UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

GABRIELA SILVA NEUBERN DE OLIVEIRA

Evaluation of *Qualea grandiflora* Mart effect on HIF-1alpha and MMP-14 modulation in cell lines of pre-osteoblasts and fibroblasts

Avaliação do efeito da *Qualea grandiflora* Mart na modulação de HIF-1alpha e MMP-14 em linhagens celulares de fibroblastos e pré-osteoblastos

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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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"Feliz aquele que transfere o que sabe

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"Se vi mais longe, foi por estar sobre ombros de gigantes".

Isaac Newton

ABSTRACT

Evaluation of *Qualea grandiflora* Mart effect on HIF-1alpha and MMP-14 modulation in cell lines of pre-osteoblasts and fibroblasts

The vegetable specie *Qualea grandiflora* (QG), popularly known as “pau-ferro”, “pau-terra-da-folha-grande”, “pau-terra” or “pau-de-tucano”, very common in the Brazilian Cerrado, is well known due to its varied therapeutic properties. Its indications include preventive actions in the appearance of lesions of gastric mucosa, analgesic, antibacterial, anti-inflammatory and antifungal effects. Thus, QG components could have some action on molecules widely involved in angiogenic and developmental / repair processes, such as Matrix metalloproteinase 14 (MMP-14) and Hypoxia-Inducible Factor-1α (HIF-1alpha). Thus, the objective of our study was to investigate the effects of QG hydroalcoholic extract on cell viability and expression of MMP-14 and HIF-1alpha in NIH/3T3 fibroblasts and MC3T3-E1 pre-osteoblasts cell lines. For the cell viability assay and expression of the molecules, concentrations of 0.1; 1.0 and 10 µg / mL of the hydroalcoholic extract of leaves of QG, were administered for periods of 24, 48, 72 and 96h. After each period, the cell viability was evaluated by MTT assay and the expression of the molecules was analyzed using the immunofluorescence technique. The results show that the QG extract does not promote reduction of the cellular viability of fibroblasts and pre-osteoblasts in concentrations up to 10 µg/mL in the initial periods (24 and 48h). However, a significant reduction in viability can be observed in 72h and 96h for fibroblasts and 96h for pre-osteoblasts exposed to the highest extract concentration (10 µg/mL). The immunofluorescence assay indicates that the extract, at concentrations of 0.1; 1.0 and 10 µg/mL was able to increase the expression of MMP-14 and HIF-1alpha in both cell types. In conclusion, our results indicate that the QG extract exerts an effect capable of increasing the expression of the two molecules under study (MMP-14 and HIF-1alpha) both for the NIH/3T3 fibroblasts as well as for the MC3T3-E1 pre-osteoblasts cells. Thus, the QG compounds could have potential to be used as angiogenesis modulating therapeutic agents, by increasing the expression of MMP-14 and HIF-1alpha.

Keywords: *Qualea grandiflora* MART. HIF-1alpha. MMP-14. Pre-osteoblasts. Fibroblasts.

RESUMO

A espécie vegetal *Qualea grandiflora* (QG), popularmente conhecida como "pau-ferro", "pau-terra-da-folha-grande", "pau-terra" ou "pau-de-tucano", muito comum no Cerrado brasileiro, é bem conhecida devido às suas variadas propriedades terapêuticas. Suas indicações incluem ações preventivas no aparecimento de lesões de mucosa gástrica, efeitos analgésicos, antibacterianos, anti-inflamatórios e antifúngicos. Assim, os componentes da QG poderiam ter alguma ação sobre moléculas amplamente envolvidas em processos angiogênicos e de desenvolvimento/reparo, como a Metaloproteinase de matriz 14 (MMP-14) e o Fator Induzido por hipóxia 1α (HIF-1alfa). Dessa maneira, o objetivo deste estudo foi investigar os efeitos do extrato hidroalcoólico das folhas de QG na viabilidade celular e expressão de MMP-14 e HIF-1alpha em culturas de fibroblastos da linhagem NIH⁄3T3 e pré-osteoblastos da linhagem MC3T3-E1. Para o teste de viabilidade celular e expressão das moléculas, concentrações de 0.1; 1.0 e 10 µg⁄mL do extrato hidroalcoólico das folhas de QG foram administrados por períodos de 24, 48, 72 e 96h. Após cada período, a viabilidade celular foi avaliada pelo método de redução de MTT e a análise da expressão das moléculas foi feita por meio da técnica de imunofluorescência. Os resultados mostram que o extrato de QG não promove redução da viabilidade celular de fibroblastos e pré-osteoblastos em concentrações até 10 µg⁄mL, nos períodos iniciais (24 e 48h). Porém, uma redução significativa da viabilidade pode ser verificada nos períodos de 72h e 96h para os fibroblastos e 96h para os pré-osteoblastos, expostos a mais alta concentração do extrato (10 µg⁄mL). O ensaio de imunofluorescência indica que o extrato, nas concentrações de 0.1; 1.0 e 10 µg/mL foi capaz de aumentar a expressão de MMP-14 e HIF-1alpha, em ambos os tipos celulares. Em conclusão, nossos resultados indicam que o extrato de QG exerce um efeito capaz de aumentar a expressão das duas moléculas em estudo (MMP-14 e HIF-1alpha), tanto para os fibroblastos da linhagem NIH⁄3T3 como para os pré-osteoblastos da linhagem MC3T3-E1. Assim, os compostos de QG podem apresentar potencial para serem utilizados como agentes terapêuticos moduladores da angiogênese, por meio do aumento da expressão de MMP-14 e HIF-1alpha.

Palavras-chave: *Qualea grandiflora* MART. HIF-1alpha. MMP-14. Pre-osteoblastos. Fibroblastos.

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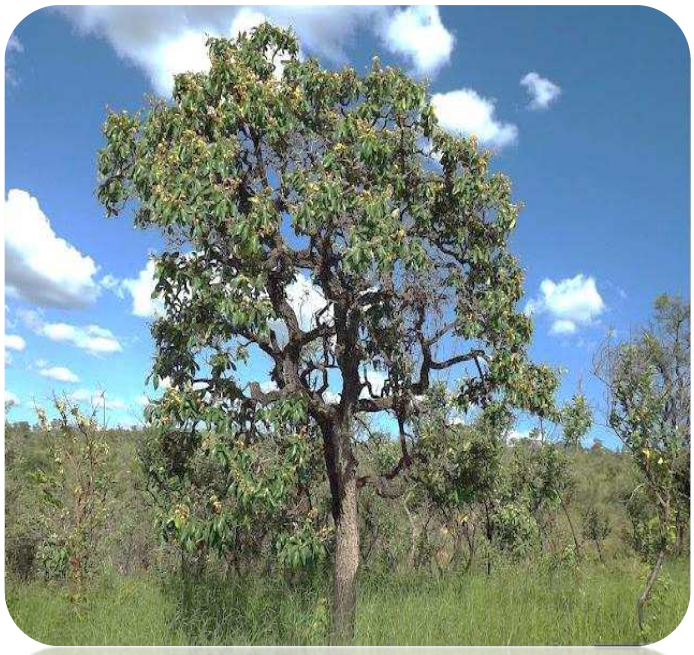


1- INTRODUCTION

1 INTRODUCTION

The use of plant species dates back to time immemorial, since primitive man had to resort to nature to seek solutions for the relief of pain and the cure of diseases (PASQUALE, 1984). The enormous diversity of compounds synthesized by plants would have formed an adaptive evolutionary defence mechanism of these plants in the face of the environmental conditions abundant in insects, micro-organisms and animals (REINBOTHE; DIETTRICH; LUCKNER, 1990). The use of medicinal plants, for a large part of the population, is seen as a historical integration with the use of synthetic drugs, since these are considered to be more expensive and aggressive to the body. The low cost and easy access were attractive enough to grab the attention of mankind to use these plants widely (OMS, 2008; SANTOS et al., 2011).

