EFFECTS OF CINNAMON WATER EXTRACT AS A CARIOSTATIC AGENT ON NICOTINE-INDUCED *STREPTOCOCCUS MUTANS* BIOFILM

by

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DEDICATION

I dedicate this work to the Almighty Allah for his blessings, graces and virtues. In addition, I dedicate this work to my beloved parents, without whose prayers and support I would never have been who I am today, and to my lovely brothers and sisters for their love and encouragement during my studies. Lastly, I dedicate this work to my mentors and instructors throughout all my schooling.

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INTRODUCTION

Dental caries is considered one of the most widespread chronic bacterial infections in the world.[1] *Streptococcus mutans* and Lactobacilli are considered the main bacteria involved in dental caries. However, other bacteria are involved in the caries process such as Actinomycetes and Veillonella species. The etiology of dental caries is attributed to differences in eating habits, especially sugar consumption, oral hygiene practices, the virulence of oral bacteria, and alterations in the oral protective mechanisms. Extracellular polysaccharide (EPS), composed of glucans, are an extracellular layer produced by oral streptococci in the presence of sucrose that enhances the adhesion and aids in dental plaque biofilm formation. *S. mutans* glucans are produced as a result of the cooperative effects of glucosyltransferase (GTF) and glucan-binding proteins (GBP).[2] Tooth demineralization is primarily due to lactic acid that is produced specifically by cariogenic bacteria present in dental plaque.[3]

According to a 2003 World Oral Health report, 60 percent to 90 percent of schoolchildren and the majority of adults have experienced dental caries. Moreover, dental caries is a major public health concern in other countries, such as in Asian countries and Latin America.[4] In 2007 around 91percent of adults in the US older than 20 years had dental caries and 27 percent had untreated carious lesions.[5] A study by Dye et al. showed that one in five children aged five years to 11 years and one in seven adolescents aged 11 years to 19 years have at least one untreated dental caries lesion.[6] Previous data showed that about 38 percent of children aged two years through eight years in the US had some dental caries, and 14 percent had untreated dental caries.[7]

Dental caries is a dynamic process of demineralization and remineralization cycles that affects the quantity of minerals in the tooth structure. If demineralization prevails, loss of minerals from the enamel occurs and teeth may become cavitated. However, if remineralization prevails, the lesion can be arrested, and teeth can gain the minerals that were lost during the demineralization process.[8,9] It was proven that non-cavitated lesions could be remineralized without the need for tooth drilling and filling.[10]

Many approaches have been used to arrest or reverse the demineralization process. Fluoride is considered the most commonly used anti-caries agent. Studies indicate that it is effective in decreasing the amount of caries incidence around the world.[11,12] In a systematic review of 70 clinical trials that compared fluoride dentifrice with a placebo, it was concluded that fluoride is capable of decreasing the DMFS score by 24 percent in permanent teeth.[13] Fluoride has two modes of action, systemic and topical.[14-16] During tooth development, systemic fluoride is incorporated into the tooth structure.[16,17] After tooth formation is completed, fluoride is absorbed into the crystalline structure of the tooth through topical agents.[16] Moreover, casein phosphopeptide (CPP) amorphous calcium-phosphate (ACP) complexes have shown the ability to inhibit demineralization and to enhance the remineralization process by increasing the amount of calcium and phosphate in the dental plaque, which in turn helps to restore the minerals that were lost during the demineralization process.[18-21]

Silver diamine fluoride (SDF) is another approach that demonstrates the ability to arrest both enamel and dentin caries processes.[18] The combined effects of silver and fluoride have the ability to arrest caries progression and prevent the development of new caries lesions.[22] Research has demonstrated that the application of SDF is more effective in preventing dental caries compared with sodium fluoride varnish. In

addition, it was found that SDF is effective whether the caries was removed or not.[23]

Aside from remineralization of caries lesions, recent focus has been on decreasing the incidence of caries by reducing the load of *S. mutans* and dental plaque. Various antibacterial compounds have been used for these purposes, such as chlorhexidine, xylitol, triclosan, cetylpyridinium chloride, sanquinarin, sodium dodecyl sulphate, and various metal ions (tin, zinc, copper).[24] With the exception of chlorhexidine, the effectiveness of these agents is still controversial.[25] Chlorhexidine has been widely prescribed; however, several side effects have been reported for this agent.[26] For that reason, research has been conducted to seek adjunctive antibacterial compounds that could prevent or reduce plaque formation on tooth surfaces.

Among the widely used herbs and spices is cinnamon, which has shown antimicrobial activity against pathogens and assists in the preservation of food.[27] Cinnamon demonstrated a strong antibacterial activity against a wide variety of bacteria including *Streptococcus faecalis* DC 74, *Pseudomonas aeruginosa* ATCC 27859, *Enterobacter cloacae* ATCC 13047 and *Staphylococcus aureus* 6538 P.[28] The antibacterial activity of cinnamon was investigated against *Enterococcus faecalis*, one of the main causative factors of pulp and periapical diseases of the oral cavity. The results indicated that there was an inhibition of bacterial growth.[29] Cinnamon essential oil, obtained from the leaves of *C. zeylanicum*, has been shown to be effective against *S. mutans* and *Lactobacillus acidophilus*, which are partially responsible for dental plaque formation and caries development.[30] A study concluded that cinnamon oil demonstrated a broad range of antimicrobial activity against bacterial strains causing dental caries.[31]

Tobacco smoke contains 7,357 different chemical substances, among which nicotine is the most abundant alkaloid chemical. Tobacco addiction is primarily

caused by nicotine as it is considered the main biobehavioral chemical compound found in tobacco that explains the reason for habitual tobacco use.[32] The relation between smoking and dental caries has been extensively investigated. A positive correlation has been found between nicotine and *S. mutans* biofilm. The presence of nicotine enhances the cariogenicity of *S. mutans* and *S. sanguinis* as nicotine enhances biofilm formation and increases biofilm metabolic activity.[33,34]

Nicotine also upregulates Gtf and glucan binding protein (GbpA) expression, Ldh, nlmC, and phosphotransferase system (PTS)-associated genes, which eventually leads to more lactic acid production.[35,36] A recent *in-vivo* study indicates that caries was higher in Wistar rats infected with *S. mutans* and treated with nicotine compared with a nicotine-untreated group.[37]

A study has investigated the effect of cinnamon water extract on *S. mutans* and demonstrated that cinnamon water extract inhibited biofilm formation at a minimum concentration of 5 mg/ml. This decrease in biofilm formation of *S. mutans* by cinnamon water extract could be due to inhibition of acid production and reduced adherence.[38] However, no study has investigated the effect of cinnamon water extract specifically on nicotine-induced *S. mutans*.

OBJECTIVES/SPECIFIC AIM

The aim of this study is to identify the effects of nicotine exposure on the inhibitory effects of cinnamon water extract on *S. mutans* biofilm formation.

Oral biofilm formation involves the aggregation of bacteria, attaching to oral tissues and to one another to form bacterial micro-colonies. The formed biofilm is continually bathed by saliva in the oral cavity. Oral microorganisms are also detected as free-floating, unattached planktonic cells in saliva.[39] This study will demonstrate the results of planktonic, biofilm and total growth assays incubated with the MIC of

cinnamon aqueous concentrate and nicotine levels corresponding with a primary level of nicotine exposure.

This project will mimic the *in-vivo* condition in which cinnamon water extract could be incorporated into oral hygiene products with the appropriate concentration for its antimicrobial effect on *S. mutans* and as a mouth refresher. In the future, a smoker could brush his or her teeth with cinnamon-containing toothpaste. Furthermore, the smoker could rinse his or her mouth with a cinnamon-containing mouthwash after smoking, which could help to inhibit or reduce nicotine-induced *S. mutans* biofilm.

HYPOTHESIS

The alternative hypothesis is that the presence of cinnamon water extract will inhibit nicotine-induced *S. mutans* biofilm formation at different concentrations of nicotine.

The null hypothesis is that the presence of cinnamon water extract will increase or have no effect on nicotine-induced *S. mutans* biofilm formation at different concentrations of nicotine.

REVIEW OF LITERATURE

Dental caries is a multifactorial disease. Diet, fermentable carbohydrates, oral microflora and host factors such as salivary disfunction are implicated in dental caries formation .[40]

DIETARY FACTORS

Fermentable carbohydrates in the diet including starches and simple sugars such as sucrose are implicated in caries causation. It was proven that sucrose has the greatest cariogenic potential.[40] The relation of diet to dental caries was first noticed by Aristotle in the fourth century B.C. He hypothesized that dental caries was developed as a result of consumption of sweet figs.[41] There is overwhelming evidence that dental caries does not develop in the absence of fermentable carbohydrates in the diet.[42] In the Hopewood House study conducted from 1947 to 1952, children were fed food with no sugar contents. This study concluded those children had fewer cases of dental caries compared with children in other schools.[43]

The sequence in which fermentable carbohydrate-containing foods are eaten also significantly influences cariogenesis. A sharp decrease in the pH of saliva has been observed following the use of a sugar rinse in which the pH returns to baseline after approximately 30 minutes. However, when cheese is consumed 5 minutes after a sugar rinse, the pH returns quickly to baseline.[44-46]

Two main dietary factors significantly influence caries formation: frequency and form of sugars consumed. In a study conducted in a mental institution in Vipeholm, Sweden, adult patients were fed a significant amount of sweets. Two important findings were concluded from that study. First, dental caries is influenced

more by frequency of sucrose intake rather than the total amount consumed. Second, solid forms of sugar, which are easily retained in teeth, are more cariogenic than liquid forms of sucrose.[47]

The association of excessive sugar intake to dental caries has been affirmed by an Expert Panel of the World Health Organization (WHO), in which the strength of evidence linking dietary factors to caries was reviewed. The Panel concluded that an increased risk of caries is associated with the amount and frequency of simple sugars consumption.[48] In a study conducted in 1976 the cariogenicity of three sweeteners was investigated: sucrose, fructose and xylitol. It was found that sucrose was associated with the highest caries rate. With regard to fructose and xylitol, they exhibited 32 percent and 85 percent lower caries rates than sucrose, respectively.[49] This study and others[50,51] demonstrated that sucrose is the most cariogenic sugar. It was proven that in order for dental caries to occur, sugar has to be available to be utilized by the cariogenic bacteria. The amount and frequency of carbohydrates have a direct correlation with the acidity of the oral cavity when carbohydrates are incorporated in the biofilm, which in turn leads to acid production and a significant demineralization of the tooth structure.[52,53]

