

# Biological Activities and Cytotoxicity of *Eperua oleifera* Ducke Oil-resin

Fernanda Torlania Alves Gomes, Ana Paula de Araújo Boleti, Lidiam M. Leandro<sup>1</sup>, Diego Squinello<sup>1</sup>, Ellen S. P. Aranha, Marne C. Vasconcelos, Paul Cos<sup>2</sup>, Valdir F. Veiga-Junior<sup>1</sup>, Emerson Silva Lima

Biological Activity Laboratory, Faculty of Pharmaceutical Sciences, Federal University of Amazonas, <sup>1</sup>Chemistry Department, Exact Sciences Institute, Federal University of Amazonas, Manaus, Amazonas, Brazil, <sup>2</sup>Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, B2610 Antwerp, Belgium

Submitted: 06-12-2016

Revised: 01-02-2017

Published: 13-11-2017

## ABSTRACT

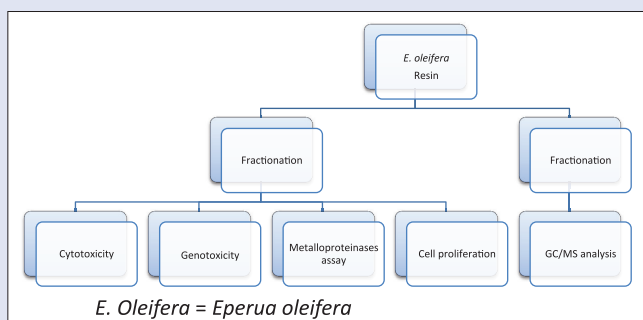
**Background:** The oil-resin of *Eperua oleifera* Ducke has been used in popular medicine similarly to the copaiba oil (*Copaifera* spp.). **Objective:** This study aimed to investigate the effects of the acid fraction of *E. oleifera* oil-resin (AFEOR) on cell proliferation, collagen production in human fibroblasts, inhibition of metalloproteinases, and cytotoxicity against tumor cell lines. **Materials and Methods:** Acid fraction of *E. oleifera* was fractionated in the ion exchange column chromatography. Cytotoxicity and genotoxicity were evaluated by Alamar Blue<sup>®</sup> and Cometa assay. The inhibition of metalloproteinases was performed by zymography and Western blotting. **Results:** The predominant acidic diterpenes in the AFEOR were copalic and hardwickiic acids. AFEOR caused morphology alteration and decrease of proliferation at concentrations higher than 5 µg/mL. It also caused significant collagen proliferation in fibroblasts. It showed cytotoxicity against tumoral and nontumoral cell lines, with IC<sub>50</sub> values ranging from 13 to 50 µg/mL, and a hemolytic activity with an IC<sub>50</sub> value of 38.29 µg/mL. AFEOR inhibited collagenase activity, with an IC<sub>50</sub> value of 46.64 µg/mL, and matrix metalloproteinase-2 (MMP)-2 and MMP-9 in HaCaT cells or MMP-1 expression in MRC-5 cells. AFEOR induced genotoxicity in MRC-5 cells with a DNA damage index between 40% and 60% when compared to the negative controls (0%–20%). **Conclusion:** For the first time, biological activities from oil-resin *E. oleifera* demonstrated ratifying somehow its popular use.

**Key words:** collagenase, *Eperua*, metalloproteinases, tumoral cell

## SUMMARY

- Analysis of crude oil-resin and fractionation of diterpenic fraction was performance using selective ion-exchange column chromatography

- Cytotoxicity analysis and morphology were performed with different cell lines
- Collagen production in human fibroblasts, inhibition of metalloproteinases were demonstrated by zymography and Western blotting.



**Abbreviations used:** AFEOR: *Eperua oleifera* oil-resin.

## Correspondence:

Dr. Emerson Silva Lima,  
Laboratório de Atividade Biológica,  
Faculdade de Ciências Farmacêuticas,  
Universidade Federal do Amazonas, Manaus,  
Amazonas, Brazil.

E-mail: eslima@ufam.edu.br

DOI: 10.4103/pm.pm\_552\_16

## Access this article online

Website: [www.phcog.com](http://www.phcog.com)

## Quick Response Code:



## INTRODUCTION

Plants produce an important structural diversity of metabolites and represent the largest source of bioactive compounds. They are used worldwide as self-prescribed home medicines and in the pharmaceutical industry.<sup>[1-3]</sup> Since compounds isolated from plants have many biological activities, the invaluable biodiversity among Brazilian plants presents a great asset in the development of novel drugs.<sup>[4]</sup> The identification of any potential toxicological activity in plant bioactive substances is primordial to evaluate their risks and potential uses.<sup>[5]</sup>

*Eperua* genus is found in the Central Amazon, with 14 species described in the literature, and is distributed in the North and Western Amazonia from Ecuador to Guyana and Venezuela. *Eperua* trees have similar biological properties as the genus *Copaifera*, which is also from Fabaceae-Caesalpinioideae family and is commonly known as copaiba oil. These species produce oil-resins used for therapeutic purposes in folk medicine. Some of these *Eperua* oil-resins have similar names to *Copaifera* species such as *Eperua oleifera* and *E. purpurea*, known as “copaiba-jacaré” and “copaibarana,” respectively.<sup>[6,7]</sup> *Eperua* oil-resin is obtained by exudation of the trunk trees and has been used as skin healing, antibacterial and antifungal agents in Amazonian folk medicine.<sup>[8-10]</sup>

Chromatographic analysis of copaiba oil-resin has revealed that it contains sesqui- and diterpenes as the major compounds described as kaurane-, labdane-, and clerodane-type diterpenes. Some of the identified diterpenes, such as kaurenoic and hardwickiic acids, have been reported to possess antitumor activity<sup>[11,12]</sup> and eperuic acid as an accelerator of collagen production.<sup>[13]</sup> Even considering its wide use in folk medicine, there are no *in vitro* studies on the biological activities of *E. oleifera* oil-resin.

Matrix metalloproteinases (MMPs) are proteinases that are involved in the breakdown and remodeling of the extracellular matrix (ECM). It plays critical roles in cell growth, angiogenesis, invasion, and

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

**For reprints contact:** [reprints@medknow.com](mailto:reprints@medknow.com)

**Cite this article as:** Alves Gomes FT, Boleti AP, Leandro LM, Squinello D, Aranha ES, Vasconcelos MC, *et al.* Biological activities and cytotoxicity of *Eperua oleifera* Ducke oil-resin. Phcog Mag 2017;13:542-52.

metastasis of cancer cells through degradation of the ECM.<sup>[14,15]</sup> Numerous studies have focused on compounds from plant species as potential inhibitors of MMPs with anticancer effects.<sup>[16,17]</sup> MMPs are also responsible for changes in skin collagenous tissues by breakdown of collagen, i.e., a major in the ECM, especially MMP-1 (interstitial collagenase-1).<sup>[18]</sup>

This study aimed to investigate the effects of the acid fraction of *E. oleifera* oil-resin (AFEOR) on cytotoxicity of normal and malignant cell lines, genotoxicity, and inhibition of metalloproteinases, cell proliferation, and collagen production.

## MATERIALS AND METHODS

### Plant material

Samples of the *E. oleifera* oil-resin were commercially available at the municipal market of Manacapuru, Amazonas, Brazil.

### Acid fraction procedure

From crude oil-resin, a fractionation was performed to separate diterpenic fraction from the apolar sesquiterpenes. The usual column chromatography is not effective to separate the sesquiterpenes from diterpenes with an acid function. Therefore, a selective ion-exchange column chromatography fractionation was performed using silica impregnated with KOH, as previously used to copaiba oils. After adding the modified silica and AFEOR, the column was submitted to dichloromethane elution to obtain the sesquiterpenes and the nonacid components separated from the diterpenic carboxylic acids. The diterpenic acids remain retained by silica impregnated with KOH during dichloromethane elution. Sequentially, methanol was used to elute the diterpenic acids as potassium salts. The methanol fraction was concentrated under low pressure and immediately acidified with HCl until pH 4–5. Dichloromethane was added and the diterpenic acids were recovered from the organic phase (dichloromethane) in a separation vessel. The solvent was evaporated in a rotary evaporator and the acid fraction obtained was stored under low temperature until the analysis. After derivatization with freshly prepared diazomethane, AFEOR was analyzed by gas chromatography using flame ionization and mass spectrometry detector. Copalic and hardwickiic acids were identified as their respective methyl esters by comparison with standards obtained previously from copaiba oils.