In Latin America, especially in tropical regions, there is an immense variety of medicinal plants (ARNOUS; SANTOS; BEINNER, 2005). In this sense, Brazil stands out. It is the country with the richest flora of the world, distributed in the ecosystems - Cerrado, Amazonia and Mata Atlântica (MMA, 2002). The Cerrado is the second largest biome in South America, occupying an area of 2,036,448 km^2, about 22% of the national territory. It is considered a global biodiversity *hotspot*, sheltering more than 11,000 species of native plants already registered (MMA, 2013), many of these species being used in popular medicine for the treatment of tropical diseases (ALVES et al., 2000; ANTINARELLI et al., 2015).

Among the diverse plant species of the Cerrado, the *Qualea grandiflora* (QG), popularly known as: “pau-ferro”, “pau-terra-da-folha-grande”, “pau-terra” or “pau-de-tucano”, from the family Vochysiace, has aroused the interest of researchers because it is known to have components which help to treat many of the known diseases. The literature in this context is although quite scarce. In popular medicine, the decoction or infusion obtained from the leaves of QG is used for the treatment for bloody diarrhoea, intestinal colic and against amoeba (RODRIGUES; CARVALHO, 2001). Other beneficial aspects include the treatment of wounds and inflammations (SOUZA; FELFILI, 2006). Apart from therapeutic role, the other species of the genus *Qualea* provide wood for the manufacture of canoes and carpentry; its fruits and bark have

tannins and provide tintorial matter, particularly the QG offers yellow paint (CORREA, 1981).

The phytochemical investigation of the components present in the hydroalcoholic extract of QG bark reveals the presence of terpenes, steroids, saponins, tannins and phenolic compounds (HIRUMA-LIMA et al., 2006). Other studies reveal the presence of phenolic compounds such as gallic acid, an important antioxidant, in the bark of the stem (MESQUITA et al., 2015). Regarding the leaves, the ethanolic extract reveals the presence of flavonoids, alkaloids, saponins, tannins and gallic acid, in addition to antioxidant capacity (LIMA NETO et al., 2015; SOUSA et al., 2007; TOKUHARA, 2016). The therapeutic role of the plant is attributed to the various compounds present therein.

The condition of hypoxia (low oxygen concentration) is an event that is linked to several pathological and physiological conditions. The main mediator of the adaptive response of cells to hypoxia is the hypoxia-inducible transcription factor-1 (HIF-1). HIF-1 is a heterodimer including HIF-1α, the oxygen-sensitive subunit, and HIF-1β; under conditions of normoxia, HIF-1alpha is ubiquitinated and subject to degradation via proteasome. Under hypoxia conditions, the fraction of HIF-1alpha that is ubiquitinated decreases dramatically, resulting in accumulation of the protein, which then migrates to the nucleus, controlling the transcription of target genes to restore the oxygen level through an angiogenic response (LUNDGREN et al., 2007; XING et al., 2015).

Recent studies show that the HIF-1alpha pathway plays a critical role in bone development and regeneration processes, through the coupling between angiogenesis and osteogenesis (LI et al, 2016; SHEN et al., 2009; STEGEN, 2016). Bone regeneration recalls processes that occur during skeletal development and requires close spatial and temporal coordination of events involving resident bone cells, bone marrow stromal elements, and associated vascular structures (WAN et al., 2008). The balance of the bone remodeling process is sensitive to aging, diseases (such as osteoporosis) and medications, presenting a very complex molecular control (BARON; KNEISSEL, 2013). Because it is linked to these events (angiogenesis and development/repair), it has been found that HIF-alpha is widely expressed in several cell types such as osteoblasts, osteocytes and chondrocytes, involved in fracture healing; therefore, of paramount importance to optimize bone growth at the fractured site (SANG et al., 2016). During repair, inflammatory cells, fibroblasts and stem cells are recruited to the site, participating in the process of forming new blood vessels

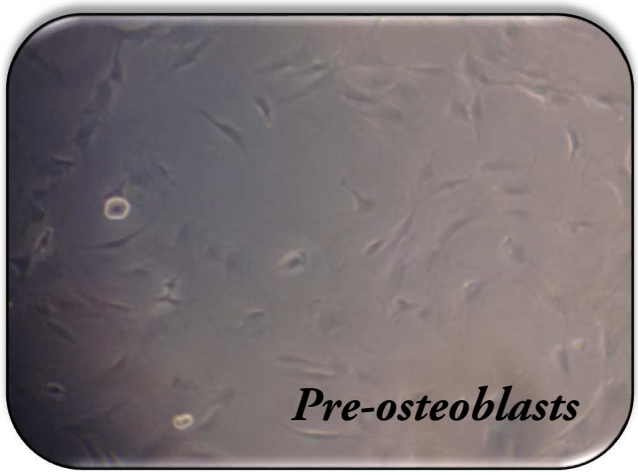
(CARANO; FILVAROFF, 2003). The inadequate supply of vessels is one of the main causes of failure in fracture healing, which has already been proven by experiments in various animal models. Therefore, treatment strategies that promote tissue vascularization may be beneficial for tissue healing and regeneration (STEGEN; VAN GASTEL; CARMELIET, 2015).

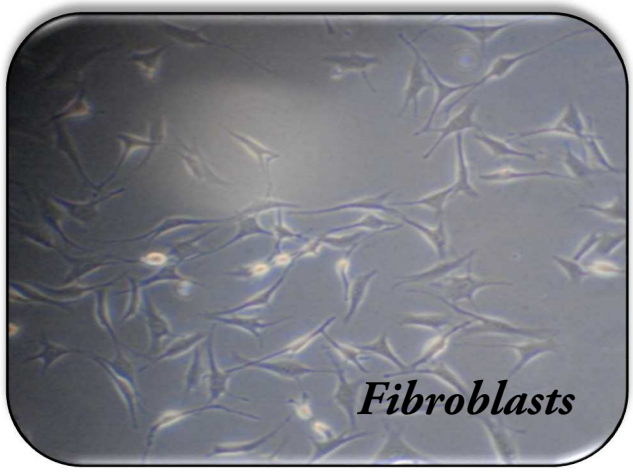
Matrix metalloproteinases (MMPs) are an important family of zinc-dependent proteolytic enzymes, responsible for the degradation of extracellular matrix components. Due to this, they play an important role in determining the tissue microenvironment and participate in a wide range of metabolic processes (MACIEJCZYK et al., 2016). Currently, more than 20 types of MMPs are known, which are classified according to their location and substrate specificity, ranging from MMP-1 to MMP-28 (VASAVADA; DHOLAKIA, 2013). MMP-14, also known as MT1-MMP (MT-MMPs - transmembrane MMPs) is expressed on the cell surface, which causes them to act in the pericellular space. This location gives these enzymes a particular property: they can alter the immediate microenvironment around the cells. Special attention is given to MT1-MMP, as it affects cellular functions in a variety of ways (ITOH; SEIKI, 2006). It has the ability to degrade the basement membrane and extracellular matrix proteins (ECMs), cell adhesion molecules, cytokines, growth factors and receptors, being expressed in several tissues and cell types, including bone cells and those of adjacent tissues, such as fibroblasts (LUCAS et al., 2016; ITOH, 2015).

