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ANTIMICROBIAL ACTIVITY WITH MIXTURE OF CALCIUM HYDROXIDE AND PROPOLIS

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ABSTRACT

The aim of this study was to compare the *in vitro* antimicrobial activity of a mixture of calcium hydroxide/propolis, propolis/calcium hydroxide ($Ca(OH)_2$) in *Enterococcus faecalis*, at different concentrations. Paper discs were impregnated with such medication and were placed in Mueller–Hinton plate agar with blood at 5 %, inoculating $1x10^5$ UFC of *E. faecalis* and incubating at 35–37 ^oC for making measurements of the inhibition halos with a vernier at 24, 48 and 72 hours of incubation. The measurements were statistically analyzed with ANOVA with a $p < 0.01$ significance. The mixture of propolis and $Ca(OH)_2$ 1:3 at 48 and 72 hours showed the largest zone of inhibition.

KEYWORDS

Calcium hydroxide, *Enterococcus faecalis,* intracanal medicaments, propolis

INTRODUCTION

Root canal treatment is a therapy that is used to preserve teeth and prevent tooth extraction. Most teeth need endodontic treatment through correct instrumentation techniques and intracanal medication between appointments and it is possible prevent root canal infection and seal teeth. Ideal intracanal medication must be compatible with dental tissues and have bactericidal and bacteriostatic effect. Therefore, endodontic treatment is very important to understand the etiology, prevention of, pathology, diagnosis, and treatment of the pulp.

It is well known that the origin of all drugs are natural, and that is why there are continuous researches about plants and their components in order to benefit human health. Interest in alternative drugs has involved several studies about propolis and its composition. It mainly contains flavonoids that have antibacterial properties and antifungal activity and hence the ability to promote healing. They also stimulate dentin formation and decrease pulpal inflammation[1,2].

The microorganism *E. faecalis* was elected because of its ability to grow and survive under adverse environments such as in the presence of calcium hydroxide. The resistance of *E. faecalis* to calcium hydroxide ($Ca(OH)_2$) allows it to survive and proliferate when its action is over, ending in the colonization and infection of the root canal,and finally resulting in the failure of the root canal treatment [3].

Therefore, consideration of propolis as an alternative intracanal drug is appropriate. Literature mentions that propolis is highly effective against *E. faecalis* [2] owing to its antibacterial characteristics and $Ca(OH)_2$ does not have the same effect. Hence, the present *in vitro* study focuses on the antimicrobial activity of a mixture of calcium hydroxide and propolis at different concentrations against *E. faecalis*.

MATERIALS AND METHODS

For this study, ATTC 29212 *E. faecalis* bacteria obtained from the Biologics National School of Science registered with the World Federation of Culture Collection (WFCC), code 449, was used to determine the effectiveness of the medication.

Medicaments with antimicrobial activity with $Ca(OH)_2$ 20% and propolis in the ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 (volume–volume relationship) were prepared. Antimicrobial activity was determined using the diffusion agar-disc method. This method considers a small amount of antibiotic in a 6 mm diameter absorbent paper disc placed over the agar plate surface previously inoculated with the microorganism to be analyzed through a concentric inhibitory zone. The zone of inhibition for most of the antimicrobial agents is determined by the concentration of minimal inhibition (CIM) of the antibiotics.

The test of the CIM of an antimicrobial agent is the minimal concentration of the antimicrobial agent that disables the multiplication and production of a visible growth of a bacterial strain given in the system of test. Concentration in the laboratory incubating a

known quantity of bacteria with definite dilutions of the antimicrobial agent was determined.

These methods of sensibility defined by the Federal Drug Administration (FDA) of the United States and the National Committee constitute the base for the methods recommended by the Clinical Laboratory and Standards Institute (CLSI)[4]. The methods must be followed with accuracy if exact and reproducible results are to be obtained.

PROCEDURE FOR DIFFUSION TEST IN AGAR BY DISC

(i) ***Determination of the antimicrobial activity.***

The method for culture preparation recommended was the agar Mueller–Hinton with blood at 5%.

(ii) ***Preparation of discs.*** Absorbent paper was used in 6 mm diameter discs; impregnated with the medication Ca $(OH)_2$ at 20% and propolis at different concentrations until dryness (the moment when the paper has totally absorbed the medication). Once prepared, the discs were kept frozen in a hermetically closed container in a refrigerator at 4 °C until its use (Fig 1).

Paper discs

Figure 1
Discs impregnated with the medication

***E. faecalis* strain**

(iii) ***Inoculum preparation.*** A night culture was obtained from the original bacteria (Fig 2), which consists of sowing a pipe with broth of Mueller–Hinton incubated at 35–37 °C for 20 hours to obtain a concentration of $1x10^5$ UFC of *E. faecalis*. The pipe was waved immediately before its utilization for a minute. An inoculum was prepared for every drug as well as for the mixture (Fig 3).

