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Full Length Research Paper

Antibacterial activity of propolis and $Ca(OH)_2$ against *Lactobacillus*, *Entrococcus facalis*, *Peptostreptococcus* and *Candida albicans*

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Propolis has been shown to be possibly an appropriate alternative as an intracanal medicament due to its antibacterial properties. The aim of present study was to compare the activity of calcium hydroxide and propolis against *Lactobacillus*, *Enterococcus faecalis*, *Peptostreptococcus* and *Candida albicans*. This experimental study was conducted to evaluate antimicrobial activity of ethanol extract of propolis and calcium hydroxide ($Ca(OH)_2$) powder mixed with saline solution. Agar diffusion test and dilution methods were used to compare the results. There were separate plates to control diffusion of two substances in agar and antimicrobial activity of solvents. Figures of diameter of inhibition zone and minimal inhibitor concentration (MIC) and minimal bacterial concentration (MBC) were calculated. Paired T-test was used to compare the MIC differences. Propolis was more effective against *Lactobacillus*, *E. faecalis* and *Peptostreptococcus* with 8.6984 mm compared with 7.0833 mm mean diameter of inhibitory zone for $Ca(OH)_2$. The difference was statistically significant ($P<0.001$) indicating that $Ca(OH)_2$ was less effective against experimental microorganisms. The inhibitory zone of the two drugs demonstrated stronger effect of propolis on contaminating microorganisms. The MIC of propolis for all studied microorganisms was at least 4 times less than calcium hydroxide. Propolis was more effective than calcium hydroxide against *Lactobacillus*, *E. faecalis* and *Peptostreptococcus*. In ADT For *C. albicans*, larger inhibition zone observed around calcium hydroxide, could be due to low diffusion potentiality of propolis in agar compared to calcium hydroxide whereas, MIC demonstrated higher antifungal activity for propolis. Propolis was more effective against *C. albicans* in spite of its smaller inhibitory zone.

Key words: Propolis, calcium hydroxide, *Lactobacillus*, *Enterococcus faecalis*, *Peptostreptococcus*, *Candida albicans*.

INTRODUCTION

Microorganisms are the most important causes of the root canal treatment failures. During endodontic treatments, mechanical and chemical methods are used to decrease the large number of bacteria, however; some irritants possibly remain in the root canal due to the complex anatomy of the region (Bystrom and Sunndqvist, 1981; Perez et al., 1993). Therefore, the use of different intracanal medicaments has been recommended for the chemical cleaning of the root canal and to provide a favorable environment for healing (Leonardo et al., 1999; Sjogren et al., 1991; Siqueira, 1997).

Calcium hydroxide [$Ca(OH)_2$] has been used for apexification of the pulpless teeth, dentine bridge formation and as intracanal medicament for many years. However, it is potentially toxic due to higher pH levels

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and capable to destruct the soft tissues, all these may possibly lead to chronic inflammation and cellular necrosis in the clinical environment (Ferreira et al., 2002).

In addition, it has been shown that the compound is not so effective against certain microorganisms in *in vivo*. This inefficacy may be related to the drug inability to catch bacteria and the buffering capacity of the blood, tissue fluids and dentin. Some antimicrobial investigations showed special bacterial species to be resistant against calcium hydroxide too (Podbielski et al., 2000; Siqueira and Uzeda, 1998). So, the research for new and safe medicaments in the endodontic treatments would be beneficial to obtain the least irritation and maximum antibacterial effectiveness.

Propolis, a beehive resinous complex, showed good capacities in this regard. Propolis is a Greek term formed of “pro” meaning “against” and “polis” which means “city” or “defender of city”. The bees take the resin back to the hives and work on it to produce a glue-like substance with which they fill cracks and seal up their hives. Higher temperature, small size and wetness of the hive make it an ideal place for bacterial growth, however; the microorganisms do not grow because the antibacterial properties of propolis. Propolis is a complex mixture of chemical components with over than 180 compounds being identified, with constituents including bioflavonoid, the highly active bio-chemical compound, and phenol acid being responsible for biological activities of propolis (Sonmez et al., 2005).