Several studies report the important involvement of MT1-MMP in developmental processes, bone repair and angiogenesis (SCHINDELER et al., 2008; ITOH, 2015; STEGEN; VAN GASTEL; CARMELIET, 2015). The extracellular bone matrix is a dynamic macromolecule mesh that exerts strong control over the fate and behavior of bone cells (including cell proliferation, survival, migration and differentiation); remodeling of this matrix during bone development and repair is essential and requires the involvement of MMP-2, MMP-9, MMP-13 and MT1-MMP (LU et al., 2016; SIMSA-MAZIEL; SELA-DONENFELD; MONSONEGO-ORNAN, 2013). The importance of MT1-MMP in bone development got confirmed when it was found that MT1-MMP deficient mice show severe defects in skeletal development and angiogenesis (ZHOU et al., 2000).

To date, no scientific research has attempted to investigate the possible effects of the extract of the leaves of the QG, which has several therapeutic properties, as

seen previously, on molecules involved in the process of bone repair. Thus, in the present study, the effect of the extract of the leaves of QG on the cell viability of fibroblasts and pre-osteoblasts and the action of the QG on the expression of MMP-14 and HIF-1alpha, were evaluated *in vitro*.





2-Article

2 ARTICLE

Article formatted according to Journal of Ethnopharmacology

Effect of *Qualea grandiflora* Mart. (Vochysiaceae) on the expression of MMP-14 and HIF-1alpha in fibroblasts and pre-osteoblasts

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Abstract

Ethnopharmacological relevance: The specie *Qualea grandiflora* (QG), popularly known as “pau-ferro”, “pau-terra-da-folha-grande”, “pau-terra” or “pau-de-tucano”, very common in the Brazilian Cerrado, is well known due to its varied therapeutic properties. Its indications include preventive actions in the appearance of lesions of gastric mucosa, analgesic, antibacterial, anti-inflammatory and antifungal effects, being used mainly in the form of tea or infusion. Thus, QG components could have some action on molecules widely involved in angiogenic and developmental / repair processes, such as Matrix metalloproteinase 14 (MMP-14) and Hypoxia-Inducible Factor-1α (HIF-1alpha).

Objective: To investigate the effects of hydroalcoholic extract from QG leaves on cell viability and expression of MMP-14 and HIF-1alpha in fibroblast and pre-osteoblast cell lines.

Materials and methods: Murine MC3T3-E1 pre-osteoblasts and NIH/3T3 fibroblasts cell lines (ATCC) were used for the experiments. Cell viability was assessed by the MTT colorimetric assay and the expression of MMP-14 and HIF-1alpha by immunofluorescence.

Results: The results show that the hydroalcoholic extract of the leaves of QG does not promote reduction of the cell viability in pre-osteoblast and fibroblasts cells in concentrations up to 10 µg/mL, in the initial periods (24 and 48h). However, a significant reduction in viability can be verified in 72h and 96h periods for fibroblasts and 96h for pre-osteoblasts upon exposing it to the highest extract concentration (10 µg/mL). Immunofluorescence assay indicates that the extract, at concentrations of 0.1, 1.0 and 10 µg⁄mL was able to increase the expression of MMP-14 and HIF-1alpha in both cell types.

Conclusion: The QG hydroalcoholic extract was able to increase the expression of the two molecules under study, MMP-14 and HIF-1alpha, in MC3T3-E1 pre-osteoblasts and NIH/3T3 fibroblasts cell lines. Thus, the QG compounds have the potential to be used as angiogenesis modulating therapeutic agents, by increasing the expression of MMP-14 and HIF-1alpha.

Keywords: *Qualea grandiflora* MART.; HIF-1alpha; MMP-14; Pre-osteoblasts; Fibroblasts.

1. Introduction

The native vegetable specie *Qualea grandiflora* (QG) of the family Vochysiaceae is a giant deciduous tree, much common in Brazilian Cerrado, already known to have several medicinal benefits. It has various popular names as “pau-ferro”, “pau-terra-da-folha-grande”, “pau-terra” or “pau-de-tucano”. It has been reported to have various therapeutic aspects such as in the cure and prevention of gastric mucosa lesion (Hiruma-Lima et al., 2006), also as depressant of the central nervous system, analgesic, potential anticonvulsant (Gaspi et al., 2006), antibacterial (Ayres et al., 2008), anti-inflammatory (Santos et al., 2011) and antifungal (Costa et al., 2008). The researches shows that the QG presents components with important pharmacological activities, however, the studies involving the plant in question are still scarce.

It is now known that both Matrix Metalloproteinase 14 (also known as membrane type-1 matrix metalloproteinase -MT1-MMP- and Hypoxia-Inducible Factor-1α (HIF-1alpha) are widely involved in physiological and pathological processes, including remodeling / repair, bone tissue development (Thompson et al., 2015; Drager and Harvey et al., 2015) and inflammation (Amalinei et al., 2010; Ng et al., 2011). MT1-MMP, which belongs to the group of transmembrane MMPs, has the ability to degrade the basement membrane and proteins of the extracellular matrix (ECM), cell adhesion molecules, cytokines, growth factors and receptors, being expressed in several tissues and cellular types, including bone cells and those of adjacent tissues, such as fibroblasts (Lucas et al., 2016; Itoh, 2015). The importance of the normal activity of MT1-MMP can be confirmed by means of its effect seen in knockout mice models. Such mice are reported to have varied defects, such as deformity in bone development, angiogenesis, fibrosis, development of the submandibular gland and lung, craniofacial abnormalities and even premature death (Itoh, 2015; Xu, 2016).

Hypoxia-Inducible Factor-1α (HIF-1alpha) is mainly involved with the angiogenic process and is essential in several situations. The hypoxia-inducible factor pathway (HIF) controls various genetic programs linked to angiogenesis (for example, vascular endothelial growth factor (VEGF)) and cellular metabolism (glucose transport). In addition, HIF can recruit inflammatory and mesenchymal cells. Not only this, it also influences cell differentiation (Wan et al., 2008). During the formation of new blood vessels, the production of angiogenic growth factors thus initiates, which causes the extracellular matrix of the basement membrane to be degraded, a process effecting by

MMPs; they also release pro-angiogenic factors (Stegen and Gastel et al., 2015). The establishment of a functional vascular system is fundamental during tissue repair process. In bone, blood vessels carry oxygen, nutrients and provide calcium and phosphate which is crucial for mineralization; without angiogenesis, otherwise osteogenesis would not occur (Xing et al., 2015). Investigations about the molecular and cellular processes involved in vascularization may contribute to the possible genetic and/or pharmacological modulation of HIF-1alpha, which could add more to the improvement of bone regeneration clinically (Hankenson et al., 2011) moreover it can also open an era towards a new therapeutic approach, inclined to the pathological disorders (Semenza, 2000).

