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Maria Luiza GIOSTER-RAMOS[a]
Patricia Milagros MAQUERA-HUACHO[b]
Natalia da Ponte LEGUIZAMON[c]
Denise Palomari SPOLIDORIO[b]
Roberto MESSIAS[d]
Milton Carlos KUGA[e]
Luis Geraldo VAZ[a]

[a] Universidade Estadual Paulista – Unesp, Araraquara School of Dentistry, Department of Dental Materials, Araraquara, SP, Brazil.

[b] Universidade Estadual Paulista – Unesp, Araraquara School of Dentistry, Department of Physiology and Pathology, SP, Brazil.

[c] Universidade Estadual Paulista – Unesp, Araraquara School of Dentistry, Department of Periodontics, Araraquara, SP, Brazil.

[d] Universidade Federal do Amapá - Unifap, Laboratory of Atomic Absorption and Bioprospection, Macapá, AP, Brazil.

[e] Universidade Estadual Paulista – Unesp, Araraquara School of Dentistry, Department of Dentistry, Araraquara, SP, Brazil.

Corresponding Author:
Maria Luiza Gioster-Ramos
E-mail: luiza.gioster@unesp.br

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Cytocompatibility and bioactive potential of *Pentaclethra macroloba* extract and $Ca(OH)_2$ as intracanal medication

Abstract: Alternative therapies using plant-derived extracts are currently being studied due to their beneficial properties. This study investigated the Amazonian extract of *Pentaclethra macroloba*, pure and in combination with calcium hydroxide, regarding its biological properties in endodontics. Osteoblast Saos-2 and Fibroblast L929 cell lines were used for evaluation of cell viability/metabolism by MTT (48h), cell proliferation with Alamar blue (1, 3, 5 and 7 days), mineralization with Alizarin Red (7 days) and alkaline phosphatase activity (7 days). In 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 cells, the lowest concentrations were not cytotoxic in the MTT assay, and there was cell proliferation on days 3, 5, and 7. *Pentaclethra macroloba* extract was not cytotoxic, it induced cell proliferation, and was able to form mineralization nodules, essential characteristics for endodontic medications.

Descriptors: Oils, Volatile. Phytotherapy.

Introduction

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

Teeth with an open apex that need canal treatment may require pulp revascularization using an endodontic medication with antimicrobial and restorative action.[7] The aim of this procedure is to preserve the remaining pulp tissue and thus continuous root development with apical closure.[8]

The use of medicinal plant extracts has become widespread in the literature as an alternative in the formulation of new natural medicines to replace synthetic ones.[9-11] *Pentaclethra macroloba*, also known as "Pracaxi", is a native plant from riverine regions in the Amazon[12] that has been studied for its antibacterial action against several strains of bacteria, including *Enterococcus spp.*[13,14] and for its potential bone healing activity.[15]

As intracanal medications must remain within the root canal system (RCS) and in direct contact with apical and periapical tissues in order to

exert their antimicrobial and tissue-repairing effects, 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.

Methods

Ethical approval

All study procedures involving human subjects were in accordance with the ethical standards of the institution, the national research committee, and the 1964 Helsinki Declaration.

Plant extract

The bark of the *Pentachletra macroloba* tree was cleaned and dehydrated in an oven at 37°C for 3 days. After dehydration, the bark was ground into powder in a mechanical mill. The powder then was covered with PA-grade ethyl alcohol at a volume 10% greater than that of the sample and stored for four days, with homogenization movements four times a day to enhance the extraction process. The powder and solvent (PA ethyl alcohol) mixture was filtered through filter paper, after which the solvent was removed and recovered by steam distillation. A vacuum pump system was used to facilitate solvent removal, and a water bath at the lowest possible temperature was applied to optimize distillation, thus obtaining the extract at 100%.

Cell culture and endodontic medication preparation

The osteoblastic cell derived from human osteosarcoma (Saos-2) lineage obtained from American Type Culture Colection (ATCC Htb-85) was cultured in Dulbecco's Medium (DMEM) (Sigma Chemical Co., St. Louis, USA) supplemented with 10% fetal bovine serum (SFB) (Gibco, Grand Island, 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 from mouse subcutaneous connective tissue (ATCC® CCL1 ™, Manassas, USA) was 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, USA) in a humidified atmosphere at 5% CO2 at 37 ºC.

