UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

CINTIA KAZUKO TOKUHARA

Cytotoxicity of *Qualea grandiflora* Mart. leaf extract (Vochysiaceae) on pre-osteoblast culture. Evaluation of cell viability and expression/activity of MMPs

Citotoxicidade do extrato de folhas da *Qualea grandiflora* Mart. (Vochysiaceae) em cultura de pré-osteoblastos. Avaliação da viabilidade celular e expressão/atividade de MMPs

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Dissertation presented to the Bauru School of Dentistry of the University of São Paulo to obtain the degree of Master in Science in the Applied Dental Science Program, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Dr. Rodrigo Cardoso de Oliveira

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"Não importa o quanto a vida possa ser ruim,
sempre existe algo que você pode fazer e triunfar".
Stephen Hawking

ABSTRACT

Cytotoxicity of *Qualea grandiflora* Mart. leaf extract (Vochysiaceae) on pre-osteoblast culture. Evaluation of cell viability and expression/activity of MMPs.

The use of medicinal plants with the purpose of treatment of many diseases is common. However, a scientific study to ensure the doses considered safe for consumption is required. *Qualea grandiflora* has been used in folk medicine to treat bloody diarrhea and nervous system depressant, antioxidant and anti-inflammatory activity and among other applications. The aim of this study was to investigate the influence of hydroalcoholic extract obtained from the leaves of *Q. grandiflora* on MC3T3-E1 pre-osteoblast lineage, correlating the effects of this plant on cell viability and expression and activity of matrix metalloproteinase. It was performed using serial dilution (1000 µg/mL, 100 µg/mL, 10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL) of different concentrations of the hydroalcoholic extract of leaves *Qualea* exposed on pre-osteoblast MC3T3-E1 cell line. Four periods (24, 48, 72 and 96 h) were stipulated in order to assess cytotoxicity of the plant. From these safe concentrations were chosen (10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL) and evaluated the expression and activity of MMPs of these cells exposed to the extract *Qualea*. We found in 70% EtOH extract of the leaves of *Q. grandiflora*, is mostly derived from gallic acid compounds and other compounds of flavonoid. From the concentrations evaluated, only the lower concentrations (0.1 µg/mL, 1.0 µg/mL and 10.0 µg/mL) showed not be cytotoxic to this cell line and for concentrations higher than 10.0 µg/mL caused decreased cell viability. An increase in MMP-9 expression in the group with the extract was found, though the activity was not confirmed by zymography assay. In conclusion, our results indicate what is already practiced by folk medicine as low extract concentrations shown to be safe for this cell line, but high concentrations showed cytotoxicity.

Keywords: *Qualea grandiflora*. Citotoxicity. Matrix Metalloproteinase. Viability.

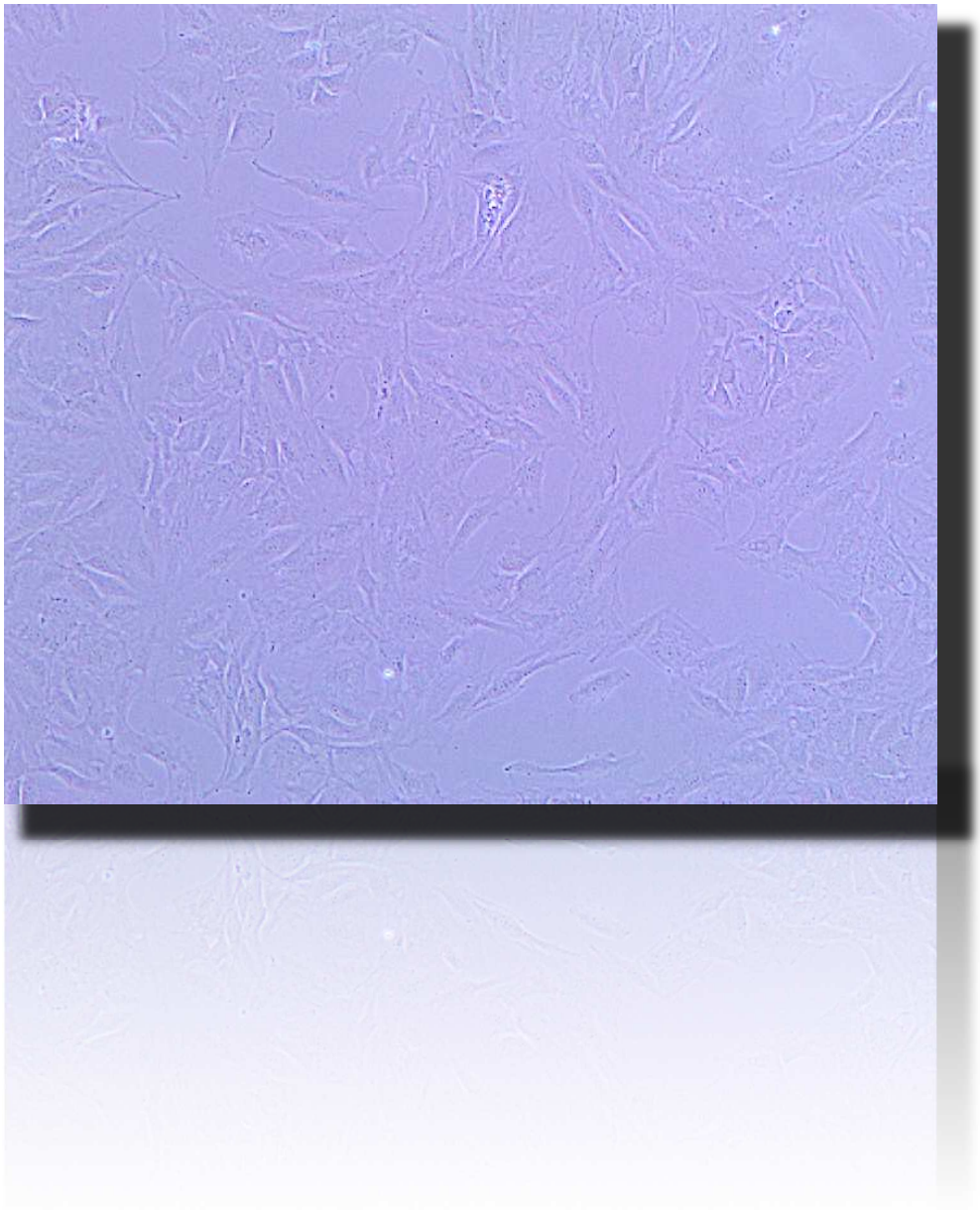
RESUMO

Citotoxicidade do extrato de folhas da *Qualea grandiflora* Mart. (Vochysiaceae) em cultura de pré-osteoblastos. Avaliação da viabilidade celular e expressão/atividade de MMPs.

O uso de plantas medicinais com o propósito de tratamento de inúmeras doenças é algo comum. No entanto, estudos científicos para garantir as doses consideradas seguras para consumo são necessários. A *Qualea grandiflora* tem sido usada na medicina popular para o tratamento de diarréia com sangue, além de depressor do sistema nervoso, atividade antioxidante e antiinflamatório entre outras aplicações. Assim, o objetivo deste estudo foi investigar a influência do extrato hidroalcoólico obtido das folhas de *Q. grandiflora* na linhagem MC3T3-E1, pré-osteoblastos, correlacionando os efeitos desta planta sobre a viabilidade celular, expressão e atividade de metaloproteinases da matriz. Foi realizada diluições seriadas (1000 µg/mL, 100 µg/mL, 10.0 µg/mL, 1.0 µg/mL e 0.1 µg/mL) de diferentes concentrações do extrato hidroalcoólico de folhas *Qualea* e deixadas em contato com os pré-osteoblastos. Quatro períodos (24, 48, 72 e 96 h) foram avaliados de modo a verificar a citotoxicidade da planta. A partir das concentrações seguras escolhidas (10.0 µg/mL, 1.0 µg/mL e 0.1 µg/mL) foram avaliados a expressão e atividade de MMPs destas células expostas ao extrato da *Qualea*. Encontramos no extrato das folhas de *Q. grandiflora*, principalmente derivados de compostos de ácido gálico e outros compostos de flavonóides. A partir das concentrações testadas, apenas as concentrações mais baixas (0,1 µg/mL, 1.0 µg/mL e 10.0 µg/mL) não mostraram ser citotóxicas para esta linhagem celular e concentrações superiores a 10.0 µg/mL, causaram uma diminuição na viabilidade celular. Um aumento da expressão de MMP-9 no grupo com o extrato foi encontrado, embora a atividade não tenha sido confirmada por ensaio de zimografia. Em conclusão, nossos resultados indicam o que já é praticado pela medicina popular, pois baixas concentrações de extrato mostraram ser seguras para esta linhagem celular, mas concentrações elevadas mostraram citotoxicidade.

