

Article

In Vitro Evaluation of the Antimicrobial Properties of Natural Toothpastes Containing Silver, Citrus, and Cranberry Extracts Against Oral Pathogenic Microorganisms

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Abstract: Natural toothpastes were introduced to limit the use of chemical ingredients commonly found in conventional toothpastes. The purpose of this in vitro study was to evaluate the antimicrobial properties of three developed natural toothpastes containing different antimicrobial agents: (a) Biosecur Organic Oral Care (BOOC), (b) Microsilver BG, and (c) Cranberry LG. These toothpastes were compared with a natural toothpaste of the same composition but without any added natural antimicrobial agent (negative control), as well as with a commercial synthetic toothpaste (positive control). The antimicrobial properties of the toothpastes were assessed using the disc diffusion test against three oral pathogens: *Candida albicans*, *Streptococcus mutans*, and *Prevotella intermedia*. Each tested toothpaste sample was placed in Petri dishes, where specific microorganisms selected for the study were cultivated. After incubation, the circular area formed around the discs (diameter), known as the inhibition zone, was measured demonstrating the inhibitory effect of the product on the microorganisms used in the efficacy test. All the experimental toothpastes exhibited higher antimicrobial properties compared to the negative control group, except for *Streptococcus mutans*, where only BOOC-containing toothpaste presented significant higher inhibition zones ($p < 0.001$). Considering the outcomes of the antimicrobial property test, the most effective natural experimental toothpaste was the BOOC-containing one, which showed better antimicrobial behavior even from the commercially available synthetic toothpaste (positive control). The tested natural antimicrobial agents were effective for enhancing the antimicrobial properties of the experimental toothpastes that were included, especially Biosecur Organic Oral Care agent.

Keywords: tooth caries; dental microbial plaque; natural cosmetics; natural toothpaste formulation; oral hygiene; periodontal disease



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1. Introduction

The market for natural products is expanding globally across a variety of industries, such as food, fashion, and cosmetics. In the past, chemicals were used to replace expensive natural ingredients, making cosmetics available and widely used by everyone. Today,

the trend towards a healthier lifestyle is changing perceptions and increasing consumer interest in natural products, including cosmetics [1]. Natural cosmetics are purchased by individuals who follow an environmentally friendly lifestyle and care about their health, beauty, and appearance [2].

Current environmental problems serve as stimuli for consumers, encouraging them to purchase natural products [3]. Natural products are developed according to ecological standards and are refined as such. They undoubtedly have various advantages, for example: less use of water, materials, and energy during their production, they are non-polluting or only slightly polluting to the environment, and their packaging can be recycled [4].

Since the time when the human population realized the importance of overall health preservation and the burden of diseases, there has been a search for therapeutic properties in the natural environment. Herbal therapy is the use of plants with medicinal properties to prevent and treat conditions that can affect general health [5]. Recently, there has been increasing interest in the use of traditional herbal therapy alongside synthetic modern drugs. Approximately 80% of the population, especially in developing countries, relies on this for healthcare [6]. According to the World Health Organization (WHO), oral health is considered an important part of general health and quality of life [7]. The use of natural medicines for managing pathological oral–dental conditions, such as caries, periodontal disease, microbial infections, oral cancers, and inflammatory conditions, can be a reasonable alternative to pharmaceutical methods due to their availability, low cost, and fewer side effects [8].

Moreover, herbal therapy has gained significant popularity over the past decade. Many researchers in the fields of dentistry and pharmacology have dedicated their time and resources to evaluating natural products, discovering their bioactive compounds, and finding applications for them in various aspects of oral health maintenance [9,10]. It is noteworthy that polyphenolic compounds, such as flavonoids and tannins, are among the common bioactive ingredients found in herbal extracts, with promising beneficial properties, such as anti-inflammatory, antimicrobial, antioxidant, and anticancer activities [11].

Natural toothpastes were introduced to limit the use of chemical ingredients commonly found in conventional toothpastes. Many natural toothpastes do not contain sodium lauryl sulfate, polyethylene glycol (PEG) derivatives, artificial colors, synthetic flavors, or sweeteners. The ingredients found in natural toothpastes vary widely but often include minerals, such as silica, kaolin, and perlite, natural sweeteners, herbal extracts, and essential oils [12]. The European standards for natural products were defined in the COSMOS Standard (Cosmetics Organic and Natural Standard) published in 2013 [13]. However, these standards depend on the manufacturer of the cosmetics or the country of origin and are not European legislation but rather the guidelines of an international, reputable, yet private body [14].

As mentioned before, herbal plants contain numerous beneficial components, including tannins, phenolic compounds, saponins, minerals, antioxidants, flavonoids, vitamins, and macronutrients [8,15]. These natural substances exhibit a diverse range of mechanisms of action, such as inhibiting ATP synthesis and energy metabolism, promoting enamel remineralization, whitening effects, preventing colony formation and adhesion, and altering pH homeostasis [16–20]. The antibacterial properties of herbal extracts arise from the ability of their active components to interact with bacterial cell walls. Additionally, these antibacterial effects are directly linked to the extracts' capacity to interact with soluble and extracellular proteins [21,22].

