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In vitro study after exposure to the aqueous extract of *Piper amalago* L. shows changes of morphology, proliferation, cytoskeleton and molecules of the extracellular matrix

Estudo *in vitro* após a exposição ao extrato aquoso de *Piper amalago* L. mostra alterações de morfologia, proliferação, citosesqueleto e moléculas da matriz extracelular

Estudio in vitro después de la exposición al extracto acuoso de *Piper amalago L*. muestra los cambios en la morfología, proliferación, citoesqueleto y moléculas de la matriz extracelular

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Abstract

Piper amalago L. is a medicinal plant traditionally used as a healing agent for wounds, burns, abscesses, boils, and insect bites. The current study aimed to evaluate the possible effects of the aqueous crude extract obtained from *P. amalago* leaves, in different concentrations and in different incubation times, using the in vitro model of mouse

fibroblasts (3T3). The extract was tested in different concentrations at the 24 h incubation time for analysis of cell viability, cytotoxicity, proliferation, cell morphology, immunostaining, adhesion and cell spreading assays, as well as to determine the hydroxyproline concentration and activity of the metalloproteinase MMP2. Morphologically, after exposure to the concentrations of 15 and 150 µg/mL, the cells maintained the morphology, yet a greater number of cells with more expansions of the cell body and larger than the control cells were observed. The treated cell culture also showed a greater number of cells, larger cells, a greater expansion of the cell body, adherent cells spread over the substrate, and a more juxtaposed, central and spherical nucleus. The treatment induced greater cell adhesion to the polymer, fibronectin, and collagen I. Biochemical results showed a significant increase in the hydroxyproline amino acid after exposure for 96 h. The extract did not induce loss of cell viability until the concentration reached 150 µg/mL, positively modulating proliferation, morphology, adhesion, degree of spreading, and organization of microfilaments. The extract also promoted a significant increase in the hydroxyproline amino acid.
Keywords: Fibroblast; Jaborandi-manso; 3T3 strain.

Resumo

Piper amalago L. é uma planta medicinal tradicionalmente usada como agente de cura para feridas, queimaduras, abcessos, furúnculos e picadas de insetos. O presente estudo teve como objetivo avaliar os possíveis efeitos do extrato bruto aquoso obtido das folhas de *P. amalago*, em diferentes concentrações e em diferentes tempos de incubação, utilizando o modelo in vitro de fibroblastos de camundongo (3T3). O extrato foi testado em diferentes concentrações no tempo de incubação de 24 horas para análise de viabilidade celular, citotoxicidade, proliferação, morfologia celular, imunomarcação, adesão e ensaios de propagação celular, bem como para determinar a concentração de hidroxiprolina e atividade da metaloproteinase MMP2. Morfologicamente, após exposição às concentrações de 15 e 150 µg/mL, as células mantiveram a morfologia, porém foi observado um número maior de células com mais expansões do corpo celular e maiores que as células controle. A cultura celular tratada também apresentou maior número de células, células maiores, maior expansão do corpo celular, células aderentes espalhadas pelo substrato e núcleo mais justaposto, central e esférico. O tratamento induziu maior adesão celular ao polímero, fibronectina e colágeno I. Os resultados bioquímicos mostraram um aumento significativo no aminoácido hidroxiprolina após exposição por 96 h. O extrato não induziu perda de viabilidade celular até a concentração atingir 150 µg/mL, modulando positivamente a proliferação, morfologia, adesão, grau de disseminação e organização dos microfilamentos. O extrato também promoveu um aumento significativo no aminoácido hidroxiprolina.
Palavras-chave: Fibroblasto; Jaborandi-manso; Linhagem 3T3.

Resumen

Piper amalago L. es una planta medicinal utilizada tradicionalmente como agente cicatrizante de heridas, quemaduras, abscesos, furúnculos y picaduras de insectos. El presente estudio tuvo como objetivo evaluar los posibles efectos del extracto crudo acuoso obtenido de las hojas de *P. amalago*, en diferentes concentraciones y en diferentes tiempos de incubación, utilizando el modelo in vitro de fibroblastos de ratón (3T3). El extracto se probó a diferentes concentraciones en el tiempo de incubación de 24 horas para el análisis de viabilidad celular, citotoxicidad, proliferación, morfología celular, inmunotinción, ensayos de adhesión y propagación celular, así como para determinar la concentración de hidroxiprolina y la actividad metaloproteinasa de MMP2. Morfológicamente, tras la exposición a concentraciones de 15 y 150 µg/mL, las células mantuvieron su morfología, pero se observó un mayor número de células con más expansiones del cuerpo celular y más grandes que las células control. El cultivo celular tratado también mostró un mayor número de células, células más grandes, mayor expansión del cuerpo celular, células adherentes difundidas sobre el sustrato y núcleos más yuxtapuestos, centrales y esféricos. El tratamiento indujo una mayor adhesión celular al polímero, fibronectina y colágeno I. Los resultados bioquímicos mostraron un aumento significativo en el aminoácido hidroxiprolina después de 96 h de exposición. El extracto no indujo pérdida de viabilidad celular hasta que la concentración alcanzó 150 µg/mL, modulando positivamente la proliferación, morfología, adhesión, grado de diseminación y organización de los microfilamentos. El extracto también promovió un aumento significativo del aminoácido hidroxiprolina.
Palabras clave: Fibroblasto, Jaborandi-manso; Linaje 3T3.

1. Introduction

Some plant species are popularly used in various regions of Brazil as healing agents (Piriz et al., 2014). Compounds and extracts from the aerial parts and roots of *Piper* species have been studied in order to elucidate their benefit in the healing process (Bastos et al., 2011; Jardim, 2011; Paco et al., 2016; Santos et al., 2020).

Piper amalago L., popularly known as jaborandi-manso, is a shrub that measures 2 to 7 m, native from Central America and Brazil (Parmar et al., 1997; Guimarães & Valente, 2001). It is an aromatic plant that produces essential oil (Santos et al., 2015). In Brazil, its leaves are used in traditional medicine as a healing agent for wounds, burns, abscesses, boils,

and insect bites (SEMA, 2003; Alves et al., 2008; Bratti et al., 2013; Salehi et al., 2019). Additionally, *P. amalago* is an analgesic and digestive aid (Facundo et al., 2008), use to treat urinary stones and heart problems (Novaes et al., 2014). Furthermore, in Puerto Rico, the masticated leaves of *P. amalago* are put on bleeding cuts; whereas in Mexico, the Huasteco-Maya tribes use *P. amalago* to treat edema, inflammations, and as an antipyretic agent (Durant-Archibold et al., 2018).

Pharmacological studies, performed with *P. amalago*, showed anti-inflammatory (Sosa et al., 2002; Novaes et al., 2014), antimicrobial (Carrara et al., 2012; Santos et al., 2016), schistosomicidal (Carrara et al., 2014), diuretic, anti-lithiasic, (Novaes et al., 2014), anxiolytic (Lopes et al., 2012), anti-Leishmania (Carrara et al., 2013), and healing activities (Guimarães & Valente, 2001). Recently, the topical application of the aqueous extract, produced from *P. amalago* leaves, aided in the healing of a lacerated wound in the left thumb of a patient with type 2 diabetes mellitus over a period of 15 days in study by Santos et al. (2020).

Phytochemical analysis of *P. amalago* leaves showed the presence of amides, alkaloids, condensed tannins, flavonoids, and triterpenes (Domínguez et al., 1985; Carrara et al., 2013; Lopes et al., 2012; Rovani et al., 2013; Novaes et al., 2014; Santos et al., 2020). Essential oils of *P. amalago* leaves evidenced the presence of monoterpenoids and sesquiterpenoids (Carrara et al., 2010; Salehi et al., 2019).

Healing involves numerous cellular and biochemical events that interact for the regeneration of injured tissue. The healing process has three phases: inflammatory; proliferation or granulation, and remodeling or maturation (Campos et al., 2007; Peng et al., 2013; Medeiros & Dantas Filho, 2016; Luciano et al., 2021). It is a complex process involving growth factors, cytokines and chemokines (Peng et al., 2011; Peng et al., 2013; De Mais et al., 2016). In a few hours after the injury, the inflammatory phase begins with increased vascular permeability, secretion of cytokines and growth factors, with activation of cell migration (Medeiros & Dantas Filho, 2016).