For the tests, three formulations were used: pure ethanolic extract of *Pentaclethra macroloba*, pure extract of *Pentaclethra macroloba* combined with $Ca(OH)_2$ PA (Synth, São Paulo, BR), and pure $Ca(OH)_2$ PA. A total of 5 mL of each solution was prepared at concentrations of 0.194% for Pracaxi, 1.09% for $Ca(OH)_2$, and 0.096 + 0.068% for Pracaxi associated with $Ca(OH)_2$. The solutions were stored at 37°C for 24 hours. Next, the supernatant was transferred to new microtubes and 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 designated as the "stock/extract solution". This solution was subsequently diluted and applied to Saos-2 and fibroblast cells.

Assessment of cell viability and metabolism by MTT

Saos-2 and L929 cells were plated in 96-well plates ($5x10^4$ cells/well and $1x10^4$ cells/well, respectively) and incubated for 24 h. Thereafter, the culture medium of the confluent cell monolayer was replaced by concentrations of 0.0030% – 0.00009% (v/v) of Pracaxi, 0.545% – 0.017% (v/v) of $Ca(OH)_2$, and 0.0030 + 0.00106% – 0.00009 + 0.00003% (v/v) of Pracaxi associated with $Ca(OH)_2$ for Saos-2 cells. In a second experiment, concentrations of 0.097% – 0.003% (v/v) of Pracaxi, 0.545% – 0.017% (v/v) of $Ca(OH)_2$, and 0.048 + 0.034% – 0.001 + 0.001% (v/v) of Pracaxi associated with $Ca(OH)_2$ were tested on L929 cells. For both experiments, positive (10 µM Camptothecin) and negative (culture medium) controls were prepared previously. 200 µL of each concentration test was added to each well and the plate was incubated for another 48 h at 37°C in a humidified atmosphere containing 5% of CO_2. 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, St. Louis, USA), prepared by dissolving 5 mg of the MTT salt in 1 mL of sterile PBS. After incubation of the cells for 4 h at 37ºC, 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, Wilmington, USA).

Cell proliferation assay

Saos-2 and L929 cells were plated in 48-well plates ($1x10^4$ cells/well and $5x10^3$ cells/well, respectively). After 24 h, the culture medium was replaced by non-cytotoxic concentrations of Pracaxi, $Ca(OH)_2$, and Pracaxi associated with $Ca(OH)_2$. The plate was incubated at 37°C and 5% CO_2 and cell proliferation was 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. An aliquot of 100 µL from each well was transferred to a new 96-well plate for spectrophotometer reading (570 and 600 nm).

Alizarin red staining (ARS)

Saos-2 cells were plated in 96-well plates (1 x 10^4 cells/well) and after 24 h the culture medium was replaced by non-cytotoxic concentrations of each test group prepared in osteogenic medium. The control group was cells treated with normal 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, Brazil). Dye that was 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, Wilmington, USA) at 562 nm.

Alkaline phosphatase activity (ALP)

Saos-2 cells were plated in 96-well plates (1 x 10^4 cells/well), and after 7 days of osteogenic induction, alkaline phosphatase 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. Absorbance was measured at a wavelength of 405 nm, and the ALP activity was calculated based on the standard curve.

