In Vitro Antimicrobial Activity of Propolis in Comparison with Calcium Hydroxide against Enterococcus Faecalis

Zohre Ahangari[1]* Gita Eslami[2] Setare Ghannad[3]

*[1]Corresponding Author: Associate Professor, Dept. of Endodontics, Dental School, Shahid Beheshti University of Medical Sciences, Tehran- Iran. E-mail:zohreahangari@sbmu.ac.ir
[2]Associate Professor, Dept. of Microbiology, Medical School, Shahid Beheshti University of Medical Sciences, Tehran- Iran.
[3]Dentist

Abstract

Objective: Propolis, a natural product of the honeybee, is currently used as an anti-inflammatory and antimicrobial agent. Using different antibacterial agents is an important step to reduce the number of microorganisms within the root canal and improve the endodontic treatment prognosis. The present in vitro study investigated the antibacterial efficacy of Propolis against *Enterococcus faecalis* compared to calcium hydroxide.

Methods: In this experimental study, 42 single-rooted human teeth were selected and their smear layer was completely removed after access cavity and canal preparation. After infecting the prepared canals with *Enterococcus faecalis* species, negative control group was used during sterilization period and in two groups of 18 each, canals were filled with 30%propolis extract and calcium hydroxide, respectively. No material was added to the positive control group. The specimens were stored in CO_2 incubator for 72 hours, 1 week and 1 month and afterwards, samples were taken from inside the canals and *Enterococcus faecalis* colonies were counted. Number of colonies at different time intervals was statistically analyzed using Kruskal-Wallis test. Mann-Whitney U test was used to compare the number of colonies after using the understudy medicaments.

Results: Number of colonies was 55,000±46,368.09 and 43,333.33±48,027.077 after incubation for 72 hours and using 30% Propolis extract and calcium hydroxide, respectively. After 1 week incubation, number of colonies was 166.67±408.25 in the Propolis group and zero in the calcium hydroxide group. No colonies were observed after 1 month incubation in both groups. No significant differences were noted between two medicaments at different time intervals.

Conclusion: In general, antimicrobial activity of Propolis against *Enterococcus faecalis* species was comparable with that of calcium hydroxide at different time intervals. Therefore, it can be used as an alternative natural material for disinfection of canals during endodontic treatment.

Key words: Antimicrobial activity, Calcium hydroxide, *Enterococcus faecalis*, Propolis

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Introduction:

During endodontic treatment, number of microorganisms within the root canals is reduced as much as possible using mechanical and chemical procedures (1, 2). However, there is a possibility that some of them are left in the canal. That is why various medications are placed inside the canals during the time period between treatment sessions (3-5). *Enterococcus faecalis* is a gram positive anaerobe and part of the mouth's normal flora. It is usually found in small numbers in root canals before preparation. Its role in the outcome of endodontic treatment has yet to be clearly determined but it seems that this strain is the most common bacteria detected in root canals that develop chronic apical periodontitis following endodontic treatment (6). This microorganism is capable of invading dentinal tubules and is resistant in various ecologic conditions. It also copes well with unfavorable conditions inside the root canal. These factors are the reason why this

microorganism is recognized as a resistant pathogen in endodontic treatments (7). The efficacy of various intracanal medicaments against *Enterococcus faecalis* has been evaluated in the literature and among the studied materials, calcium hydroxide has been introduced as the standard drug of choice against this pathogen (8-10). Although chlorhexidine, iodine, potassium iodide or their combination have also been used for this purpose.

Use of calcium hydroxide has some limitations because this drug is not capable of removing all microorganisms from the canal (11) and requires a long time to confer its antimicrobial activity (4). Due to its high pH, calcium hydroxide is potentially toxic and can also result in soft tissue destruction which per se can cause chronic inflammation and cell necrosis in the clinical setting (12). Propolis is a resinous mixture collected by the honey bees from botanical sources and is used for reinforcement of the structural stability of the hive and for disinfection and inhibition of bacterial growth in the beehive. Propolis is a complex mixture of various chemical substances with known biologic effects like antibacterial and antifungal activity and restorative characteristics (13).

Also, it has been revealed that calcium hydroxide in some cases has not been able to eliminate *Enterococcus faecalis* species from the root canal (14,15).This can result in higher bacterial colonization in root apex and periapical tissue and prevent/impair the process of healing which has a negative impact on endodontic treatment prognosis (16).

This study aimed at determining the antibacterial efficacy of Propolis in comparison with calcium hydroxide against *Enterococcus faecalis* in vitro.

Methods:

This study was conducted in vitro on 42 single-rooted and single canal human extracted teeth.

