Antimicrobial effect of natural kinds of toothpaste on oral pathogenic bacteria

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Abstract

Introduction: Because of the adverse effects on human health of some antimicrobial ingredients in traditional toothpaste, consumers are increasingly turning to toothpastes with natural ingredients. This study evaluates the antimicrobial effect of toothpastes containing different natural active agents against three oral pathogens: *Streptococcus mutans*, *Streptococcus sanguinis*, and *Enterococcus faecalis*.

Methodology: This study tested one traditional toothpaste and seven different natural toothpastes containing theobromine, aloe vera, miswak, propolis, chitosan, enzymes and probiotics. The agar-well diffusion method was used to test the antimicrobial effect. Inhibition zones formed around toothpastes after 24 hours of incubation were measured and the data collected were statistically analyzed.

Results: Toothpastes containing theobromine and chitosan and the traditional toothpaste showed antimicrobial efficacy for all tested bacteria. Toothpastes containing aloe vera, miswak, and propolis were only effective on *S. mutans*, while toothpastes containing probiotics and enzymes did not show any antimicrobial effect on the bacteria. Among toothpastes with natural ingredients, the theobromine-containing toothpaste showed the highest efficacy on *S. mutans*, while the aloe vera- and propolis-containing toothpastes had the lowest efficacy \((p < 0.05)\).

Conclusions: Theobromine- and chitosan-containing toothpastes, which showed antimicrobial activity against all bacteria, can be recommended as alternatives to traditional toothpastes.

Key words: Toothpaste; *Streptococcus mutans*; *Streptococcus sanguinis*; *Enterococcus faecalis*; antimicrobial effect.

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Introduction

Dental caries is a local, progressive and infectious bacterial dynamic disease caused by acidic byproducts of bacterial metabolism that lead to the dissolution of dental hard tissues [1]. The formation of caries typically depends on multiple factors, including cariogenic microflora, fermentable carbohydrates, plaque formation and time [2]. Dental plaque, an oral microbial biofilm formed on dental surfaces, consists of a wide variety of oral microbial strains and species [3]. *Streptococcus mutans* and *Streptococcus sanguinis* are two of the main bacteria involved in dental biofilm formation and responsible for dental hard tissue destruction [4]. *S. mutans*, a member of the group of mature dental plaques, has the ability to synthesize soluble and insoluble glucans from dietary sucrose mediated by glucosyltransferase enzymes, allowing extracellular aggregation for stable biofilm formation on the tooth surface [5]. *S. sanguinis* is one of the first colonizing streptococci in the course of biofilm formation. These two species may be more dominant in certain environments due to bacteriocins produced by *S. mutans* and hydrogen peroxide produced by *S. sanguinis* [6]. *Enterococcus faecalis* has not been considered as part of the normal oral microbiota and is regarded an infectious microorganism associated with unsuccessful endodontic treatments. However, *E. faecalis* has been found in different layers of the oral biofilm [7]. In addition, *E. faecalis* can contribute to collagen and periodontal destruction, leading to the progression of the disease in patients with chronic periodontitis [8]. Due to its resistance to effective antibiotic groups such as vancomycin, ciprofloxacin and erythromycin, the World Health Organization has reported *E. faecalis* as a “high priority resistant pathogen” for the development of antibiotics [9].

Reducing the number and activity of cariogenic microorganisms assists in the prevention of plaque-related dental and gingival diseases. Therefore, the use of mechanical plaque control and oral hygiene products containing antimicrobial agents is recommended [10]. Toothpastes are a widely used agent for dental biofilm control [11]. However, toothpastes can contain potentially harmful components that may lead to local...
side effects such as irritation and desquamation of the oral mucosa (stomatitis, glossitis, gingivitis, buccal mucositis) and systemic side effects such as allergic and toxic reactions (acute or chronic). These effects have mainly been associated with fluoride, sodium lauryl sulfate (SLS) and triclosan [12].