Flavonoids are known herbal compounds with the approved antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory activities and anesthetic actions (Al-Shaher et al., 2004). Furthermore, it is toxic against tumoral cells, develops cartilaginous and bone tissue regeneration, prohibits stomach ulcer and tooth decay, and has immunomodulatory properties (Marcucci, 1995).

Park et al. (1998) concluded the existence of different flavonoids within the propolis composition and its inhibitory effect against tooth decay inducing bacteria (Park et al., 1998). The product inhibits prostaglandin synthesis, activates thymus gland, and improves immune system by means of increased phagocytes activities. It has also analgesic and anti-tumoral properties (Park et al., 1998). In addition, some elements like iron and zinc have been identified as its constituents participating in the collagen synthesis and improving healing properties of epithelial tissues in turn (Al-Shaher et al., 2004).

Due to the known properties of propolis and its lower cellular cytotoxicity compared to calcium hydroxide; the compound may be an appropriate alternative as an intracanal medicament. Al-Shaher et al. (2004) reported cytotoxicity of calcium hydroxide on the pulp and periodontal fibroblasts to be ten times higher than propolis.

The objective of the present study was to determine the antibacterial and antifungal effect of propolis and calcium hydroxide on *Peptostreptococcus*, *E. faecalis*, *Lactobacillus* and *C. albicans* species using microbiologic dilution and agar diffusion tests (ADT).

MATERIALS AND METHODS

The tested medicaments in this study were calcium hydroxide (BP, Germany) solution and propolis. *Peptostreptococcus* strain was obtained from dental abscesses of patients referred to Dental school of Shahid Beheshti Medical Sciences University. The next three standard bacterial strains were obtained from the microbial bank of Medical School; Shahid Beheshti University of Medical Sciences, Tehran, Iran.

The solid propolis, obtained from the province of Azerbaijan, northwest Iran, was stored desiccated in the dark till it was used. Then, the propolis was crashed into small pieces using a shaver. Its small pieces were mixed with 96% ethanol by shaking for 3 days in a shaker. The ethanol extract of the propolis was obtained with creamy appearance and brownish color using distillation technique in vacuum. Calcium hydroxide solution was also used together with physiologic serum in this study.

Agar diffusion test (ADT)

In this test, wells were created in the blood agar with 6 mm diameter and 5 mm depth. The wells were filled with propolis extract or calcium hydroxide as it is used for the rootcanal treatments according to the manufacturer (BP Germany) instructions. After 24 and 48 h, the maximum inhibitory zone of the wells was calculated. For *Peptostreptococcus* strains, anaerobic conditions were used. Control plates with both compounds, were used for diffusion potentiality determination.

Dilution test

In this method, the effect of the serial concentrations of both compounds, prepared with standardized techniques, was assessed on the bacteria strain suspension with 0.5 MacFarland standard concentrations (1.5×10^6 CFU/ml of bacteria in TS Broth 0.5 ml of prepared serial compound concentrations were spread on Mueller Hinton and Blood agar plates, the compound concentrations were tagged at the back of each plate, before incubation in 37°C for 24 h. 0.5 ml of McFarland microbial suspension were added to prepared serial compound plates, before incubation in 37°C for 24 h. The bacterial growth was determined by observation, without using any equipment. The minimum inhibitory concentration (MIC) was defined as the lowest concentrations that did not result in any visible growth of the microorganisms compared with the growth in the control plates. The minimum bactericidal concentration (MBC) was also determined by spreading samples from each compound with a concentration equal or higher than the MIC onto the surface of blood agar plates. MIC and MBC were calculated for calcium hydroxide and Propolis extract. The plates were anaerobically incubated at 37°C for 48 h to establish the MBC. Control plates were used together with the experimental plates. The MBC was determined to be the lowest concentration that precluded bacterial growth on the agar plate.