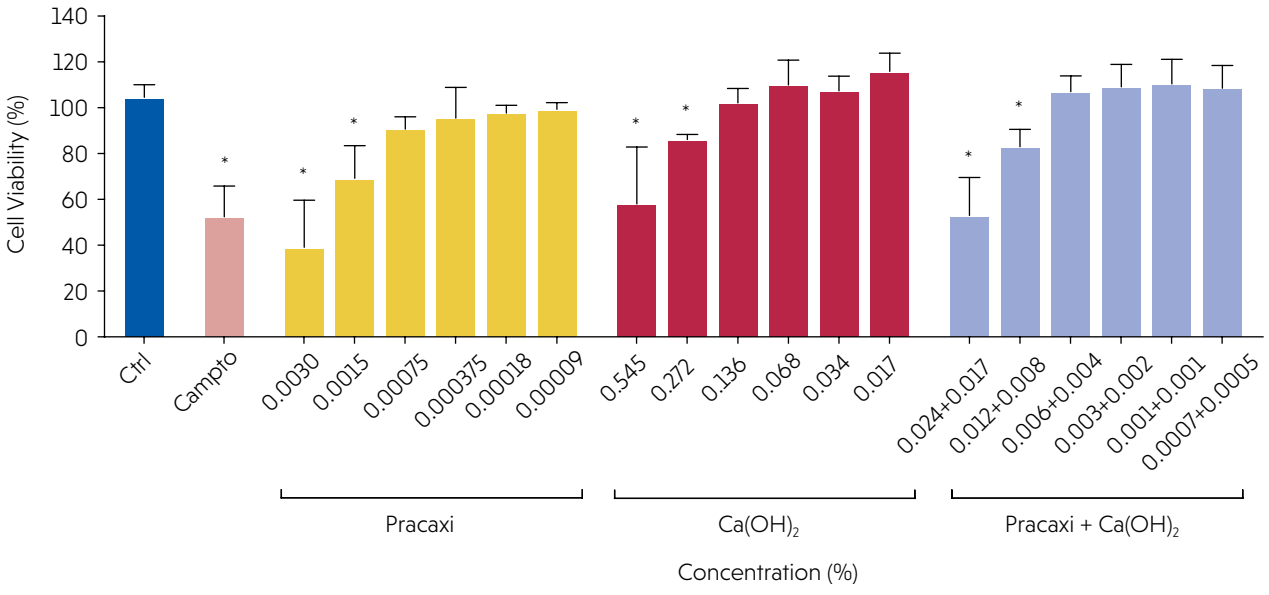
Statistical analysis

All assays were performed in triplicate in three independent experiments (n=9), and the means ± standard deviations (SD) were calculated. The significance level was set at 95% ($p < 0.05$). A one-way ANOVA followed by Dunnett's multiple comparison test and two-way ANOVA with Tukey's post-hoc test were used to analysis the data.

Results

Cell viability

Figure 1 shows the results of the cytotoxicity of Pracaxi, $Ca(OH)_2$, and Pracaxi + $Ca(OH)_2$ in Saos-2 osteoblasts after 48 hours of treatment. The two highest concentrations of Pracaxi (0.0030 and 0.0015%), $Ca(OH)_2$ (0.545 and 0.272%), and Pracaxi + $Ca(OH)_2$ (0.024+0.017 and 0.012+0.08%) showed cytotoxicity compared to the negative control group (culture medium) ($p < 0.01$). These concentrations showed a decrease in cell viability of 64.9 and 34.8% in Pracaxi groups, 46 and 17.9% in $Ca(OH)_2$ groups, and 51 and 21.2% in Pracaxi associated with $Ca(OH)_2$ when compared to the control group. At lower concentrations, the three groups evaluated did not present a statistically significant decrease compared



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

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

to the negative control group ($p > 0.01$). The percentage of cell viability of these non-cytotoxic concentrations of each group showed values between 90.6 and 110.4 %.

In L929 fibroblasts, cellular toxicity was observed 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 (0.048 + 0.034, 0.024 + 0.017 and 0.012 + 0.008%) of the Pracaxi + $Ca(OH)_2$ group compared to the control group (culture medium) ($p < 0.01$) (Figure 2). With these concentrations, a cell viability of 89.8 and 82.2% was found in Pracaxi groups, of 24.3% in $Ca(OH)_2$ group, and 25.7, 21.2 and 20.7% to Pracaxi + $Ca(OH)_2$ when compared to the control group. At lower concentrations, cell viability in the three evaluated groups was significantly lower when compared to the control group ($p > 0.01$), with values between 94.4 and 108.1%.