In addition, some of these active components that contain herbal plants exhibit antioxidant activities, which play a key role in preventing the oxidation of specific substances, a process that can generate free radicals capable of causing cell damage [23]. Antioxidant compounds may neutralize these free radicals, effectively halting the oxidation process [24].

Similar findings were reported in a previous study [25], which observed a correlation between antioxidant levels and the total phenolic content. Another antimicrobial mechanism of action was reported in a study on *Staphylococcus aureus*, in which soybean isoflavones were found to inhibit DNA unwinding by affecting topoisomerase I and II enzymes. This interference reduced nucleic acid production, increased the presence of supercoiled DNA, and ultimately inhibited bacterial cell division [26].

The purpose of this *in vitro* study was to evaluate the antimicrobial properties of three developed natural toothpastes containing different antimicrobial agents: (a) Biosecure Organic Oral Care, (b) Microsilver BG, and (c) Cranberry LG. The first one includes mandarin, bitter orange, and sweet orange peel extracts that contain flavonoids, which have been used in toothpastes due to their antimicrobial properties [27]. The second one includes silver microparticles that have been recognized to exhibit valuable antimicrobial properties in dental products [28], and the third one includes cranberry extracts, which includes proanthocyanidins and have been used in various oral health products [29]. These toothpastes were compared with a natural toothpaste of the same composition but without any added natural antimicrobial agent, as well as with a commercial synthetic toothpaste. The antimicrobial properties of the toothpastes were assessed using the disc diffusion test against three oral pathogens: *Candida albicans*, *Streptococcus mutans*, and *Prevotella intermedia*. The null hypothesis of the study was that all the tested toothpastes would exhibit the same antimicrobial properties.

2. Materials and Methods

2.1. Preparation of the Natural Toothpaste

The preparation of 500 g natural toothpaste at the laboratory, which was the base for the developed antimicrobial toothpastes, was conducted according to the following procedure.

The process began by adding deionized water to a beaker. The beaker was then placed on a heated magnetic stirrer, where the water was gently warmed to a temperature of 35 °C. This controlled heating created an optimal environment for the dissolution of subsequent additives. Once the water reached the desired temperature, sodium benzoate was added to the beaker. Using the magnetic stirrer, the solution was mixed thoroughly until the compound was completely dissolved. This phase was carefully monitored, and after 5 min, a dissolution check was conducted to ensure full integration of the sodium benzoate.

Following the successful dissolution of sodium benzoate, sodium fluoride was added to the mixture. The stirring process continued until the sodium fluoride was fully dissolved, maintaining consistent monitoring. After another 5-min period, a dissolution check was performed to confirm the homogeneity of the solution. Subsequently, the addition of 5% *w/w*. Xylitol, as a sweetener, was used in powder form, and the mixture was stirred until it was fully dissolved.

Next, sorbitol was introduced into the main beaker. The solution was stirred vigorously until sorbitol was fully dissolved, a process that typically requires between 5 and 10 min. A subsequent dissolution check ensured that the mixture remained uniform and free of any undissolved particles. Then, blanose 7M1F, was added to the beaker. To achieve even dispersion and form a viscous gel, an external homogenizer was employed. The homogenizer operated at 3500 rpm for 10 min, ensuring that the Blanose 7M1F was uniformly distributed throughout the solution. A final check was conducted to verify complete dissolution and uniform gel formation.

The thickening agent Tixosil 73 was gradually introduced into the mixture using an external homogenizer. It was essential to disperse it evenly throughout the entire mass to achieve a homogeneous solution. During this process, the homogenizer was set to operate between 4000 and 5000 rpm, with dispersion checks conducted at regular intervals

(15–20 min). Stirring was briefly halted to remove any residual Tixosil 73 powder that may have accumulated on the blades of the homogenizer, ensuring consistent particle distribution. Following the successful dispersion of Tixosil 73, Tixosil 43 was slowly and incrementally added. The external homogenizer, operating at 4000–5000 rpm, ensured that this thickening agent was uniformly distributed throughout the sample. Given that the addition of Tixosil 43 causes a marked increase in viscosity, careful control of the stirring speed was crucial to prevent damage to the mechanical equipment. Dispersion checks were carried out after 20–30 min to confirm uniformity. Periodically, stirring was paused to remove any remaining powder from the homogenizer blades, which helped maintain a consistent mixture.

Due to the mechanical energy input, the temperature of the formulation increased significantly. To prevent thermal degradation and maintain the integrity of the product, the sample was cooled to a stable temperature of 35 °C before proceeding. Once the mixture was cooled, Medialan LD PF10, a surfactant, was added. This step required gentle stirring without the use of the external homogenizer. This precaution minimized air entrapment, ensuring a smoother and more stable formulation. A final dissolution check was performed after 5 min to ensure that the surfactant was fully integrated. The addition of the flavor “Natural Strong Mint” (1% *w/w*) was made at 38 °C and stirred until it was fully dissolved in the toothpaste. The composition of the natural toothpaste is shown in Table 1.

Table 1. The composition of the natural toothpaste.

