DEVELOPMENT OF ANTIBACTERIAL TOOTHPASTE FORMULATION USING NATURAL RAW MATERIALS

Duygu ŞAHİN, Burak SAKA
Evyap Sabun Yağ Gliserin San. ve Tic. A.Ş., Istanbul, Turkey
dozfidan@evyap.com.tr, bsaka@evyap.com.tr

ABSTRACT
New treatment methods are very important in the treatment of oral cavity diseases such as tooth decay, gingivitis, and general oral infections. Essential oils have long been used as antibacterial agents due to having biologically active compounds with important health effects. This study aimed to assess the antibacterial and antibiofilm effects of toothpastes with clove and thyme essential oils and propolis extract against bacteria associated with oral diseases. Toothpastes were formulated with 3% concentrations of the clove and thyme essential oils and propolis extract and evaluated for antibacterial and antibiofilm activities. For this purpose, antibacterial activity of toothpastes against Staphylococcus aureus ATCC6538 bacteria were determined and maximum antibacterial activity was observed with clove oil.
Keywords: Toothpaste; clove oil; thyme oil; propolis; oral flora; Staphylococcus aureus.

Introduction

Dental and gum diseases are among the most important health issues in the world. It is important that oral-dental health is closely related to main health and wellbeing. Many problems such as bad breath, caries, plaque and tooth structures, gum diseases, and tooth sensitivity may occur due to the lack of dental cleaning. In order to prevent these problems and to have a good oral health, functional oral care products should be used correctly, and daily preventive care should be applied.

The oral cavity creates favorable conditions for the reproduction of aerobic, facultative, and anaerobic microorganisms with 35-36℃ temperature, humidity, various nutrients, and variable oxygen exposure. Therefore, it is a highly heterogeneous environment with optimal physicochemical conditions and high nutrient availability for bacterial communities (Carvalho et. al, 2020).

The presence of commensal bacteria is very important for the physiology of the oral cavity; however, the formation of pathogenic microorganisms can lead to serious health problems, particularly biofilm-related infections. *Actinomyces* and *Streptococcus* biofilms may trigger some of the most common oral cavity diseases such as dental caries, gingivitis, and periodontitis (Verkaik et. al, 2011). Therefore, the development of antibiofilm agents represents an important strategy in combating oral cavity infections and attracts considerable attention.

Oral microbial flora is the region with the highest number of microorganisms compared to other regions of the human body. However, according to the data obtained by molecular biological methods, it contains more than 700 microorganism species. These microorganisms are members of colonies of microorganisms that cover the oral surface, also called dental plaque or oral biofilm. *Streptococcus salivarus* which constitutes 50% of *Streptococci* on the tongue, cannot attach to the hard tissues of the teeth. On the other hand, *Streptococcus mutans* that can adhere to tooth hard tissues very well, cannot hold onto the tongue surface. The tooth is one of the most suitable places for *Streptococcus sanguis* and *Streptococcus mutans* to settle. They constitute the dominant streptococcal flora on the mouth and tooth surface (Hepdeniz et. al, 2017). The list of the main bacteria found in the oral flora is presented in Table 1. Oral bacteria are *Streptococcus, Lactobacillus, Staphylococcus, Corynebacterium* and especially bacteroides group anaerobes (Özan et. al, 2015).

Streptococcus mutans is the primary oral colonizer. Bacteria adhere to the tooth surface and create favorable conditions to adhere secondary colonizers such as *Lactobacillus species* (Verkaik et. al, 2011).

Dental biofilm formation is a natural process in the oral environment, but it should be controlled with regular brushing to prevent the development of caries and periodontal diseases. Brushing alone is not enough to prevent the development of periodontal diseases and caries and to destroy oral biofilm (Verkaik et. al, 2011).

