UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Maria Luiza Gioster Ramos

Avaliação do extrato amazônico de pracaxi (*Pentaclethra macroloba*) puro e associado com hidróxido de cálcio como medicação intracanal

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Tese apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara para obtenção do título de Doutor em Ciências do Programa de Odontologia, na Área de Biociências aplicadas à Odontologia.

Orientador: Prof. Dr. Luis Geraldo Vaz
Coorientador: Prof. Dr. Roberto Messias Bezerra

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DADOS CURRICULARES

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“Respeite os problemas alheios, sem interferir neles, a menos que a sua cooperação seja solicitada. Não pronuncie palavras que ofendam e depreciem. Quando possível, dê sempre alguma frase de consolo e esperança a quem sofre. Não se faça estação de pessimismo ou desânimo. Esqueça o mal que receba e nunca faça a cobrança do bem que tenha podido distribuir. Não impulsione para a frente qualquer questão desagradável. O trabalho no desempenho do seu dever é o capital que lhe valoriza as orações. Lembre-se da parcela de socorro que sempre devemos aos companheiros mais necessitados que nós mesmos. Quanto possível faça algo ou algo aprenda de útil para que seu dia de hoje seja melhor que o de ontem. Nunca se esqueça de que todas as vantagens ou benefícios que desfrutemos da vida são empréstimos de Deus.”

André Luiz*

André Luiz. Nosso lar: a vida no mundo espiritual. Psicografado por Francisco Cândido Xavier. Rio de Janeiro: FEB; 1944.

Ramos MLG. Avaliação do extrato amazônico de pracaxi (*Pentaclethra macroloba*) puro e associado com hidróxido de cálcio como medicação intracanal [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

RESUMO

A *Pentaclethra macroloba*, da família Fabaceae, comumente conhecida como "pracaxi" é uma árvore nativa da Amazônia, e o seu extrato tem sido estudado quanto a suas inúmeras propriedades, tais como: anti-inflamatórias, cicatrizantes e antimicrobianas. O presente estudo tem como objetivo avaliar uma inovadora medicação intracanal contendo o extrato de pracaxi (*Pentachletra macroloba*), puro ou associado ao hidróxido de cálcio. **Manuscrito 1:** 3 grupos foram determinados: ULT (hidróxido de cálcio com metilcelulose); HCP (hidróxido de cálcio associado com extrato de *Pentaclethra macroloba*); e EPM (extrato de *Pentaclethra macroloba* puro). Para avaliar sua atividade antimicrobiana, foi realizado o teste de macrodiluição e avaliação intratubular em Microscopia Confocal de Varredura a Laser (CLSM) com cepas padrão de *Enterococcus faecalis*. Para a análise dos resultados obtidos o teste de Shapiro-Wilk foi aplicado com o objetivo de averiguar se houve distribuição normal da amostra, e dependendo do tipo de distribuição os testes ANOVA e Tukey ou Kruskal Wallis e Dunn, com nível de significância de 5%. O extrato em associação com hidróxido de cálcio apresentou menor Concentração Bactericida Mínima. No CLSM todas as medicações testadas foram efetivas contra *E. faecalis* (*P >0,05*). **Manuscrito 2:** 3 grupos foram determinados: extrato etanólico de *Pentaclethra macroloba* puro, associação de hidróxido de cálcio pa com o extrato e hidróxido de cálcio pa puro. Para avaliar a citotoxidade foi escolhida cultura de células de fibroblastos L929 e osteoblasts Saos-2, onde foi observado a viabilidade/metabolismo celular por ensaio de MTT, proliferação celular com alamar blue e para se analisar se a medicação é bioativa, os testes de fosfatase alcalina (ALP) e vermelho de alizarina (ARS) foram utilizados. Para a análise dos resultados obtidos o teste ANOVA One-way /teste de Dunnett e ANOVA Two-way/ Tukey foram utilizados. Em Osteoblastos Saos-2, concentrações menores das medicações foram menos citotóxicas no ensaio de MTT. Houve proliferação celular no dia 3 e 5. E observou-se presença de nódulos de mineralização nos 3 grupos. Em Fibroblastos L929 as menores concentrações não se mostraram citotóxicas no ensaio de MTT, e houve proliferação celular nos dias 3, 5 e 7. **Conclusões:** A nova droga intracanal à base de extrato de *P. macroloba* tem efeito antimicrobiano contra *E. Faecalis* e é pouco citotóxica às células testadas, além de serem bioativas.

Palavras chave: Óleos Vegetais. Endodontia. Hidróxido de Cálcio.

Ramos MLG. Evaluation amazonian extract of pracaxi (*Pentaclethra macroloba*) purê and associated with calcium hydroxide with intracanal medication [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

ABSTRACT

The *Pentaclethra macroloba*, from the Fabaceae family, commonly known as "pracaxi" is a tree native to the Amazon, and its extract has been studied for its numerous properties, such as: anti-inflammatory, healing and antimicrobial. The present study aims to evaluate an innovative intracanal medication containing pracaxi extract (*Pentachletra macroloba*), pure or associated with calcium hydroxide. **Manuscript 1:** 3 groups were determined: ULT (calcium hydroxide with methylcellulose); HCP (calcium hydroxide associated with *Pentaclethra macroloba* extract); and EPM (pure *Pentaclethra macroloba* extract). To evaluate its antimicrobial activity, the macrodilution test and intratubular evaluation in Confocal Laser Scanning Microscopy (CLSM) were performed for standard strains of *Enteroccus faecalis*. For the analysis of the results obtained, the Shapiro-Wilk test was applied to verify the normal distribution of the sample, and there was the type of distribution of the ANOVA and Tukey or Kruskal Wallis and Dunn tests, with a significance level of 5%. The extract in association with calcium oxide presented the lowest Minimum Bactericidal Concentration. In CLSM all tested medications were effective against faecalis (P >0.05). **Manuscript 2:** 3 groups were determined: ethanolic extract of *Pentaclethra macroloba* pure, association of calcium hydroxide pa with the extract and calcium hydroxide pure pa. To evaluate the cytotoxicity, a culture of osteoclast cells Saos 2 was chosen, where the cell viability/metabolism was observed by MTT assay, cell proliferation with alamar blue and to analyze whether the medication is bioactive, the alkaline phosphatase (ALP) and alizarin red tests (ARS) were used. For the analysis of the results, the One-way ANOVA/Dunnett's test and Two-way ANOVA/Tukey were used (P >0.05). In Osteoblasts Saos-2, lower concentrations of medications were less cytotoxic in the MTT assay. There was cell proliferation on days 3 and 5. And we observed the presence of mineralization nodules in the 3 groups. In L929 Fibroblasts the lowest concentrations were not cytotoxic in the MTT assay, and there was cell proliferation on days 3, 5 and 7. **Conclusions:** The new intracanal drug based on *P. macroloba* extract has an antimicrobial effect against *E. Faecalis* and is not very cytotoxic to the tested cells, in addition to being bioactive.

Keywords: Plant Oils. Endodontics. Calcium Hydroxide.

SUMÁRIO

1 INTRODUÇÃO

O tratamento endodôntico tem por finalidade a eliminação de micro-organismos, restos pulpares e necróticos presentes no sistema de canais radiculares (SCR), modelando-os[1] e assim favorecendo a ação da solução irrigadora[2] além de proporcionar um espaço adequado para o preenchimento com material obturador[3]. A limpeza e modelagem do SCR são fundamentais para o sucesso clínico[4].

Durante o preparo ocorre a remoção mecânica da dentina infectada e alargamento das paredes do canal o que propicia a penetração de irrigantes através do canal principal, melhorando o processo de descontaminação[5]. No entanto, microrganismos e restos de tecido necrótico ou pulpar permanecem no SCR, presentes nas superfícies do canal radicular que permaneceram intocadas, independente dos instrumentos utilizados durante o preparo[6]. Diversos estudos relataram que o preparo químico-mecânico não elimina todas as bactérias do canal radicular[7-9].

A presença de micro-organismos no SCR desencadeia a persistência ou reinfecção caso obtenham acesso aos tecidos perirradiculares após a obturação dos canais radiculares[10], e é a principal causa de fracasso no tratamento endodôntico[11]. Este infortúnio provoca uma intensa resposta inflamatória[12], que leva à destruição dos tecidos periapicais, conhecido como periodontite apical[13,14].

O *Enterococcus faecalis* é a espécie microbiana mais comumente encontrada em canais radiculares após falha do tratamento endodôntico[15]. A infecção endodôntica abriga uma microbiota polimicrobiana composta por microrganismos aeróbios, anaeróbios, gram-positivos e gram-negativos que se organizam sob a forma de biofilme[16]. Biofilmes são comunidades microbianas envoltas por matriz extracelular de polissacarídeo e proteínas na qual as células ficam aderidas[17] e apresentam grande resistência à antissépticos e antibióticos[18]. A eliminação do biofilme e a destruição dos micro-organismos do sistema de canais radiculares são objetivos importantes na desinfecção endodôntica[19].

Para efetivar a remoção de micro-organismos resistentes ao preparo químico-mecânico o uso da medicação intracanal, deve ser utilizado como forma complementar ao preparo na desinfecção do SCR[20]. Um dos principais avanços nas medicações utilizadas foi o advento da pasta antibiótica tripla, contendo metronidazol,

ciprofloxacina e minociclina, que apesar de sua eficácia, apresenta a possibilidade de descoloração dos dentes devido à presença de minociclina[21,22]. Outras alternativas foram surgindo, como o uso de medicamentos associados à combinação do hidróxido de cálcio e veículos bioativos[22-25]. Por outro lado, nenhuma medicação é capaz de eliminar todos os micro-organismos presentes no SCR.