Ingredients	Commercial Name	INCI Name	% (<i>w/w</i>)
Deionized water	-	Aqua	11.78
Purox S	Emerald Chemical	Sodium Benzonate	0.40
Sodium fluoride powder	Honeywell	Sodium Fluoride	0.32
Meritol 160	TereosSyrat	Sorbitol	60.00
Xivia C	Danisco	Xylitol	5.00
Blanose 7M1F	Ashland	Sodium Carboxymethylcellulose	1.50
Tixosil 73	Solvay	Hydrated Silica	8.00
Tixosil 43	Solvay	Hydrated Silica	9.00
Medialan LD PF10	Clariant	Aqua, Sodium LauroylSarcosinate	3.00
Flavor Natural Strong Mint	Vioryl	Aroma	1.00

INCI name: International Nomenclature Cosmetic Ingredient.

2.2. Preparation of the Developed Natural Antimicrobial Toothpastes

The preparation of the three experimental natural toothpastes was conducted by incorporating three different antimicrobial agents including Biosecur Organic Oral Care (BOOC), Microsilver BG, and Cranberry LG. The first one (BOOC) was added directly to the toothpaste with simple stirring until fully incorporated. The powder of Microsilver BG was dispersed in glycerin (at a 1:7 ratio) after the water solution (in the same ratio) was discarded. This was done because the substance was insoluble in water, and complete separation of the mixture occurred after a short time at room temperature. Wetting the silver powder in glycerin produced a gray paste. This was then added to the natural toothpaste using a homogenizer to achieve better dispersion. The Cranberry LG was added directly to the natural toothpaste with simple stirring until fully incorporated, same as the first one. An excellent dispersion of the powders and homogeneity were observed, with a few trapped air bubbles in all three samples during microscopic imaging. Their physical characteristics and their content (% *w/w*) in the toothpaste are presented in Table 2.

Table 2. The composition, content (% *w/w*) and physical characteristics of the tested antimicrobial agents.

Commercial Name of the Natural Antimicrobial Agent (Manufacturer)	INCI Name—Composition	Content (% <i>w/w</i>)	Physical Characteristics of the Natural Antimicrobial Agent
Biosecur Organic Oral Care (Sharon)	Glycerin, Citrus Reticulata Fruit Extract, Citrus Aurantium Amara Fruit Extract, Citrus Aurantium Sinensis Peel Extract, Ascorbic Acid, Citric Acid, Lactic Acid, Water	0.1%	Brown, slightly viscous liquid consisting of a mixture of citrus extracts in glycerin (extracts from: mandarin fruit, bitter orange fruit, sweet orange peel)
Microsilver BG (BioGate)	Silver	0.1%	Grey powder
Cranberry LG (Givaudan)	Glycerin, Vaccinium Macrocarpon Extract, Water	0.5%	Colorless solution of cranberry extract in glycerin

The samples were centrifuged for 60 min at 4000 rpm and observed under a microscope to adequately check the proper dispersion of the powders in the three natural cosmetic products and the homogeneity of the final product. The physical–chemical parameters of the developed natural antimicrobial toothpastes are presented in Table 3. A microbiological test was also conducted on all three samples, as required for cosmetic products by legislation (European Regulation EC 1223/2009 [30]). The tests were carried out in accordance with the European Pharmacopoeia, 10th edition [31].

Table 3. The physical–chemical characteristics of the developed natural antimicrobial toothpastes with antimicrobial agents.

Antimicrobial Natural Toothpaste	Appearance/Color	Flavor/Odor	pH	Viscosity (Brookfield DVI+, Spindle: F–96, rpm: 1.5, T: 20 °C)
Biosecur Organic Oral Care (0.1% <i>w/w</i>)	Yellowish gel	Characteristic of flavor (mint)	6.89	313.000 cPs
Microsilver BG (0.1% <i>w/w</i>)	Grey gel	Characteristic of flavor (mint)	6.85	289.000 cPs
Cranberry LG (0.5% <i>w/w</i>)	Transparent gel	Characteristic of flavor (mint)	6.78	285.000 cPs

2.3. Evaluation of the Antimicrobial Properties of the Developed Toothpastes

The development of a natural toothpaste has to be acceptable in terms of its organoleptic characteristics (appearance, color, flavor), meaning it must meet the physicochemical and microbiological specifications set for this type of product (pH, viscosity, dispersion of silica particles, total aerobic microbial count, molds, yeast, and absence of pathogens). For this reason, the newly developed natural products successfully passed both accelerated and long term stability tests according to protocol by ICH Topic Q1 A(R2): 40 ± 2 °C/75% RH for 3 months, 50 ± 2 °C/75% RH for 1 month, 25 ± 2 °C/60% RH for 12 months, 5 ± 3 °C for 6 months, and Freeze/Thaw Cycle Tests (4) to be considered stable over time, before conducting the antimicrobial property test.

Three equally stable versions of natural toothpaste were achieved, each with a different natural antimicrobial agent. The natural toothpastes, SAMPLE A (0.1% BOOC), SAMPLE B (0.1% Microsilver BG), and SAMPLE C (0.5% Cranberry LG), met all the required specifications for such a formulation. Therefore, a comparative *in vitro* efficacy study of the three natural antimicrobial agents compared to a negative control (SAMPLE D) as well as a commercial synthetic toothpaste as a positive control (SAMPLE E) was conducted.