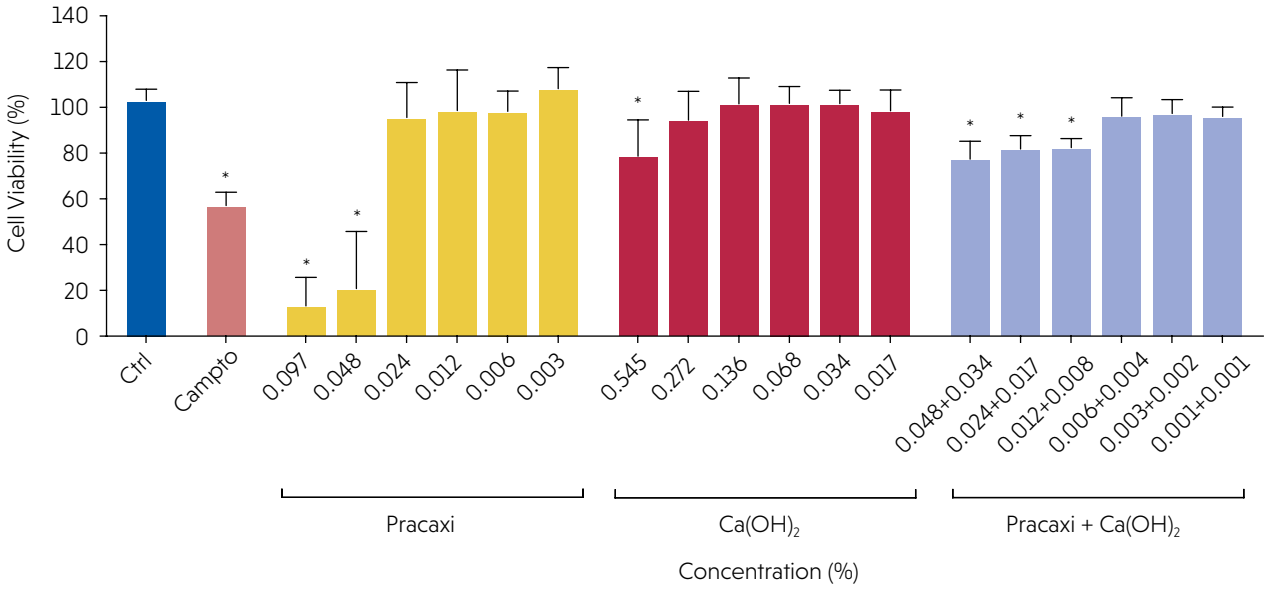
Cell proliferation assay

Cell proliferation of human osteoblasts treated with non-cytotoxic concentrations of Pracaxi, $Ca(OH)_2$, and Pracaxi + $Ca(OH)_2$ was tested at 1, 3, 5, and 7 days. Figure 3 shows a significant increase in cell proliferation in all groups at 1, 3, and 5 days ($p < 0.05$). After 7 days, cell proliferation did not increase 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, all groups evaluated showed a significant increase in cell proliferation ($p < 0.05$) compared to the control group, being similar among groups ($p > 0.05$), and only the highest concentration of the Pracaxi + $Ca(OH)_2$ 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 of days 3 and 5. However, the concentration of 0.034% $Ca(OH)_2$ and 0.0007 + 0.0005% Pracaxi + $Ca(OH)_2$ showed the highest cell viability when compared to the control group at day 7 ($p < 0.05$).

On the other hand, in L929 fibroblast cells, there was no cell proliferation in any of the groups tested on day 1 and at any concentration 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 ($p < 0.05$), and cell viability was increased in all concentrations of the 3 groups compared to the control group ($p<0.05$). On day 7, there was still a difference in cell proliferation compared to the other days ($p<0.05$). All groups, with the exception of 0.006% Pracaxi, showed an increase in cell viability compared to the control group ($p < 0.05$) (Figure 4).

