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Antibacterial Efficacy of Novel Eastern Medicine-Inspired Toothpastes Compared to Commercial Formulations

Joshua S. Goldfaden
University of the Pacific, Arthur A. Dugoni School of Dentistry, j_goldfaden@u.pacific.edu

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Antibacterial Efficacy of Novel Eastern Medicine-Inspired Toothpastes Compared to Commercial Formulations

Abstract

Toothpaste is an oral health care agent that dates back to the 5th Century B.C. in East Asia. Following the conception of dentistry in the 7th Century B.C. and the field's subsequent growth, the embodiment of toothpaste has evolved from containing natural substances such as tea leaves to abrasive chemicals, particularly: fluoride, triclosan, and sodium lauryl sulfate (SLS). Regarding how each respective reagent functions, fluoride forms a complex with calcium ions in the teeth to prevent erosion of the protective enamel coating. This ion also disrupts metabolic activities of *Streptococcus mutans* (*S. mutans*), which are the bacteria that cause cavities. Similarly, triclosan is involved in countering gingivitis and limiting bacterial growth. SLS serves as a foaming agent in many toothpastes for texture. Although these chemicals are generally effective in maintaining oral health and are present in many mainstream toothpastes, they also impose various health complications in humans. In particular, fluoride disrupts a vital metabolic pathway in odontoblasts (teeth cells), resulting in cellular death. Regarding triclosan, even in small concentrations within toothpaste, has been found to downregulate anticancer genes. Concerning SLS, this ingredient contributes to the formation and prolongation of mouth ulcers and also diminishes oral epithelial tissue thickness. In place of these ingredients, I have formulated three toothpastes that contain different combinations of the following, minimally abrasive Eastern medicine ingredients: coconut oil, sodium bicarbonate, bamboo charcoal powder, xylitol, matcha, and cinnamon oil. After conducting a minimum inhibitory concentration test to determine the efficacy of these formulations against *S. mutans*, my pilot data suggest that one of the formulations exhibits antimicrobial properties that are qualitatively comparable to three mainstream toothpastes from Colgate®, Crest®, and Sensodyne®.

Keywords

Eastern Medicine, Pharmaceutical Sciences, Dental Hygiene, Dental Materials

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Introduction

Toothpaste is an oral health care agent that is believed to date back to the 5th Century B.C. in East Asia. Following the conception of dentistry in the 7th Century B.C. and the field's subsequent growth, the embodiment of oral care products has evolved from natural substances such as tea leaves and oils to abrasive chemicals, in particular: fluoride, triclosan, and sodium lauryl sulfate (SLS). Regarding how each respective reagent functions, fluoride forms a complex with calcium ions in the teeth to prevent erosion of the protective enamel coating. Additionally, this ion also disrupts the metabolic activities of *Streptococcus mutans* (*S. mutans*), which is the primary strain of bacteria responsible for causing cavities [1]. With respect to triclosan, this potent antibacterial agent is involved in countering gingivitis through limiting bacterial growth [2]. As for SLS, this chemical is both an anionic detergent and surfactant, which serves as a foaming agent in many commercial toothpastes for texture [3].

Although these three chemicals are generally effective in maintaining oral health, they also impose various health complications in humans. In particular, therapeutic concentrations of fluoride such as in toothpaste are not only effective in disrupting the metabolic processes of bacteria, but exhibit the same effects in odontoblasts (teeth cells) via a JNK-dependent mechanism – this ultimately induces cellular death via apoptosis [4]. Additionally, fluoride was found to be cytotoxic to cultured human pulp cells by inhibiting cellular growth and proliferation, mitochondrial metabolism, as well as protein synthesis [5]. With respect to triclosan, even in low concentrations such as within toothpastes, this bactericide is responsible for the downregulation of genes coding for the protein p21 as well as BAX proteins, both of which are involved in the prevention of ovarian cancer [6]. Coincidentally, upon determining an association between triclosan and osteoporosis in women, the Food and Drug Administration

banned commercial toothpastes containing triclosan in 2019, just after the beginning of this study [7]. Concerning SLS, this ingredient contributes to the formation and prolongation of mouth ulcers [3]. Furthermore, SLS has been found to diminish the thickness of oral epithelial in vitro models by interfering with cellular adhesion, a process by which neighboring cells attach to each other [8]. In place of these ingredients, I propose a toothpaste embodiment that is composed of natural, minimally abrasive Eastern medicine ingredients: matcha, coconut oil, cinnamon oil, bamboo charcoal powder, in addition to xylitol, sodium bicarbonate, and calcium carbonate.