The method that was used in this experiment was the disc diffusion test based on Kirby–Bauer Test [32]. The tested toothpaste was placed in Petri dishes in which specific microorganisms selected for the study had been selectively cultivated. After incubation, the circular area formed around the discs (diameter), known as the inhibition zone, was measured, demonstrating the inhibitory effect of the product on the microorganisms used in the efficacy test. The larger the inhibition zone, the greater the antimicrobial action of the product, always compared with the reference systems and control samples [33–35].

More specifically, a sample of the product diluted with water at a 1:1 ratio (to simulate the clinical conditions) was added to sterile filter paper discs and placed on top of a pre-inoculated agar plate surface with bacteria or fungi. This test relies on the diffusion of the test product from the filter paper into the pre-inoculated solidified agar. If the test formulation inhibited or killed the bacteria or fungi, a circular area around the discs, known as the inhibition zone, was formed, demonstrating its inhibitory effect on the microorganisms under test.

The microorganisms used for this test along with the nutrient ingredients used for the preparation of their suspensions and the period of incubation are presented in Table 4.

Table 4. The characteristics, incubation conditions, and nutrient ingredients of the three microorganisms used in the test for evaluating the antimicrobial properties of the toothpastes.

Name (Lot Number)	Characteristics	Incubation Conditions	Nutrient Ingredients
<i>Candida albicans</i> (ATCC 10231)	Fungus, one of the main microorganisms of the normal oral flora, which, however, can grow, multiply easily, and cause fungal infections in the oral cavity	2 days at 25 °C	Sabouraud Dextrose Agar
<i>Streptococcus mutans</i> (ATCC 25175)	Gram-positive, anaerobic, round bacterium (coccus), commonly found in the human oral cavity and significantly contributes to the formation of dental caries	2 days at 37 °C	Brain Heart Infusion Agar
<i>Prevotella intermedia</i> (ATCC 25611)	Gram-negative, anaerobic pathogenic bacterium involved in periodontal infections such as gingivitis and periodontitis	2 days at 37 °C	Soybean-Casein Digest Agar

The discs made of filter paper were processed accordingly before the test in order to remove any background flora. Specifically, all filter discs were immersed in a 70% *v/v* isopropyl alcohol solution and allowed to dry covered, protected from cross-contamination. Standard suspensions of all microorganisms used during the test were prepared and inoculated onto the surface of suitable agar plates to form a uniform layer.

1. Test product: The filter paper discs were impregnated with 25 µL of the product being tested, which was diluted 50% with water. The discs were placed on the surface of pre-inoculated agar plates. The same procedure was repeated for all microorganisms.
2. Negative test: The filter papers were impregnated with 25 µL of sterile water. The discs were aseptically placed on the surface of pre-inoculated agar plates. The same procedure was repeated for all the tested microorganisms.
3. Each test product was compared with a negative control product, which did not contain an antimicrobial agent, and with a positive control product, which was a commercially available synthetic toothpaste (Instant Whitening Blue Toothpaste, Frezyderm SA, Athens, Greece), whose antimicrobial action was proven. The composition of this toothpaste is: deionized water, blue covasorb, hydrated silica, Cymenol, 0.32% *w/w* sodium fluoride (1450 ppmF).

Three independent repetitions were performed for each product and each microorganism to increase accuracy and minimize bias. The evaluation of the antimicrobial activity

of the product was conducted by measuring the diameter of the inhibition zones around the filter discs. Three measurements were taken per paper disc at three different points. The diameter was recorded in mm, and the average value was calculated per disc. The study was conducted using a double-blind design to enhance the objectivity of the research. This implies that neither the researchers conducting the experiments nor the statisticians analyzing the data were aware of the group assignments.

2.4. Statistical Analysis

The statistical analysis of the data was conducted using IBM SPSS Statistics version 23.0 (SPSS Inc., Chicago, IL, USA) software. Normality and homogeneity of the data were evaluated using the Shapiro–Wilk and Levene tests, respectively. To compare the mean diameter of the inhibition zone among the tested toothpastes for each microorganism (with Bonferroni corrections), one-way ANOVA and Tukey’s post hoc test were employed. Moreover, two-way ANOVA was used to ascertain significant interactions between toothpastes and microorganisms. The level of significance was preset at $\alpha = 0.05$.

3. Results

Two-way ANOVA showed a statistically significant main effect for the toothpaste used ($p < 0.05$), a statistically significant main effect for the microorganism used ($p < 0.05$) and a statistically significant interaction “toothpaste $\times$ microorganism” ($p < 0.05$). The means and standard deviations of the diameter of the inhibition zone in mm that formed by the six experimental groups of the study are presented in the Table 5 and illustrated in Figure 1. Representative samples of Petri dishes of all the experimental groups that indicate the inhibition zones that were formed for each microorganism’s cultivation are shown in Figures 2–4.

Table 5. Means and standard deviations of the inhibition zone in mm that formed by the six experimental groups of the study for each tested microorganism. The same uppercase superscript in rows indicates no statistically significant difference ($p > 0.05$).