Even though the risk posed by carbohydrates has decreased compared with the pre-fluoride era, people should still be aware of the importance of dietary content in relation to dental caries.[54] Furthermore, the US Dept. of Agriculture's Food Guide Pyramid stated that consumed foods should contain a small amount of sugar.[55]

ORAL MICROFLORA

The oral cavity is a moist nutrient-rich environment that facilitates the growth of microorganisms.[56] The role of oral microflora in cariogenesis has been extensively investigated. It has been found that rats delivered by Caesarean section under sterile

conditions were caries-free from birth and remained so, even when fed fermentable carbohydrates.[57]

S. mutans and the Lactobacilli species are the species most commonly associated with cariogenesis. Previous data indicate that the number of *S. mutans* is 70 times higher in caries-infected subjects compared with caries-free subjects.[58] Cariogenic bacteria have the ability to produce acid (acidogenic), which in turn dissolve the enamel layer and the ability to survive in a high acidity environment (aciduric). The byproduct of dietary carbohydrate fermentation is the acid that causes demineralized non-cavitated enamel surfaces to become cavitated.[8] These cariogenic bacteria have the ability to form a biofilm on tooth surfaces and can readily utilize fermentable carbohydrates to produce lactic acid, which demineralize the tooth structure and initiate the carious process.[59,60]

S. mutans is one of the most cariogenic bacteria with the ability to adhere to hard tooth structures and to proliferate, leading to the formation of a mature cariogenic biofilm. In addition, *S. mutans* can attach to the tooth structure by two main mechanisms: initial sucrose-independent adherence in which antigen I/II are involved, and sucrose-dependent adherence in which glucans are produced as a result of the cooperative effects of glucosyltransferase (GTF) and glucan-binding proteins (GBP).[61]

Biofilms are crucial in the human body as they are responsible for the majority of microbial infections. Microorganisms in biofilms demonstrate increased resistance to antimicrobial agents and host immune defense as well as environmental stresses.[62] Dental biofilm (dental plaque) formation involves four stages including salivary acquired pellicle formation, initial adhesion, maturation, and a dispersion phase.[63]

In the first stage, within five minutes following toothbrushing, salivary glycoproteins are adsorbed to hydroxyapatite on enamel tooth surfaces by electrostatic interactions forming the acquired enamel pellicle.[64,65] In the second stage, early colonizers may recognize and attach to specific binding sites in salivary-acquired pellicle utilizing specific receptors.[63] *S. mutans* plays a major role in the initial sucrose-independent adherence involving antigen I/II, which is a bacterial surface protein adhesin that interacts specifically with salivary agglutinin glycoprotein (SAG) in the acquired enamel pellicle. Once the early colonizing bacteria attach to acquired enamel pellicle, they produce an extracellular polysaccharide matrix (EPS) as a result of the cooperative effects of glucosyltransferase (GTF) and glucan-binding proteins (GBP) which bind cells together and to the tooth surface.[66, 67]

The third stage of dental biofilm formation involves the maturation of biofilm in which early colonizing bacteria provide binding sites for the later colonizers for subsequent attachment. The dispersion phase is the last stage of dental biofilm formation, in which bacteria leave the biofilm due to lack of nutrients and attach to new sites for more nutrients for growth.[68,69]

Acidogenic bacteria produce large amounts of lactic acid as a byproduct of dietary carbohydrate fermentation, which results in lowering pH and the establishment of an aciduric biofilm.[70] That acid diffuses through the tooth enamel and causes the demineralization process to begin and develop tooth decay.[8]

HOST FACTORS

The composition and the rate of salivary flow significantly influences cariogenesis.[71] Saliva contains high levels of calcium and phosphate and low levels of fluoride, which facilitates remineralization of the demineralized tooth structure.[72]

Functions of saliva include cleansing, lubrication, mucosal integrity, buffering, remineralization, taste, digestion and poses anti-microbial properties.[73] Salivary mucins are well recognized as an important factor in the preservation of the health of the oral cavity. Moreover, mucin is one of the main lubricants in the oral cavity which facilitates eating, talking and swallowing. In addition, mucins reduce the colonization of fungal and bacterial species. Based on macromolecular characteristics, there are two forms of mucin in saliva: MG1 and MG2. MG1 is a high molecular weight, highly glycosylated mucin, whereas MG2 is a slightly lower molecular weight, single-glycosylated peptide chain mucin. MG1 tightly adheres to the tooth structure and forms dental pellicle. On the other hand, MG2 could be removed easily from the enamel and enhances the clearance of the bacteria. Previous data show that MG1 levels are higher in caries-susceptible subjects, while MG2 is higher in caries-free individuals. Overall, dental pellicle containing MG1, MG2 and other salivary proteins forms a protective barrier against acid attack, which in turn minimizes mineral loss from enamel.[74,75]

Bicarbonate, phosphate and urea are salivary components that can buffer and neutralize the acidity of the oral cavity. Data show that oral pH drops to a value under six after 5 minutes of eating. The pH returns to normal 15 minutes following food intake.[76] The salivary buffering effect inhibits the demineralization process, thereby protecting the tooth structure against acid attack. In addition, saliva acts as a reservoir for calcium and phosphate. Salivary proteins participate in the incorporation of calcium and phosphate into the tooth structure to increase tooth maturation and limit mineral loss from the tooth structure.[77,78]

Saliva has several antimicrobial agents that interfere with the cariogenicity of bacteria, such as IgA, lactoferrin and lysozyme. IgA binds to bacterial antigens that

interfere with bacterial attachment. Lactoferrin is capable of limiting nutrient availability to cariogenic bacteria by binding ferric iron, a main nutrient for cariogenic bacteria. Lactoferrin also is considered a sensitive material for *S. mutans*. Lysozyme limits bacterial growth in host tissues because it has a destructive effect on the bacterial cell wall.[77,79-81]

Salivary gland dysfunction results in a significant decrease in the buffering capacity and increases the risk of acidity and demineralization of tooth structure.[82, 83] Therefore, monitoring salivary flow is paramount in those patients who are categorized as high caries risk. There are different means to increase salivary flow such as using medications or hydration tools.

THE RISK OF SMOKING

Smoking or tobacco use is one of the biggest health concerns in the world. In 2012 the World Health Organization (WHO) reported that globally, about 23 percent of people older than age 15 years were smokers. Moreover, in 2015 the WHO published its report on the prevalence of tobacco smoking, which estimates 6 million deaths each year as a result of tobacco use, either by smoking or by the use of smokeless tobacco.[84,85] Also, in 2012 approximately 42.1 million adults in the US were smokers.[86]

Smoking harms nearly every organ of the body, causes many diseases, and reduces the health of smokers in general. Smoking is responsible for 30 percent of cancer-related deaths.[87,88] Moreover, a strong association has been found between smoking and lung cancer, which is the leading cause of cancer-related deaths in the US. In addition, cigarette smoking is a major cause of cardiovascular disease (CVD).[89] Also, smoking is the primary cause of chronic obstructive pulmonary disease (COPD) in the U.S.[90,91] Smoking also has been linked to reproductive

abnormalities and low birth weight. More recently, studies indicate that smoking during pregnancy could cause orofacial clefts. Smoking impairs immune function, resulting in an increased risk of pulmonary infections and rheumatoid arthritis.[87,89] Moreover, smoking and smokeless tobacco weaken the immune system, especially in the oral cavity, and can cause gingival and periodontal diseases.[92-95] Furthermore, smoking has been recognized as a risk factor for peri-implantitis and implant failure.[96]

Tobacco smoke contains 7,357 different chemical substances, among which nicotine is the most abundant alkaloid chemical.[97] Tobacco addiction is primarily caused by nicotine as it is considered the main biobehavioral chemical compound found in tobacco, which explains the reason for the habitual tobacco use.[32] The relation between smoking and dental caries has been extensively investigated. It has been found that there is a positive correlation between nicotine and *S. mutans* biofilm. In 2012 Huang et al. conducted a study to investigate the effects of nicotine on seven *S. mutans* strains, UA159, UA130, 10449, A32-2, NG8, LM7, and OMZ175. They found that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for nicotine was 16 mg/ml and 32 mg/ml, respectively. In addition, it has been found that the minimum biofilm inhibitory concentration (MBIC) for nicotine was 16 mg/ml for all strains except for ATCC strain 10449, which was 8 mg/ml.[34]

Another study concluded that the presence of nicotine enhances the cariogenicity of *S. mutans* and *S. sanguinis* as nicotine increases biofilm formation and biofilm metabolic activity.[33] A recent *in-vivo* study indicates that caries was higher in Wistar rats infected with *S. mutans* and treated with nicotine compared with a nicotine-untreated group. Also, the severity of caries increased with nicotine treatment.[37] Studies show that nicotine load is higher in the oral cavity of smokers.[98,99]

A recent review shows that smoking and nicotine alters microbial composition in the oral cavity and increases the pathogenicity of several oral microorganisms.[100] Nicotine also upregulates Gtf and glucan binding protein (GbpA) expression,[35] Ldh, nlmC, and phosphotransferase system (PTS)-associated genes and increases lactate production.[36]

In a metabolic study, it has been found that nicotine increases lactic acid production of *S. mutans* by two-fold.[36] In addition, it has been reported that smoking during pregnancy or in the postnatal period could increase the risk of caries in primary dentition.[101] For those people who cannot quit smoking or those who spend time with smokers, the question arises how the effects of nicotine on the growth of biofilm could be diminished.

HOW TO LIMIT THE CARIOGENICITY OF NICOTINE?