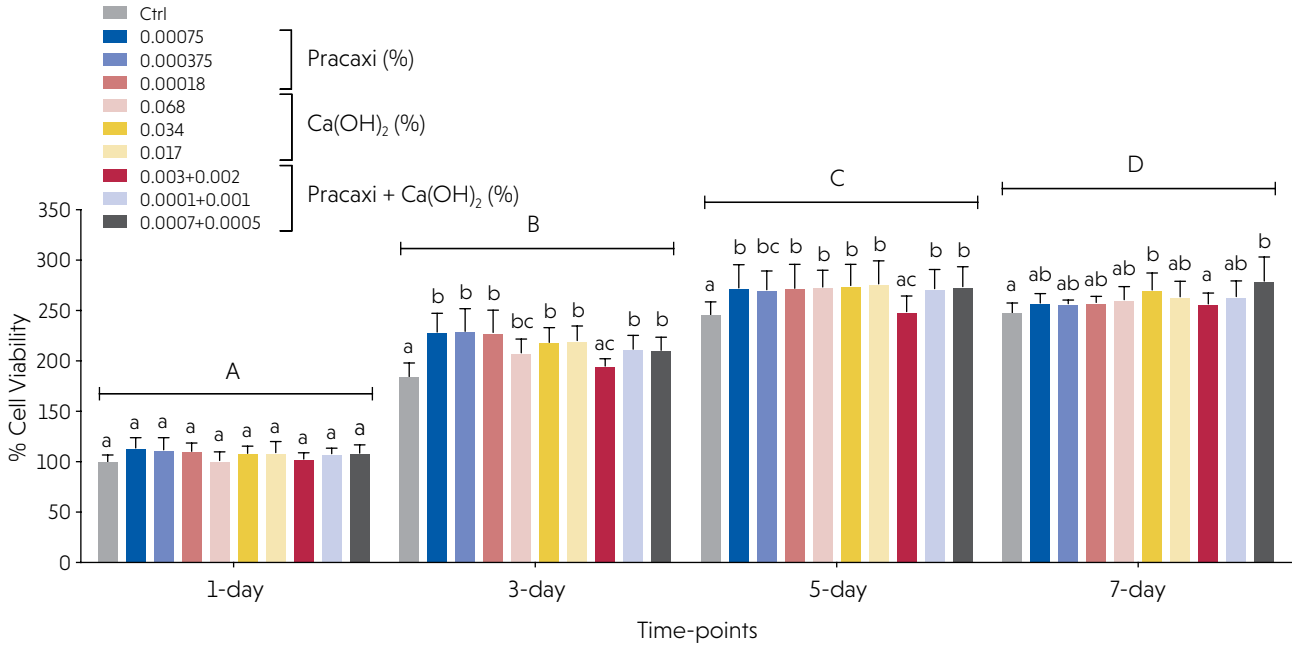
Alizarin red staining

Figure 5 shows 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. In all the three groups tested, there was an increase in mineralization deposits when compared to the groups treated with normal and osteogenic medium ($p < 0.01$). In the Pracaxi group, the two highest concentrations tested showed a higher