Table 1. Microorganisms frequently isolated from the oral cavity

ANAEROBIC BACTERIA	
Gram-negative rod-shape bacteria	
Porphyromonas	*P. gingivalis, P. endodontalis, P. catoniae*
Prevotella	*P. oralis, P. oris, P. buccae, P. corporis, P. denticola, P. loescheii, P. intermedia, P.nigrescens, P. melaninogenica*
Fusobacterium	*F. nucleatum spp. nucleatum, spp. vincentii, spp. polymorphum*
Mitsuokella	*M. dentalis*
Selenomonas	*S. sputigena, S. noxia*
Campylobacter	*C. sputorum, C. rectus, C. curvus*
Treponema	*T. denticola, T. vincentii, T. socranski*
Bacteroides	*B. forsythus*
Gram-positive rod-shape bacteria	
Eubacterium	*E. alactolyticum, E. lentum, E. yurii*
Propionibacterium	*P. acnes, P. propionicus, P. jensenii, P. granulosum, P. avidum*
Lactobacillus	*L. catenaforme, L. crispatus, L. oris, L. uli, L. grasseri*
Actinomyces	*A. israelii, A. odontolyticus, A. meyeri*
Arachnia	*A. propionica*
Gram-negative spherical bacteria	
Veillonella	*V. parvula, V. alcalescens*
Gram-positive spherical bacteria	
Peptostreptococcus	*P. asaccharolyticus, P. magnus, P. micros, P. anaerobius P. prevotii*
FACULTATIVE ANAEROBIC BACTERIA	
Gram-negative rod-shape bacteria	
Eikenella	*E. corrodens.*
Capnocytophaga	*C. ochracea, C. sputigena, C. gingivalis, C. haemolytica, C. granulosa*
Actinobacillus	*A. actinomycetemcomitans.*
Haemophilus	*H. aphrophilus H. influenzae, H. parainfluenzae, H. paraphrophilus, H. segnis*
Gram-positive rod-shape bacteria	
Corynebacterium	*C. xerosis, C. matruchotii*
Actinomyces	*A. naeslundii, A. viscosus*
Rothia	*R. dentocariosa*
Lactobacillus	*L. acidophilus, L. brevis, L. buchneri, L. casei, L. salivarius, L. fermentum*
Gram-negative spherical bacteria	
Neisseria	*N. flavescens, N. mucosa, N. sicca, N. subflava*
Branhamella	*B. catarrhalis*
Gram-positive spherical bacteria	
Streptococcus	*S. mutans, S. sanguis, S. salivarius, S. sobrinus, S. rattus, S. downei, S. mitis,S. milleri, S. oralis, S. intermedius, S. constellatus*
Staphylococcus	*S. aureus, S. epidermidis*
Enterococcus	*E. faecalis, E. faecium*

Herbal toothpastes containing natural antibacterial agents are a good alternative to reduce the risk of oral diseases in both children and adults. Developing natural antibiofilm and antibacterial agents which are safe alternatives that support oral health, may be an effective method for the prevention and control of oral cavity pathologies. In the study, the herbal toothpaste formulations as safe and natural antibiofilm alternatives against cariogenic and periodontal diseases which contained thyme oil, clove oil and propolis extract were developed and tested for their antibacterial efficacy to evaluate the antibacterial and antibiofilm effects.

Materials and Methods

The thyme oil, clove oil and propolis extract added formulations were prepared by using the base formulation given in Table 2.

Table 2. Toothpaste base formulation (%)

Sorbitol	30-35%
Aqua	20-30%
Silica	15-20%
Glycerin	10-15%
PEG 8	2-5%
Sodium Lauryl Sulfate	1-3%
Dicalcium Phosphate Dihydrate	1-3%
Cellulose Gum	1-3%
Xanthan Gum	1-3%
Sodium Fluoride – Active Ingredient 0.32% (1470 ppm F)	0.32%
Sodium Saccharin	0.20%

The formulations by adding 3% concentration of thyme oil, clove oil and propolis extract to the base formulations were developed and named as in Table 3.

Tablo 3. Newly developed Formulations

F1	Base Formulation
F2	Base Formulation + 3% Thyme oil
F3	Base Formulation + 3% Clove oil
F4	Base Formulation + 3% Propolis Extract

Thyme oil, clove oil and propolis extract were purchased from Talya Herbal Products Industry and Trade Inc. The antibacterial tests EN 1276 were performed by accredited laboratories.