### Cell culture

Human primary fibroblast (HPF) and human keratinocytes (HaCaT) cells were kindly provided by Dr. Silvyia Stuchi Maria-Engler, from the Department of Clinical Analysis and Toxicology, Faculty of Pharmaceutical Sciences, University of São Paulo. Human fibroblasts (MRC-5) and tumor cells of melanoma (SKMEL19), human colorectal carcinoma (HCT116), breast adenocarcinoma (MCF-7), gastric carcinoma (ACP-02), and ovarian adenocarcinoma (ES-2 and NHOVCAR) were provided by human Cytogenetics Laboratory of the Federal University of Para. The cells were grown in culture flasks of 75 cm<sup>2</sup> in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) bovine serum fetal (GIBCO), penicillin (100 U/ml), and streptomycin (100 U/ml) and kept in an incubator at 5% CO<sub>2</sub> and 37°C. After reaching 70%–80% confluence, the cells were transferred in sterile bottles. After reaching confluence, cells were detached with a 0.05% trypsin-EDTA solution and neutralized with the same volume of DMEM medium. Then, the cells were centrifuged twice for 10 min at 1500 rpm and resuspended in the culture medium. The cell concentration was adjusted in a Neubauer chamber, with >97% viability.

### Cell viability assay

The cytotoxicity of AFEOR was determined by the Alamar Blue method as described by Nakayama *et al.*<sup>[19]</sup> The cell lines were seeded at a density of  $5 \times 10^3$  cells/well in a 96-well microtiter plate and incubated overnight. Cells were treated with and without AFEOR (at a concentration range of 0.7–50 µg/mL) in serum-containing DMEM for 24, 48, and 72 h. After incubation, the Alamar Blue solution (10 µL of 0.4% Alamar blue [resazurin] in phosphate-buffered saline [PBS]) was added and the cells were incubated for 3 h at 37°C. Fluorescence was measured (excitation at 545 nm and emission at 595 nm) and expressed as a percentage of the cells in the control after subtraction of background fluorescence. Doxorubicin at 5 µg/mL was used as a positive control and all the assays were performed in triplicate.

### Morphological analysis

The overall organization of the layering of the fibroblasts from the treatment period (24–72 h) was monitored using phase-contrast microscopy. The cells were viewed under phase illumination with an inverted microscope and imaged. Differential morphology of cultures was observed to analyze the AFEOR treatment effects.

### Cell counts and growth curve

Cells were seeded in a 12-well plate, with approximately 1 mL ( $\sim 1 \times 10^4$ ) cells in each well. After 24 h, when the fibroblasts and HaCaT cells attached to the walls of the wells, the experiment began. AFEOR was dissolved in serum-free DMEM, yielding different concentrations ranging from 1.25 to 5.0 µg/mL. In the treated wells, the different concentrations of AFEOR were added and incubated for 24, 48, and 72 h. As control, wells were filled with DMEM and dimethyl sulfoxide (DMSO) 0.5% for the same period. The wells were then rinsed with PBS and filled with culture media. Cell counts were performed in triplicate and growth curves of the fibroblasts were determined by the Trypan Blue exclusion method. Cell morphology was observed by inverted microscope.

### Matrix metalloproteinase-2 and matrix metalloproteinase-9 inhibition

To study the effect of AFEOR on MMP-2 and MMP-9 inhibition, MRC-5 and HaCaT cells were seeded in a 12-well plate on reaching confluence, washed twice with PBS, and then treated with or without AFEOR (1.25, 2.5 and 5 µg/mL). After 24 h, the media were collected, centrifuged to avoid cellular debris, mixed with 4X sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% sodium dodecyl sulfate (SDS), and 0.00625% bromophenol blue, and then loaded for electrophoresis on a 10% SDS-polyacrylamide gel for zymography studies.

### Zymography of matrix metalloproteinase-2 and matrix metalloproteinase-9 inhibition

In the zymography assay, gelatine was used as a substrate for MMP-2 and MMP-9.<sup>[20]</sup> Gelatin at a concentration of 0.1% was incorporated into 10% polyacrylamide gel containing 0.4% SDS. Electrophoresis under nonreducing conditions was performed using a Bio-Rad mini-gel system at 120 V for 90–120 min. After electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100 (v/v) to remove the SDS, and then incubated overnight in the developing buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM CaCl<sub>2</sub>, and 10 mM ZnCl<sub>2</sub>) at 37°C. Digestion bands were quantified by Quantity One (Bio-Rad Laboratories, Hercules, CA, USA).

## Western blot analysis of matrix metalloproteinase-2 and matrix metalloproteinase-9 inhibition

MRC-5 and HaCaT cells were cultured in 6-well plates ( $1 \times 10^6$  cells per well) and incubated with AFEOR in concentrations of 2.5, 5, and 10  $\mu\text{g}/\text{mL}$  and incubated for 24 h. After incubation, cells were washed with PBS and lysed with lysis buffer consisting of Tris-HCl (50 mM, pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , 10% glycerol, and proteases inhibitors (Cocktail of proteases inhibitors EDTA-free, Roche; 1 mM phenylmethanesulfonyl fluoride). After 1 h at 40°C, cells lysates were obtained by centrifugation at 10,000 g for 10 min. The total protein concentration in the lysates was measured by the Bradford method with bovine serum albumin as standard. Samples containing equal amounts of protein concentration were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding was blocked with Tris-buffered saline with Tween 20 (1 M Tris-HCl [pH 7.5], 2.5 M NaCl, and 0.5% Tween 20) containing 5% nonfat milk for 2 h at room temperature. The membranes were incubated overnight with the primary antibody (MMP-1 and  $\beta$ -actin) diluted in Tris-buffered saline with Tween 20 (1:1.000 and 1:2.000, respectively), then washed with the same buffer, and incubated with horseradish peroxidase-conjugated anti-immunoglobulin G antibody (goat anti-rabbit immunoglobulin G) as secondary antibody for 1 h at room temperature. The immunoblots were visualized with chemiluminescence detection kit according to the manufacturer's recommendations (kit Pierce). Western blots are representative of three independent experiments.

## Hemolysis test

The hemolytic activity of the AFEOR was evaluated according to Fischer *et al.*<sup>[21]</sup> with modifications. Blood from Swiss mice was collected in heparinized tubes and centrifuged at 700 g for 10 min. The pellet was washed three times with cold PBS pH 7.4 by centrifugation at 700 g for 10 min and resuspended in the same buffer. This suspension of red blood cells was always freshly prepared and used within 24 h after collection. Different concentrations of AFEOR were prepared in PBS buffer, added to the erythrocytes, and incubated for 60 min at 37°C in a shaking water bath. The release of hemoglobin was determined after centrifugation (700 g for 10 min) by spectrophotometric analysis of the supernatant at 540 nm. Complete hemolysis was achieved using 0.2% Triton X-100 yielding the 100% control value. Less than 10% hemolysis was considered as nontoxic effect level in our experiments. The experiments were run in triplicate.

## Comet assay

The Comet assay with MRC-5 cells was used to detect DNA damage. Before each experiment, frosted microscope slides were precoated with two layers (100  $\mu\text{l}$ ) of normal agarose (1% in Milli-Q water) and left at room temperature to allow agarose to dry. The cells were treated during 3 h with different concentrations of the test samples. The cell dilution ( $5 \times 10^5$  cells in 60  $\mu\text{l}$ ) was mixed with an equal volume of low-melting point agarose (1.2% in PBS). This agarose cell suspension (120  $\mu\text{l}$ ) was spread onto each precoated slide and covered with a coverslip. After 10 min on ice, the cover slip was gently removed, and the slides were placed in a tank filled with the lysate buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% sodium sarcosinate pH 10, 1% of Triton X-100, and 10% DMSO). They were immersed for 1 h in this buffer (4°C, in the dark). The slides were then transferred into the electrophoresis buffer (NaOH 10 N, EDTA 200 mmol/l, and pH 13 in distilled water) for 20 min at room temperature in darkness. Electrophoresis was carried out for 15 min at 25 V, 300 mA. Finally, the slides were gently rinsed with neutralization solution (0.4 M Tris-HCl,

pH 7.5) 3 times for 5 min each. Staining of DNA was accomplished using 50  $\mu\text{l}$  of ethidium bromide solution (20  $\mu\text{g}/\text{mL}$  in PBS) per slide. The slides were examined using an epifluorescence microscope.

## Quantification of the comet assay

A total of 100 comets on each scored slide for each sample concentration were visually scored according to the relative intensity of fluorescence in the tail and classified as belonging to one of five classes. We utilized three slides for each extract concentration, and the experiments were performed in triplicate. Each comet class was given a value of 0, 1, 2, 3, or 4 (from undamaged, 0 to maximally damaged, 4) as described by Cavalcanti *et al.*<sup>[11]</sup> The total score of DNA damage was calculated by the following equation: Total DNA damage = (number of cells in class 0  $\times$  0) + (number of cells in class 1  $\times$  1) + (number of cells in class 2  $\times$  2) + (number of cells in class 3  $\times$  3) + (number of cells in class 4  $\times$  4).

## Statistical analysis

Results are expressed as the means and standard deviations of triplicate measurements. Each experiment was performed at least three times. Differences between groups were assessed by one-way analysis of variance followed by the Bonferroni, Dunnett's, and Tukey's posttest.  $P < 0.05$  indicated significance.