Figure 2
Original bacteria E. faecalis

Mueller–Hinton agar inoculated with *E. faecalis*

Figure 3

Inoculum prepared for every drug from the original bacteria

(iv) ***Medium inoculation.*** A sterile hyssop was introduced in the suspension of the inoculum after 20 hours of incubation. The excess broth was suppressed by squeezing or rotating the hyssop against the interior wall of the pipe of the suspension (Fig 4). Then the content from the hyssop was distributed uniformly in the surface of the plate of the agar Mueller–Hinton with blood at 5 % (Fig 5). Finally, the hyssops were made to touch all around the edge of the agar. It was left to dry and inoculated for 3 or 5 minutes and later, the discs were applied manually using sterile tweezers.

Tubes prepared with the Mueller–Hinton agar

Figure 4

Sterile hyssop introduced in the suspension of the inoculum

Surface of the plate of the agar Mueller–Hinton

Figure 5

Content from the hyssop distributed uniformly in the surface of the plate of the agar Mueller–Hinton with blood at 5 %

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Paper discs and the surface of the agar plate

Figure 6

Disc already impregnated with the medication were placed in the agar plate

After its placement, the discs were pressed carefully against the agar surface. They were placed 15 mm beyond the edge of the plate and were distributed by avoiding the overlap halos of inhibition.

In the plates of 150 mm diameter more than 12 discs cannot be placed, and in the plates of 100 mm diameter, it is not advisable to place more than 6. Therefore, 3 discs were placed in each plate (Fig 6). After 15 minutes, the plates were turned and placed into an incubator at 35–37 °C, in CO_2 conditions for 24, 48, and 72 hours.

(v) Quality assurance. Quality must be assured in all phases of the procedure. The Mueller–Hinton agar medium with 5% blood must have 4 to 6 mm depth or thickness, and it must be prevented from drying off (have to remain fresh and be in use for 2 weeks). The discs must remain frozen before its use; the lot of the discs used has to remain in a packing desiccated at 4 °C (refrigeration), and must be allowed to reach ambient temperature before its utilization everyday. Bacterial strains that are to be used as the control must be prepared.

The surfaces of the plates of agar Mueller–Hinton with 5% blood that are to be inoculated separately are covered with cotton to ensure the purity of the inoculum for every lot of the inoculated plates.

The inhibition halos were measured in mm.

STATISTICAL ANALYSIS

We used the SPSS version 16. Continuous variables were expressed in means ± standard deviation (SD). We used ANOVA and T test as suitable. P value ≤ 0.05 was considered statistically significant.

RESULTS

Pure $Ca(OH)_2$ showed a 1.95 mm inhibition halo, being the greatest at 24 hours exposure; but at 48 hours and 72 hours it was overcome by the combination of propolis/$Ca(OH)_2$1:3. At 48 hours, the second greatest inhibition halo was the combination propolis/$Ca(OH)_2$ ratio of 1:4 (Fig 7). The differences observed in these two combinations of propolis and $Ca(OH)_2$ were statistically significant and were more potent than all other groups ($p < 0.05$).

