RESEARCH

Antimicrobial Effect of Propolis Drops On Oral Pathogens: An *In Vitro* Study

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ABSTRACT

Antimicrobial Effect of Propolis Drops On Oral Pathogens: An *In Vitro* Study

Background: The aim of this *in vitro* study was to evaluate the antimicrobial effectiveness of various propolis drops found easily in Turkey against two oral pathogens; Streptococcus mutans and Enterococcus faecalis.

Methods: Seven different propolis drops (Group 1 to 7: Umay Herbal Organic Propolis, Bee'o Up [15%], Propoli EVSP, Brazilian Green Liquid Propolis, Eğriçayır Propolis, Bee'o Up [30%], Biostore Propolis) were included in the study. Antimicrobial effect of drops was investigated by the agar plate diffusion method. Petri dishes were prepared with Brain-Heart Infusion broth. 100 µl of microorganisms and 10 µl of each propolis were planted to these petri dishes from a broth culture. Inhibition zones formed on the 24th hour were measured in mm's and each experimental group was photographed. Data were compared by Mann Whitney-U test.

Results: Only water-based propolis of the present study (Group 1) formed no inhibition zone against both oral pathogens and alcohol-based groups 4 and 5 formed no zone only against the latter. Group 5 showed significantly the highest antimicrobial effect against S. mutans, however that group showed no influence on E. faecalis. Group 6 was found significantly more effective on E. faecalis than other propolis drops ($p<0.05$).

Conclusion: All of the groups showed antimicrobial effect on either one of the oral pathogens except for the water-based propolis drop. Compared to E. faecalis, tested propolis drops had affected S. mutans more. Thus, propolis extracts could be used as a more natural and harmless prevention method to dental caries specifically.

KEYWORDS

Antimicrobial effectiveness, Inhibition zone, Oral pathogens, Propolis

ÖZ

Farklı Propolis Damlalarının Oral Patojenlere Karşı Antimikrobiyal Etkinliğinin Değerlendirilmesi: Bir *İn Vitro* Çalışma

Amaç: Bu *in vitro* çalışmanın amacı, Türkiye'de kolaylıkla bulunan çeşitli propolis damlalarının iki oral patojene (*Streptococcus mutans ve Enterococcus faecalis*) karşı antimikrobiyal etkinliklerinin değerlendirilmesidir.

Gereç ve Yöntemler: Çalışmaya yedi farklı propolis damlası (1'den 7'ye gruplar: Umay Herbal Organic Propolis, Bee'o Up [%15], Propoli EVSP, Brazilian Green Liquid Propolis, Eğriçayır Propolis, Bee'o Up [%30], Biostore Propolis) dahil edildi. Bakteriler 100 µl, her bir propolis örneği ise10 µl olacak şekilde yayma ekim tekniği ile Brain-Heart Infusion agar besiyerine ekildi. Petri kutuları bakteri üremesi için 24 saat inkübasyona bırakıldı ve sonuçta oluşan zon çapları ölçüldü. Her deney grubu fotoğraflandı. İstatistiksel analiz Mann Whitney-U testi kullanılarak yapıldı.

Bulgular: Su bazlı propolis (Grup 1) iki oral patojene karşı da inhibisyon bölgesi oluşturamamıştır. Alkol bazlı propolislerden ise, yalnızca 4. ve 5. gruplar *E. faecalis*' e karşı etkinlik gösterememiştir. S. mutanslara karşı en yüksek antimikrobiyal etki 5. grup'ta görülürken, aynı grup *E. faecalis* üzerinde herhangi bir etki gösterememiştir. 6. grup, *E. faecalis* üzerinde diğer propolis damlalarından anlamlı olarak daha etkili bulunmuştur ($p<0,05$).

Sonuç: Test edilen propolis damlaları *E. faecalis* ile karşılaştırıldığında, *S. mutans*ları daha fazla etkilemiştir. Su bazlı propolis grubu hariç, grupların tümünün, en az bir oral patojen üzerinde antimikrobiyal etkiye sahip olduğu görülmüştür. Bu nedenle, propolis ekstreleri özellikle diş çürüğünü önlemede daha doğal ve zararsız materyaller olarak kullanılabilir.