Secondary metabolites from plants, microorganisms and seafood have long been recognized as valuable sources of new molecules with drug development potential in many biomedical fields [13]. They show anti-inflammatory, analgesic [14], antimicrobial [15], antineoplastic [16] and anticariogenic [17] effects. The development and introduction of oral care products claiming to be prepared from natural agents has been continually increasing [18]. Despite the recent increase in consumption of oral care products that claim to be healthy and contain natural agents, there is scant evidence of their benefits [19]. It has also been reported that the antimicrobial activities of these natural ingredients may be altered due to their interactions with other ingredients in commercial preparations [20].

Although many studies have investigated the remineralization and antiplaque effects of natural toothpastes, few studies examined their antimicrobial efficacy. Therefore, this study aims to determine and compare the antimicrobial efficacy of natural toothpastes that have been in use in recent years.

**Methodology**

Eight toothpastes were tested in this study: toothpastes containing theobromine (Theodent, LLC, Louisiana, USA), aloe vera (Urtekram, International A/S, Mariager, Denmark), miswak (Eyüp Sabri Tuncer, Istanbul, Turkey), propolis (Aagaard Propolis, Sanhelios, Bremen, Germany), chitosan (Chitodent, Helmut Focken Biotechnik e.K., Sindelfingen, Germany), probiotics (PerioBiottic, Designs for Health, Inc., Suffield, Canada), enzymes (Curaprox Enzycal Zero, Curaden AG, Kriens, Switzerland), and a traditional toothpaste (Colgate Total 12, Colgate-Palmolive Co., Ltd., China). Table 1 lists the contents of the tested toothpastes. Furthermore, a mouthwash containing 0.2% chlorhexidine digluconate (Klorhex

| Table 1. Contents of the toothpastes tested in the study. |
|-----------------------------------------------|
| **Toothpaste** | **Product name (Manufacturer)** | **Contents** |
|----------------|--------------------------------|--------------|
| **Theobromine-containing toothpaste** | Theodent (LLC, Louisiana, USA) | Active Ingredients: theobromine, calcium acetate and sodium hydrogen phosphate. Inactive Ingredients: purified water, hydrated silica, sorbitol, xylitol, glycerin, sodium laurylsarcosinate, xanthan gum, titanium dioxide, citric acid, and spearmint oil, sodium benzoate, stevia extract, sodium bicarbonate, and sugar-free vanilla extract. |
| **Aloe Vera-containing toothpaste** | Urtekram (International A/S, Mariager, Denmark) | Calcium carbonate, aqua, glycerin, Aloe barbadensis leaf extract, xanthan gum, citrus reticulata peel oil* (aroma), commiphoramryrrha oil, Magnolia officinalis bark extract, limonene, linalool. (ingredient from organic farming, made using organic ingredients). Natural chalk, water, glycerine**, aloe vera*, polyaccharides, mandarin oil*, myrrh, magnolia bark extract. * = Organic farming. ** = Made using organic ingredients. |
| **Miswak-containing toothpaste** | Eyüp Sabri Tuncer (İstanbul,Turkey) | Aquas, Sorbitol, Hydrated Silica, Glycerin, Xylitol, Polysorbate 20, Cocamidopropyl Betaine, Disodium Phosphate, Aroma, Xanthan Gum, Menthol, Thymol, Stevia rebaudiana Extract, Mentha piperita Oil (Nanjeyâg), Eucalyptus globulus Leaf Oil, Salvadora persica bark / root extract, Phenylpropanol, Caprylyl Glycol, Propanediol, Tocopherol). |
| **Propolis-containing toothpaste** | Aagaard Propolis (Sanhelios, Bremen, Germany) | Aqua (Water), sodium metaphosphate, sorbitol, silica, alcohol, sodium alginate, capryl/capramidopropyl betaine, aroma (fragrance), Propolis cera, sodium chloride, Chamomilla recutita flower extract (matricaria), ascorbic acid, sodium hydrogen, sodium saccharin, Salvia officinalis Leaf Extract, CI 77891 (titanium dioxide). |
| **Chitosan-containing toothpaste** | Chitodent (Helmut Focken Biotechnik e.K., Sindelfingen, Germany) | Aqua, sorbitol, hydrated silica, glycerin, coccoamidopropyl betaine, chitosan, lactic acid, aroma, hydroxypropyl guar, Sodium, saccharin, CI 42051 (colorant bleu), natrium hydrixoid. |
| **Probiotic-containing toothpaste** | PerioBiottic (Designs for health, Inc. Suffield, Canada) | Water, hydrated silica, calcium glycerophosphate, glycerin, xylitol, spearmint oil (Mentha viridis), carrageenan, Dental-Lac (Lactobacillus paracasei), potassium sorbate, yucca (Yucca filamentosa) powder, stevia leaf extract (Eupatorium rebaudianum, zinc chloride. |
| **Enzymes-containing toothpaste** | Curaprox Enzycal Zero (Curaden AG, Kriens, Switzerland) | Aqua, sorbitol, hydrated silica, glycerin, steareth-20, titanium dioxide, aroma, sodium hydrophosphonate, carrageenan, sodium chloride, citric acid, sodium benzoate, potassium thiocyanate, glucose oxidase, amyloglucosidase, lactoperoxidase. |
| **Traditional toothpaste** | Colgate Total 12 White (Colgate-Palmolive Co., Ltd. China) | Hydrated silica, water, glycerin, sorbitol, PVM/MA copolymer, sodium lauryl sulfate, flavor, sodium hydroxide, carrageenan, propylene glycol, sodium fluoride, sodium saccharin, triclosan, cellulose gum, CI 77891 (titanium dioxide), CI 77019 (sericite mica), CI 42090 (Colorant Bleu brillant FCF). Active Ingredients: Sodium Fluoride 0.32% w/w (1450 ppm F), Triclosan 0.3% w/w. |
mouth rinse fluid, Drogsan İlaçları Sanayi ve Ticaret A.Ş., Ankara, Turkey) was used as the positive control group, while 0.85% NaCl dilution fluid sterile saline (ID Broth, Becten, Dickinson and Company, USA) was used as the negative control group.