A *Pentaclethra macroloba,* da família Fabaceae, é comumente conhecida como "árvore Gavil", "pracaxi" ou "pracachy" e é uma árvore nativa da Amazônia que vem ganhando atenção devido sua exploração eco-sustentável[26]. Suas sementes contêm aproximadamente 30% de óleo de pracaxi[27]. Este óleo é composto por ácidos graxos que se dividem em: ácido arelaúrico (0,4%), ácido mirístico (0,3%), ácido palmítico (2,9%), ácido palmioleoleico (0,1%), ácido esteárico (3,6%), ácido oleico (61,4%), ácido linoléico (16,5%), ácido linolênico (0,8%), ácido araquídico (1,2%), ácido beênico (8,4%) e ácido lignocérico (4,1%)[28]. *Pentaclethra macroloba* possui efeito anti-hemorrágicos, antiproteolíticos[29], antibacteriano contra bactérias Gram-positivas (*Staphylococcus spp. E Enterococcus spp*.) e Gram-negativas (*Pseudomonas aeruginosa, Acinetobacter spp. e Klebsiella pneumoniae*)[30] e cicatrizante[31]. Sendo assim, o seu emprego como medicação intracanal pode ser um benéfico e promissor tratamento para auxiliar no combate da infecção endodôntica, devido às suas propriedades biológicas e microbianas.

A eliminação de micro-organismos resistentes ainda hoje é um grande desafio na terapia endodôntica e o uso de óleos essenciais como o ``pracaxi`` objetivando o desenvolvimento de uma nova medicação intracanal eficaz frente aos patógenos endodônticos torna-se necessário. O projeto visa trabalhar em parceria da UNESP (Faculdade de Odontologia-FOAr e Faculdade de Ciências Farmacêuticas-FCF) com as escolas agrícolas do Amapá (Universidade Federal do Amapá-UNIFAP), Laboratório de Bioprospecção e Absorção Atômica - LAAB) através do trabalho em conjunto com o Prof. Dr. Roberto Messias Bezerra no intuito de propagar o conhecimento do potencial emprego dos extratos naturais de ``pracaxi`` extraídos de forma sustentável do bioma Amazônico e sua aplicabilidade na Odontologia, agregando valor.

2 PROPOSIÇÃO

O objetivo do presente estudo foi avaliar a proposta de uma nova formulação para uso como medicação intracanal contendo o extrato de pracaxi (*Pentachletra macroloba*) no canal radicular, por meio de:

- Avaliação de atividade antimicrobiana sobre *Enterococcus faecalis* por análise de microdiluição e microscopia confocal de varredura a laser (MCVL);
- Análise da citotoxidade, avaliando a viabilidade/metabolismo celular por ensaio de MTT e proliferação celular com Alamar blue;
- Análise da bioatividade através do teste de fosfatase alcalina (ALP) e vermelho de alizarina (ARS).

3 MANUSCRITOS

O presente trabalho resultou em dois manuscritos.

3.1 Manuscrito 1*

Antimicrobial effect of new intracanal medicament of *Pentaclethra macroloba* against *Enterococcus Faecalis*

ABSTRACT

Enterococcus faecalis is one of the main causes of failure in endodontic therapy.This aim study evaluates the antimicrobial efficacy of a new intracanal medicament based on *Pentaclethra macroloba* extract, a plant of Amazonian origin, against *Enterococcus faecalis* using macrodilution test and intratubular evaluation in Confocal Laser Scanning Microscopy (CLSM). In the macrodilution test the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) was be determined of the 3 groups: pure extract of *Pentaclethra macroloba*, extract of *Pentaclethra macroloba* in association with calcium hydroxide and Ultracall. In the intratubular evaluation, thirty-three dentin cylinders were prepared with ProTaper F5 and inoculated with *E. faecalis.* After 24 hours of contamination, the intracanal medications for the 3 groups tested were inserted for 7 days, and thus the antibacterial effect of the medications on the dentinal tubules against *E. faecalis* was be evaluation in CLSM. The data were analyzed using the Kruskal-Wallis and Dunn tests. The extract in association with calcium hydroxide showed a lower MBC. And in the intratubular test all tested medications were effective against *E. faecalis* (P >0.05). The new intracanal medicament based on *P. macroloba* extract has an antimicrobial effect against *E. Faecalis.*

Keywords: Antimicrobial effect, Herbal medicine, Intracanal medicament, Endodotics, *Pentaclethra macroloba.*

* Manuscrito escrito segundo as normas do periódico *Brazilian Dental Journal*, ao qual foi submetido.

INTRODUCTION

The chemical-mechanical procedures performed during endodontic treatment are important to obtain cleaning, shaping, debridement and, consequently, bacterial reduction for periradicular tissue repair to occur (1). But sometimes only the action of endodontic files in association with irrigants is not enough to eliminate microorganisms from the root canal system (RCS) (2). Thus, additional methods, such as the use of intracanal medication with antibacterial action, are necessary to optimize the disinfection of the RCS (3). Mainly in cases where the infection is persistent even after the end of the endodontic therapy, and there is presence of pain or constant exudation (4).

Calcium hydroxide ($Ca(OH)_2$) is one of the most used endodontic medications (5) . Due to its biological induction of mineralized tissue deposits (6) and antimicrobial properties against a wide range of bacteria present in root canal (7), which makes it the medication of choice for use as an intracanal dressing (8). Calen (SS White Artigos Dentários, Rio de Janeiro, RJ, Brazil) and UltraCal XS (Ultradent Products, South Jordan, UT, USA) are medication with $Ca(OH)_2$ used in endodontic routine, as they have antimicrobial activity, induce mineralization, has activity against bacterial endotoxin and to be biocompatible (9). However, they are not able to completely eliminate the microorganisms present in the RCS (9,10).

Facultative anaerobic bacteria such as *Enterococcus faecalis* (Gram-positive bacteria) *Actinomyces naeslundii, L. rhamnosus, L. casei, Streptococcus sanguinis, S. mitis* and *Candida albicans* are microorganisms presents in fail endodontic treatment (11) . E. faecalis is observed in asymptomatic and persistent therapies, being a bacteria resistant to endodontic treatments, whtat becoming challenge for sucess the therapy endodontic (12).

With the increase in bacterial resistance due to the indiscriminate use of antibiotics, note an increase in the development of other classes of antimicrobials for infection control (13). Products extracted from roots, barks, seeds and seeds have been an important source of research to formulate new medications with antimicrobial action (2,14,15). *Pentaclethra macroloba* is a native Amazonian tree commonly known as "pracaxi" that stands out due to its eco-sustainable exploitation (16). It have antibacterial action against Gram-positive (Staphylococcus spp. and Enterococcus spp.) and Gram-negative (Pseudomonas aeruginosa, Acinetobacter spp. and Klebsiella pneumoniae) (17) and healing action (18) .

Due to this need to search for to new herbal medicines products, the aim of the present study was to analyze a new intracanal medicament of natural extract of *Pentaclethra macroloba*, pure and associated with the $Ca(OH)_2$ and compare it with UltraCal XS (ULT) to verify its antibacterial activity against *Enterococcus faecalis*. The null hypothesis was that there would be no difference in antimicrobial activity between the groups.

MATERIALS AND METHODOS

Extraction

Pentachletra macroloba peels were cleaned and dehydrated in an oven at 37°C for 3 days. After dehydration, was performed grinding in a mechanical mill taking the sample to powder dimensions. The bark powder was covered with ethyl alcohol PA ten percent above the sample volume, stored for four days, with homogenization movements being carried out to enhance the extraction process, four times a day. The powder and solvent (PA ethyl alcohol) passed through a filtering system with filter paper. Where the solvent was eliminated and recovered by steam distillation, with a vacuum pump system to promote the drag of the solvent, for optimizing the distillation was made water bath in the lowest temperature, thus obtaining the extract at 100% (19).

Determination of the minimum inhibitory concentration and the minimum bactericidal concentration

Before the antibacterial activity was tested by macrodilution test to determine the concentration needed to kill the reference bacterial strains of *Enterococcus faecalis* (ATCC 29212), which were obtained from Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. *E. faecalis* was subcultured in brain heart infusion (BHI) broth (Difco) as a facultative microorganism.

The intracanal medication tested were:

PME (*Pentaclethra macroloba* extract): ethyl extract from the bark of the plant, 25 mL extract with 75 mL propylene glycol

PCH (*Pentamacroloba macroloba* extract associated with Calcium hidroxid): The calcium hydroxide PA (Synth, São, SP, BR) 17.5 mg associated with the *Pentaclethra macroloba* extract 12.5 mg in 50 mL propylene glycol; and

ULT (Calcium hidroxid with methylcellulose): The medication prepared with calcium hydroxide and methylcellulose (Ultracal XS, Salt Lake, USA) 25 mL ULT with 75 mL propylene glycol;

For the macrodilution test (20), screw-capped tubes containing 3 mL of BHI broth were used, and precise volumes of antimicrobials were added to the broths and repeaters in triplicate. The inoculum was obtained after 24 hours of incubation in BHI broth at 37°C under anaerobic conditions. Tube turbidity was read in a spectrophotometer (Ultrospec 1000; Amersham Pharmacia Biotech, Cambridge, UK) at 540 nm. Then, cultures were diluted to MacFarland 3 standard and diluted to 9x108 UFC/mL and distributed in volumes of 3 mL to each tube containing diluted broth and intracanal medication.

Turbidity readings were taken on the spectrophotometer before and after anaerobic incubation to detect tubes with bacterial growth. The tube readings determine the minimum inhibitory concentration (MIC) for the tested medication, the time required for growth bacteria was 24 hours in BHI broth. Negative and positive bacterial growth controls were used.

