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In vitro antibacterial effects of glass-ionomer cement containing ethanolic extract of propolis on *Streptococcus mutans*

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ABSTRACT

Objective: The aim of this study was to evaluate the antibacterial property of glass-ionomer cement (GIC) containing propolis against *Streptococcus mutans* and its effect on the *in vitro S. mutans* biofilm formation.

Methods: Ethanolic extract of propolis (EEP) was prepared at two concentrations as 25 and 50%. Three different experimental GIC disks were prepared using pure liquid and liquid solutions diluted with 25 and 50 percent of EEP concentrations. Minimum inhibitory concentration (MIC) of EEP on the growth of *S. mutans* ATCC 25175 was determined by using agar dilution method. Agar diffusion test and an *in vitro S. mutans* biofilm assay for GIC disks with and without EEP were performed.

Results: MIC values of Turkish propolis for *S. mutans* ATCC 25175 was found as 25 µg/mL. Experimental GICs containing propolis exhibited inhibition zones and their dry biofilm weights were less than the pure GIC. The bacterial density was lower in the GIC containing 50% EEP.

Conclusions: A distinct antibacterial and antibiofilm efficacy of propolis containing GIC on *S. mutans* has been observed. Although further research is needed to show clinical results, antibacterial GIC containing propolis would be a promising material for restoration. (Eur J Dent 2012;6:428-433)

Key words: Propolis; *Streptococcus mutans*; glass-ionomer cement; biofilm formation; antibacterial effect

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INTRODUCTION

Over the last few decades, a worldwide increase has been observed in the use of natural products for pharmacological purposes. Propolis, which is a natural product widely consumed in the folk medicine since ancient times, is a serious candidate to be added to topical formulations due to its antioxidant properties.[1] Besides antioxidant activity, epidemiological studies have also detected that propolis has many pharmacological prop-

erties, such as antibacterial, antifungal, antiviral, antitumor and anti-inflammatory among others.[2,3] In addition, this material is still used as a remedy in modern medicine due to a general "back to nature trend".

The antibacterial and antifungal properties of propolis have been extensively investigated and, although its chemical composition is linked to the phytogeographic origin, the activity of bee glue has always been reported.[4] Propolis may act against a wide range of bacteria, fungi, yeasts and viruses.[2,4]. The antimicrobial properties of this mixture of natural substances are mainly attributed to the flavonone pinocembrin, to the flavonol galangin and to the caffeic acid phenethyl ester, with a mechanism of action probably based on the inhibition of bacterial RNA-polymerase.[5]

Although propolis has shown variable activity against different bacteria and there are many products containing propolis on the world market such as ethanol extracts, toothpastes and mouth rinses, few studies have been made for the antibacterial activity of propolis on oral microorganisms.[6,7] Recent studies have shown the anti-caries potential of propolis.[7-9] Propolis samples reduced the incidence of caries and dental plaque accumulation *in vivo*.[9,10] Two action mechanisms have been associated with the anti-caries/anti-plaque properties of propolis: (i) antimicrobial activity against cariogenic bacteria (such as *Streptococcus mutans* and *Streptococcus sobrinus*), and (ii) inhibition of glucosyltransferase enzymes (GTFs) activity.[10,11]

The dental biofilm formation on tooth surfaces is a prerequisite for development of both dental caries and periodontal disease. The level of mutans streptococci (*S. mutans* and *S. sobrinus*) in dental biofilm is associated with caries development.[12] Caries is one of the primary causes of cast restoration failure, so an ideal luting agent would actively prevent caries at the restoration-tooth interface. The popularity of the glass ionomer luting cements is undoubtedly due to the fluoride release associated with these materials and the presumed benefit of reduced caries.[13] Glass ionomer cement has been shown to increase the fluoride ion concentration in the saliva in the short-term, *in vivo*.[14] When used as a restorative material, both conventional and the resin-modified glass ionomers have been shown *in vitro* to reduce artificial caries[15] and *in vivo* to remineralize carious lesions[16] and to enhance fluoride uptake by underlying dentin.[17] Under these circumstances, we hypothesize that propolis, a well known anticariogenic material, could augment the anticariogenic properties of GIC. The aims of the present study were to investigate: (i) antibacterial effect of Turkish propolis on *S. mutans*; (ii) antibacterial efficacy of GIC containing propolis on *S. mutans* and (iii) effect of GIC containing propolis on *S. mutans* biofilm formation.