The mean values of inhibitory zone, MIC and MBC were calculated for each compound and analyzed by paired t test.

RESULTS

ADT showed similar inhibitory zone for propolis and calcium hydroxide against *Lactobacillus* strains with the

Table 1. Mean and standard deviation of inhibitory zone of propolis and calcium hydroxide in different microorganisms and times.

Time (h)	Material	Bacterial strain	Mean	Standard deviation
24	Propolis	*Enterococcus faecalis*	9.7661	0.38925
	Calcium hydroxide		6.0	0.0
	Propolis	*Peptostreptococcus*	8.7619	0.63365
	Calcium hydroxide		6.7661	0.38925
	Propolis	*Lactobacillus*	9.7667	0.65134
	Calcium hydroxide		9.7667	0.88763
	Propolis	*Candida albicans*	7.125	0.43301
	Calcium hydroxide		6.0	0.0
48	Propolis	*Enterococcus faecalis*	9.5833	0.46872
	Calcium hydroxide		6.0	0.0
	Propolis	*Peptostreptococcus*	8.1667	0.38925
	Calcium hydroxide		5.8333	0.38925
	Propolis	*Lactobacillus*	8.4167	0.66856
	Calcium hydroxide		7.0	1.04447
	Propolis	*Candida albicans*	6.875	0.22613
	Calcium hydroxide		10.0	0.85280

mean values of 9.6667 mm (p=1) after 24 h. However, the inhibitory zone of propolis was significantly greater than calcium hydroxide solution after 48 h (n=12; 8.4167 mm vs. 7.0 mm: p<0.0001).

For *Peptostreptococcus* strains, the mean inhibitory zone of propolis was also significantly greater than calcium hydroxide on 24 (8.9167 mm vs. 6.1667 mm; p<0.0001) and 48 h (8.0 mm vs. 6.1667 mm; p<0.0001).

Similarly, for *E. faecalis* strains, the mean inhibitory zone of propolis was significantly greater than calcium hydroxide on 24 (9.1667 mm vs. 6.0 mm; p<0.0001) and 48 h (9.5833 mm vs. 6.0 mm; p<0.0001).

The mean inhibitory zone of propolis for *C. albicans* was 7.1250 and 6.8750 mm after 24 and 48 h which was significantly smaller than the mean inhibitory zone of calcium hydroxide with mean values of 10.0 and 9.0 mm for 24 and 48 h (p<0.0001).

Mean and standard deviation of inhibitory zone of propolis and calcium hydroxide in different microorganisms and times are depicted on Table 1.

After calculation of the inhibitory zone, the cultures of both agents surrounding area were made again to control absolute microorganism killing effect on blood agar medium. Therefore, the complete cleaning of the inhibitory zone and the strain inability to growth were assessed. As suggested by the results, re-culturing of the inhibitory zone created by calcium hydroxide were positive for all microorganisms. On the contrary, it was negative for the microorganisms in the inhibitory zone developed by propolis extract. Each plate contained two wells of propolis and calcium hydroxide simultaneously.

The experiments were repeated in some cases for bacterial contamination. In these cases, the inhibitory zone of Propolis extract did not show the presence of medium contaminant microorganisms, however, medium contaminant microorganisms did grow in the inhibitory zone of calcium hydroxide. Gram staining test demonstrated contaminant microorganisms to be predominantly *Staphylococcus*, gram positive *bacillus* and different fungal strains.

As shown by dilution test, all microorganisms did grow in the dishes containing calcium hydroxide with the concentrations of up to 512 µg/ml, which are four folds higher than maximum MIC for propolis extract (Table 2). In the control plates, the solvents of alcohol and physiologic serum used for preparing serial concentrations did not affect the bacterial growth.

DISCUSSION

The results suggested propolis extract to be more effective than calcium hydroxide regarding inhibition of *lactobacillus* strains growth after 48 h. Furthermore,

Table 2. Dilution test results regarding MIC and MBC of propolis and calcium hydroxide.