Addressing the first proposed ingredient, matcha is a powder derived from green tea leaves that effectively reduces plaque and gingivitis thus enhancing freshness of breath [9]. Additionally, matcha is an effective antibacterial agent that counters oral pathogens with its antioxidant properties [10]. Further, these antioxidant properties of matcha are effective in preventing the development of cancer within the oral cavity [11]. Relatedly, coconut oil has been found to have similar antibacterial properties and is a safer alternative to SLS. That is, coconut oil has been proven to reduce the number of *S. mutans* in saliva [12]. Moreover, coconut oil has the ability to increase mucous levels of epithelial tissue that line body cavities as well as reduce acid levels in the body that are responsible for inducing and perpetuating ulcers [13]. As such, coconut oil ultimately diminishes oral inflammation and also has been found to exhibit teeth whitening properties [14]. Similarly, bamboo charcoal powder functions as a tooth whitening agent, acts as a bactericide against *S. mutans*, and is also an effective toxin absorbent [15].

As both coconut oil and bamboo charcoal lack flavors that complement matcha's naturally sweet flavor, the proposed toothpaste embodiment includes cinnamon oil to enhance flavor. Moreover, this antioxidant is a strong inhibitor of many periodontal pathogens,

particularly *S. mutans* [16]. An additional proposed flavor enhancer in this formulation is xylitol, a naturally occurring sugar alcohol present in some plants. Not only does this biomolecule provide the formulation with a sweet taste, but xylitol also inhibits the growth of *S. mutans* as they are unable to metabolize it [17]. One reagent adapted from commercial toothpastes that is incorporated into this embodiment is sodium bicarbonate. This particular compound is minimally abrasive in nature as it is relatively soft when compared to tooth enamel and dentin (calcified tissue underneath the enamel), yet this compound is harder than dental plaque and thus easily removes it from the surfaces of teeth [18]. Moreover, sodium bicarbonate diminishes mouth odor and raises the pH of the mouth, thus disrupting the ideal, acidic environment in which *S. mutans* amongst other oral bacteria thrive [18]. As a complement to sodium bicarbonate, calcium carbonate, a modern component of toothpaste, also acts as an abrasive through effectively lifting stains from teeth [19].

In order to assess the quality of the experimental toothpaste formulations, two criteria must be evaluated: antibacterial efficacy and consumer safety. In assessing the former criterion, a minimum inhibitory concentration (MIC) test will be implemented to determine the efficacy of both the proposed toothpaste as well as those from mainstream brands in countering the growth of *S. mutans*. Specifically, this assay entails exposing *S. mutans* to a spectrum of toothpaste concentrations, which are uniform between each experimental group. These identical toothpaste mixtures create a means of comparing product efficiency [20]. To test the consumer safety of each toothpaste, in vitro human fibroblasts derived from healthy gingival tissue will serve as the model in the Cell Counting Kit-8 (CCK-8) assay. Particularly, this method conveniently quantifies cellular proliferation and cytotoxicity of drugs by utilizing the highly water-soluble tetrazolium salt, WST-8 [21]. When absorbed by cells, WST-8 is reduced to formazan dye, a

yellow-colored substance, by dehydrogenase enzymes [21]. Since the absorbance of formazan is at 460 nm, a calibration curve can be generated to determine the absorbance value of each experimental sample. This value is proportional to the number of viable cells and thus the safety of the drug being studied [21].

By incorporating the proposed natural ingredients into novel toothpaste formulations and subsequently implementing the experimental methods described previously to compare the efficacy as well as the safety of these experimental toothpastes with commercially-available products, I am confident that a safer, equally effective line of toothpaste can be introduced to the dental field.