MATERIAL AND METHODS

Preparation of Ethanolic Extract of Propolis

Propolis samples were produced by honeybees (*Apis mellifera* L.) in the region of Yomra, Trabzon, Turkey, rich in Picea orientalis, *Fagus orientalis,Castanes sativa, Rhodddendron ponticum, Rhododendron luteum, Rubus caucasicus.*[18] Propolis was provided by Trabzon Agricultural Development Cooperative. Hand collected propolis were kept desiccated and in the dark up to their processing. The samples were grinded (Ultra-Centrifugal Mill ZM 200, Retsch GmbH, Germany) and bottled in 10 g portions. They were then dissolved in 20 mL of ethanol (70%, w/v) by magnetic mixer for 48 h at room temperature. Lastly, rough particles were removed from propolis extract by filter.

Bacterial Strain and Inoculum Preperation

Streptococcus mutans ATCC 25175 type strain was used throughout the study. Bacteria were cultured overnight at 37°C in the Brain Heart Infusion Broth (BHI, Merck KGaA 64271 Darmstadt, Germany) and used as inoculum. The turbidity of the suspension was adjusted to the McFarland 0.5 turbidity standard (Densimat, BioMerieux, France).

Determination of the Minimal Inhibitory Concentration (MIC)

The agar dilution method was used as recommended by the Clinical and Laboratory Standards Institute.[19] Serial two-fold dilutions of EEP were prepared in Mueller-Hinton agar (MHA, Oxoid Ltd, Basingstone, Hempshire, UK) supplemented with 5% defibrinated sheep blood. Agar dilutions ranged from 0.1 to 50 µg/mL. Each antimicrobial test was also re-performed with plates containing the culture medium plus ethanol as solvent control. Each plate was then inoculated with a multipoint inoculating device (Steers replicator), which delivered a final inoculum of approximately 10^5 CFU per spot. The inoculum size was verified

by plating serial dilutions of the inoculum and performing colony counts. All experiments were performed in duplicate while the MIC of EEP was determined.

Preparation of GIC Containing Propolis

The conventional GIC (Kavitan Pro, SpofaDental, Czech Republic) was used in this study. The test materials were: (i) GIC (Powder^{GIC}: Liquid^{GIC} ratio=1:1), (ii) 25% EEP added GIC (PGIC: LGIC:LEEP ratio=1:0.75:0.25) and (iii) 50% EEP added GIC (P^{GIC}: L^{GIC}:LEEP ratio=1:0.5:0.5). EEP was added after mixing powder and liquid of GIC. After mixing the powder and liquid of each cement, pastes were put into cylindrical molds (10 mm in diameter and 2 mm thickness), and the upper surface was flattened by pressing down with a glass slide. At 230C and 50% humidity, a Vickers needle (300 g, 1.12 mm in diameter) was placed onto the surface of the cement every 15 s, and the surface was examined for any imprint left by the needle. Tests were repeated three times for each material.

Agar Disk Diffusion Test

The GIC disks were placed onto a MHA supplemented with 5% defibrinated sheep blood agar plate and inoculated with 0.1 mL of inoculum. Plates were incubated at 37°C for 48 h, and diameters of inhibition zones produced around specimens were measured using a digital caliper (Mitsutoyo, Tokyo, Japan) at three different points. Sizes of inhibition zones were calculated by subtracting the diameter of the specimen (10 mm) from the average of the three halo measurements. The procedure was modified from Takahashi et al.[20] Tests were repeated three times for each material.