After reading the final absorbances, 25 µL of the solution from each tube was transferred to reinforced clostridial medium (RCM) blood agar plates measuring 15 x 60 millimeters. These plates were anaerobically incubated at 37°C for 48 hours to establish the minimum bactericidal concentration. MBC was considered the lowest concentration of the medication that was able to inhibit bacterial growth on the plates. The testes were made in triplicate.

Intratubular viability assessment

Thirty-three human teeth extracted after approval by the Ethics Committee for Research on Human Beings (CAAE: 39393620.7.0000.5416), were selected from the tooth bank and divided into the three intracanal medication groups to be tested (n=10), with one teeth of control negative in each group, which was processed like the others but only with contamination, without treatment. Contamination was performed with the microorganism *E. faecalis* and eight images were obtained for intratubular evaluation of each tooth (four images of each half).

The extracted teeth were stored for 48 hours in a 1% sodium hypochlorite (NaOCl) solution for initial decontamination, then stored in distilled water. The crowns of the

teeth were cut and patterned on a dentin cylinder using Isomet (Isome 1 standardized, IL, USA) with a diamond blade at 250 rpm, under activated irrigation.

Root canals were prepared using ProTaper F5 rotary files (Dentsply, Tulsa Especialidades Odontológicas) in an endodontic appliance (X-Smart Plus Maillefer Dentsply) according to the manufacturer's instructions. During preparation Irrigation with 2.5% NaOCl was carried, and after irrigation out for 5 min with etilenodiaminotetraacético acid 17% (EDTA) (Chemical and Pharmaceutical Biodynamics, Ibiporã, PR, Brazil), then the channels were washed with deionized water and dried for 24 hours before beings autoclaved at 121°C.

E. faecalis (ATCC 29212) was reactivated in infusion broth (BHI, Brain Disc, Kansas City, MO, USA) and kept at 37°C for 24 hours. the cultured bacteria were transferred to a new BHI infusion broth and cultured for 24 hours for exponential growth. This culture was ajusted in McFarland Standard Nº 3 (9x 108 UFC/mL) using an spectrophotometer SF325NM (Bel Photonics do Brazil Ltda, Osasco, SP, Brazil).
For the intratubular contamination test (21) 800 µL of BHI was inserted into an Axygen Scientific microtube (Axygen Scientific, Union City, CA, USA) containing the dentin cylinder. A 15-minute ultrasonic bath was performed to allow maximum penetration of the culture medium into the dentine tubes. The contaminated was carried out in five days, with centrifugation on alternate days. The inoculum (800 µL) was inserted into the microtubes with the dentin cylinders and centrifuged (Eppendorf 5417R centrifuge, Eppendorf, Hamburg, Germany). The inoculum ewas renewed at each centrifugation cycle. On day fifths, the samples were taken from microtubes and observed CLSM using LIVE/DEAD® BacLightTM fluorescent identification (Molecular Probes, Eugene, OR, USA) to verify bacterial penetration and viability.

The specimens were longitudinally sectioned with a diamond disk in Isomet. Half of the dentinal tubes were stained with 30 µL of LIVE/DEAD® bacterial viability measurement kit (Eugene Molecular Probes, OR) for 20 minutes, thus allowing the identification of viable bacteria. Samples were examined with a Leica Confocal Microscope in TCS-SPE (Leica, Baden-Württemberg, Germany) using a 40X amplied lens. Eight sequential images were taken from each dentinal tube, four from the cervical third and four from the medial third. As images were taken in fragments using the Leica Application Suite Advanced Fluorescence Software (LAS AF, Leica, Mannheim, Baden-Würberg, Germany).

These data were statistically evaluated using the Kruskal-Wallis and Dunn tests. A p-value <0.05 was considered statistically significant.

RESULTS

Minimum inhibitory concentration and the minimum bactericidal concentration

All tested intracanal medications were able to inhibit and eliminate all strains of *E. faecallis*. However, the pure extract of pracaxi required a higher concentration than the other substances tested (Table 1). The vehicle used, propylene, did not influence the antimicrobial effect. The concentration necessary to eliminate *E. faecallis* was 25% for pure *P. macroloba* extract, and when associated, 3.12% for *P. macroloba* extract and 4.37% for calcium hydroxide. Ultracall was considered as a control group, as it is already a drug with proven antimicrobial action, and got 6.25% of MBC.

Table 1 - MIC and MBC in percentage (%) of the extract of all endodontic medications obtained by the macrodilution method in brain-heart infusion broth in *Enterococcus faecalis*

EPM		**ULT**		**HCP**	
CIM	CBM	CIM	CBM	CIM	CBM
12,5	25,0	1,56	6,25	-	3,12 + 4,37

EPM, *Pentaclethra macroloba* extract pure; *ULT*, Calcium hydroxide with metilcelulose; *HCP*, Calcium hydroxide with *Pentaclethra macroloba* extract.

Intratubular viability assessment

The CLSM images obtained showed that there was bacterial penetration in all groups and throughout the root canal. A higher concentration of dead than viable bacteria was observed in all groups except the control, which had only intratubular contamination (Figure 1).

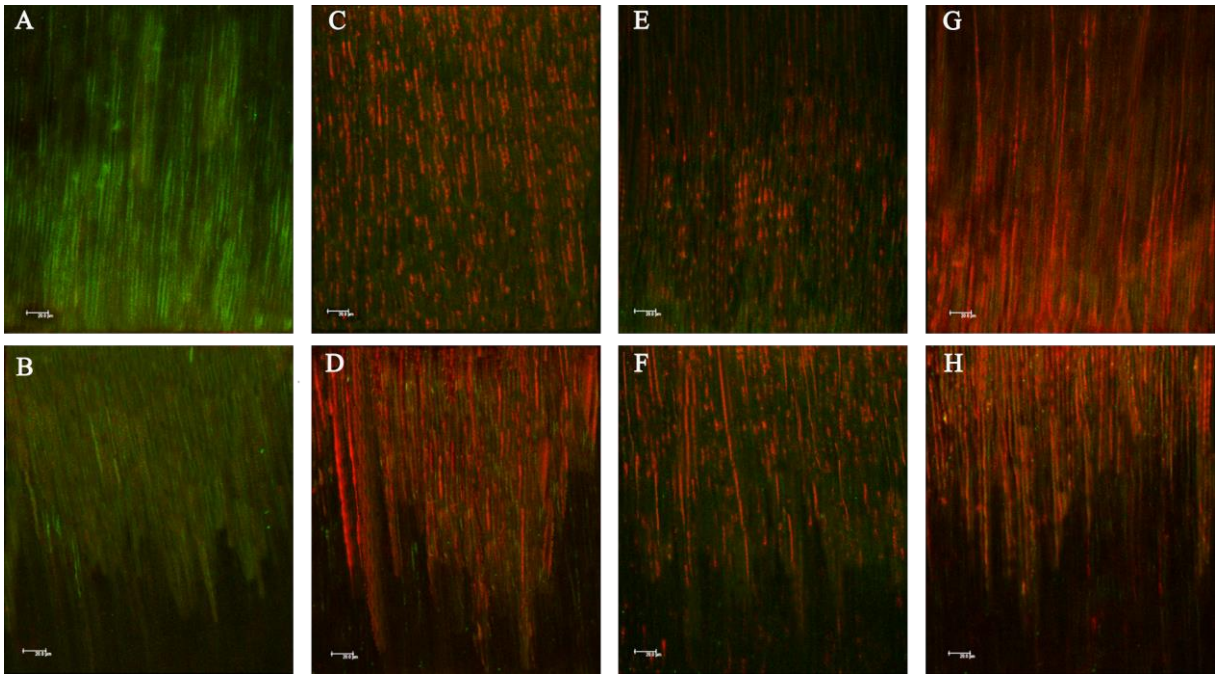

Figure 1 - Confocal laser scanning microscopy (CLSM) images following contamination with E. faecalis. Longitudinal views of the cervical and middle third of root canal. Live bacteria can be observed in green and dead bacteria an be observed in red. **A,B** Control grup in the middle and cervical third respectively. **C,D** Ultracal group in the middle and cervical third respectively. **E,F** Pracaxi group middle and cervical third respectively. **G,H** Pracaxi with calcium hydroxide in the middle and cervical third respectively.

There was a statistically significant difference between the control group and the others in relation to total, cervical, middle, superficial and deep *(p>0.05)* (Table 2). The pure extract when compared to the one associated with $Ca(OH)_2$ showed no significant difference in any analysis *(p<0.05)*. And when compared with ULT, only statistics differences were observed in deep *(p>0.05).*

Table 2 – Median percentage (95% confidence interval) of viable bacteria in the dentinal tubules after treatment in each group.

Group	Total	Cervical	Middle	Superficial	Deep
Control	51.64 (32.02-76.64)^{Aa}	45.39 (25.86-70.03)^{Aa}	55.67 (28.26-85.22)^{Aa}	58.23 (29.61-78.40)^{Aa}	45.39 (23.82-79.00)^{Aa}
Pracaxi 25%	32.40 (8.49-62.14)^{Ba}	41.19 (6.26-66.84)^{Aa}	25.53 (10.48-48.86)^{Ba}	23.38 (5.08-45.14)^{Ba}	38.11 (13.72-79.59)^{BCa}
Ultracal 25%	14.04(2.31-47.53)^{Ba}	12.83 (1.34-49.44)^{Aa}	23.36 (2.24-46.33)^{Ba}	15.32 (1.80-47.57)^{Ba}	13.03 (2.49-47.02)^{Ca}
Pracaxi 6,25% + $Ca(OH)_2$ 8,75%	29.56 (10.71-63.97)^{Ba}	30.85 (6.82 – 61.20)^{Aa}	28.95 (11.55-64.15)^{Ba}	36.44 (10.38-63.97)^{ABa}	23.73 (10.71-61.49)^{BCa}

Test Kruskal-Wallis and Dunn ($p < 0,05$). Different capital letters superscript in a column represent significant differences between groups; different superscript lowercase letters in a line represent significant differences within groups

DISCUSSION

The present study was carried out using strains of *E. faecalis,* as this is the Gram-positive facultative bacterium most resistant to endodontic treatment, being frequently present when endodontic treatment fails (3). In addition to having a capacity for deep intratubular penetration, biofilm formation in the periapical region, which contributes to its resistance to chemical-mechanical root preparation protocols (4), and also the ability to proliferate after an incubation period (22). The bacterial reference strain *E. faecalis* 29212 from the American Type A Culture Collection (ATCC) used in this study has been tested in several studies because of its virulence factors that pose a challenge to current antimicrobial agents (21,23,24).