Bacterial strain	Propolis MBC (mg/ml)	Propolis MIC (mg/ml)	Calcium hydroxide MBC (mg/ml)	Calcium hydroxide MIC (mg/ml)
Enterococcus faecalis	128	64	512	512
Peptostreptococcus	64	32	512	512
Lactobacillus	16	8	512	512
Candida albicans	16	8	512	512

propolis showed statistically higher inhibitory effect than calcium hydroxide for *Peptostreptococcus* and *C. albicans* species after 24 and 48 h, although both medicaments were shown to inhibit and eliminate the tested strains.

According to ADT, calcium hydroxide was significantly more effective than propolis after 24 and 48 h, however, re-culture results of inhibitory zone developed on the calcium hydroxide surrounding areas together with its staining showed the area did not completely cleaned, being contaminated with *C. albicans*. Microorganism growth was not observed in the contaminated cultures for the propolis adjacent area; however, calcium hydroxide surrounding areas were subjected to microorganism growth. Furthermore, both materials diffused slightly in the agar medium while the propolis showed the least diffusion. In ADT technique, the drug toxicity and diffusion into the medium both affects the inhibitory zones of medicaments. Higher inhibitory zone of calcium hydroxide in the *C. albicans* culture medium than propolis can be justified in terms of its better diffusion in the agar. In spite of this fact, microscopic contamination of calcium hydroxide inhibitory zone suggests its incomplete antimicrobial properties. On the contrary, propolis showed complete antimicrobial efficacy in its inhibitory zone.

The present study showed antibacterial superiority of propolis than calcium hydroxide on three different aspects. Firstly, propolis had significantly higher inhibitory zone than calcium hydroxide regarding *E. faecalis* and *Peptostreptococcus* strains on both time intervals. It was so on *Lactobacillus* stains after 48 h too. Control plates containing both compounds, used for assessing compunds' diffusion potentiality, demonstrated calcium hydroxide medicament to be diffused more easily than propolis in the agar medium as shown by the control plates in the present study. That is to say, propolis was able to develop wider inhibitory zone than calcium hydroxide despite weak diffusion potentiality concerning *E. faecalis*, *Peptostreptococcus* and *Lactobacillus* species (after 48 h). Secondly, MIC results measured for propolis was significantly lower than calcium hydroxide for all microorganism strains (at least four folds). Thirdly, the re-culture results of *C. albicans* growth inhibitory zone demonstrated calcium hydroxide not to completely remove all *C. albicans* strains compared to propolis extract which effectively removed the microorganisms in its limited inhibitory zone. In addition, medium contaminant microorganism's inability to growth in the propolis surrounding area was definitely suggestive of propolis effectiveness against most of the remaining bacteria. Calcium hydroxide potential to easily diffuse in the agar is possibly the main cause of some differences being observed regarding ADT and MIC results; as the diffusion is not a determinant factor in MIC technique. Then, it failed to be a positive property for calcium hydroxide. Therefore, Propolis demonstrated higher antibacterial activity in terms of MIC results.

However, both agents demonstrated inadequate diffusion in the agar medium. In MIC technique, exact estimation of medicament inhibitory concentrations was not possible by observation due to the turbidity of the medium, so that, the tube containing must be cultured for bacterial growth assessments.

Al-Shaher et al. (2004) concluded that toxicity of calcium hydroxide to be 10 times more than propolis extract; as more than 75% of PDL fibroblasts and 90% of pulp fibroblasts were killed in its 0.4 µg concentrations. Propolis toxicity was evident in the concentrations of 4 µg/mL and lower being apparently higher than our findings. It seems that propolis application in its bacteria-effective concentrations to be significantly lower than its toxic concentrations.

Oncag et al. (2006) approved propolis efficacy as an intracanal medicament against *E. faecalis* strains in the root canals of extracted teeth resembling our findings and suggesting that it could be used as an alternative intracanal drug.