Results

The use of natural active ingredients in cosmetic products has taken attention. The toothpaste formulations with different oils and extracts were developed to analyze their antibacterial and antibiofilm effects. Therefore, 3% thyme oil, clove oil and propolis extract were added to the base formulation.

GC/MS (Gas chromatography/mass spectrometry) analysis taken from the supplier shows the major components of thyme oil and clove oil and their percentages in Table 4.

Table 4. Major components of thyme oil and clove oil

	Components	Amounts (%)
Thyme Oil	Thymol	2.36
	Carvacrol	41.45
Clove Oil	Eugenol	64.53
	Caryophyllene	8.21
	Eugenyl acetate	5.48

Antibacterial efficacy performances are evaluated by EN 1276 standard test method. The logarithmic reductions of *Staphylococcus aureus* ATCC 6538 are tested. Minimum 5 log reduction is needed for a positive test result for *Staphylococcus aureus* strains. The contact duration of the antibacterial formulation is standardized as 5 minutes and incubation temperature is 37°C. The results were compared in Table 5.

Table 5. Antibacterial efficacy results of the formulations on *Staphylococcus aureus* strain

			Logarithmic reduction (cfu/ml)			
Bacteria	***Contact duration (min)***	***Activity range***	**F1**	**F2**	**F3**	**F4**
Staphylococcus aureus ATCC 6538	1	>5	5.50	5.89	6.03	5.70
Efficacy result			(+)	(+)	(+)	(+)

The logarithmic reduction values of all formulations are higher than 5 log after 5 minutes contact duration. It has been observed that F2 and F4 are very close to base formulation F1. However, F3 containing clove oil has an obvious difference than others with 6.03 log reduction in bacterial count. It may be because of high content of Eugenol as 64.53% of clove oil. In addition, thyme oil contains 41.45% carvacrol, it may have affected the antibacterial activity performance in F2 succeeding F3.

It has been known that thyme plant has antibacterial, antiviral, antioxidant, antilipidemic and antifungal effects. Thymol in thyme essential oil is a terpenic substance and has antiseptic, antibacterial, antispasmodic, antiasthmatic, expectorant and fungicidal effects, it has 30 times more antiseptic effect and 4 times less toxic effect than phenols. Thymol is also used in dentistry as an antioxidant for temporary fillings (Çetin et. al, 2013).

Recently, thymol and its derivatives have received a lot of attention in applications such as various biological functions, phyto-pharmaceutical preparations, food preservatives. It was found that the most effective compound in essential oils was thymol with the lowest minimum inhibitory concentration (MIC, 35-128 µg / ml) followed by eugenol and carvacrol. The Botelho group tested the antimicrobial activity of thymol against cariogenic bacterial species of the genus *Streptococcus* and *Candida albicans* with MIC values ranging from 0.625 to 10.0 mg /mL. In another study, the aerial parts of *Origanum vulgare L.* were tested against 26 MSS and 21 MRS using an agar dilution method. The results showed that the best MIC values were for thymol (0.03-0.06% v/v) and derivatives of thymol 2- (allyloxy) -1-isopropyl-4-methylbenzene, 4-allyl-2-isopropyl-5-methylphenol, 2-isopropyl-5-methyl-4-propylphenol (Dheer et. al, 2019).

Clove oil helps reduce stomatitis and gingivitis that contains eugenol substance. The eugenol substance is a natural anesthetic, analgesic (pain reliever) and antibacterial. In addition, clove oil shows antibacterial activity by preventing the growth of gram positive and gram-negative bacteria. In addition, it is known to have fungistatic and antiviral effects (Mohammadı Nejad et. al, 2017).

In a study, when clove essential oil was analyzed using GC-MS, 18 basic chemical compounds were extracted and tested for potential antioxidant activities. Eugenol is an important active ingredient representing 90% of clove essential oil and has been approved by the FDA for use as a food additive. In addition to its antioxidant activity, clove oil has bactericidal properties against many types of bacteria such as *Salmonella, Staphylococcus, Listeria* and *Escherichia* (Abdelkhalek et. al, 2020).

