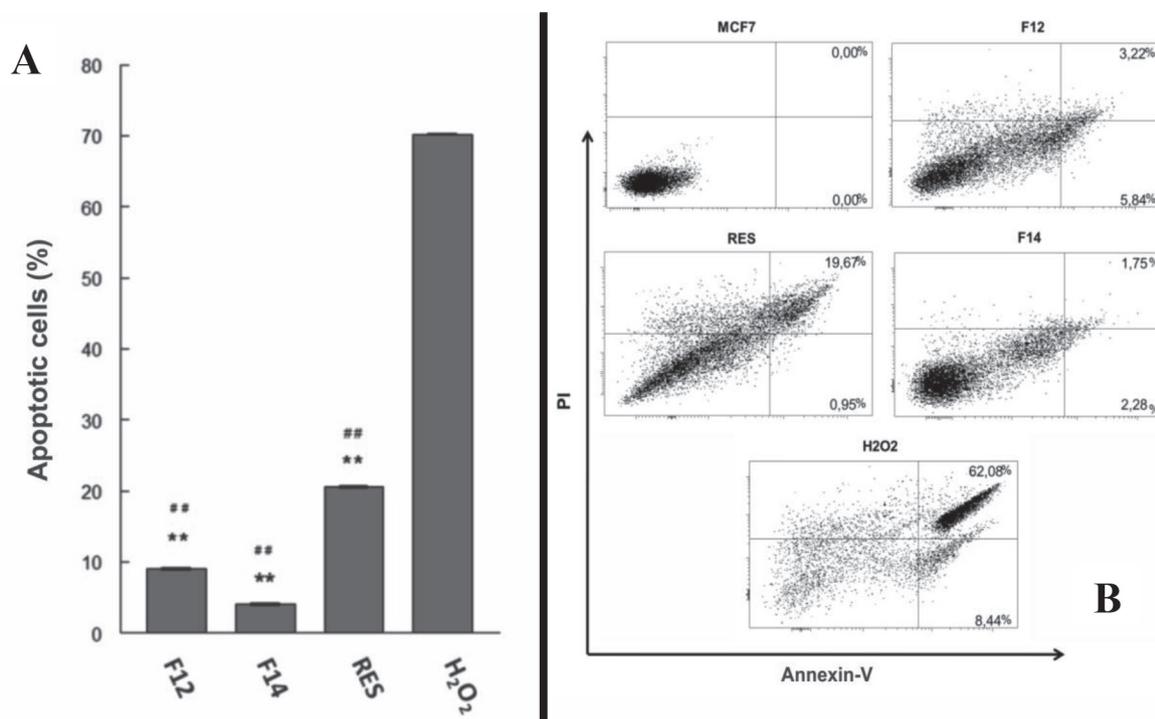


Figure 3 – Analysis of apoptosis by flow cytometry in a breast cancer cell line (MCF-7). The cells were treated with the *Protium heptaphyllum* crude resin (RES) and the fractions enriched in α - and β -amyryn (F12 and F14) at 40 μ g/ml, and hydrogen peroxide (H₂O₂) for 72 h. After the treatment period of 72 h, the cells were labeled with Annexin V-FITC and propidium iodide (PI) and then analyzed by flow cytometry. Cells in the initial stage of apoptosis were defined as Annexin-V (+) / PI (-), while late apoptotic cells were defined as double positive. **A**, representative data from three independent experiments. **B**, percentage of apoptotic cells (the sum of Annexin-V (+) / PI (-) and double positive). The values represent the means \pm SEM; ** p < 0.01 compared to H₂O₂. ## p < 0.01 between the different fractions.

caspases-7 and -6. Another proposed mechanism is the modulation of apoptosis through certain signaling proteins, such as NF- κ B, Akt, and p53, which act simultaneously through various pathways (Reed, 2003). Furthermore, the resin could induce apoptosis independently from caspase activation through factors such as endonuclease G (EndoG) and apoptosis inducing factor (AIF). EndoG and AIF are two mitochondrial mediators of apoptosis that have the capacity to produce DNA fragmentation on a large scale when translocated into the nucleus (Kroemer et al., 2007).