There are few reports in the literature on the use of *Pentaclethra macroloba*, but its antimicrobial effect was highlighted in a study by Leal et al. in 2011 (17).Due to this search for a medication with antimicrobial effect and that is extracted from the Brazilian biome, a macrodilution test was performed for the use of the extract against the main microorganism resistant to endodontic treatment, *E. faecalis*. New intracanal medication was formulated where the extract de pracaxi was evaluated pure and in associated with $Ca(OH)_2$, which has bactericidal action comproved and was compared with the Ultracall paste. Where it was proved that the extract has bactericidal action on *E. faecalis*, and thus its MBC (25%) when pure and MBC (3.12%) when associated with $Ca(OH)_2$ was determined. The PCH needed a lower concentration of the extract to have the bactericidal effect, this is justified by the addition of $Ca(OH)_2$ the extract in has too effect bacteciridal, confirming what the literature has already about its isolated action (25) .

The intratubular analysis was performed in order to simulate the most serious situation that occurs in endodontic infections in the oral environment. To approximate how the procedures that occur during an endodontic treatment, teeth extracted from humans were standardized at 12 mm, forming cylinders of dentin. They were prepared with ProTaper F3 rotary instrumentation and irrigated with 2.5% NaOCl and final irrigation with 17% EDTA. To promote an effective bacterial contamination by E. faecalis inside the dentinal tubules, the centrifugation protocol suggested by Ma et al. in 2011 (26) was used. This ensured better intratubular proliferation for the study than that provided by previous methods (21).

A specimen with contamination and without treatment was used as a control in each group, demonstrating that the dentinal tubules were contaminated both in the

total and in the cervical, middle, superficial and deep. And after statistical testing, a significant difference was demonstrated between the control groups and the 3 medications under study. The Ultracall group served as a parameter, as it is an intracanal medication with proven effects against the microorganism under study. Having presented a statistically significant difference regarding the control group in all analyzes ($p>0.05$), which proves its effect and is in agreement with the literature (27) .

Several Amazonian medicinal herbs are completely unknown scientifically regarding their medicinal properties. But the native population, with traditional knowledge passed from generation to generation, knows how to use different herbs according to their symptoms (28). The use of medicinal herbs has gained strength in research for new medications, as they are natural and do not cause microbial resistance (17*). P. macroloba* is a plant native to riverside regions of the Amazon Rainforest, which has been little investigated in the scientific literature, but has been widely used by the native population as an antimicrobial, anti-inflammatory and healing agent. Studies have shown its effect as healing (18,29), anti-hemorrhagic (30) and antimicrobial against *E. Faecallis* (17). This is in agreement with the results found in the study, which confirm the antimicrobial action in both the macrodilution and intratubular assays against *E. Faecallis*.

The association with $Ca(OH)_2$ was proposed because $Ca(OH)_2$ has been widely used in endodontic therapy as an intracanal medication. It is considered a root channel dressing that acts as a physical barrier, preventing RCS reinfection and thus interrupting the arrival of nutrients for the remaining bacteria (31). Its antimicrobial effect is associated with its high pH (25), but no study has proven that it alone is capable of eliminating microorganisms from the RCS. Its association with camphorated paramonochlorophenol is necessary, which is a substance toxic to living tissues (32). Search your association with natural plants is very important, to minimize toxic effects of other products already used. A study that made an association of chitosan and propolis, a herbal medicine, showed that the natural extract when associated obtained better antimicrobial results, which is in agreement with the finding that *P. macroloba* extract associated with $Ca(OH)_2$ required a lower concentration of the extract to have a action against *E. faecallis* (33).

In conclusion, within the limitations of our in vitro experiments, the null hypothesis tested was partially confirmed, as the new medication, pracaxi 25% and pracaxi 6.25% with $Ca(OH)_2$ 8.75%, presented similar results to the Ultracall group.

Being statistically equal in total, medium, cervical, superficial and deep. This proves the antimicrobial effect of the tested medication and indicates the *P. macroloba* extract as a promising intracanal medication. More studies should be carried out to evaluate other characteristics of the extract in terms of in vitro and in vivo tissue repair, a property necessary for an ideal intracanal medication.

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3.2 Manuscrito 2*

Cytocompatibility and bioactive potential of the Amazonian *Pentaclethra macroloba* extract, pure and in association with calcium hydroxide

RESUMO

Objectives This study aimed to investigate *Pentaclethra macroloba* extract, pure and associated with calcium hydroxide, regarding its biological properties as a new endodontic medication.

Methodology Osteoblast SaOS-2 and Fibroblast L929 were used for evaluation of cell viability/metabolism by MTT (48h), cell proliferation analysis with Alamar blue (1, 3, 5 and 7 days), mineralization assay with Alizarin Red (7 days) and Alkaline Phosphatase activity (7 days).

Results In Osteoblasts SaOS-2, lower concentrations of the medications were less cytotoxic in the MTT assay. There was cell proliferation on days 3 and 5. Mineralization nodules were observed in the 3 groups. In L929 Fibroblast cells the lowest concentrations were not cytotoxic in the MTT assay, and there was cell proliferation on days 3, 5 and 7.

Conclusion *Pentaclethra macroloba* extract was not cytotoxic, it induced cell proliferation, and was able to form mineralization nodules.

Clinical relevance New medication showed cytocompatibility and bioactive potential, essential characteristics for endodontic medications.

Keywords *Pentaclethra macroloba* . Calcium hydroxide . Intracanal medication . Cytotoxicity.

* Manuscrito escrito segundo normas do periódico *Clinical Oral Investigations* para o qual foi submetido.

Introdução

Calcium hydroxide ($Ca(OH)_2$) is one of the most used components as intracanal medication for endodontic treatment [1,2] due to its ability to inactivate bacterial toxins [2] and contribute to bone and tissue repair [3]. However, bacteria such as *Enterococcus faecalis*, which are present in 80% of endodontic lesions, are resistant to $Ca(OH)_2$ [4,5]. In order to increase its antimicrobial action and repairing effect, the association of $Ca(OH)_2$ with several substances has been studied [6,7].

The use of extracts of medicinal plants has spread in the literature as an alternative in the formulation of new natural medicines to replace synthetic ones [8,9]. *Pentaclethra macroloba,* also known as "Pracaxi", is a native plant of riverside regions in the Amazon [10], which has been studied for presenting antibacterial action against several strains of bacteria, including Enterococcus spp. [11] and also for presenting potential healing activity [12].

Considering that intracanal medications must remain within the root canal system (RCS), as well as in direct contact with apical and periapical tissues in order to exert an antimicrobial and tissue repairing effect, it is essential that biocompatible, bioactive and antimicrobial materials are used. Thus, the objective of this study was to evaluate in vitro the cytocompatibility and the bioactive potential of the Pentaclethra macroloba extract, pure and in association with $Ca(OH)_2$, for the formulation of a new intracanal medication.

Materials and methods

Cell culture and endodontic medication preparation

Osteoblastic cell derived from human osteosarcoma (Saos-2) American Type Culture Colection (ATCC Htb-85) lineage were cultured in Dulbecco's Medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (SFB) (Gibco, Grand Island, NY, USA) and 100 µg/mL penicillin G-streptomycin. In order to stimulate osteoblastic differentiation, the medium was also supplemented with 50 µg/mL of ascorbic acid and 10 mM of β-glycerophosphate. Cell culture was maintained at 37°C with 5% CO_2.

The fibroblast cell line L929 of subcutaneous connective tissue mouse (ATCC® CCL1 TM, Manassas, VI, USA) were cultured and maintained in DMEM supplemented with 10% FBS and 1% penicillin, streptomycin and glutamine (100 UT/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine) (Gibco, Grand Island , NY, USA) in a humidified atmosphere at 5% CO_2 at 37 ºC.

For the accomplishment of the tests, it was used pure ethanolic extract of *Pentaclethra macroloba*, pure extract of *Pentaclethra macroloba* associated with $Ca(OH)_2$ pa (Synth, São Paulo, SP, BR) and pure $Ca(OH)_2$ pa. 100 ml of each material was placed in a 1.5 ml microtube (Eppendorf, Hamburg, Germany), and 1.2 ml of DMEM was added. The microtubes were housed in an oven at 37°C for 24 hours. The supernatant was transferred to new microtubes and then centrifuged for 10 min at 20,800 g (5430, Eppendorf AG, Hamburg, Germany) to decant the material particles. The supernatant was transferred to a new tube and considered as the “stock/extract solution”. It was then diluted and contacted with Saos-2 and fibroblast cells.

Assessment of cell viability/metabolism by MTT

Saos-2 and L929 cells were plated in 96-well plates (5x104 cells/well and 1x104 cells/well respectively). Concentrations of 0.0035 of Pracaxi, 0.544 of $Ca(OH)_2$ and Pracaxi associated with $Ca(OH)_2$ (0.0012 + 0.008 respectively) and their positive (10 µM Camptothecin) and negative (culture medium) controls were prepared 24h before. 200 µL of each concentration was added to each well and the plate was incubated for another 7 days. The medium was changing every 48 hours. After the period, cytotoxic effects were evaluated using the MTT (methyltetrazolium) assay.