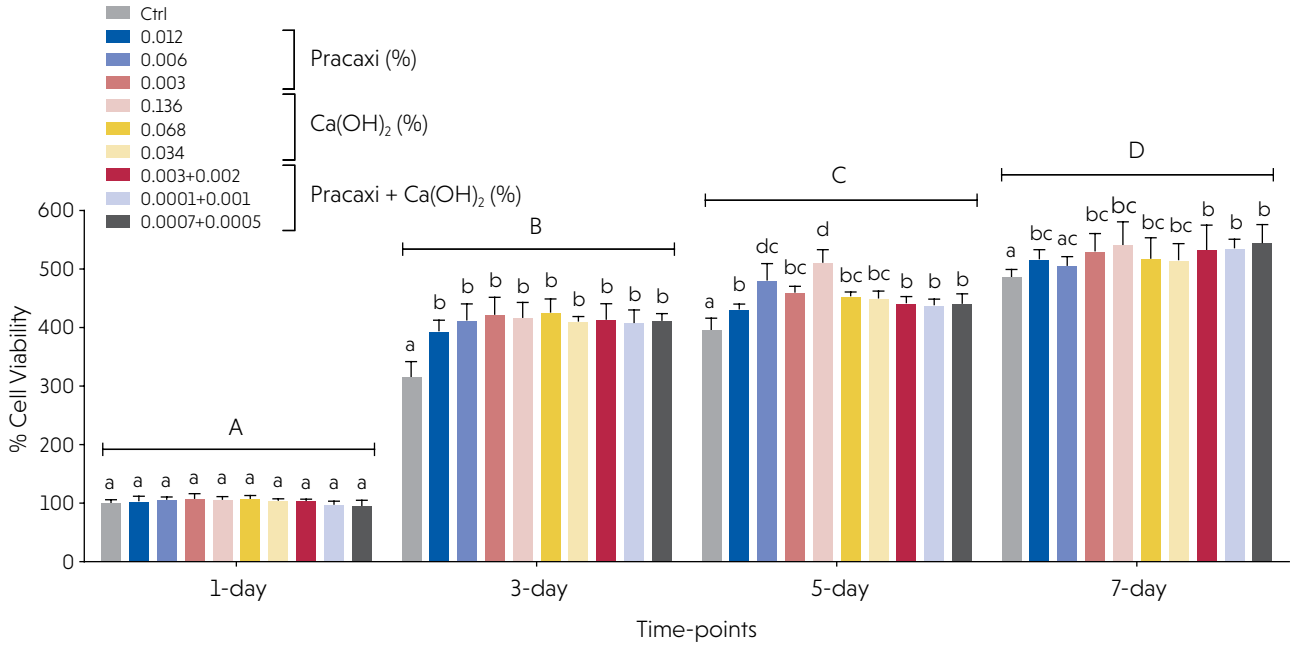
*Significant decrease in comparison with the control group (culture medium) (one-way ANOVA/Dunnett's test, $p < 0.01$).

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



*Different letters indicate statistically significant differences (two-way ANOVA/Tukey's test, $p < 0.05$).

Figure 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 means ± SD of triplicate assays from three independent experiments. Uppercase letters indicate comparison among time points for each group; lowercase letters indicated comparison among groups at each time point.



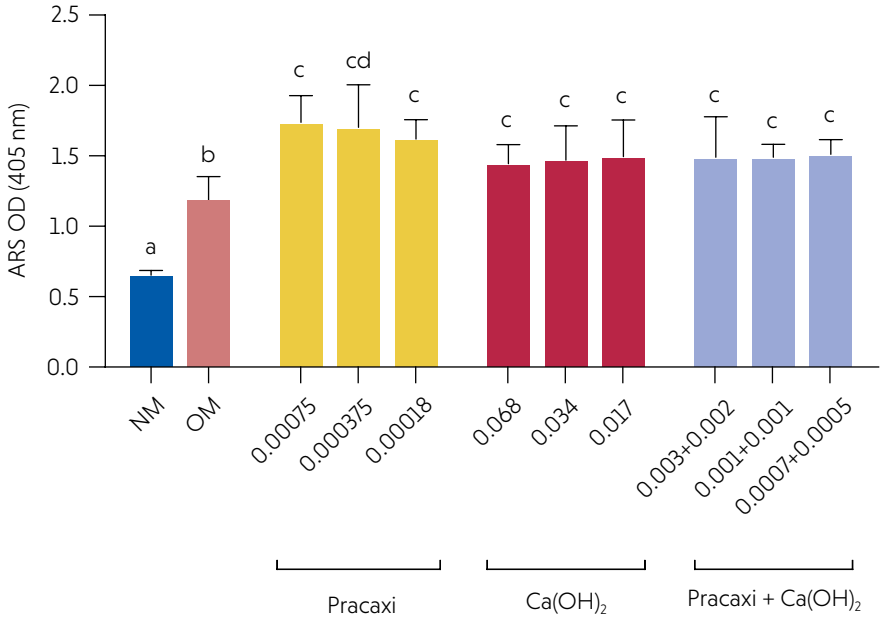
*Different letters indicate statistically significant differences (two-way ANOVA/Tukey's test, $p < 0.05$).

