



Effect of Herbal Dentifrice Without Fluoride Against Oral Microbial

: An *in vitro* Study

Natrapee Akarathanakrit

**A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master
of Science in Oral Cell Biology and Biomaterials Science**

Prince of Songkla University

2025

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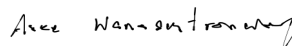
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Author	Miss Natrapee Akarathanakrit
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ABSTRACT

Objective: This study aims to evaluate the antimicrobial effects of fluoride-free herbal toothpaste on *Candida albicans* (*C. albicans*) and *Streptococcus mutans* (*S. mutans*). Despite its limitations, the findings offer valuable insights for both consumers and dental professionals, helping to guide informed decisions in toothpaste selection.

Material and Methods: *C. albicans* ATCC 90028 was cultured in Sabouraud's dextrose agar, and *S. mutans* ATCC 25175 was cultured at 37°C with 5% CO₂ for 18 hours. Twenty-seven commercially available herbal toothpaste formulations were diluted with 2% Dimethyl Sulfoxide (DMSO) at a 1:1 ratio and mixed using a vortex mixer. Antibiotic sensitivity testing was performed using SDA for *C. albicans* and BHI for *S. mutans*, with results recorded by measuring the diameter of the clear zone. The minimum inhibitory concentration (MIC) was determined using a 96-well plate with resazurin, while the minimum bactericidal concentration (MBC) was assessed by culturing on agar and counting CFUs. Biofilm formation was analyzed using crystal violet staining and OD measurement at 560 nm. All tests were performed in triplicate.

Results: The MIC and MBC tests showed no microbial growth, confirming that all 27 herbal toothpaste formulations at a concentration of 1 g/ml effectively inhibited and killed *C. albicans*. Five formulations (A005, A018, A025, A004, and A023) demonstrated notable antifungal activity. In the biofilm formation assay, all 27 formulations exhibited biofilm formation, while 15 formulations completely inhibited *C. albicans* and *S. mutans* biofilm formation.

Conclusion: The study confirms the antifungal and antibacterial potential of herbal toothpaste against *C. albicans* and *S. mutans*. Variations in effectiveness highlight the need for further research on the active ingredients. Inhibiting biofilm formation is crucial for preventing persistent infections. These findings support the use of herbal toothpaste as a preventive measure for *C. albicans* and *S. mutans*-related oral infections.

Keyword : Herbal toothpaste, *Candida albicans*, *Streptococcus mutans*, fluoride

ชื่อวิทยานิพนธ์	ผลของยาสีฟันสมุนไพรที่ไม่มีฟลูออไรด์ต่อเชื้อในช่องปาก
	: การศึกษาในห้องปฏิบัติการ
ผู้เขียน	นางสาวณัฏฐ์ระพี อัครธนกฤต
สาขาวิชา	เซลล์วิทยาช่องปากและวิทยาศาสตร์ชีววัสดุ
ปีการศึกษา	2567

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินฤทธิ์ต้านจุลชีพของยาสีฟันสมุนไพรที่ไม่มีฟลูออไรด์ต่อเชื้อแคนดิดา อัลบิแคนส์ และสเตรปโตค็อกคัส มิวแทนส์ แม้ว่าจะมีข้อจำกัดบางประการ แต่ผล การศึกษานี้ให้ข้อมูลเชิงลึกที่มีประโยชน์ต่อผู้บริโภคและทันตแพทย์ เพื่อช่วยในการตัดสินใจเลือกใช้อยา สีฟันอย่างมีข้อมูลถูกต้องแก่ผู้ป่วยในการรักษาโรคในช่องปาก

เพาะเลี้ยง แคนดิดา อัลบิแคนส์ สายพันธุ์ เอทีซีซี 90028 ในอาหารเลี้ยงเชื้อซาโบโรด์ เด็กซ์ โทรสชนิดแข็ง และเพาะเลี้ยงสเตรปโตค็อกคัส มิวแทนส์ สายพันธุ์ เอทีซีซี 25175 ในอาหารเลี้ยง เชื้อเบรน ฮาร์ท อินฟิวชัน ชนิดแข็งที่อุณหภูมิ 37 องศาเซลเซียส ภายใต้บรรยากาศที่มี คาร์บอนไดออกไซด์ร้อยละ 5 เป็นเวลา 18 ชั่วโมง นำยาสีฟันสมุนไพรที่วางจำหน่ายในท้องตลาด ทั้งหมดจำนวน 27 สูตร มาเจือจางด้วย ร้อยละ 2 ของไดเมทิล ซัลฟอกไซด์ ในอัตราส่วน 1:1 และ ผสมด้วยเครื่องวอร์เท็กซ์ การทดสอบความไวต่อยาปฏิชีวนะทดสอบในอาหารเลี้ยงเชื้อซาโบ โรด์ เด็กซ์โทรสชนิดแข็ง และสเตรปโตค็อกคัส มิวแทนส์ ทดสอบในอาหารเลี้ยงเชื้อเบรน ฮาร์ท อินฟิว ชัน และบันทึกผลโดยการวัดเส้นผ่านศูนย์กลางของบริเวณที่เกิดวงใส การทดสอบความเข้มข้นต่ำสุดที่ สามารถยับยั้งเชื้อได้ ทำโดยจานเลี้ยงเชื้อ 96 หลุม โดยใช้สารเรซาซูลิน และการทดสอบความเข้มข้น ต่ำสุดที่ฆ่าเชื้อได้ตรวจทำโดยการเพาะเลี้ยง แคนดิดา อัลบิแคนส์ บนอาหารเลี้ยงเชื้อซาโบโรด์ เด็กซ์ โทรสชนิดแข็ง และเพาะเลี้ยงสเตรปโตค็อกคัส มิวแทนส์ ในอาหารเลี้ยงเชื้อเบรน ฮาร์ท อินฟิวชัน และ ตรวจนับจำนวนปริมาณจุลินทรีย์ในหน่วยโคโลนีฟอร์มมิงยูนิต ทำการวิเคราะห์การสร้างไบโอฟิล์มโดย การย้อมด้วยสีคริสตัลไวโอเล็ตและวัดค่าการดูดกลืนแสงที่ความยาวคลื่น 560 นาโนเมตร โดยทำการ ทดสอบทั้งหมดซ้ำ 3 ครั้ง

การทดสอบความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อและความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อได้ แสดงผลให้เห็นว่าไม่มีการเจริญเติบโตของจุลชีพในการทดสอบของยาสีฟันสมุนไพรทั้ง 27 สูตร ที่มี ความเข้มข้น 1 กรัมต่อมิลลิลิตร สามารถยับยั้งและฆ่าแคนดิดา อัลบิแคนส์ และสเตรปโตค็อกคัส มิว

แทนส์ได้อย่างมีประสิทธิภาพ ยาสีฟันที่มีฤทธิ์ด้านเชื้อราอย่างมีประสิทธิภาพเด่นชัดที่สุด 5 สูตร ได้แก่ A005, A018, A025, A004 และ A023 ในการทดสอบการยับยั้งการสร้างไบโอฟิล์มของยาสีฟันทั้ง 27 สูตร แสดงให้เห็นว่ามียาสีฟัน 15 สูตรสามารถยับยั้งการสร้างไบโอฟิล์มของแคนดิดา อัลบิแคนส์ได้อย่างสมบูรณ์

การศึกษานี้ยืนยันถึงศักยภาพในการต้านเชื้อราและด้านแบคทีเรียของยาสีฟันสมุนไพรต่อแคนดิดาอัลบิแคนส์ และสเตรปโตค็อกคัส มิวแทนส์ ความแตกต่างในประสิทธิภาพชี้ให้เห็นถึงความจำเป็นในการศึกษาต่อเกี่ยวกับสารออกฤทธิ์ การยับยั้งการสร้างไบโอฟิล์มเป็นสิ่งสำคัญในการป้องกันการติดเชื้อเรื้อรัง ผลการศึกษานี้สนับสนุนการใช้ยาสีฟันสมุนไพรเป็นมาตรการป้องกันการติดเชื้อในช่องปากที่เกี่ยวข้องกับแคนดิดา อัลบิแคนส์ และ สเตรปโตค็อกคัส มิวแทนส์

คำสำคัญ : ยาสีฟันสมุนไพร, แคนดิดา อัลบิแคนส์, สเตรปโตค็อกคัส มิวแทนส์, ฟลูออไรด์

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degrees Celsius
AIDs	=	Acquired immunodeficiency syndrome
ALS	=	Agglutinin-like sequences
BHI	=	Brain heart infusion
CFUs	=	Colony forming unit
CHX	=	Chlorhexidine
cm	=	centimeter
CO ₂	=	Carbon dioxide
DMSO	=	Dimethyl sulfoxide
g	=	gram
GTF	=	Glycosyltransferase
h	=	hour
HIV	=	Human Immunodeficiency Virus
KDa	=	kilodalton
LDH	=	Lactate dehydrogenase
M	=	meter
ml	=	milliliter
MBC	=	Minimum bactericidal concentration
MBSA	=	Mitis Salivarius Agar
MSB	=	Mitis Salivarius Broth

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUE)

Mg	=	milligram
MIC	=	Minimum inhibitory concentration
min	=	minutes
Mm	=	millimeter
nm	=	nanometer
NaCl	=	Sodium chloride
OD	=	Optical Density
PBS	=	Phosphate Buffer Saline
pH	=	Potential of Hydrogen ion
rpm	=	Revolutions per minute
SAPs	=	Secreted aspartic proteinases
SDA	=	Sabouraud's Dextrose Agar
SDB	=	Sabouraud's Dextrose Broth
TIS	=	Thai Industrial Standard
μL	=	microliter
μM	=	micrometer

CHAPTER 1

INTRODUCTION

Dental caries is a major global public health and economic problem worldwide¹. The cause of oral caries is often a disruption of the oral homeostasis, leading to tooth loss², which has a significant impact on quality of life and work, and involves relatively high medical expenses. The risk factors for dental caries include eating foods or beverages high in sugar, smoking, and a lack of oral hygiene, such as scaling and brushing with toothpaste³.

Toothpaste is a product used to remove plaque, food debris, and maintain oral hygiene. It is used together with a toothbrush to clean the mouth and teeth and helps inhibit the growth of microorganisms in the mouth. The most important components of toothpaste are fluoride, antimicrobial agents, and various tooth-whitening agents⁴.

Nowadays, many studies try using various herbs as ingredients in toothpaste. Most of which were found to have the effect of inhibiting germs and effectively reducing microbial plaque. Herbs that have been tested as ingredients in toothpaste include licorice (*Glycyrrhiza glabra* L.), which inhibits the growth of bacteria, prevents tooth decay, and reduces sticky saliva⁵, aloe vera (*Aloe vera* L.) inhibits bacteria in the mouth and reduces plaque accumulation⁶, clove (*Syzygium aromaticum* L.) has antimicrobial properties against *Enterococcus faecalis*, helps relieve toothache, relieves inflammation, and helps reduce bad breath⁷⁻¹⁰, mangosteen peel (*Garcinia mangostana* L.) has the effect of inhibiting the growth of bacteria in the mouth, reducing the chance of gingivitis¹¹⁻¹³, peppermint oil (*Mentha Piperita*) has a refreshing scent and helps inhibit microorganisms in the mouth¹⁴. There is also a lot of research on herbal toothpaste to inhibit microorganisms in the mouth to compare the effectiveness of various herbal toothpastes found in the market with general toothpastes that have the effect of inhibiting microorganisms. It was found that herbal toothpaste has the same antimicrobial effect as regular toothpaste. Toothpastes containing different types, amounts and quantities of herbal ingredients have different antimicrobial effects¹⁵.

The oral cavity is an organ connected to the respiratory and digestive systems, which is rich in nutrients, and the appropriate temperature and humidity create conditions that are conducive to the growth of microorganisms. Moreover, more than 700 types of microorganisms were found in the mouth, including *Candida albicans* (*C. albicans*) , *Streptococcus mutans* (*S. mutans*) , *Streptococcus hemolyticus*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *E. faecalis*^{16,17}. These microorganisms are resident microorganisms (normal flora) in the oral cavity, helping to maintain balance in the oral cavity. and resist the invasion of adverse factors from outside. Conversely, when the oral ecosystem is disrupted or there are predisposing factors, these microorganisms have the ability to cause oral diseases, such as dental caries, periodontal disease, and bad breath^{18, 19}.