ANAHTAR KELİMELER

Antimikrobiyal etkinlik, İnhibisyon alanı, Oral patojenler, Propolis

The oral cavity contains various kinds of species including bacteria and fungi.[1] All of these compromises the oral microbiota on equilibrium and the excessive amount of any species could create oral diseases. Gathering of species to form oral biofilm is an important virulence factor in the oral cavity, where many microbial species share the same ecological field, compete for resources and thus, influence each other. To explain the importance, it is clear that oral biofilm is mainly responsible for many oral diseases.[2]

Regarding dental caries, the main etiological agent is *Streptococcus mutans* (*S. mutans*) among other Streptococcus groups which have been described and divided by gene sequence comparisons as pyogenic, mitis, anginosus, mutans, salivarius, and bovis.[2] *S. mutans* plays a central role in the development of cariogenic biofilms, mainly due to its acid-tolerant and acidogenic characteristics. This microorganism uses dietary sucrose for the carbohydrate fermentation process that creates a low-pH oral environment resulting in enamel and dentin demineralization.[3] Apart from *Streptococcus* species, *Enterococcus faecalis*

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(*E. faecalis*), which is the predominant human enterococcus, is also important for oral health, since it is mainly an indicator of failures of endodontic treatments.[4] *E. faecalis* localizes in root canals of devitalized teeth and is responsible for apical inflammation.[4,5] Moreover, *E. faecalis* has also related to oral diseases, such as caries, periodontitis, and peri-implantitis in certain studies as well.[6,7] Recently, Anderson et al. have reported that oral isolates had the highest percentages of virulence genes as well as extracellular enzymes and a capacity to form biofilms.[8] Due to *E. faecalis*' resistance to common endodontic irrigants, chlorhexidine (CHX) is generally used for root canal therapies instead of calcium hydroxide and it showed significant microbial reduction.[9]

Since oral diseases, such as dental caries, are among the most common non-communicable diseases, great importance on any prevention method should be taken into consideration by health care providers.[10] As there are more than 700 species of bacteria in the oral environment that could cause demineralization reactions and persistent inflammations, it is advisable to use natural products that have been contributing significantly to drug development as well. Among these, different kinds of propolis are such products that are valuable for their biological properties, including antimicrobial, antifungal and antitumor effects with direct application in dentistry.[3,11,12] Propolis is a resinous hive product collected by Apis mellifera bees. Additively, it contains bioactive molecules and generally used for oral ulcers.[13] However, regarding its effects on inhibiting glucosyltransferase enzymes and diminishing acid production of *S. mutans*, it could be used as an anticaries agent as well.[14] For instance, Duarte et al. showed less acid production and tolerance of cariogenic Streptococci after propolis extracts in smooth surface caries of rats.[15] De Luca et al. had also found in an in vivo study that propolis varnish prevented smooth-surface enamel caries and showed low cell toxicity.[3] Hence, propolis could affect cariogenic bacteria directly and help to prevent not only oral ulcers but also other oral diseases as well.

Considering that there is a lack of studies including commonly consumed propolis products in Turkey, the aim of the present *in vitro* study is to compare the antimicrobial effect of these products against leading oral pathogens, *S. mutans* and *E. faecalis*. CHX and chloramphenicol were used as positive controls. The null hypothesis was that there is no significant difference among the propolis groups.

MATERIALS AND METHODS

Material selection

Seven different propolis drops that were commonly consumed in Turkey were chosen to be used in the present study. Numbers of the groups and contents of each propolis drop are given in Table 1.

Table 1.

Group numbers and content of the propolis drops used in the study

Group No.	Propolis	Content	Type
1	Umay Herbal Organic Propolis	Water, organic propolis extract, crude propolis (44.4%)	Drop
2	Bee'o Up	Water, glycol, propolis (15%)	Drop
3	Propoli EVSP	Water, glycerol, L-ascorbic acid, propolis extract (%20), green tea and grapes aroma	Drop
4	Brazilian Green Liquid Propolis	Water, propolis (10%)	Drop
5	Eğriçayır Propolis	Water, alcohol (67%), crude propolis (30%)	Drop
6	Bee'o Up	Water, grain alcohol, propolis (30%)	Drop
7	Biostore Propolis	Water, Glycerol, propolis (10%)	Drop

Microorganisms

Microorganisms used in the present study were Streptococcus Mutans ATCC 25175 and Enterococcus Faecalis ATCC 29212. Bacterial strains were supplied by Scientific Research Projects Coordination Unit of Istanbul University (Project No: 30073).

In vitro experiment

Properly lyophilized bacterial strains were diluted according to Mac-Farland 0.5 and incubated at 37°C for 24 hours. 18 standard petri dishes were prepared with Brain-Heart Infusion broth. 100 µl of each microorganism were planted to these petri dishes from a broth culture. Then, 10 µl of each propolis was planted to 6 mm diameters of discs. As a negative control, 10 µl of saline solution, and as the positive controls, 10 µl of chloramphenicol and 2% CHX were impregnated in each disc. The total amount of antimicrobials was calculated to be lower than 30 µg. Both propolis and antimicrobial discs were placed on petri dishes with a sterilized forceps. For each bacterial and propolis sample, the experiment was performed in duplicate. Then, the dishes were incubated in the oven at 37°C for 24 hours. Eventually, inhibition zones were measured in mm's. and each experimental group was photographed.