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

number of mineralized nodules when compared with group treated with osteogenic medium only ($p < 0.01$). A similar behavior was found in the Pracaxi + $Ca(OH)_2$ group at 0.003 + 0.002% concentration ($p < 0.01$).

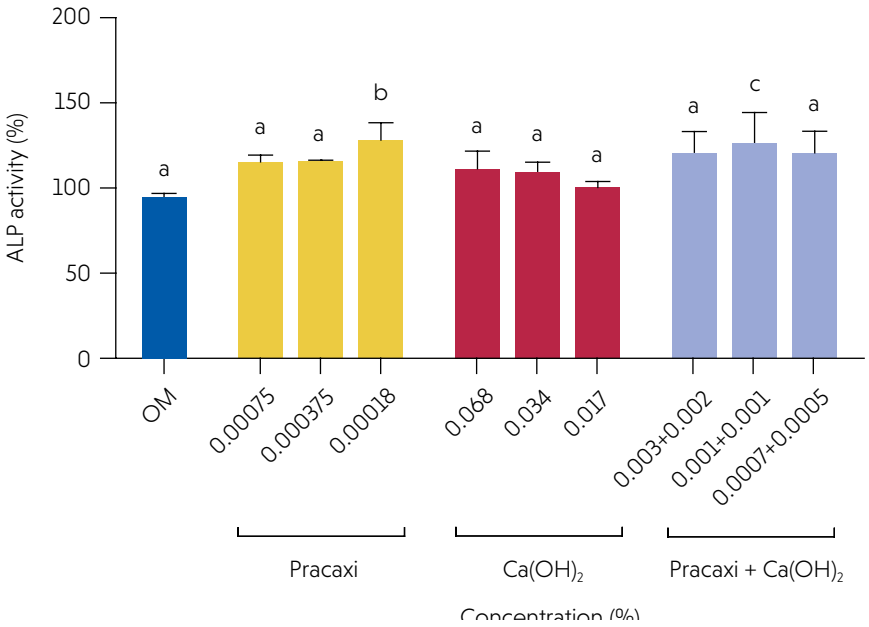
Alkaline phosphatase activity

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



*Different letters indicate statistically significant differences (One-way ANOVA/Dunnett's test, $p < 0.01$).

Figure 5. Quantification of mineral deposition by 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 means ± SD of triplicate assays for three independent experiments.



*Different letters indicate statistically significant differences (one-way ANOVA/Dunnett's test, $p < 0.05$).

Figure 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 means ± SD of triplicate assays from three independent experiments.

Discussion

This study was designed with the aim of providing new evidence of the biocompatibility and biological activities of *Pentaclethra macroloba* extract, pure and in combination with $Ca(OH)_2$. Calcium hydroxide is a medication with proven action, being recommended by the European Society of Endodontics.[16] Medications were evaluated over a 1-week period, as this is the minimum application time for intracanal medications recommended by the American Association of Endodontists for endodontic regeneration procedures (2017).[17]

$Ca(OH)_2$ has proven to be effective for revascularization.[18] However, its high pH can destroy cells of the apical papilla and periapical tissues that are fundamental for tissue repair.[19] In addition, $Ca(OH)_2$ may inhibit the proliferation of soft tissues with odontogenic potential and interfere with the bleeding necessary for revascularization.[20]

The Saos-2 cell line was selected because they are mature osteoblasts and widely used in research.[21,22] These cell can express markers of early osteogenic differentiation, such as ALP and COL-1, and can be analyzed at a shorter culture time, even in non-osteogenic media.[22] In the present study, parameters were evaluated at 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 enhance the validity 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.[23] The results in fibroblast cells were similar to those in osteoblast cells, where small concentrations of the pure extract and the extract associated with $Ca(OH)_2$ were not toxic to cells. Cell proliferation occurred on days 1, 3, 5, and 7, demonstrating a longer proliferation time 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 was no statistical difference between Pracaxi and Pracaxi + $Ca(OH)_2$ in toxicity and cell proliferation, both products showed promising results, indicating a lower toxicity when evaluated in osteoblast cells and enhancing cell proliferation in fibroblast cells. In a previous study that associated $Ca(OH)_2$ with *Propolis* (another phytotherapic), the association was better than the pure extract in terms of cytotoxicity.[24] This result was also found in the association of $Ca(OH)_2$ with *Myracrodruon urundeuva Allemão* (aroeira), which was also cell-compatible.[11]