Materials and Methods

a. Formulation of Proposed Toothpaste

In developing a novel Eastern medicine-inspired toothpaste, the embodiment of "Total Advanced" Toothpaste by Colgate ® was used loosely as a model [22]. Three formulations were prepared in a pharmaceutical compounding laboratory within the Chapman University School of Pharmacy. It is noteworthy that all reagents were transferred into weigh boats using an assortment of spatulas and were subsequently massed using an analytical balance. Each of the reagents were then transferred and mixed in an eight-ounce glass mortar and were pestled. Prior to measuring out each ingredient, each spatula as well as the glass mortar and pestle were wiped down with a 70% ethanol solution to minimize contamination. The three formulations prepared are as follows: Formulation I: 25.000 grams of coconut oil, 6.250 grams of calcium carbonate, 6.250 grams of sodium bicarbonate, 7.000 grams of bamboo charcoal powder, 4.000 grams of xylitol, 2.000 grams of matcha powder, and 1.000 grams of cinnamon oil; Formulation II: 27.000

grams of coconut oil, 3.250 grams of calcium carbonate, 4.250 grams of sodium bicarbonate, 10.000 grams of bamboo charcoal powder, 3.000 grams of xylitol, 1.500 grams of matcha, and 1.500 grams of cinnamon oil; and Formulation III: 29.500 grams of coconut oil, 1.600 grams of sodium bicarbonate, 10.700 grams of bamboo charcoal powder, 2.700 grams of xylitol, 1.000 grams of matcha, and 2.700 grams cinnamon oil. These formulations were each transferred into three individual ointment tubes, each of which were previously rinsed with a 70% ethanol solution.

b. Minimum Inhibitory Concentration (MIC) Assay

The objective of this portion of the experiment was to assess the antibacterial efficacy of the three experimental toothpaste formulations relative to three commercial toothpastes: Sensodyne ® "Extra Whitening Toothpaste," Colgate ® "Total Advanced Whitening Toothpaste," and Crest ® "Cavity Protection Toothpaste." First, 8 mL of a 1x brain heart infusion (BHI) growth medium was decanted into a 15-mL conical tube under a lab workbench fume hood. Then, *S. mutans* ATCC25715 (serotype c) frozen at -80°C were transferred into the 15-mL conical tube using an inoculation loop. The conical tube was subsequently vortexed for approximately 5 seconds and placed in a 37°C incubator for 24 hours. It is worth noting that both prior to using the workbench as well as after use, the area was sterilized with a 70% ethanol solution.

After incubating the *S. mutans* for 24 hours, the 15-mL conical tube was vortexed for roughly 5 seconds to disperse the plethora of bacterial growth present at the bottom of the tube. Next, under the fume hood, 1 mL of a normal saline solution was decanted into a 2-mL glass vial. Additionally, 30 µL of the overnight *S. mutans* culture was successively added to the 2-mL glass vial until the cloudiness of the solution visually matched that of a 0.5 McFarland Latex

Standard. Once the desired 0.5 McFarland turbidity was achieved, two 50-mL conical tubes containing horse blood were centrifuged at 3,000 rotations per minute (rpm) for 20 minutes. As the horse blood was being centrifuged, each toothpaste was diluted as follows: for the three experimental formulations, 1.000 gram of each toothpaste was massed in separate 15-mL conical tubes using an analytical balance. Similarly, 0.100 grams of each commercial toothpaste were massed in separate 15-mL conical tubes using the same analytical balance for consistency. Based on the protocol and findings of Dongol, Gautam, and Shrestha [20], each toothpaste was diluted under the fume hood in the following manner: 500 µL of a 5% DMSO solution in sterilized DI water was decanted into each of the 15-mL conical tubes containing 1.000 gram of experimental toothpaste. After manually shaking each 15-mL conical tube, a 2:1 homogenous mixture of toothpaste using 5% DMSO was prepared. The same process was repeated for the three 0.100-gram commercial toothpaste samples, except 800 µL of 5% DMSO was decanted into each 15-mL conical tube to ultimately create a 1:8 homogenous mixture of toothpaste using 5% DMSO [20].

200 µL of each toothpaste mixture were then decanted into the wells of the first and last columns of a 96-well plate. In the remaining 10 wells of each row, 100 µL of 1x BHI growth medium was decanted using a multichannel pipette. From the wells containing 200 µL of toothpaste mixture, 100 µL of each mixture was transferred to the neighboring well containing 100 µL of 1x BHI growth medium. The toothpaste mixtures and the growth medium were pipetted together several times using a multichannel pipette to thoroughly combine both solutions. This process was repeated such that five sequential serial dilutions were created from each original toothpaste mixture. Further, these serial dilutions were duplicated in neighboring rows. It is noteworthy that each of the wells had a final volume of 100 µL. Regarding the two

remaining rows of the 96-well plate, the wells of one row were filled with 100 µL of 1x BHI growth medium to serve as a negative control. As for the last row, 200 µL of 1x BHI growth medium was decanted into each well to serve as a blank to account for any contamination.

