# Antimicrobial activity and pH evaluation of Calcium Hydroxide associated with natural products

## *Avaliação da atividade antimicrobiana e do pH do Hidróxido de Cálcio associado a produtos naturais*

**Yuri Wanderley CAVALCANTI**
Undergraduate student - Universidade Federal da Paraíba – João Pessoa - PB – Brazil.

**Leopoldina de Fátima Dantas de ALMEIDA**
Graduate Student - Universidade Federal da Paraíba – João Pessoa - PB – Brazil.

**Mariana Machado Teixeira de Moraes COSTA**
PhD – UNESP – Univ Estadual Paulista – Faculdade de odontologia de Araçatuba – Araçatuba – SP – Brasil.

**Wilton Wilney Nascimento PADILHA**
Associate Professor - Universidade Federal da Paraíba – João Pessoa - PB – Brasil.

### Abstract

New intra-canal medications can be developed by the combination of natural products with calcium hydroxide (Ca(OH)2). The aim of this study was to evaluate the antimicrobial activity and pH values of Ca(OH)2 associated with propolis tincture 5% (S1); pomegranate tincture 5% (S2); essential oil from eucalyptus 0.5% (S3); inert solution (S4) and with CPMC - Camphorated Paramonochlorophenol (S5) on strains of *Enterococcus faecalis* (ATCC29212); *Aggregatibacter actinomycetecomitans b* (ATCC29522), *Eikenella corrodens* (ATCC23834) and *Candida albicans* (ATCC40277). Sterile paper cones were immersed for 1 minute in the solutions under test. The agar diffusion test was carried out and the plates were incubated at 37 °C in bacteriological incubator for 48 h. The mean diameter of growth inhibition (MDGI) produced by the formulations were calculated in millimeters and statistically analyzed by ANOVA and Tukey's tests. The tests were performed in triplicate. The pH of substances was measured at room temperature. For *E. faecalis, A. actinomycetecomitans b; E. corrodens and C. albicans* MDGI for each substance was, respectively: 1.6, 3.1, 4.6 and 2.3 mm (S1), 1.5, 3.1, 8.8 and 0.3 mm (S2), 1.5, 3.5, 5.5 and 0.5 mm (S3), 1.5, 5.3; 11.6 and 3.1 mm (S4) and 3.0, 8.5, 17.3 and 7.3 mm (S5). The inhibition zones produced by S5 were larger than the ones from S1, S2 and S3 ($p<0,05$). The pH-values found were: 11.55 (S1); 11.52 (S2); 11.48 (S3); 11.54 (S4); and 11.65 (S5). Commercial formulations of Ca(OH)2 showed better antimicrobial performance than the associations with natural products. The pH of tested formulations did not change significantly.

### Uniterms

Biological Products; Calcium Hydroxide; Root Canal Therapy.

## INTRODUCTION

The Calcium Hydroxide (Ca(OH)2) is a substance used in different clinical situations, represented by the dentin-pulp complex protection and by the treatment of infections of the root canal system [1]. The versatility of Calcium Hydroxide is attributed to its antimicrobial activity, biocompatibility and ability to induce remineralization [1,2].

Infection of the root canal system occurs as a result of multiple activity of microorganisms, among them, Gram-positive or Gram-negative, facultative aerobic or anaerobic bacteria [3,4,5]. It can also be found fungi and viruses [4,5]. The Enterococcus faecalis is the most common microorganism involved in endodontic changes, especially with regard to secondary infections and to the appearance of periapical lesions [3,4,5].

In order to assist the root canal system sanitization process, it was necessary to develop products used as intracanal medication [1,6]. Due to the antimicrobial potential of Ca(OH)2, which is related to its alkaline

pH, this substance is used to reduce inflammation and infection [7]. According to Leonardo et al.[7] (2004), Calcium Hydroxide is able to inactivate the toxic effects of bacterial endotoxins, translated by Lipopolysaccharides (LPS) present in the cell walls of Gram-negative bacteria.

Thus, aiming to enhance the antimicrobial and anti-inflammatory activities of Ca(OH)2, it has been tested the association of this substance with antibiotics (e.g.: Camphorated Paramonochlorophenol - CPMC, Iodoform, among others) [6,7,8]. These products have low biocompatibility, being suggested the replacement of these compounds in case of association with Calcium Hydroxide [9].