S. mutans is a bacterium found in the mouth, pharynx, and intestines²⁰. It has an important role in dental disease^{21,22}. *S. mutans* is acid tolerant and produces acid. When we ingest sugar, particularly table sugar or sucrose, the bacteria ferment the sugar into lactic acid, creating an acidic environment in the mouth. The enamel is composed of hydroxyapatite, which the bacteria use to remove hydroxyapatite from the enamel²³. It is also a bacterium that produces the enzyme glucosyltransferase (GTF), which plays an important role in the pathogenesis of dental caries. This bacterium produces GTF by using sucrose to synthesize glucan, which is one of the factors that cause plaque on the tooth surface and ultimately leads to dental caries²⁴.

C. albicans is a normal flora in the oral cavity that does not normally cause disease. However, when there are predisposing factors, it causes Candida-associated denture stomatitis, which is the most common oral lesion²⁵, and is more common in elderly denture wearers²⁶⁻²⁹. In addition, *C. albicans* also has an important pathogenic factor, which is the formation of a biofilm, which can resist the elimination of bacteria and become resistant to antifungal drugs³⁰⁻³². Therefore, treatment consists of eliminating risk factors that cause infection together with oral hygiene care, including the use of antifungal drugs if a concomitant fungal infection is found³³.

From the above information, the researcher considers that controlling oral homeostasis is extremely important in preventing oral disease. Brushing teeth with toothpaste is one of the basic oral hygiene methods that are widely used. There are many types of toothpaste available in the Thai market, both general toothpaste and herbal toothpaste, which each brand has different ingredients and ratios. Herbal toothpaste, according to the Thai Industrial Standard (TIS 41-2562), does not cover toothpaste containing fluoride and synthetic coloring³⁴. In foreign countries, there have been studies on the inhibition of oral microorganisms of herbal toothpaste of various brands. However, in Thailand, studies on herbal toothpaste are still limited and there is not much data. In addition, entrepreneurs and manufacturers continuously produce new and diverse toothpaste formulas. Therefore, the researcher wants to study the inhibition of oral microorganisms of herbal toothpaste without fluoride against pathogenic microorganisms, such as *S. mutans*, to provide basic information and help consumers decide on choosing herbal toothpaste. It can also provide information for the dentists, and to be used to advise patients on the correct selection of toothpaste. This study was an in vitro study. There are still several limitations of this study, such as the experimental conditions which are not similarly the real oral conditions of humans, the study of the different types of microorganisms for inhibition when in actual oral microorganisms are in a biofilm, etc., and various other factors which will be discussed

CHAPTER 2

REVIVE OF LITERATURE

1. Dental caries

Dental caries is considered as one of the most common oral diseases that can occur in both children and adults, caused by the breakdown of food for energy, acid formation, tooth demineralization, and bacterial destruction of the tooth structure, which results in a hole or cavity on the tooth. If left untreated, it will spread, expand, and become deeper, causing pain and suffering, and eventually tooth loss, which may have to be extracted. Dental caries is considered a contagious disease because they are caused by germs and transmitted through saliva. The disease process requires three important elements: the tooth, the microorganisms, and the acidic environment in the mouth³⁵.

Dental caries is caused by bacteria producing organic acid, such as lactic acid, acetic acid, etc., by the process of glycolysis of bacteria, which uses carbohydrates as an energy source. The acid produced from glycolysis will reduce the pH in the mouth. Acid produced by glycolysis will reduce the acidity in the mouth.

The oral system is normally neutral to maintain the pH balance. But if too much acid is produced to the point where the acidity is lower than the critical value for tooth decay, which is 5.2-5.5, it will result in mineral loss. (demineralization) of tooth enamel³⁶.

1.1 Factors causing dental caries

1.1.1 Physical characteristics of teeth

The physical characteristics of each person's teeth, including the position, shape, composition, structure, and age of the teeth, all influence tooth decay. It was found that the location of tooth decay is most found in the upper primary teeth and has the least

effect on the lower primary teeth because the lower primary teeth are close to the opening of the salivary glands. There is a tongue to help clean and prevent milk residue from sticking to the teeth. In addition, it was found that the glucose leaching from the tooth surface by saliva is lowest at the lip of the upper incisors and near the cheek of the lower molar. Therefore, nutrients are retained in this area more than other areas in the mouth³⁷.

Saliva has an important role in preventing dental caries. It functions to help wash food out of the mouth, balance the acidity and alkalinity of the mouth, and inhibit the growth of bacteria, which helps reduce the incidence of dental caries in the teeth and roots³⁸. Saliva contains proteins that inhibit bacteria, such as lysozyme, lactoferrin, peroxidase, and agglutinin. Studies have found that the level of lysozyme activity in preschool children without dental caries is higher than that in those with dental caries³⁹. Therefore, children with impaired immune systems are at higher risk of dental caries than normal children⁴⁰.

The food-washing and buffering properties of saliva and the inhibition of bacterial growth depend on the flow rate of saliva. It was found that the salivary flow rate is reduced during sleep⁴¹. Normally, children sleep more than adults. If children consume sugary foods during this time or have food residue in their mouths, they are at risk of dental caries⁴². In addition, it was found that the salivary flow rate in children increases with age. The amount of sodium, potassium chloride, and protein is low in the first year, which may be the cause of dental caries because the oral conditions are more conducive to tooth surface mineral loss than remineralization⁴⁰.

1.1.2 Eating behavior

Consuming carbohydrates and sugars in foods is broken down by bacteria to produce acids that destroy tooth enamel. Sucrose is a sugar that has a very important role in the development of dental caries in all age groups because bacteria can use it to create extracellular glucan, a substance that bacteria use to attach themselves and allow them to multiply⁴².

Previous studies have found that sucrose is more cariogenic than other sugars, depending on the type of microorganism and the type of animal study and the area of tooth decay involved⁴³. Part of the caries potential of sucrose is because microorganisms can use sucrose to synthesize extracellular polysaccharides, which facilitate *S. mutans* attachment to dental plaque⁴⁴.