To date, no scientific research has attempted to investigate the possible effects of QG leaf extract on the molecules MMP-14 and HIF-1alpha, widely involved in angiogenic and development / repair processes. Thus, in the present work, the effect of the extract of the leaves of QG on the cell viability of fibroblasts and pre-osteoblasts and the action of the same on the expression of MMP-14 and HIF-1alpha, was evaluated in vitro.

2. Materials and Methods

2.1. Preparation of the extract

The collection of the fresh leaves of QG was carried out in the Municipal Ecological Park Jardim Botânico de Bauru, São Paulo, Brazil. The samples were prepared, identified by A. L. Dokkedal and deposited in the Herbarium of the Department of Biological Sciences (UNBA) of the Paulista State University "Júlio de Mesquita Filho", Bauru, Brazil, under nº. 39825-1. The processing of the plant to obtain the hydroalcoholic (methanol) extract, powder form, was carried out following the method described previously by Machado et al. (2016).

2.2. Cell Culture

For the cell expansion, murine MC3T3-E1 pre-osteoblasts (ATCC) and NIH/3T3 fibroblasts cell lines (ATCC) were grown, respectively, in Minimum Essential Medium Eagle - Alpha Modification (α-MEM) and Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For subculture, they were incubated with trypsin (0.25%) for 5 minutes at 37°C, followed by trypsin inactivation with medium containing 10% FBS. After centrifugation at 1,200 rpm for 5 minutes, the pellet was resuspended in the respective media and the cells were cultured in bottles for further experiments. Cells were incubated at 37ºC in a humid atmosphere containing 5% CO_2.

2.3. Assay for analysis of cell viability

2.3.1. MTT reduction

For viability assays, $3x10^3$ cells / well were plated in 96-well plates. After 24h of incubation, the culture medium was replaced with concentrations of 0.1; 1.0 and 10 µg/mL of QG crude hydroalcoholic extract for periods of 24, 48, 72 and 96h (Machado et al., 2016). Positive and negative control groups were also carried out, the cells being treated, respectively, with: 10% FBS medium and 1% FBS medium. Control groups were not exposed to the extract. After treatment times, the MTT assay was performed according to the method of Mosmann (1983). Absorbance was determined by optical density spectrophotometer with 562nm filter (FluoStar OPTIMA, microplate fluorescence reader) (Pagin et al., 2014; Pacheco et al., 2013).

2.4. Assay for the analysis of MMP-14 and HIF-1alpha expression

2.4.1. Immunofluorescence

For the expression analyses of MMP-14 and HIF-1alpha, $3x10^3$ cells / well were plated in 24-well plates containing coverslips. After the treatment time (24, 48, 72 and

96h), the medium with the extract at the concentrations of 0.1; 1.0 and 10 µg / mL was removed, cells thus washed with PBS three times, fixed in methanol for 6 min at 4°C and then were with washed three times for five minutes with PBS-Tween (Sigma-Aldrich). After removal of PBS-Tween, the permeabilization of the membrane was done with triton Triton X-100 (Sigma-Aldrich) for 10 minutes at 4ºC, followed by washing with PBS-Tween 3 times of 5 minutes each. In order to block the nonspecific sites, 5% skimmed MOLICO® milk was added which was let to stand for 30 minutes. The sample was then incubated with the primary antibody - anti-MMP14 (1:250) and anti-HIF-1alpha - (1:250) (SANTA CRUZ, Biotechnology) overnight at 4 ºC. After the incubation period, the cells were washed with PBS-Tween 3 times for 10 minutes each; 1% skimmed MOLICO® milk was added for 15 minutes and, later, the cells was incubated with FITC-conjugated secondary antibody or PE-conjugated secondary antibody (1:250) for 1h30 min at room temperature. After removal of the secondary antibody, the sample was washed in PBS-Tween 3 times for 10 minutes each. Prolong gold (LIFE TECHNOLOGIES) was added onto glass slides, on which the coverslips were placed and subsequently examined by the fluorescence confocal microscope (Leica DMIRBE Inverted Microscope).

Regarding the control groups, the positive means presence of the primary and secondary antibodies; the negative control consisted of absence of primary antibody. Osteosarcoma cells (SaOS-2) were used as a positive control for labeling, since they have high expression of MMP-14 and HIF-1alpha (Futamura et al., 2014; Wei et al., 2016).

2.5. Statistical analysis

Data is presented as a percentage of the mean and standard deviation (SD). The parameters were analyzed by analysis of variance (ANOVA) coupled with Tukey's post-hoc test; for all analyzes, values of $p < 0.05$ were considered statistically significant. All statistical tests were performed using GraphPadInStat and Prisma (GraphPad, San Diego, CA).

3. Results

3.1. MTT reduction

Figures 1 and 2 demonstrate the effect of the different dilutions of the hydroalcoholic extract of the QG on the viability value (MTT) of the fibroblasts and pre-osteoblasts, respectively, after periods of: 24, 48, 72 and 96h. For the production of the graphs and statistical analysis, the average of three isolated experiments (biological triplicate), for fibroblasts and for pre-osteoblasts, was averaged.

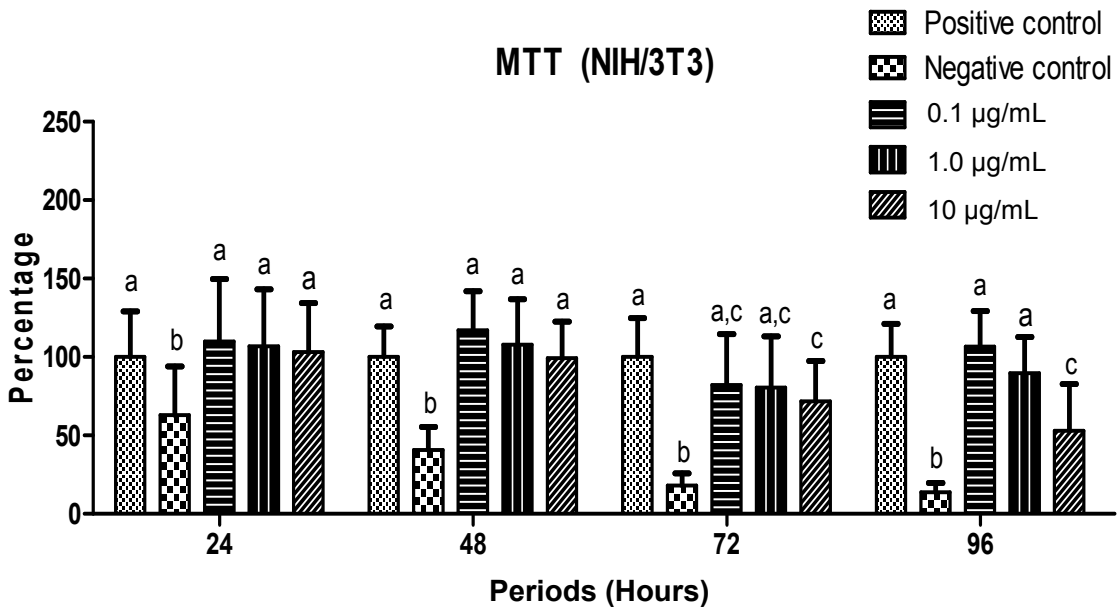

Fig.1. Effect of the extract dilutions on the viability of fibroblasts of the NIH/3T3 line after exposure for: 24, 48, 72 and 96h. Different letters indicate a statistically significant difference ($p<0.05$) within each period.