On the other hand, the use of natural products in dentistry has been justified by popular use, low cost and appropriate antimicrobial and anti-inflammatory activities. Therefore, the combination of natural products with Ca(OH)2 should be evaluated in order to investigate new possibilities for the development of intra-canal medications.

In this perspective, the purpose of this study was to evaluate the pH and the antimicrobial activity of Calcium Hydroxide (Ca(OH)2) associated with propolis tincture at 5% (S1); pomegranate tincture at 5% (S2), essential oil from eucalyptus at 0.5% (S3); inert solution (S4), and with the CPMC (S5) on Enterococcus faecalis (ATCC 29212); Aggregatibacter actinomycetecomitans b (ATCC 29522), Eikenella corrodens (ATCC 23834) and Candida albicans (ATCC 40277).

## Material And Methods

The products tested were obtained from a compounding pharmacy and made from the composition reported in the Calcium Hydroxide paste's leaflet + CPMC, available in the commercial formulation - Calen PMCC® (SS White Dental Articles Ldt, Rio de Janeiro-RJ, Brazil) . Then, in the composition of the formulations, Calcium Hydroxide was used in the concentration of 49.38% and the vehicle was Polyethylene glycol 400 (PEG 400).

Propolis (S1) and pomegranate (S2) tinctures were used at a concentration of 5.0% for association with Ca(OH)2. The essential oil from eucalyptus (S3) was used at concentration of 0.5%. For S4 and S5, were used commercially available formulations (Table 01). The substance corresponding to the association of Calcium Hydroxide with Camphorated Paramonochlorophenol - CPMC (S5), in commercial formulation, was considered positive control. The list of substances used in the present study, and their respectively composition, are shown on Table 01.

For antimicrobial evaluation were used reference strains of Enterococcus faecalis (ATCC 29212); Aggregatibacter actinomycetecomitans b (ATCC 29522); Eikenella corrodens (ATCC 23834) and Candida albicans (ATCC 40277). It was carried out the agar diffusion method in which sterile paper cones were immersed for 1 minute in the solutions under test and then transferred to Petri plates seeded with the microorganisms [9].

For doing so, nine Blood agar plates and three Sabouraud Dextrose agar plates were prepared for bacteria and fungi growth, respectively. The sowing of the plates was made from fungal and bacterial inoculums at concentrations of 1.5 x 106 CFU/mL and 1.5 x 108 CFU/mL, compared to McFarland scale. The plates were incubated at 37° C in bacteriological incubator for 48h. The higher diameter of the inhibition areas of microbial growth around the paper cone was measured with a millimeter caliper, for each microorganism, which corresponded to the substances' antimicrobial potential [9].

The mean diameter of growth inhibition (MDGI) produced by the formulations were calculated in millimeters analyzed statistically. The data were tabulated in GraphPad Prism 5.0 (GraphPad for Windows, San Diego, CA - USA), by which it undertook statistical analysis by ANOVA and Tukey post-test.

The pH measurement occurred at room temperature, using a pHmeter (Orion 4star Benchtop pH / ISE meter, Thermo Scientific - Singapore) with electrode calibrated in standard-solutions (pH4 and pH7). The pH values were analyzed descriptively.

## Results

The values of the mean diameters of growth inhibition (MDGI), standard-deviation (SD) and the statistical differences for the tested products are presented in Table 02.

The substance S5 presented antimicrobial activity statistically different from S1, S2 and S3 ($p<0,05$). It was not observed difference statistically significant for the antimicrobial activity of S4, when compared to other tested formulations ($p>0,05$). There was no difference statistically significant between the antimicrobial activity of S1, S2, and S3 ($p>0,05$).

The pH values of the tested substances are observed in Table 03.

## Discussion

In this study, a modification of the agar diffusion method was used to evaluate the antimicrobial activity of tested products [9]. As described by other studies, for the diffusion of the substances tested in solid medium, wells are made with diameters of 6 to 4mm [8], or else are used absorbent paper disks [10]. Based on comparison with the positive control and the methodology proposed by Tanomaru et al. [9] (2007) were used absorbent paper cones for agar diffusion of the substances evaluated.