For centuries, herbs and spices have not only demonstrated their effects as antioxidants and flavoring agents, but most importantly, they have shown antimicrobial activity against pathogens and aid in the preservation of food. Cinnamon, dated as far back as the 16th century, has become a popular spice and food flavor additive.[27] Cinnamon has been planted in some islands in the Indian Ocean, primarily in Sri Lanka, and East Asia mainly in China.[102-104] Cinnamon also has been cultivated on a small scale in both India and Vietnam.[102,105]

There are two main important species of cinnamon in the genus Cinnamomum. The first one is *Cinnamomum verum*. It is also called true cinnamon, Sri Lankan, Ceylon cinnamon or *Cinnamomum zeylanicum*. The other main species of cinnamon is cassia cinnamon which is further subdivided into *Cinnamomum burmannii* (also called Korintje, Padang cassia, Java, or Indonesian cinnamon),

Cinnamomum loureiroi (also known as Vietnamese or Saigon cinnamon) and *Cinnamomum aromaticaum* (also called cassia or Chinese cinnamon).[106]

Polyphenols and volatile phenols are the main chemical compounds in cinnamon. With regard to polyphenols, cinnamon contains mainly caffeic, vanillic, gallic, p-coumaric, and ferulic acids.[107] Among volatile components cinnamaldehyde is the most abundant substance in cinnamon bark essential oil.[108] Furthermore, the other minor volatile compounds are hydrocarbons and oxygenated compounds (e.g. β-caryophyllene, linalool, eugenyl acetate, and cinnamyl acetate).[109-111]

Cinnamon alleviates nausea and vomiting and can be used to treat both bacterial and fungal infections. It also can be used as an insecticide as well as pesticide to kill plant-parasitic nematodes.[112-118] In addition, cinnamon demonstrated a strong antibacterial activity against a wide variety of bacteria including *S. faecalis* DC 74, *P. aeruginosa* ATCC 27859, *E. cloacae* ATCC 13047 and *S. aureus* 6538.[28] Also, cinnamon has been used to treat skin diseases.[119]

According to Ayurvedic literature, cinnamon has been used to control diarrhea and to alleviate gases in the alimentary canal.[120] Moreover, cinnamon can be used to stop hemorrhages as it has a coagulant effect.[121] Additionally, cinnamon improves tissue renewal and growth as it increases the blood flow in the uterus.[122] Recently, it has been proven that cinnamon is an anti-inflammatory and has potent antioxidant properties. It is effective in preventing damage to vital organ cells including liver, heart and nerve cells and can also prevent gastropathy.[123,124]

Cinnamon and its metabolite sodium benzoate upregulate neurotropic factors such as brain-derived neurotropic factors (BDNF) as well as neurotrophin-3 (NT-3) in the mouse central nervous system, which indicates that cinnamon has activities against neurological disorders.[125] A recent study found a reduction in the total

cholesterol, triglycerides, and lipoproteins in rats administered *Cinnamomum cassia* powder (15 percent).[126] It has been reported that trans-cinnamaldehyde exhibited potential effects in restraining tumor cell growth and in enhancing tumor cell apoptosis.[127]

It has been proven that cinnamon has the potential to be used to treat cardiovascular diseases. Cinnamic aldehyde and cinnamic acid, isolated from *C. cassia* have an effect against myocardial ischemia.[128] A recent study indicates that cinnamon can be used to control diabetes because a linalool chemotype was found to enhance insulin secretion.[129] Cinnamon essential oil is also used in massage therapy. Studies have shown benefits from the use of cinnamon oil in massage to ease menstrual pain in high school girls.[130]

Some studies showed that cinnamon extracts and essential oils could be active against oral cavity infections. A study conducted by Chaudhari et al. in 2012 demonstrated that cinnamon was active against *S. mutans* and concluded that the use of cinnamon can be a good alternative to other antibacterial compounds against the bacteria responsible for oral infections.[131]

More recently, the antibacterial activity of cinnamon was studied against *E. faecalis*, one of the main causative factors of recurrent pulp and periapical diseases of the oral cavity. The results indicated that cinnamon has a strong inhibitory effect on *E. faecalis*.[29] Recent research has demonstrated that cinnamon is active against *S. mutans* and *L. acidophilus*, which are involved in dental plaque formation and caries development.[30] A study concluded that cinnamon oil demonstrated a broad range of antimicrobial activity against the bacteria causing dental caries.[31] A recent study demonstrated that cinnamon water extract inhibited biofilm formation at a minimum

concentration of 5 mg/ml. This decrease in biofilm formation of *S. mutans* by cinnamon could be due to inhibition of acid production and reduced adherence.[38]

MATERIALS AND METHODS

PRELIMINARY EXPERIMENT

Before starting the main study, a preliminary experiment was conducted to determine the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC) of cinnamon water extract alone on the growth of *S. mutans* in tryptic soy broth supplemented with 1.0-percent sucrose (TSBS). A recent pilot study report indicated that the MIC and MBIC of cinnamon water extract was 5 mg/ml.[38] An overnight culture of *S. mutans* UA159 (ATCC 700610) was grown in TSB at 37°C in 5.0-percent CO_2 and stored with 20-percent glycerol at -80^{o}C (Figure 1). This was accomplished by mixing 20 mg/ml of *C. burmannii* powder, also known as Indonesian cinnamon, or korintje (Morton & Bassett Spices, Rohnert Park, CA) in sterile deionized water, the solution was transferred into a test tube and heated for 60 minutes at 121°C at 15 PSI of pressure (Figure 2) in an autoclave. The sterility of the solution was confirmed by streaking on a blood agar plate (Figure 3). The solution was centrifuged (Beckman GS-6R Refrigerated Centrifuge) for 10 min (Figure 4) to clarify the preparation. Serial dilutions of cinnamon water extract in the supernatant (ranging in concentration from 0 mg/ml to 10 mg/ml) were prepared in TSBS. Ten µl of an overnight culture of *S. mutans* (approximately 10^6 colony-forming units [CFU]/ml, determined by spiral plating) in TSB was treated with 190 µl of the cinnamon water extract dilutions and incubated at 37°C for 24 h in sterile 96-well flat-bottom microtiter plates (Fisher Scientific, Newark, DE, USA). The optical density (OD) values of the bacterial cultures were measured at 595 nm in a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA). The MIC was determined by the concentration where there was an obvious clear-cut decrease in the

absorbance. After incubation, the unbound planktonic cells (120 µl) were gently aspirated and transferred to a new 96-well plate and the OD at 595 nm was determined in order to calculate the effect on planktonic cells. The remaining planktonic cells were removed from the biofilm microtiter plate wells (leaving attached biofilm), and 200 µl of 10-percent formaldehyde was added to each well for 30 min to fix the cells. After 30 min, the formaldehyde was removed, and the biofilm cells were washed 3 times with deionized water. Two hundred µl of 0.5-percent crystal violet dye was added to each well and the cells stained for 30 min. The wells were rinsed three times and 200 µl of 2-isopropanol was placed into each well for 1 h to lyse the cells and extract the crystal violet. The plates were read in a spectrophotometer at 490 nm to measure biofilm formation.[34]

EFFECTS OF CINNAMON ON NICOTINE-TREATED *S. MUTANS* BIOFILM

The MIC for cinnamon water extract was determined to be 5 mg/ml. A concentration of 2.5 mg/ml (sub-MIC) one dilution below the MIC of cinnamon in TSBS was used from the preliminary experiment to treat nicotine-treated *S. mutans*. This dilution was selected because use of the MIC of cinnamon water extract would obscure any potential effect of nicotine. Stock solutions of cinnamon water extract (sub-MIC) 2.5 mg/ml were prepared using the following protocol:

A stock solution of a concentration of 5 mg/ml of cinnamon water extract was prepared by mixing 200 mg of cinnamon powder in 40 ml deionized water, then 20 ml of that solution was mixed with 20 ml of TSBS to form a new stock solution of 40 ml of a concentration of 2.5 mg/ml (Figure 5).

The solution was transferred into a test tube and heated for 60 minutes at 121°C at 15 PSI of pressure (Figure 2) in an autoclave. The sterility of the solution

was confirmed by streaking on a blood agar plate (Figure 3). The solution was centrifuged (Beckman GS-6R Refrigerated Centrifuge) for 10 min (Figure 4).

In order to measure the effect of cinnamon water extract on nicotine-treated *S. mutans* initial biofilm formation, serial dilutions of TSBS were prepared to yield 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml nicotine (Sigma-Aldrich Chemical Co., St. Louis, MO) without cinnamon water extract, and 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml nicotine with the sub-MIC dilution of cinnamon water extract (determined by the results of the preliminary experiment) (Figure 6). One hundred ninety μl of TSBS at each nicotine concentration was aliquoted into wells of a sterile 96-well flat bottom microtiter plate. Ten μl of a fresh overnight TSB culture of *S. mutans* was added to each well (Figure 7). The microtiter plate was incubated in 5 percent CO_2 at 37°C for 24 h. The following day, the total absorbance (biofilm and planktonic growth) was measured in a spectrophotometer (SpectraMax 190; Molecular Devices Inc., Sunnyvale, CA) at 595 nm. Next, 120 μl from each well was transferred to the corresponding well of a new microtiter plate. The absorbance of each well was read at 595 nm to measure planktonic growth. The remaining planktonic cells were removed from the biofilm microtiter plate wells (leaving attached biofilm), and 200 μl of 10-percent formaldehyde was added to each well for 30 min to fix the cells. After 30 min, the formaldehyde was removed, and the biofilm cells were washed 3 times with deionized water (Figure 8). Two hundred μl of 0.5-percent crystal violet dye was added to each well and the cells were stained for 30 min (Figure 9). The wells were rinsed 3 times (Figure 10) and 200 μl of 2-isopropanol was placed into each well for 1 h to lyse the cells and extract the crystal violet (Figure 11). The plates were read in a spectrophotometer at 490 nm to measure biofilm formation (Figure 12).

CONTROLS

Controls included biofilms of *S. mutans* without nicotine and with or without cinnamon water extract as well as a sterile control.

STATISTICAL ANALYSES

Each experiment was repeated three times. A two-way ANOVA was used to compare the effects of cinnamon water extract exposure (each dilution individually), on nicotine concentration (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mg/ml) and their interaction on planktonic, biofilm, and total growth. Pairwise comparisons were made between nicotine concentrations to the zero nicotine both with and without cinnamon water extract. Pairwise comparisons were also made comparing each nicotine concentration with and without cinnamon water extract. The comparisons involving cinnamon water extract exposure were of primary interest to test the study hypotheses. The distribution of the measurements was investigated and found to be non-normal, and thus a rank transformation was used to satisfy the ANOVA assumptions prior to analysis.

SAMPLE SIZE CALCULATIONS

Based on prior studies, the within-group standard deviation of the absorbance measurements for biofilm formation was estimated to be 0.15. With samples of each cinnamon water extract/nicotine dilution in each of three replicates of the study, the study had 80-percent power to detect a difference of 0.2 between samples with and without cinnamon water extract for each nicotine concentration, assuming two-sided tests were conducted at an overall 5-percent significance level.