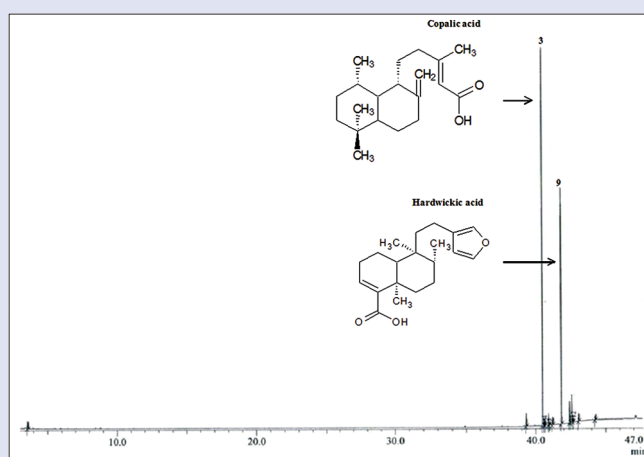
## RESULTS

### Chemical composition of *Eperua oleifera* oil-resin

A selective fractionation was performed to separate an acidic diterpenic fraction from the apolar sesquiterpenes, all naturally present in the *E. oleifera* oil-resin and observed after derivatization with diazomethane by gas chromatography-mass spectrometry. In this analysis, two main diterpenes acids were identified: copalic and hardwickiic acids [Figure 1]. Unfortunately, it was not possible to perform the quantification of these in the fraction obtained yet their presence as major components can partly explain the biological activity observed in this study.

### Effect of *Eperua oleifera* oil-resin on cell viability

To investigate the cytotoxicity of AFEOR on human HaCaT keratinocytes, normal FPH and MRC-5 fibroblasts, and the malignant cells lines MCF-7, HCT116, ACP-02, ES-2, SKMELL 19, and NHOVCAR, the cells were seeded into wells of 96-well culture plates at a density of  $0.5 \times 10^4$  cells/



**Figure 1:** Representative gas chromatography-mass spectrometry chromatogram of acid fraction of oil-resin of *Eperua oleifera* can be identified such as main compounds copalic acid (peak 3) and hardwickiic acid (peak 9)

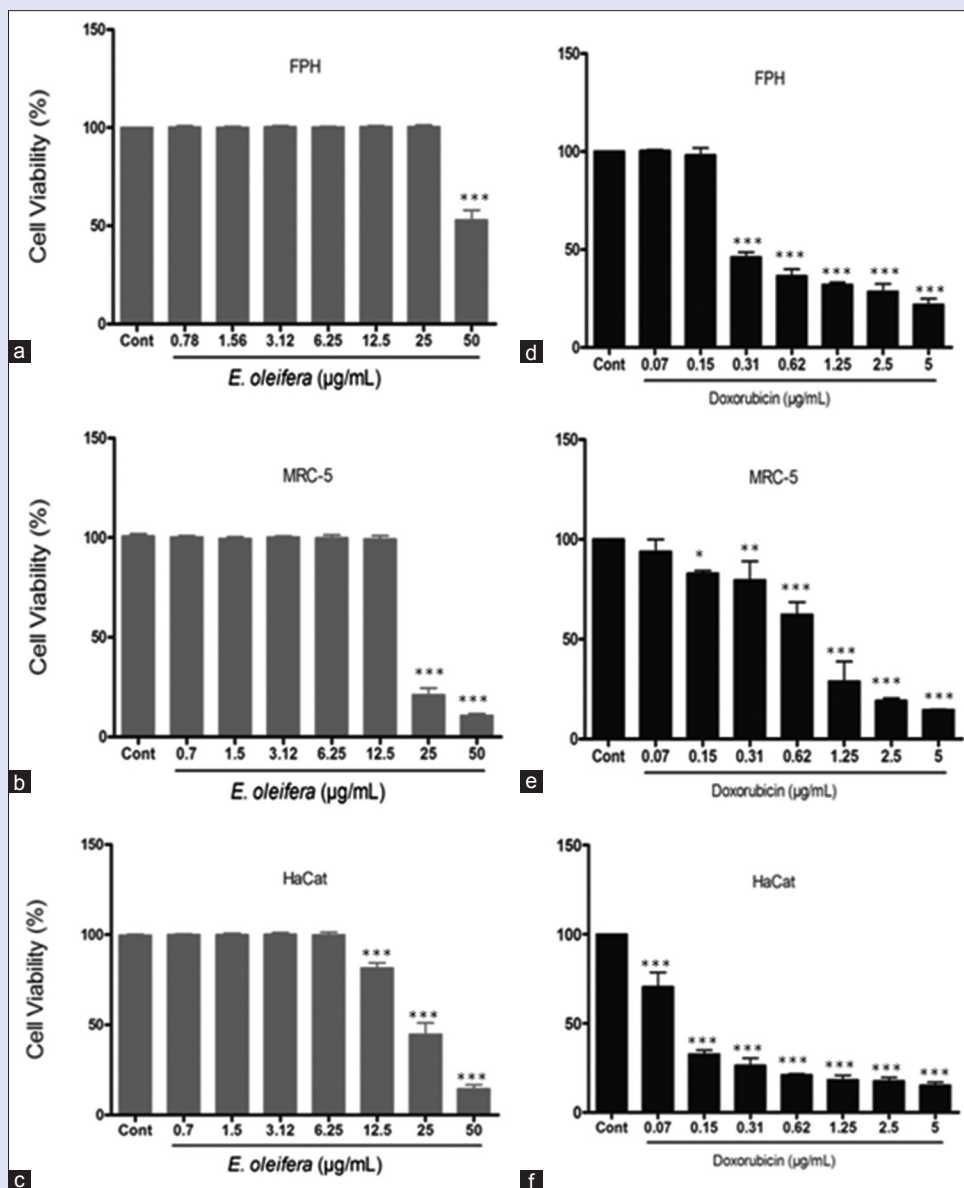
well. The influence of AFEOR at various concentrations on different cellular toxicity was analyzed using the Alamar Blue<sup>®</sup> assay.

Together with the increase in incubation time, we observed a concentration-dependent reduction in the cell viability after 72 h. A decrease of FPH cell viability was only observed at the highest concentration (50 µg/mL). However, the HaCaT cells showed a significant loss in viability of about 56%–86% observed at concentrations ≥25 µg/mL. After 72 h, we determined >85% of viable cells at a concentration of 12.5 µg/mL. Below this concentration, the cell viability did not change in comparison with control cells. Independent from the time of exposure, incubation with 3.12 µg/mL of AFEOR, all cells were viable. As a negative control, we considered cells that were not treated with AFEOR, and as a positive control, the cells that were treated with doxorubicin.

The cytotoxicity effects of AFEOR on MRC-5 cell, after 72 h, are described in Figure 2a-f. Results showed a significant loss in viability of about 40%–80% observed at concentrations higher than 25 µg/mL, with several cell deaths at a maximum concentration of 50 µg/mL and IC<sub>50</sub> value of ~25 µg/mL. On the other hand, below this concentration, cells were 100% viable, as well as control cells.

### Morphology of human fibroblasts and keratinocytes incubated with acid fraction of *Eperua oleifera* oil-resin

Substantial changes in MRC-5 cell morphology were detected microscopically after 24 h and exposure with the oil-resin and becoming more prominent after 72 h of incubation. The AFEOR at 3.12 µg/mL



**Figure 2:** Cell viability using an Alamar Blue<sup>®</sup> cytotoxicity assay was performed for the cells FPH, MRC-5, and HaCaT. (a-c) Treatment with *Eperua oleifera* oil-resin (at a concentration range of 0.7–50 µg/mL) in Dulbecco's modified Eagle medium for 72 h. (d-f) Doxorubicin treatment as a positive control, in a concentration range of (0.07–5 µg/mL). The negative control (dimethyl sulphoxide solvent 0.1%). Each bar represents the mean ± standard deviation of triplicate determinations from three independent experiments. \*\*\**P* < 0.001, \*\**P* < 0.01, \**P* < 0.05 (one-way analysis of variance with Dunnett's multiple-comparison posttest)

showed no or little damage when compared with control cells [Figure 3a-c]. However, up to 6.25 µg/mL was notorious, a decrease of the cell number and at 50 µg/mL we observed cellular debris and severe changes in morphology, such as the decrease of cell density [Figure 3d-f]. The morphology of FPH and HaCaT cells was also studied after AFEOR treatment [Figure 4a-f]. There were no morphological differences between cultures with and without treatment at low concentrations (1.25–2.5 µg/mL) on FPH cells.

### Effect of *Eperua oleifera* on proliferation and synthesis of collagen of FPH and MRC-5 cells

In this study, the effect of AFEOR on cell growth was investigated for two different fibroblast cell lines. After treatment with AFEOR, FPH and MRC-5 cell proliferation [Figure 5a and b] decreased when cells were incubated at a concentration of 5 µg/mL.