Three lyophilized microorganism strains from the American Type Culture Collection (ATCC) were used to test the antimicrobial effectiveness of the toothpastes. These were: *S. mutans*-ATCC 25175, *S. sanguinis*-ATCC 10556, and *E. faecalis*-ATCC 29212. Microorganisms stored inactively at 4 ± 1 °C were inoculated into separate media and activated and multiplied by keeping in an incubator at 37 ± 1 °C for 24 hours. Mueller Hinton Fastidious Agar plates (MH-F, Becton Dickinson GmbH, Heidelberg, Germany) with a diameter of 9 cm and a thickness of 5 mm as recommended by the European Antibiotic Susceptibility Committee (EUCAST) were used to determine antimicrobial activity, and the agar-well diffusion method was preferred. For each bacteria and toothpaste one separate agar plate was used. In each agar plate a sterile cork borer (Cork Borer-Isolab, İnterlab. Ürünleri San. Tic. A.Ş., Istanbul, Turkey) was used to bore 3 wells with 6mm diameter and 5mm depth, equidistant from each other and from the edges. One well each was used for the toothpaste sample, the positive control and the negative control (Figure 1.A). Microorganism suspension adjusted to 0.5 McFarland (1.5 × 10⁸ CFU/mL) in activated fresh bacterial cultures 0.85% NaCl solution was inoculated by spreading over the entire surface of the agar plates in all directions with the help of sterile swabs. Each well was filled with an equal amount (0.2ml) of toothpaste and control group materials using sterile syringes and incubated for 24 hours at 37 ± 1 °C in 5% CO₂ (Figure 1.B). This procedure was repeated three times. At the end of 24 hours, the inhibition zone diameters around the tested materials were measured using a digital caliper (Insie 1108-200 digital caliper, Insie Co. Ltd., Jiangsu Province, PRC) randomly and the readings were recorded (Figure 1.C).