The proliferation phase is responsible for the closing of the injury, in which epithelialization, fibroplasia and angiogenesis occur. Fibroblasts and specialized cells attract chemically attracted cells to the inflammation site in fibroplasia, proliferation, biosynthesis and secretion of the extracellular matrix components occur (Medeiros & Dantas Filho, 2016).

The fibroblasts act in the remodeling phase producing collagen and depositing it in an organized way. The collagen produced initially is Type III collagen, and it is gradually replaced by Type I collagen (Peng et al., 2011; Peng et al., 2013; Cunha et al., 2015). Fibroblasts also produce metalloproteinase inhibitors (TIMPs). Metalloproteinases (MMPs) are enzymes that catalyze extracellular matrix proteins. Together, MMPs and TIMPs form a complex biological system that maintain extracellular matrix homeostasis (MEC) (Trengove et al., 1999; Gill & Parks, 2008).

Metalloproteinases are enzymes that degrade proteins in the extracellular matrix. The MMP family includes approximately 25 proteins, being gelatinase B (MMP-9) and gelatinase A (MMP-2), two closely-related members of the MMP family that degrade collagen denatured or gelatins (Visse & Nagase, 2003). In addition, metalloproteinases have important functions in several biological processes, for example, in morphogenesis, and in all stages of healing, such as tissue repair and remodeling in response to injury. In addition to these important functions, it is noteworthy that metalloproteinases are very important in structural and functional tissue maintenance, so they do not lose their normal architecture (Araújo et al., 2011).

In wound healing, the main effects induced by the active principles present in plant extracts are those that have antimicrobial, and antioxidant activities, as well as those that induce cell proliferation, angiogenesis; in addition to those activities that contribute to the increase in collagen production (Budovsky et al., 2015).

Considering the traditional uses of leaves of *P. amalago* as a healing agent, the present study aimed to evaluate the possible effects of the aqueous crude extract obtained from *P. amalago* leaves on cell viability, cytotoxicity, proliferation, cell morphology, immunostaining, adhesion, and cell spreading assays. In addition, this study aimed to determine the

hydroxyproline concentration and activity of the metalloproteinase MMP2, using an *in vitro* model of mouse fibroblasts (3T3) in different concentrations and incubation times, in order to indicate a possible validation of its popular use as a healing agent.

2. Methodology

To carry out this study, a quantitative methodology was used in an experimental model (Pereira et al., 2018).

2.1 Plant material

Aerial vegetative parts of *Piper amalago* L., Piperaceae, were collected in Curitiba, Paraná (24º 18' S and 49º 37' W), in May 2016. The vegetal material was identified by a taxonomist and compared with the voucher specimens deposited in the Municipal Botanical Museum of Curitiba, and was registered under number 71947.

The access to the plant material was permitted and licensed by Genetic Heritage Administration Council (CGEN/SISGEN) and was registered under number A14F6AF.

2.2 Obtaining the aqueous extract

The aqueous extract of the leaves of *P. amalago* was obtained according to the methodology described by Viana et al. (2006). The dried leaves were ground for 30 seconds; and then, 75 g of crushed material were mixed in 500 mL of distilled water for homogenization under agitation for 1 min. The mixture was stored in glass containers kept at rest for 24 h at 5ºC. After the cooling period, the liquid extract was filtered to remove residues and particulate material.

2.3 Obtaining the lyophilized product

The aqueous extract was frozen at -65ºC, after being subjected to negative pressure (vacuum), to remove the water by sublimation. The product obtained has a porous structure free of moisture, capable of being reconstituted in water. A Virtis SP Scientifica freeze dryer, BenchTop Pro 8L - XL70 model, was used (Viana et al., 2006).

2.4 Cell culture

3T3 mouse fibroblasts (ATCC® CRL-1658TM) were maintained in Dulbecco's Modified Eagle's Medium – DMEM, supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM Hepes, 0,25 µg/mL penicillin-streptomycin in 0,85% saline, 3.7 g/L sodium bicarbonate at 37°C in 5% CO_2 in humidified atmosphere (George et al., 2010).

2.5 Cell viability, cytotoxicity and proliferation assays

3T3 cells were exposed to the aqueous extract of *P. amalago*, in a time-dependent concentration. Cell viability, cytotoxicity and proliferation were measured using neutral red as described by Borenfreund and Puerner (1985), MTT (Thyazolyl Blue Tetrazolium Bromide), as described by Mosmann (1983) and Crystal Violet, Crystal Violet Gillies et al. (1986) assays, respectively. All experiments were compared to cells in the absence of aqueous extract of *P. amalago* (control condition).

2.6 Cell morphology

Cell morphology was analyzed by light microscopy (LM) and scanning electronic microscopy (SEM). Cells (1×104) cultured in 24-well plates over glass coverslips (Corning), exposed or not for 24 h to 15 and 150 µg/mL aqueous extract of *P. amalago*, were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Washington, NY, USA), for 30 min, at 22°C, washed with PBS, dehydrated in an ethanol series and stained with hematoxylin and eosin. Coverslips were mounted in

Entelan® and examined by LM. For SEM, cells were fixed for 1 h in Karnovski solution (2% glutaraldehyde (Electron Microscopy Sciences, Washington, NY, USA), 4% paraformaldehyde, $CaCl_2$ 1 mM in sodium cacodylate buffer 0.1 M (Merck). Washed and post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Washington, NY, USA). Washed in sodium cacodylate buffer 0.1M for 1 h (in the dark at room temperature) and then dehydrated using increasing ethanol concentrations. The samples were dried to critical point and metallized using gold. Images were acquired on TESCAN VEGA3 LMU microscope of UFPR Electronic Microscopy Center (Biscaia et al., 2017).

2.7 Fluorescence cytochemistry assays

Cells (1×10^4) were grown on 13-mm-diameter glass coverslips in 24-well plates (TPP, Trasadingen, Switzerland) and were exposed or not (the negative control) for 24 h with 15 and 150 µg/mL aqueous extract of *P. amalago*. Cells were then rapidly washed with PBS and fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for 30 min at 22°C, subsequently washed with PBS. For confocal microscopy, marking of cell surface carbohydrates, cells were incubated for 1 h with Lectin WGA conjugated to FITC (4 mg/mL) in the presence of 1% BSA in PBS at room temperature. The cell's cytoskeleton was marked with Alexa Fluor® 546 Faloidina- (1:500 in 0,01% de saponin-PBS) for 30 min at 22°C. After, cells were washed three times with PBS. For all nuclear fluorescent using Fluoromount-G® mounting medium with DAPI (Electron Microscopy Sciences, Cat. 17984-24, Hatfield, PA, USA). Images were acquired using the laser scanning confocal electron microscopy A1R MP+ microscope (Nikon Instruments Inc, Tokyo, Japan) (Biscaia et al., 2017).

2.8 Adhesion and cell spreading assay

Tissue culture plates (24 wells, Costar Corp., Cambridge, MA) were coated for 2 h with fibronectin and collagen Type I (10 µg/mL in PBS). Non-adhesive substrate was prepared by coating the wells with 1% BSA (Sigma) for 60 min at 37ºC. Plates were washed with PBS and blocked with 1% BSA in PBS for 1 h. Cells exposed or not (the negative control) for 24 h with 15 and 150 µg/mL aqueous extract of *P. amalago* were suspended (5 x 10^5 cells in 0.5 mL medium) and allowed to attach to the substrate for 1 h at 37ºC under 5% atmosphere CO_2. Following incubation, unattached cells were removed by washing with PBS. Attached cells were fixed in methanol for 20 min, stained with 0.8% crystal violet (Sigma) dissolved in 20% ethanol and washed five times in PBS. Cells were photographed with an inverted microscope (Nikon TE-300). The dye was eluted with 50% ethanol in 0.1 M sodium citrate, pH 4.2 and the optical density measured at 540 nm. The degree of spreading was determined based on the images, using the program Image J Fiji Software, taking as parameter the nucleus area x cytoplasmic area ratio (proportion of the cell body expansion) (Nowatzki et al., 2010).