RESULTS

RESULTS OF THE PRELIMINARY EXPERIMENT

From the results of the preliminary experiment, the MIC of cinnamon water extract was 5 mg/ml (Figure 13). The results also indicated that cinnamon was able to inhibit biofilm formation significantly ($p < 0.05$). There was significant reduction ($p < 0.05$) in biofilm formation between 2.5 mg/ml to 10 mg/ml. It was seen clearly that 10 mg/ml is bactericidal, while 5 mg/ml cinnamon water extract exhibited significant biofilm formation inhibition (Figure 14). For this reason, 5 mg/ml of cinnamon water extract was recognized as the MIC and the MBIC for *S. mutans*.

RESULTS OF THE MAIN EXPERIMENT

Overall, there was a significant effect for cinnamon water extract presence, nicotine concentration, and their interaction in all measures (biofilm, planktonic, total absorbance) (Table VI). The results were categorized in the following sections.

RESULTS OF COMPARING NICOTINE CONCENTRATION WITH AND WITHOUT CINNAMON WATER EXTRACT

For total absorbance: there was a significant inhibitory effect of cinnamon water extract at nicotine concentrations 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 8 mg/ml. However, a slight increase in total absorbance can be seen at nicotine concentrations 1 mg/ml and 2 mg/ml, but these were not statistically significant. In addition, a non-statistically significant decrease in total absorbance was noted at nicotine concentrations 4 mg/ml, 16 mg/ml and 32 mg/ml (Figure 15). Regarding planktonic growth: there was a significant inhibitory effect of cinnamon water extract at nicotine concentrations 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml and 4 mg/ml.

Also, at nicotine concentrations of 8 mg/ml, 16 mg/ml and 32 mg/ml, there was a decrease in planktonic growth, but these were not statistically significant (Figure 16). As for biofilm growth: there was a significant inhibitory effect of cinnamon water extract at nicotine concentrations 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml and 8 mg/ml, whereas a significant increase in biofilm growth was observed at nicotine concentrations 2 mg/ml and 4 mg/ml. At nicotine concentrations 16 mg/ml and 32 mg/ml, there was a decrease in biofilm growth, but these were not statistically significant (Figure 17).

S. MUTANS BIOFILM WITH CINNAMON WATER EXTRACT AT NICOTINE CONCENTRATIONS VERSUS ZERO-NICOTINE CONCENTRATION WITH CINNAMON WATER EXTRACT

For total absorbance: there was a significant increase with the presence of cinnamon water extract at nicotine concentrations 0.5 mg/ml, 1 mg/ml, 2 mg/ml and 4 mg/ml compared with the zero-nicotine concentration with cinnamon water extract. On the other hand, there was a significant decrease with the presence of cinnamon water extract at nicotine concentrations 8 mg/ml, 16 mg/ml and 32 mg/ml compared with the zero-nicotine concentration with cinnamon water extract. Also, there was a slight increase at the nicotine concentration of 0.25 mg/ml, but this was not statistically significant (Figure 15). As for planktonic growth: there was a significant increase with the presence of cinnamon water extract at nicotine concentrations 4 mg/ml, 8 mg/ml, 16 mg/ml and 32 mg/ml compared with the zero-nicotine concentration with cinnamon water extract. However, a non-statistically significant slight increase was noted with the presence of cinnamon water extract at the nicotine concentration of 2 mg/ml compared with the zero-nicotine concentration with cinnamon water extract. In addition, there was no statistical significance with the presence of cinnamon water extract at nicotine concentrations 0.25 mg/ml, 0.5 mg/ml

and 1 mg/ml compared with the zero nicotine concentration with cinnamon (Figure 16). Regarding biofilm growth, there was a significant increase with the presence of cinnamon water extract at nicotine concentrations 1 mg/ml, 2 mg/ml and 4 mg/ml compared with the zero-nicotine concentration with cinnamon water extract; whereas, there was a significant decrease with the presence of cinnamon water extract at nicotine concentrations 8 mg/dl, 16 mg/dl and 32 mg/ml compared with the zero-nicotine concentration with cinnamon water extract. In addition, there was a slight increase at the nicotine concentration of 0.5 mg/ml and a slight decrease at the nicotine concentration of 0.25 mg/ml, but this was not statistically significant (Figure 17).

S. MUTANS BIOFILM GROWTH WITHOUT CINNAMON WATER EXTRACT AT NICOTINE CONCENTRATIONS VERSUS ZERO-NICOTINE CONCENTRATION WITHOUT CINNAMON WATER EXTRACT

Regarding total absorbance: there was a significant decrease without cinnamon water extract at nicotine concentrations 16 mg/ml and 32 mg/ml compared with the zero-nicotine concentration without cinnamon water extract. However, there was a significant increase without the presence of cinnamon water extract at nicotine concentrations 2 mg/ml, 4 mg/ml and 8 mg/ml compared with the zero-nicotine concentration without cinnamon water extract. Also, there was a decrease at nicotine concentrations 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml, but these were not statistically significant (Figure 15). As for planktonic growth: there was a significant decrease without cinnamon water extract at all nicotine concentrations 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml compared with the zero-nicotine concentration without cinnamon water extract (Figure 16). For biofilm growth, there was a significant decrease without the presence of cinnamon water extract at nicotine concentrations 16 mg/ml and 32 mg/ml compared with the zero-nicotine concentration without cinnamon water

extract. On the other hand, there was a slight increase without cinnamon water extract at nicotine concentrations 4 mg/ml and 8 mg/ml and a slight decrease at nicotine concentrations 1 mg/ml and 2 mg/ml. Both were not statistically significant compared with the zero-nicotine concentration without cinnamon water extract. In addition, there was no statistically significant difference at nicotine concentrations of 0.25 mg/ml and 0.5 mg/ml (Figure 17).

FIGURES AND TABLES

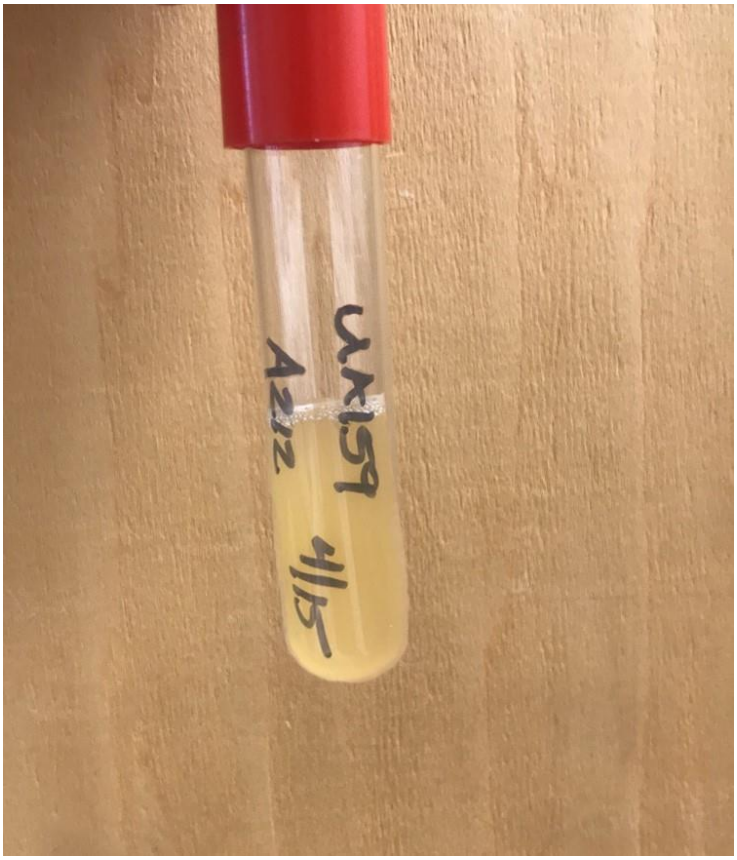

FIGURE 1. *S. mutans* UA159 was grown in TSB at 37°C in 5% CO_2 for 24 hours and stored with 10% glycerol at -80°C.

FIGURE 2. Cinnamon powder was mixed at 20 mg/ml with sterile deionized water, transferred into a test tube, placed in the autoclave and heated for 60 minutes at 121°C at 15 PSI of pressure.

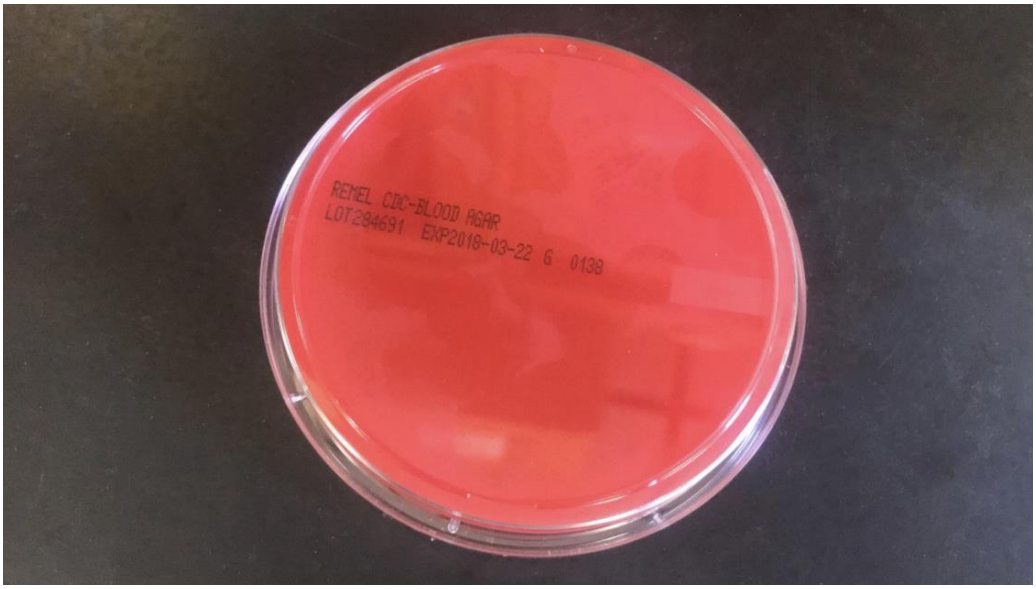

FIGURE 3. The sterility of the cinnamon solution was confirmed by streaking on a blood agar plate.