The results showed that the use of *Pentaclethra macroloba* extract, either pure or combined with calcium hydroxide, in small concentrations was not cytotoxic for Saos-2 and L929 cells after 48 h of contact compared to the control group (culture medium) evaluated by MTT assay. $Ca(OH)_2$ was also not cytotoxic at low concentrations. Higher concentrations of the 3 intracanal medications were able to cause a slight decrease in the metabolism of Saos-2 and L929 cells, similar to other previously studied natural extracts.[25,26]

As expected, because of the healing action of the extract[15], the Alamar Blue assay showed increased cell proliferation with the use of *Pentaclethra macroloba* extract, pure or in association with $Ca(OH)_2$, on days 3, 5, and 7 in the two types of cells tested. Saos-2 cells showed proliferation stagnation only from day 5 to 7. *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 diabetic wounds,[15] as an effect of the fatty acids in its composition,[13] which are known to improve wound closure and improve healing.[27]

ARS staining identifies calcium deposits in cell cultures.[28] Mineralization nodules were similarly observed in all groups, with the 0.000375% Pracaxi group having a further formation of mineralization nodules in relation to the other concentrations of the same group and to the other groups, indicating a potential repairing effect of this extract when pure. The ability of $Ca(OH)_2$ to form mineralization nodules was found in this study and it is consistent with other studies.[29] In our study, we prepared extracts of each solution and discarded the sediment through a centrifugation process. In clinical applications, considering the biological structures in which intracanal medications are applied, the release of the medicament into the root canal plays an important role in treatment success. Calcium phosphate compounds are water soluble and dissolve into calcium and hydroxide ions[30]. Previous studies showed that calcium ions have the ability to improve the remineralization process,[30-32] which corroborate our results.

The evaluation of alkaline phosphatase activity, an enzyme that is expressed throughout the process of early maturation of osteoblasts, indicates the potential of medication to induce mineralized tissue formation.[33] The highest ALP activity was observed in Saos-2 cells after a 7-day exposure to Pracaxi + $Ca(OH)_2$ in one of its concentrations, followed by exposure to Pracaxi alone. Studies that evaluated ALP in natural products such as curcumin and icariin also found a greater ALP activity.[25,34]

Pentaclethra macroloba extract, pure and combined with $Ca(OH)_2$, in small concentrations, did not have cytotoxic effects in Saos-2 and L929 cells. The compounds caused cell proliferation and formation of mineralization nodules,

indicating a potential use of the tested medications in cases of pulpal revascularization. The antimicrobial action of *Pentaclethra macroloba* extract against *Enterococcus faecalis* has already been reported in the literature.[13,14] A recent study showed that a new medication for endodontic use based on *Pentaclethra macroloba* extract associated or not with $Ca(OH)_2$ showed antimicrobial action against *E. faecalis* when compared to the medication used in clinical practice. This biological action demonstrated in the present study added to the antimicrobial action that is already verified, indicating a promising endodontic medication.

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 for future use in clinical practices. While not being cytotoxic to cells, it stimulated cell proliferation and formation of mineralization nodules. Nevertheless, the present study was performed in vitro, which may not fully represent a clinical situation. In view of the findings of the present study, in vivo studies should be performed to confirm and validate the results found for this potential future endodontic medication.

Conclusions

Pentaclethra macroloba extract showed bioactive potential and cytocompatibility, and was able to induce cell proliferation in Saos-2 osteoblasts and L929 fibroblasts. It also induced mineralization nodules in Saos-2 cells, which are essential characteristics of endodontic medications for traditional endodontic treatment and revascularization.

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Data availability: The authors declare that all data generated or analyzed during this study are included in this published article.