2.9 Determination of hydroxyproline concentration

3T3 cells were plated on circular coverslips within 24 well-plates. Subsequently, 15, 50 and 150 µg/mL of the crude aqueous extract of *P. amalago* were treated for 96 h. The supernatant was removed and the cells were subjected to alkaline hydrolysis with NaOH (10M) at 80°C for 20 min, and then the hydrolysate was oxidized with chloramine T. Finally, Ehrlich's reagent was added, which complexed hydroxyproline in a colorimetric reaction, with the intensity of coloration being proportional to the amount of hydroxyproline tissue. Absorbance was measured at 550 nm (Soley et al., 2016).

2.10 Quantification and activity of MMP2 metalloproteinase

3T3 cells were plated on circular coverslips within 24-well-plates. Subsequently, 15, 50 and 150 µg/mL of the crude aqueous extract of *P. amalago* leaves were treated for 96 h. The supernatant was removed and used for the evaluation of MMP2 metalloproteinase quantification and activity.

2.11 Protein concentration

The cell culture supernatant was concentrated from 6 mL to 100 µL of volume in a SavantTM SPD1010 SpeedVac. To this volume, 400 µL of methanol was added, followed by homogenization and centrifugation at 11,000 rpm for 15 s. Later, 100 µL of chloroform and 300 µL of ultra-pure water were added, each step with homogenization and centrifugation as described above. Three phases were formed: the upper phase, which was discarded and 300 µL of methanol was added; followed by homogenization; and centrifugation at 11,000 rpm for 2 min. The protein pellet was air-dried and re-suspended with RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0) to be submitted to zymography (Bradford, 1976).

2.12 Zymography

The samples were subjected to dosage total protein by the Bradford method and added in a sample buffer (2% SDS, 30% glycerol, 0.01% bromophenol blue, 60 mM Tris pH 6.8). The samples were loaded on 12% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was washed in a 10 mM Tris solution (pH 8.0) containing 2.5% Triton X-100 for SDS removal and protein renaturation. Later, the gel was incubated in a solution containing 5 mM $CaCl_2$, 150 mM NaCl and 1 mM $ZnSO_4$ for 48 h at 37ºC. Afterwards, the gel was stained with Coomassie Brilliant Blue R-250 and decolorized (40% methanol, 10% acetic acid). The bands resulting from gelatin degradation were analyzed by densitometry using the Image J software (Bradford, 1976).

2.13 Immunoblotting

3T3 cells were cultured in 100 mm^2 plates until reaching 80% confluence, and treated under the conditions described above. The cells were washed with ice-cold PBS and lysed with RIPA buffer, with protease inhibitor (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher), and 1 mM EDTA. The cell lysate was centrifuged at 10,000 g at 4ºC, and the supernatant was subjected to the determination of total proteins by Bradford method (Bradford, 1976). Equal amounts of protein were added to sample buffer (2% SDS, 10% glycerol, 0.01% bromophenol blue, 0.0625 mol/L Tris-HCl pH 6.8, 5% β-mercaptoethanol), and heated at 100°C for 10 min. The samples were loaded to the electrophoresis gel and then transferred to polyvinylidene difluoride (PVDF) membrane (Hybond ECL-GE Healthcare). After this, the membrane was blocked with 5% skim milk in tris-buffered saline (TBS; 20 mM tris, 150 mM NaCl, pH 7,6) with 0.1% tween 20 for 1 h at room temperature. The membrane was incubated with anti-MMP-2 antibodies (Thermo Fischer Scientific Inc. 2C1-1D12; 1:500) in the block solution for 18 h at 4°C. Next, the membrane was incubated with the secondary anti-mouse IgG HRP antibody (Sigma) of concentration 1 mg/mL (1:5000). The washes between the steps were done with TBS containing 0.05% tween 20 and TBS. The revelation was performed by chemiluminescence with Luminata Forte Western HRP substrate (Millipore) reagent using X-ray film. The intensity of the bands was analyzed with ImageJ software (Bradford, 1976).

2.14 Statistical analysis

Statistical analyses were performed by analysis of variance (ANOVA: single factor) of the data obtained. Tukey's test was also be used, allowing significant differences to be identified between groups. GraphPad software was used in these aspects.

3. Results

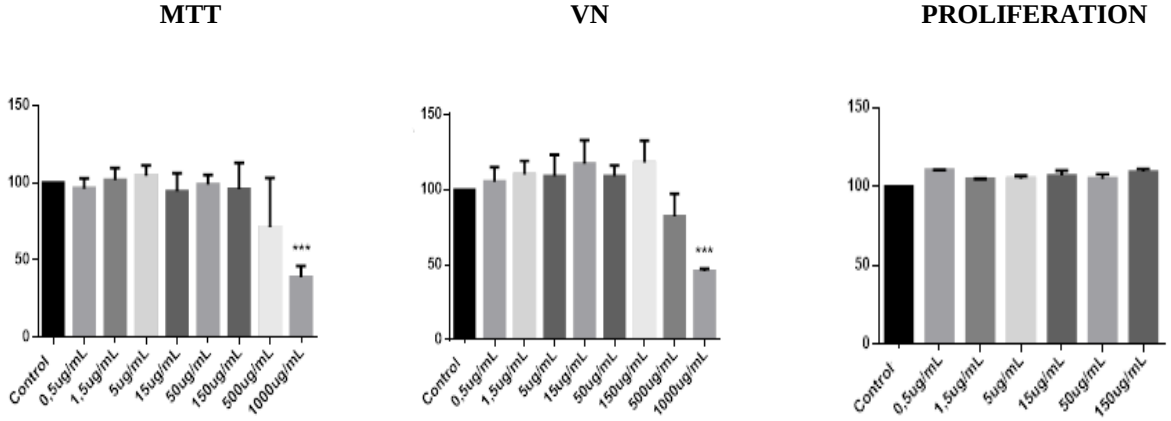
3.1 Aqueous extract up to a concentration of 150 µg/mL does not induce cytotoxic action, loss of viability and induces cell proliferation.

As shown in Graph 1 for the cell cytotoxicity assay using the MTT method (A), and cell viability using the neutral red method (B), the results presented demonstrate that the aqueous extract neither induce cytotoxicity action nor loss of cell viability to a concentration of 150 µg/mL within 24 h of exposure. Using these methods, cytotoxicity and loss of viability were observed in the highest concentrations of 500 and 1000 µg/mL, being in this last concentration, performing significantly.

Compound induced cell proliferation can be seen in the graph on cell proliferation assay (C), at all concentrations tested, the largest being 150 µg/mL.

In the concentrations of 500 and 1000 µg/mL, the tests were not performed because the assess impairment of cell viability (MTT and VN) proved to be cytotoxic. Thus, the cell proliferation assay was performed only at concentrations that maintained cell viability.

Graph 1. Cytotoxicity, viability and proliferation assays.



These tests were carried out in biological quadruplicates. Source: Authors.

The 3T3 cells were exposed to the crude extract of *Piper amalago* in concentrations of 0.5, 1.5, 5, 15, 50, 150, 500 and 1000 µg/mL for a 24h treatment period. Letter A shows cell cytotoxicity assay, by the MTT method, letter B the cell viability assay, by the Neutral Red method and letter C the cell proliferation assay, using the Violet Crystal method.