Once the 96-well plate was prepared, 60 µL of the 0.5 McFarland overnight growth solution created previously was decanted into a 15-mL conical tube under the fume hood. Additionally, 180 µL of horse blood and 8.760 mL of 1x BHI growth medium were added to the tube, which was then vortexed for approximately 5 seconds. The homogenized solution was next decanted into a sterile multichannel reservoir. After, a multichannel pipette was used to transfer 100 µL of the solution into all wells of the 96-well plate that had a volume of 100 µL (i.e. all wells of the 96-well plate have a final volume of 200 µL). The lid of the 96-well plate was secured using tape to prevent contamination, and the plate was left in the 37°C incubator for 24 hours. As the toothpaste solutions interfered with visually interpreting results, 96 individual inoculation loops were used to transfer the solution of each well to marked, 5-mL BHI agar petri dishes. The lids of each dish were secured with tape, and all dishes were incubated at 37°C for 24 hours. The growth or inhibition of *S. mutans* was qualitatively determined by visually examining each petri dish.

c. Cell Counting Kit-8 (CCK-8) Assay

i. Culturing and Subculturing of Fibroblasts

In order to quantitatively compare the cytotoxicity of the experimental toothpaste formulations with the commercially available toothpastes, frozen, healthy human fibroblasts derived from gingival tissue were first thawed. In particular, the cryovial containing the frozen cells was first removed from liquid nitrogen storage and immediately placed into a 37°C water bath for approximately 30 seconds. While the cryovial was heated in the bath, it was gently

swirled until only a small amount of ice remained in the vial. The vial was then taken under a sterile workbench covered by a fume hood and was wiped down with a 70% ethanol solution. The volume of cells in addition to 10 mL of pre-warmed growth medium was transferred into a sterile 50-mL conical tube. The cells were then suspended by centrifuging the tube at 600 rpm for 5 minutes. After evaluating the clarity of the supernatant and the visibility of a complete pellet, the supernatant was removed using a vacuum and Pasteur pipette without disturbing the pellet. The pellet was then broken up with 10 mL of fresh fibroblast growth medium, and the suspended cells were transferred to the appropriate culture vessel. The culture vessel was placed in a humidified incubator (37°C, 5% CO_2) for 48 hours.

Upon analyzing the fibroblasts under a microscope with 1000X magnification and determining that their confluency was 80-90%, the cells were subcultured. Specifically, the fibroblast growth medium within the culture vessel was removed using a vacuum and Pasteur pipette, and the cells were then rinsed with 10 mL of sterile, calcium and magnesium-free HBSS. The HBSS was subsequently removed using a vacuum and Pasteur pipette, and 2 mL of a 1x 0.5% trypsin/EDTA solution was added to the culture vessel. The culture vessel was immediately placed in the humidified incubator for 2 minutes. After this brief incubation, the 1x 0.5% trypsin/EDTA solution was immediately deactivated by adding 10 mL of fibroblast growth medium to the culture vessel. Cells were re-suspended by gently pipetting the liquid suspension up-and-down to break up cellular clumps. The cell suspension was next transferred to a sterile 50-mL conical tube and centrifuged for 5 minutes at 600 rpm. The supernatant was removed using a vacuum and Pasteur pipette, and the complete pellet at the bottom of the conical tube was broken up with 10 mL of fresh fibroblast growth medium. The cell suspension was again gently pipetted up-and-down to break up cellular clumps. The cellular suspension was finally

transferred to a 100 mm dia x 15 mm H sterile petri dish, which was incubated in the humidified incubator for 48 hours. This subculturing process was continuously repeated to maintain the healthy fibroblasts.