**Statistical Analyses**

SPSS v22.0 was used for statistical analysis of the data obtained in the study. The normality of the distribution was tested using the Shapiro-Wilk test. Intergroup comparisons of non-normally distributed data was performed with Kruskal-Wallis and post-hoc tests. The descriptive statistics are given as mean value ± SD. A p-value of < 0.05 was considered significant.

**Results**

Inhibition zone diameters formed by the tested toothpastes against *S. mutans*, *S. sanguinis* and *E. faecalis* after 24 hours of incubation are shown in Table 2. The traditional toothpaste and theobromine- and chitosan-containing toothpastes showed antimicrobial efficacy for all tested bacteria, while probiotic- and enzyme-containing toothpastes did not have antimicrobial efficacy. The 0.2% chlorhexidine digluconate positive control group showed the highest antimicrobial efficacy on all bacteria (p < 0.05). The negative control group showed no antimicrobial efficacy on any of the bacteria.

The toothpaste with traditional ingredients showed the highest antimicrobial efficacy against *S. mutans*, followed by toothpastes containing theobromine, miswak, chitosan, aloe vera and propolis, respectively. No statistically significant differences were found between the traditional toothpaste and the theobromine-containing toothpaste (p > 0.05).

The traditional toothpaste and theobromine- and chitosan-containing toothpastes showed antimicrobial efficacy on *S. sanguinis*. The traditional toothpaste...
showed the highest effect, followed by the chitosan- and theobromine-containing toothpastes. Statistically significant differences were found between all groups ($p < 0.05$).

The traditional toothpaste and theobromine- and chitosan-containing toothpastess showed antimicrobial efficacy on $E. faecalis$. The highest antimicrobial efficacy on $E. faecalis$ was seen in the toothpaste with traditional ingredients ($p < 0.05$), followed by toothpastess containing theobromine and chitosan, respectively. Statistically significant differences were found between the theobromine- and chitosan-containing toothpastes ($p > 0.05$).

**Discussion**

This study was designed to evaluate the antimicrobial efficacy of toothpastess containing natural ingredients on $S. mutans$, $S. sanguinis$ and $E. faecalis$. The results showed that theobromine- and chitosan-containing toothpastess had antimicrobial efficacy on all three pathogenic microorganisms; however, toothpastess containing aloe vera, miswak and propolis were effective on $S. mutans$ only. Toothpastess containing probiotics and enzymes did not have any antimicrobial efficacy on the tested bacteria.

A 0.2% chlorhexidine digluconate solution was used as a positive control group. This solution is accepted as the gold standard due to its ability to bind microorganisms to negatively charged cell walls and disrupt their osmotic balance, its prolonged broad-spectrum antimicrobial activities, and its plaque prevention potential [21]. The highest antimicrobial efficacy on $S. mutans$, $S. sanguinis$ and $E. faecalis$ was seen in the positive control group, followed by traditional toothpaste. In the positive control group and all other groups, $S. sanguinis$ and $E. faecalis$ were more resistant against the antimicrobial agents tested in the study compared with $S. mutans$. Previously published studies support this conclusion, and this resistance could be associated with the ability of these strains to block the entry of antimicrobial molecules due to outer cell layers that form an impermeable barrier with bacterial spores [22,23].

In this study, theobromine-containing toothpastess showed antimicrobial efficacy for all tested bacteria. Theobromine is a natural alkaloid of white crystalline structure and bitter taste, found mainly in cocoa beans and hence in chocolate, of which it is an active ingredient, but also in tea, guarana, cola and mate leaves [24]. The anti-caries effect of theobromine has been associated with active ingredients in its structure such as anti-glycosyltransferase, oleic and linoleic acid, flavonoids and polyphenols. The anti-glycosyltransferase could inhibit the glycotransferase substance produced by $S. mutans$ by adhering to the plaque [25]. Flavonoids and polyphenols could perform a bacteriostatic effect on $S. mutans$ and diminish its activity, thus reducing caries formation by preventing the development of biofilm [26]. Similar to the present study, Lakshmi et al. [27] reported that theobromine-containing toothpaste showed antimicrobial efficacy. They also reported that theobromine was effective on inhibition of $S. mutans$, Lactobacillus acidophilus, $E. faecalis$, and Candida albicans.