Different from other studies [8,10,11], this research have found lower values of mean diameters of growth inhibition (MDGI). However, the findings of this research are validated by comparison with the positive control and by the results reported by Tanomaro et al. [9] (2007) .

The selection of microorganisms for this study was based on the frequency related to the development of endodontic infections and severe periodontitis, like Enterococcus faecalis [3,4,5,12], Candida albicans [4,5,12], Aggregatibacter actinomycetecomitans b [5,13,14,15] and Eikenella corrodens [4,13].

Estrela et al. [1] (1995) indicate that the mechanism of action of Calcium Hydroxide is directly linked to alkaline pH, with approximate value of 12.6. Therefore, the release of hydroxyl ions alters the integrity of the microorganisms' cell membrane, causing them toxic effect [1]. Accordingly, the association with natural products or any other adjuvant substances should not lead to changes in pH of Calcium Hydroxide.

The natural products that composed the tested formulations have potential for constituting intracanal medication, once that they did not expressively alter the pH of Calcium Hydroxide (Table 03). It was also identified the lack of expressive variation of pH of the formulations tested, even with the use of tinctures (hydrophilic) and essential oils (hydrophobic) in association with Ca(OH)2. This fact is supported by the study of Pereira et al. [16] (2009) which states that oily vehicles do not cause change in pH.

The study by Pereira et al. [16] (2009) aimed to evaluate the pH of Calcium Hydroxide and Iodoform, associated or not with a neutral oily vehicle in solutions of distilled water, saline and alcohol. The significant variation in pH of Calcium Hydroxide (SD=1.5) was observed only when associated to the oily vehicle in distilled water solution [16]. In this study, the pH values varied less than 0.1 between samples.

Given these findings, the same authors considered that the change of medium or the combination to oily vehicles didn't alter the behavior of Calcium Hydroxide [16]. In this sense, this study's results corroborate the findings of Pereira et al. [16] (2009), once it wasn't verified significant change in the pH of Calcium Hydroxide. This fact becomes important insofar as this substance, by inducing a strongly alkaline medium, may become aggressive not only to microorganisms but also to tissue components.

As regards the nature of the hydrophobic or hydrophilic substances associated with Ca(OH)2, a study by Gomes et al. [17] (2002) concluded that diffusion and antimicrobial activity were affected by the type of vehicle used. Accordingly, the authors found that the Calcium Hydroxide pastes formed in oily vehicles produced zones of growth inhibition greater than those formed in aqueous or viscous vehicles.

Thus, once the substances S1 and S3 showed similar antimicrobial activity (Table 02), the results of this study agree with the findings of Gomes et al. [17] (2002), because the concentration of eucalyptus essential oil used was ten times lower than the concentration of propolis tincture. Thereby, it is suggested to carry out further studies to compare the antimicrobial activity of these products at the same concentration.

It was found that S5 had the highest values of MDGI, which represented higher antimicrobial performance in relation to the other substances tested. The highest antimicrobial power of S5 is justified by the association of CPMC with Ca(OH)2, which provides increased antimicrobial activity and slow dissociation of hydroxyl ions[17].

The effect of tested substances on the microorganisms of this study was not uniform. In other words, there was variation in the size of the halos of inhibition and antimicrobial activity against the different specimens. This fact is supported by the study of Gomes et al. [17] (2002) who reported that Calcium Hydroxide has no uniform antimicrobial activity against all microorganisms present in the root canal, becoming necessary its association with antimicrobial substances.

Many studies have evaluated the antimicrobial activity of natural products based on propolis against microorganisms found in root canal infection [8,10,18,19,20,21,22]. Similarly, it was identified effective activity of propolis, associated or not with Ca(OH)2, in controlling endodontic infection [18,20], being suggested the use of this substance as an alternative intracanal medication [18], which justifies the use of propolis tincture on the evaluation proposed by this study.