Microorganism	Toothpaste Sample A (mm)	Toothpaste Sample B (mm)	Toothpaste Sample C (mm)	Control-Sample D (mm)	Control+ Sample E (mm)	Sterilized Water (Negative Test) (mm)
<i>Candida albicans</i>	20.0 $\pm$ 1.0 ^A	19.3 $\pm$ 1.2 ^A	18.7 $\pm$ 1.5 ^A	14.3 $\pm$ 2.3 ^B	22.3 $\pm$ 2.1 ^C	0.0 $\pm$ 0.0 ^D
<i>Streptococcus mutans</i>	97.7 $\pm$ 17.0 ^A	70.3 $\pm$ 3.1 ^B	69.7 $\pm$ 4.5 ^B	74.3 $\pm$ 14.6 ^B	81.0 $\pm$ 13.1 ^C	0.0 $\pm$ 0.0 ^D
<i>Prevotella intermedius</i>	59.7 $\pm$ 1.2 ^A	53.0 $\pm$ 8.9 ^B	57.0 $\pm$ 3.6 ^A	52.0 $\pm$ 1.7 ^B	52.7 $\pm$ 7.6 ^B	0.0 $\pm$ 0.0 ^C

SAMPLE A: Biosecur Organic Oral Care-containing toothpaste; SAMPLE B: Microsilver BG-containing toothpaste; SAMPLE C: Cranberry LG-containing toothpaste; SAMPLE D: baseline toothpaste; and SAMPLE E: Instant Whitening Blue toothpaste.

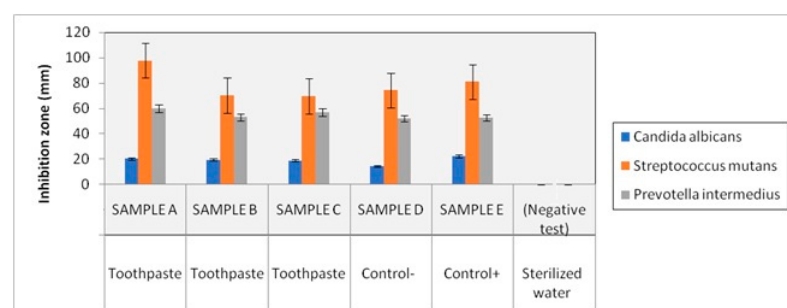


Figure 1. Means and standard errors of the inhibition zone in mm that formed in the six experimental groups of the study for each tested microorganism. SAMPLE A: Biosecur Organic Oral Care-containing toothpaste; SAMPLE B: Microsilver BG-containing toothpaste; SAMPLE C: Cranberry LG-containing toothpaste; SAMPLE D: baseline toothpaste; and SAMPLE E: Instant Whitening Blue toothpaste.

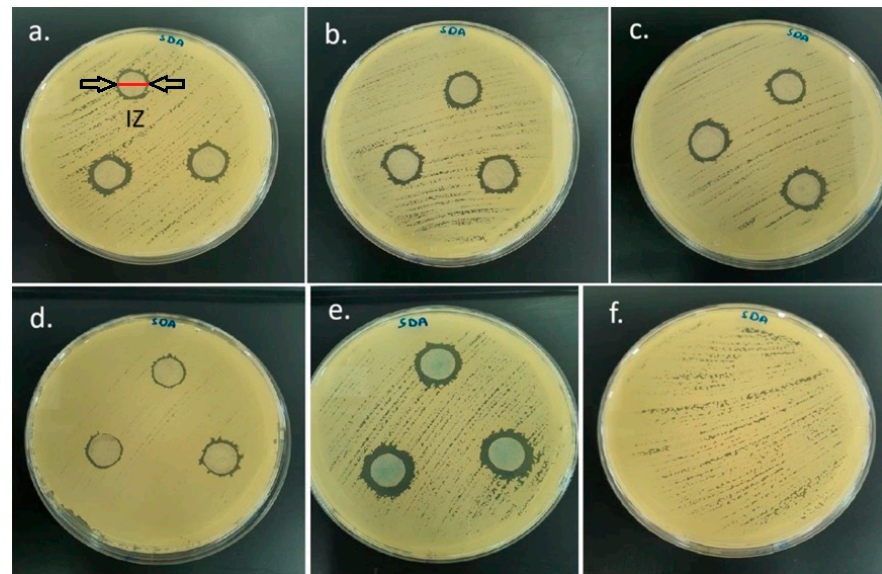


Figure 2. Representative samples of Petri dishes of all the experimental groups that indicate the inhibition zones (red line—IZ) that were formed in *Candida albicans* cultivations. (a): 0.1% Biosecur organic oral care-containing, (b): 0.1% Microsilver BG-containing toothpaste, (c): 0.5% Cranberry LG-containing toothpaste, (d): baseline toothpaste (control−), (e): synthetic toothpaste (control+), (f): water (negative test).