Chitosan is a natural polycationic linear polysaccharide derived from the partial deacetylation of chitin. The polysaccharide structure of chitosan consists of $\beta$- (1-4) linked D-glucosamine and N-acetyl-D-glucosamine randomly distributed in the polymer [28]. Chitosan is used in medicine due to its diverse characteristics such as biocompatibility, non-toxicity, low allergenicity and biodegradability. In addition, chitosan has been reported to have other biological properties such as antitumoral, antimicrobial, and antioxidant efficacy [29]. Chitosan is a beneficial ingredient in toothpaste that prevents streptococci from adhering to tooth surfaces and is less abrasive [30]. Furthermore, in patients with poor oral hygiene,

**Table 2.** Mean values of inhibition zones (mm).

| Groups          | Mean (Std. Deviation) | Mean (Std. Deviation) | Mean (Std. Deviation) |
|-----------------|-----------------------|-----------------------|-----------------------|
| **S. mutans**   |                       |                       |                       |
| Theobromine     | 16.30 (0.29)$^b$      | 11.65 (0.07)$^d$      | 11.36 (0.03)$^c$      |
| Aloe vera       | 10.64 (0.08)$^d$      | 0                     | 0                     |
| Miswak          | 13.16 (0.13)$^c$      | 0                     | 0                     |
| Propolis        | 10.49 (0.06)$^d$      | 0                     | 0                     |
| Chitosan        | 12.53 (0.09)$^c$      | 13.04 (0.04)$^c$      | 10.99 (0.01)$^d$      |
| Probiotic       | 0                     | 0                     | 0                     |
| Enzymes         | 0                     | 0                     | 0                     |
| **S. sanguinis**|                       |                       |                       |
| Theobromine     | 17.04 (0.03)$^b$      | 16.96 (0.03)$^b$      | 12.61 (0.02)$^b$      |
| Aloe vera       | 24.24 (0.53)$^a$      | 19.99 (0.17)$^d$      | 19.82 (0.67)$^a$      |
| Miswak          | 0                     | 0                     | 0                     |
| Propolis        | 0                     | 0                     | 0                     |
| Chitosan        | 0                     | 0                     | 0                     |
| Positive control|                       |                       |                       |
| Negative control|                       |                       |                       |

Different superscript letters (a,b,...) mean statistically differences in same column (Group comparison) ($p < 0.05$).
chitosan can reduce the demineralization of enamel and prevent excessive dentin sensitivity and tooth decay [31]. In this study, chitosan-containing toothpaste showed a significant antimicrobial effect on the microorganisms tested. This effect can be explained by the ability of chitosan to disrupt the cell structure by binding to the bacterial cell wall and changing membrane permeability or by binding to bacterial DNA and inhibiting DNA replication, causing cell death [32]. In addition, one of the suggested mechanisms for its antimicrobial efficacy is that chitosan acts as a chelating agent that selectively binds metal elements that cause toxin production and inhibit microbial growth [33].

In this study, toothpastes containing aloe vera, miswak, and propolis showed an antimicrobial effect only on S. mutans. Although different results have been reported in the literature regarding the antimicrobial activity of these three natural ingredients, most of the researchers agree that they are effective on S. mutans. One study reported that a toothpaste containing aloe vera had a high antimicrobial effect on S. mutans, C. albicans, S. sanguinis and Actinomyces viscosus [34]. This result, which runs contrary to our study, may be related to the presence of different active agents (SLS, sodium lauroyl sarcosinate) in the aloe vera-containing toothpaste. Surfactants such as SLS, cocamidopropyl betaine and sodium lauroyl sarcosinate added to toothpastes have been reported to increase the antibacterial effect [20]. Carvalho et al. reported no antimicrobial efficacy of aloe vera-containing toothpaste in their study [35]. However, the concentration of the antimicrobial agent has been shown to significantly affect its antimicrobial efficacy [36].