ii. Evaluation of Cell Proliferation and Cytotoxicity Using CCK-8 Reagent

Once the fibroblasts achieved the desired confluency within the petri dish, the cells were re-suspended as described previously and were transferred into a sterile multichannel reservoir. The dilution scheme from the MIC assay was repeated for this experiment, except each toothpaste sample was diluted using a 5% DMSO solution in fibroblast growth medium [20]. Next, 200 µL of each toothpaste mixture were decanted into the first three wells of each row of the 96-well plate. The remaining wells in each row were filled with 100 µL of 5% DMSO in fibroblast growth medium via a multichannel pipette. From the wells containing 200 µL of toothpaste mixture, 100 µL of each mixture was transferred to the neighboring well containing 100 µL of 5% DMSO in fibroblast growth medium. The toothpaste mixtures and 5% DMSO growth medium solution were pipetted together several times using a multichannel pipette to thoroughly combine both solutions. This process was repeated such that triplicate serial dilutions were created from the three wells containing each original toothpaste mixture. Regarding the two remaining rows of the 96-well plate, the wells of one row were filled with 100 µL of fibroblast growth medium to serve as a negative control. As for the last row, 100 µL of 5% DMSO in fibroblast growth medium were decanted into each well to account for any potential cytotoxicity of DMSO. Using the multichannel pipette, 100 µL of fibroblast suspension were added to all the wells of the 96-well plate. An identical plate was prepared; however, in place of the 100 µL of fibroblast suspension was 100 µL of fibroblast growth medium. This blank plate was intended to account for any product interference detected by the microplate spectrophotometer.

The lids of both plates were secured with tape, and the plates were then placed in a humidified incubator for 48 hours to allow for adequate drug uptake by the cells. After this incubation period, 10 µL of CCK-8 reagent was added to each well of the 96-well plate containing the fibroblasts under a darkened fume hood. The plate was then wrapped in aluminum foil to prevent light from disturbing the CCK-8 reagent and was placed back in the humidified incubator for 1 hour. Once the fibroblasts had an adequate period of time to metabolize the CCK-8 reagent, the plate containing fibroblasts as well as the blank plate were placed in a microplate spectrophotometer. Finally, the absorbance of both plates at 460 nm was measured and recorded.

Results

With respect to the experimental data collected from the two assays described previously, below in Table 1 are the MIC assay results of the duplicate novel toothpaste embodiments that were recorded based on visual inspection of BHI agar plates incubated at 37°C for 24 hours. Likewise, Table 2 renders the MIC assay results of the duplicate commercially available toothpastes, and Table 3 corresponds to the observed *S. mutans* growth in the negative control group as well as the blank. It is noteworthy that the "+" symbol indicates a moderate amount of *S. mutans* growth, "+/-" signifies a relatively small amount of growth, and the "-" symbol denotes no observed *S. mutans* growth. In addition to this efficacy data are observations regarding the physical appearance, flavor, and sensation of the experimental toothpaste formulations when used.

Table 1. MIC Assay Findings of Duplicate Experimental Formulations

Formulation Duplicates	**Dilution Scheme**					
	1:1	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**
Formulation I	+	+	+	+	+	+
Formulation I	+	+	+	+	+	+
Formulation II	+	+	+	+	+	+
Formulation II	+	+	+	+	+	+
Formulation III	-	+/-	+/-	+/-	+/-	+/-
Formulation III	-	+/-	+/-	+/-	+/-	+/-

Table 2. MIC Assay Findings of Duplicate Commercially Available Toothpastes

Commercial Toothpaste Duplicates	**Dilution Scheme**					
	1:16	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
Colgate ®	-	+	-	-	+/-	-
Colgate ®	-	-	-	-	-	-
Sensodyne ®	-	-	-	-	-	+/-
Sensodyne ®	-	-	-	-	-	+/-
Crest ®	-	-	-	-	+	+
Crest ®	-	-	-	-	+/-	+

Table 3. Results of Negative Control Group and Blank Corresponding to MIC Assay

Negative Control	+	+	+	+	+	+	+	+	+	+	+	+
Blank	-	-	-	-	-	-	-	-	-	-	-	-

Observation 1: Formulation I had a dull black color and thick consistency that was reminiscent of any conventional toothpaste when squeezed from the ointment tube. When lightly rinsed with water, the toothpaste exhibited minimal water solubility. Regarding the sensation of the toothpaste in the mouth, the product slightly foamed and felt creamy, similar to commercially available toothpastes. The toothpaste was moderately sweet in flavor and had a bitter, cinnamon aftertaste. After use, the teeth appeared to have less plaque, and the mouth felt refreshed.