In a study by Carvalho and colleagues, toothpastes were formulated with different concentrations of the most active essential oils and evaluated for their antibacterial and antibiofilm activities. Minimum inhibitory concentration (MIC) and antibiofilm activity was determined against *Staphylococcus aureus, Streptococcus mutans*, *Lactobacillus lactis* and *Enterococcus faecalis*. The MIC was determined as the lowest concentration of essential oil that does not cause bacterial growth. The antibiofilm activity of clove, thyme, and cinnamon essential oils at 1×, 2×, and 4×MIC against *S. mutans* did not differ from that of the control (0.12 % chlorhexidine gluconate mouthwash) and has been found to completely disrupt *S. mutans* biofilms. Microbial inhibition was categorized as strong (MIC < 0.5 mg/mL), moderate (0.5≤MIC ≤ 1.5 mg/mL), and weak (MIC > 1.5 mg/mL). When the results were examined, the MIC values of the control were found as follows: 0.05 for *Streptococcus mutans*, 0.05 for *Staphylococcus aureus*, 0.25 for *Lactobacillus lactis*, 0.05 for *Enterococcus faecalis*. The MIC values of clove essential oil were found as 0.315 for *Streptococcus mutans*, 0.625 for *Staphylococcus aureus*, 1.25 for

Lactobacillus lactis, 1.25 for *Enterococcus faecalis*. Clove essential oil caused strong and moderate inhibition against *S. mutans* and *S. aureus* and weak inhibition of *L. lactis* and *E. faecalis*. The MIC values for thyme essential oil are as follows: 0.625 for *Streptococcus mutans*, 0.625 for *Staphylococcus aureus*, 0.315 for *Lactobacillus lactis*, 0.625 for *E. faecalis*. In MIC tests, thyme essential oil showed moderate inhibition against *S. mutans, S. aureus, E. faecalis* and strong inhibition against *L. lactis* (Carvalho et. al, 2020).

Propolis; a honeybee hive product contains numerous active compounds such as phenolic acids, esters and flavonoids. Thus, it has a wide pharmacological potential, including anti-bacterial, anti-fungal, anti-protozoal, hepatoprotective, antioxidant, anti-inflammatory, anti-viral, anticancer, and anti-tumor properties (Anjum et al, 2019). Also, adding ethanolic propolis extract to the composition of mouthwashes and toothpastes enhances the prevention of microbial infection and is effective in treating gingivitis. The antibacterial activity of propolis is thought to be due to flavonoids and aromatic acids and esters in the resin. Galangin, pinocembrin and pinobanksin have been identified as the most effective flavonoids against bacteria. Ferulic and caffeic acid also play a role in the bactericidal effect of propolis (Carvalho et. al, 2020).

In an antibacterial study on Staphylococcus aureus strain, propolis was found to interact severely with streptomycin and cloxacillin, and moderately synergistically with chloramphenicol, cephradine, and polymyxin B. It has been observed that propolis solution shows antibacterial activity in vitro, inhibits the adhesion of cells and formation of water-soluble glucan. According to the data obtained from the studies conducted, it has been observed that propolis solutions prepared in appropriate concentrations have inhibitory properties on oral microorganisms and have no toxic effects on oral tissues. It is also known that propolis prevents the formation of tooth decay and periodontal disease (Özan et. al, 2015).

Conclusion

New effective methods of herbal toothpastes supported natural antibacterial and antibiofilm agents provide to prevention and control of oral cavity pathologies.

The results of this study pointed out that developed herbal toothpastes exhibit statistically higher antibacterial activity against *Staphylococcus aureus* than their initial forms after the addition of essential oils.

As can be seen from the results of EN 1276, thyme oil, clove oil and propolis extract components affected the logarithmic reduction values. The plant that oil is obtained from has some parameters such as collection time, drying patterns, drying temperature, and duration that affect the volatile composition of the plants. For this reason, the parameters should be taken into consideration, and work with oils and extracts with appropriate composition.

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