Each well of the experimental and control groups received a 100 µL solution, 90 µL of culture medium and 10 µL of MTT solution (Sigma, MO, USA), prepared by dissolving 5 mg of the MTT salt in 1 ml of sterile PBS. After incubation of the cells for a period of 4 h at 37 ºC, the culture medium with the MTT solution was aspirated, to solubilize the crystals, and 100 µL of isopropanol solution acidified in 0.04 N HCL was added. The staining produced was quantified, and cell viability was evaluated by spectrophotometry (570 nm) using a spectrophotometer (Synergy H1 Multi-Mode Reader-BioTek, USA).

Cell Proliferation assay

Saos-2 and L929 cells were plated in 48-well plates (1x104 cells/well and 5x103 cells/well respectively). After 24 h, the culture medium was replaced by non-cytotoxic concentrations of 0.00075 of Pracaxi, 0.068 of $Ca(OH)_2$ and Pracaxi associated with $Ca(OH)_2$ (0.0003 + 0.002 respectively) for Saos-2 cells and 0.012 of Pracaxi, and 0.136 of $Ca(OH)_2$ and Pracaxi with associated $Ca(OH)_2$ (0.003 + 0.002 respectively) for L929 cells. The plate was incubated at 37 °C and 5% CO2 and cell proliferation were analyzed at 1, 3, 5 and 7 days using the Alamar Blue assay. In each period, 10% Alamar Blue® solution, diluted in culture medium, was added to the wells and the plates were incubated for 4 h, and an aliquot of 100 µL from each well was transferred to a new 96-well plate for spectrophotometer reading (570nm and 600nm).

Alizarin red

Saos-2 were plated in 96-well plates (1 x 104 cells/well) and after 24 h the culture medium was replaced by non-cytotoxic concentrations of NE-HT prepared in osteogenic medium, but the control group received only culture medium. After 7 days, the adhered cells were washed twice with PBS and fixed with 100 µL of 70% ethanol for 30 min. The wells were washed and stained with 150 µL of alizarin red solution (40 mM, pH 4.2 - Sigma-Aldrich) for 20 minutes at room temperature and under gentle agitation (VDRL Shaker, Biomixer, Ribeirão Preto, SP, Brazil). Dye not incorporated into cells was aspirated and wells were washed twice with distilled water. The mineralization nodules were dissolved with 200 µL of 10% cetylpyridine (Sigma-Aldrich) for 15 minutes under agitation. Then, the absorbance was measured in a microplate reader (Synergy H1 Multi-Mode Reader-BioTek, USA) at 562 nm.

Alkaline phosphatase activity

Saos-2 were plated in 96-well plates (1 x 104 cells/well) and after 7 days of osteogenic induction, alkaline phosphatase (ALP) activity was evaluated using the Alkaline Phosphatase kit (ABCAM, ab83369) following the manufacturer's instructions. Samples and pNPP (p-nitrophenyl phosphate) substrate were mixed and reacted for 60 minutes, and the reaction was stopped with stop solution. The plate was read at a

wavelength of 405 nm and the ALP activity calculated based on the standard curve plot.

Results

Assessment of cell viability/metabolism by MTT

Figure 1 shows the results of cytotoxicity in Saos-2 osteoblasts of Pracaxi, $Ca(OH)_2$ and their association (Pracaxi + $Ca(OH)_2$) after 48 hours of treatment. It can be seen that the two highest concentrations of Pracaxi (0.0030 and 0.0015%), $Ca(OH)_2$ (0.545 and 0.272%) and Pracaxi associated with $Ca(OH)_2$ (0.0024+0.0017 and 0.0012+0.008%) showed toxicity in cells compared to the negative control group (culture medium) (*p<0.01*), showing a decrease in cell viability between 13.83 and 60.9%. At lower concentrations, the three groups evaluated did not present a statistically significant decrease when compared to the negative control group (*p>0.01*).

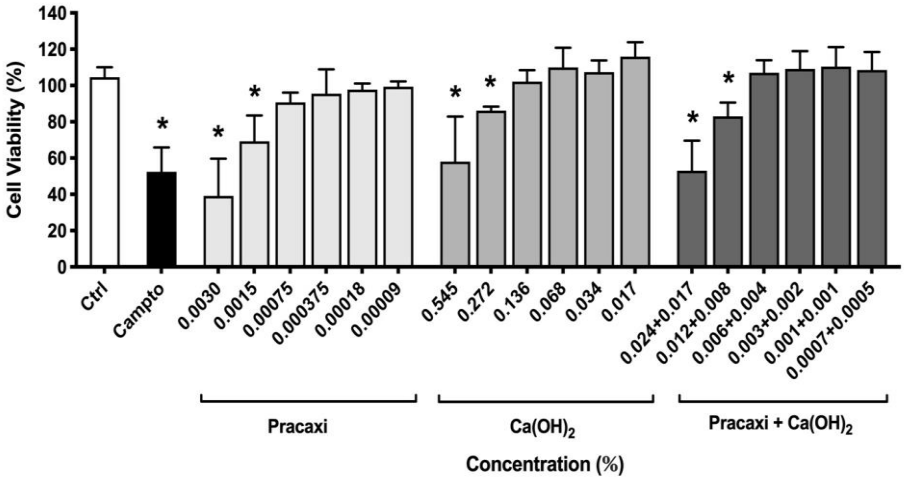

Fig.1 Viability percentage of Saos-2 osteoblast-like cells after 48 hours of exposure with concentrations of Pracaxi, $Ca(OH)_2$and Pracaxi associated with $Ca(OH)_2$.

*Statistically significant difference compared to the control group (One-way ANOVA/Dunnett's test, p<0.01).

In L929 fibroblasts there was presence of cellular toxicity in the two highest concentrations (0.097 and 0.048%) of the Pracaxi group, in the highest concentration

(0.545) of the $Ca(OH)_2$ group, and in the three highest concentrations (4.8+3.4, 2.4+1.7 and 1.2 +0.8%) of the Pracaxi associated with $Ca(OH)_2$ group, compared to the control group (culture medium) ($p<0.01$). At lower concentrations, the three evaluated groups did not present a statistically significant decrease when compared to the negative control group ($p>0.01$) (Figure 2).

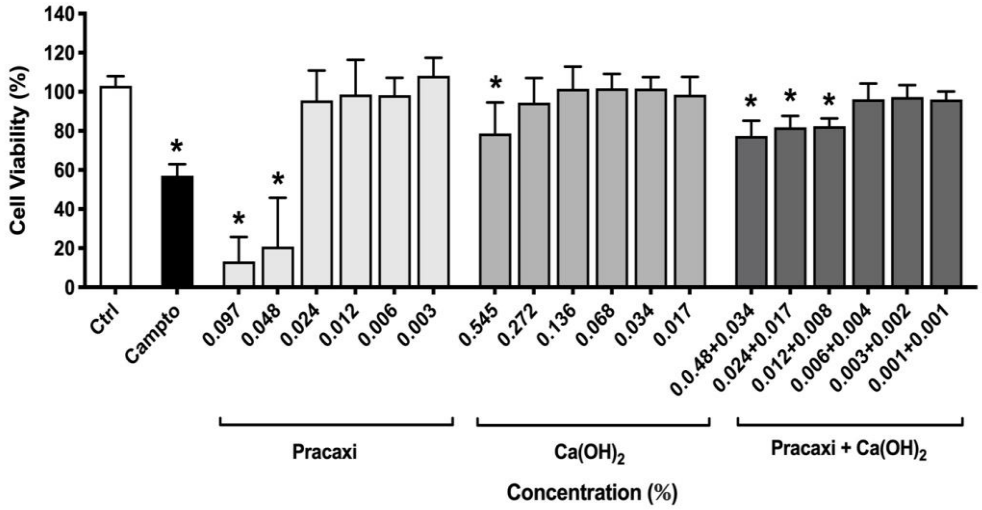

Fig. 2 Viability percentage of L929 fibroblast cells after 48 hours of exposure with concentrations of Pracaxi, $Ca(OH)_2$, and Pracaxi associated with $Ca(OH)_2$.

*Significant decrease in comparison with the control group (culture medium) (One-way ANOVA/Dunnett's test, $p<0.01$).

Cell proliferation assay

Non-cytotoxic concentrations of Pracaxi, $Ca(OH)_2$ and their association were tested on human osteoblasts at 1, 3, 5 and 7 days, and cell proliferation was determined. Figure 3 shows an increase in cell proliferation in all groups at 1, 3 and 5 days, showing statistically significant differences (*$p<0.05$*). After 7 days, the cells did not show an increase in cell proliferation when compared to the 5-day period (*$p>0.05$*). On day 1 there was no increase in cell proliferation in relation to the control group (*$p>0.05$*), in any of the concentrations evaluated in the different test groups. On days 3 and 5, we can see that all groups evaluated showed a significant increase in cell proliferation

(*p<0.05*) being similar amoung groups (*p>0.05*) and only the highest concentration of the Pracaxi associated with $Ca(OH)_2$ group showed no statistically significant difference when compared to the control group (*p>0.05*). On day 7, the cell behavior was similar to that shown on days 3 and 5; however, the concentration of 0.034% $Ca(OH)_2$ and 0.0007%+0.0005% Pracaxi associated with $Ca(OH)_2$ showed a greater tendency in cell proliferation with a statistically significant difference when compared to the control group (*p<0.05*).