Palavras-chave: *Qualea grandiflora*. Citotoxicidade. Metaloproteinase de Matriz. Viabilidade.

TABLE OF CONTENTS



1-INTRODUCTION

1 INTRODUCTION

Medicinal plants

It is known that medicinal plants are used for millennia as an alternative the main sources of therapeutic agents for humanity. The drug discovery from medicinal plants in the 19th century, this led to purification of various drugs such as codeine, digitoxin, morphine and quinine. Drugs discovery leads from medicinal plants may be aid by ethnopharmacology which is a mode of scientific investigation relating to the indigenous medicinal uses of a particular species (Kinghorn *et al.*, 2011).

However, it is known that contrary to popular belief, the use of medicinal plants is not without risk. In addition to the therapeutic active ingredient, the plants may contain toxic substances, and may be contamination by pesticides or heavy metals (aluminum, lead, mercury, manganese, etc.) and the interaction with other medications can cause health damage (Brasil. Ministério Da Saúde. Secretaria De Ciência, 2009).

According Kinghorn *et al.*, (2011), the terms “natural product” and “secondary metabolite” now have a common meaning, with such compounds evolving to enhance the survival of the producing organism.

Among the various plants in Brazil, one of the species of the savannah that interest is the *Qualea grandiflora* Mart. (*Q. grandiflora*) from *Vochysiaceae´s* family, an occurrence plant in gallery forest, cerrado and cerradão, considered cerrado’s symbol (Almeida *et al.*, 1998) and investments to promote studies about the Cerrado gained importance only in the last decade, when it was included in the list of world biodiversity hotspots (Myers *et al.*, 2000; Mittermeier *et al.*, 2005).

Hiruma-Lima *et al.*, (2006) to investigate the effectiveness of hydroalcoholic extract of *Q. grandiflora* in the prevention and healing of gastric ulcers, suggest that efficacy is based on the ability of it to stimulate mucus synthesis, an important factor for gastric protection. Moreover, the presence of isoprenoid-derived compounds and tannins may be responsible for the antiulcerogenic effect on the surface of the gastric mucosa.

The bark and leaves of the *Q. grandiflora* are medicinal and fruit give yellow dye matter. Studies have reported that infusion or decoction of the leaves of *Q. grandiflora* mart. They are used to popularly for bloody diarrhea, intestinal colic and against amoeba (Rodrigues and Carvalho, 2001).

Thus, they become more than necessary to conduct further research in this area in our native flora, and ethnobotany, pharmacology and biochemistry, important tools to work on this goal. In this sense a number of actions have been proposed and put into practice, as rules established by the National Health Surveillance Agency for registration of Herbal Medicines (ANVISA) and incentives for the SUS (Health Unic System - National Program of Medicinal Plants and Herbal medicines) through the Health Ministry (Brasil. Ministério Da Saúde. Secretaria De Ciência, 2009).

The *Q. grandiflora* has shown positive results in research involving the microorganism control related to oral diseases and inflammatory conditions. It is also known that matrix metalloproteinases are involved in inflammation conditions. Thus, it would be appropriate to assess whether the *Q. grandiflora* extract can act in the modulation of MMP, relating this information with applications used in folk medicine.

Matrix Metalloproteinases

MMPs are proteolytic enzymes, zinc family and calcium dependent able to degrade extracellular matrix components (Peres and Line, 2005; Nagase *et al.*, 2006; Navarro *et al.*, 2006; Zhao *et al.*, 2016) and basement membrane (Navarro *et al.*, 2006) and have specific inhibitors known as tissue inhibitors of metalloproteinase (TIMPs).

MMPs are expressed in many cell and tissue types, including cells of the vascular smooth muscle, endothelium, fibroblasts, osteoblasts and inflammatory cells (Guimarães *et al.*, 2010). Specifically MMP-9 participates in the remodeling of extracellular matrix during tooth development (Peres and Line, 2005).

The matrix metalloproteinases are considered responsible for the remodeling of extracellular matrix (Vu and Werb, 2000; Zuo *et al.*, 2014) and this process required for normal development (Vu and Werb, 2000; Yang *et al.*, 2013) and turnover of the matrix extracellular in physiological processes such as angiogenesis,

embryogenesis, morphogenesis and wound healing. On the other hand, the MMPs are related to the development/progression of disease states such as cancer, myocardial infarction, rheumatism, osteoarthritis and fibrotic diseases (Zuo *et al.*, 2014; Zhao *et al.*, 2016).

MMP-2 (72kDa) and MMP-9 (92kDa) are gelatinases A and B respectively, that specifically degrade collagen type IV regulating the remodeling of the basal membranes. Feature role in extracellular matrix degradation, and MMP-2 expressed constitutively presenting a so-called domain "fibronectin like", responsible for the identification of collagen type I, IV, V, VII and X, gelatin (denatured collagen) laminin and elastin (Guimarães *et al.*, 2010; Vandooren *et al.*, 2013).

In this context the TIMPs have an essential role therefore control the activity of MMPs to the extracellular matrix degradation does not occur overly (Nagase *et al.*, 2006; Guimarães *et al.*, 2010). They´re multifunctional proteins with regulatory endogenous property of MMPs and have fundamental influence on the extracellular matrix, acting on cell adhesion molecules and other cytokines, chemokines and growth factors (Avădani *et al.*, 2015).

The use of medicinal plants in folk medicine is an ancient, traditional Chinese medicine uses plants to treat numerous diseases for thousands of years (Junqueira, 2007; Mazzolin *et al.*, 2010; Lao *et al.*, 2014), furthermore extracts of plants can to inhibit the actives of snake venoms (Mourão De Moura *et al.*, 2014).

Despite the use of the plants are not recent, the study and research are necessary to be safely administered.

2-Article

2 ARTICLE

Article formatted according to Journal of Ethnopharmacology

Qualea grandiflora **Mart. effect on pre-osteoblast behavior and matrix metalloproteinase.**

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Abstract

Ethnopharmacological relevance

Qualea grandiflora has been used in folk medicine to treat bloody diarrhea and nervous system depressant. In addition, antioxidant and anti-inflammatory activity.

Aim of the study

To investigate the influence of hydroalcoholic extract obtained from the leaves of *Q. grandiflora* on MC3T3-E1 pre-osteoblast lineage, correlating the effects of this plant on cell viability, differentiation and mineralization, expression and activity of matrix metalloproteinase and determination of species reactive of oxygen.

Materials and Methods

It was performed using serial dilution of different concentrations of the hydroalcoholic (70% EtOH) extract of leaves *Qualea* exposed on pre-osteoblast cells. The periods evaluated to assess cytotoxicity of the plant. From these safe concentrations were chosen and evaluated the expression and activity of MMPs of these cells exposed to the extract *Qualea*.

Results

We found in extract of the leaves of *Q. grandiflora*, is mostly derived from gallic acid compounds and other compounds of flavonoid. From the concentrations evaluated, only the lower concentrations (0.1 µg/mL, 1.0 µg/mL and 10.0 µg/mL) showed not be cytotoxic to this cell line and for concentrations higher than 10.0 µg/mL caused decreased cell viability. We found an increase in MMP-9 expression in the group with the extract, though the activity was not confirmed by zymography assay. Concentrations of 1.0 and 10. 0 µg/ mL promoted nodules mineralization with significant difference compared to control.

Conclusion

In conclusion, our results contribute towards direct to a safe dose range that should be further studied. *Q. grandiflora* showed an osteogenic potential in osteoblast MC3T3-E1 cell.

Keywords: *Qualea grandiflora*; Cell viability; Medicinal plant; Cytotoxicity; Matrix metalloproteinase.

1. Introduction

Potential of medicinal plants

Plants candidate for medicinal purposes can be found in various biomass in the world. In Brazil, we have the cerrado, the second largest biome in South America, occupying an area of 2,036,448 km^2, about 22% of the national territory. In relation of biological diversity, the Brazilian cerrado is recognized as the richest savanna in the world, home to 11.627 species of native plants already cataloged (Brasil, 2013).