We can verify that in the 24 and 48h periods the three dilutions of the extract (0.1, 1.0 and 10 µg/mL) were similar to the positive control (no treatment), which was thus used as a comparison parameter (100% cellular viability). The only statistically significant difference was observed in the negative control group when compared to the other groups, and this difference remained in all four periods. In the periods of 72 and 96h, exposure to the highest extract concentration (10 µg/mL) resulted in a decrease in cell viability when compared to the positive control ($p <0.05$). Comparing the groups treated with the extract, we can observe that the highest concentration (10 µg/mL) differs statistically from the lowest concentrations (0.1 µg/mL and 1.0 µg/mL)

to a lower percentage in the last period (96h). In general, we conclude that the lower dilutions were similar to the positive control, in all periods. However, the higher concentration (10 µg/mL) reduced cell viability in the two final periods (Fig. 1).

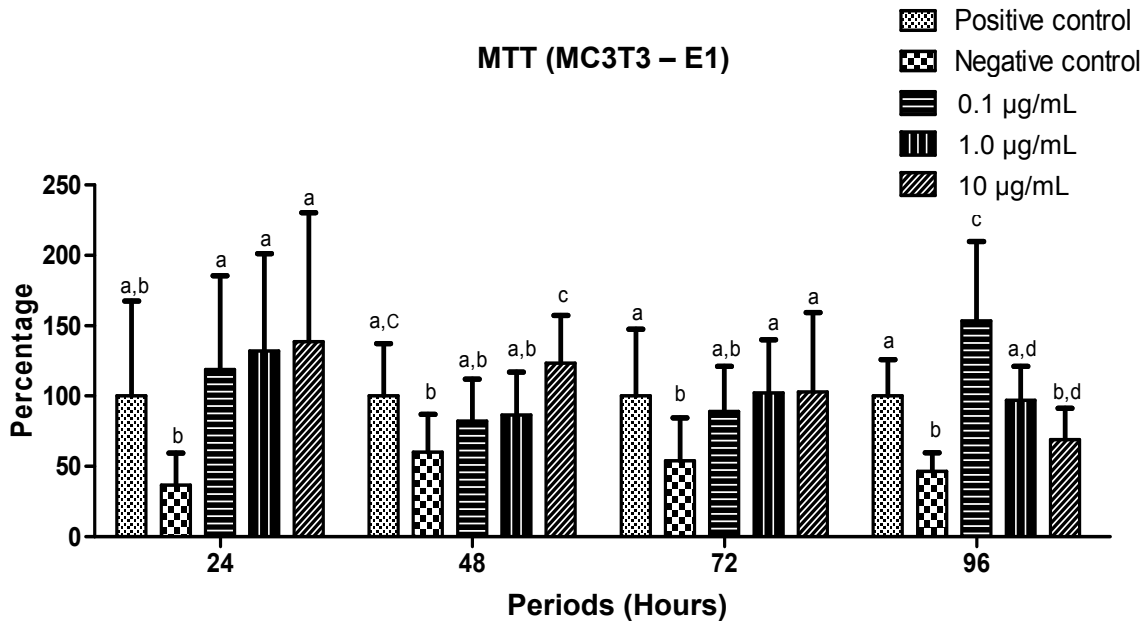

Fig. 2. Effect of the extract dilutions on the viability of pre-osteoblasts of the MC3T3-E1 line after exposure for: 24, 48, 72 and 96h. Different letters indicate a statistically significant difference ($p<0.05$) within each period.

For the pre-osteoblasts we can verify that the significant difference occurs in the period of 96h, in which the lowest concentration of the extract (0.1 µg/mL) presents a significant difference for a higher percentage value and the highest concentration (10 µg /mL) difference for a lower value, when compared to the positive control. The negative control group, in general, presented a decrease in viability in comparison with the other groups. Comparing the groups conditioned with the extract, we see that the differences appear in the highest concentration (10 µg / mL) in the 48h period and, in the 96h period, in the highest dilution (0.1 µg/mL). Analyzing the profile of the graph, we conclude that only the higher concentration of the extract (10 µg/mL), in the last period (96h), reduced cell viability for this lineage (Fig. 2).

3.2. Immunofluorescence

Figures 3 and 4 demonstrate the effect of the highest concentration (10 µg/mL) of the hydroalcoholic extract of the QG on the expression of MMP-14 and HIF-1alpha after 24, 48, 72 and 96h periods, in NIH/3T3 fibroblasts and MC3T3-E1 pre-osteoblasts. The images related to the marking of the molecules exposed to the other concentrations (0.1 µg/mL and 1.0 µg/mL) are not exposed, as they showed a marked similarity to the untreated group.