FIGURE 4. The cinnamon solution was clarified by centrifugation (Beckman GS-6R Refrigerated Centrifuge) for 10 min.

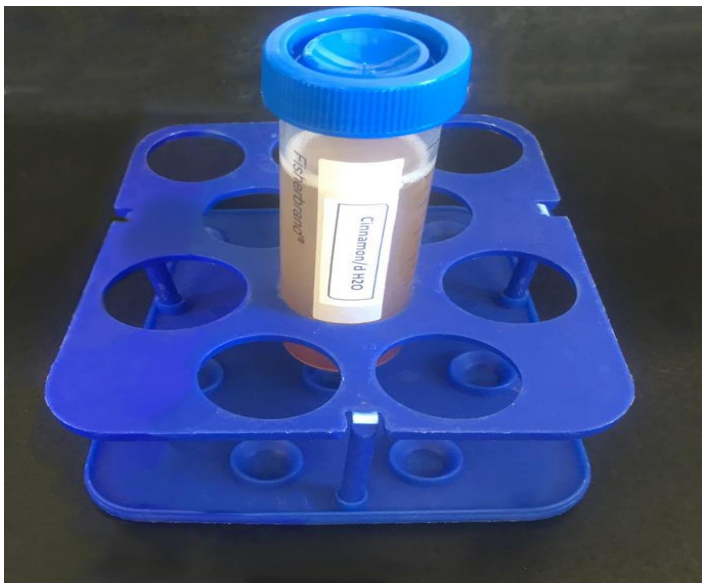

FIGURE 5. Cinnamon powder was mixed with sterile deionized water.

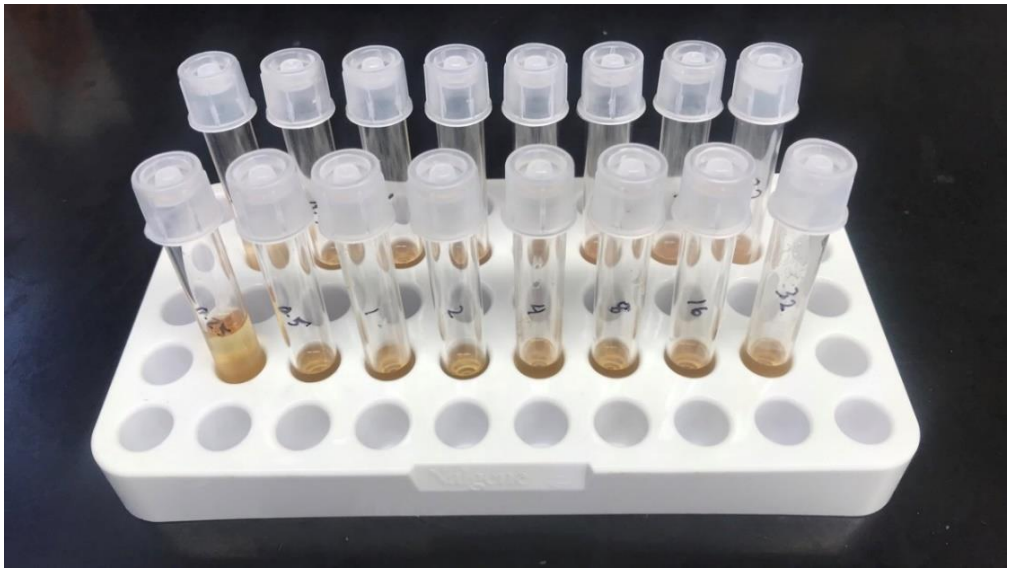

FIGURE 6. Dilutions of 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/ml nicotine in TSBS with and without cinnamon water extract.

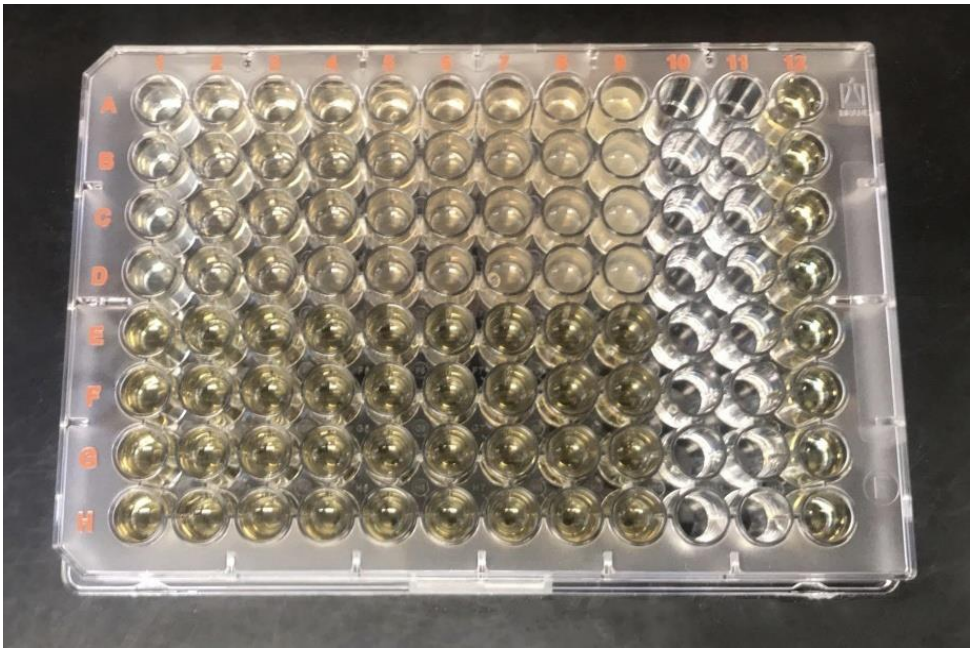

FIGURE 7. Each nicotine concentration was aliquoted by pipetting 190 µl of TSBS containing the nicotine/cinnamon water extract into wells of a sterile 96-well flat bottom microtiter plate. Then, 10 µl of the fresh overnight TSB culture of *S. mutans* was added.

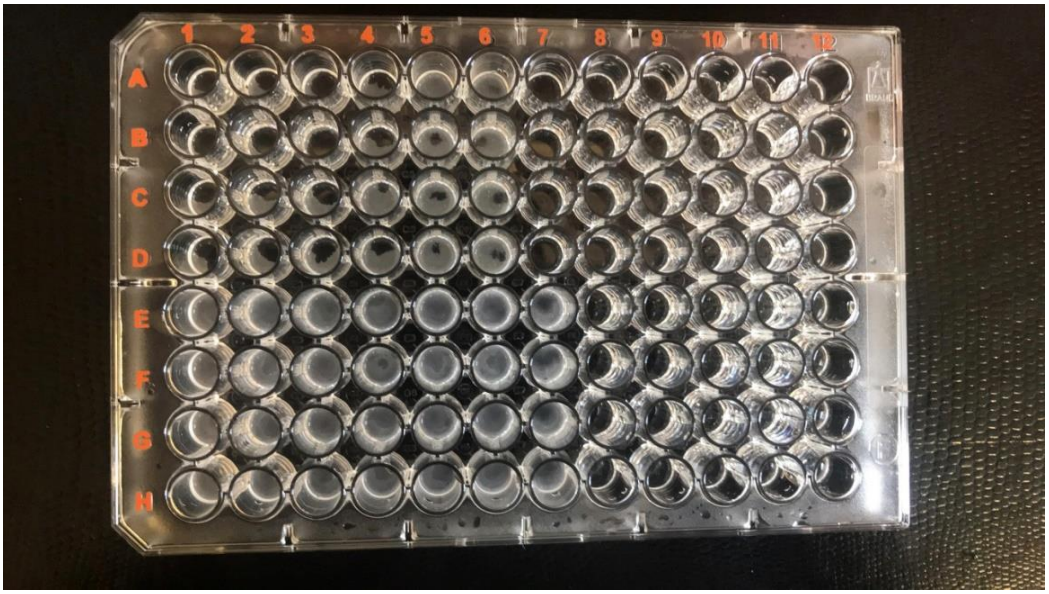

FIGURE 8. Biofilm formation can be seen clearly in the control group (rows E, F, G and H/columns 1-7) and in the test group (rows A, B, C and D/ columns 5-6) after 30 minutes following the application of formaldehyde.

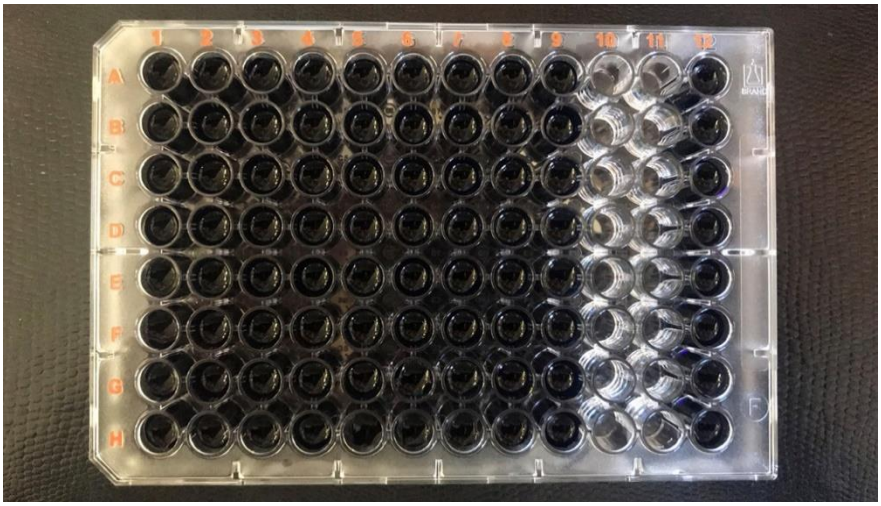

FIGURE 9. 200 µl of 0.5% crystal violet dye was added to each well and the biofilm cells stained for 30 min.