Plants rich in phenolic have encouraged the interest in the food industry by retarding the oxidative degradation of lipids and thereby improving the quality and nutritional value of food (Wojdylo et al., 2007). In addition, several species of herbs are known for their medicinal properties and the health benefits, such as antioxidant activity, digestive stimulant, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effect and anticarcinogenic and antitumor potential (Aaby et al., 2004; Ribeiro et al., 2010).

The *Qualea grandiflora* Mart (*Q. grandiflora*), popularly known pau-terra, pau-terra-do-campo, pau-terra-do-cerrado, pau-terra-da-folha-larga, ariavá and others (Corrêa, 1978), species of the family *Vochysiaceae*, has been studied and used in folk medicine with some benefits (Silva Júnior, 2005).

Some authors have reported that infusion or decoction of the leaves of *Q. grandiflora* Mart. are used popularly for bloody diarrhea, intestinal colic and against amoeba (Rodrigues and Carvalho, 2001). Other results demonstrate that the extract of *Qualea´s* leaves have depressant activity of central nervous system, analgesic and anticonvulsant potential (Gaspi et al., 2006). The ethanolic extract of the leaves have antioxidant effect and antibacterial action especially against strains of *Staphylococcus spp*. (Ayres et al., 2008).

Already the bark of the trunk has anti-inflammatory action and as an infusion is used for cleaning wounds and external ulcers (Almeida et al., 1998). The ethanolic extract of the bark of *Q. grandiflora* displays antiulcer action (Hiruma-Lima et al., 2006). There are also reports that the ethanolic extract of the root has been studied and showed antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* (Alves et al., 2000).

Lima Neto et al. (2015) evaluated the phytochemical profile of ethanolic extract of many plants, including the *Q. grandiflora* (bark and leaves). In the DPPH test, the same showed significant scavenging activity of free radicals, that is, the extract has an antioxidant capacity and these retard or prevent the oxidation of lipids (Chanwitheesuk et al., 2005; Moreira et al., 2012) and to prevent and repair damage caused to cells by reactive oxygen species (Chanwitheesuk et al., 2005; Lima Neto et al., 2015).

According Santos et al. (2011), research using *Q. grandiflora* are insufficient in the scientific literature, requiring further studies involving its, and compounds derived from this plant are still unknown and literature describes that many plants have mutagenic compounds.

Thus, the study of *Q. grandiflora* is necessary to understand the effects on specific cell types. The aim of this study was to investigate the influence of hydroalcoholic extract obtained from the leaves of *Q. grandiflora* on pre-osteoblast MC3T3-E1 lineage, correlating the effects of this plant on cell viability and expression and activity of matrix metalloproteinase.

2. Materials and Methods

2.1 Pre-osteoblast cell line

MC3T3-E1 (subclone 4) pre-osteoblast cell line from mice calvaria was obtained from ATCC (Manassas, USA), grown at 37ºC in α-MEM (Minimum essential medium alpha - Gibco) supplemented with 10% FBS (Fetal Bovine Serum), 100 U/mL of penicillin, and 100 µg/mL of streptomycin under a humidified 5% CO_2 atmosphere. The medium was changed every 3 days and when the flask was confluent, the cells were sub-cultured with 0.25% trypsin-EDTA and plated for the assays.

2.2 *Qualea grandiflora* Mart

The leaves of *Q. grandiflora* Mart were collected in Bauru Municipal Ecological Park Botanic Garden, in the winter (Gobbo-Neto and Lopes, 2007). The hydroalcoholic extract of *Q. grandiflora* was kindly provided by Profª Drª. Anne Ligia Dokkedal Bosqueiro (Professor, Department of Biological Science, State University of São Paulo "Júlio de Mesquita Filho" UNESP / Bauru); the voucher specimen

(chemical voucher) is in the herbarium of the Department of Biological Sciences (UNBA) and collections have authorization issued by SISBIO under #39825-1.

To determine the concentrations of the extract, made an adaptation of the ISO 10993-5, due solubility of it, using 1mg/mL, the same used by (Machado et al., 2016) with *Myracrodruon urundeuva* extract. From this concentration held the serial dilutions of the hydroalcoholic extract of *Q. grandiflora*, 100 µg/mL; 10.0 µg/mL; 1.0 µg/mL and 0.1 µg/mL. Then a small portion (about 5 mL) of medium prepared with the extract was used for measure pH meter. The results are shown in Table 1.

Table 1. Experimental group and their pH

Groups	pH
1000 µg/mL *Q. grandiflora*	7,48
100 µg/mL *Q. grandiflora*	7,55
10.0 µg/mL *Q. grandiflora*	7,54
1.0 µg/mL *Q. grandiflora*	7,50
0.1 µg/mL *Q. grandiflora*	7,50
CP- Positive control 10% SFB	7,40
CN- Negative control 1% SFB	7,40

In relation to pH, different concentrations of the extract did not modify the pH value of the culture medium. Both control groups have similar value of pH compared to experimental groups.

Furthermore, we observed that the pH did not affect the viability of cells exposed to the extract.

2.3 Chemical profile by HPLC-DAD

The chemical profile of 70%EtOH extract of the leaves of *Q. grandiflora* was held in high performance liquid chromatograph (HPLC) coupled with diode array detector (DAD) (Jasco®, Tokyo, Japan). An aliquot of 5mg of 70%EtOH extract was solubilized in acetonitrile (ACN): H_2O (7:3, v/v) and filtered through a PTFE syringe filter membrane with 0.45 µm pore. Chromatographic separations were performed with a reverse phase column, Phenomenex® Luna C18 (250 x 4.6 mm id, 5 µm) and a mobile phase consisting of a mixture ACN: H_2O acidified (0.1% Formic Acid) in

analytical method 5-40% ACN gradient manner in H_2O in 120 min. Injection volume: 5 µL.

2.4 Cell viability

The cell viability was assessed after experimental time (24, 48, 72 and 96 h) by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide and CV (Crystal Violet) assays. Cells were plated in 96-well plates at density $3x10^3$ cells in α-MEM 10% FBS, after 24 h of cellular adhesion, different concentrations of *Q. grandiflora* (1000 µg/mL, 100 µg/mL, 10.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL) were administered and positive and negative control (10% FBS and 1% FBS, respectively).

For MTT assay, the cells were washed by phosphate-buffered saline (PBS) followed by the addition of MTT solution (0.5 mg/mL of medium without serum). After incubation at 37º C for 4 h protected from light, DMSO reduced MTT dye was diluted by and the absorbance was evaluated in microplates reader (Fluostar OPTIMA, BMG Labtech, Offenburg, Germany) at 562 nm. For CV assay the cell were washed with PBS and then added 100% methanol for 10 minutes. It´s removed and CV solution (0.2% Crystal Violet in 2% ethanol) was added to the cells for 3 minutes followed by additional wash and incubation with sodium citrate 0.05 mol/L in 50% ethanol solution for 10 minutes. The plate was read at 540 nm.

2.5 Real Time RT-PCR

For this assay, $5x10^5$ cells were plated in 6-well plates. After 24 h of adhesion the concentrations of extract (0.1 µg/mL, 1.0 µg/mL and 10.0 µg/mL) and control group were administered for 24 h and 48 h. The mRNA extracted by Trizol and transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer´s recommendations after 24 h and 48 h of culture. The cDNA samples were incubated with SYBR® Green PCR Master Mix (Applied Biosystems) and read in the Real-Time RT-PCR System ViiATM7 (Applied Biosystems). PCR amplification was performed using the following primers sets: MMP-2 ACAGGACATTGTCTTTGATG (forward), TACACAGCGTCAATCTTTTC (reverse), MMP-9 CTTCCAGTACCAAGACAAAG (forward) ACCTTGTTCACCTCATTTTG (reverse), TIMP-2 CATCGAGTTTATCTACACGG

(forward) CTTTCCTGCAATTAGATACTCC (reverse), GAPDH TGGCAAAGTGGAGATTGTTGC (forward) AAGATGGTGATGGGCTTCCCG (reverse). The GAPDH expression was used a control.