In vitro S. mutans Biofilm Formation Assay

Bacterial Culture: The stock culture was prepared in 60 mL Tripticase Soy Broth (Merck KGaA 64271 Darmstadt, Germany) supplemented with 5% (w/v) sucrose. S. *mutans* ATCC 25175 type strain concentration of the suspension was determined as 1x10^5 cfu/mL.

Processed Saliva: Unstimulated whole human saliva was used to coat the cement disks. The saliva was collected from the same person and stored at -20°C before preparation. The saliva was centrifuged at 12.000xg for 15 minutes. The saliva supernatants were decanted, treated at 60°C for 30 minutes and filtered by passage through a 0.2-µm cellulose acetate membrane filter.[21] The efficacy of filtration was assessed by culture methods.

Biofilm Formation: To allow the formation of salivary pellicle, each disk was placed in sterile plastic Petri dishes containing the processed saliva and incubated by shaking at 37°C for one hour. The pellicle-coated disks were then rinsed twice in sterile PBS. The disks were placed in Petri dishes containing 20 mL of the stock culture and incubated in 5% CO_2 at 37°C for 42 hours. The non-adherent cells were removed by washing the disks with sterile saline solution. To collect the biofilm, the deposits were carefully removed with sterile scalpels.

Assessment: To measure the dry biofilm weight (biomass), the collected biofilm was placed on the pre-weighed glass microcoverslips. The final weight was recorded after incubation at 60°C for 5 minutes. The dry weight was obtained by subtracting the weight of coverslip from the final weight.

To measure the number of viable bacteria, the collected biofilm was suspended in 4 mL 0.1 N NaOH. The suspension was vortexed for two minutes and sonificated for one minute. The optical density of the biofilm was determined by a spectrophotometer (Pharmacia LKB-Ultrapec II, UK) at 640nm.

Statistical Analysis

Statistical analysis was performed by Kruskal – Wallis test, at 95% confidence level using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The MIC value of the EEP was found as 25 µg/mL. According to disk diffusion test results, the experimental GICs containing EEP exhibited inhibition zones (Table 1). The inhibition zone sizes were not dependent upon the concentration of propolis. The pure conventional GIC did not show any antibacterial efficacy against *S. mutans*.

According to the *in vitro* biofilm formation assay, the experimental GICs containing EEP developed less biomass (dry-weight) on their surface than the conventional GIC (P<.001). The mean biomass amount of biofilm formed on the 50% EEP added GIC was lower than the 25% EEP, but the difference was not statistically significant (Table 2). Ac-

cording to the optical density measurement of the formatted biofilm, the number of viable bacteria was lower in the GIC containing 50% EEP than the 25% and the conventional GIC (P<.001) (Table 3). As can be seen in Table 3, the mean bacteria count in the biofilm on the 25% EEP added GIC was lower than the conventional GIC but the difference was not statistically significant.

DISCUSSION

GICs are capable of releasing fluoride, which contributes to some reduction in the number of residual bacteria in cavities[6,7,17] as well as remineralization of softened dentin.[22-25] Several attempts in developing GICs with antibacterial effects by the addition of antibacterial solutions such as chlorhexidine (CHX) have been reported.[25-30] With regard to these studies, we decided to use EEP, which showed remarkable antimicrobial activities against several oral microorganisms such as *mutans* streptococci in recent studies.[7,8,11,31]