The production of collagen by MRC-5 and FPH cells was also investigated, using the Sirius red assay [Figure 5e]. To measure the effects of AFEOR on collagen synthesis, cells were incubated for 24, 48, and 72 h with AFEOR [Figure 5c and d]. Results of MRC-5 cells showed that collagen synthesis increased significantly after 24 h when incubated with 1.25 µg/mL AFEOR. Thus, on FPH cell line, the collagen production was increased at about 50%, after 24 h of treatment, and at 25% after 48 h of treatment with 5 µg/mL of AFEOR, in comparison with nontreated cells.

### Determination of the activity and expression of metalloproteinases

To determine the inhibitory effects of AFEOR on collagenases activities, different concentrations of the oil-resin were incubated with 25 µg/mL of collagenase from *Clostridium histolyticum* and applied to gelatin zymography. Negative controls contain DMSO (0.1%). As shown in Figure 6a-e, treatment with AFEOR was capable of inhibiting collagenase activity in 88% at the maximum concentration of 200 µg/mL. It exhibited an IC<sub>50</sub> value of 46.64 µg/mL.

To determine if the AFEOR could inhibit the MMP-2 and MMP-9 enzymatic activities, HaCaT and MRC-5 cells were treated with 5, 2.5 and 1.25 µg/mL of AFEOR in serum-free medium and incubated for 24 h. Then, the conditioned media of the cells were collected, and their MMP-2 and MMP-9 enzymatic activities were assessed using the gelatin

zymography method. As shown in Figure 6b and f, MMP-2 and -9 activities were detected in the conditioned media and zymography quantitative analysis did not show a significant decrease of MMP-9 activity compared with the control. In addition, there was only a small decrease in MMP-2 activity when the cells were treated with AFEOR.

To evaluate the effects of the AFEOR on MMP-1 protein expression, MRC-5 cells were exposed to the oil-resin treatment (1.25–5 µg/mL) for 24 h. Levels of MMP-1 expression were measured by Western blot assay [Figure 6c] after protein dosage of total cell lysates. AFEOR treatment decreased protein expression in a dose-dependent manner, i.e. 39%, 47%, and 86% at, respectively, 1.25 µg/mL, 2.5 µg/mL, and 5 µg/mL [Figure 6d].

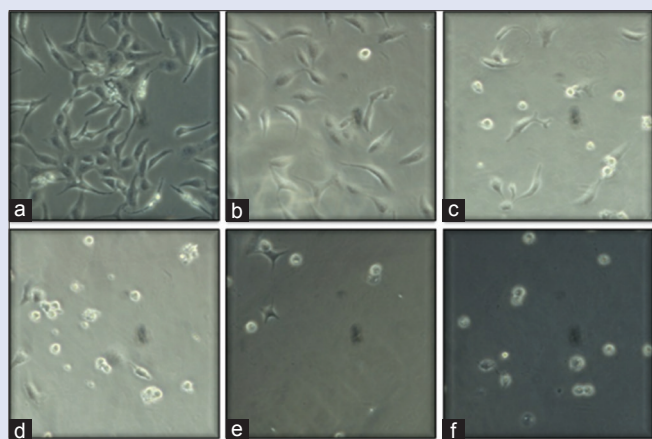
### Measuring and comparing the cytotoxicity of acid fraction of *Eperua oleifera* oil-resin in multiple malignant cell lines

The cytotoxicity of AFEOR on malignant cell lines was also tested with the Alamar Blue<sup>®</sup> assay. Treatment at the maximum concentration of 50 µg/mL caused a significant cytotoxicity in HCT116, MCF-7, and SKMELL19 cell lines [Figure 7a-f]. After 72 h, cell viability was significantly reduced on the malignant cell lines, with IC<sub>50</sub> values showing significant cytotoxic effects [Table 1].

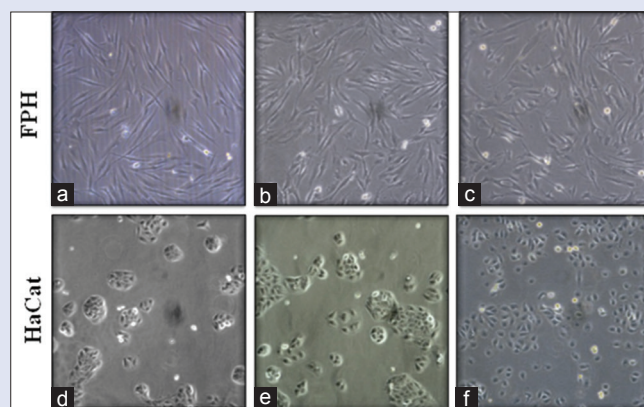
In addition, other three malignant cell lines were also used in this assay, i.e., ES-02, NHOVCAR, and ACP-02. AFEOR at 50 µg/mL exhibited a high cytotoxicity against these tested malignant cell lines, in comparison with doxorubicin at 5 µg/mL. The only exception was that ACP-02, ES-2, and NHOVCAR malignant cell lines that showed more resistant toward AFEOR treatment.

### Hemolytic activity

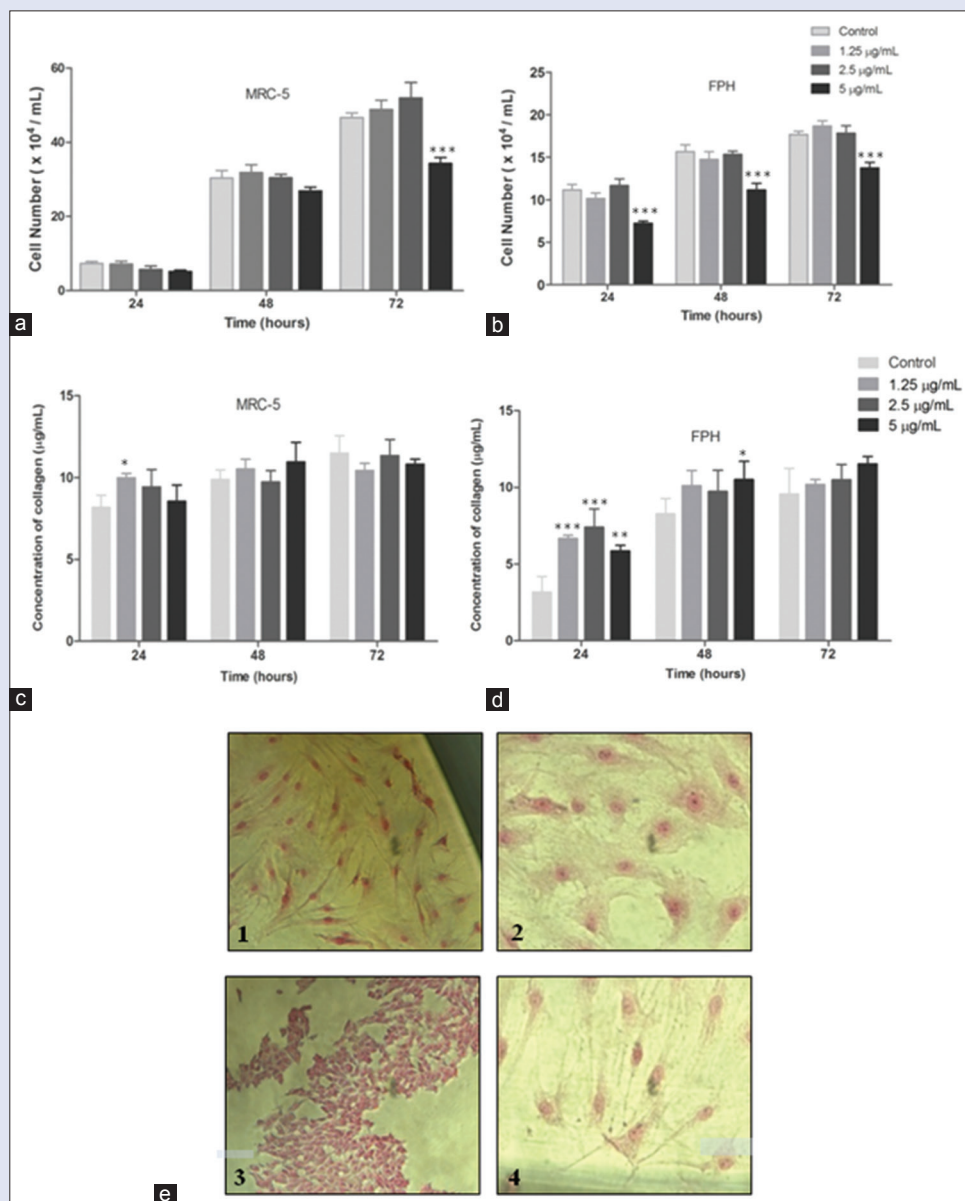
To evaluate the *in vitro* hemolytic activity, Swiss mice erythrocytes were exposed to various concentrations of AFEOR and the release of hemoglobin was used to quantify the membrane-damaging properties. The IC<sub>50</sub> values were calculated, and AFEOR showed hemolytic effects up to 62.5 µg/mL, with 64% of hemolysis indicating significant damage of the red blood cell membranes. Concentrations of AFEOR in a range of 125–500 µg/mL caused 82%–84% of hemolysis, decreasing to 73% at a maximum concentration of 2000 µg/mL. As 100% values, we used Triton X-100 and found IC<sub>50</sub> values of 38.28 (29.60–49, 50) µg/mL [Figure 8].