3.2 Aqueous extract induces morphological and ultrastructural changes

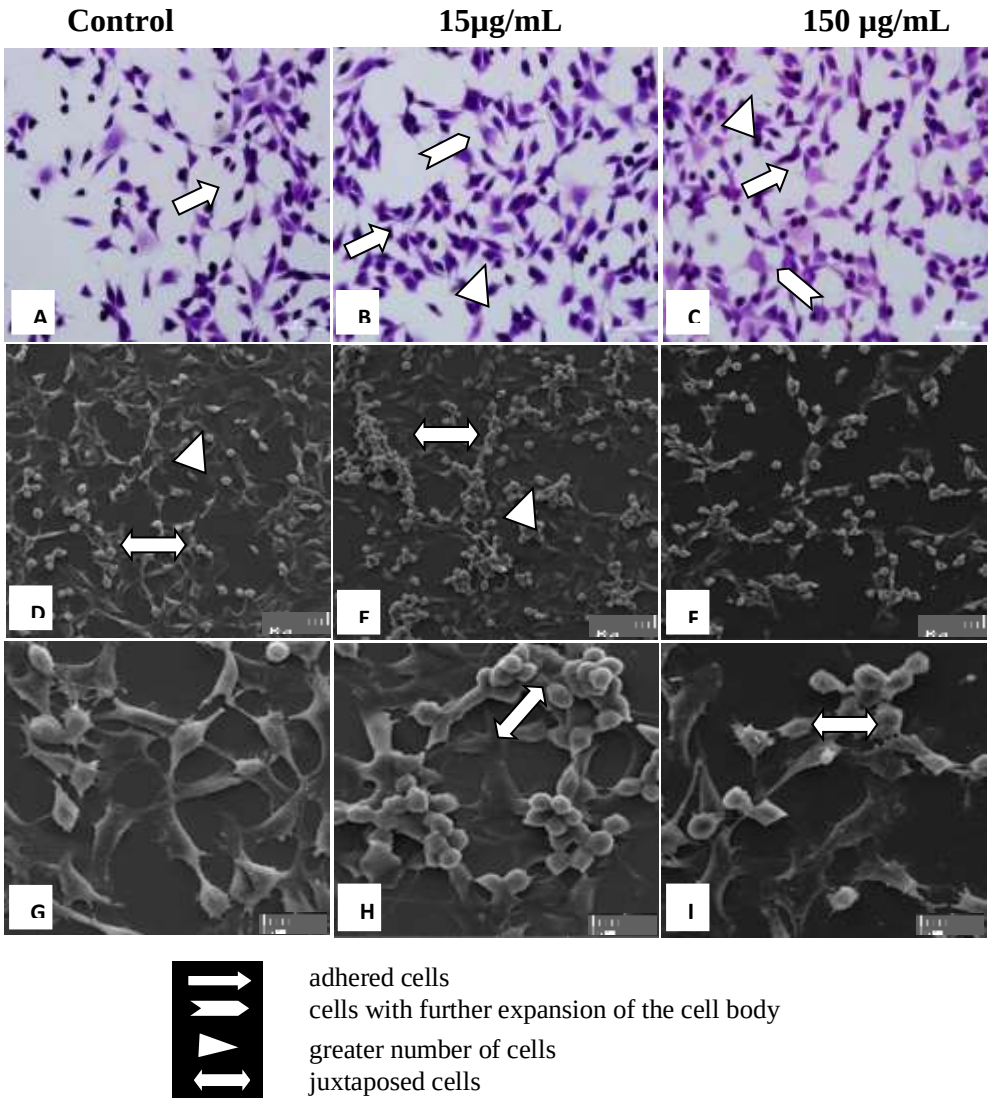
Since the cytotoxicity, cell viability and proliferation assays showed similar results in the lowest exposure concentrations 0.5 to 150 µg/mL, and that only in concentrations above 150 µg/mL, the previous assays demonstrated evident cytotoxicity with impaired cell viability. In order for the other tests to be analyzed, we only standardize the concentrations of 15 and 150 µg/mL, to be imposed for all other subsequent tests.

The morphological analysis of 3T3 control cells (CTR), cells treated with 15 µg/mL and with 150 µg/mL of crude *P. amalago* extract are shown in Figure 1 (A to I) for a period of 24 h of exposure.

It is noted that, in Figure 1A, controls cells observed under an optical microscope comply with the standard established for that line. They are cells with different morphologies, with characteristic cell body expansions and membrane projections. Rounded and central nucleus show contact inhibition. It can also be noted that these cells are adhered to the substrate. There is a predominance of elongated cells with clear membrane extensions, central and spherical nucleus. Treated

cells 15 µg/mL (1B) and 150 µg/mL (1C) show a greater number of cells in cell culture. These treated cells are larger with greater expansion and degree of cell spread. Images (D to I) represent ultrastructural analysis in SEM: control cells (D and G), exposed to 15 µg/mL (E and H) and 150 µg/mL (F and I), with the same exposure time. SEM results showed a greater number of cells after treatment; these are greater due to the greater expansion of the cell body. Adhered cells, were more spread over the substrate. There is a predominance of rounded cells forming cellular structures in strands, and juxtaposed cells. The pattern of cell culture was positively modulated in both morphological analyses obtained through LM and SEM.

Figure 1. Morphological examination.



These tests were performed in quadruplicates, in two different tests. Source: Authors.

The light microscope shows in (A) 3T3 control cells (maintenance of cell culture in the absence of crude *Piper amalago* extract), exposed to 15 µg/mL (B) and 150 µg/mL (C), for a period of 24 hours of exposure. 400x magnification images. These tests were performed in quadruplicates, in two different tests. The scanning electron microscopy shows the control cells (D and G), exposed to 15 µg/mL (E and H) and 150 µg/mL (F and I) for a period of 24 hours of exposure. The images D, E and F are at 300X magnification and G, H and I images at 6000X magnification.

3.3 Aqueous extract induces greater cell body expansion and intercellular adhesion

The detection of specific cell surface carbohydrates was performed using Lectin WGA conjugated to FITC. To detect the actin microfilaments, the phalloidin probe conjugated with Alexa Fluor 594 was used.

Image 2A shows 3T3 control cells not imposed on treatment with crude *P. amalago* extract. Image B shows cells exposed at a concentration of 15 µg/mL, and image C shows cells imposed at a concentration of 150 µg/mL. All of these images show glycocalyx through the immunodetection of carbohydrate (N-acetyl-glucosamine) by lectin WGA.

The (2D) image of control cells, (3E) of cells exposed to 15 µg/mL and (2F) of cells exposed to 150 µg/mL of the crude extract of *P. amalago* show the actin microfilaments of the cytoskeleton evidenced by phalloidin.

It can be observed in the immunofluorescence images that the control cells have a more elongated morphology when compared to the treated cells (2B and 2C). These cells presented with less expansion of the cell body volume (2A). In the detection for surface carbohydrates, less intensity of staining is observed for control cells. In these cells, when the nucleus/cell cytoplasm correlation is observed, they present the cell body (cytoplasm) with less expansion. Cells with different morphologies adhered to the substrate and smaller cells are observed.

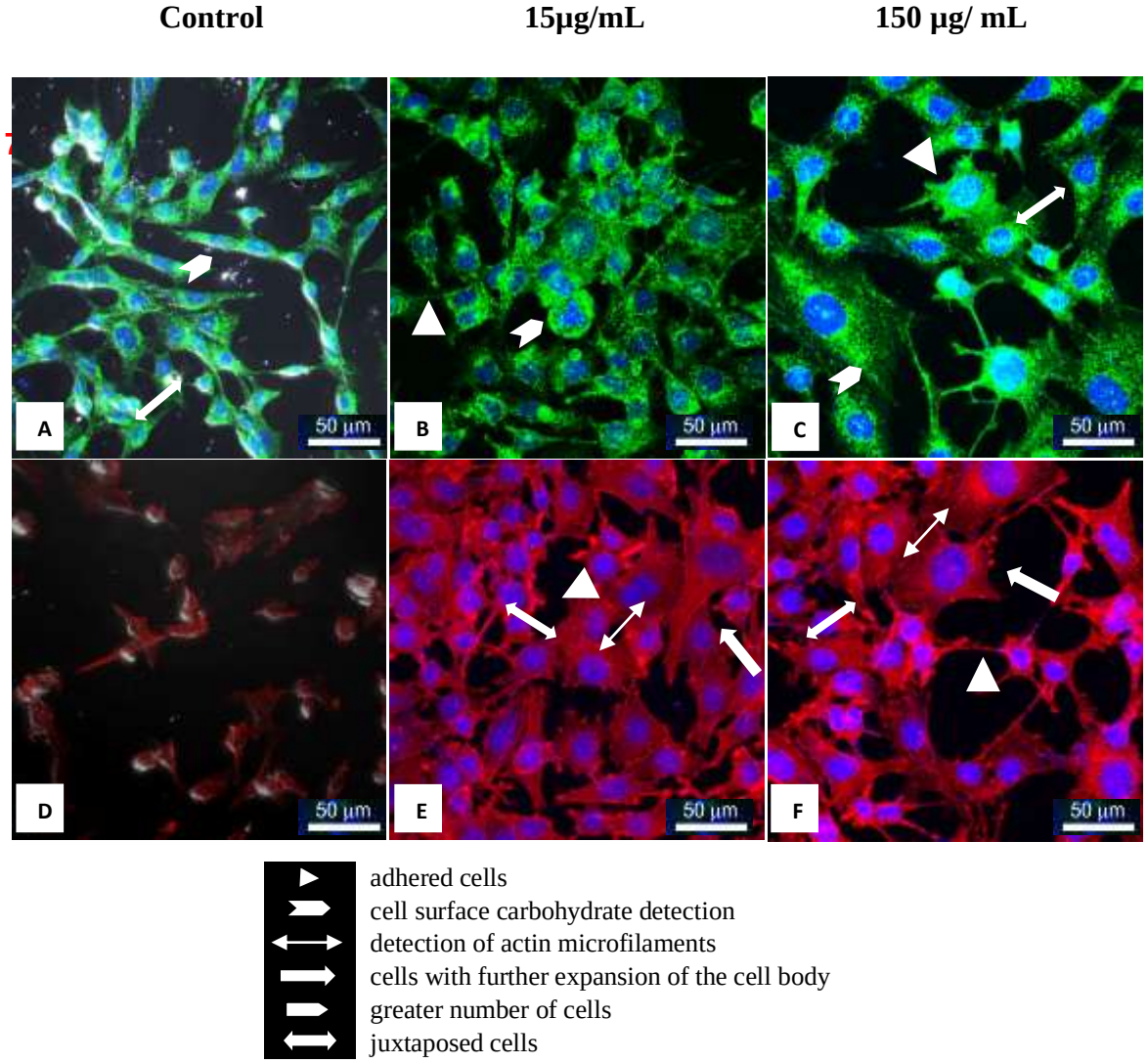
Interestingly, the exposure of the cells to the aqueous extract of *P. amalago* (2B, 2C, 32 and 2F) 15 and 150 µg/mL respectively show, when compared with the control cells (2A), greater expansion of the cell body. In particular, cells after exposure to 15 µg/mL are typically rounded, juxtaposed, maintaining intercellular adhesion forming aggregates, by cellular juxtaposition. The marking for cell surface carbohydrates shows intense dispersed marking over the entire length of the cell surface. It can be observed that treated cells showed morphological changes and the largest being juxtaposed in particular for the treatment with 15 µg/mL.