Statistical Analysis

Data were analyzed through SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). The normality of data was examined by Kolmogorov-Smirnov and Shapiro-Wilk tests. The data from zones of inhibition of each bacterium related to groups were compared by a

non-parametric Mann Whitney-U test. The level of significance was set to 0.05.

RESULTS

Mean zones of microbial growth inhibition by propolis drops are shown in Table 2.

Table 2.

Mean area of zones (mm) and statistical significance of bacterial growth by propolis drops and antibacterial agents. *

No.	Groups	S. Mutans	E. Faecalis
1	Umay Organic Propolis	0^{a}	0^{A}
2	Bee'o Up 15%	20^{b}	16^{B}
3	Propoli EVSP	14^{c}	14^{C}
4	Brazilian Green Liquid	15^{d}	0^{A}
5	Eğriçayır	30^{e}	0^{A}
6	Bee'o Up 30%	24^{f}	16^{B}
7	Biostore	12^{g}	8^{D}
Pos. Control	CHX	34^{h}	34^{E}
Pos. Control	Chloramphenicol	22^{i}	22^{F}

**Different superscripsts showed statistical significance between groups (p<0.05). Lower and upper cases are used for different bacteria.*

It is shown on the table that positive control groups demonstrated the widest inhibitory zones. All of the propolis groups showed lower inhibition of bacteria than positive control groups. Besides, CHX had the highest inhibition and this result is statistically significantly different than all of the groups including chloramphenicol (p=0.000). The first experimental group (Umay Organic) presented no growth of either *S. mutans* or *E. faecalis* (Figure 1). Similarly, experimental groups 4 and 5 showed no inhibitory zone against *E. faecalis*.

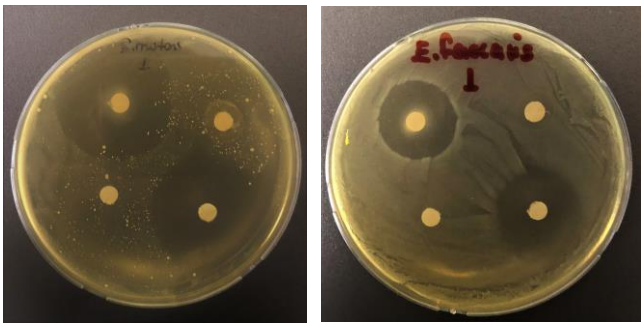

Figure 1

Cultivation of S. mutans and *E. faecalis* in the same medium with experimental group 1 and positive control groups.*

All experimental propolis drops formed statistically significantly different inhibition zones against *S. mutans* (p<0.05). Propolis drops tested against E. faecalis showed significantly different results except for the groups 2 and 6 (p=0.362) and 4 and 5 (p=0.186). Reactions of groups 2 and 4 to *E. faecalis* are shown in Figure 2.

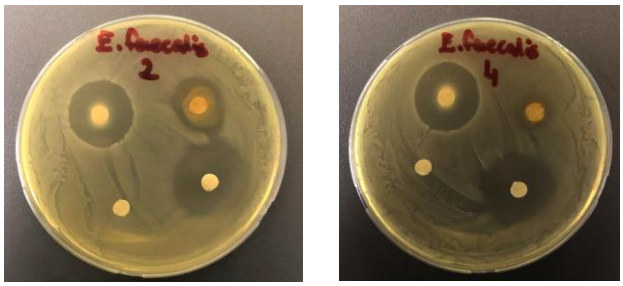

Figure 2

Example of bone level measurements on ImageJ programme.

DISCUSSION

Oral diseases are both the most common and also easily preventable pathologies amongst non-communicable diseases. Dental caries constitute a great part of these diseases and there are many methods for preventing caries and protecting risky teeth such as fluoride intakes, diet analysis, gels and mousses, oral hygiene motivation and others.[16] On the other hand, other oral diseases such as periodontitis or endodontic problems usually need treatment or anti-microbial agents. Nowadays, herbal extracts and natural products are on great demand due to their easy usage, lower impact on systemic health and lack of possibility of drug resistance.[17] As it is previously reported that a natural product, propolis, had reduced the cariogenic activity[18] and showed certain effects on oral pathogens such as *S. mutans* and *E. faecalis*.[13] Therefore, propolis types with various extract ratios were investigated in the present study. CHX, which is used as an irrigation solution for infected root canals and as gels for cavity disinfection19 and chloramphenicol, which is an antibiotic and used usually as pasta for infected primary molars[20], were tested as positive controls. Tested propolis drops showed significantly different inhibition zones, thus the null hypothesis is rejected.