There are reports that suggest that MCF-7 cells are incapable of activating caspase-3 by genetic deletion (Mooney et al., 2002; Simstein et al., 2003). However, other studies have demonstrated cytotoxicity in MCF-7 cells with substances that interact with caspase-3 during apoptosis (Yang et al., 2006; Abu Bakar et al., 2010), confirming the data obtained in this study.

Reduction of TNF- α level by resin and fractions of *P. heptaphyllum*

Pinto et al. (2008) demonstrated the anti-inflammatory potential of the isomeric mixture of α - and β -amyryn in an animal model of acute peritonitis showing that TNF- α levels are reduced after treatment. The results of the present study show that treatment with the crude resin and its fractions

is capable to reduce the TNF- α level, thereby inhibiting preexisting inflammatory processes in MCF-7 cells (Fig. 4).

The local administration of low concentrations of TNF- α shows a potent anti-tumor effect with antiangiogenic action (Lejeune et al., 1998). The combination of doxorubicin with target-TNF- α on H22 allografted tumor showed a stronger antitumor effect than the single doxorubicin agent alone (Jiang et al., 2014). However, its endogenous production increases the development and proliferation of tumor (Ryuto et al., 1996; Samaniego et al., 1997; Yoshida et al., 1997; Leek et al., 1998; Relf et al., 1997). In the present study, doxorubicin decreases the level of endogenous TNF- α .

Alvarez-García et al. (2012) demonstrated that TNF- α may be produced endogenously by MCF-7 breast cancer cells, which was also observed in the results of this study (Fig. 4). Shishodia et al. (2003) suggested that ursolic acid is able to reduce the production of inflammatory cytokines such as TNF- α , thereby inhibiting the activation of nuclear transcription factor (NF- κ B), a regulatory factor for several genes that mediate the process of tumorigenesis. Thus, the cytotoxic and pro-apoptotic activities of the doxorubicin, resin and its fractions may be related to the reduction of TNF- α in MCF-7 cells, all of them with the same potency.

Recent research showed that doxorubicin can induce cell death in MCF-7 cell by Akt/ERK-mediated and Fas/FasL-

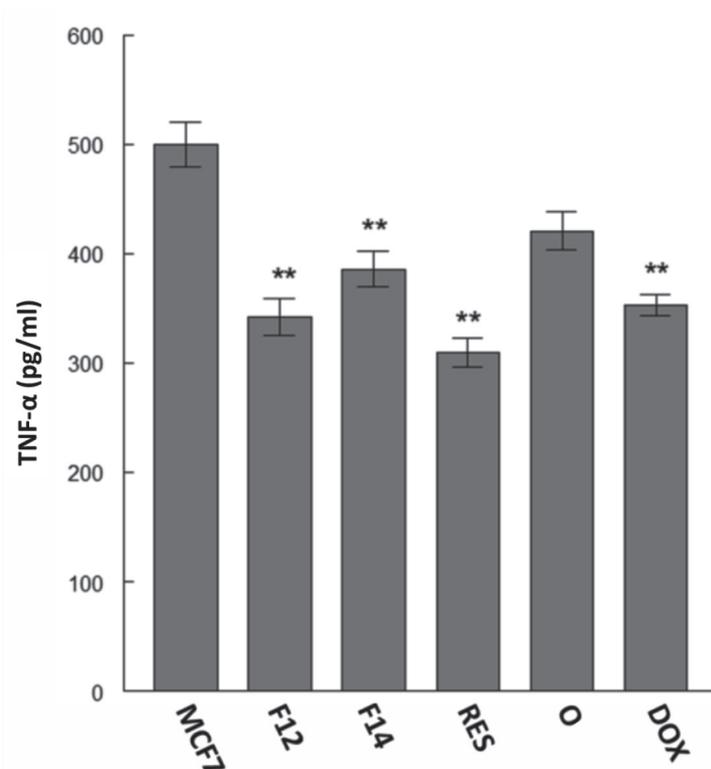


Figure 4 – Quantification of TNF- α by ELISA in a breast cancer cell line (MCF-7). The cells were treated with *Protium heptaphyllum* crude resin (RES) and its fractions enriched with α - and β -amyrin (F12 and F14) at 40 μ g/ml and doxorubicin (DOX) for 72 h. The values represent the means \pm SEM; ** $p < 0.01$ compared to MCF-7 cells.

mediated caspase-8 activation, this can indicate a probable mechanism in which resin and fractions reduce TNF- α levels (Liu and Chang, 2011), whereas the doxorubicin showed a lower potency than F12, F14 and resin.