The study by Sanchez-Ayala, Silveira and Santos [22] (2008) reported the antimicrobial evaluation of Ca(OH)2 associated with propolis ethanolic extracts at 20% and 40% against Enterococcus faecalis, Staphylococcus aureus, Staphylococcus saprophyticus, Pseudomonas aeruginosa and Escherichia coli. Whereupon, it was found that propolis extracts decreased the antibacterial activity of Ca(OH)2. The reasons for this result may be related to the resinous characteristics of the natural product and to the difficulty of diffusion of the substance [22]. Given this, the present study observed that the association of Ca(OH)2 with propolis tincture 5% (S1) showed antimicrobial activity lower than Calen® and Calen CPMC® (Table 02).

Complementing these findings, the study by Costa et al. [8] (2008) found that the propolis extract, analyzed isolated or in association with Ca(OH)2 or Iodoform, showed low antimicrobial activity against Enterococcus faecalis [8]. Thus, according to the results of this study and the literature [18,20], the association of Ca(OH)2 with propolis tincture at 5% must be investigated in higher concentrations of the natural product, in order to confirm or repudiate the potential for the development of an intracanal medication.

When evaluated separately on microbial control of Enterococcus faecalis, the study by Maia-Filho et al. [10] (2008) identified that the Ca(OH)2 produced halos of growth inhibition of 16.2mm, while the propolis Extract at 1% was responsible for halos of 10.9mm [10]. Therefore, the concentration of propolis tincture used in this study provides adequate antimicrobial activity against endodontic microbiota. Considering the results of this study, the antimicrobial properties of propolis tincture in association with Calcium Hydroxide should be evaluated at concentrations higher than 5%.

Differently from the studies about the use of propolis in endodontics, were not found researches or evidences that address the association of Calcium Hydroxide with the natural products from pomegranate or eucalyptus.

In dentistry, the use of Pomegranate (Punica granatum) has been more frequently reported in the control of forming biofilm microorganisms [23,24,25]. In this sense, it is necessary to investigate the antimicrobial activity of this natural product on other microorganisms involved in oral diseases.

Vasconcelos et al. [25] (2006) evaluated the antimicrobial activity of pomegranate extracts and determined the minimum inhibitory concentration at the concentrations of 5% and 10% against strains of Candida albicans, Streptococcus mutans and Streptococcus mitis.

Study by Sharma et al. [26] (2009) reported the antibacterial efficacy of the aqueous, ethanolic and acetic extracts of pomegranate (Punica granatum), in the concentration of 5% against resistant clinical strains of Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Enterococcus faecalis. Accordingly, the pomegranate tincture used at a concentration of 5% is indicated in the control of root canal microorganisms. However, when associated with Calcium Hydroxide, the present study did not identified satisfactory antimicrobial activity when compared to other tested formulations.

The use of natural products from eucalyptus (Eucalyptus globulus) for the treatment of root canal system is most often reported in cases of removal of filling material at root canal retreatment [27]. Thus, the use of eucalyptus essential oil as antimicrobial agent has been poorly reported in the literature [28,29].

Studies have reported the antimicrobial activity of eucalyptus essential oil only in pure concentration on Pseudomonas aeruginosa and Escherichia coli [28] as well as on Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus subtilis and Staphylococcus aureus [29]. Studies reporting antimicrobial activity of eucalyptus essential oil on Enterococcus faecalis, Aggregatibacter actinomycetecomitans b, Eikenella corrodens and Candida albicans were not found in the literature.

Considering the antimicrobial potential of eucalyptus essential oil against resistant microorganisms [28,29], it's expected that the association with Ca(OH)2 contributes to the control of endodontic microbiota. However, the present study considers that more laboratorial steps are to be conducted in order to confirm the potential for constituting intracanal medication and to consider the clinical applicability of these associations.

## Conclusion

Among the substances tested, the commercial formulations of Ca(OH)2 showed better antimicrobial performance when compared to associations with natural products. The combination of natural products with Calcium Hydroxide provided no significant change in pH.

Thus, it is suggested that, when combined with Calcium Hydroxide, the concentration of natural products should be increased and new laboratorial tests are to be produced in order to test new Ca(OH)2 formulations with natural products.