**Figure 3:** Representative photograph of human fibroblast (HPF) and Keratinocyte morphology (HaCat). The HPF (a-c) and HaCat (d-f) cells were incubated with two different concentrations of *E. oleifera* oil-resin in DMEM media for 24 h. Morphological changes were observed and compared with non-treated cells. The cell culture was examined and photographed using an inverted microscope (×40)



**Figure 4:** Representative photograph of human fibroblast and keratinocyte morphology. The FPH (a-c) and HaCaT (d-f) cells were incubated with acid fraction of *Eperua oleifera* oil-resin in Dulbecco's modified Eagle medium media for 24 h. Morphological changes were observed and compared with nontreated cells. The cell culture was examined and photographed using an inverted microscope (×40)



**Figure 5:** (a and b) Effects of acid fraction of *Eperua oleifera* oil-resin on MRC-5 and FPH cell proliferation. Cells were treated for 24, 48, and 72 h with 0.625–5 µg/mL. Growth curve of fibroblasts was assessed by the Trypan blue exclusion assay. (c and d) The analysis of collagen production of fibroblasts performed by Picosirius-Red assay. (e) The top panel shows staining of cells in culture, with a typical fibroblastic morphology treated with acid fraction of *Eperua oleifera* oil-resin, as the intensity of the staining based on the uptake of the dye in deposited collagen. (1 and 2) MRC-5, (3 and 4) FPH cells stained with sirius red and photographed under a light microscope at (x40–x100). As negative control was used (dimethyl sulfoxide solvent 0.1%). Each bar represents the mean ± standard deviation of triplicate determinations from three independent experiments. \*\*\**P* < 0.001, \*\**P* < 0.01, \**P* < 0.05 (two-way analysis of variance analysis, Bonferroni posttest)

In addition, other three malignant cell lines were also used in this assay, i.e., ES-02, NHOVCAR, and ACP-02. AFEOR at 50 µg/mL exhibited a high cytotoxicity against these tested malignant cell lines, in comparison with doxorubicin at 5 µg/mL. The only exception was that ACP-02, ES-2, and NHOVCAR malignant cell lines that showed more resistant toward AFEOR treatment.

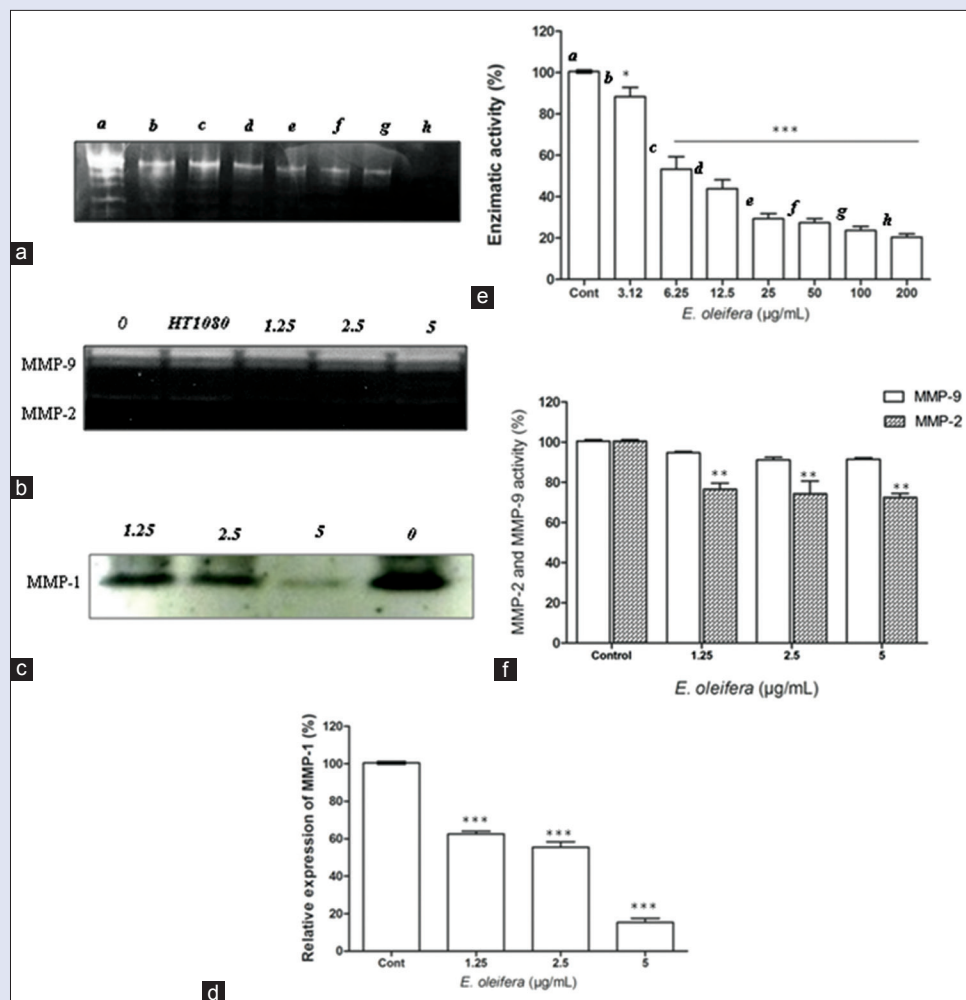
### Comet assay

The induction of DNA damage in MRC-5 cells after exposition to different AFEOR concentrations was investigated using the Comet assay. Data are reported as total DNA damage in Figure 9a and damage

frequency in Figure 9b. It was indicated that the oil-resin-induced genotoxicity (18%–60%) at concentrations of 7.5–30 µg/ml with a significant difference between the total DNA damage of the negative control (DMSO 0.2%). The frequency of DNA damage in MRC-5 cells was described as [Figure 9b] the different levels of tail comets extents increased by AFEOR treatment, compared with DMSO (0.1%) as a negative control and doxorubicin (10 µg/mL) as a positive control.

### DISCUSSION

This research aimed to study *E. oleifera* biological activities. Here, we have explored its activity on normal and tumor cell viability, cell proliferation,



**Figure 6:** Effects of *E. oleifera* oil-resin on metalloproteinase activity and expression. (a) Gelatin zymography was used to evaluate the enzymatic activity of collagenases from *Clostridium Histolyticum*, (b) MMP-2 and MMP-9 activity. The HaCat cells were cultured with 1–5 µg/mL *E. oleifera* oil-resin for 24 h, and conditioned media subjected to 10% SDS-PAGE. HT1080 cells were used as control, with no AFEOR treatment. (c) MMP-1 expression in MRC-5 cell lysates subjected to Western blot analysis with a primary antibody of MMP-1. (d-f) the bar graphs (means ± S.D, n = 3) represent quantitative densitometric results of at least three independent experiments. (\*\*P < 0.01, \*\*\*P < 0.001) compared with the control (DMEM, DMSO 0.1%). Densitometric analysis was performed using ImageJ® software (National Institutes of Health, Bethesda, Maryland). Data statistics (by one-way analysis of variance with Dunnett’s multiple-comparison post-test)

metalloproteinase activity and expression, and collagen production. The present study is the first report demonstrating biological activities of *E. oleifera* on human cell lines.