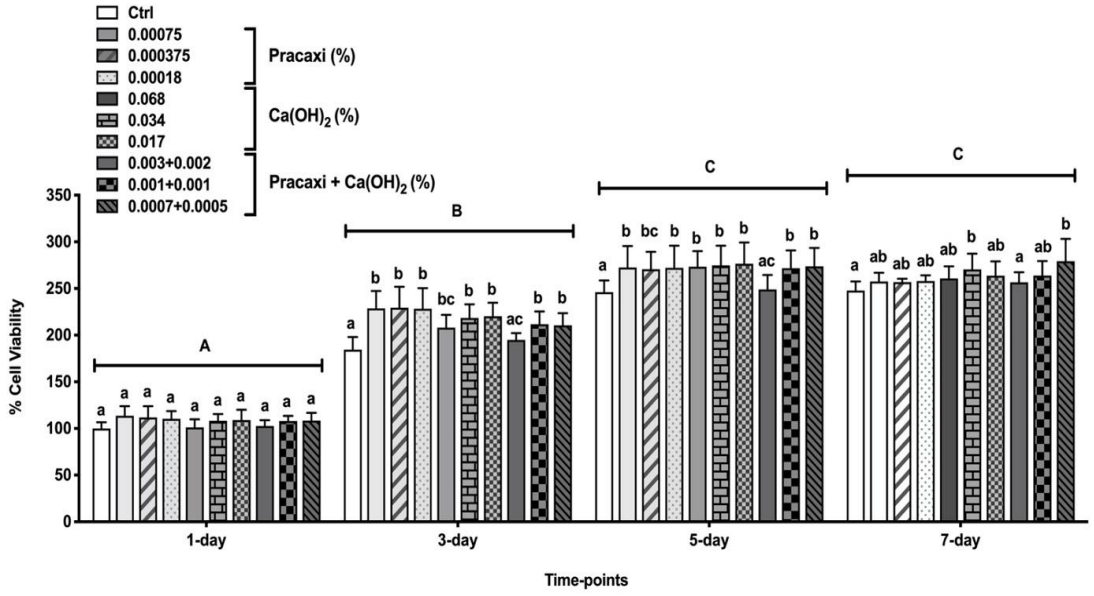

Fig. 3 Cell proliferation assay. Effect of Pracaxi, $Ca(OH)_2$ and Pracaxi associated with $Ca(OH)_2$ on Saos-2 osteoblast-like cells. Results are expressed as the means ± SD of triplicate assays from three independent experiments. Uppercase letters allow for comparison among time points for each group; lowercase letters allow for comparison among groups at each time point.

*Different letter indicates statistically significant differences (Two-way ANOVA/Tukey's test, p<0.05).

On the other hand, in L929 fibroblast cells, on day 1, there was no cell proliferation in any of the groups tested, at any of the different concentrations compared to the control group (*p>0.05*). On day 3, all tested groups were different from the control group, showing that there was cell proliferation (*p<0.05*). On day 5 there was greater cell proliferation compared to day 1 and 3 (*p<0.05*), all concentrations of

the 3 groups obtained different results from the control group (*p>0.05*). On day 7, there was still a difference in cell proliferation compared to the other days (*p<0.05*), only with the concentration (0.006%) of the Pracaxi group similar to the control group (p>0.05) (Figure 4).

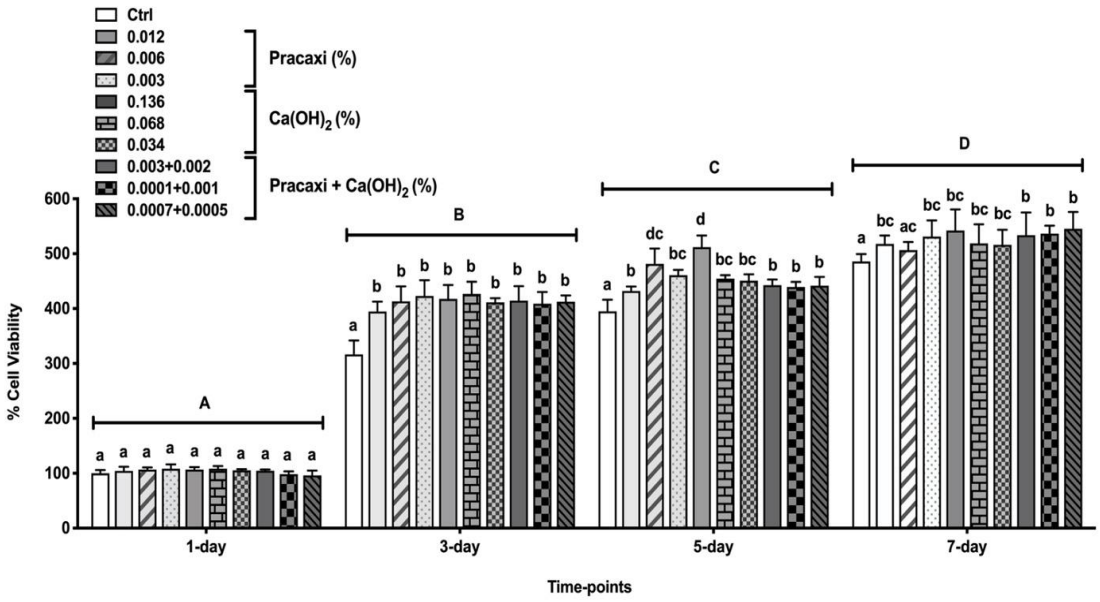

Fig. 4 Cell proliferation assay. Effect of Pracaxi, $Ca(OH)_2$ and Pracaxi associated with $Ca(OH)_2$ on L929 fibroblast cells. Results are expressed as the means ± SD of triplicate assays from three independent experiments. Uppercase letters allow for comparison among time points for each group; lowercase letters allow for comparison among groups at each time point.

*Different letter indicates statistically significant differences (Two-way ANOVA/Tukey's test, *p<0.05*).

Alizarin red

Figure 5 represents the results of the quantification of mineralization nodules from human osteoblasts treated with different concentrations of Pracaxi, $Ca(OH)_2$ and their association after 7 days of treatment. It can be observed that in all three groups tested there was an increase in the presence of mineralization deposits when compared to the groups treated with normal and osteogenic medium (*p<0.01*). In the Pracaxi group, the two high concentrations tested showed a greater tendency in the formation of mineralization nodules (*p<0.01*). A similar behavior can be observed in the Pracaxi

associated with $Ca(OH)_2$ group at a concentration of 0.003%+0.002%, showing a greater tendency in the formation of mineralization nodules (*p<0.01*).

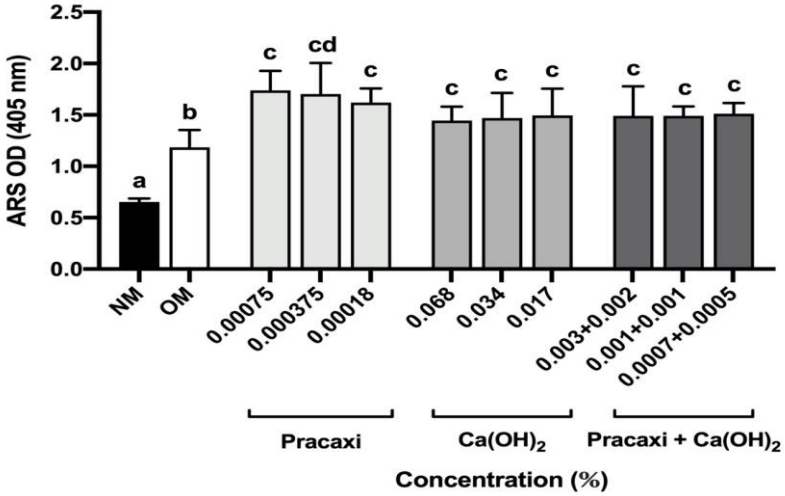

Fig. 5 Quantification of mineral deposition by detecting absorbance of ARS extracts from Saos-2 osteoblast-like cells cultured for 7 days with Pracaxi, $Ca(OH)_2$ and Pracaxi associated with $Ca(OH)_2$ treatments. Results are expressed as the means ± SD of triplicate assays for three independent experiments.

*Different letter indicates statistically significant diferences (One-way ANOVA/Dunnett's test, *p<0.01*).

Alkaline Phosphatase activity

Higher alkaline phosphatase activity was observed in Saos-2 cells in the concentration of 0.001% of Pracaxi associated with 0.001% of $Ca(OH)_2$, followed by the concentration of 0.00018% of Pracaxi (*p>0.05*) (Figure 6).

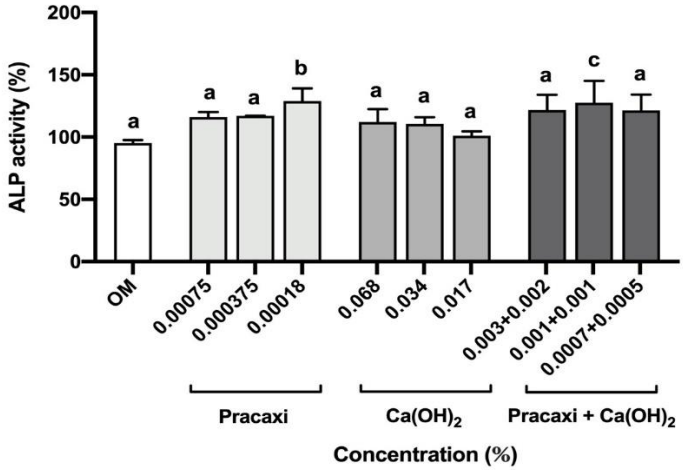

Fig. 6 Alkaline phosphatase (ALP) activity in Saos-2 osteoblast-like cells after 7 days of treatment with Pracaxi, $Ca(OH)_2$ and Pracaxi associated with $Ca(OH)_2$ treatments. Results are expressed as the means ± SD of triplicate assays from three independent experiments.

*Different letter indicates statistically significant differences (One-way ANOVA/Dunnett's test, *p<0.05*).