The detection images of the actin microfilaments of the cytoskeleton showed that the control cells (2D) organize the microfilaments keeping the cells adhered to the substrate by organizing them. Smaller cells with little organization of these constituents are observed, forming few stress fibers. These cells have different morphologies.

The images (2E and 2F), 15 and 150 µg/mL respectively show a clear organization of the actin microfilaments, in stress fibers occupying the entire cell cytoplasm. It is observed with this detection that the nucleus/cytoplasm ratio becomes evident that the treatment increases when compared to the cells that control the expansion of the cell body.

Similar to what was observed with the other techniques (SEM and detection of surface carbohydrates), the treatment with 15 µg/mL was the one that induced the greatest intercellular adhesion, being the cells more juxtaposed. In both treatments, these cells remained with a greater degree of adhesion to the substrate, spreading and an increase in the cell body.

Figure 2. Immunomarking analysis using confocal microscopy.



The experiments were carried out in triplicate in three independent experiments. The images are at 600X magnification. Source: Authors.

The images A and D represent control cells maintained in culture only in the middle presence. Images B and E are of cells exposed to 15 µg/mL of crude *P. amalago* extract and images C and F, cells exposed to 150 µg/mL of extract. Exposure time of 24 hours. The images A, B and C show N-acetyl-glucosamine carbohydrates on the cell surface of the control and treated cells. Images D, E and F show detection of actin microfilaments.

3.4 Aqueous extract induces greater cell adhesion

Control 3T3 cells, treated with 15 and 150 µg/mL after 24 h of exposure, were imposed on the cell adhesion assay. Different MEC fibronectin (FN) was purified from fresh human plasma (obtained at the General Hospital, Federal University of Parana, Brazil) by gelatin–Sepharose affinity chromatography (Sigma, St Louis, MO, USA) as described by Engvall and Ruoslahti (1977) and Collagen I (Col. I) commercially purchased from Sigma, as well as adhesion to the polymer (substrate of the cell culture plate). For negative control of cell adhesion, bovine albumin (BSA 1%) was used.

Graph 2 shows that in all analyzed substrates there was an increase in cell adhesion after treatment. For the treatment with the concentration of 15 µg/mL, can be observed that there was a percentage increase of 113% adhesion to plastic and 111% adhesion to fibronectin when compared to the control. With this concentration on, both the substrates had a stimulus

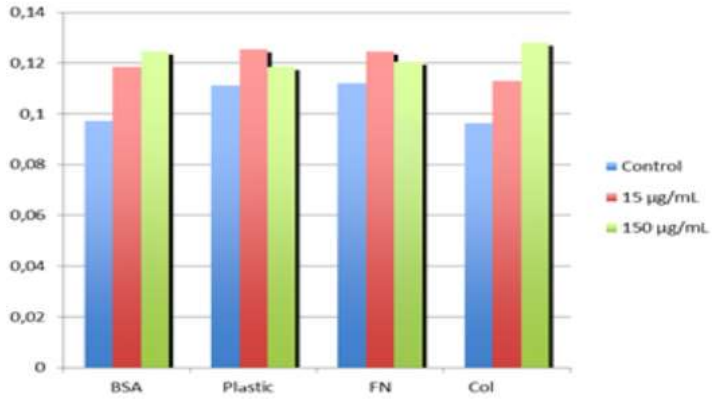
higher than that found for the concentration of 150 µg/mL (adhesion to plastic with an increase in adhesion of 106% and fibronectin of 107.5% in relation to the control).

It was also observed that for the concentration of 150 µg/mL, the collagen I substrate, was the one that induced 133% greater adherence compared to the control. It is also noticed that the control cells adhere similarly to BSA and to Col. I. This result shows that the cells have less affinity for the referred substrates. Adhesion similarity of control cells was also observed for the plastic substrate and for fibronectin.

Light microscopy images of control cells (3A, 3D and 3G), treated with 15 µg/mL (3B, 3E and 3H) and 150 µg/mL (3C, 3F and 3I), after the cells were imposed on the cell adhesion assay (Figure 3), show that in all analyzed substrates fibronectin, (3D, 3E and 3F) collagen I (3G, 3H and 3I) and BSA 1% negative control (3A, 3B and 3 C), after treatment the cells increased the expansion of the cell body, the cells are bigger, more adhered and spread by on the substrate.

Among all the analyzed substrates, it is observed that fibronectin is the substrate of greatest affinity for this cell line. Especially the FN substrate, both for control cells (3D), for cells treated with 15 µg/mL (3E) or for cells treated with 150 µg/mL (3F), this matrix protein induced not only adhesion of cells to this substrate as well as a higher degree of spreading that can be observed for both treatment concentrations.

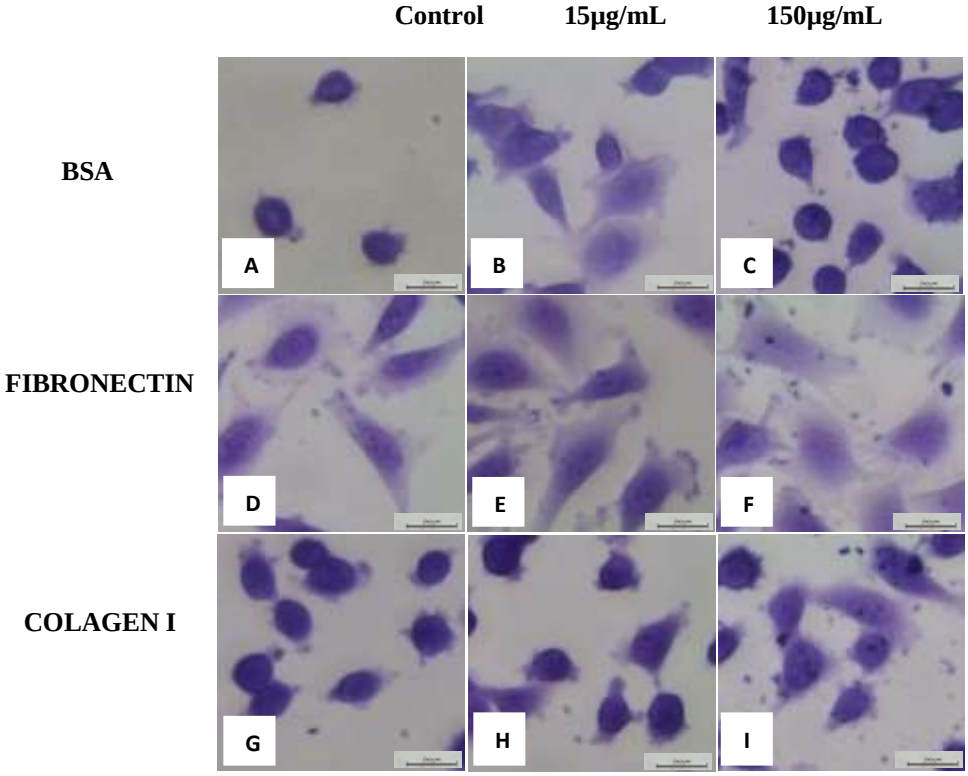
Graph 2. Cell adhesion assay of 3T3 cells on different MEC proteins.