2.6 Western Blotting

Cells were plated in petri dish 100 x 20 mm at density $1x10^6$ cells. After 24 h and 48 h with the concentrations of extract (0.1 µg/mL, 1.0 µg/mL and 10.0 µg/mL) and control group, the cells were lysed with buffer solution containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.2% Nonidet P-40 supplemented with protease inhibitors (Sigma-Aldrich) and phosphatase inhibitor (0.2M sodium orthovanadate-Calbiochem). The lysate was pooled, sonicated and centrifuged at 1000 rpm for 10 minutes at 4º C. Protein samples (50 µg) were applied to electrophoresis in Tris-HCl 10% polyacrylamide gel and subsequently transferred to PVDF membrane. It was immune-labeled with goat polyclonal anti-MMP-2 and anti-MMP-9 primary antibody (Santa Cruz Biotechnology) followed by secondary anti-goat IgG conjugated to HRP (Horseradish Peroxidase – Santa Cruz Biotechnology), rabbit polyclonal anti-TIMP-2 primary antibody (Santa Cruz Biotechnology) followed by secondary anti-rabbit IgG conjugated to HRP (Horseradish Peroxidase – Santa Cruz Biotechnology) and rabbit polyclonal anti-β-actin was used a control and finally the ECL reagent (enhanced chemiluminescence) detection (Amersham Biosciences). The bands densities were determined by densitometrical analysis using the Image Studio Lite software from LI-COR Corporate Offices-US (Lincoln, Nebraska USA) and ImageJ software (National Institutes of Health, NIH Image). The density values obtained were corrected by subtraction of the backgrounds values.

2.7 Zymography

$2x10^6$ cells were plated in petri dish 100 x 20 mm and after 24 h of adhesion, concentrations of 0.1 µg/mL, 1.0 µg/mL and 10.0 µg/mL of extract were administered, beyond control group. After experimental time 24 h and 48 h, the supernatant was collected. From this conditioned supernatant were obtained 50 µg of protein were used for zymography gel loading. As controls, molecular weight standards and recombinants hrMMP-2 and hrMMP-9 proteins (Calbiochem; Merck, Darmstadt, Germany) were used. The gels were stained with Coomassie Blue G-250 (0.5%),

scanned using the Loccus Biotecnologia DS-6000, followed Lab Image 1D software for analysis. The relative densities of the degradation´s gel was determined by densitometry analysis using Image J software (National Institutes of Health, NIH Image) (Oliveira et al., 2016).

2.8 Alkaline Phosphatase Activity

$2x10^4$ cells were plated in 24-well plate. After 24 h of adhesion, the medium was then changed to osteogenic medium, α-MEM + 10% FBS, supplemented with 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate. The cells were washed with PBS and lysis buffer (10 mM Tris pH 7.5, 0.5 mM $MgCl_2$ and 0.1% Triton x-100) was added 200 µL/well. The cell lysate was collected and centrifuged 14.000 g for 15 minutes. The supernatants were used to determine the alkaline phosphatase activity (Pathomwichaiwat et al., 2015).

2.9 Mineralization Assay

The mineralization was evaluated by Alizarin Red S Staining (Sigma) and $4x10^5$ cells were plated in 12-well plates and cultivated in osteogenic medium and it´s changed every 3 days. After 21 days of culture, the supernatants were removed and cells were washed with PBS followed by fixation with 4% formaldehyde for 5 minutes at room temperature. So, cells were incubated with 2% alizarin solution at pH 4.2 for 10 minutes and followed by three consecutives washes with ultrapure water. Then, quantitative analyses were evaluated by the colorimetric method. Dried microplate was added 10% acetic acid for 30 minutes under agitation at room temperature. The content of each well was heated at 85° C for 10 minutes and kept on ice for 5 minutes followed by centrifugation at 20.000g, for 15 minutes. 100 µL of content was transferred to 96-well plates and 10% ammonium hydroxide was added to neutralize the acid. The absorbance was measured at 405 nm (Oliveira et al., 2016; Petri et al., 2010).

2.10 Reactive Oxygen Species

$4x10^5$ cells were plated in 12-well plate and after 24 h, 48 h and 72 h of treatment, the cells were washed with PBS and 5 µg/mL of DCFH-DA

(dichlorofluorescin diacetate) were added. The cells were incubated at 37º C for 30 minutes. The relative fluorescence units were quantified in spectrophotometer at 485/535 nm.

2.11 Statistical Analysis

The results were analyzed by GraphPad Prism 5 Software (La Jolla, USA). The groups were subjected to nonparametric ANOVA one way, followed Tukey´s test and passed to normality assessed by Kolmogorov test. The significance was given when $p<0.05$.

3. Results

Analysis by HPLC-DAD of 70% EtOH extract of the leaves of *Q. grandiflora* (Figure 1) allows us to identify the classes of compounds present in the extract. The analysis of the UV-vis spectra obtained by the DAD detector scanning 200-600 nm of the eluted peaks show a pattern typical maximum absorption of flavonoids (F) (Figure 2a) in 200-230 nm and 250-280 nm (Rt = 55-60 min) and benzyl system with absorption maxima at 210-220 nm and 260-280 nm suggesting the presence of compounds derived from gallic acid (DAG) (Figure 2b) (Merken and Beecher, 2000).

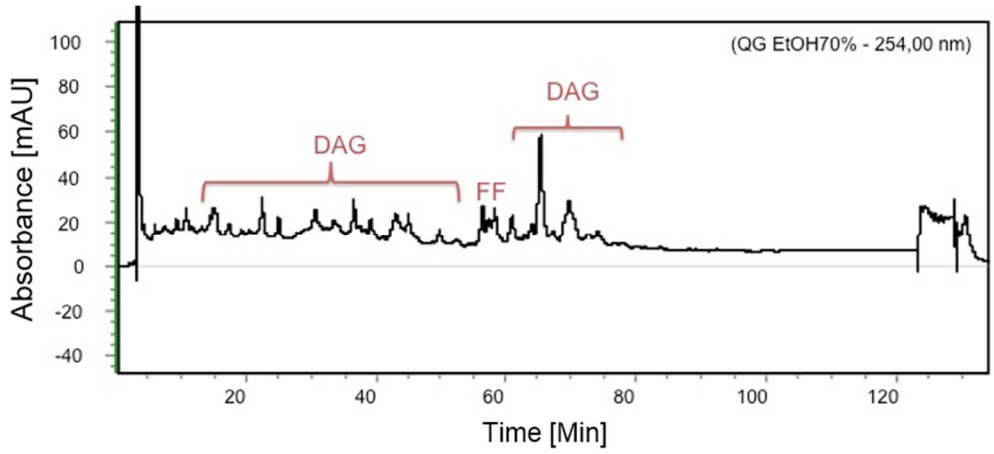

Figure. 1 Analytical chromatogram HPLC-DAD 70%EtOH extract leaves of *Q. grandiflora*. Phenomenex® Luna C18 column (250 x 4.6 mm i.d., 5 µm). Gradient: 5-40% ACN in H_2O for 120 min. Monitoring at 254 nm. DAG = compounds derived from gallic acid. F = flavonoid compounds derived.

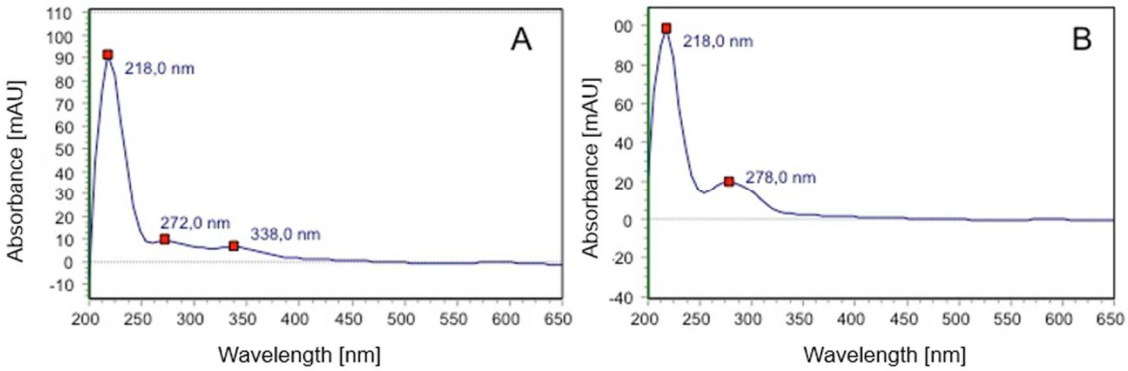

Figure 2. UV-vis spectra obtained by HPLC-DAD of the peaks present in the chromatogram of the 70%EtOH extract of the leaves of *Q. grandiflora*. A - standard spectrum with typical absorption of flavonoids. B - absorption spectrum with standard compounds derived from gallic acid.