Standard preparation of specimens:

A total of 42 single root and single canal intact human teeth that had been recently extracted in the process of orthodontic treatment or because of periodontal problems were evaluated. Immediately after extraction, teeth were debrided and cleaned and all hard and soft tissues that were still attached to the teeth were carefully removed using scaling curettes. For topical disinfection, specimens were placed in 5.25% sodium hypochlorite (Household Bleach, Shamin Chemistry Co. Tehran, Iran) for 30 minutes. Then the teeth were stored in sterile 0.9% physiological saline (0.9% sodium chloride solution, DarooPakhsh Co. Tehran, Iran) at room temperature until the time of study. At first, a standard access cavity was prepared on the teeth. Then, endodontic files sizes 10 and 15 (Maillefer, Switzerland) were used to make sure that the roots had only one canal and the canal was patent. In order to decrease the effects of confounding factors all canals were primarily prepared using similar mechanical and chemical procedures. In order to do so, first a coronal preflaring was done using Gates Glidden drills numbers 2 and 3 (Maillefer, Switzerland) in an orderly fashion with no lateral pressure and passive up and down motion. The full working length of the canals was then prepared up to size 45 with hand instrumentation (Maillefer Swiss). Recapitulation was performed between every 2 files with hand file size 15. Irrigation was done thoroughly with 5.25% sodium hypochlorite.

Smear layer removal:

In order to completely eliminate the smear layer, samples were placed in an ultrasonic bath (Ultrasonic Cleaner, Vector 55, Jelenko, Jelcraft). In this process, teeth were first placed in EDTA (ethylene diaminetetraacetic acid) 17% at a pH of 7.8 for 4 minutes and then another 4 minutes in 5.25% sodium hypochlorite (NaOCl) and were eventually irrigated with sterile

distilled water for 10 minutes.

Sterilization of samples:

The teeth were separately placed in 2 ml micro tubes containing 0.5 ml brain heart infusion broth (BHI) and then were sterilized in an autoclave for 20 minutes at 121°C and 15 psi pressure. Samples in aseptic condition were separately incubated in micro tubes containing BHI for 24 hours in aerobic conditions at 37°C to reassure sterilization. After that, 3 samples were randomly selected and a microbial culture was prepared from their BHI. No bacterial growth was considered as the proof of correct sterilization (negative control). After confirmation of sterilization, all phases of the experiment were performed in aseptic conditions using sterile gloves and instruments.

Procurement of *Enterococcus faecalis*:

In this study, in order to induce a controlled and standard infection in all samples a resistant bacterium that is mainly responsible for the failure of endodontic treatments named *Enterococcus faecalis* was employed. This gram positive facultative anaerobic microorganism was procured from the Microbiology Department of Shahid Beheshti University of Medical Sciences with the ID number ATCC29212.

Infecting the samples:

Enterococcus faecalis was cultured in BHI medium in CO_2 incubator at 37°C for 48 hours. A pure suspension of *Enterococcus faecalis* bacteria with a concentration of $1.5X10^8$ CFU/ml was prepared using spectrophotometry with a turbidity adjusted to be equivalent to a 0.5 McFarland BaSo4 standard close to the flame. Using sterile insulin syringes, the standardized bacterial suspension was injected into the canals in similar portions (0.5CC). Then, all microtubes were stored in an incubator at 37°C in aerobic conditions for 72 hours.

Some other 2 ml micro tubes containing sterile BHI were prepared in numbers equal to that of samples. These micro tubes were used at the phase of taking samples from inside the canals.

Preparation of the understudy materials (Propolis 30% and calcium hydroxide):

For preparing Propolis 30% 7 gr ethanol 96% was combined with 3 gr Propolis and Chromafil CA-20/25 filter was used for elimination of the impurities of the obtained 30% solution (Figure1). Calcium hydroxide was also prepared in combination with normal saline and with creamy consistency.

Figure 1- Propolis 30%

Evaluation of the effect of understudy materials on the specimens:

After completion of the incubation period, the teeth specimens were divided into 6 groups of 6 teeth each and one positive control group of 3 teeth. Samples were extracted from the micro tubes using sterile gloves and instruments and were fixed using a hemostat. Understudy materials (Propolis 30% and calcium hydroxide) were then inserted into the canals according to the following grouping. Insulin syringe was used for injection of Propolis, and calcium hydroxide was carried into the canal using a hand file.