Observation 2: Formulation II was an obsidian color and had a thick texture that was similar to those of mainstream toothpastes. When gently rinsed with water, the toothpaste exhibited no water solubility. When used to clean the teeth and gums, the product had moderate foaming and had a buttery consistency. In particular, the consistency of this formulation was thinner than that of a typical, commercial toothpaste. The toothpaste had a slightly bitter flavor and a mild cinnamon aftertaste. After use, plaque build-up on the teeth was successfully abraded, and the oral cavity felt cleaner.

Observation 3: Similar to Formulation II, Formulation III had an obsidian color and a thick texture that was comparable to those of commercial toothpastes. When rinsed with water, this formulation was insoluble. When applied to the teeth and gums using a toothbrush, the toothpaste's consistency was thick and felt no different than a commercial toothpaste. Further,

the texture was somewhat gritty due to grains of bamboo charcoal that separated from the formulation. The flavor of the product was bitter and a zesty, cinnamon aftertaste was present. After use, plaque build-up was efficiently eroded away by the bamboo charcoal, and the mouth felt effectively cleaned.

**Based upon a preliminary assessment of the CCK-8 assay, no interpretable data was obtained. Specifically, the microplate spectrophotometer readings of the wells containing the serial dilutions of each toothpaste mixture obtained at an absorbance of 460 nm ranged from 0.1704 to 3.5727. However, as a majority of the measurements surpassed the typical CCK-8 background absorbance of 0.1-0.2 absorbance units, these values were unable to be elucidated (3). Even after subtracting the absorbance values of the blank 96-well plate measured at an absorbance of 460 nm, which ranged from 1.4188 to 35764, the CCK-8 assay results were unable to be assessed.*

Discussion and Conclusion

The aim of the first portion of this study was to develop novel, Eastern medicine-inspired toothpaste formulations that exhibit antimicrobial properties comparable to those of three commercial toothpastes, specifically: Sensodyne ® "Extra Whitening Toothpaste," Colgate ® "Total Advanced Whitening Toothpaste," and Crest ® "Cavity Protection Toothpaste." With respect to Table 1, neither Formulation I nor Formulation II exhibited effective antimicrobial properties. However, Formulation III, particularly when compared to the two previous formulations, displayed significant antimicrobial efficacy. These findings are attributable to the fact that calcium carbonate, which comprises 12.1% of Formulation I and 6.4% of Formulation

II, has no known antibacterial properties. Rather, in commercial toothpastes at least, calcium carbonate functions as a water- insoluble abrasive that is effective in removing plaque as well as food buildup on the surfaces of teeth [23]. As such, the presence of calcium carbonate in both Formulation I and Formulation II takes the place of effective antimicrobial ingredients, in particular coconut oil, bamboo charcoal powder, and cinnamon oil.

Regarding the activity of coconut oil, this lipid has been found to significantly reduce *S. mutans* count in the mouth relative to distilled water; moreover, its antibacterial efficacy is not significantly different than that of 0.2% chlorhexidine, a common component of commercial mouthwashes [13]. Formulation I is comprised of only 48.5% (w/w) coconut oil, Formulation II's composition is 53.5% (w/w) coconut oil, and Formulation III is 61.2% (w/w) coconut oil. That is, Formulation III has, on average, approximately 10.2% (w/w) more coconut oil than Formulation I and Formulation II. With respect to bamboo charcoal powder, this naturally-occurring ingredient minimizes *S. mutans* growth even in concentrations ranging from 2% (w/v) to 5% (w/v) [15] Though proven to be effective at low concentrations, Formulation I and Formulation II are comprised of only 13.6% (w/w) and 19.8% (w/w) bamboo charcoal, respectively. However, Formulation III consists of 22.2% (w/w) bamboo charcoal, thus this ingredient's antibacterial properties are further optimized. Addressing the last ingredient listed, cinnamon oil, this organic substance has been determined to have strong inhibitory effects against *S. mutans* {MIC value of 0.08% (v/v)} [24]. Similar to bamboo charcoal powder, cinnamon oil also exhibits significant antimicrobial efficacy at low concentrations. With regards to the experimental formulations, Formulation I and Formulation II are respectively comprised of 2% (w/w) and 3% (w/w), whereas Formulation III consists of 5.6% (w/w). Thus, increasing the

amount of coconut oil, bamboo charcoal, and cinnamon oil in the experimental formulations will further enhance the antibacterial efficacy of the formulation holistically.