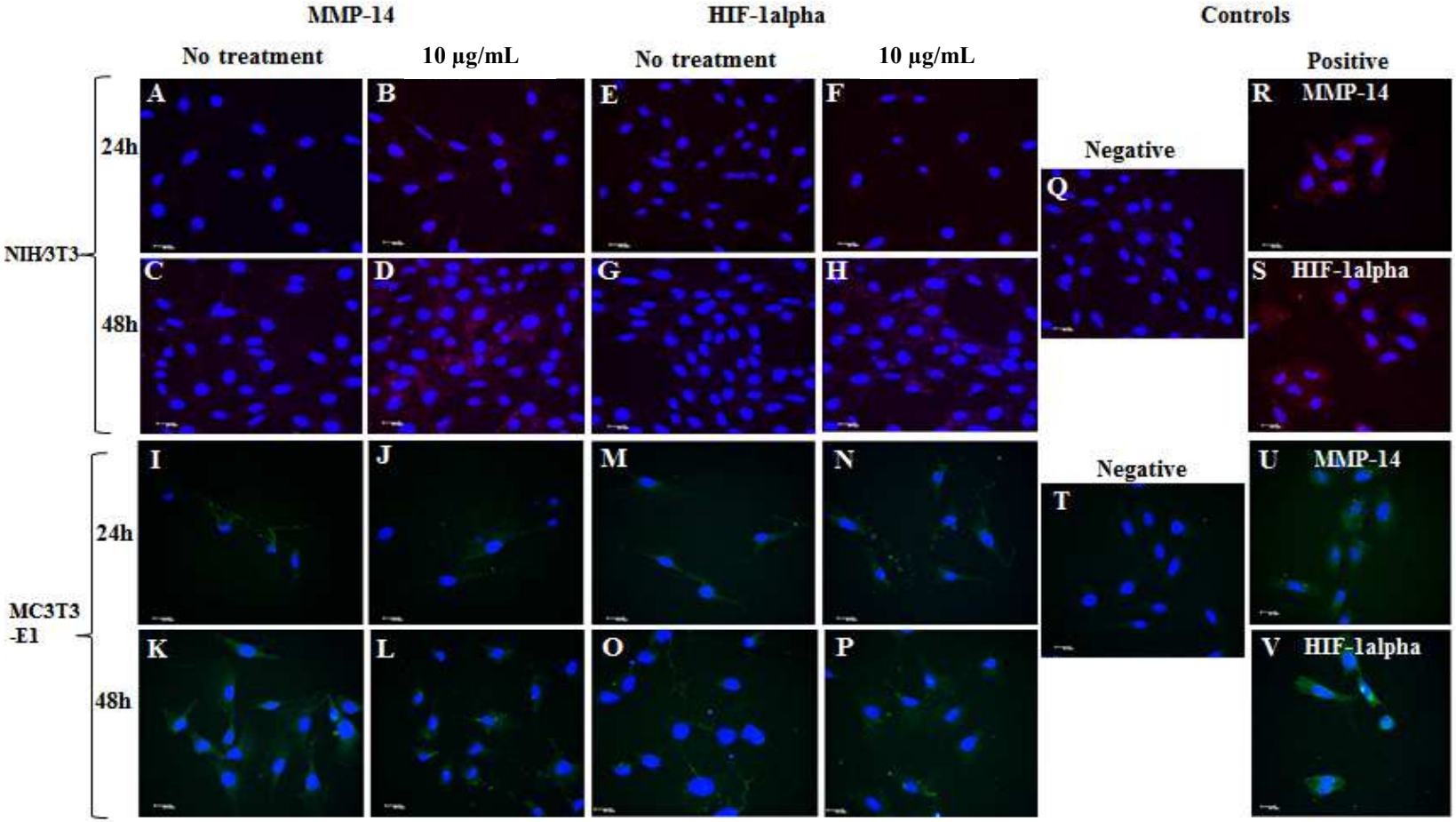

Fig. 3. Immunofluorescence reactions. Expression of MMP-14 and HIF-1alpha in NIH/3T3 fibroblasts and MC3T3-E1 pre-osteoblasts after 24 and 48h exposure to the 10 µg/mL concentration of the QG hydroalcoholic extract. Figures A, B, C, D, I, J, K and L represent the labeling for MMP-14 and E, F, G, H, M, N, O and P, for HIF-alpha. Figures A, B, E, F, I, J, M and N represent 24h of exposure to the extract and C, D, G, H, K, L, O and P, 48h. (A / C; E / G; I / K; M / O) Absence of extract. (B / D and F / H) Expression of MMP-14 and HIF-1alpha (red) after exposure to 10 µg/mL extract for fibroblasts (J / L and N / P) Expression of MMP-14 and HIF-1alpha (green) after exposure to the extract at the concentration of 10 µg/mL, for the pre-osteoblasts (Q / T) Negative control, absence of primary antibody. (R / S / U / V) Positive control (SaOS-2). (R) MMP-14 (red) (S) HIF-1alpha (red) (U) MMP-14 (green) (V) HIF-1alpha (green). The nuclei were stained with DAPI (blue).

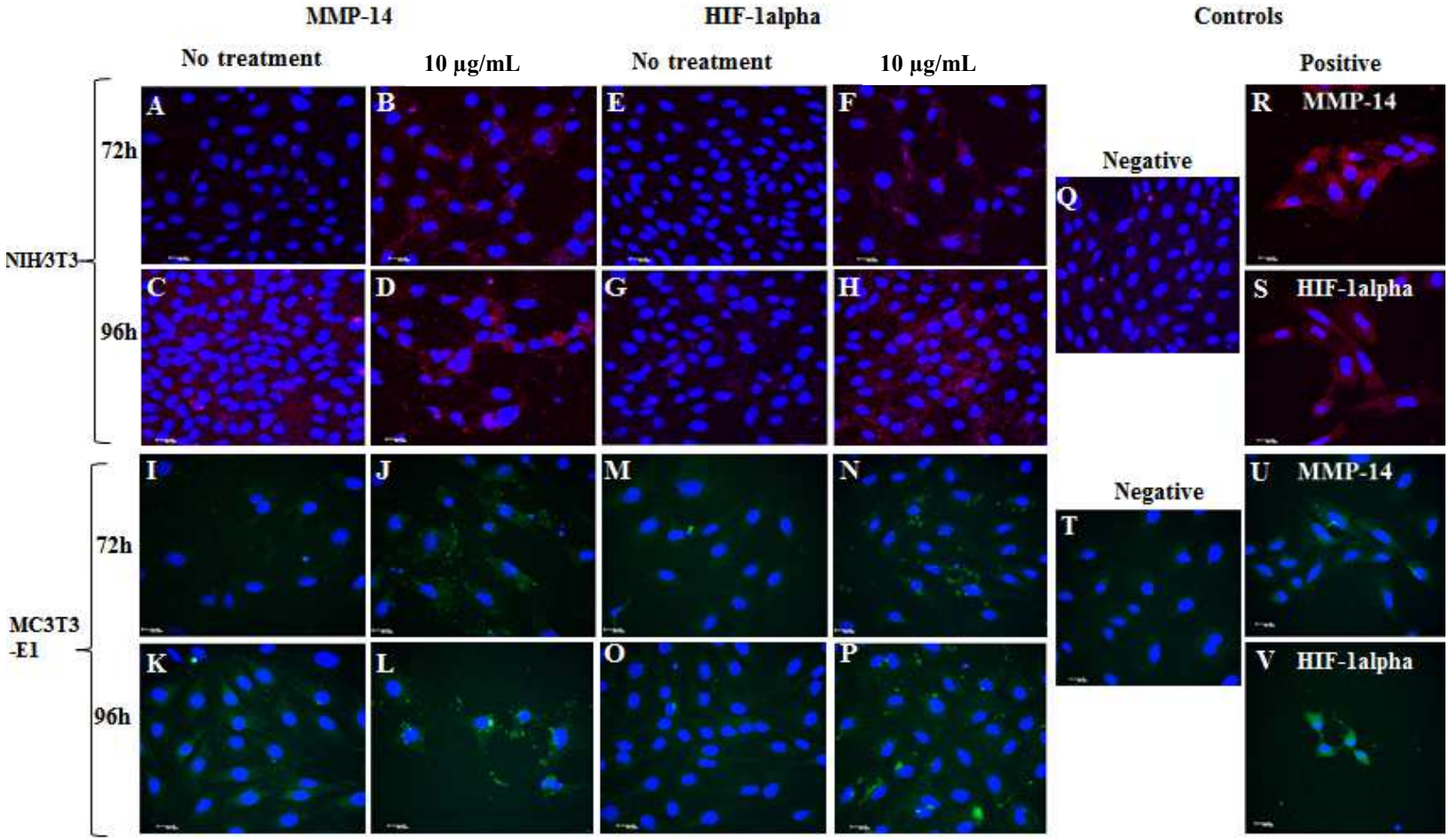

Fig. 4. Immunofluorescence reactions. Expression of MMP-14 and HIF-1alpha in culture of NIH/3T3 fibroblasts and MC3T3-E1 pre-osteoblasts after 72 and 96h of exposure to the 10 µg/mL concentration of QG hydroalcohol extract. Figures A, B, C, D, I, J, K and L represent the labeling for MMP-14 and E, F, G, H, M, N, O and P, for HIF-alpha. Figures A, B, E, F, I, J, M and N represent 72h of exposure to the extract and C, D, G, H, K, L, O and P, 96h. (A / C; E / G; I / K; M / O) Absence of extract. (B / D and F / H) Expression of MMP-14 and HIF-1alpha (red) after exposure to 10 µg/mL extract, for fibroblasts (J / L and N / P) Expression of MMP-14 and HIF-1alpha (green) after exposure to the extract at the concentration of 10 µg / mL, for the pre-osteoblasts (Q / T) Negative control, absence of primary antibody. (R / S / U / V) Positive control (SaOS-2). (R) MMP-14 (red) (S) HIF-1alpha (red) (U) MMP-14 (green) (V) HIF-1alpha (green). The nuclei were stained with DAPI (blue).