Previous studies using conventional GICs demonstrated conflicting results about antibacterial effects observed by the addition of CHX. Some of the studies reported that antimicrobial activity was dependent upon the concentration of disinfectant added to GICs, [25,27,30] and others indicated no dose-response effects.[26] Beside its antimicrobial activity, propolis is considered to be safe in low doses. The cytotoxicity tests have showed that the propolis solutions, even the propolis used in our study, are safe for gingival fibroblasts.[32] The chemical composition of propolis is complex and highly variable depending on its geographical origin. There are several studies on antibacterial activity of Turkish propolis.[33-36] The antimicrobial assays in this study demonstrated that the propolis collected from Trabzon/ Turkey has significant activity against *S. mutans* growth, showing a MIC value of 25 µg/mL. EEP should be kept as low as possible, as the EEP does not contribute to the formation of the glass ionomer network, and therefore, high amounts of EEP would weaken the scaffold and compromise the physical properties of the antibacterial glass ionomer. According to disk diffusion test, we found that sizes of inhibition zones produced against *S. mutans* were not dependent upon the concentration of EEP, which means that antibacterial activities were not effectively enhanced by increasing concentrations of the agent. So it would be more appropriate to use 25% EEP addition for development in antibacterial GICs.

Bacterial adherence to tooth and restoration surface is a very important stage in the pathogenesis of dental caries and soft tissue inflammations. The adherence mechanism of bacteria to the tooth

Table 1. The inhibition zone diameters of the GIC disks (mm).

GIC type	Control	25% EEP	50% EEP
n	7	7	7
Mean	0,3	0,7	0,8
Standard Deviation	0,1	0,1	0,1
Median	0,3	0,7***	0,8***

***Statistically significant (P<.001)

Table 2. The dry biofilm weights of the GIC disks (mg).

GIC type	Control	25% EEP	50% EEP
n	13	14	14
Mean	0,5846	0,2429	0,1643
Standard Deviation	0,3262	0,1555	0,1336
Median	0,5	0,2***	0,2***

***Statistically significant (P<.001)

Table 3. The optical densities of the biofilms (OD_{640}).

GIC type	Control	25% EEP	50% EEP
n	13	14	15
Mean	0,028	0,018	0,01
Standard Deviation	0,016	0,007	0,008
Median	0,026	0,0175	0,009***

***Statistically significant (P<.001)

surface occurs with the formation of acquired salivary pellicle on the surface and adherence of bacteria the acquired pellicle.[37] A sucrose-mediated mechanism is a main route that facilitates bacterial adherence to surfaces. *S. mutans* is capable of synthesizing mainly glucans from sucrose, which promotes a high adhesion and eventually contributes to the formation of dental biofilm.[12] Because of the fact that the bacteria in biofilms are more resistant than their planktonic forms, the MIC does not provide information on the efficacy of antimicrobial agents against infections involving biofilms. *In vitro* biofilm assays can easily be used as a relevant model for testing the antiplaque properties of the oral restorative materials.

Duarte et al[31] showed that subinhibitory concentration of EEP was used to evaluate its action on biofilm formation. According to our biofilm formation assay, the GICs containing EEP developed less biomass on their surface than the conventional GIC. Although the dry biofilm weights were not significantly different on each EEP concentration, the bacterial density was lower in the GIC containing 50% EEP. The result means the concentration is important in terms of the bacterial load on dental biofilm. In general, GICs containing EEP with each concentration showed a potential antibacterial and anti-GTFs activity *in vitro,* but more EEP concentration is essential to reduce *S. mutans* in biofilms.

EEP solutions have been widely used commercially on the market as toothpaste, mouth wash etc. However, it is still an unofficial drug in pharmacy. A further step should be taken to verify if a sufficient dose to kill the target microorganisms can be reached within the oral cavity, without causing major local or systemic adverse effects. Even though we studied on the cariogenic microorganism, *S. mutans*, more studies with other bacterial strains which take place for the dental biofilm formation are still needed. The effective antimicrobial activity of propolis gives hope in the treatment of oral cavity diseases.

CONCLUSION

This is the first report that evaluated antibacterial efficacy of propolis containing GIC on *S. mutans*. By the results of this preliminary study, it can be concluded that GIC containing propolis would be a promising material for restoration. Reduction in bacterial counts obtained by placing GIC in a cavity probably due to the release of fluoride, is not reliable; therefore, antibacterial GICs containing propolis provides an alternative approach. However, further studies should be performed on its physicochemical aspects to know how the presence of EEP interferes with the mechanical and adhesive properties of GIC.

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