These tests were performed in quadriplicates in two independent tests. Source: authors.

The cell adhesion of control cells and treated with crude *P. amalago* extract at concentrations of 15 and 150 µg/mL for a 24h treatment period, in 24-well plates. A concentration of 10 µg/mL was used for the fibronectin and collagen I substrates. As a negative control, 1% BSA was used.

Figure 3. Light microscopy analysis of cells imposed to the cell adhesion assay on different MEC proteins.



All images are in 400X magnification. Crystal Violet dye was used. These tests were performed in triplicates in two independent tests. Source: Authors.

The image A represents control cells adhered to the BSA (negative control); B cells treated with 15 µg / ml adhered to BSA and C, cells treated with 150 µg/mL adhered to BSA. Image D represents control cells adhered to the fibronectin (FN) substrate, E cells treated with 15 µg/mL adhered to FN and F, cells treated with 150 µg/mL adhered to FN. Image G shows control cells adhered to the substrate Collagen I (Col. I), H, the cells treated with 15 µg/mL adhered to Col. I and I, cells treated with 150 µg/mL adhered to Col. I.

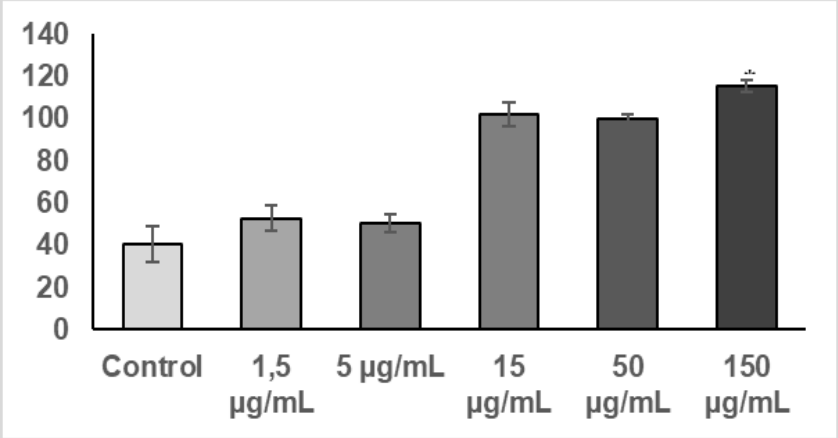
3.5 Aqueous extract induced an increase in hydroxyproline concentration

The hydroxyproline concentration is used as an indirect indicator to determine the collagen content (Reddy & Enwemeka, 1996). For the quantification of hydroxyproline, a standard curve comprising concentrations between 1.5 to 150 µg/mL was constructed. 99% N-acetyl hydroxyproline (Sigma 01192) was used as standard. The results obtained correspond to the average of samples from analyzes performed in quadruplicate in two independent tests (Reddy &d Enwemeka, 1996).

As shown in Graph 3, the crude extract of *P. amalago* promotes a statistically significant increase in the hydroxyproline content in the concentrations of 15, 50 and 150 µg/mL, when compared to the control. A smaller increase was observed at concentrations of 1.5 and 5.0 µg/mL.

Biochemical results, obtained for the determination of hydroxyproline concentration after 48 and 72 h of exposure, were not significant. However, after exposure for 96 h, there was a significant increase in the hydroxyproline amino acid, showing greater collagen biosynthesis by the cells after treatment.

Graph 3. Evaluation of hydroxyproline concentration.



These tests were performed in quadruplicate in two independent tests. Source: Authors.

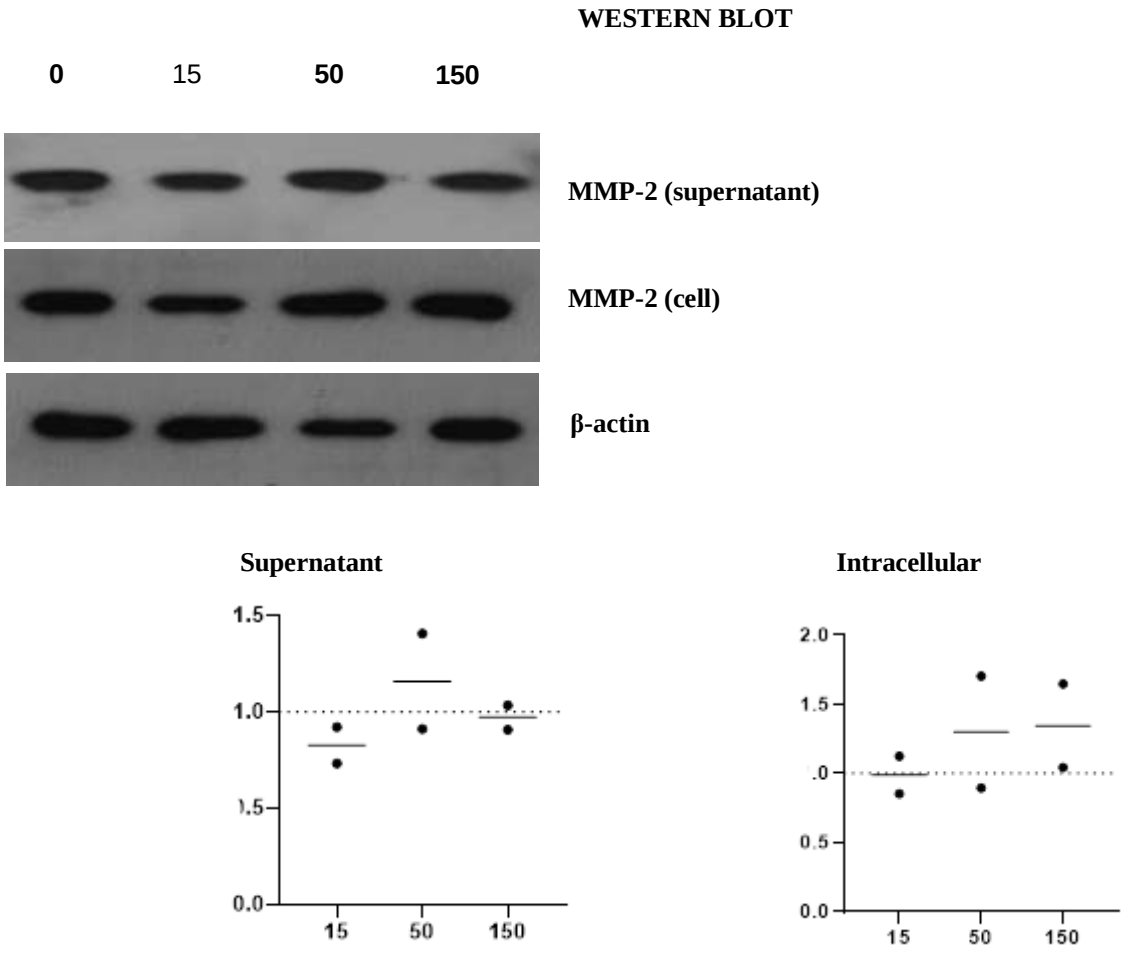
The evaluation of hydroxyproline concentration in control cells and treated with crude extract of *P. amalago* in concentrations of 1.5; 5.0; 15 and 150 µg/mL for a 96-hour treatment period. These tests were performed in quadruplicate in two independent tests.

3.6 Quantification and metalloproteinase activity (MMP2)

Western blotting showed no modulation of MMP2 after 96 h of exposure to the aqueous extract of *P. amalago* at the concentrations employed.