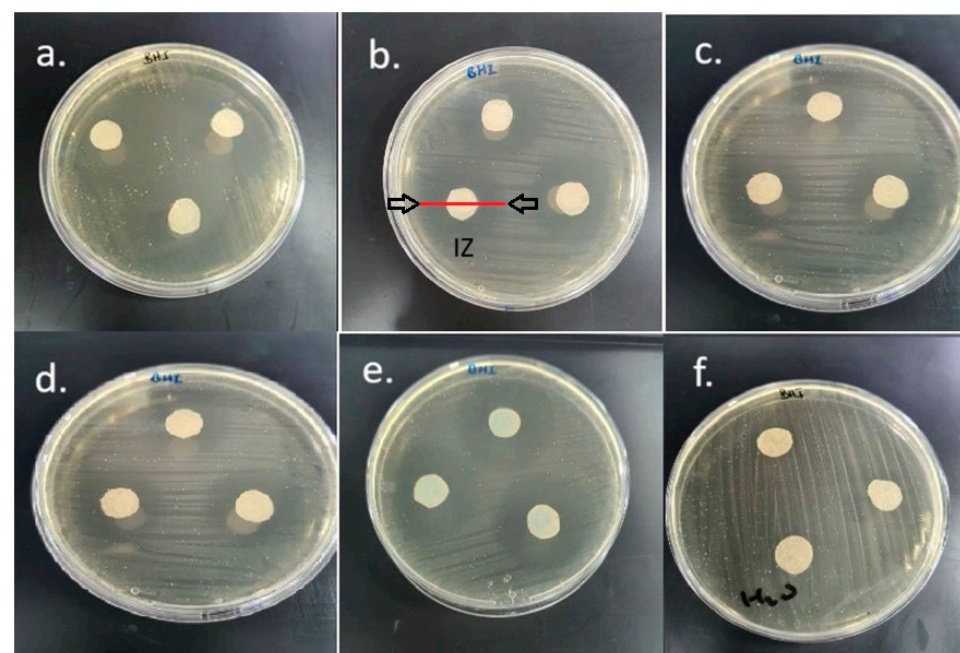


Figure 3. Representative samples of Petri dishes of all the experimental groups that indicate the inhibition zones (red line—IZ) that were formed in *Streptococcus mutans* cultivations. (a): 0.1% Biosecur organic oral care-containing, (b): 0.1% Microsilver BG-containing toothpaste, (c): 0.5% Cranberry LG-containing toothpaste, (d): baseline toothpaste (control−), (e): synthetic toothpaste (control+), (f): water (negative test).

SAMPLE A (BOOC-containing toothpaste) presented the highest inhibition zones among the other experimental groups ($p < 0.05$), except for *Candida albicans*, where SAMPLE E (control+) showed the highest values ($p < 0.05$), followed by SAMPLES A, B, and C, which did not differ from each other ($p > 0.05$). All the experimental toothpastes exhibited higher antimicrobial properties compared to the negative control group, except for *Streptococcus mutans*, for which only SAMPLE A presented significant higher inhibition

zones ($p < 0.001$). Considering the outcomes of the antimicrobial property test, the most effective natural experimental toothpaste was the BOOC-containing one (SAMPLE A), which showed better antimicrobial behavior even from the commercially available synthetic toothpaste (positive control).

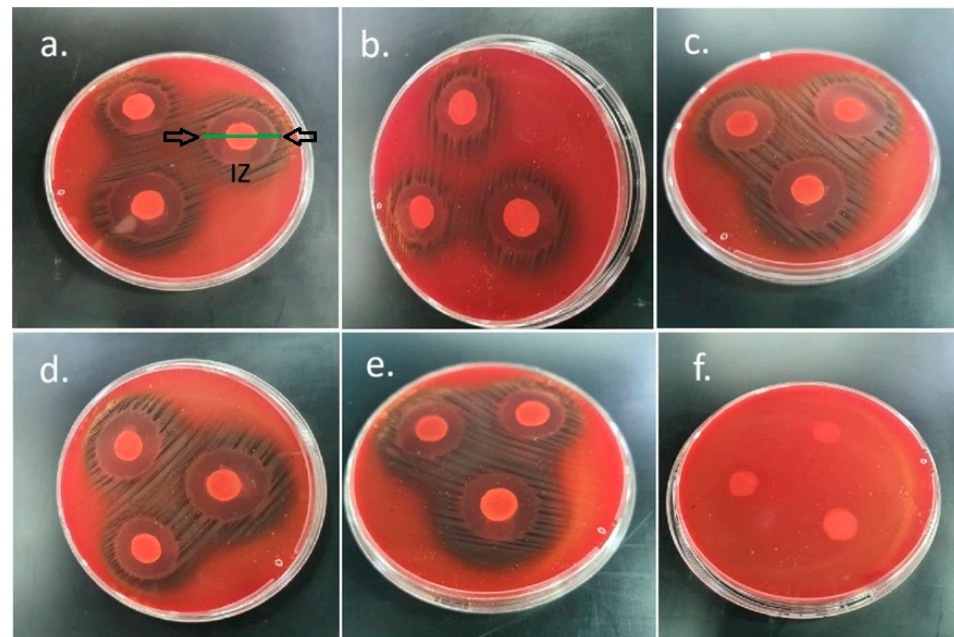


Figure 4. Representative samples of Petri dishes of all the experimental groups that indicate the inhibition zones (green line—IZ) that were formed in *Prevotella intermedia* cultivations. (a): 0.1% Biosecur organic oral care-containing, (b): 0.1% Microsilver BG-containing toothpaste, (c): 0.5% Cranberry LG-containing toothpaste, (d): baseline toothpaste (control−), (e): synthetic toothpaste (control+), (f): water (negative test).