In the present study, toothpaste containing miswak showed an antimicrobial effect on S. mutans, a result which is in line with the literature [37,38]. This efficacy may be due to substances such as fluoride, silica, resins, sulfur compounds, sodium bicarbonate, tannic acid, alkaloid, chloride and vitamin C [39]. Fatin-Majdina et al. [38] reported that a miswak-containing mouthwash was effective also on S. sanguinis, although to a lesser extent than its efficacy on S. mutans. Researchers proposed that this might be because S. sanguinis, as a hydrophobic bacteria, adheres more tightly to the biofilm.

Smoralek et al. [40] evaluated the antimicrobial efficacy of a combination of propolis and tea tree oil in toothpastes and a mixture of propolis, SLS, and NaF on S. mutans, E. faecalis and Pseudomonas aeruginosa. They reported that a combination of propolis and tea tree oil in toothpaste had an antimicrobial effect on only S. mutans, while toothpaste containing propolis, SLS, and NaF had an antimicrobial effect on S. mutans and E. faecalis. Taken together, the previous and current data suggest that different concentrations and origins of propolis in toothpastes might affect its antimicrobial efficacy.

In the current study, toothpastes containing probiotics and enzymes did not show antimicrobial efficacy on any of the three microorganisms. Probiotics prevent pathogenic microorganisms from adhering to surfaces in the mouth and from forming biofilm by competing with existing pathogenic microorganisms and by producing chemicals (such as bacteriocin and hydrogen peroxide) that inhibit them [41]. Furthermore, in in-vivo plaque control studies, toothpaste containing probiotics has been reported to reduce the incidence of gingivitis and the number of pathogenic microorganisms [42]. These studies suggest that probiotics may be more effective together with mechanical plaque control.

The enzyme-containing toothpaste used in this study contained lactoperoxidase, amyloglucosidase, and glucose oxidase enzymes. Lactoperoxidase, an enzyme secreted primarily in saliva, milk and other body fluids, has an antimicrobial effect against bacteria, fungi and viruses [43]. Amyloglucosidase and glucose oxidase work together to produce hydrogen peroxide from polyglycans in the environment, and when sufficient hydrogen peroxide is formed, lactoperoxidase shows an antimicrobial effect by catalyzing the conversion of thiocyanate to hypothiocyanite [44]. Accordingly, the antimicrobial effect of the toothpaste may differ in the oral environment. Studies have reported that lactoperoxidase is not a bactericide but rather is bacteriostatic on streptococci and lactobacilli [45].

This study has some limitations. Toothpastes are compound products consisting of more than one ingredient. When their effectiveness is tested, it is possible to achieve different results depending on the concentration of active ingredients, surfactants or the agent with antimicrobial efficacy. Although products with only one active agent listed by the manufacturer were selected when choosing toothpastes with natural ingredients, when the content of the tested pastes is examined, different active agents are found, for instance chamomilla in addition to propolis, magnolia in addition to aloe, and mentha in addition to miswak. These agents, which may not be explicitly stated by the manufacturer, may increase the antimicrobial activity of the toothpaste. Additionally, sodium bicarbonate [46], titanium dioxide [47], glycerin [48] and xylitol
[49] have been identified in toothpastes and may also show antimicrobial activity. For this reason, different results regarding antimicrobial efficacy can be found in literature. Therefore, to protect and maintain oral and dental health, it is important to test the antimicrobial efficacy of these products that are used as alternatives to traditional toothpastes.

**Conclusions**

Toothpaste is a personal hygiene product that is widely used to ensure and maintain oral and dental health. Natural ingredients toothpastes are increasingly being used to prevent caries formation and to maintain gingival and periodontal health. This study evaluated the effectiveness of toothpastes with natural ingredients on pathogenic microorganisms that cause dental and oral health deterioration. According to the obtained data, toothpastes containing theobromine and chitosan showed antimicrobial activity against all bacteria and can be recommended as an alternative to traditional toothpastes. However, additional studies are needed to evaluate their effectiveness in clinical practice.

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