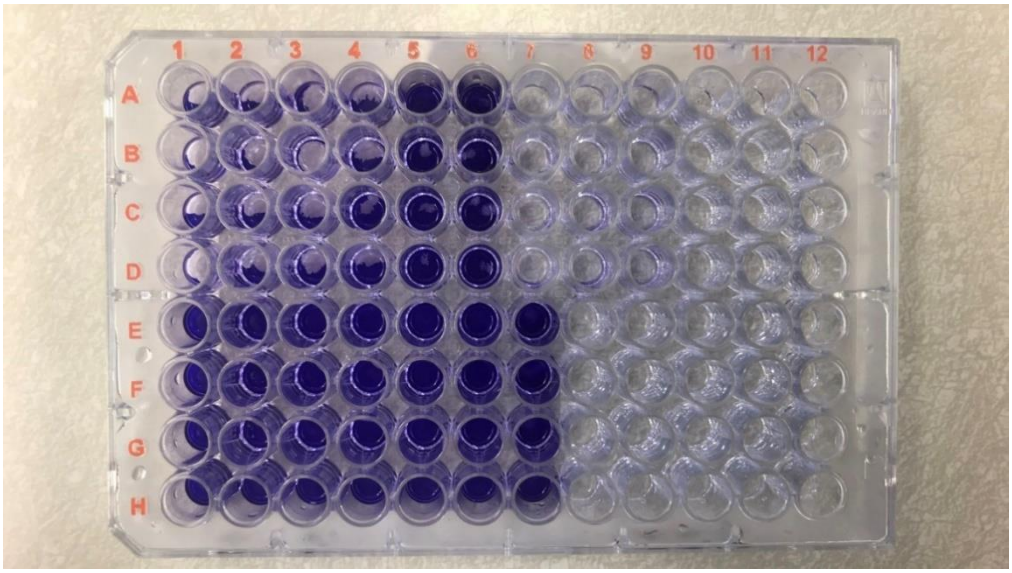

FIGURE 10. After crystal violet dye application, the wells were rinsed 3 times. The heavily stained wells are associated with more biofilm formation.

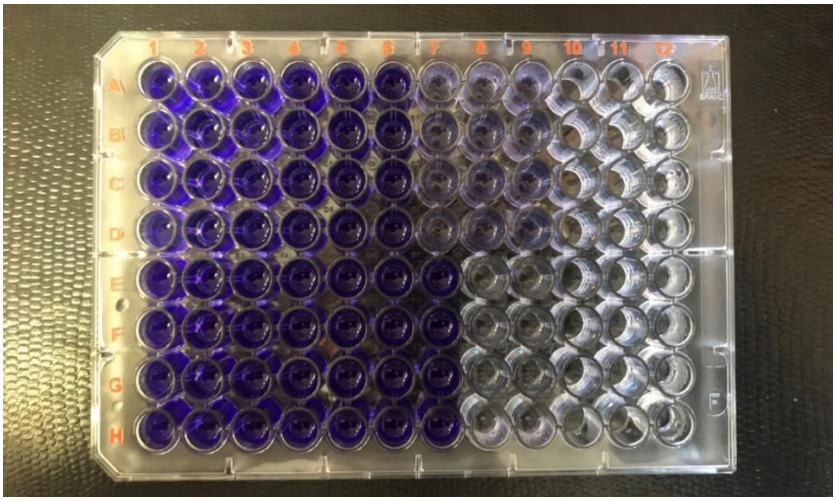

FIGURE 11. Two hundred (200) µl of 2-isopropanol was placed into each well for 1 h to lyse the biofilm cells and extract the crystal violet.

FIGURE 12. The absorbance of the microtiter plate wells were read using a spectrophotometer.

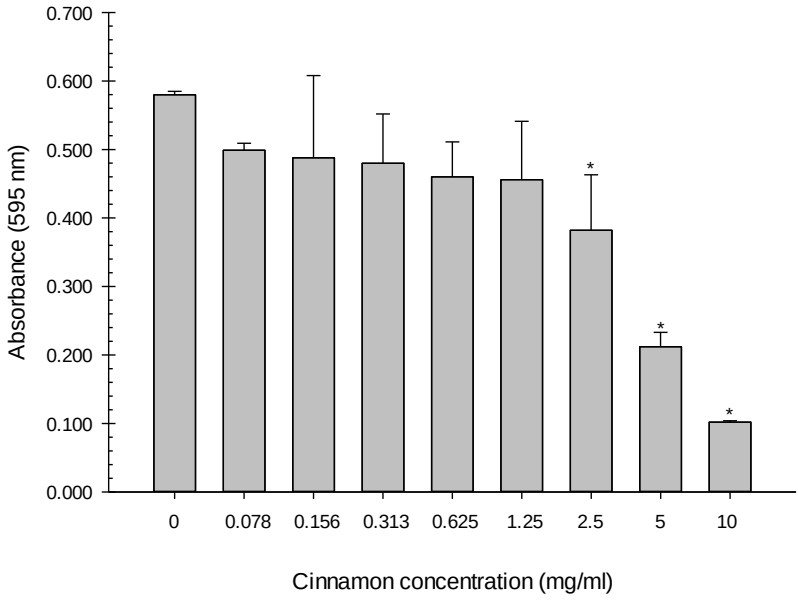

FIGURE 13. Effect of cinnamon water extract on total absorbance of *S. mutans*. Asterisks indicate significant differences ($p < 0.05$) compared with samples without cinnamon.

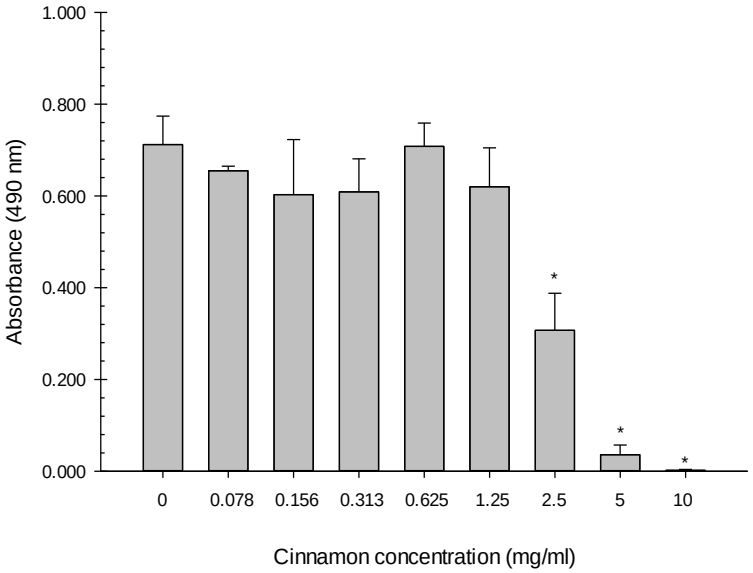

FIGURE 14. Effect of cinnamon water extract on biofilm formation of *S. mutans*. Asterisks indicate significant differences ($p < 0.05$) compared with samples without cinnamon.

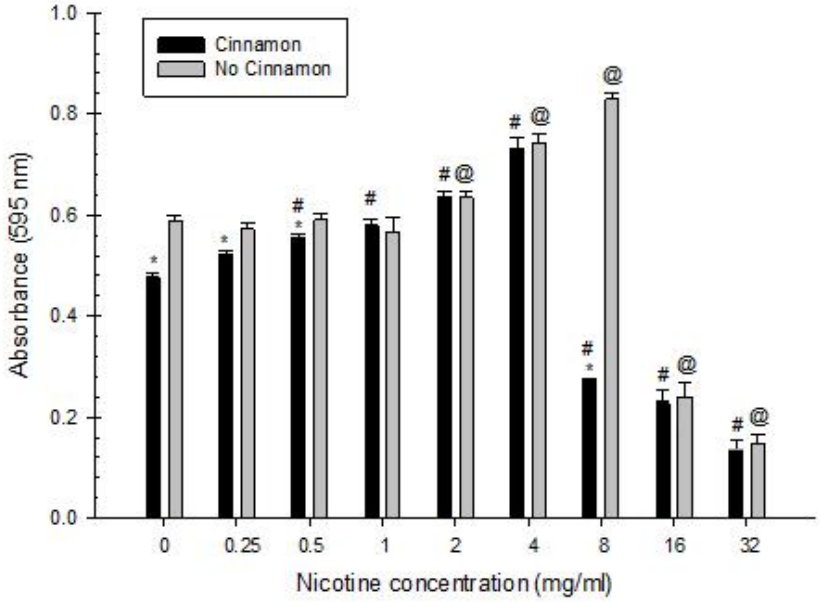

FIGURE 15. Combined effect of cinnamon water extract (2.5 mg/ml) and nicotine on *S. mutans* total absorbance. Asterisks indicate significant differences ($p < 0.05$) with cinnamon water extract compared to samples without cinnamon water extract. The # symbol indicates significant differences ($p < 0.05$) between *S. mutans* total absorbance with cinnamon water extract at different nicotine concentrations and the zero-nicotine concentration with cinnamon water extract. The @ symbol indicates significant differences ($p < 0.05$) between *S. mutans* total absorbance without cinnamon water extract at different nicotine concentrations and the zero-nicotine concentration without cinnamon water extract.

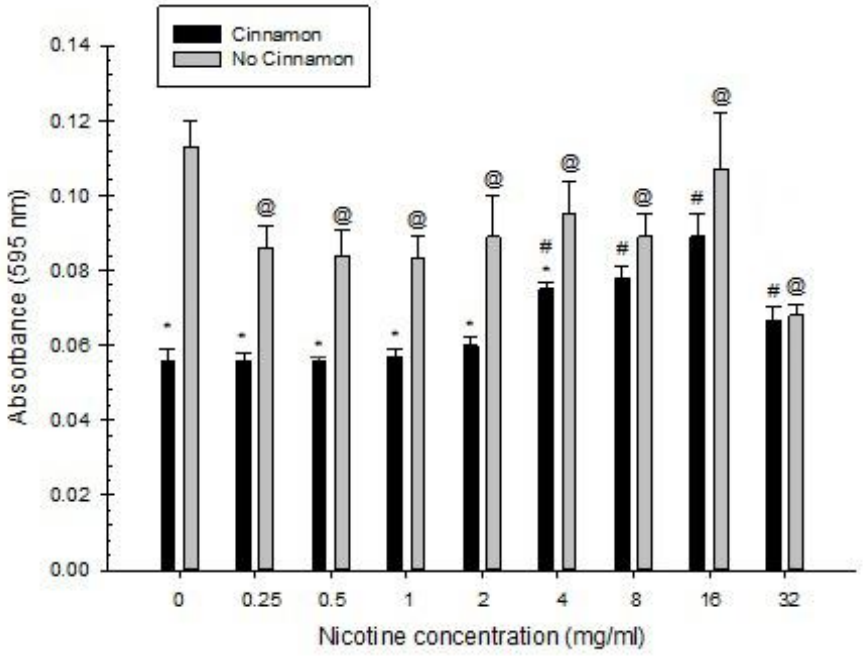

FIGURE 16. Combined effect of cinnamon (2.5 mg/ml) and nicotine on *S. mutans* planktonic growth. Asterisks indicate significant differences ($p < 0.05$) with cinnamon water extract compared to samples without cinnamon water extract. The # symbol indicates significant differences ($p < 0.05$) between *S. mutans* planktonic growth with cinnamon water extract at different nicotine concentrations and the zero-nicotine concentration with cinnamon water extract. The @ symbol indicates significant differences ($p < 0.05$) between *S. mutans* planktonic growth without cinnamon water extract at different nicotine concentrations and the zero-nicotine concentration without cinnamon water extract.