Group 1: Addition of Propolis 30% and storing the specimens in the CO_2 incubator for 72 hours

Group 2: Addition of calcium hydroxide and storing the specimens in the CO_2 incubator for 72 hours

Group 3: Addition of Propolis 30% and storing the specimens in the CO_2 incubator for 1 week

Group 4: Addition of calcium hydroxide and storing the specimens in the CO_2 incubator for 1 week

Group 5: Addition of Propolis 30% and storing the specimens in the CO_2 incubator for 1 month

Group 6: Addition of calcium hydroxide and storing the specimens in the CO2 incubator for 1 month

Three specimens in the positive control group were stored in the CO_2 incubator without any additives according to the following timing:

Sample 1 for 72 hours
Sample 2 for 1 week
Sample 3 for 1 month
Taking samples from inside the canals:

After completion of the required time period, #2 peeso reamer was used with the aim of observing and evaluating the microorganisms that had penetrated into the dentin. Peeso reamer was guided down until reaching the working length in one move. By doing so, we were able to collect debris from the whole length of the canal. This phase was also performed under same sterile conditions and close to the heat. Debris on the peeso reamer was quickly transferred into the microtubes containing sterile BHI that had been prepared earlier using a sterile hand file. Microtubes were stored for 48 hours in CO2 incubator. After this time period, samples were removed and cultured in bile esculin agar medium (Figure2). After 72 hours, colonies were counted, *Enterococcus faecalis* was observed under the light microscope and type of bacteria was confirmed (Figure3). Presence of other bacteria was also evaluated for detection of any possible contamination during the process the result of which was negative (17,22).

Figure 2- *Enterococcus faecalis* on bile esculin agar medium

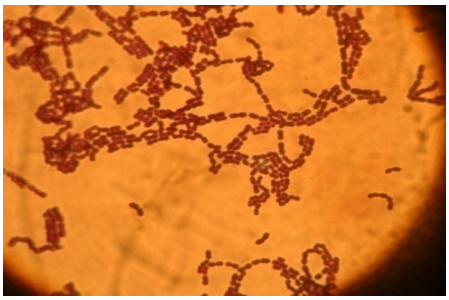

Figure 3- Confirming the presence of *Enterococcus faecalis* species under light microscopy

Number of counted colonies was compared statistically based on the factor of time using non-parametric Kruskal-Wallis test. Comparison of the antimicrobial effect of calcium hydroxide and Propolis in the mentioned time periods was done using non-parametric Mann-Whitney U test.

Results:

Number of counted colonies when using Propolis 30% for 72 hours in the incubator was 55, 000±46,368.09 CFU. This number was 43, 333.33±48,027/077 CFU when using calcium hydroxide in the same conditions. One week after storing the specimens in the incubator,

number of colonies was 166.67±408.25 CFU when using Propolis and zero when using calcium hydroxide. No *Enterococcus faecalis* colonies were detected 1 month after storing specimens in the incubator in either of Propolis extract or calcium hydroxide treated teeth (Table1).

Table 1: Number of counted colonies when using propolis and calcium hydroxide in different time periods of storage in the incubator

Time duration	Drug	Mean	Standard deviation	Minimum	Maximum
72 hours	Propolis	55,000	46368.09	0	100,000
72 hours	Calcium hydroxide	43333.33	48027.077	0	100,000
1 week	Propolis	166.67	408.25	0	1000
1 week	Calcium hydroxide	0	0	0	0
1 month	Propolis	0	0	0	0
1 month	Calcium hydroxide	0	0	0	0

Results of Kruskal-Wallis test showed a significant difference in the number of *Enterococcus faecalis* colonies based on the time of storing the specimens in the incubator ($P<0.001$) in a way that by increasing the incubation time from 72 hours to 1 week and 1 month, number of colonies decreased significantly.

Statistical comparison of the number of counted colonies after each incubation time and based on the type of drug used inside the canal is demonstrated in Table 2. In none of the incubation times of 72 hours (Mann-Whitney U test, $P> 0.613$), 1 week (Mann-Whitney U test, $P> 0.317$) and 1 month (Mann-Whitney U test, $P=1$) a significant difference was observed between Propolis extract 30% and calcium hydroxide in terms of number of *Enterococcus faecalis* colonies.

Discussion:

The present study showed that Propolis extract 30% collected from Azerbaijan province districts was similar to calcium hydroxide, the standard intracanal medication, in terms of antimicrobial activity against *Enterococcus faecalis* strains although its antimicrobial properties was slightly lower than that of calcium hydroxide. Therefore, if all properties of Propolis extract are proved safe, we can use this natural material in root canals with no specific complication or side effects. Calcium hydroxide has many applications as an intracanal medicament. However, it has been demonstrated that *Enterococcus faecalis* shows resistance against it (10). This was also approved in a study by Awawdeh et al, in 2009. Also, *Enterococcus faecalis* species can stay for long inside dentinal tubules (9). At the same time, *Enterococcus faecalis* can stay alive as a single microorganism without the help of any other species inside the root canal (21). This microorganism can stay alive in dentinal tubules and along with invading the tubules, it can be attached to the collagen in presence of human serum (27). Elimination of smear layer can also result in penetration of *Enterococcus faecalis* deep into the dentinal

tubules (28).