Participation of RAS in the pro-apoptotic activity of *P. heptaphyllum*

The role of the Renin-Angiotensin System (RAS) in the specific context of tumor cells has been discussed in a recent review (George et al., 2010). The role of RAS in angiogenesis, apoptosis and tumor proliferation is large, complex, and sometimes paradoxical (George et al., 2010). Tumor cell lines that possess activated RAS may respond to Angiotensin II (Ang II) stimuli, expressing cytokines that assist in angiogenesis, such as interleukin-8 (IL-8) (George et al., 2010).

However, the tumor cell response to TNF- α requires the action of Ang II to increase apoptosis (Wang et al., 2000). Several studies have reported that cells with high Angiotensin Converting Enzyme (ACE) activity produce higher levels of Ang II, increasing the rate of apoptosis (George et al., 2010). This mechanism may have been verified in the present study because (Herr et al., 2008) showed the expression of RAS components and their receptors in MCF-7 cells.

In this study, ACE activity was evaluated in MCF-7 cells in the presence and absence of treatment with triterpene (Herr et al., 2008). It was also observed that treatment with the resin increases ACE activity (Fig. 5). The high pro-apoptotic rate observed after treatment with the resin and the F12 and F14 fractions may be related to increased enzymatic activity after treatment (Fig. 5), whereas the doxorubicin showed a lower potency than F12, F14 and resin. However, the role of doxorubicin in RAS activation is not yet described.

Altogether, it may be concluded that treatment of MCF-7 cells with resin and the fractions enriched with α - and β -amyrin was able to promote pro-apoptotic effects, most likely by decreasing the levels of TNF- α and increasing ACE activity. Further studies are required for a detailed evaluation of the mechanisms and pathways to better understand the apoptotic processes under study.

Authors' contributions

DCE and TUA, conceived and designed the experiments; EML and AMN, performed the experiments; DL, DCE and TUA, analyzed the data; RS, SSM and GAPB, contributed with reagents/materials/analysis tools; and EML, DCE and TUA, wrote the manuscript.

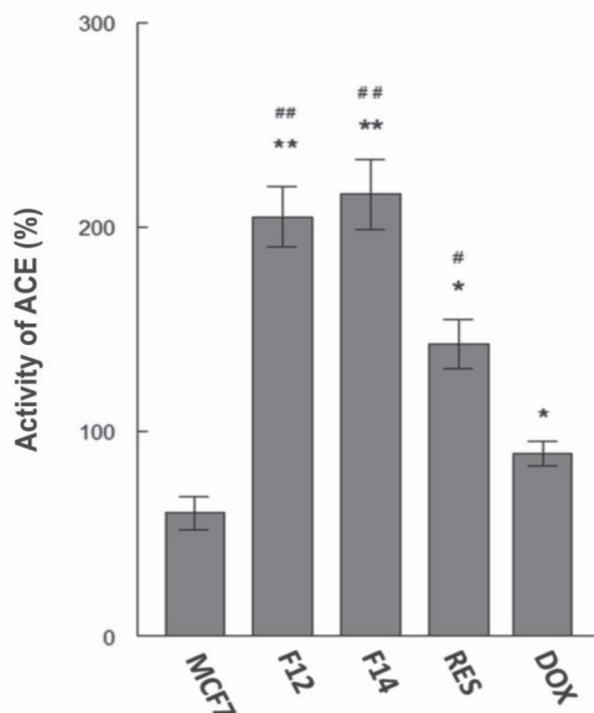


Figure 5 – Percentages of ACE activity determined by ELISA in a breast cancer cell line (MCF-7). The cells were treated with *Protium heptaphyllum* crude resin (RES) and its fractions enriched with α - and β -amyirin (F12 and F14) and doxorubicin (DOX) at 40 $\mu\text{g/ml}$ for 72 h. The values represent the means \pm SEM; ** $p < 0.01$ and * $p < 0.05$ compared to MCF-7 cells. ## $p < 0.01$ and # $p < 0.05$ compared to DOX.

Conflicts of interest

The authors declare no conflicts of interest.

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