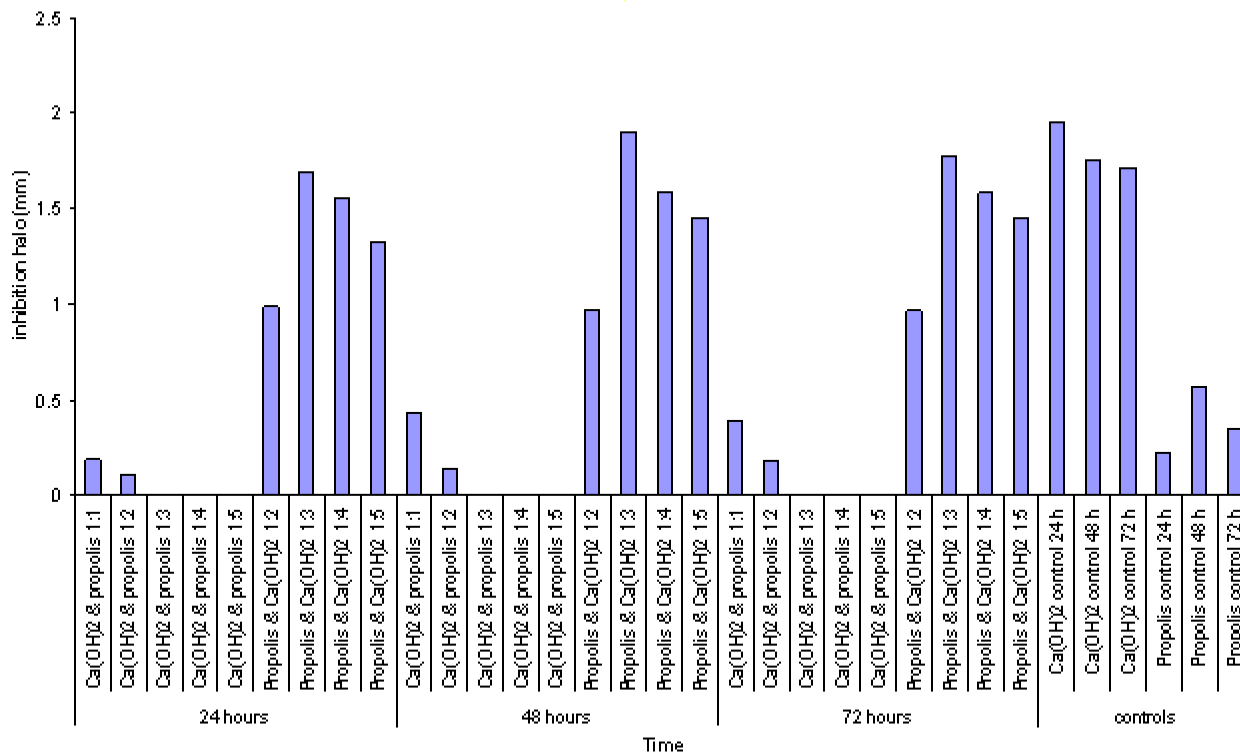

Figure 7
Inhibition halos of different mixtures of propolis and $Ca(OH)_2$

DISCUSSION

To our knowledge, this is the first time that a mixture of propolis and $Ca(OH)_2$ has been evaluated as a medicament against *E. faecalis*.. As written above, propolis is a nontoxic natural product with multiple pharmacological effects and a complex chemical composition which exhibits a wide range of biological activities, including antimicrobial, anti-inflammatory, anesthetic, and cytostatic properties [5]. Some other studies on flavonoid contents have been evaluated using ethanolic extracts of propolis against *Staphylococcus aureus* and *Micrococcus luteus* showing high antibacterial activity [6]. Howeve,r the inhibition of growth with propolis against some bacteria depends on the type of microorganism and the solvent employed. B. Tosi et al.[7] found that extracts of propolis were particularly active against *Sarcina lutea*, *Candida albicans*, *Rhodotorula glutinis*, *Schizosaccharomyces pombe* and *dermatophytes*. In addition, its composition can vary depending on the area from where it is collected. For example, propolis from Turkey and Brazil are effective against *Peptostreptococcus anaerobius* and *micros*, *Lactobacillus acidophilus*, *Actinomyces naeslundii*, *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Veillonella parvula* [8].

Several *in vitro* assays, like that in our study have been made to determine the antibacterial activity of propolis against some microorganism. In the case of C*andida albicans*, in which the microbiological method

was agar dilution, rendered the clearest results in accordance with the results of the chromatographic analysis [9]. Other researchers have compared the antimicrobial activity by microdilution methods using propolis and other substances like $Ca(OH)_2$, camphorated paramonochlorophenol, and formocresol against anaerobic bacteria and found that *E. faecalis* was the less susceptible strain which coincides with the present study[10].

Anaerobic bacteria dominate the root canal microbiota with some facultative strains such as *E. faecalis* in persistent infections, thereby influencing the failure of the treatment. Shveta Gupta et al.[11] used two different formulations of propolis extract (30% propolis in dimethyl sulfoxide and 30% propolis in ethyl alcohol) as irrigants in the root canal system against *E. faecalis* and the results have clearly indicated that propolis alone was not very effective against *E. faecalis.*

Clinically, $Ca(OH)_2$ is routinely used as an intracanal medicament [12]. However, several published studies have showed that *E. faecalis* is resistant to $Ca(OH)_2$, and are not in agreement with this *in vitro* study, wherein it is shown that $Ca(OH)_2$ has the highest inhibitory action against *E. faecalis* at 24 hours exposure. According to our results, the combination of propolis/$Ca(OH)_2$ 1:3 offers the best results at 48, and 72 hours exposures, against *E. faecalis*.

Table 1
ANOVA STATISTICS

	Sig. (2-tailed)
24 hours Propolis/Calcium hydroxide 1:3 Propolis/Calcium hydroxide 1:4	,257
48 hours Propolis/Calcium hydroxide 1:3 Propolis/Calcium hydroxide 1:4	,031
72 hours Propolis/Calcium hydroxide 1:3 Propolis/Calcium hydroxide 1:4	,073

	Sig. (2-tailed)
24 hours Propolis/Calcium hydroxide 1:3 Propolis/Calcium hydroxide 1:5	,027
48 hours Propolis/Calcium hydroxide 1:3 Propolis/Calcium hydroxide 1:5	,012
72 hours Propolis/Calcium hydroxide 1:3 Propolis/Calcium hydroxide 1:5	,030

CONCLUSION

$Ca(OH)_2$ gives a better inhibitory reaction compared to propolis against *E. faecalis* at the first 24 hours of exposure but at 48 hours and 72 hours it is exceeded by the combination of propolis/$Ca(OH)_2$ 1:3.

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