Normally, when we eat sugary foods frequently and there are leftover food particles, the bacteria will decompose them to produce acid and dissolve the tooth surface. However, the amount of saliva and minerals in the saliva reduces the acidity and replenishes the tooth with minerals. Therefore, if we eat frequently or have less saliva, the acidity of the microbial plaque will increase, causing more *S. mutans* bacteria and more loss of minerals in the tooth enamel, and the remineralization will not be sufficient, so dental caries will continue⁴².

1.1.3 Oral microorganisms

Streptococcus mutans (MS) is an important causative agent of dental caries. The most common species in humans include *S. mutans* and *S. sobrinus*⁴³. The most common species in humans are *S. mutans* and *S. sobrinus*⁴³. *S. mutans* colonization is related to other behavioral factors, such as sugary diet, snack consumption, and oral hygiene⁴⁵. Bacterial acidosis is caused by bacteria that accumulate in dental plaque on the lateral surfaces of the teeth converting glucose, fructose, and sucrose to acid via glycolysis. The primary energy source for bacteria. *S. mutans* has been implicated in dental caries, and it could further break down pyruvate, thus generating more energy and acid⁴⁶.

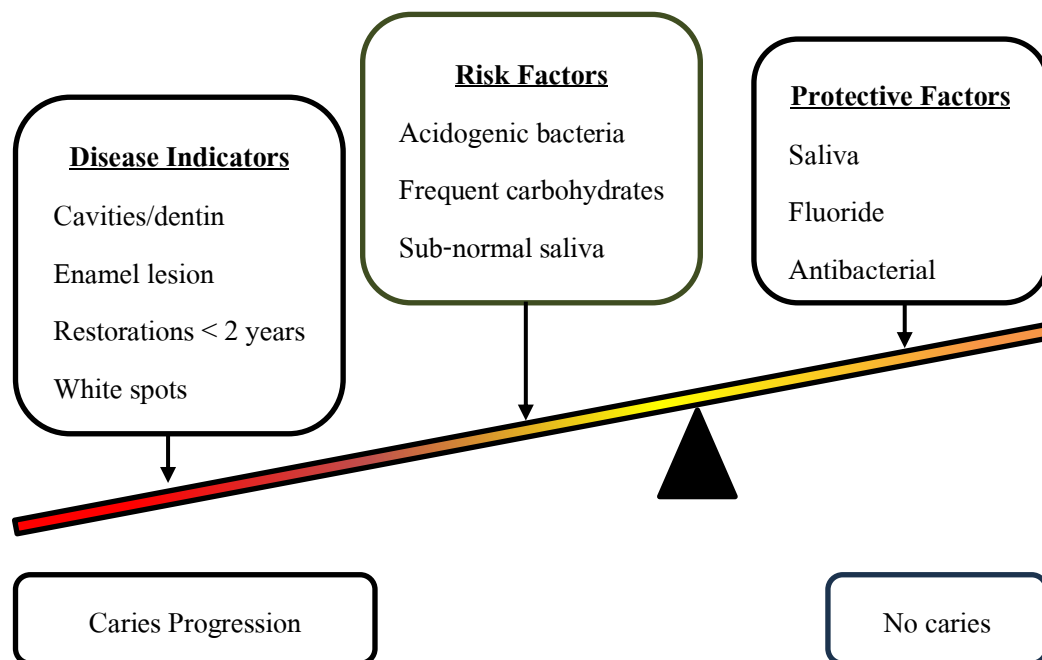
A study by Seow et al. (2003) examined the effect of dental education and tooth brushing instruction on MS levels and found that children who initially tested negative for MS had a higher frequency of tooth brushing and ate fewer snacks than children who tested positive for the virus⁴⁵. Most previous studies have not detected MS in infants whose primary teeth have not yet erupted because MS requires a rough surface to settle. Thus, MS can begin to be detected in infants whose primary teeth have erupted

in the oral cavity or in infants with cleft lip and palate who require an acrylic palate prosthesis⁴⁷.

Another important group of organisms is the *Candida* fungi, which are the most isolated from the oral cavity. It is an opportunistic infection which often infects the human body while the immune system is impaired. Numerous studies have shown that these fungi are involved in the pathogenesis of oral diseases. In addition, human immunodeficiency virus (HIV) infection and AIDS have been reported to increase the incidence of oral *Candida* infections⁴⁸. Other risk factors include medical conditions that reduce salivary production, such as diabetes and diabetes insipidus, and certain medications that reduce salivary production, such as antihistamines and antidepressants. Smoking is also a risk factor for tooth decay.

1.2 Severity level of dental caries

1. When white plaque occurs, bacteria react with starch or sugar. It produces acids that can destroy tooth enamel. This is the beginning of the loss of minerals (calcium), which will appear as cloudy white stains on the teeth. This stage is still easily treated.
2. Enamel decay: Calcium loss continues until the enamel surface is broken down. This stage should be treated by a dentist.
3. Dentin decay: The decay from the enamel spreads deep into the dentin, which can spread to other teeth.
4. Dental pulp decay: If tooth decay is not treated, the decay will spread deep into the dental pulp, which is the collection of many nerves. If the pulp becomes infected, an abscess may form at the root tip⁴⁹.

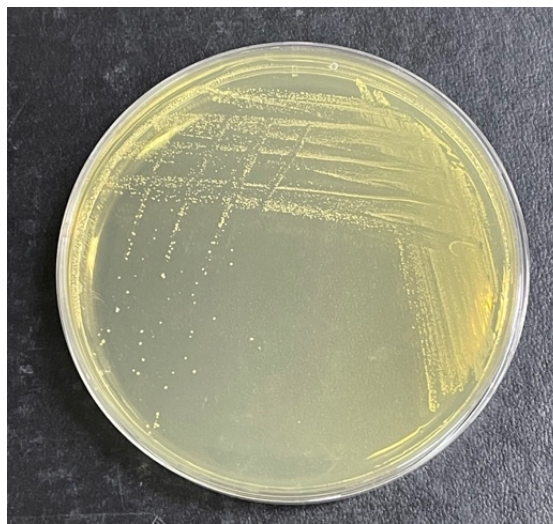


Figures 1 Factors affecting the development of dental caries.