Calcium hydroxide has favorable biological properties as well. It plays a role in neutralizing bacterial lipopolysaccharides and anti-resorption process and also helps the formation of hard tissue (29). On the other hand, it has been determined that inefficiency of calcium hydroxide occurs only in-vitro and may be different from what occurs in a clinical setting. Various clinical and para-clinical methods have been employed for evaluation of the antimicrobial effects of intracanal medications. Although it is not possible to completely generalize the results of laboratory methods to the clinical setting, using these results for comparison of different drug regimens, screening of dental materials and practical techniques is the focus of attention in many researches. This methodology can also explain the related controversies mentioned in various studies (21).

Due to the presence of numerous confounding factors in relation with the results, intense quality control is necessary during antimicrobial susceptibility testing. In our study, this was achieved by using reference microorganisms including *Enterococcus faecalis* species (ID # ATCC29212). Control microorganisms at ideal conditions possess susceptible MIC end points and show little desire to change their susceptibility patterns in time (26). Therefore, in the present study, only one microorganism with a specific ID was used.

Propolis extract contains compounds like aldehyde, aliphatic acid ester, carboxylic acids, cinnamic acid and its esters, ketone, terpene, alcohol, ether, hydrocarbon, and phenolic resin each having their own antibacterial effect (17). In addition, synergy between these compounds along with unique properties of each constituent is effective in occurrence of the antibacterial effects of Propolis. Also, it has been found that each of the constituents of Propolis alone is effective against microorganisms but Propolis itself has greater antibacterial activity against pathogenic strains when compared to its constituents (18-20).

Awawdeh et al. (2009) showed that Propolis extract 30% (commercial product) has optimal effects against endodontic microorganisms when used as an intracanal medication (21). In this study, no bacterial growth was observed in the first and second day following application of Propolis. Since the ingredients and constituents of Propolis used by Awawdeh in his study are not clearly known, we cannot attribute the mentioned antimicrobial effects to Propolis alone. Propolis's antimicrobial properties have also been evaluated in other studies the results of which, overall, are in accord with those of ours (13,22,23).

Oncag et al. (2006) showed that Propolis was efficient against *Enterococcus faecalis* (22) which is in agreement with our finding. In his study, ethanolic extract of Propolis was used in root canals of human teeth contaminated with *Enterococcus faecalis*. Based on the results of this study, propolis was the most efficient intra-canal medication after 10 days. Its effects were similar to those of chlorhexidine gel 1% and significantly higher than that of calcium hydroxide. Kayaoglu et al. (2011) demonstrated that antimicrobial activity of Propolis against *Enterococcus faecalis* strains was lower than chlorhexidine 2% and more than calcium hydroxide (17). When comparing the reported results of the recent studies it is found that antibacterial properties of Propolis against *Enterococcus faecalis* strains in our study were slightly limited. In the previous studies, Propolis had antimicrobial effects greater than calcium hydroxide; whereas, in our study Propolis's antimicrobial activity was similar to that of calcium hydroxide and no significant difference was detected between their activities against

Enterococcus faecalis strains. These differences can be due to the ingredients and constituents of the Propolis used and generally the high content of flavonoids in the mentioned studies. In Oncag et al, study, the flavonoid content was estimated as 52% (13, 22).

In Awawdeh et al, (2009) study, antibacterial effects of calcium hydroxide were less than Propolis 30%. This finding is in contrast with our finding (21). It seems that the criteria for comparison of calcium hydroxide and Propolis in the mentioned study are not acceptable because sufficient time was not given to the medicament to work against microorganisms. However, the minimum time period required for the calcium hydroxide to reach its maximum antimicrobial effect has yet to be determined (4). Safavi et al. (1990) demonstrated that infected dentinal specimens were free from *Enterococcus faecalis* strains 24 hours after application of calcium hydroxide (24). On the other hand, Sjorgren et al. (1991) reported that calcium hydroxide as intracanal dressing should stay for 7 days in order to enhance its antimicrobial activity (4). That is why in the present study, we evaluated the antimicrobial effects of Propolis 30% and calcium hydroxide up to 1 month after storage in the incubator. Similar to what was reported by Sjorgren et al, (1991), in our study, 1 week after application of calcium hydroxide as intracanal dressing no *Enterococcus faecalis* strain colony was observed (4). On the other hand, it has been reported that dentin powder in vitro had preventive effects on calcium hydroxide's function and totally inactivated it (25). No such phenomenon was observed in our study.

Given all of the above, after approving all characteristics and properties of Propolis and evaluation of its benefits in a clinical setting, this substance may be appropriate for use alone or in combination with calcium hydroxide in root canal treatments.

Conclusion:

Antibacterial properties of Propolis against *Enterococcus faecalis* strains were similar to those of calcium hydroxide in different incubation times. Therefore, if all of its characteristics are approved, this natural material can be used for root canal treatments with no specific complication or side effects.

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