As shown in Graph 4, when the concentration of 15 µg/mL of *P. amalago* extract is used, it has a tendency to reduce the activity of metalloprotease (MMP-2) in the supernatant secreted by the cells. The concentration of 50 µg/mL tends to increase in both the supernatant and intracellular. The concentration of 50 µg/mL suggests only intracellular growth.

Graph 4. Quantification and metalloproteinase activity (MMP2).



These tests were performed in quadruplicate in two independent tests. Source: Authors.

The evaluation of the quantification and activity of MMP2 in control cells and treated with crude extract of *P. amalago* at concentrations of 15, 50 and 150 µg/mL for a treatment period of 96 hours.

4. Discussion

To the best of our knowledge, there is no *in vitro* study in the literature with *P. amalago,* using the *in vitro* model of mouse fibroblasts (3T3).

In wound healing, the main effects of the chemical compounds present in plant extracts are antimicrobial and antioxidant activities, as well as active components that promote mitotic activity. These active compounds contribute to cell proliferation, angiogenesis and increased collagen production and DNA synthesis (Budovsky et al., 2015).

Bioactive compounds, such as alkaloids, flavonoids, essential oils and terpenoids, have several activities, such as antimicrobial, antioxidant, anti-inflammatory and healing (Budovsky et al., 2015; Thakur et al., 2011; Tsala et al., 2013; Nascimento Júnior et al., 2020). The antimicrobial, antioxidant, anti-inflammatory and mitogenic activities of phytochemicals are essential in healing, and can positively favor one or more phases of the tissue restructuring process (Ghosh & Gaba, 2013). As they increase the rate of wound contraction, the number of fibroblasts and the disposition of collagen fibers also increase as well as reduce the number of inflammatory cells (Nayak et al., 2013; Dogoury et al., 2015). Additionally, natural chemical

compounds also influence the level of cytokines and levels of hydroxyproline and hexosamine (Roy et al., 2012), as well as angiogenesis (Parente et al., 2012).

Several chemical studies have been performed with *P. amalago* leaves and indicated the presence of alkaloids, condensed tannins, flavonoids and triterpenes. Piperidine, pyrrolidine amides, isobutylamides, chalcones, flavonols and phenolic compounds were also found in *P. amalago* (Domínguez et al., 1985; Carrara et al., 2013; Lopes et al., 2012; Novaes et al., 2014; Santos et al., 2020). In addition, Rovani et al. (2013) analyzed the crude ethanolic extract by high performance liquid chromatography (HPLC) and found the presence of vitexin and lupeol; while Carrara et al. (2012) evidenced pyrrolidine alkaloid in the leaves of *P. amalago*, using HPLC analysis of supercritical carbon dioxide and compressed propane extracts.

With regards to biological activities, vitexin and its derivatives have already been reported as potent antioxidants with anticarcinogenic and antimutagenic potential (Deladino et al., 2017). Lupeol is a triterpene found in several medicinal species and has antidiabetic, anti-inflammatory and antioxidant activities (Geetha & Varalakshmi, 2001; Prasad et al., 2008). The treatment of wounds with lupeol-based cream effectively improved the healing process in hyperglycemic rats induced with streptozotocin with modulating effects on inflammation, oxidative stress and angiogenesis (Harish et al., 2008). In a study carried out with rat melanoma cells, lupeol inhibited cell proliferation by inducing cell differentiation (Hata et al., 2010). A pyrrolidine alkaloid showed antileishmanial activity with strong activity on promastigotes and selective action against intracellular amastigotes (Carrara et al., 2012). Amides present in the ethanol extract of *P. amalago* showed anti-hyperalgesic, antinociceptive and anti-arthritic activities in models of acute and chronic pain in rodents (Arrigo et al., 2016).

In general, tannins are used to treat wounds, burns and inflammations (Funari & Ferro, 2005). They have the ability to promote the proliferation of epithelial cells and their migration from the periphery of the lesions, a phenomenon regulated by genes, growth factors, integrins, and matrix metalloproteinase-type enzymes (MMPs) (Hernandes et al., 2010), in addition to having high antioxidant activity (Batista et al., 2010).

In addition to stimulating healing, flavonoids have microbial and immune-modulating, anti-inflammatory, analgesic activities (Vieira et al., 2008; Coutinho et al., 2009; Gazola et al., 2014). Flavonoids also have antioxidant activity (Rovani et al., 2013; Torres et al., 2018) and antimicrobial activity (Santos et al., 2019). In antioxidant activity, flavonoids regulate the production of superoxide anions and the blood flow of the injured region, promoting angiogenesis, thus reducing free radicals in the wound (Mittal et al., 2013).

Additionally, the majority of analyses of essential oil of *P. amalago* leaves showed the prevalence of sesquiterpene hydrocarbons such as bicyclogermacrene, β-phellandrene and germacrene D, with substantial amounts of monoterpene hydrocarbons, such as α-pinene, and some sesquiterpenoids such as spathulenol (Salehi et al., 2019). The essential oil played important biological roles, such as antimicrobial (Moqrich et al., 2005; Santos et al., 2016) anti-inflammatory (Jeong et al., 2014; Bahi et al., 2014; Passos et al., 2014; Carneiro et al., 2017) antioxidant (Nogueira Neto et al., 2013; Santos et al., 2016; Oliveira et al., 2016), and antihyperalgesic (Carneiro et al., 2017).

Fractions obtained from stems and roots of *Piper hayneanum* C.DC were tested for healing activity in rats, with dorsal lesions infected with *Staphylococcus aureus* and *Candida albicans,* and showed better wound healing compared to those treated with gentamicin (*S. aureus*) and miconazole (*C. albicans*) (Bastos et al., 2011).

Bacterial contamination can increase the inflammatory phase, causing prolonged production of pro-inflammatory cytokines; if this condition persists and bacterial elimination is not achieved, the wound becomes chronic and does not heal. Therefore, the elimination of bacterial contamination in the wound microenvironment is essential for optimal wound repair (Edwards & Harding, 2004; Guo & DiPietro, 2010).

The aqueous extract of *P. amalago* in concentrations ranging from 0.5 to 150 µg/mL did not cause damage to 3T3 cells and induced cell proliferation (Graphs 1 A and B).

In the case of mitosis and fibroblast migration, it is necessary to have a solid substrate that serves as a support base for the cells. The construction of this substrate is done by the lysis of inert constituents of the extracellular matrix followed by its replacement with new characteristics. Any lack of control in the lithic process, instead of benefits, can result in damage to the evolution towards repair (Balbino et al., 2005).

Since fibroblasts are the main targets in the design of therapeutic drugs, bioactive compounds that can stimulate the proliferation of fibroblasts may be able to stimulate the healing process (Singh et al., 2014), as observed in the present study. Also, the results indicated that none of the extracts exhibited cytotoxic activity in 3T3 fibroblasts. In that sense, ethnopharmacological studies have indicated that extracts obtained from species of *Piper* presented the ability to promote the proliferation of fibroblasts during the healing process (Panah et al., 2014; Varga et al., 2015). Khosravitabar et al. (2017) have suggested that the effects of flavonoids on fibroblast proliferation depend on their structure and concentration, and confirm that flavonoids stimulate fibroblast proliferation, especially at lower concentrations (Khosravitabar et al., 2017).

The ethanolic extract and the dichloromethane and hexane fractions obtained from the leaves of *Piper arboreum* Aubl. showed high toxicity at concentrations of 100 and 500 µg/mL, with fibroblast mortality between 79% and 100% (Figueredo et al., 2014).

The hydroethanolic extract obtained from the leaves of the species *Piper aduncum* L. increased the proliferation and migration of human fibroblasts (HDFA), and in addition, increased the expression of growth factors (FGF, EGF and PDGF), important in the healing process. The concentrations used were 103.5 µg/mL after 24 h of treatment and 39.54 µg/mL after 48 h of treatment (Paco et al., 2016).

3T3 fibroblasts treated with 125 µg/mL of the methanolic fraction obtained from the leaves of *Jatropha gossypiifolia* L. exhibited an increase in migration, at 12 h and 24 h, when compared to cells treated with culture media (Silva et al., 2018).

Study carried out with a fraction composed of long-chain hydrocarbons (LCHCs) obtained from the crude methanolic extract of *P. cubeba* L. fruit showed cytotoxic activity against breast cancer cells (IC_{50} of 2.69 ± 0.09 µg/mL) and low toxicity against fibroblasts (IC_{50} of 2.69 ± 0.09 µg/mL) (Graidist et al., 2015).