2. Significant microorganisms causing oral diseases

2.1 *S. mutans*

S. mutans is a Gram-positive, spherical cocci that can grow both in aerobic and anaerobic conditions (facultative anaerobic bacterium), acid-tolerant and acid-producing. It is a normal flora in the human oral cavity, but when it grows in large numbers and undergoes metabolism, it forms a plaque on various parts of the oral cavity and is an important pathogen causing dental caries⁵⁰. The morphology of *S. mutans* on Mitis Salivarius Agar (MSB agar) shows that the colonies are rough, piled (Figure 2), and in many cases, bead-like or liquid-like colonies, which are caused by the organism producing insoluble extracellular polysaccharide or glucan around the colony surface, but sometimes they may be smooth or slimy⁵¹.



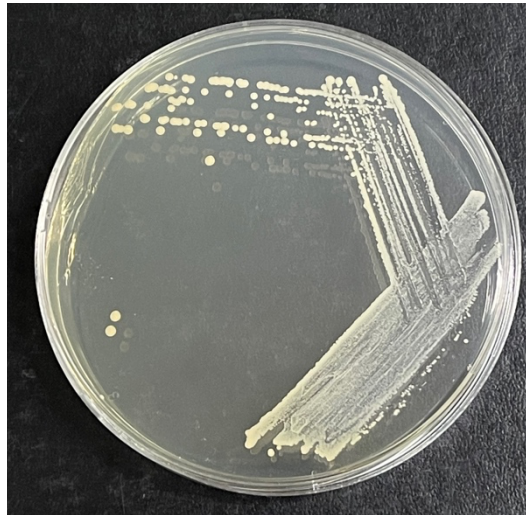
Figures 2 Colony morphology of *S. mutans* on BHI agar

2.2 *C. albicans*

Candida is normally a normal flora found in the human body without causing disease. However, when the body's immune system is abnormal, *Candida* can cause disease. *Candida* can be divided into 2 groups: *C. albicans* and non-*albicans*, including *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*. However, at present, *C. albicans* is found to be the cause of more than 50% of diseases. Common diseases caused by *Candida* include *Candida* skin diseases in the toenails, groin, oral thrush (thrush), which is common in diabetic patients and AIDS. *Candida* can cause diseases that invade deep tissues, such as esophagitis and endocarditis⁵².

C. albicans is a fungus that has two forms: yeast and long-chain fungus. It has an oval shape and has the ability to create pseudo hyphae, chlamydospore and germ tube. It can grow at temperatures of 25°C and 37°C and can cause disease in both humans and animals. This yeast is usually found on mucous membranes and skin, especially the gastrointestinal tract and genital tract. Most

infections are caused by immunodeficiency, antibiotic use, and malnutrition, etc. During growth, it can penetrate tissues and transform into a hypha without a septum⁵³.



Figures 3 Colony morphology of *C. albicans* on SDA medium.

3. Pathogenic mechanisms of oral bacteria

3.1 Oral pathogenic mechanism of *S. mutans*

3.1.1 Adhesion: ability to adhere

The main factor that causes *S. mutans* to cause dental caries is its ability to adhere to the tooth surface. which has 3 main factors as follows

3.1.1.1 Sucrose-independent adhesion

Adhesion is mediated by the antigen I/II protein. This protein is found in streptococci and has the ability to bind to various proteins in saliva, leading to plaque formation⁵⁴. It has been reported that a mutant strain of *S. mutans* P1, which is unable to produce the protein, has reduced hydroxyapatite that coats the saliva. This study

suggests that if the organism is deficient in protein, it will have reduced adherence to the salivary proteins that coat the tooth surface. In addition, the deficiency of the P1 protein has been shown to impair plaque formation in vitro, which may result in altered pathogenicity⁵⁵.

3.1.1.2 Sucrose-dependent adhesion

S. mutans is a bacterium that can produce up to three types of Glucosyltransferases (GTFs) enzymes: GtfB, GtfC, and GtfD, using sucrose as a precursor to degrade glucose to connect to form a glucan polymer. GtfB synthesizes insoluble proteins as glucans with an $\alpha(1-3)$ structure. GtfC synthesizes both insoluble and soluble proteins, and GtfD synthesizes mainly water-soluble proteins (dextran). These glucans are important components of bacterial biofilm formation. Biofilm promotes the accumulation of bacterial cells until they spread into a wide-area microbial plaque that can protect against external aggressors, i.e., can make bacteria more resistant to stimuli⁵⁶.

3.1.1.3 Glucans binding of proteins (Gbps)

Gbps are the proteins that mediate the binding of bacteria to glucan. There are four types of these proteins: GbpA, GbpB, GbpC, and GbpD. These four proteins are located on the bacterial cell wall and act as specific receptors for glucan and play important roles in bacterial adhesion and biofilm formation⁵⁷⁻⁵⁹.

3.1.2 Acidogenicity

Acid production by *S. mutans* is dependent on sugars through glycolytic and fermentation processes, yielding various acids. In the presence of abundant glucose, lactic acid is the most abundant⁶⁰.

Studies have shown that *S. mutans* with reduced lactate dehydrogenase (LDH) levels have a reduced caries potential, and if LDH is completely absent, *S. mutans* will eventually die, because the process of converting pyruvate to lactic acid and obtaining

energy requires LDH. The absence of this enzyme will affect acid production and the survival of *S. mutans*⁶¹.

3.2 Oral pathogenic mechanism of *C. albicans*

C. albicans possesses several virulence factors, such as the ability to change cell shape (morphological switching) in response to environmental conditions, the ability to adhere to epithelial cells and other surfaces, and the ability to produce a variety of enzymes that are potent in degrading proteins and substances in human connective tissue, potentially facilitating tissue invasion.