4. Discussion

Based on the results of the current study, the null hypothesis, which stated that all the tested toothpastes would exhibit the same antimicrobial properties, was rejected. More specifically, SAMPLE A (with 0.1% Biosecur Organic Oral Care) was more effective against two of the three microorganisms studied (*Streptococcus mutans* and *Prevotella intermedia*) compared to the positive control (synthetic toothpaste). For the third strain (*Candida albicans*), the results were comparable. It was clearly more effective than the other two toothpastes, SAMPLE B (with 0.1% *w/w* Microsilver BG) and SAMPLE C (with 0.5% *w/w* Cranberry LG). On the other hand, SAMPLE B exhibited nearly the same performance as the positive control against all the microorganisms and performed better than SAMPLE C, while SAMPLE C exhibited slightly lower activity against all the microorganisms compared to the positive control.

It was therefore concluded that the flavonoids found in the mandarin fruit, the bitter orange fruit, and the sweet orange peel (Biosecur Organic Oral Care) were more effective against the three microorganisms studied compared to silver and the proanthocyanidins contained in cranberry extract. These differences in the antimicrobial properties may be attributed to the concentration of the active agents and the different mechanisms of antimicrobial action that they exhibit. Based on the above, the composition selected for the development of SAMPLE A (0.1% Biosecur Organic Oral Care) could serve as a viable proposal, in combination with the other natural ingredients, for the development of a natural toothpaste.

Flavonoids, which were included in the SAMPLE A, are a diverse group of plant-derived polyphenolic compounds and exhibit remarkable antimicrobial properties that make them valuable in dental products. These compounds target oral pathogens, such as *Streptococcus mutans*, *Candida albicans*, and *Prevotella intermedia*, which are associated with dental caries, periodontal diseases, and oral infections [36,37]. Flavonoids disrupt bacterial cell membranes, inhibit enzymes critical for microbial metabolism, and prevent biofilm formation, thereby reducing plaque accumulation and the risk of oral diseases [38]. Additionally, their antioxidant and anti-inflammatory properties help protect gum tissues from oxidative damage and inflammation caused by bacterial toxins [39]. By incorporating flavonoids from sources, such as citrus fruits, tea, and herbs, dental products, like toothpastes and mouthwashes, offer a natural, effective means to enhance oral hygiene and health [40].

Silver, which was included in SAMPLE B, has long been recognized for its potent antimicrobial properties, making it a valuable ingredient in dental products [41]. In its nanoparticle form, silver releases ions that disrupt microbial cell membranes, inhibit vital enzymatic functions, and interfere with DNA replication, effectively neutralizing a wide range of bacteria, fungi, and viruses commonly found in the oral cavity [42]. This broad-spectrum antimicrobial activity helps reduce dental plaque formation, combat periodontal pathogens, and prevent oral infections, such as gingivitis and periodontitis [43]. Additionally, silver exhibits a prolonged antimicrobial effect due to its sustained ion release, enhancing its efficacy in dental products, like toothpastes, mouthwashes, and dental coatings. Its biocompatibility and effectiveness at low concentrations further underscore its suitability for use in maintaining oral health [44].

Proanthocyanidins, a class of polyphenolic compounds found in various plants, such as cranberries, have demonstrated significant antimicrobial properties, making them a valuable component in dental products [45]. These compounds inhibit the adhesion of bacteria, such as *Streptococcus mutans*, to tooth surfaces, thereby reducing the formation of dental plaque and the risk of caries [16]. Moreover, it has been reported that A-type cranberry proanthocyanidins by affecting the adherence properties of *Candida albicans* and attenuating the inflammatory response induced by this pathogen, represent potential therapeutic agents for the prevention/treatment of oral candidiasis [46]. Additionally, proanthocyanidins disrupt biofilm formation and neutralize bacterial toxins, contributing to their efficacy in managing periodontal diseases [47]. Their antioxidant properties further support oral health by reducing inflammation and protecting gum tissues from oxidative stress caused by bacterial activity [48]. The inclusion of proanthocyanidins in dental products, like toothpastes and mouthwashes, provides a natural and effective approach to confront oral pathogens and promote overall dental hygiene [49].

In the present study, three different microorganisms were tested (*Streptococcus mutans*, *Candida albicans*, and *Prevotella intermedia*), which has been documented to constitute a crucial role in the most common oral diseases (dental caries, periodontitis, and oral candidiasis, respectively). It is beyond doubt that not only the tested microorganisms are responsible for the aforementioned oral diseases. However, useful information can be derived when evaluating antimicrobial properties of such dental products using the tested microorganisms. In particular, the cariogenic potential of *Streptococcus mutans* biofilm has been well-established [50] and is attributed to three key factors: (i) its ability to produce acid, (ii) its acid resistance allowing it to metabolize various carbohydrates into organic acids and persist under low pH conditions, and (iii) its capacity to synthesize extracellular polymers. These polymers promote biofilm growth, protect bacterial cells, and enable their survival in hostile environments [51]. The matrix production associated with cariogenic biofilm formation is facilitated by three glucosyltransferases (GtfBCD) produced by *Streptococcus mutans* [52]. However, advancements in the prevention and treatment

of dental caries have shown that focusing solely on targeting *Streptococcus mutans* and reducing sugar intake is insufficient for effective caries prevention. Therefore, while the experimental toothpastes may demonstrate activity against *Streptococcus mutans*, this does not necessarily translate to clinical inhibition of dental caries.