Cell Viability

When comparing the *Q. grandiflora* groups each other in MTT assay (Fig.3), the lower concentrations of the extract (0.1 µg/mL, 1.0 µg/mL, 10.0 µg/mL) were different statistically than higher concentrations (1000 µg/mL and 100 µg/mL) in 72h and 96 h. Generally, we can see a similar profile in all periods, showing that the five concentrations of the *Qualea* extract, the lowest no demonstrated cytotoxicity to pre-osteoblasts, however were not more efficient than the positive control. In contrast, the crude extract (1000 µg/mL) and 100 µg/mL were considered cytotoxic with significant statistical difference ($p < 0.05$).

The colorimetric assay Crystal Violet (Fig.4), we find some variations among the groups, however at the end of the last period, the lowest concentrations of hydroalcoholic extract of *Qualea* leaves were similar to the control group, in contrast, higher concentrations had lower absorbance values with a significant difference ($p < 0.05$).

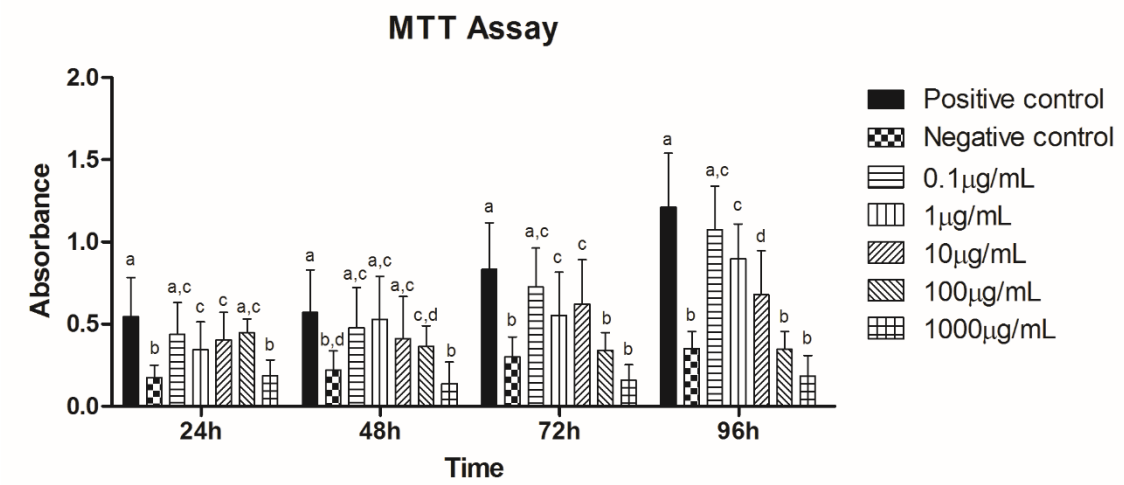

Fig. 3. Viability of MC3T3-E1 cell murine exposed to different concentrations of *Q. grandiflora* by MTT assay. Different letters represent statistically significant difference ($p < 0.05$) within each period.

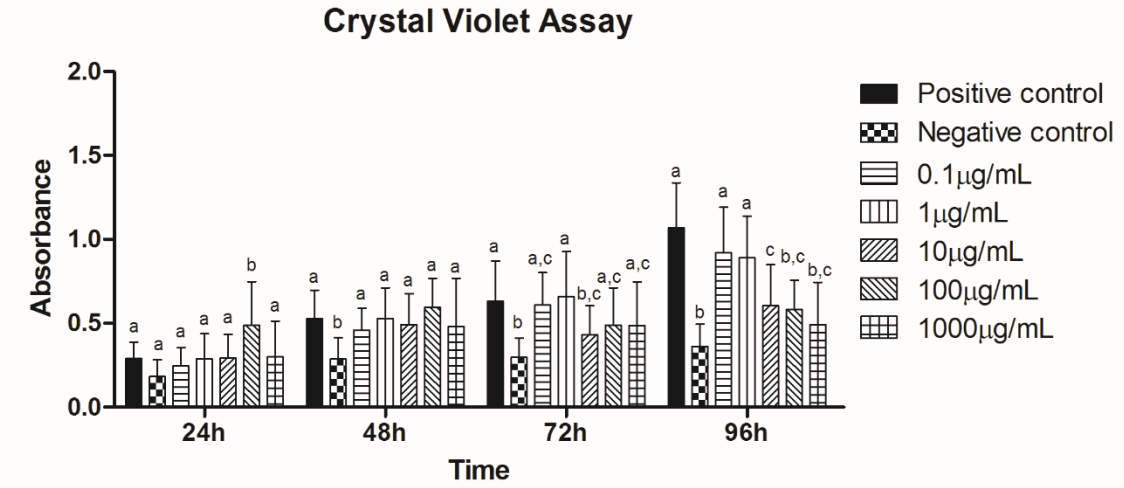

Fig. 4. Viability of MC3T3-E1 cell murine exposed to different concentrations of *Q. grandiflora* by CV assay. Different letters represent statistically significant difference ($p < 0.05$) within each period.

From the results of viability assay, in which there was a significant decrease in cell groups with the highest concentrations of the extract, it was decided to molecular assays, the three lower concentrations of *Qualea* extract (0.1 µg/mL, 1.0 µg mL and 10.0 µg/mL).

Gene expression – MMP-2, MMP-9 and TIMP-2

Analyzing the gene expression of MMP-2, MMP-9 and TIMP-2 (Fig.5a), on pre-osteoblast treated with different concentrations of *Qualea´*s extract, for MMP-2, the groups with or not extract were similar in 24 h, but in following time, 0.1 µg/mL

showed significant increase of expression (Fig.5b). For MMP-9, there was a significantly higher expression of 0.1 µg/mL group in both periods (Fig.5ab) and for TIMP-2, in the first period all groups were similar (Fig. 5a), but in the following period, the groups 0.1 µg/mL and 1.0 µg/mL showed a significant lower difference expression from control group ($p < 0.05$) (Fig.5b).

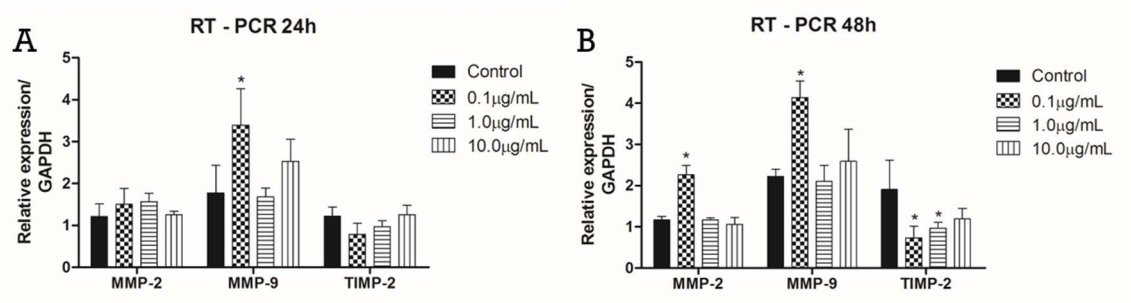

Fig. 5. Gene expression of MMP-2, MMP-9 and antagonist TIMP-2 in periods of 24 h and 48 h. GAPDH was used a control. * represent statistically significant difference ($p < 0.05$) with control group.

Western blotting

The expression of MMP-2, MMP-9 and TIMP-2 (Fig. 6c) were evaluated in 24 h and 48 h and the expression of MMP-2 and -9 for the groups exposed to the extract showed no significant difference (in each period) compared to the control group in both periods (Fig. 6ab). For TIMP-2, only 10.0 µg/mL group was statistically different compared to the control with lower expression in the 24 h (Fig. 6a), but in 48 h, all the groups were similar (Fig. 6b). It was observed a decrease in the expression of the metalloproteinase and its inhibitor, in 48 h (Fig. 6b).