As for the remaining components of the experimental toothpaste formulations, the amount of matcha powder, xylitol, and sodium bicarbonate were each decreased from Formulation I to Formulation III. Interestingly, each of these ingredients have antibacterial properties; yet, Formulation III demonstrates the greatest antimicrobial efficacy of the experimental formulations. Regarding matcha powder, the primary antimicrobial component of this substance is epigallocatechingallate (EGCG). Particularly, EGCG has demonstrated inhibition of growth and glucosyltransferase activity in most serotypes of *S. mutans* at an MIC of 31.25 µg/mL [25]. However, based on the findings within this study, it is possible that *S. mutans* serotype c may have evolved a resistance mechanism to this polyphenol. With respect to xylitol, this polysaccharide was found to have no significant effect on growth nor viability of *S. mutans* serotype c in the presence of glucose, a component of BHI medium [26]. This is quite logical as glucose is a preferential source of energy for all organisms; that is, the bacteria selectively metabolized this monosaccharide as opposed to xylitol. As for sodium bicarbonate, this salt is the principal buffer of both extracellular fluid as well as saliva [27]. The fermentation of carbohydrates by oral bacteria produces acids, which lower the pH of the mouth. The protons responsible for the decrease in pH are neutralized by salivary bicarbonate to produce water and carbon dioxide; in fact, the alkaline conditions created by sodium bicarbonate have shown bactericidal effects [27]. Yet, within oral health care products such as toothpaste, the antibacterial properties of sodium bicarbonate are primarily activated through mechanical cleaning [28]. Essentially, the effects of sodium bicarbonate in the experimental formulations were not fully optimized as the MIC assay was performed in vitro.

In analyzing the antibacterial efficacy of the three commercial toothpastes selected for this study, the growth detected in the first duplicates of the Colgate ® "Total Advanced Whitening Toothpaste" diluted in both a 1:32 ratio and a 1:256 ratio with 5% (v/v) DMSO in sterile water is likely due to contamination. Specifically, the inoculation loops used to transfer these bacterial suspension samples from the 96-well plate to their respective BHI agar petri dishes may have made contact with the work bench surface. Regarding the 1:32 ratio sample, there was an excess of bacterial growth observed – this contamination may have been from an inadequately cleaned spill within the incubator. Aside from these discrepancies, Colgate ® "Total Advanced Whitening Toothpaste" exhibited antibacterial efficacy against *S. mutans*, even in concentrations as low as 1:512. These findings are contributable to the toothpaste's quintessential active ingredient, sodium fluoride {0.24% (0.15% fluoride ion)}. The fluoride ion inhibits bacterial metabolic enzymes, like enolase, thus reducing phosphoenolpyruvate (PEP) levels – this ultimately deprives the cell of glucose [29]. Even in small concentrations, such as within drinking water {1.0 part per million (ppm)} has been determined to have some inhibitory effects on the growth of *S. mutans* serotype c [30]. Sodium fluoride is also the active ingredient of Sensodyne ® "Extra Whitening Toothpaste" (0.15% w/v fluoride ion) and Crest ® "Cavity Protection Toothpaste" {0.243% (0.15% w/v fluoride ion)}. Colgate ® "Total Advanced Whitening Toothpaste" also contains the active ingredient triclosan (0.30%). This antibacterial agent was found to be an effective inhibitor of the F(H+)-ATPase enzyme in *S. mutans*; specifically, in concentrations as low as 0.1 mmol/L at physiological pH is lethal to these bacteria [31].