3.2.1 The ability to change the shape of cells

C. albicans can grow in both yeast, pseudo hyphae, and true hyphae. Previous studies reported on genetic modification of *C. albicans* to prevent cell morphology and maintain a single hypha, indicating that whether the organism is in yeast or fungal form, its pathogenicity is greatly reduced. Therefore, the ability to change morphology is more important for pathogenicity than having a single hypha. In addition, it was found that *C. albicans* can undergo multiple high-frequency phenotypic switching. This morphology allows the organism to adapt to its environment better and results in changes in cell surface antigens, allowing the organism to evade immune recognition.

3.2.2 The ability to adhere to the surface

Adhesion to surfaces, both epithelial and non-epithelial, is an important factor in pathogenesis. Several proteins on the cell surface of *C. albicans* play a role in adhesion, most of which are glycoproteins, such as fibrinogen binding manno protein and agglutinin-like sequences (ALS). These proteins can bind to extracellular matrix substances and human cell surface substances, facilitating adhesion. There is also a protein on the cell surface of the fungus that is a precursor to the enzyme transglutaminase (transglutaminase) of humans causes cross-linking (crosslinking) between fungal cells and human epithelial cells. Previous studies have shown that some

adhesion proteins are essential for pathogenesis, and if they are not produced, the pathogenicity is reduced.

Biofilms allow the organism to adhere better to the surface and are more difficult to eliminate. In addition, the extracellular matrix that encases the organism and the growth conditions in the biofilm also increase the organism's resistance to antifungal drugs. For the dentist, bases are a major problem in denture stomatitis because denture bases are often made of plastic and have a rough surface.

3.2.3 The ability to produce enzymes

C. albicans is capable of producing and secreting a variety of enzymes that can hydrolyze human tissue metabolites and denature immune proteins that have antimicrobial activity, particularly enzymes belonging to the hydrolase family, such as secreted aspartic proteinases (SAPs), phospholipases, and lipases. Many of these enzymes play important roles in disease progression⁶².

4. Herbal toothpaste

Herbal toothpaste refers to products made from herbs such as Phaya Yo, guava leaf, neem, and mangosteen peel, either one or a mixture., mixed with tooth polishing agents, such as salt, sodium bicarbonate (baking soda), sea pangolin, flavorings such as essential oils, borneol, camphor, menthol. Other ingredients may be added such as surfactants, thickeners, and moisturizing agents such as glycerin. The S Industry Standard covers only herbal toothpastes that contain herbs as ingredients, in the form of liquids, thick liquids, semi-solids, and powders, used for cleaning teeth by using with a toothbrush, but does not cover toothpastes that contain fluoride and synthetic colors³⁴.

4.1 The role of herbal toothpaste in maintaining oral hygiene

Herbal oral care is a primary practice in rural areas of developing countries⁶³. Natural products derived from herbal plants provide the basis for many biocomponents that could potentially lead to the development of new chemical compounds in the

future. The antibacterial, antiviral, and anti-inflammatory activities of herbal products have long been introduced into the dental field. Some studies have investigated the activity of plant extracts and plant products specifically against oral pathogens, while other studies have focused specifically on inhibiting the formation of biofilm by pathogens, and it has been found that herbal extracts can reduce the adhesion of microorganisms that are the main drivers of plaque formation on the teeth⁶⁴, and, in addition, the ability of plant extracts, including essential oils and phytochemicals, to prevent bacterial adhesion has been studied²⁰. The use of essential oils in herbal toothpastes has been shown to have antibacterial activity against a wide range of bacteria, including *S. mutans*⁶⁵.

In 2004, Sean S. Lee D.D.S. et al. studied the inhibition ability of 14 brands of herbal toothpastes against *S. mutans*, *S. Sanguis*, *Actinomyce Viscosus*, and *C. albicans* using the standard disk diffusion method. Colgate Total toothpaste was used as the positive control and sterile distilled water was used as the negative control. By testing toothpaste at normal concentration and toothpaste diluted with distilled water (1:1), measuring the diameter of the germ-free area at 24 and 48 hours after inoculation, it was found that 6 brands were able to inhibit microorganisms. All 4 types were obtained: there were 6 brands that had more areas that were not infected with *Actinomyces viscosus* than the positive control, and there were 6 brands that were able to inhibit *C. albicans*, but when using toothpaste diluted (1:1) to test, there was only one brand that was able to inhibit all 4 types of microorganisms. Only one brand was able to inhibit *A. viscosus* more than the positive control, and only three brands were able to inhibit *C. albicans*.

In 2006, a study by Ozaki F et al.⁶⁶ investigated the efficacy of herbal toothpaste in 48 patients with gingivitis. The patients were asked to use the toothpaste three times a day for 28 days and used a toothpaste containing triclosan and fluoride as a positive control. The toothpaste in both the test and control groups was found to be effective in reducing plaque and gingivitis, and no other adverse effects were found.

In 2010, a study by Jarunyong et al.⁶⁷ investigated the effectiveness of three herbal toothpastes and one toothpaste containing triclosan on controlling plaque and

gingivitis in 82 volunteers. All volunteers underwent scaling and root planning and started brushing their teeth using the specified toothpaste. One week after scaling, the gingival condition index, bleeding index, and plaque index on representative teeth/index before scaling, one week after scaling, and one and two months after using the toothpaste. The results showed that there were no significant differences in the gingival condition index, bleeding index, and plaque index between the different toothpastes at any time points. In 2010, a study by Jarunyong et al.⁶⁷ investigated the effectiveness of three herbal toothpastes and one toothpaste containing triclosan on controlling plaque and gingivitis in 82 volunteers. All volunteers underwent scaling and root planning and started brushing their teeth using the prescribed toothpaste. One week after scaling, the gingival condition index, bleeding index, and plaque index on representative teeth/index before scaling, one week after scaling, and one and two months after using the toothpaste. The results showed that there were no significant differences in the gingival condition index, bleeding index, and plaque index between the different toothpastes at any time points. From this study, it can be concluded that herbal toothpaste is just as effective at controlling plaque and gingivitis as toothpaste with triclosan.

In 2014, a study by Izabela⁶⁸ was conducted on the imbalance of oral microflora that can lead to dental caries and periodontal disease. It was found that bacteria use sucrose products to produce acids that can lower the pH of the mouth to less than 5.5 and cause the formation of biofilms or plaques on the tooth surfaces, which eventually damages the tooth structure and enamel.