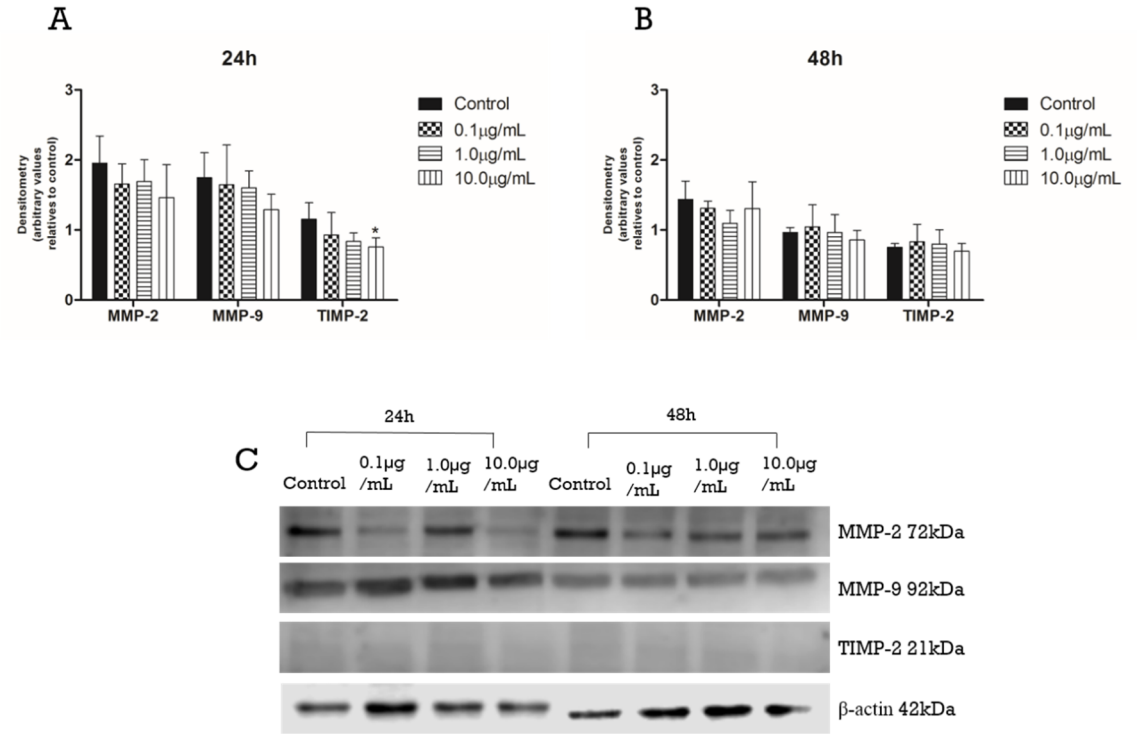

Fig. 6. Expression of MMP-2, MMP-9 and TIMP-2 by western blotting. β-actin was used a control. * represent statistically significant difference ($p < 0.05$) within each period.

Matrix Metalloproteinase activity

The *Q. grandiflora* extract was evaluated in the activity of gelatinases MMP-2 and MMP-9 (pro-enzyme and active enzyme forms) by zymography for pre-osteoblasts (Fig. 7ef). The results of densitometry of the pro and active enzymes MMP-2 showed no significant difference when compared to the control group in both periods analyzed (Fig. 7ab). This was repeated with gelatinase MMP-9 (pro-enzyme and active enzyme forms), showing that the *Qualea* extract did not alter the activity of the pro-enzyme and active enzyme forms (Fig. 7cd).

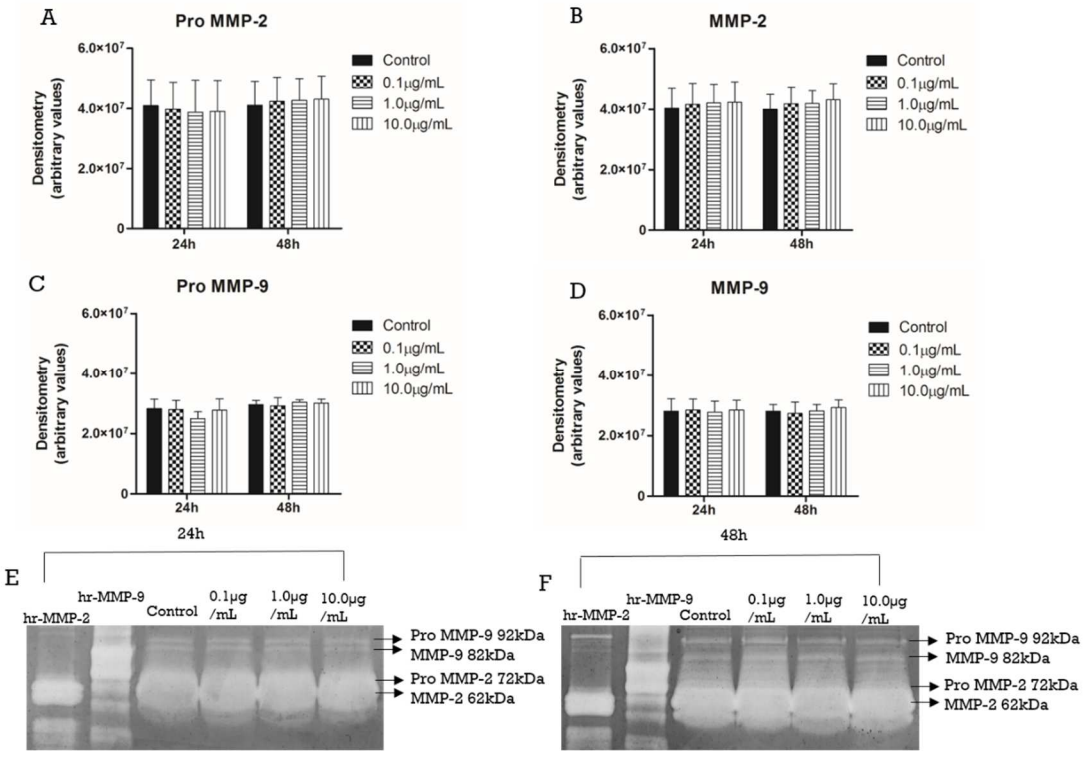

Fig. 7. Activity of pro-enzyme (A and C) and active enzyme (B and D) forms MMP-2 and MMP-9. The supernatant of control group and different concentrations of extract were collected after 24 h and 48 h of exposition of Q. *grandiflora* and eletrophorese gel was done. The degradation of gelatin (substrate to MMP-2 and MMP-9) were evaluated.

Alkaline Phosphatase (ALP)

Alkaline phosphatase activity was assessed on days 7, 10 and 14 (Fig. 8). We can observe a decrease of the activity of the alkaline phosphatase on days 10 and 14. Over time, we observed that already 10 days, the presence of mineralized nodules.

There was no significant difference among the groups, within each time.

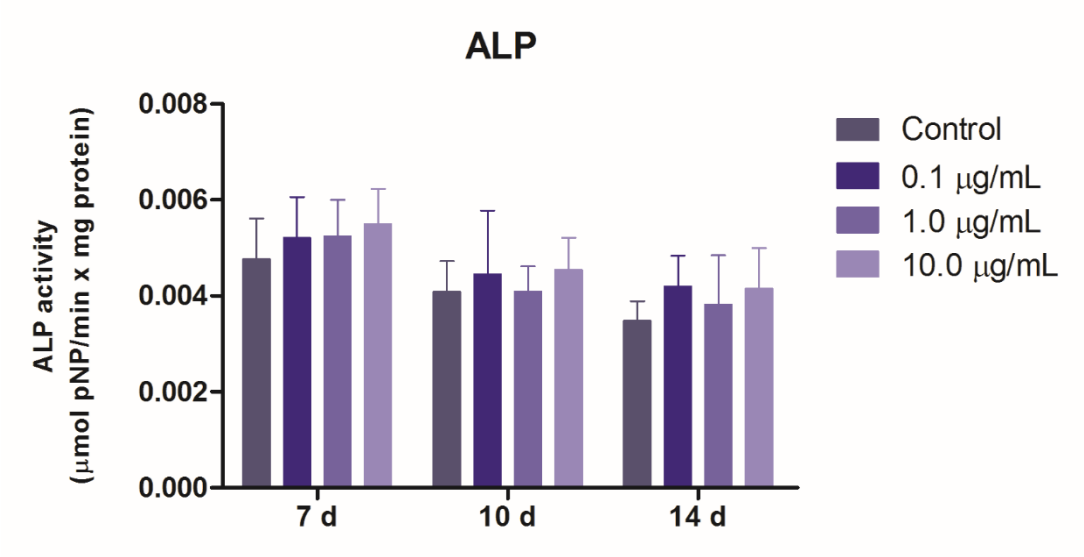

Fig. 8. Alkaline phosphatase activity of different concentrations of Q. *grandiflora* and control group. Three experimental time were evaluated on days 7, 10 and 14. There was no difference significant among groups in each time.