Santos et al. (2002) studied the antibacterial efficacy of propolis against oral anaerobic bacteria using a technique similar to ours and demonstrated all strains to be sensitive to propolis among which *Peptostreptococcus, Porphyromonas gingivalis* and *Prevotella intermedia* were the most sensitive strains. These results are similar to our findings.

Antibacterial properties of propolis had been justified in different methods. It possibly prevents bacterial cell division breaking down the bacterial walls and cytoplasm as it is so in some antibiotics (Takaisi-Kikuni and Schilcher, 1994). Kujumgiev et al. (1999) concluded the antibacterial, antifungal and the antiviral properties of propolis to be for the presence of flavnoids and esters of phenolic acids in its composition (Kujumgiev et al., 1999).

The use of different antimicrobial methodologies without standardization is associated with findings that cannot be compared. The agar diffusion test is not proposed for anaerobes due to their complex and slow growth; however, some investigators did use it (Stevens and Grossman, 1983; Gomes et al., 2002; Ohara et al., 1993). Calcium hydroxide results given by this method are poor (Stevens and Grossman, 1983; Gomes et al., 2002), probably due to its weak diffusion.

The agar diffusion test is viable when testing anti-microbials having a similar diffusion gradient to enable the comparison of the medicaments. However, in some tests using antibiotics, there is a poor correlation between diffusion-test inhibition zones and dilution-test MIC values (Koneman et al., 1997).

Calcium hydroxide showed good biologic and antimicrobial properties, being the chosen medicament for the intracanal use whenever possible. However, it does have some disadvantages such as its minimal period to act (Sjogren et al., 1991) and no inhibition of *E. faecalis* (Stevens and Grossman, 1983; Gomes et al., 2002), a microorganism that is able to develop in an alkaline pH up to 11.5 (Sjogren et al., 1991). Furthermore, the dentinal-tubule disinfection methodology presented poor $Ca(OH)_2$ results (Haapasalo and Qrstavik, 1987; Gomes et al., 2003), because the solutions and the hydroxyl ions must diffuse through the dentin and suppress its capacity to inactive alkaline pH. In *in vivo* studies, the results are more favorable when canal disinfection is done using $Ca(OH)_2$ (Sjogren et al., 1991; Bystrom et al., 1985).

In addition to antibacterial properties, calcium hydroxide possesses good biologic characteristics, for example, it participates in the bacterial lipo-polysaccharides neutralization and anti-resorption activities which is an important aid to form hard tissue (Siqueira and Uzeda, 1997). However, calcium hydroxide may show poor antibacterial characteristics in laboratory conditions which are significantly different than clinical environments. Some clinicians attempted to improve its antibacterial shortcomings by adding other medicaments such as chlorhexidine or camphorated monochlorophenol (CMPC) (Lin et al., 2003), although CMPC has been shown to be toxic in nature and chlorhexidine failing to possess adequate biologic properties as an intracanal medicament (Lin et al., 2003). The findings of the present study suggests propolis could be mixed with calcium hydroxide as an antibacterial agent and used as an intracanal dressing material ,provided its other properties be approved with further researches. Different forms of commercial propolis like powder, tablet and irrigating solutions with different concentrations are available in the market all could be used together with calcium hydroxide in endodontic treatments in the future. It must be said that lower concentrations of the medicaments are required for the inhibition of isolated bacterial growth than different strains of the associated bacteria, which is an important difference between the clinical and laboratory environments.

Organic and inorganic compositions of propolis have been reported to differ greatly depending on the area when bees collect the samples. Because of the changing plant variety and limited bee travels distance from the collected propolis places of deposit, the composition of propolis may alter in the same areas (Seidel et al., 2008). As a result, before testing propolis in laboratory and clinical studies, chemical analysis of propolis must be done.

Is spite of approved antibacterial efficacy of propolis, more laboratory and clinical studies are needed to be carried out to validate findings of the beneficial use of propolis in root canal treatments as other endodontic agents.

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Conclusion

The present study demonstrated higher antibacterial efficacy of Propolis compared to calcium hydroxide.

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