Propolis extracts have numerous benefits such as antibacterial, antifungal, antitumoral and antioxidative.[13,21] Its direct mechanism is not known however it showed that propolis' could directly damage the cell membrane[13] and the main active ingredients, phenolic compounds.[16] These compounds are responsible for the inhibition of glucosyltransferase enzyme which creates the sticky layer to initiate biofilm formation.[15] The ingredients of propolis products depend on both its water or alcohol content and also its year of collection. Although differences from the collection year distinctively inhibited the biofilm formation of *S. Mutans*, it did not influence bacterial growth.[18] The present study had investigated the bacterial growth

in terms of inhibition zone so, phenolic contents of the groups had not evaluated. Generally, propolis extracts are solved in alcoholic contents but except for the alcohol-based drops, water-based propolis (Group 1) had included in the present study to distinguish the effect of alcohol.

Similar to our study, Agüero et al. found that alcohol-based propolis was more effective.[22] In the present study, only water-based propolis could not inhibit any of the oral pathogens. Even though that group had the highest propolis ratio among all groups, it showed no effect. That result could also support the outcomes from some studies evaluating water and alcohol-based propolis'.[22-24]

According to results, group 5 (Eğriçayır) formed the largest inhibition zone against *S. mutans* compared to positive control groups. That group contains not only one of the highest propolis ratios (30%), but it also had the highest alcohol content (67%) among others. One of the limitations of the study is that there is not an alcohol group (i.e. ethanol) used as negative control so, the individual contribution of propolis extract and alcohol toward inhibition of bacteria cannot be determined. Although group 5 showed great inhibition to *S. mutans*, it could not affect *E. faecalis*. Besides, the same reaction was also seen in group 4 (Brazilian Green Liquid) as well. Propolis extracts showed superior inhibitory effects against *E. faecalis* in many studies.[12,13,25] However, the contra-results on inhibition of propolis drops could be attributed to the content of groups. There are conflicting results over Brazilian green propolis against *E. faecalis*. Although Brazilian Green propolis contains astaxanthin, which is 10 times effective than strong antioxidants,[26] carotenoids found in many propolis formulae, inferior results were found in the present study. Vasconcelos et al. showed a 16±0.5 mm inhibition zone against *E. faecalis* and a 22±0.5 mm zone against *S. mutans* with Brazilian green propolis.[27] The propolis extract used in that study consisted of 40% crude propolis so, the difference between the ratios of the content may lead to higher inhibition zones for both bacteria. However, a study verified that Brazilian green propolis was only susceptible to *E. faecalis*[28] and another recent study showed that green propolis was effective on *E. faecalis* as irrigant solution, but not able to inhibit bacteria.[29] 10% propolis ratio may not form an inhibitory zone so, the present study is in accordance with those researches. Low propolis level in the drop used in the study could be the reason for inferior results on *E. faecalis*.

The main factors of propolis that serve as antioxidants and show antibacterial and antiinflammatory properties are mainly flavonoids. Ketones, acids, esters and other phenolics may contribute to those actions.[30] The more propolis extract involves in solutions, the more beneficial activities reveals. Therefore it is obvious that group 7 with 10% propolis extract had the least inhibition against *S. mutans* growth and lower inhibition against *E. faecalis*. Group 3 had also 20% of propolis extract however with the addition of L-ascorbic acid which is an antioxidant as well. Results of group 3 on both bacteria may appear from the synergic effect of ascorbic acid and propolis itself. The importance of the propolis ratio was revealed between the similar results of groups 2 and 6. All the ingredients were the same except for half of the propolis ratio of group 2 (15%) and it showed statistically significantly lower inhibition than group 6 (30%).

CONCLUSION

The present study determined the effect of various types of propolis drops, which are found in the Turkish market, against the growth of common oral pathogens by measuring inhibition zones. Drops with higher propolis ratios could able to inhibit *S. mutans* and *E. faecalis* more. Instead of water-based propolis drops, alcohol-based groups showed higher inhibition of the growth of oral pathogens.

The molecular level of propolis' was not investigated in the present study even so, instead of dental forms such as kinds of toothpaste and mouthwashes, oral drops that were highly retained to hard and soft tissues were found effective on especially *S. mutans*. Within the limitations of the present study, propolis extracts could be used as a more natural and easily accessible prevention method to dental caries specifically. Further investigations are needed on improving the standardization of intensity and type of propolis formulas that can be used in dentistry areas.

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