Mineralization Assay

In this assay, we analyzed if the different concentrations of extract affect the mineralization (Fig. 9A-C). Calcium deposition was quantitatively measured through colorimetric analysis on day 21 (Fig. 9B).

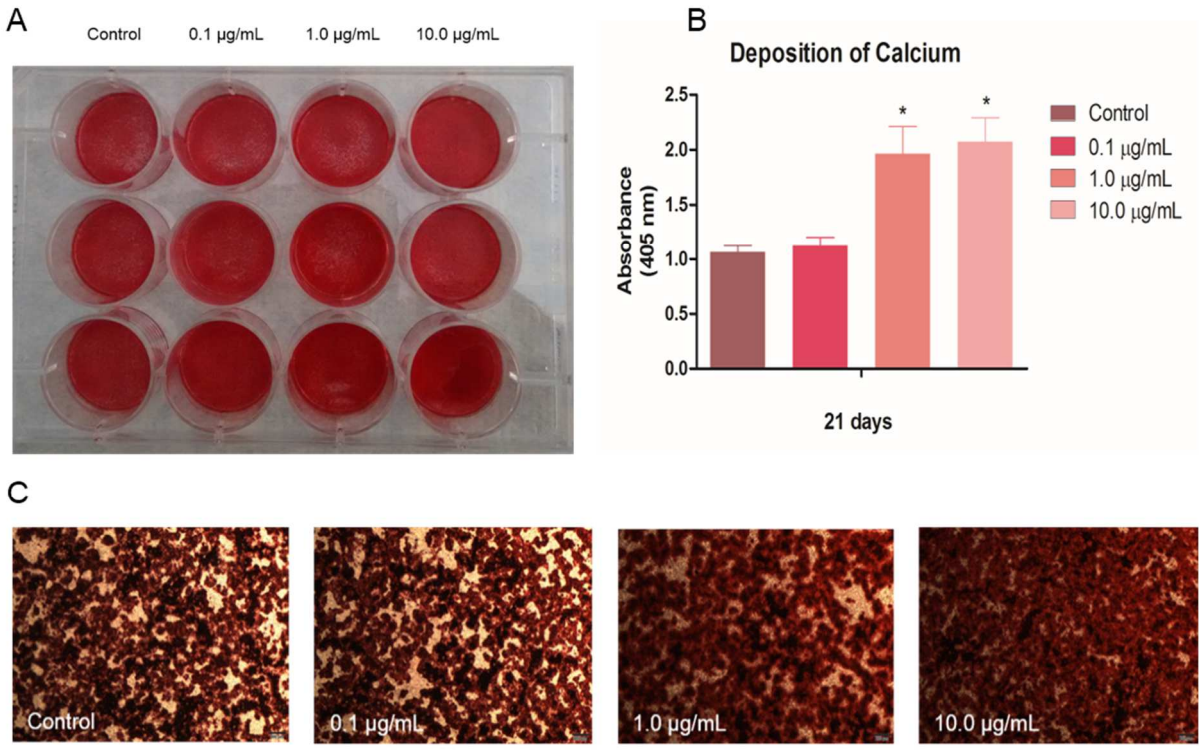

Fig. 9. Mineralization assay was assessed by alizarin red. The cells were cultivated for 21 days and the culture osteogenic medium with extract or not was changed every 3 days (A). Nodules of mineralization were found in all analyzed groups (C).

Reactive Oxygen Species (ROS)

In our study, the ROS assay did not show an antioxidant activity of *Qualea´s* extract in contact MC3T3-E1 line. Experimental time (24 h, 48 h and 72 h) were analyzed and there was no significant difference among the groups, within each period (Fig. 10).

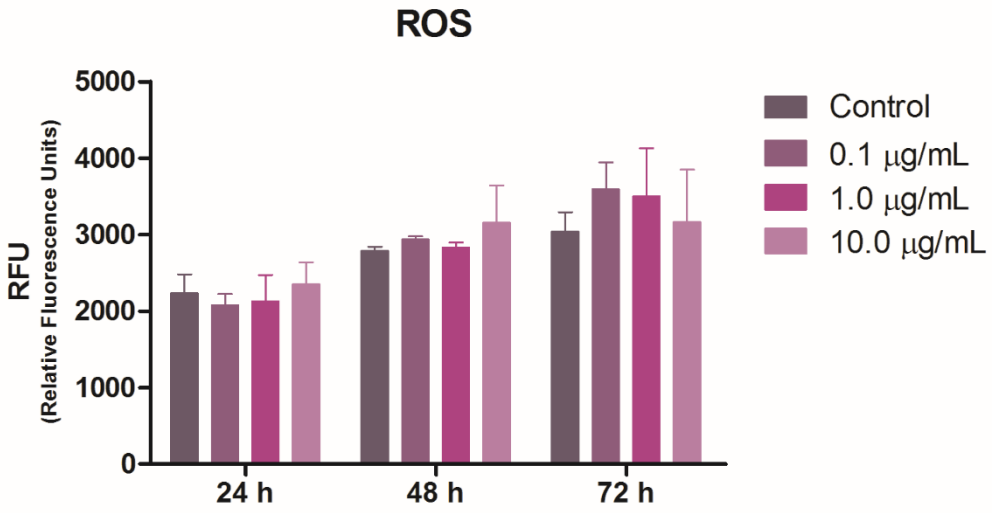

Fig. 10. Relative fluorescence units were quantified in 24 h, 48 h and 72 h. The extract of Q. *grandiflora* didn´t show antioxidant activity in this study. There was no difference significant among groups in each time.

4. Discussion

The literature shows that the use of plant extracts has been used to treat many diseases because of its beneficial effects (Junqueira, 2007; Mazzolin et al., 2010). However, it must be checked constituents and effects of these plants, as some may have genotoxic properties, causing changes in DNA (Marques et al., 2003).

There are no data in the literature demonstrating the use of *Q. grandiflora* in pre-osteoblasts and osteoblasts. According authors (Ayres et al., 2008) the ethanol extract of the leaves of *Q. grandiflora*, has tannins, triterpenes, flavonoids, benzoquinones, anthraquinone and other chemical constituents in species belonging to the family *Vochysiaceae.* (Hiruma-Lima et al., 2006) found in phytochemical investigation tannins, catechins, steroids, terpenoids and saponins in bark of *Q. grandiflora.*

Tannins and flavonoids have in their composition a variety of phenolic hydroxyl groups and show the strongest antioxidant capacity and free radical-scavenging

activity among around a hundred phenolic compounds (Bonacorsi et al., 2013). Flavonoids are widely found in plants and known for antioxidant activity. Moreover, several have been isolated and shown to be excellent antioxidants in pharmacologically activities (Kim et al., 2010).

The anti-inflammatory action of plants is largely related to two secondary metabolites which are the tannins and flavonoids (Asongalem et al., 2004; Hosseinzadeh and Younesi, 2002; Manga et al., 2004; Onwukaeme, 1995; Rane and Mengi, 2003) present in the composition of the *Q. grandiflora* leaves, which can relate to the effect used in folk medicine.

In our study, we found in 70% EtOH extract of the leaves of *Q. grandiflora*, is mostly derived from gallic acid compounds and other compounds of flavonoid. Gallic acid interest for chemical and pharmaceutical industry due to properties and applications like food additives as antioxidants (Owv and Stupans, 2003). The antioxidant activity reported in the literature may be related to the presence of gallic acid because its protects cells from oxidative damage (Bajpai and Patil, *2008*).

Furthermore, the presence considerable of gallic acid in *Q. grandiflora* could be promising in tumor cells, because the compound exhibits cytotoxicity on this cell type (Bajpai and Patil, *2008*). For the pre-osteoblast, in this study, concentrations of 1000 µg/mL and 100 µg/mL presented cytotoxicity with significant difference. In addition, it´s known that MMP-9 may be involved in tumor development as degradation of basement membrane and extracellular matrix, facilitates invasion, metastasis, growth and angiogenesis (Yang et al., 2014).