The results show that there was an increase in the expression of MMP-14 and HIF-1alpha both for the NIH/3T3 fibroblasts (Fig.3 and 4 -B, D, F and H - red marking) and for the MC3T3-E1 pre-osteoblasts (Fig. 3 and 4 – J, L, N e P- green marking) as the time of exposure of the cells to the extract increases (from 24 to 96h) and to the highest concentration (10 µg/mL) comparing with the non-exposed groups (Figure 3 and 4 - A, C, E and G for NIH/3T3 and I, M, K and O for MC3T3-E1) and with the positive control, SaOS- 2 (Fig. 3 and 4) (R / U) MMP-14; (S / V) HIF-alpha. The marking of the molecules exposed to the other concentrations (0.1 µg/mL and 1.0 µg/mL) was very similar to the positive control; for this reason, the images of these were omitted from the results. The expression, for both MMP-14 and HIF-1alpha, is localized to the cytosol.

4. Discussion

Qualea grandiflora Mart is an angiosperm widely found in the Brazilian Cerrado, which is known as "Brazilian savannah". This one presents a very rich and characteristic flora, and it's one of those many of the plants found in the region which are being used to treat several tropical diseases (Gaspi et al., 2006). Some studies have shown that bark and leaves of QG have medicinal properties (Martins et al., 2015). However, the studies on its leaves are pretty scarce. Thus, the present study evaluated the effect of different concentrations of the crude hydroalcoholic extract of the leaves of QG on the cell viability and expression of MMP-14 and HIF-1alpha in fibroblasts and pre-osteoblasts cultures.

The results of the MTT assay showed that the hydroalcoholic extract of the leaves of QG has the capacity to decrease the cell viability only in the last periods of exposure in the group having highest concentration of the extract, for both cell types (Fig. 1 e 2). Other studies carried out in our research group evaluated the effects of leaves extracts of QG on the cell viability of fibroblasts and pre-osteoblasts, using the same periods and concentrations of this work, but with an increase of higher concentrations. In the MTT assays, concentrations above 10 µg/mL (100 and 1000 µg/mL) showed significant decreases in viability from the initial periods for both cell lines when compared to the positive control group (Santesso et al., 2016; Tokuhara et al., 2016). The choice of the 10 µg/mL concentration was based on previous results obtained from our group, adopting it as the threshold concentration for the viability

reduction. These effects are time and concentration dependent and possibly are associated with components present in the plant. We also found, means of HPLC-DAD analysis, of ethanolic extract (EtOH) 70% of leaves of QG, flavonoids and compounds derived from gallic acid (Tokuhara et al., 2016). The literature reveals that the leaf extract not only has flavonoids, alkaloids, saponins and tannins, rather it also has antioxidant capacity (Lima Neto et al., 2015; Sousa et al., 2007).

The results of immunofluorescence assays reveal that the QG extract has the ability to increase the expression of MMP-14 and HIF-1alpha in both cell types. Osteosarcoma cells (SaOS-2) which are known to have increased labeling for the two molecules under study (Futamura et al., 2014; Wei et al., 2016) (Fig. 3 e 4) were used as a positive control for the experiment. Tokuhara (2016) evaluated the influence of the hydroalcoholic extract obtained from the leaves of QG on the expression and activity of matrix metalloproteinases in cultures of pre-osteoblasts. Upon comparing control with the treated group, an increase in the expression of MMP-9 gene was noticed; however, enzyme activity was not confirmed by zymography assays (Tokuhara, 2016). In addition to the present work, these results demonstrate that the extract of the QG has the ability to interfere in the expression of MMP-9 and MMP-14.

HIF-1alpha, one of the subunits of the HIF-1 heterodimeric nuclear transcription factor, is an important molecule involved in response to hypoxia conditions. Its activation is capable of triggering the expression of 100 genes, including angiogenesis and oxygen transport (Ferns and Heikal, 2016). Numerous studies report the effects of increased HIF-1alpha expression. Some of them cite the essential role of the lumen in processes of healing a fracture, bone development and regeneration (Drager and Harvey et al., 2015; Tomlinson and Silva, 2015; Stegen et al., 2016). In fact, the genetic overexpression of HIF-1alpha in mouse osteoblasts has been reported as a way to improve angiogenesis and bone healing (Stegen and Gastel et al., 2015), and may be pharmacologically targeted to increase regeneration (Wan et al., 2008). On the other hand, several studies pointed out the negative effects related to under-expression of the HIF pathway. These include, inflammatory processes such as rheumatoid arthritis (Park et al., 2015), periodontal tissue inflammations (Ng et al., 2011) and osteosarcoma (El Naggar et al., 2012). Therefore, depending on the disease or physiological condition, the activation or inhibition of HIF may provide the desired therapeutic effect (Sholz and Taylor, 2013). The angiogenic process is closely related to the inflammatory process and both are involved in tissue regeneration. The HIF path,

among others, is a key factor for both of the processes and should be finely regulated (Szade et al., 2015). Our results show that the QG extract can increase the expression of the HIF pathway, which possibly could benefit the tissue regeneration processes.

The relationship between the HIF pathway and MMPs is pretty narrow. MMPs actively participate in all phases of angiogenesis, starting with initial degradation of the basement membrane until the final degradation of excess vasculature (Kachgal et al., 2012); among the MMPs involved in the process, MT1-MMP has been identified as a key factor (Genís et al., 2006). Because it is closely related, MT1-MMP (or MMP-14) also appears to have a role in the bone development, remodeling (Paiva and Granjeiro, 2014), carcinogenic processes (Pahwa and Stawikowski et al., 2014) and periodontal diseases (Sapna and Gokul et al., 2014). The close relationship between molecules justifies why both are being investigated in the present work.

This is the first study investigating the effect of the QG extract on the expression of MMP-14 and HIF-alpha in cell cultures, which makes it almost impossible to compare results. It is worth mentioning that the present research did not use individual components of the extract of leaves of QG, because otherwise the effects noted could be the result of the interaction between two or more components. The information we have available so far is that the components present in the plant knowingly exhibit various therapeutic properties.

In view of the foregoing, we may suggest that, after further research, it may be possible to use QG as one of the therapeutic agent that mediates angiogenesis by increasing the expression of MMP-14 and HIF-1alpha respectively.

5. Conclusion

The QG hydroalcoholic extract was able to increase the expression of the two molecules under study, MMP-14 and HIF-1alpha, in MC3T3-E1 pre-osteoblasts and NIH/3T3 fibroblasts cell lines. Thus, the QG compounds could have potential to be used as angiogenesis modulating therapeutic agents, by increasing the expression of MMP-14 and HIF-1alpha.

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3-Discussion

3 DISCUSSION

This work evaluates the effect on the cell viability, through the MTT assay, of different concentrations of the crude hydroalcoholic extract of the leaves of *Qualea grandiflora* in fibroblasts and pre-osteoblast strains of mice. The evaluation of the effect of plant extract on the modulation of HIF-1alpha and MMP-14, in the same cell lines, was also studied. All tests were performed *in vitro* using cell culture, since this is an accepted substitute for animal models, because its application reduces the number of experiments with them, which is a relevant feature of this method (TOROPAINEN et al., 2001). Cell culture, in regard to the discovery and analysis of drugs, is an advantageous means, as it allows the evaluation of the properties of natural and chemical substances (PADUCH; WOZNIAK, 2015).