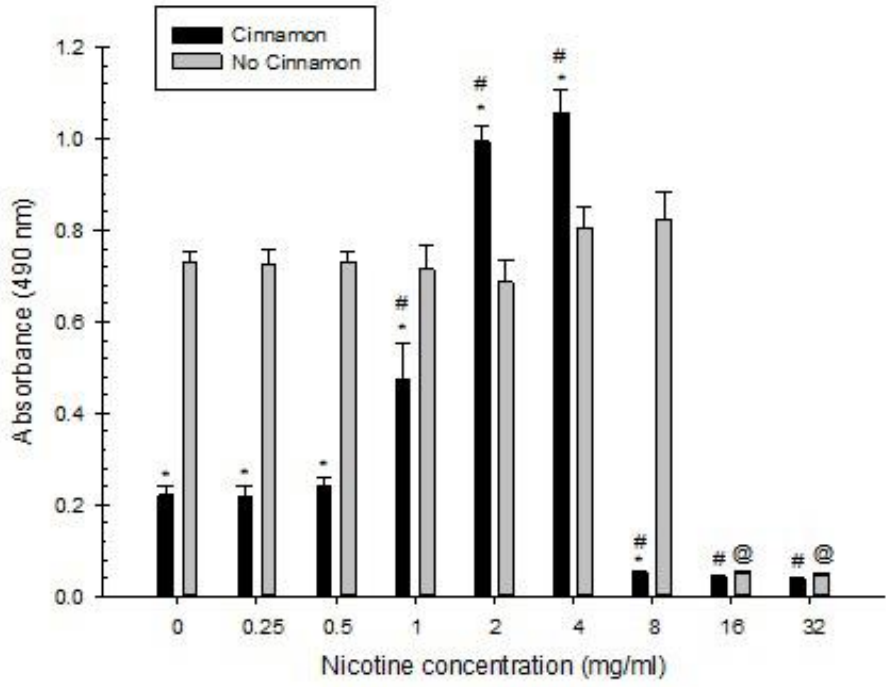

FIGURE 17. Combined effect of cinnamon water extract (2.5 mg/ml) and nicotine on *S. mutans* biofilm growth. Asterisks indicate significant differences ($p < 0.05$) with cinnamon water extract compared to samples without cinnamon water extract. The # symbol indicates significant differences ($p < 0.05$) between *S. mutans* biofilm growth with cinnamon water extract at different nicotine concentrations and the zero-nicotine concentration with cinnamon water extract. The @ symbol indicates significant differences ($p < 0.05$) between *S. mutans* biofilm growth without cinnamon water extract at different nicotine concentrations and the zero-nicotine concentration without cinnamon water extract.

TABLE I

Basic descriptive statistics for *S. mutans* total absorbance, by cinnamon water extract (2.5 mg/ml) and nicotine concentration

Group	Nicotine [mg/ml]	N	Mean	SD	SE	Min	Max
W/ Cinnamon water extract (2.5 mg/ml)	0	12	0.476	0.032	0.009	0.437	0.528
	0.25	12	0.521	0.031	0.009	0.481	0.582
	0.5	12	0.555	0.028	0.008	0.51	0.596
	1	12	0.58	0.04	0.012	0.513	0.629
	2	12	0.634	0.048	0.014	0.555	0.715
	4	12	0.731	0.073	0.021	0.659	0.857
	8	12	0.274	0.009	0.003	0.26	0.294
	16	12	0.24	0.021	0.006	0.261	0.323
	32	12	0.148	0.014	0.004	0.254	0.302
W/O Cinnamon water extract	0	12	0.589	0.034	0.01	0.541	0.643
	0.25	12	0.572	0.042	0.012	0.518	0.65
	0.5	12	0.59	0.049	0.014	0.525	0.682
	1	12	0.568	0.092	0.027	0.374	0.668
	2	12	0.634	0.049	0.014	0.527	0.711
	4	12	0.743	0.056	0.016	0.651	0.863
	8	12	0.829	0.039	0.011	0.746	0.91
	16	12	0.293	0.094	0.027	0.129	0.393
	32	12	0.278	0.065	0.019	0.105	0.314

TABLE II

Basic descriptive statistics for *S. mutans* planktonic growth, by cinnamon water extract (2.5 mg/ml) and nicotine concentration

Group	Nicotine [mg/ml]	N	Mean	SD	SE	Min	Max
W/ Cinnamon water extract (2.5 mg/ml)	0	12	0.056	0.009	0.003	0.044	0.077
	0.25	12	0.056	0.006	0.002	0.048	0.066
	0.5	12	0.055	0.005	0.001	0.049	0.063
	1	12	0.057	0.006	0.002	0.051	0.071
	2	12	0.06	0.008	0.002	0.041	0.071
	4	12	0.075	0.009	0.002	0.054	0.091
	8	12	0.078	0.011	0.003	0.06	0.095
	16	12	0.089	0.022	0.006	0.049	0.111
	32	12	0068	0.015	0.004	0.054	0.103
W/O Cinnamon water extract	0	12	0.113	0.025	0.007	0.088	0.164
	0.25	12	0.086	0.019	0.006	0.058	0.114
	0.5	12	0.084	0.024	0.007	0.058	0.139
	1	12	0.083	0.02	0.006	0.06	0.115
	2	12	0.089	0.039	0.011	0.063	0.209
	4	12	0.095	0.033	0.009	0.061	0.189
	8	12	0.089	0.022	0.006	0.052	0.132
	16	12	0.107	0.051	0.015	0.045	0.223
	32	12	0.087	0.01	0.003	0.043	0.082

TABLE III

Basic descriptive statistics for *S. mutans* biofilm growth, by cinnamon water extract and nicotine concentration

Group	Nicotine [mg/ml]	N	Mean	SD	SE	Min	Max
W/ Cinnamon water extract (2.5 mg/ml)	0	12	0.221	0.074	0.021	0.141	0.399
	0.25	12	0.22	0.072	0.021	0.114	0.299
	0.5	12	0.243	0.05	0.015	0.172	0.32
	1	12	0.475	0.273	0.079	0.16	0.897
	2	12	0.995	0.114	0.033	0.699	1.115
	4	12	1.058	0.177	0.051	0.804	1.458
	8	12	0.053	0.006	0.002	0.043	0.062
	16	12	0.052	0.01	0.003	0.044	0.075
	32	12	0.048	0.014	0.004	0.049	0.095
W/O Cinnamon water extract	0	12	0.728	0.095	0.027	0.59	0.865
	0.25	12	0.725	0.106	0.031	0.556	0.866
	0.5	12	0.731	0.082	0.024	0.588	0.868
	1	12	0.714	0.185	0.054	0.415	0.961
	2	12	0.687	0.16	0.046	0.498	0.978
	4	12	0.806	0.156	0.045	0.651	1.043
	8	12	0.824	0.216	0.062	0.577	1.295
	16	12	0.056	0.006	0.002	0.045	0.066
	32	12	0.061	0.004	0.001	0.045	0.056

TABLE IV

Two-way ANOVA tests comparing the effects of cinnamon water extract on nicotine concentrations and their interaction on *S. mutans* total absorbance, planktonic cells and biofilm

Total absorbance			
Effect	Num DF	F Value	Pr > F
Nicotine level	8	148.62	<.0001
Group	1	92.4	<.0001
Nicotine level*group	8	48.65	<.0001

Planktonic absorbance			
Effect	Num DF	F Value	Pr > F
Nicotine level	8	6.41	<.0001
Group	1	84.72	<.0001
Nicotine level*group	8	11.26	<.0001

Biofilm absorbance			
Effect	Num DF	F Value	Pr > F
Nicotine level	8	121.81	<.0001
Group	1	68.66	<.0001
Nicotine level*group	8	41.3	<.0001

DISCUSSION

The preliminary experiment was designed for two reasons: 1) To confirm the antimicrobial activities of cinnamon water extract, and 2) To confirm the MIC of cinnamon water extract. In this study, *C. burmannii* powder (also called Korintje, Padang cassia, Java, or Indonesian cinnamon) was used because it is the most common and least costly type of cinnamon sold in the US, including in grocery stores.

Even though the antimicrobial activity of cinnamon was discussed in many previous studies, there was a lack of evidence regarding the direct effect of cinnamon on *S. mutans* biofilm growth. In addition, *S. mutans* is considered one of the normal flora species in the oral cavity. So, it is crucial to measure the MIC of cinnamon water extract on *S. mutans* to not negatively affect the normal ecology of the oral flora. However, in this study a sub-MIC (2.5 mg/ml) one dilution below the MIC (5 mg/ml) of cinnamon water extract in TSBS was used to treat nicotine-treated *S. mutans*. This dilution was selected because use of the MIC of cinnamon water extract would obscure any potential effect of nicotine. An overnight culture of *S. mutans* was placed in 96-well microtiter plates with TSBS to stimulate *S. mutans* growth and biofilm formation. Nicotine was added to both study and control groups to increase biofilm formation as this was confirmed in previous studies.[33-36]

The results of this study indicate that cinnamon water extract alone is able to significantly diminish the biofilm formation of *S. mutans* and also significantly limits the ability of nicotine to increase the growth of *S. mutans* at very low concentrations of nicotine (0.25 mg/ml, 0.5 mg/ml and 1 mg/ml) as well as also at very high levels of nicotine (8 mg/ml, 16 mg/ml and 32 mg/ml). However, the results indicate that cinnamon water extract is able to significantly enhance the ability of nicotine to

increase the growth of *S. mutans* at certain nicotine concentrations (2 mg/ml and 4 mg/ml). This was interpreted to indicate that any nicotine concentration above 8 mg/ml, when combined with 2.5 mg/ml of cinnamon water extract can strongly inhibit *S. mutans* biofilm formation and any nicotine concentrations below 1 mg/ml also inhibit *S. mutans* biofilm formation. However, nicotine concentrations of 2 mg/ml and 4 mg/ml can enhance *S. mutans* biofilm formation.