The morphological analysis of the cells suggests that the treatment with the aqueous extract in the concentrations of 15 and 150 µg/mL after 24 h of exposure, did not cause cytotoxic effects on the cells when compared to the control cells (Figures 1 A and B).

Fibroblasts have fusiform morphology with numerous cytoplasmic extensions, with a central and rounded nucleus, evident nucleolus and fine chromatin. They are the cells responsible for the synthesis and degradation of connective tissue (fibrous and non-fibrous) and synthesis of numerous mediators (Kanitakis, 2002; Tobin, 2006; Gartner & Hiatt, 2017).

The fibroblast motion also shows stress fibers and lamellipodia, and changes a cell body from extended to fan-shaped or elongated (Vallenius, 2013). Filopodia and lamellipodia form immature adhesions within the substrate of the extracellular matrix, as they contain integrins and signaling molecules (Czuchra et al., 2005; Pankov et al., 2005).

These cells *in vitro* are linked by communicating and adhesion junctions, an indication that these cells are metabolically associated (Arenas & Zurbarán, 2002). In the substrate adhesion tests, Graph 4 shows that for both control cells and cells treated with the crude extract of *P. amalago*, there was an increase in adhesion to fibronectin, collagen I and BSA proteins.

Through the cell adhesion assay by optical microscopy analysis, it is noted that the FN substrate was the one with the highest affinity for both control and treated cells (Figures 2 D, E and F).

Since the α5β1 integrin membrane receptor modulates adhesion to this substrate, possibly the treatment may be making an expressive modulation of this substrate. Integrins are responsible for many of the interactions between cells and the extracellular matrix (ECM), in addition to serving as a union between ECM and the cytoskeleton (Arenas & Zurbarán, 2002;

Savino et al., 2003). One ECM component is fibronectin (FN), an important molecule in migration and cell adhesion processes (Anjos & Alvares-Silva, 2000; Kunz-Schughart et al., 2001).

Fibronectin and hyaluronic acid are the predominant components of the matrix during the early stages of wound repair (Suzuki et al., 2005).

Activated inflammatory cells are producers of reactive oxygen and nitrogen species with known lytic potential on cell membranes. Some of the inflammatory mediators, such as TNFs and TGF-b, can induce apoptosis of cells by death receptors (Moore et al., 2001).

In the present study, the results of scanning electron microscopy, optical microscopy and immunostaining show cells with cellular integrity, greater expansion of the cell body adhered to the substrate, juxtaposed, and high number of cells. These cells increased the adhesive capacity of extracellular matrix molecules, especially fibronectin, showing that the crude *P. amalago* extract does not induce loss of viability or changes in cell integrity or in cell adhesion dynamics at the evaluated times and concentrations.

The results of the carbohydrate detection assay suggest the presence of surface glycosaminoglycan scattered throughout the cell body. When compared to control cells, the treated cells show greater distribution of these glycoconjugates containing residues of N-acetyl-glucosamine (detected using WGA) (Figures 3 A, B and C). N-acetyl glucosamine regulates cellular responses to hormones, such as insulin; initiates a protective stress response; modulates growth and cell division capacity; and regulates gene transcription (Liu et al., 2004).

An important tool for the investigation of the cytoskeleton dynamics is the fluorescent marking of actin, using fluorescents derived from phallotoxins, as they allow the identification of whole cell culture with minimal cell disruption (Devod et al., 2001).

In the results obtained, the treated fibroblasts show a clear organization of the actin microfilaments compared to control cells. Stress fibers occupy the entire cell cytoplasm with a greater degree of adhesion to the substrate and spreading of the cell body. The treatment with 15 µg/mL was the one that induced the greatest intercellular adhesion (Figures 3 D, E and F).

The strength of a scar is given by the amount of collagen deposited and the way the fibers are organized. In the fiber ripening process, lysines, hydroxylysines and glycosylated lysines, constituents of the tropocollagen molecule, are oxidized (Frazier et al., 1996). In healing, when the lesion space is filled with granulation tissue, the margins move towards each other, due to the differentiation of some fibroblasts from the wound margins to myofibroblasts that have contractile capacity (Peacock Jr., 1984). In that case, the immunostaining results for the constituents of the cytoskeleton, specifically the actin microfilaments marked with the phalloidin probe, showed a clear increase in the quantity and organization of these constituents after exposure to the crude extract of *P. amalago.*

Important steps in the new phase of tissue formation are the migration and proliferation of keratinocytes and fibroblasts (Gurtner et al., 2008). The cells move, rearranging their actin framework. In order for cells to migrate, the long chain of actin must be deranged, thus being able to change shape and then migrate to other sites (Natérica, 2005). A change in shape of the actin cytoskeleton is a prerequisite for cell migration (Friedl & Wolf, 2010).

The hydroxyproline quantification method shows that the cells treated with the aqueous extract of *P. amalago* produced a greater amount of this amino acid when compared to the control cells, with the concentration of 150 µg/mL being the most statistically significant ($p < 0.05$) (Graph 3).

Collagen is the most abundant protein in the body, comprising 30% of the mass of all proteins. It is an important structural protein of connective tissues (Sandhu et al., 2012). Among the types of collagen, collagen I is the most abundant, being found mainly in the skin. This type of collagen has about 20% proline (Pro) and 4- or 3-hydroxyproline (Krane, 2008; Gordon & Hahn, 2010; Mienaltowski & Birk, 2014).

The collagen protein is directly involved in the reconstitution of the extracellular matrix and in the healing of wounds. Its deposition and collagen remodeling contribute to increase the tensile strength of the injured tissue (Vancheri et al., 2005; Sandhu et al., 2012).

Human dermal fibroblasts treated with extracts of *Piper cambodianum* P. Fourn. showed an increase in the expression of extracellular matrix genes, such as collagen and elastin; decreased expression of the metalloproteinase-3 gene; a dose-dependent increase in RNAm expression levels of collagen, elastin and hyaluronic acid synthase-2 (hyaluronan synthase-2); and decreased levels of expression of the metalloproteinase-1 gene. The extract also showed positive effects on wound healing in mice (Lee et al., 2016).

As shown by the results obtained in the evaluation of MMP-2 secretion by treated 3T3 fibroblasts and the gelatinolytic activity of this metalloprotease, there was no difference between the treated cells when compared to the control cells (Graph 4).

After analyzing the antitumor effects of the aqueous extracts of *Aloe vera* (L.) Burm.f., *Annona muricata* L. and black tea in three serial dilutions on the gelatinolytic activity of MMPs 2 and 9, the results suggest that the antitumor effects can be partially explained by their ability to inhibit the proteolytic activity of MMPs (Ribeiro et al., 2010).

The results obtained with the cell adhesion and spreading tests on extracellular matrix molecules showed an increase in both the degree of adhesion and the spread of these 3T3 cells after exposure to the crude extract of *P. amalago*.

Fibroblasts are cells that undergo phenotypic changes; the cytoplasm becomes bulky and has an abundant rough endoplasmic reticulum. Thus, fibroblasts secrete large amounts of collagen (Balbino et al., 2005). In the present study, the results of biochemical tests to assess hydroxyproline biosynthesis reveals an increase in the production of this molecule after treatment with *P. amalago* extract. Subtle modulation of MMPs can also be assessed.

5. Conclusion

According to the results found in the present study, the aqueous extract obtained from the leaves *of P. amalago* on the 3T3 line did not prove to be cytotoxic until the concentration reached 150 µg/mL after 24 h of exposure. The cells after treatment showed greater expansion of the cell body, more juxtaposed; there is a greater number of cells with greater adhesion and spreading. The extract induced increased cell adhesion and spreading especially in fibronectin and type I collagen. The extract also induced a significant increase in the concentration of hydroxyproline (collagen production) and subtle modulation of metalloproteinase (MMP2) activity. Thus, the results revealed positive modulation in the degree of cell spreading, number of cells, adhesion, intercellular cohesion, and collagen production with promising results of the action of this compound in the healing model, in the times and concentrations studied in the 3T3 fibroblast lineage.

These results endorse the ethnopharmacological use of *P. amalago* as a healing agent for wounds and burns by Central American and Brazilian communities, and may be important in the development of new healing phytomedicines.

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