The chemical composition of *E. oleifera* oil-resin is a dispersion of diterpene acids in a mixture of mono- and sesquiterpenes. The main components found in AFEOR were labdane, clerodane, and kaurane diterpenes. Some of the diterpenes reported in *Eperua* species are eperuic, kaurenoic, and hardwickiic acids, mostly present in oil-resin of the genus *Copaifera*.<sup>[7]</sup> Kaurenoic acid, i.e., a diterpene isolated from *Copaifera langsdorffii* oil-resin, has been reported by Costa-Lotufo *et al.*<sup>[37]</sup> for its cytotoxic and embryotoxic effects. Ohsaki *et al.*<sup>[12]</sup> described the antitumor activity of hardwickiic acid against IMC carcinoma in Swiss mice. Copaiba oil-resin is generally used in traditional medicine for its anti-inflammatory, antitumor, antimicrobial, wound healing, and antiseptic properties.<sup>[12]</sup>

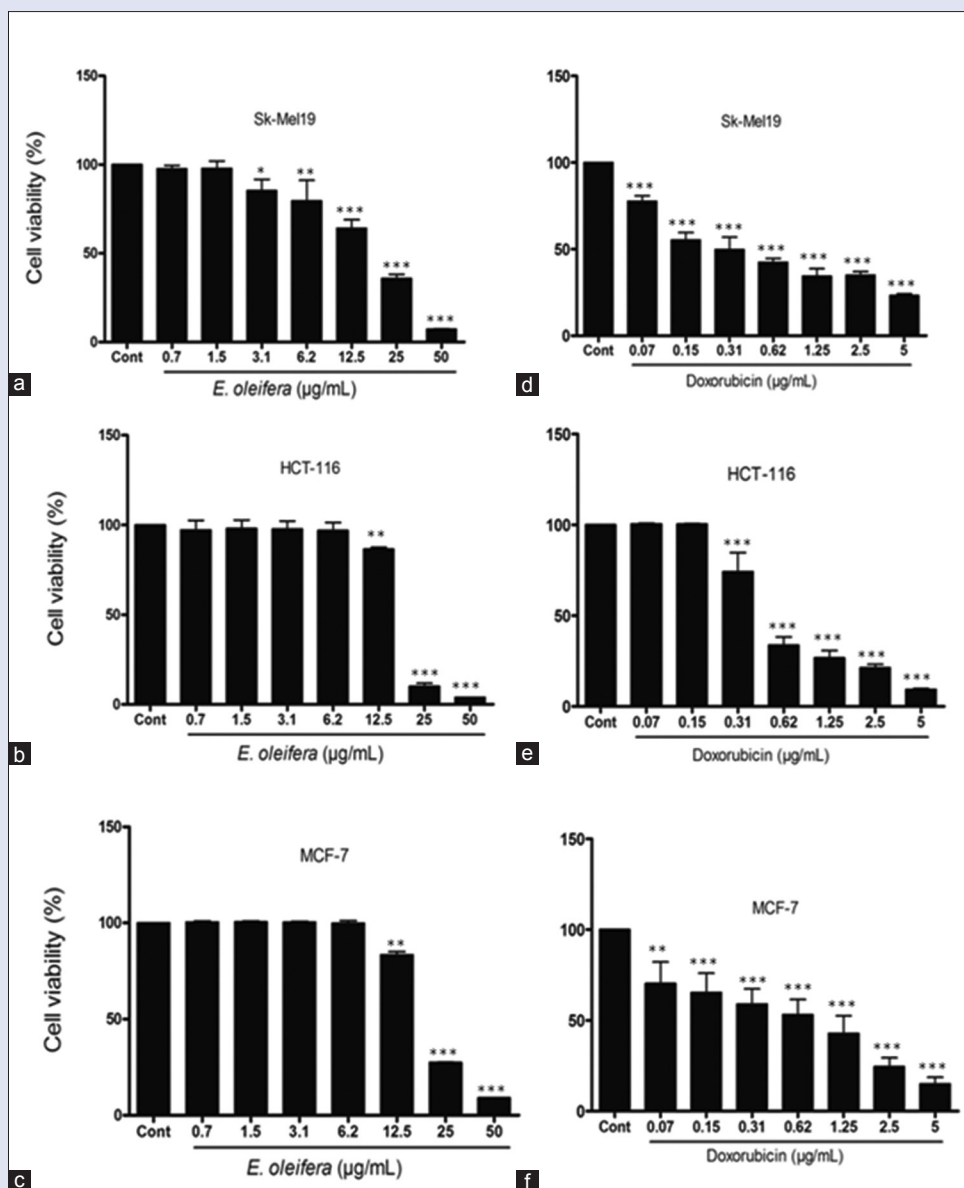
Diterpenes and sesquiterpenes are well known for their antitumor and anti-inflammatory properties.<sup>[22-24]</sup> Paclitaxel, a known diterpene isolated from *Taxus brevifolia*, is used for treating solid tumors. It works

**Table 1:** Cytotoxicity of *Elaeis oleifera* oil-resin in different cells lines compared with standard doxorubicin (IC<sub>50</sub> value µg/mL)

Treatment cell line	<i>Elaeis oleifera</i>	Doxorubicin
MRC-5	14.65 (13.79-15.55)	3.2 (2.6-4.0)
FPH	>50	2.14 (1.10-4.18)
HaCaT	22.92 (18.16-28.94)	0.58 (0.40-0.83)
HCT116	17.22 (14.60-20.30)	0.42 (0.37-0.78)
MCF-7	19.49 (16.95-22.40)	0.95 (0.73-1.24)
SK-Mel-19	15.99 (13.97-18.31)	0.779 (0.57-1.03)

\*Average values for IC<sub>50</sub> (µg/mL); results are represented by the means±SD of three experiments. SD: Standard deviation

by increasing microtubules stability and thus blocking progression of mitosis.<sup>[3,25]</sup> A recent study has investigated cytotoxic, mutagenic, and genotoxic effects of copalic acid, isolated from *C. langsdorffii*.<sup>[26]</sup> To the best of our knowledge, there are no data available on the cytotoxicity of AFEOR. In this work, we reported an in-depth analysis of its cytotoxic effects on a variety of cell lines and compared it with the known drug doxorubicin.<sup>[27]</sup>



**Figure 7:** Cytotoxicity of *Eperua oleifera* oil-resin in malignant cells Sk-Mel19, HCT-116, and MCF-7. (a-c) Treatment with *Eperua oleifera* oil-resin (at a concentration range of 0.7–50 µg/mL) in Dulbecco's modified Eagle medium for 72 h. (d-f) Doxorubicin treatment as a positive control, in a concentration range of 0.07–5 µg/mL. The negative control (dimethyl sulfoxide solvent 0.1%). Each bar represents the mean ± standard deviation, of triplicate determinations from three independent experiments. \*\*\**P* < 0.001, \*\**P* < 0.01, \**P* < 0.05 (one-way analysis of variance with Dunnett's multiple-comparison posttest)

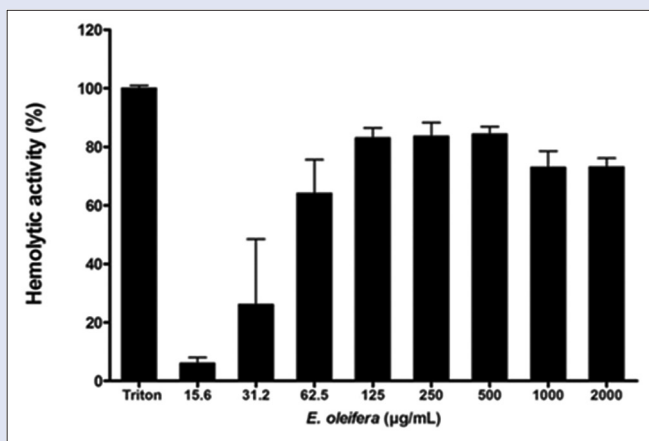
Fibroblasts have a higher basal proliferative capacity and its proliferation is one of the early processes during dermal wound healing.<sup>[28]</sup> Several studies on the molecular mechanisms of cellular carcinoma have revealed that deregulation of proliferation plays an important role in tumorigenesis.<sup>[29]</sup> In addition, the normal fibroblasts derived from a primary cell culture were used together with MRC-5 fibroblasts and human keratinocytes (HaCaT) to evaluate the effects of AFEOR on MMP activity and expression, cell proliferation, and production of collagen.

Morphological changes of fibroblasts corresponded well with the cytotoxicity tests. There were clear differences in cytotoxicity and morphology between cells with and without AFEOR treatment ( $\geq 10$  µg/mL). However, the significant reduction on cell viability of normal fibroblasts and keratinocytes by AFEOR treatment hampers its use as a potential antitumor drug.