In 2015, Sabiha S et al.⁶⁹ tested the inhibitory activity of 10 Indian toothpaste brands against *S. mutans*, *S. sanguinis*, *Actinomyces viscosus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *C. albicans* using the standard disk diffusion method. The test was conducted on two types of toothpaste: toothpaste diluted with distilled water at a ratio of 1:1 and toothpaste at normal concentration. The results showed that each toothpaste had different inhibitory activity, with Danth Kanthi toothpaste being the most effective in inhibiting all types of bacteria.

In 2016, a study by Mangilal T⁷⁰ evaluated the efficacy of a new herbal toothpaste compared to commercial toothpastes. The antibacterial activity of the toothpaste containing guava extract was tested using the well-diffusion method using Mueller Hinton culture media. Five-millimeter wells were drilled to place the tested toothpaste in the wells. The culture media were left to dry for 3-5 minutes and then grown at 37°C for 24 hours. After that, the clear zone was measured. The tested toothpaste was found to be as effective as commercial toothpaste.

In 2017, from the study of Gautam B et al.⁷¹ to examine the antimicrobial activity of toothpaste by determining the minimum inhibitory concentration, minimum bactericidal concentration and minimum fungicidal concentration in the study of 8 toothpastes, 2 were herbal toothpastes, 3 were general toothpastes and 3 were toothpastes mixed with herbs and chemicals against 6 types of microorganisms, namely *Lactobacillus* spp. *C. albicans* *S. aureus* *S. epidermidis* *P. aeruginosa* and *Escherichia coli*, it was found that after incubating in the incubator at 37°C for 24 hours for bacteria and culturing at 27°C for 3 days for fungi, the three herbal toothpastes studied showed more inhibition of the growth of bacteria and fungi than the negative control.

CHAPTER 3

RESEARCH METHODOLOGY

Microorganisms

The microorganisms used in this study included *S. mutans* and *C. albicans*.

Culture Preparation

First, standard cultures of *S. mutans* ATCC 25175 and *C. albicans* ATCC 90028 were obtained from the Faculty of Dentistry laboratory, Prince of Songkla University, in 50 ml culture flasks with 30 ml of culture media, using Brain heart infusion broth (BHI) for *S. mutans* and Sabouraud's dextrose broth for *C. albicans*, and incubated in an incubator with 5% carbon dioxide at 37°C for 18 hours. Then, the cultures were adjusted for turbidity by using BHI medium to adjust the turbidity of *S. mutans* and SDA medium to adjust the turbidity of *C. albicans* to make both types of cultures turbidity equal to 0.5 McFarland turbidity standard⁷² by using a turbidity detector, by adjusting the wavelength to 600 nm, which will result in the amount of bacteria used for testing approximately 1.5×10^8 colonies per milliliter (CFU/ml)⁷³.

Toothpaste Preparation

Table 1 the 27 toothpaste formulas studied were:

No.	Types of toothpaste
A001	Twin Lotus Herbal Toothpaste Original Formula
A002	Twin Lotus Herbal Toothpaste Everfresh Formula
A003	Goodgarnic Herbal Toothpaste
A004	Threeherb herbal toothpaste
A005	Sparkle Triple White Toothpaste
A006	Sparkle Double White Toothpaste Lemon Soda
A007	Sparkle White Toothpaste
A008	Sparkle Naturals Himalaya Pink Salt Toothpaste
A009	Sparkle Natural Complete Care Toothpaste
A010	Twin Lotus Herbalist Active Charcoal Toothpaste
A011	Doctor V Whitening System Toothpaste - Diavee 01
A012	I-Vish Gentle Powerful Whitening and Anti-Cavity Toothpaste
A013	Toothpaste Wisut Pure Extra Gum Protection & Gentle Powerful Whitening Toothpaste
A014	MYSEPTIC MYBACIN Toothpaste with Andrographis paniculata formula
A015	Denticon Q10 total care toothpaste
A016	Denticon Q10 rose refresh toothpaste
A017	Blooms Nature Love Natural Fresh & Gum Care Toothpaste
A018	Herbal Den Plus Neem Toothpaste
A019	Veldent Extreme Awake Tooth Paste
A020	Thep Thai Herbal Toothpaste, Concentrated Formula, Spearmint Flavor
A021	Thep Thai herbal toothpaste, concentrated formula, mixed fruit flavor
A022	Thep Thai Herbal Toothpaste, concentrated formula, original flavor
A023	Thep Thai Herbal Toothpaste, concentrated formula, salt flavor

A024	Dentiste toothpaste original formula
A025	Dentamate Herbals Toothpaste
A026	Colgate Total Advanced Fresh Toothpaste
A027	Colgate Total 12 Professional Clean Toothpaste

Table 2 Toothpaste Ingredients Used in the Test.

Sort by the Order Used in the Test Type A001-A027.

Types of Toothpastes	Ingredients
A001 Twin Lotus Herbal Toothpaste Original Formula	Sorbitol (Natural Origin from Cassava), Calcium Carbonate (Mineral Abrasive), Cuttlefish Bone, Toothbrush Tree, Clinacanthus nutans , Sodium Lauryl Sulfate (Natural Origin from Coconut Oil), Hydrated Silica (Mineral Thickener), Orange Jessamine, Peppermint Oil, Menthol, Eucalyptus Oil , Sodium Benzoate
A002 Twin Lotus Herbal Toothpaste Everfresh Formula	Calcium Carbonate (Mineral Abrasive), Sorbitol (Natural Origin from Cassava), Para Cress, Water, Sodium Lauryl Sulfate (Natural Origin from Coconut Oil), Hydrated Silica (Mineral Thickener), Glycerin (Natural Origin from Vegetable Oil, Toothbrush Tree , Clinacanthus nutans, Orange Jessamine, Menthol, Wintergreen Oil, Eucalyptus Oil , Tetrasodium Pyrophosphate (Mineral Antitartar Ageni), Xanthan Gum (Natural Thickener), Sodium Benzoate.