Discussion

The outlined of this study was design with the aim of providing a comprehensive understanding of the biological aspects of Pentaclethra macroloba extract, pure and its association with $Ca(OH)_2$. It is a medication with proven action recommended by the European Society of Endodontics [15]. Medications were evaluated within a period of 1 week, as this is the minimum time of application of intracanal medications recommended during endodontic regeneration procedures according to the American Association of Endodontists [16].

Saos-2 osteoblast cells were chosen because they are mature osteoblasts and are widely used in the literature [13,14] that can use markers that identify early osteogenic differentiation, such as ALP and COL-1, and can be analyzed at shorter intervals of culture time even in non-osteogenic media [14]. In the present study, different periods were used; 1, 3, 5 and 7 days. Cell lines were evaluated by supplementing the osteogenic medium with ascorbic acid and β-glycerophosphate to stimulate osteoblastic differentiation.

L929 fibroblast cells were chosen to have a greater validation of the results found. Fibroblasts play a key role in the angiogenesis of the dental pulp by producing and releasing growth factors and participating in tissue regeneration [17]. The results in fibroblast cells were similar to those found in osteoblast cells, where small concentrations of the pure extract and extract associated with $Ca(OH)_2$ were not toxic to cells. Cell proliferation occurred on days 1, 3, 5 and 7, demonstrating a pattern of proliferation more long-lasting than in osteoblasts, which showed stagnation on days 5 and 7. This confirms the cytocompatibility of the pure extract and the extract associated with $Ca(OH)_2$ for endodontic medication.

Despite the fact that there is no statistical difference between Pracaxi and Pracaxi associated with $Ca(OH)_2$ in toxicity and cell proliferation, both extracts showed a tendency that points to be less toxic when evaluated in osteoblast cells and stimulates greater cell proliferation in fibroblast cells. In a previous study that associated $Ca(OH)_2$ with another phytotherapic named Propolis, it was observed that the association was better than the pure extract in terms of cytotoxicity [18]. This result was also found in the association of $Ca(OH)_2$ with Myracrodruon urundeuva Allemão (aroeira), which was also cytocompatible [9].

The results showed that the use of *Pentaclethra macroloba* extract, either pure or associated with calcium hydroxide, in small concentrations did not cause cytotoxic effects for Saos-2 and L929 cells after 48h of contact in relation to the control group that was only culture medium evaluated by MTT assay. $Ca(OH)_2$ was also not cytotoxic at low concentrations. Higher concentrations of the 3 intracanal medications studied were able to cause a slight decrease in the metabolism of Saos-2 and L929 cells.

As expected, in view of the healing action of the extract previously reported [12], the Alamar Blue assay showed that there was cell proliferation with the use of Pentaclethra macroloba extract, pure and in association with $Ca(OH)_2$ on days 3, 5 and 7 in the two types of cells tested. In Saos-2 cells showed stagnation in this proliferation from day 5 to 7 only. *Pentaclethra macroloba* extract has not yet been studied for its effects on Saos-2 cells, but it is known that its oil has a healing action in people with diabetic wounds [12] and this action is justified by the presence of fatty acids in its composition [10] which are known to improve wound closure and improve healing [19].

ARS staining identifies when calcium deposits are present in cell cultures [20]. Mineralization nodules were observed in all groups, with similarity among the groups,

with of the concentration 0.000375% of the Pracaxi group having a further formation of mineralization nodules in relation to the other concentrations of the same group and the other groups, indicating a potential repairing effect of this extract when it is pure. The ability to form mineralization nodules by $Ca(OH)_2$ was found in this study and it is consistent with other studies [21].

The evaluation of Alkaline Phosphatase activity, an enzyme that is expressed throughout the process of early maturation on osteoblasts, makes it possible to determine the potential that medication has to induce formation of mineralized tissue [22]. The highest ALP activity was observed at day 7 in Saos-2 cells after Pracaxi associated with $Ca(OH)_2$ exposure in one of its concentrations, followed by Pracaxi group. Studies that evaluated ALP in natural products such as curcumin and icariin also found to have greater ALP activity [23,24].

The combination of the extract with $Ca(OH)_2$ was superior to the pure extract only in the cell proliferation assay in L929 fibroblast cells and only in one of the concentrations tested. This indicates that the pure extract may be a new endodontic medication to be used in the future in clinical practices. As it was not cytotoxic to cells, it stimulated cell proliferation and formation of mineralization nodules. In view of the findings of the present study, in vivo studies in animals should be performed to confirm and validate the results found for this future endodontic medication.

Conclusion

Pentaclethra macroloba extract was bioactive potential and cytocompatibility, was able to induce cell proliferation in Saos-2 osteoblast and L929 fibroblasts cells. It was also able to form mineralization nodules in Saos-2 cells, which are essential characteristics of endodontic medications.

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4 CONCLUSÃO

O extrato de *Pentaclethra macroloba* mostrou ser um medicamento endodôntico promissor, tanto puro como em associação com o hidróxido de cálcio. Tendo características primordiais para uma medicação intracanal, por apresentar efeito antimicrobiano para cepas de *Enterococcus faecalis*, comuns em casos de insucesso de tratamento endodôntico. Além de uma potencial bioatividade, ajudando na regeneração tecidual e por não ser citotóxico aos tecidos quando em menores concentrações.

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APÊNDICE A - METODOLOGIA EXPANDIDA

1 Extração

1. 1 Coleta das amostras

A casca de *Pentaclethra macroloba* foram recolhidas diretamente da planta no bioma Amazônico, no estado do Amapá, em áreas de várzea, cerrado, altas florestas e de transição do platô guianense. E foram armazenadas na Universidade Federal do Amapá e depositadas nos herbários do Instituto de Pesquisas Científicas e Tecnológicas do Amapá (IEPA).

1. 2 Produção do extrato

As cascas de *Pentachletra macroloba* foram limpas e desidratadas em estufa à 37°C por 3 dias. Após a desidratação foi realizada a moagem em moinho mecânico levando a amostra a dimensões de pó. O pó da casca foi recoberto com álcool etílico PA dez por cento acima do volume da amostra, armazenado por quatro dias, sendo efetuado movimentos de homogeneização para potencializar o processo de extração, quatro vezes ao dia. O conjunto pó e solvente (álcool etílico PA) passou por um sistema de filtragem com papel de filtro. Onde foi eliminado e recuperado o solvente por destilação à rota vapor, procurando equilibrar o processo otimizando a destilação com a temperatura do banho de água na menor temperatura, com sistema da bomba de vácuo para promover o araste do solvente, assim se obtendo o extrato à 100%.

2 Atividade Antimicrobiana

2. 1 Grupos experimentais

ULT (hidróxido de cálcio com metilcelulose): A medicação previamente preparada com hidróxido de cálcio e metilcelulose (Ultracal XS; Ultradent, Salt Lake, UT, USA);

HCP (hidróxido de cálcio associado com extrato de *Pentaclethra macroloba*): A medicação composta por hidróxido de cálcio pa (Synth, São Paulo, SP, BR) associado ao extrato de pracaxi

EPM (extrato *Pentaclethra macroloba*): extrato etílico puro da casca da planta

2. 2 Determinação concentração inibitória mínima e concentração bactericida mínima

Cepas bacterianas de referência de *Enterococcus faecalis* (ATCC 29212) foram obtidas da Fundação Oswaldo Cruz, Rio de Janeiro, Brasil. *E. faecalis* foi subcultivado em caldo de infusão cérebro coração (BHI) (Difco) como microrganismo facultativo.

Para o teste de macrodiluição, tubos com tampa rosqueada contendo 3 mL de caldo BHI foram usados, e volumes precisos de antimicrobianos foram adicionados aos caldos e repetidos em triplicatas. O inóculo foi obtido após 24h incubado em caldo BHI à 37°C sob condições anaeróbicas. A turbidez do tubo foi lida em espectrofotômetro (Ultrospec 1000; Amersham Pharmacia Biotech, Cambridge, Reino Unido) a 540 nm. Em seguida, as culturas foram diluídas para o padrão de 3 MacFarland e diluído para a concentração de $9x10^8$ UFC/mL e distribuídos em volumes de 3 mL para cada tubo contendo caldo diluído e drogas.

Foi comparada as leituras de turbidez no espectrofotômetro antes e depois da incubação anaeróbica para detectar os tubos com crescimento bacteriano. As leituras do tubo determinaram a concentração inibitória mínima (CIM) para cada medicamento testado, o tempo necessário para promover o crescimento foi de 24 horas em caldo BHI. Controles negativos e positivos de crescimento bacteriano foram utilizados.

Após a leitura das absorbâncias finais, 25 µL da solução de cada tubo foi transferido para placas de ágar RCM de sangue medindo 15 x 60 milímetros. Estas placas foram anaerobicamente incubadas à 37°C por 48 horas para estabelecer a concentração bactericida mínima (CBM). O CBM foi considerado a concentração mais baixa do medicamento que foi capaz de inibir o crescimento bacteriano nas placas. Os testes foram feitos em triplicatas.

2. 3 Intratubular

2. 3. 1 Preparo das amostras

Trinta e três dentes humanos extraídos CAAE: 39393620.7.0000.5416, foram divididos nos três grupos das medicações testadas (n=10), sendo 1 controle negativo em cada grupo, que foi processado como os demais mas sem contaminação. A contaminação foi feita com o microrganismo *Enterococcus faecalis*. Oito imagens CLSM foram obtidas para cada dente (quatro imagens de cada metade).

Os dentes extraídos foram armazenados por 48 horas em solução de hipoclorito de sódio 1% para descontaminação inicial, e posteriormente imersos em

água destilada. As coroas dos dentes foram removidas e as amostras foram padronizadas em cilindros de dentina usando uma serra Isomet (Isomet 1000, Buehler Ltd, Lake Bluff, IL, EUA) com um disco diamantado à 250 rpm, sob irrigação.