Our experiment shows that AFEOR inhibits fibroblast cells proliferation even in low concentrations (5 µg/mL). These effects of AFEOR were only detected at concentrations  $\geq 25$  µg/mL in the cell viability assays. Our studies also aimed to investigate whether AFEOR increases the stimulation of collagen production in human fibroblasts. At 5 µg/mL, AFEOR stimulated the collagen production, even when there was a small decrease in cell number. It is an important result because collagen stimulation is ideal to counteract skin aging and to improve healing repair.<sup>[30,31]</sup>

A patent application has been filled for isolated compounds of *Eperua falcate* resin, as an accelerator of collagen production. This product comprises a mix of labd-8(17)-en-15-oic acid (eperuic acid) and labd-8-en-15-oic acid, obtained by chemically treating labdenoic acid.<sup>[13]</sup> These findings are important since the species of *E. oleifera* contain the



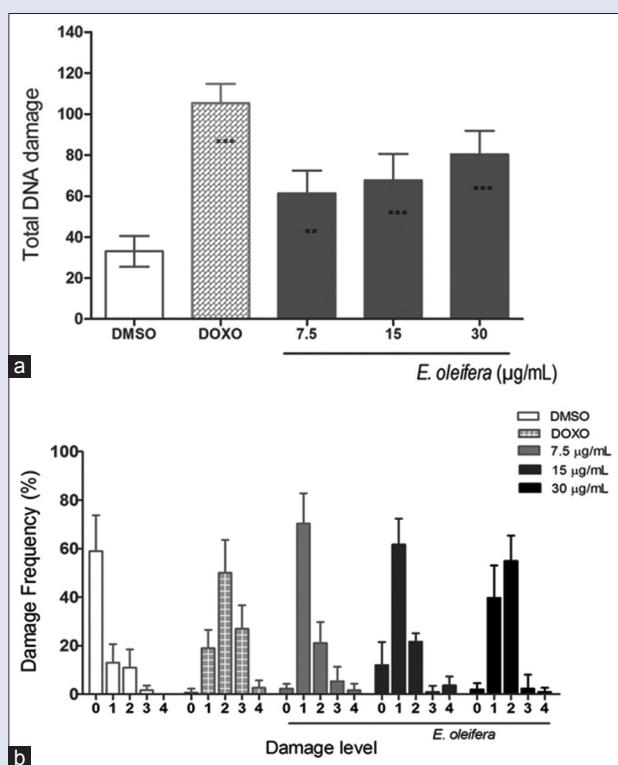


**Figure 8:** Hemolytic activity of *Eperua oleifera* oil-resin. Freshly prepared erythrocytes (from Swiss mice) were incubated with various concentrations of acid fraction of *Eperua oleifera* oil-resin and the supernatants were transferred to 96-well microplates and the absorbance was measured to quantify the extent of red blood cell lysis. Positive control (100% hemolysis) was determined by treating erythrocytes with 1% Triton X-100 (in PBS). The results are expressed as mean  $\pm$  standard deviation ( $n = 3$ ).  $IC_{50}$  values of 38.28 (29.60–49.50)

same compounds. It is noteworthy that, in traditional medicine, AFEOR is used for promoting wound healing.<sup>[8]</sup>

Natural products that inhibit MMPs can be beneficial in the prevention and/or treatment of cancer metastasis, skin aging, and wound repair.<sup>[32–34]</sup> In this study, zymographic analysis revealed inhibitory effects of AFEOR on collagenases activity. The possible mechanisms by which AFEOR inhibits the MMPs are presently unknown. Analysis of our data showed a significant inhibition by AFEOR of collagenases activity from *C. histolyticum*, with an  $IC_{50}$  value of 46.64 µg/mL. Therefore, we analyzed in cell culture whether AFEOR could have any direct effects on MMP-2 and MMP-9 activity, which are known to play key roles in several pathological conditions.<sup>[34–36]</sup> Indeed, in low concentrations from 1.25 to 5 µg/mL, AFEOR weakly inhibited gelatinase activity of HaCaT cells. Unfortunately, the applicability of the AFEOR on cell lines at concentrations up to 10 µg/mL was hampered by its cytotoxicity.

AFEOR exhibited a significant cytotoxicity *in vitro* against all tested cancer cell lines, but the highest cytotoxicity was observed for SKMELL19 ( $IC_{50} = 15.99$  µg/mL), HCT116 ( $IC_{50} = 17.22$  µg/mL), and MCF-7 ( $IC_{50} = 19.49$  µg/mL) cell lines, but  $IC_{50}$  is still high than the reference drug doxorubicin. Besides the cytotoxicity, the hemolytic activity of AFEOR in Swiss mice erythrocytes was evaluated. A high hemolytic activity with an  $IC_{50}$  value of 38.28 µg/mL was found for AFEOR after 30 min of exposure. This result suggests that the high cytotoxicity could be related to the membrane-damaging properties of AFEOR. This is confirmed by literature data, showing that diterpenes cause hemolysis and membrane damage of erythrocytes.<sup>[37,38]</sup> Cytotoxic effects of AFEOR are mediated not only by interaction of AFEOR compounds with cell membranes but also by cellular uptake and subsequent activation of necrosis or apoptosis. Therefore, more research is needed by isolating the pharmacologically active compounds from this medicinal oil. The challenge will be to isolate compounds that show almost no cytotoxicity on normal cells but a significant cytotoxic effect on tumor cells. For example, synthesis of the sesquiterpene (-)-hyrtiosal from copalic acid, i.e. a characteristic diterpene of copaiba oil-resin, led to a patent describing its selective cytotoxic activity against melanoma and leukemic cell lines.<sup>[39]</sup>



**Figure 9:** (a) Total and (b) frequency of DNA damage in human fibroblast MRC-5 cells, treated with *Eperua oleifera* for 2 h determined by the comet assay. The vehicle used to dissolve the drug, dimethyl sulfoxide (0.1%) was used as a negative control, and doxorubicin (10 µg/mL) was used as a positive control. Undamaged to maximal damage levels (0, 1, 2, 3, and 4). Data are mean  $\pm$  standard deviation of three independent experiments. \*\*\* $P < 0.001$  compared to negative control by analysis of variance followed by Tukey

This is the first report on the genotoxicity of *E. oleifera* oil-resin, whereas, in literature, diterpenes were found in *Copaifera* oil-resin with a genotoxic activity.<sup>[11]</sup> Some studies with the oil-resin of *Eperua* have described the presence of various diterpenes also found in *Copaifera* oil-resin, as kaurenoic acid, hardwickiic, copalic, and eperuic acids.<sup>[6,7]</sup> Cavalcanti *et al.*<sup>[11]</sup> reported that low concentrations of kaurenoic acid, i.e., a bioactive diterpenoid isolated from *C. langsdorffii*, did not induce DNA damage in V79 cells. *In vivo* studies with copaiba oil-resin and its fractions by Almeida *et al.*<sup>[24]</sup> revealed no toxicity, cytotoxicity, and genotoxicity of the oil even in doses of 2000 µg/mL. Gonçalves *et al.*<sup>[40]</sup> reported in a study with nine sesquiterpene compounds, including trans-caryophyllene, that none of the compounds were mutagenic. Taking into account that efficacy of some chemotherapeutic agents arises from its ability to generate extremely cytotoxic to DNA, we also evaluated genotoxicity of AFEOR.

The alkaline single-cell gel electrophoresis (comet) assay was performed evaluating the extent of DNA damage detected with the increase in DNA tail of fibroblasts. In the present study, AFEOR tested at 7.5–30 µg/mL increased DNA damage in fibroblasts by, respectively, 18%–60% when compared with the negative control. On the other hand, comparing with the positive control (doxorubicin), it was observed a significantly less DNA damage. Our results indicated that the *E. oleifera* oil-resin induced genotoxicity, with ( $P < 0.001$ ) mainly at the higher concentration of 30 µg/mL.

The mechanism through which the *E. oleifera* oil-resin acts in this cellular model is unclear. The bioassays indicated that the oil-resin of *E. oleifera*

could be useful in the search for new anticancer agents since it possesses an important cytotoxicity and inhibition of metalloproteinases. Further studies are needed to isolate the pharmacologically active compounds from AFEOR and to search for compounds with a lower cytotoxicity on normal cells. Taken together, our data also suggest that AFEOR in low concentrations could be a potential agent to develop effective MMP-1 inhibitors as chemotherapeutic agents and for skin photoprotection.

## CONCLUSION

Our results showed that oil-resin from *E. oleifera* have cytotoxic effects on tumor cells, hemolytic activities, and genotoxicity on fibroblasts and exhibits significant anticollagenase activity. Further investigations, such as screening of cytotoxic effects of the isolated compounds from AFEOR, *in vivo* studies, and research on mechanisms of action will be necessary to better understand the biological effects of *E. oleifera* oil-resin.