Prevotella intermedia is a Gram-negative, anaerobic bacterium commonly associated with periodontal diseases and other oral infections. It is considered a key player in the progression of periodontitis due to its ability to invade gingival tissues and trigger inflammatory responses [37,53]. *P. intermedia* thrives in the subgingival environment, where it contributes to the formation and maturation of dental biofilms. It is particularly adept at utilizing hemoglobin and other heme-containing molecules as growth factors, giving it a competitive advantage in inflammatory conditions [54]. The presence of such bacteria triggers immune system activation, which leads to the generation of pro-inflammatory cytokines and, ultimately, reactive oxygen species (ROS). Periodontal structure decays are destroyed because of this chronic inflammatory illness [55].

Candida albicans is a common opportunistic fungal pathogen that resides as part of the normal microbiota in the oral cavity. Under conditions of immune suppression, dysbiosis, or other environmental shifts, it can transition from a commensal organism to a pathogenic state, causing infections, such as oral candidiasis [56]. In the oral cavity, *Candida albicans* can form biofilms on mucosal surfaces, dentures, and dental implants, enhancing its resistance to antifungal agents and the host immune response. Its pathogenicity is driven by factors such as hyphal formation, adhesion to host tissues, and the production of enzymes, like secreted aspartyl proteases [57].

The synergy between active components in the natural toothpastes, as well as their content in these components, are crucial for preventative effects, stimulating the regulatory action of the defensive processes of the body, and preparing the body for potential activity against external agents, making certain herbal agents, in some cases, more successful at healing the body than pharmaceuticals. These combinations in active agents and their concentrations determine the antimicrobial efficacy and interpret the differences among the products. This is inconsistent with the outcomes of the present investigation, where a natural toothpaste (SAMPLE A) presented better antimicrobial behavior compared to the other natural toothpastes and the synthetic toothpaste that were investigated.

In the current study, the selected method for evaluating the antimicrobial properties of the products was the disc diffusion test. This method, while widely used for evaluating antimicrobial properties, has several limitations that can affect its accuracy. Some of these limitations are the qualitative nature of the method, diffusion limitations, influence of agar composition, limited tested microorganisms, non-uniform growth of the organisms, inability to test bactericidal and bacteriostatic effects, concentration gradient, the absence of simulated oral environment factors, such as the presence of saliva, etc. Additionally, it is important to mention that in the current study, only one representative causal microorganism was investigated for each oral disease. In clinical conditions, the pathogenic process of these diseases include more microorganisms. To address these limitations, it is suggested the disc diffusion test to be complemented with other methods, such as broth microdilution, time-kill assays, or molecular techniques, for a more comprehensive evaluation of antimicrobial properties.

It is beyond doubt that not all bacteria present in the oral cavity are pathogenic. In fact, many of these bacteria play a crucial role in maintaining the balance of the oral microbiota and the composition of the dental biofilm. Although the development and use of toothpastes with antimicrobial activity is an interesting proposal, it is essential to consider the possible adverse effects of an indiscriminate use of these compounds, such as the imbalance of the oral microbiota. For this reason, the use of targeted antimicrobial

agents that selectively inhibit pathogenic bacteria while preserving beneficial species is of great importance. Moreover, the indiscriminate and prolonged use of antimicrobial toothpastes, particularly in individuals with a healthy oral microbiota, should be avoided. Further research on the long-term effects of antimicrobial compounds on oral health is necessary to provide more insights for the use of these toothpastes.

The findings of this study on the antimicrobial properties of natural toothpastes offer several promising clinical implications and directions for future research, such as a key role in preventive dentistry, particularly for patients seeking more biocompatible, eco-friendly alternatives to conventional oral care products. Their ability to reduce pathogenic oral microorganisms while preserving the oral microbiome could improve oral health outcomes. With further research, natural toothpaste formulations could be tailored to target specific oral pathogens, enhancing their therapeutic efficacy while minimizing side effects or resistance development. Future studies should assess parameters, such as plaque control, microbial load reduction, enamel remineralization, and patient-reported outcomes, like taste and tolerability.

5. Conclusions

Within the limitations of the present in vitro study, it can be concluded that the tested natural antimicrobial agents incorporated into the developed toothpastes were effective forenhancing antimicrobial activity against the tested microorganisms, particularly the Biosecur Organic Oral Care agent. These findings are promising for the potential use of these antimicrobial agents in natural dental products to combat oral diseases. However, further laboratory tests and clinical studies are required to evaluate the effectiveness of these developed products and their potential role in preventive dentistry.

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