Os canais radiculares foram preparados usando limas rotatórias ProTaper F5 (Dentsply, Tulsa Especialidades Odontológicas) em aparelho endodôntico (X-Smart Plus Maillefer Dentsply) de acordo com instruções do fabricante. Irrigação com Hipoclorito 2,5 % foi realizada durante a instrumentação e depois 5 min com agitação manual de ácido etilenodiaminotetraacético 17% (EDTA) foi realizada (Biodinâmica Química e Farmacêutica, Ibiporã, PR, Brasil), então os canais foram lavados com água deionizada. Eles foram secos por 24 h antes de serem autoclavados à 121°C.

2. 3. 2 Teste de contaminação intratubular

E. faecalis (ATCC 29212) foi reativado no caldo de infusão cérebro coração (BHI, Disco, Kansas City, MO, EUA) e mantido à 37°C por 24 h. A bactéria em cultura foi transferida para um novo caldo BHI e incubada por mais 24 h para crescimento exponencial. Essa cultura foi ajustada para Padrão McFarland Nº 3 (9x 10^8 UFC/mL) usando um espectrofotômetro SF325NM (Bel Photonics do Brasil Ltda, Osasco, SP, Brasil).

Para o teste de contaminação intratubular 800 µL de BHI esterilizado foi inserido em cada microtubo (Axygen Scientific, Union City, CA, EUA) contendo o espécime de dentina. Foi realizada banho ultrassônico de 15 min para permitir a penetração máxima do meio de cultura nos túbulos dentinários. A contaminação foi realizada em cinco dias, com centrifugação em dias alternados. O inóculo (800 µL) foi inserido nos microtubos com as amostras e centrifugadas (Eppendorf 5417R centrífuga, Eppendorf, Hamburgo, Alemanha). O inóculo foi renovado a cada ciclo de centrifugação. No quinto dia, as amostras foram retiradas do microtubos e observadas com Microscopia Confocal de Varredura a Laser (CLSM) utilizando coloração fluorescente LIVE/DEAD® BacLightTM (Molecular Probes, Eugene, OR, EUA) para verificar a penetração e viabilidade bacteriana.

Os corpos de prova foram seccionados longitudinalmente com disco diamantado em Isomet. As metades dos tubos dentinários foram corados com 30 µL de coloração LIVE/DEAD® kit de viabilidade bacteriana (Invitrogen Molecular Probes, Eugene, OR) por 20 minutos, permitindo assim a fácil identificação de bactérias viáveis. As amostras foram examinadas com um Microscópio confocal Leica invertido

TCS-SPE (Leica Microsystems GmbH, Mannheim, Baden-Württemberg, Alemanha) usando uma lente de óleo de ampliação de 40X. Oito imagens sequenciais foram tiradas de cada tubo dentinário; quatro do terço cervical e quatro do terço medial. foram adquiridos e fragmentados usando o Software de Fluorescência Avançado do Conjunto de Aplicativos Leica (LAS AF, Leica, Mannheim, Baden-Württemberg, Alemanha).

Esses dados foram avaliados estatisticamente com os testes de Kruskal-Wallis e Dunn. Foi considerado um valor de $p < 0,05$ estatisticamente significativo.

3 Citotoxidade e Bioatividade Celular

3. 1 Cultura celular

Os fibroblastos de camundongos da linhagem L929 foram cultivados em garrafas, com DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich St. Louis, MO, USA), suplementado com soro fetal bovino (SFB) a 10% (Invitrocell, Campinas, SP, Brasil), penicilina (100 U/m) e estreptomicina (100 mg/mL). As células serão mantidas em estufa a 37ºC, com atmosfera umidificada contendo 5% de CO_2 e 95% de ar. Para a realização dos experimentos, os fibroblastos serão destacados das garrafas com tripsina, o sobrenadante será centrifugado a 1000 RPM por 10 minutos a 5oC, o pellet será re-suspendido em DMEM com SFB 10% e as células contadas em hemocitômetro (Reichert, Bufallo, NY).

Osteoblastos Humanos da linhagem Saos-2, foram cultivadas em meio Dulbecco’s Medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) suplementado com 10% de soro fetal bovino (Gibco, Grand Island, NY, EUA) e com 100 µg/mL de penicilina G-estreptomicina. Para que houvesse estímulo da diferenciação osteoblástica o meio foi suplementado também com 50 µg/mL de ácido ascórbico e 10 mM de β-glicerofosfato. A cultura celular foi mantida à 37 °C com 5% de CO_2.

3. 2 Preparo das medicações

Pracaxi: extrato da casca da *Pentaclethra macroloba*;

Pracaxi com $CaOH_2$: extrato da casca da *Pentaclethra macroloba* com hidróxido de cálcio pa (Synth, São Paulo, SP, BR); e

$CaOH_2$: Hidróxido de cálcio pa (Synth, São Paulo, SP, BR).

Para a realização dos ensaios foi utilizado extrato etanólico de *Pentaclethra macroloba* puro, associação de hidróxido de cálcio pa (Synth, São Paulo, SP, BR) com o extrato de *Pentaclethra macroloba* e hidróxido de cálcio pa puro. 100 mL de cada material foi colocado em um microtubo de 1,5 mL (Eppendorf, Hamburgo, Alemanha), e 1,2 mL de DMEM foi adicionado, os microtubos foram alojados em um forno a 37°C por 24 horas. O sobrenadante foi transferido para novos microtubos e então centrifugados por 10 min a 20.800 g (5430, Eppendorf AG, Hamburgo, Alemanha) para decantar as partículas do material. O sobrenadante foi transferido para novo tubo e foi considerado como a “solução estoque/extrato”. Foi então diluído e colocado em contato com células.

3. 3 Avaliação da viabilidade/metabolismo celular por MTT

Saos-2 e L929 foram cultivados em placas de 96 poços ($5x10^4$ células/well e $1x10^4$ células/well respectivamente). Concentrações NE-HT 8200 µM - 0,25 µM e os seus controles positivos (Camptotecina 10 µM) e negativo (meio de cultura) foram preparados 24h antes. Foi então adicionado 200 µL de cada concentração teste em cada poço e a placa incubada por mais 7 dias, realizando-se a troca de meio de 48h em 48h. Após o período, os efeitos citotóxicos foram avaliados utilizando o ensaio de MTT (metiltetrazolium).

Cada poço dos grupos experimentais e controle receberam uma solução de 100 µL, sendo 90 µL de meio de cultura e 10 µL de solução de MTT (Sigma, MO, EUA), preparada por meio da dissolução de 5 mg do sal de MTT em 1 mL de PBS estéril. Após a incubação das células pelo período de 4 h, a 37 ºC, o meio de cultura com a solução de MTT foi aspirado, para solubilizar os cristais foi adicionado 100 µL de solução de isopropanol acidificado em HCL 0.04 N. A coloração produzida foi quantificada e a viabilidade celular foi avaliada por espectrofotometria (570 nm) utilizando espectrofotômetro (Synergy H1 Multi-Mode Reader-BioTek, EUA).

Foram usados os testes One-way ANOVA e teste de Dunnett com nível de significânica de 1%.

3. 4 Alamar Blue

Saos-2 e L929 foram cultivados em placas de 48 well ($1x10^4$ células/well e $5x10^3$ células/well respectivamente). Após 24 h o meio de cultura foi substituído por concentrações não citotóxicas de NE-HT. A placa foi incubada a 37 ºC e 5% de CO2

e a proliferação celular foi analisada nos períodos de 1, 3, 5 e 7 dias pelo teste de Alamar Blue. Em cada período foi adicionado aos poços solução Alamar Blue® 10%, diluída em meio de cultura e as placas foram incubadas por 4 h, e uma alíquota de 100 µL de cada poço foi transferida para uma nova placa de 96 poços para leitura espectrofotômetro (570 nm e 600 nm).

Foram utilizados os testes ANOVA two-way e Tukey com nível de significância de 5%.

3. 5 Vermelho de alizarina

Saos-2 foram cultivadas em placas de 96 poços (1 x 10^4 células/well) e após 24 h o meio de cultura foi substituído por concentrações não citotóxicas de NE-HT preparadas em meio osteogênico e o grupo controle recebeu apenas meio de cultura. Após 7 dias, as células aderidas foram lavadas duas vezes com PBS e fixadas com 100 µL de etanol 70% por 30 min. Os poços foram lavados e corados com 150 µL da solução de vermelho de alizarina (40 mM, pH 4,2 - Sigma-Aldrich) por 20 minutos em temperatura ambiente e sob leve agitação (VDRL Shaker, Biomixer, Ribeirão Preto, SP, Brasil). O corante não incorporado às células foi aspirado e os poços lavados duas vezes com água destilada. Os nódulos de mineralização foram dissolvidos com 200 µL de cetilpiridineo 10 % (Sigma-Aldrich) 15 minutos sob agitação. Então, a absorbância foi medida numa leitora de microplacas (Synergy H1 Multi-Mode Reader-BioTek, EUA) a 562 nm.

Foram utilizados os testes ANOVA one-way e Teste de Dunnett com nível de significância de 1%.

3. 6 Atividade de fosfatase alcalina

Saos-2 foram cultivadas em placas de 96 poços (1 x 10^4 células/well) e após 7 de indução osteogênica, a atividade de fosfatase alcalina (ALP) foi avaliada usando o kit Alkaline Phosphatase (ABCAM, ab83369) seguindo as instruções do fabricante. As amostras e o substrato de pNPP (p-nitrofenil fosfato) serão misturados e reagirão por 60 minutos, e a reação será interrompida com solução stop. A placa será então lida no comprimento de onda de 405 nm e a atividade de ALP calculada com base no gráfico da curva padrão.

Foram utilizados os testes ANOVA one-way e Teste de Dunnett com nível de significância de 5%.

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