## Acknowledgements

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) for financial support of this research. APAB received a grant from DCR/CNPq/FAPEAM. FAT received a grant from FAPEAM. Paul Cos is a visitant professor and received a grant from CNPq.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

- Miller JS. The discovery of medicines from plants: A current biological perspective. *Econ Bot* 2011;65:396-407.
- Samwald M, Dumontier M, Zhao J, Luciano JS, Marshall MS, Cheung K. Integrating findings of traditional medicine with modern pharmaceutical research: The potential role of linked open data. *Chin Med* 2010;5:43.
- Viegas C, Bolzani VS, Barreiro EJ. Os produtos naturais e a química medicinal moderna. *Quim Nova* 2006;29:326-37.
- Barreiro EJ, Fraga CA. *Química Medicinal: As Bases Moleculares da ação dos Fármacos*. 1<sup>st</sup> ed. Artmed: Porto Alegre; 2001.
- Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 2007;70:461-77.
- Leandro LM, Vargas Fde S, Barbosa PC, Neves JK, da Silva JA, da Veiga-Junior VF. Chemistry and biological activities of terpenoids from copaiba (*Copaifera* spp.) oleoresins. *Molecules* 2012;17:3866-89.
- Veiga-Junior VF, Pinto AC. Gênero *Copaifera* L. *Quim Nova* 2002;25:273-86.
- Grenand P, Moretti C, Jacquemin H. Guanaco tradicional farmacopoeia. Créoles, Pallkui; Wayäpi. *Editions de l'ORSTOM*. Collection Mémoires 108. Paris. 177/261/347/393; 1987.
- Gournel D, Vidal-Tessier AM, Delaveau P. The resin of *Eperua grandiflora* (Aublet) Benth (leguminous Caesalpinaceae). *Ann Pharm Fr* 1985;43:565-72.
- Ducke A. Notes on the wallaba trees (*Eperua* Aubl.). Yale University: School of Forestry. Trop Woods New Haven 1940;62:21-8.
- Cavalcanti BC, Costa-Lotufu LV, Moraes MO, Burbano RR, Silveira ER, Cunha KM, et al. Genotoxicity evaluation of kaurenoic acid, a bioactive diterpenoid present in Copaiba oil. *Food Chem Toxicol* 2006;44:388-92.
- Ohsaki AY, Yan LT, Ito S, Edatsugi H, Iwata H, Komoda Y. The isolation and *in vivo* potent antitumor activity of clerodane diterpenoid from the oilresin of the Brazilian medicinal plant, *Copaifera langsdorffii* Desfon. *Med Chem Lett* 1994;4:2889-92.
- Yamamoto T, Yamamoto K. Accelerator of Collagen Production. US 2005/0004217 A1. United States: Patent Application Publication; 2005.
- Artacho-Cordón F, Ríos-Arrabal S, Lara PC, Artacho-Cordón A, Calvente I, Núñez MI. Matrix metalloproteinases: Potential therapy to prevent the development of second malignancies after breast radiotherapy. *Surg Oncol* 2012;21:e143-51.
- Hour MJ, Tsai SC, Wu HC, Lin MW, Chung JG, Wu JB, et al. Antitumor effects of the novel quinazolinone MJ-33: Inhibition of metastasis through the MAPK, AKT, NF- $\kappa$ B and AP-1 signaling pathways in DU145 human prostate cancer cells. *Int J Oncol* 2012;41:1513-9.
- Liao YF, Rao YK, Tzeng YM. Aqueous extract of *Anisomeles indica* and its purified compound exerts anti-metastatic activity through inhibition of NF- $\kappa$ B/AP-1-dependent MMP-9 activation in human breast cancer MCF-7 cells. *Food Chem Toxicol* 2012;50:2930-6.
- Koppikar SJ, Choudhari AS, Suryavanshi SA, Kumari S, Chattopadhyay S, Kaul-Ghanekar R. Aqueous cinnamon extract (ACE-c) from the bark of *Cinnamomum cassia* causes apoptosis in human cervical cancer cell line (SiHa) through loss of mitochondrial membrane potential. *BMC Cancer* 2010;10:210.
- Kim MS, Lee S, Rho HS, Kim DH, Chang IS, Chung JH. The effects of a novel synthetic retinoid, seletinoid G, on the expression of extracellular matrix proteins in aged human skin *in vivo*. *Clin Chim Acta* 2005;362:161-9.
- Nakayama GR, Caton MC, Nova MP, Paradoosh Z. Assessment of the Alamar Blue assay for cellular growth and viability *in vitro*. *J Immunol Methods* 1997;204:205-8.
- Ratnikov BI, Deryugina EI, Strongin AY. Gelatin zymography and substrate cleavage assays of matrix metalloproteinase-2 in breast carcinoma cells overexpressing membrane type-1 matrix metalloproteinase. *Lab Invest* 2002;82:1583-90.
- Fischer D, Li Y, Ahlemeyer B, Kriegelstein J, Kissel T. *In vitro* cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003;24:1121-31.
- Tappin MR, Pereira JF, Lima LA, Siani AC, Mazzei JL, Ramos MF. Chemical analysis for Standardization of copaiba oil by high resolution gas chromatography. *Quim Nova* 2004;27:236-40.
- Comelli Júnior E, Skinovski J, Sigwalt MF, Branco AB, Luz SR, Baulé Cde P. Rupture point analysis of intestinal anastomotic healing in rats under the action of pure Copaiba (*Copaifera langsdorffii*) oil. *Acta Cir Bras* 2010;25:362-7.
- Almeida MR, Darin JD, Hernandez LC, de Souza Ramos MF, Antunes LM, de Freitas O. Genotoxicity assessment of copaiba oil and its fractions in Swiss mice. *Genet Mol Biol* 2012;35:664-72.
- Corrêa AG. Taxol: From discovery to therapeutic use. *Quim Nova* 1995;18:460-7.
- Alves JM, Munari CC, de Azevedo Bentes Monteiro Neto M, Furtado RA, Senedese JM, Bastos JK, et al. *In vivo* protective effect of *Copaifera langsdorffii* hydroalcoholic extract on micronuclei induction by doxorubicin. *J Appl Toxicol* 2013;33:854-60.
- Finn NA, Findley HW, Kemp ML. A switching mechanism in doxorubicin bioactivation can be exploited to control doxorubicin toxicity. *PLoS Comput Biol* 2011;7:e1002151.
- van der Smissen A, Hintze V, Scharnweber D, Moeller S, Schnabelrauch M, Majok A, et al. Growth promoting substrates for human dermal fibroblasts provided by artificial extracellular matrices composed of collagen I and sulfated glycosaminoglycans. *Biomaterials* 2011;32:8938-46.
- Dumont N, Liu B, Defilippis RA, Chang H, Rabban JT, Karnezis AN, et al. Breast fibroblasts modulate early dissemination, tumorigenesis, and metastasis through alteration of extracellular matrix characteristics. *Neoplasia* 2013;15:249-62.
- Gopinath D, Ahmed MR, Gomathi K, Chitra K, Sehgal PK, Jayakumar R. Dermal wound healing processes with curcumin incorporated collagen films. *Biomaterials* 2004;25:1911-7.
- Man XY, Finnson KW, Baron M, Philip A. CD109, a TGF- $\beta$  co-receptor, attenuates extracellular matrix production in scleroderma skin fibroblasts. *Arthritis Res Ther* 2012;14:R144.
- Huang ST, Yang RC, Wu HT, Wang CN, Pang JH. Zinc-chelation contributes to the anti-angiogenic effect of ellagic acid on inhibiting MMP-2 activity, cell migration and tube formation. *PLoS One* 2011;6:e18986.
- Jouneau S, Leveiller G, Caulet-Maugendre S, Brinchault G, Belleguic C, Desrues B, et al. Role of matrix metalloproteinases (MMPs) in cystic fibrosis. *Matrix Metalloproteinases in Tissue Remodelling and Inflammation*. Switzerland: Birkhauser Verlag Basel; 2008. p. 71-80.
- Okada M, Suzuki A, Yamawaki H, Hara Y. Levosimendan inhibits interleukin-1 $\beta$ -induced cell migration and MMP-9 secretion in rat cardiac fibroblast. *Eur J Pharmacol* 2013;718:332-9.
- Park G, Kim HG, Sim Y, Sung SH, Oh MS. Sauchinone, a lignan from *Saururus chinensis*, protects human skin keratinocytes against ultraviolet B-induced photoaging by regulating the oxidative defense system. *Biol Pharm Bull* 2013;36:1134-9.
- Fullár A, Kovalszky I, Bitsche M, Romani A, Scharntinger VH, Sprinzl GM, et al. Tumor cell and carcinoma-associated fibroblast interaction regulates matrix metalloproteinases and their inhibitors in oral squamous cell carcinoma. *Exp Cell Res* 2012;318:1517-27.

37. Costa-Lotufo LV, Montenegro RC, Alves APNN, Madeira SVF, Pessoa C, Moraes MEA, *et al.* The Contribution of Natural Products as a Source of New Drugs Anticancer: Studies In the National Laboratory of Experimental Oncology of the Federal University of Ceará. *Rev Virtual Quim* 2010;2:47-58.
38. Sekiguchi Y, Mano H, Nakatani S, Shimizu J, Kobata K, Wada M. Anti-proliferative effects of *Salacia reticulata* leaves hot-water extract on interleukin-1 $\beta$ -activated cells derived from the synovium of rheumatoid arthritis model mice. *BMC Res Notes* 2012;5:198.
39. Lunardi I, Santiago GM, Imamura PM. Synthesis of (-)-and (+)-hyrtiosal and their C-16 epimers. *Tetrahedron Lett* 2002;43:3609-11.
40. Gonçalves O, Pereira R, Gonçalves F, Mendo S, Coimbra MA, Rocha SM. Evaluation of the mutagenicity of sesquiterpenic compounds and their influence on the susceptibility towards antibiotics of two clinically relevant bacterial strains. *Mutat Res* 2011;723:18-25.