**Table 1 – Demonstrative of the substances used in the present study and their compositions, obtained from a compounding pharmacy**

| Substances | Composition |
|---|---|
| **S1** | Calcium Hydroxide + Propolis tincture at 5% |
| **S2** | Calcium Hydroxide + Pomegranate tincture at 5% |
| **S3** | Calcium Hydroxide + Essential Oil of Eucalyptus at 0,5% |
| **S4** | Calcium Hydroxide + Inert solution (q.s.) (Calen®) |
| **S5** | Calcium Hydroxide + CPMC (Calen CPMC®) |

**Table 2 – Distribution of absolute values (in millimeters) and standard deviation (SD) of the Mean Diameter of Growth Inhibition (MDGI) for S1, S2, S3, S4, and S5 against the strains analyzed**

| Microorganisms | S1 | | S2 | | S3 | | S4 | | S5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **MDGI** | **SD** | **MDGI** | **SD** | **MDGI** | **SD** | **MDGI** | **SD** | **MDGI** | **SD** |
| *E. faecalis* | 1.6 a | *0.28* | 1.5 a | *0.00* | 1.5 a | *1.5* | 1.5 a, b | *0.5* | 3.0 b | *0.00* |
| *A. actinomycetecomitans b* | 3.1 a | *0.76* | 3.1 a | *0.28* | 3.5 a | *0.5* | 5.3 a, b | *0.28* | 8.5 b | *4.92* |
| *E. corrodens* | 4.6 a | *1.52* | 8.8 a | *4.64* | 5.5 a | *1.80* | 11.6 a, b | *4.04* | 17.5 b | *1.52* |
| *C. albicans* | 2.3 a | *1.44* | 0.3 a | *0.57* | 0.5 a | *0.86* | 3.1 a, b | *0.76* | 7.3 b | *2.88* |

Different letters on the same line indicate difference statistically significant (p<0,05 – ANOVA test and Tukey Post-Test).

**Table 3 – Distribution of pH absolute values S1, S2, S3, S4, and S5. Values were obtained at room temperature through a calibrated electrode**

| Substances Tested | pH absolute values |
|---|---|
| S1 | 11.55 |
| S2 | 11.52 |
| S3 | 11.48 |
| S4 | 11.54 |
| S5 | 11.65 |

## Resumo

Novas medicações intra-canal podem ser desenvolvidas a partir da combinação de produtos naturais com hidróxido de cálcio (Ca(OH)2). Objetivou-se avaliar o pH e a ação antimicrobiana do Ca(OH)2 associados a tintura de própolis 5% (S1); tintura de romã 5% (S2); óleo essencial de eucalipto 0,5% (S3); solução inerte (S4); e ao PMCC – Paramonoclorofenol Canforado (S5) sobre cepas de *Enterococcus faecalis* (ATCC 29212); *Aggregatibacter actinomycetecomitans* b (ATCC 29522); *Eikenella corrodens* (ATCC 23834) e *Candida albicans* (ATCC 40277). Cones de papel estéreis foram imersos durante 1min nas soluções testadas. Realizou-se o teste de difusão em agar e as placas foram incubadas a 37 ºC em estufa bacteriológica por 48 h. Os diâmetros médios de inibição do crescimento (DMIC) produzido pelas substâncias foram calculados em milímetros e analisados estatisticamente pelos testes ANOVA e de Tukey. Os testes foram realizados em triplicata. A aferição do pH se deu em temperatura ambiente. Para *E. faecalis; A. actinomycetecomitans b; E. corrodens* e *C. albicans* a DMIC para cada substância foi, respectivamente: 1,6; 3,1; 4,6 e 2,3mm (S1); 1,5; 3,1; 8,8 e 0,3mm (S2); 1,5; 3,5; 5,5 e 0,5mm (S3); 1,5; 5,3; 11,6 e 3,1mm (S4) e 3,0; 8,5; 17,3 e 7,3mm (S5). Os halos de inibição produzidos por S5 foram maiores do que os de S1, S2 e S3 (p<0,05). Os valores de pH encontrados foram: 11,55 (S1); 11,52 (S2); 11,48 (S3); 11,54 (S4); 11,65 (S5). As formulações comerciais de Ca(OH)2 mostraram melhor desempenho antimicrobiano do que as associações com produtos naturais. O pH das soluções testadas não variou significativamente.

## Unitermos

Produtos Biológicos; Hidróxido de Cálcio; Tratamento do Canal Radicular

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Corresponding author:
Mariana Machado Texeira de Moraes COSTA
moraes_mari@hotmail.com