A cinnamon water extract was investigated using microtiter plates. The plates contained the study group involving the cinnamon water extract/nicotine combination and a control group of nicotine without cinnamon water extract, because it was better to include both study and control groups in the same microtiter plate to standardize the environmental and preparation conditions. A sterility group was added to assure that contamination was not present, because if there was contamination, the sterility wells will exhibit some kind of bacterial growth. A two-way ANOVA was used to compare the effects of the presence of 2.5 mg/ml cinnamon water extract and nicotine (concentrations ranging from 0 mg to 32 mg) on *S. mutans* biofilm, planktonic cells, and total absorbance. Pairwise comparisons were made between nicotine concentrations to the zero-nicotine both with and without 2.5 mg/ml cinnamon water extract. Pairwise comparisons were also made comparing each nicotine concentration with and without cinnamon water extract. Since the experimental trial was repeated 3 times (with 4 samples per group per repeat), a random effect for the multiple trials was used. Due to non-normality, a rank transformation was used prior to analysis.

There are different bacterial growth phases (planktonic and biofilm) among which the biofilm form is considered the most important phase, the most favorable phase for oral bacteria to grow *in vivo* and cause disease. Moreover, protein expression in biofilm cells differs from the expression that is observed in planktonic

cells.[62] So, even though cinnamon water extract demonstrated a minor restriction of nicotine activity when total absorbance and planktonic growth were measured, this restriction was recognized clearly when biofilm formation was assessed. In the nicotine group (with no cinnamon), it was observed with increasing nicotine concentrations, there were increases in biofilm formation. Meanwhile with the 16 mg/ml and 32 mg/ml nicotine concentrations, there were strong inhibitory effects on *S. mutans* growth and viability, respectively. These results confirm a previous study with seven strains of *S. mutans*, including the UA159 strain tested in this study, exposed to varying concentrations of nicotine.[34] It was theorized previously that any nicotine concentration above 8 mg/ml is toxic to *S. mutans*, and furthermore any concentration at or above 32 mg/ml is bactericidal.

It is understood that 2.5 mg/ml cinnamon water extract with 16 mg or 32 mg of nicotine demonstrated more inhibition than nicotine alone and this is likely attributable to the combined antimicrobial effect of 2.5 mg/ml cinnamon water extract and nicotine. At 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml and 8 mg/ml nicotine concentrations, it was observed that with no cinnamon water extract, there was more biofilm formation than for those with 2.5 mg/ml cinnamon water extract. However, a significant increase in bacterial growth was clearly seen when cinnamon water extract was added with concentrations of 2 mg/ml and 4 mg/ml nicotine. A reasonable explanation is that a synergistic effect developed between cinnamon water extract and nicotine at these specific concentrations of nicotine.

The clinical implication of these results can be related to generally incorporating cinnamon water extract with the appropriate concentration to oral hygiene products such as toothpaste, mouth washes and dental floss to inhibit *S. mutans* biofilm, and thereby inhibit or reduce caries incidence. Cinnamon is

commonly added to oral hygiene products just as a flavoring agent and mouth refresher. However, based on the results of this study, it can be added at an appropriate concentration to affect *S. mutans* biofilm formation.

It is known that one cigarette contains nearly 1 mg of nicotine, which is partially absorbed into the bloodstream through the mucosal linings in the mouth with the remaining nicotine accumulating in saliva. According to a study, which measured the amount of nicotine in samples of saliva collected from smokers and non-smokers, the amount of nicotine in non-smokers' saliva who are affected by secondary or tertiary hand smoke falls in the range of 0 mg/ml to 0.31 mg/ml. In addition, it has been found that the amount of nicotine found in light or medium smokers' saliva ranges from 0 mg/ml to 1.33 mg/ml. Also, the amount of nicotine measured in heavy smokers' saliva was in the range of 0 mg/ml to 2.27 mg/ml.[99] Another study reported that the amount of nicotine in human saliva ranges from 0.07 mg/ml to 1.56 mg/ml in saliva samples that were collected from smokers who have smoked for at least 10 years.[98] If we suppose that the average nicotine level in human saliva is 1 mg/ml, the cinnamon water extract demonstrated the ability to inhibit nicotine induced *S. mutans* biofilm at certain nicotine concentrations (0.25, 0.5, 1 ,8,16 and 32 mg/ml), so it could be said that cinnamon water extract added to oral hygiene products would be beneficial for smokers. In addition, some commercially available smoking cessation products such as chewing gum or lozenges contain cinnamon added as a flavoring agent and nicotine in amounts ranging from 2 mg to 4 mg of nicotine per lozenge and piece of chewing gum. The product is recommended to be used every two hours during the smoking cessation course and it is assumed that cinnamon water extract and nicotine accumulate in saliva and in turn is absorbed in biofilm. However, the last statement could be refuted because no prior study has investigated the amount of

nicotine in smoker's biofilm, which is what is needed. Previous studies only reported the amount of nicotine in saliva. *In-viv*o studies are needed to determine the amount of nicotine in biofilm taken from smokers to assess the amount of nicotine absorbed in their biofilm. In addition, further research may be needed to investigate how cinnamon specifically inhibits *S. mutans* biofilm formation, and what effect cinnamon water extract may have on extracellular polysaccharide synthesis, glucosyltransferase synthesis, glucan-binding protein synthesis and acid production. Nicotine was found to increase extracellular polysaccharide (EPS) synthesis, GbpA expression, Gtfs expression, and lactic acid production in *S. mutans*.[35] Cinnamon water extract may interfere with EPS synthesis, GbpA, Gtfs expression or lactic acid production. In conclusion, *in-vivo* studies are needed to confirm the anti-microbial effect of cinnamon and its biocompatibility with oral tissues. There are number of limitations to this study: only one strain of *S. mutans* was tested in this study. In other words, other strains of *S. mutans* may interact differently with the cinnamon water extract. In addition, there were no saliva or salivary components utilized in this study. Moreover, only one species of cinnamon was tested in this study.

SUMMARY AND CONCLUSION

This study indicates that cinnamon water extract at 2.5 mg/ml demonstrated strong anti-*S. mutans* properties by inhibiting *S. mutans* biofilm formation. Cinnamon water extract can be incorporated into oral hygiene products with the appropriate anti-*S. mutans* concentration, so people may gain a dual benefit of being a mouth-refreshing and a caries-preventing agent. The growth of nicotine-induced *S. mutans* could be diminished in the presence of cinnamon. Therefore, cinnamon oral hygiene products may be beneficial for both smokers and non smokers. *In-vivo* studies are needed to confirm this benefit.

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ABSTRACT

EFFECTS OF CINNAMON WATER EXTRACT AS A CARIOSTATIC AGENT ON NICOTINE-INDUCED *STREPTOCOCCUS MUTANS* BIOFILM

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Dental caries is considered one of the most prevalent chronic bacterial infections in the world. *Streptococcus mutans* is considered to be one of the main etiological microorganisms implicated in the caries disease. The relation between smoking and dental caries has been investigated extensively. It has been found that nicotine enhances the cariogenicity of *S. mutans* and biofilm formation. Recently, the focus has been on decreasing the incidence of caries by reducing the load of *Streptococcus mutans* and dental plaque. Various antibacterial compounds have been used for these purposes. Among the widely used herbs and spices is cinnamon, which has demonstrated a strong antibacterial activity against a wide variety of bacteria including *S. mutans* and showed the ability to inhibit *S. mutans* biofilm formation.

Objective: The aim of this study was to investigate the effects of cinnamon water extract on nicotine-induced *Streptococcus mutans* biofilm. This study utilized *S.*

mutans biofilm assays with varying concentrations of nicotine/cinnamon water extract levels. First-hand exposure to nicotine has been demonstrated to significantly increase biofilm formation, while cinnamon water extract has been shown to reduce *S. mutans* biofilm formation.

Materials and Methods: A preliminary experiment was carried out to confirm the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC) of cinnamon water extract on the growth of *S. mutans* in tryptic soy broth (TSB) supplemented with 1.0-percent sucrose (TSBS). A recent pilot study indicated that the MIC and MBIC of cinnamon water extract was 5 mg/ml. In order to identify the effect of cinnamon water extract on initial biofilm formation, a 24-hour culture of *S. mutans* UA159 in microtiter plates was treated with varying nicotine concentrations (0-32 mg/ml) in TSBS at the same time with or without the optimum cinnamon water extract concentration. A spectrophotometer was used to determine total growth absorbance and planktonic growth. The microtiter plate wells were washed, fixed and stained with crystal violet dye and the absorbance measured to determine biofilm formation.

Results: The results indicated that cinnamon water extract was able to inhibit biofilm formation significantly ($p < 0.05$) at 5 mg/ml cinnamon water extract; therefore, 5 mg/ml of cinnamon water extract was recognized as the MIC for *S. mutans* biofilm formation.

When combined with nicotine, cinnamon water extract demonstrated a significant inhibitory effect ($p < 0.05$) in biofilm and total absorbance measures at high concentrations of nicotine (8 mg/ml and above). In addition, cinnamon water extract showed a significant effect ($p < 0.05$) at very low concentrations of nicotine (0.25 mg/ml and 0.5 mg/ml) in all measures (biofilm, planktonic and total

absorbance). However, at low concentrations of nicotine (2 mg/ml and 4 mg/ml), there was a significant increase ($p < 0.05$) in biofilm growth, whereas planktonic growth was significantly ($p < 0.05$) decreased at the same concentration.

Conclusion: This study indicates that cinnamon water extract demonstrated strong anti-cariogenic properties by inhibiting *S. mutans* biofilm formation. Cinnamon water extract can be incorporated into oral hygiene products with the appropriate anti-*S. mutans* concentration, so people may gain a dual benefit of a mouth-refreshing and a caries-preventing agent. In addition, these results provided more evidence regarding the negative effects of nicotine and also the positive influence of cinnamon water extract in reducing nicotine-induced biofilm formation. Cinnamon oral hygiene products may be beneficial for both smokers and non-smokers. *In-vivo* studies are needed to confirm this benefit.

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