Furthermore, fluoride and triclosan has been found to function synergistically with both SLS and zinc oxide, inactive ingredients of Colgate ® "Total Advanced Whitening Toothpaste,"

to inhibit the growth of *S. mutans* in vitro [32]. Though Crest ® "Cavity Protection Toothpaste" contains both sodium fluoride and SLS, the duplicate dilution of this toothpaste in ratios of 1:256 and 1:512 with 5% DMSO in sterile water displayed little inhibition of bacterial growth. This indicates that triclosan and zinc oxide are essential in the synergistic combination described previously, especially triclosan as it is a potent bactericide [31]. Sensodyne ® "Extra Whitening Toothpaste" does not contain triclosan, SLS, or zinc oxide; thus, it is very possible that the small amount of observed growth in this toothpaste sample diluted in a 1:512 ratio with 5% DMSO in sterile water is attributable to the lack of these ingredients. However, one inactive ingredient within Sensodyne ® "Extra Whitening Toothpaste," cocamidopropyl betaine (CAPB), may account for the lower MIC of this toothpaste compared to Crest ® "Cavity Protection Toothpaste." Specifically, CAPB is a derivative of coconut oil and possesses similar antimicrobial properties to its parental compound [33].

As the antibacterial components of the commercially available toothpastes discussed previously have marginally smaller MIC values compared to those of the well-studied natural ingredients within the experimental toothpaste embodiments, it is quite sensible that the mainstream embodiments have holistically smaller MIC values than the novel Eastern medicine-inspired formulations. Even so, it is important to consider that effective consumer use involves applying a pea-sized amount of toothpaste to the bristles of the toothbrush and brushing the teeth with the undiluted toothpaste for 3 minutes with a moderate amount of pressure, starting from interproximal space of the molar region [34]. Therefore, the MIC values of all toothpastes determined from this assay should be interpreted with some reservations. It is worth noting that one critical limitation to this study was the necessity to perform an in vitro analysis of the antibacterial efficacy of toothpastes versus an in vivo approach with human participants. Yet,

this project is only in the preliminary stages of its clinical development. Concerning the assessment of the cytotoxicity of each toothpaste using the CCK-8 assay, the standard protocol for this experiment posed many challenges for this project. Specifically, as each toothpaste possessed a color, this characteristic of each sample inevitably impacted the recorded absorbance values that quantify cellular viability in this colorimetric assay. Even after rinsing each well of the 96-well plate with HBSS four sequential times, each toothpaste "caked," a pharmaceutical term that describes a powder or cream's tendency to form clumps. That is, residue from each toothpaste remained stuck at the bottom of their respective wells, which continually interfered with absorbance readings produced by the microplate spectrophotometer. Hence, no collected data for this portion of the study was analyzable.

To conclude, Formulation III of the novel, Eastern medicine-inspired toothpaste exhibited the most promising efficacy in limiting the growth of *S. mutans*. Specifically, this embodiment displayed no growth when diluted in a 1:1 ratio with 5% DMSO in sterile water. Further, only a small amount of growth was observed when this formulation was diluted in ratios of 1:2, 1:4, 1:8, 1:16, and 1:32 with 5% DMSO in sterile water. These findings are attributable to the antibacterial properties of coconut oil, bamboo charcoal powder, and cinnamon oil. As for the antibacterial efficacy of the three commercially available toothpastes selected for this study, Colgate ® "Total Advanced Whitening Toothpaste" exhibited the greatest degree of inhibiting *S. mutans* growth, even when diluted in ratio as small as 1:512 with 5% DMSO in sterile water. This observation is due to the synergistic relationship between the active ingredients of this product, sodium fluoride and triclosan, in combination with two inactive ingredients, zinc oxide and SLS, has been determined to be a highly effective bactericidal concoction against *S. mutans* [32]. As a follow-up study to these findings, Formulation III could be modified such that the

matcha powder, which was determined to have no significant effect on inhibiting the growth of *S. mutans*, could be substituted with another reagent. One such reagent is Wu Mei powder, a Chinese herbal extract from the plum blossom [35]. This particular reagent has been found to have an MIC against *S. mutans* of 0.0781 g/ml [35]. With respect to re-approaching the CCK-8 assay, the 48-hour incubation period in which the fibroblasts are exposed to the toothpaste should be segmented into 6 hour intervals. Between each interval, each well should be lightly rinsed with HBSS to counter the caking of each toothpaste. In this way, minimal toothpaste will be retained in each well by the time the plate is ready to be analyzed using the microplate spectrophotometer. Additionally, once CCK-8 reagent is added to each well, the 96-well plate should be incubated for 4 hours as opposed to 1 hour – this ensures that fibroblasts have enough time to absorb and metabolize the reagent necessary for determining their viability.

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