The vegetable specie *Qualea grandiflora*, considered the woody species of greater distribution in the Cerrado, presents great ecological and medicinal importance (COSTA; SANTOS, 2011). According to studies, it is used for the treatment of several diseases and as an antimicrobial agent, which demonstrates that its components must have important therapeutic properties (HIRUMA-LIMA et al., 2006; GASPI et al., 2006; AYRES et al., 2008; SANTOS et al., 2011; ASSIS, 2013). However, the scientific studies involving the specie are still scarce, especially those related to the identification of leaf-related properties, and it is necessary to develop more studies involving the compounds derived from the plant, and it's also one of the reasons which pulled us to focus on this plant species.

The results of the MTT assay showed that the hydroalcoholic extract of the leaves of QG has the capacity to decrease the cell viability only in the last periods of exposure to the highest concentration of the extract, for both cell types (Fig. 1 e 2). Other studies carried out in our research group evaluated the effects of leaves extracts of QG on the cell viability of fibroblasts and pre-osteoblasts, using the same time periods and concentrations, but with slight variations. For MTT assays, concentrations above 10 µg/mL (100 and 1000 µg/mL) showed significant decreases in viability from the initial periods for both cell lines compared to the positive control groups (untreated cells) (SANTESSO et al., 2016; TOKUHARA et al., 2016). Considering the previously obtained results, we selected 10 µg/mL, as the maximum concentration to be focused

during the course of our study as this is the threshold concentration where a noticeable decrease in viability was noticed for both cell lines.

The results concerning the viability assays can be attributed to the composition of the extract. According to the literature, the phytochemical investigation of QG leaves extract reveals the presence of flavonoids, alkaloids, saponins and tannins, as well as antioxidant properties (LIMA NETO et al., 2015; SOUSA et al., 2007). The studies lastly done by our research group were focused on HPLC-DAD analysis of the EtOH extract 70% of leaves of QG, flavonoids and compounds derived from gallic acid (TOKUHARA, 2016).

Tannins and flavonoids represent groups of molecules that are present in most plants; the tannins present an ample capacity to participate in biochemical and pharmacological activities, including antitumor properties, antioxidants, antimicrobials and inhibitors of enzymes. Its presence has been associated with the curative efficacy of various herbal medicines (HIRUMA-LIMA, 2006; PADUCH; WOJCIAK□KOSIOR; MATYSIK, 2007). Flavonoids constitute one of the largest groups of secondary compounds in plants. They have potential health benefits, which are mainly associated with their antioxidant capacity (SANTOS-BUELGA; GONZÁLES-PARAMÁS, 2016). These have activities against various diseases, including anticancer and anti-inflammatory effects (XIAO et al, 2016). Saponins are surfactant glycosides produced by plants and several pharmacological properties have been reported for these compounds, such as immunostimulatory, anti-inflammatory, hypoglycemic, hypocholesterolemic, antifungal, cytotoxic and anticancer activities (MARRELI et al., 2016; XU et al., 2016). Gallic acid is widely distributed in plants and has several biological activities, including: antioxidant, antitumor and anticarcinogenic effects (SOURANI et al., 2016).

As far as we know, this is the first research that investigates the effect of the QG extract on the cell viability of fibroblasts and pre-osteoblasts in cell cultures, using the MTT assay, which makes it impossible to compare results. In addition, the present research did not use individual components of the QG leaves extract, since the effects may be the result of the interaction between two or more components. In this context, it is worth noticeable that the biological effect of the natural compounds strongly depends not only on their concentration, but also on the type of cell they influence,

their mode of interaction with the cell membrane or time of intracellular uptake (PADUCH, WOJCIAK- KOSIOR and MATYSIK, 2007).

The results of the immunofluorescence assays show that the extract of QG increased the expression of MMP-14 and HIF-1alpha to the pre-osteoblasts, mainly in the last periods (72 and 96h) and the highest concentration of the extract (10 µg/ mL). To determine if the compounds could also increase the expression of the molecules in other cells, NIH/3T3 fibroblasts were used, same way pre-osteoblasts were employed in our current study while mimicking the parameters we lastly had. According to scientific research, osteosarcoma cells have increased labeling for both MMP-14 and HIF-1alpha, and it's one of the reasons to use them as a positive control for labelling (FUTAMURA et al., 2014; WEI et al., 2016).

HIF-1alpha is an important factor involved in response to hypoxia. Its activation is capable of triggering the expression of 100 genes, including those related to the angiogenic process and oxygen transport (FERNS; HEIKAL, 2016). Membrane matrix metalloproteinase type 1 (MT1-MMP), also known as MMP-14, is closely related to HIF-1alpha factor, actively participating in the angiogenic process (KACHGAL et al., 2012; GENÍS et al., 2006). Both are widely involved in pathological conditions such as osteosarcoma, a situation in which they have increased expression (EL NAGGAR et al., 2012; LIU et al., 2014) and physiological conditions, such as bone development, in which the expression of both is beneficial (STEGEN; VAN GASTEL; CARMELIET, 2015; HOLMBECK, 1999). Thus, depending on the disease or physiological condition, the activation or inhibition of the molecules can provide the desired therapeutic effect.

Several studies reported the important role played by different plant compounds which has ability to decrease the expression of HIF-1alpha and MT1-MMP, a fact relevant to the discovery of new drugs for the treatment of cancer (FERNANDO; RUPASINGHE; HOSKIN, 2015; LU, 2009; BYAMBARAGCHAA et al., 2013). Other studies, documented previously, reported the potential of using molecules derived from natural products as potential candidates for therapeutic neovascularization; these include resveratrol, ginseng, curcumin and sokotrasterol sulphate (ZHANG et al., 2011). Our results show that the QG extract has the ability to increase the expression of HIF-1alpha and MMP-14 in fibroblasts and pre-osteoblasts but, as mentioned previously, it is not possible to infer about the possible component of the extract which is causing these effects.

Other works cite the important effects of increased HIF-1alpha expression. These are related to processes of fracture healing, bone development and regeneration (DRAGER; HARVEY; BARRALET, 2015; TOMLINSON; SILVA, 2015; STEGEN et al., 2016). In fact, the genetic overexpression of HIF-1alpha in mouse osteoblasts has been reported as a way to improve angiogenesis and bone healing, and can be pharmacologically targeted to increase regeneration (STEGEN; VAN GASTEL; CARMELIET, 2015; WAN et al., 2008). Obviously, regeneration / remodeling processes involve, in addition to resident bone cells, other cell types, such as fibroblasts, that actively participate in the process, through the secretion of several factors (PERES; LAMANO, 2011). MT1-MMP, as it is closely related to the HIF pathway, as previously mentioned, also appears in work related to bone development and remodeling (VU; WERB, 2000; PAIVA; GRANJEIRO, 2014). The fact that HIF-1alpha and MT1-MMP are related, justifies the fact that both are being studied in the present research.

In summary, our results show that the hydroalcoholic extract of the leaves of QG has the capacity to increase the expression of MMP-14 and HIF-1alpha in two cell types. In view of the foregoing, we may suggest that, after further research, it would be possible to use the QG extract as a possible therapeutic agent that modulates angiogenesis by increasing the expression of MMP-14 and HIF-1alpha.

CONSIDERAÇÕES

O relatório encontra-se formatado de acordo com as normas da Pós-graduação da Faculdade de Odontologia de Bauru, para dissertação de mestrado.

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