Plants containing substances like quercetin and gallic acid are effective in preventing ulcers, mainly because of their antioxidant properties, according literature (Bonacorsi et al., 2013). The same authors selected 10 Brazilian medicinal plants applied in the treatment of gastritis and ulcer, which determined the antioxidant activity of these extracts, including *Q. grandiflora* and its showed inhibition of more than 80% to *H. pylori* at concentrations of 100 µg/mL. In our study, this concentration of the extract showed cytotoxicity to pre-osteoblasts, with a significant difference in the control group.

The effect of *punica granatum* on osteoblast cell demonstrated that concentrations 10 – 100 µg/mL increased the proliferation moreover 50 and 100 µg/mL induced cell proliferation in 39.64 and 62.59%, respectively. Therefore *punica*

granatum extract induce cell proliferation in a dose dependent manner (Siddiqui and Arshad, 2014).

In a study Ribeiro et al. (2012), the cytotoxic profile was investigated in nine types of plants found in Brazil, in tumor cell lines, these, five were considered promising in the search for a new anticancer agent because they have IC_{50} values below 30 µg/mL, as *G. blepharophylla*; *G. hispida; J. curcas; K. rugosa and L. gracilis,* different of our study, safe concentration showed below 10 µg/mL.

We observed that the concentration of 0.1 µg/mL modulated gene expression for MMP-9, increasing its expression. However, this modulation is not repeated in protein expression and the activity of MMP-9, we observed lower expression and activity of this molecule. When found irregular levels of metalloproteinases and their inhibitors, to attenuate this imbalance, the MMP bind to TIMP to regulate the proteinase activity (Mwaura et al., 2006).

According to Mwaura et al. (2006) high levels of enzymatic activity of MMP-2 confirmed the presence of chronic leg ulcer and high levels of TIMP demonstrated greater healing in groups evaluated. This occurred in our study, which the use of *Qualea* proved not to be harmful in relation to homeostasis of the matrix metalloproteinases. MMPs can be considered pro and anti-inflammatory (Page-McCaw et al., 2007) therefore facilitate the recruitment and the clearance of inflammatory cells by cleaving inflammatory mediators and consequently a highly regulated response (Page-McCaw et al., 2007).

The MMP proteolysis can create space for cells migrate and produce specific substrate-cleavage fragments with independent biological activity, can regulate tissue architecture through effects on the ECM and intercellular junctions and can activate and deactivate or modify the activity of signaling molecules, directly and indirectly (Page-McCaw et al., 2007).

The alkaline phosphatase assay did not show significant differences among groups, within each period, different from (Pathomwichaiwat et al., 2015) who observed that the *Cissus quadrangulares* hexane extract (CQ) stimulates osteoblastic differentiation by administering CQ (10 µg/mL) in MC3T3-E1 cells, compared to control group over to day 5 to day 9, before decreasing on day 14.

On the other hand, a prolonged culture with Q. *grandiflora* extract demonstrated (21 day), stimulate deposition of calcium and consequently formation

of mineralization nodules. A great difference of the groups 1.0 and 10.0 µg/mL compared to the control was observed.

Yamaguchi et al. (2008) observed that the phytocomponent p-hydroxycinnamic acid stimulates differentiation and mineralization on MC3T3-E1, causing a significant increase in calcium content.

Siddiqui and Arshad (2014) observed that concentrations 10, 25, 50 and 100 µg/mL of *punica granatum* extract induced ALP level to 9.66, 22.49, 34.67 and 43.95 %, respectively, compared to control. In addition, *punica granatum* group increased significantly mineralized nodule formation and concentration of 100 µg/mL increased in 82.81 % compared to control.

Determination of ROS activity of *Cissus quadrangulares* Linn extract was assessed and concentrations of 10 - 50 µg/mL was reduced without distorcing cell native morphology and 75 – 100 µg/mL, induced ROS intensity and deformed morphology. In relation quantitative data, 75 and 100 µg/mL induced significant intracellular ROS level and 10 µg/mL was observed a little changed (Siddiqui et al., 2015). Different of our results, we could find difference among the groups with extract of *Qualea* compared to control.

Natural compounds displaying biological activities associated with low cytotoxicity encourage the researchers. High levels of free radicals and reactive oxygen species (ROS) produced *in vivo* are extremely reactive and they have been associated with severe pathological conditions such as cancer, inflammation, atherosclerosis and others (Gouveia-Figueira et al., 2014).

5. Conclusion

In our study, we found high concentrations of gallic acid e flavonoid compounds. In relation, the dose, high concentrations of *Q. grandiflora* shown to be cytotoxic in contrast low concentrations showed no cytotoxicity to this cell line, showing to be dose dependent manner. There was no effect of Q. *grandiflora* in ROS activity. And the group of 0.1 µg/mL in contact with pre-osteoblasts modulates gene expression to metalloproteinase -9, however this modulation not reflected in the activity of this enzyme. We didn´t observe effect of *Q. grandiflora* extract in ALP activity but in the final stage of differentiation, in mineralization, nodules of calcium formation were higher in concentrations of 1.0 and 10.0 µg/mL. Thus, *Q. grandiflora*

showed an osteogenic potential in osteoblast MC3T3-E1 cell and suggest further studies and prove the benefits that *Qualea* may provide in bone remodeling.

Acknowledgment

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3-Discussion

3 DISCUSSION

Monsef-Esfahani *et al.* (2014)) isolated two compounds *of Scrophularia striata*, nepitrin and acteoside, and viewed an effective inhibition of MMP activity at low compound concentrations in WEHI-164 cells, in contrast to acteoside inhibited at high concentrations, respectively. Fermented red ginseng extract (FRGE) at concentration of 1 – 100 μg/mL no toxicity effect demonstrated on MC3T3-E1 cell for 72 h compared to control group and can increase bone formation by stimulating cell growth and differentiation, according (Siddiqui *et al.*, 2015).

Already in our study, doses of *Q. grandiflora* (0.1, 1.0 and 10.0 μg/mL) were similar to the control group to evaluate the activity of MMPs, the treatment does not increase or decrease the activity of these molecules.

Matrix Metalloproteinase can be considered pro-inflammatory and anti-inflammatory. MMPs facilitate inflammatory cell recruitment and clearance of inflammatory cells by cleaving inflammatory mediators, resulting in a tightly regulated inflammatory response (Page-Mccaw *et al.*, 2007).

Machado *et al.* (2016) to assess the viability of human gingival fibroblasts treated with *Myracrodruon urundeuva* extract, the concentration of 100 μg/mL and 1000 μg/mL showed cellular cytotoxicity, as well as in our study that same concentrations induced cell death with statistically difference and concentrations below 10 μg/mL were no cytotoxicity.

Figueirôa *et al.* (2013), assess the cytotoxicity of *Eugenia uniflora* and *Eugenia malaccensis* L. L. and in spleen cells from BALB/c and fractions of the leaves in ethyl acetate and butanol showed toxicity profile over 25 μg/mL. In addition, fractions aqueous in butanol showed toxicity above 50 μg/mL. We found that concentrations over 100 μg/mL was cytotoxicity, but between 10-100 μg/mL, were not analyzed.

Berberine is a compound of Chinese herb and showed it can reduce MMP-9 expression while no effects on MMP-2 and minimal effect on TIMP-2, in hepatocellular carcinoma. We analyzed MMPs and TIMP expression, but the extract of *Q. grandiflora* did not show any difference of control group. In addition, in our study we work with normal cell but it motivates us in the application in tumor cells because

its compound shows gallic acid and flavonoids compounds and according literature these compounds have good results for tumor cells. (Wang *et al.*, 2016)

The polyphenols, for example, are found in various medicinal plants and herbs in traditional medicine and various studies show its antiproliferative and antitumor activity. As the flavanoids from green tea, for example, have been shown to possess cancer chemopreventive effects in a wide range of target organs in rodent carcinogenesis models (Queires *et al.*, 2006).

In conclusion, low concentrations of *Q. grandiflora* extract not showed cytotoxicity to this cell line, on the other hand, high concentrations caused decreased cell viability. In addition, despite the modulation in gene expression of MMP-9, not reflected in the enzymatic activity.

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