A003 Goodgarnic Herbal Toothpaste	Dicalcium phosphate, Sorbital, Glycerrin, Saccharin, Sodium chloride. Mentha spicata herb oil, Cinnamomum cassia leaf oil, Sodium benzoate, Water, <i>Eugenia caryophyllus</i> (clove) leaf oil, Glycerin glabra extract, Sodium lauryl sulfate, Carboxymethyl cellulose Borneol, Merthol
A004 Threeherb herbal toothpaste	Siamese rough bush, Guava leaf extract, Clove oil, Borneol, Camphor
A005 Sparkle Triple White Toothpaste	Aqua, Sorbitol, Hydrated Silica, Sodium Lauroyl Sarcosinate, Sodium Benzoate, hydroxyapatite (nano), Cellulose Gum. Magnesium Aluminum Silicate, Menthol, Menth Piperita (Peppermint) Oil, Glycerin, Flavor, Sodium Saccharin, Xylitol, Eugenia Caryophyllus (Clove) Leaf Oil, Fucus Vesiculosus Extract, Pentasodium Triphosphate, Dicalcium Phosphate.
A006 Sparkle Double White Toothpaste Lemon Soda	Water (Aqua), Sorbitol, Silica, Glycerin, Sodium Coco- Sulfate. Calcium Lactate, Flavour, Cellulose Gum, Menthol, Sodium Benzoate, Xylitol, Xanthan Gum, Sodium Saccharin, Sodium Methyl Cocoyl Taurate, Sodium Lauroyl Sarcosinate, Carrageenan. Citrus Limon Peel Oil, Methyl Disopropyl Propionamide, <i>Eugenia</i> <i>Caryophyllus</i> (Clove) Bud Oil, <i>Salvia Officinalis</i> (Sage) Leaf Extract, <i>Commiphora Myrrha</i> Resin Extract. <i>Krameria Triandra</i> Root Extract, Propolis Extract. Cyclodextrin Ascorbic Acid (nano), Caprylic / Capric

	Triglyceride, Polysorbate 20, Polysorbate 80, Ubiquinone
A007 Sparkle White Toothpaste	Aqua(Water), Hydrated Silica. Sorbitol. Glycerin. Sodium Coco-Sulfate. Cellulose Gum, Aroma (Flavor), Potassium Nitrate, Xanthan Gum. Sodium Benzoate, Xylitol, Menthol, Lauryl Glucoside, Sodium Saccharin, <i>Eugenia Caryophyllus</i> (Clove) Bud Oil, <i>Mentha Piperita</i> (Peppermint) Oil, Ascorbic Acid, Sodium Ascorbyl Phosphate, C12-15 Alkyl Benzoate, Tocopheryl Acetate, Ubiquinone
A008 Sparkle Naturals Himalaya Pink Salt Toothpaste	Aqua (Water), Sorbitol, Glycerin, Hydrated Silica, Sodium Chloride, Sodium Coco- Sulfate, PVP, Cellulose Gum, Titanium Dioxide , Aroma (Flavor), Xanthan Gum, Sodium Benzoate, Menthol, <i>Mentha Arvensis</i> Leaf Oil, Cocamidopropyl Betaine, Xylitol, Sodium Saccharin, Eucalyptol, Linseed Acid, <i>Mentha Viridis</i> (Spearmint) Leaf Oil, <i>Eugenia Caryophyllus</i> (Clove) Bud Oil, Propanediol, Propolis Extract, Citric Acid
A009 Sparkle Natural Complete Care Toothpaste	Water (Aqua). Sorbitol, Glycerin, Hydrated Silica, Sodium Coco-Sulfate, Flavour, Eucalyptol, <i>Mentha Piperita</i> Oil, Cellulose Gum, Sodium Methyl Cocoyl Taurate, Sodium Lauroyl Sarcosinate, Xanthan Gum, Xylitol, Sodium Benzoate, Sodium Saccharin, Carrageenan, <i>Eugenia Caryophyllus</i> Bud Oil, <i>Calendula Officinalis</i> Flower Extract, <i>Leontopodium Alpinum</i> Flower / Leaf Extract, Potassium Sorbate, Cyclodextrin,

	Potassium Nitrate, Menthol, <i>Quercus Salicina</i> Leaf Extract
A010 Twin Lotus Herbalist Active Charcoal Toothpaste	Sorbitol, Hydrated Silica, Water, Glycerin, Cocamidopropyl Betaine, <i>Psidium Guajava</i> Leaf Extract, <i>Eugenia Caryophyllus</i> Flower Extract, Flavor, Sodium Lauroyl Sarcosinate, Charcoal Powder, <i>Eugenia Caryophyllus</i> Flower Oil, Menthol, Cellulose Gum, Xanthan Gum, Sodium Saccharin, Disodium EDTA, Titanium Dioxide.
A011 Doctor V Whitening System Toothpaste - Diavee 01	Dicalcium phosphate dihydrate, aqua, sorbitol, glycerin, sodium lauryl sulfate, sodium chloride, menthol, cellulose gum, <i>Mentha arvensis</i> leaf oil, sodium benzoate, <i>Panax ginseng</i> root extract, sodium saccharin, dipropylene glycol, <i>Mentha piperita</i> (peppermint) oil, xanthan gum, Borneol, camphor, <i>Glycyrrhiza glabra</i> extract, <i>Psidium guajava</i> leaf extract, <i>Eugenia caryophyllus</i> flower oil, <i>Ganoderma lucidum</i> extract, <i>Mentha viridis</i> leaf oil, <i>Aloe barbadensis</i> leaf powder, o-cymen-5-ol, polysorbate 20, aluminum sulfate, <i>Garcinia mangostana</i> peel extract, propolis extract, <i>Leontopodium alpinum</i> flower extract.

A012 I-Vish Gentle Powerful Whitening and Anti-Cavity Toothpaste	Water, Calcium Carbonate, Sorbitol, Hydrated Silica, Sodium Cocoyl Glutamate, Glycerin, Menthol, Sodium Bicarbonate, Cellulose Gum, Rebaudioside A (Stevia Extract), Xanthan Gum, Mentha Piperita (Peppermint) Oil, Propanediol, Ethyl Mentane Carboxamide, Borneol, Sasa Kurilensis Water, Punica Granatum Fruit Extract, Cetylpyridinium Chloride, Mentha Viridis (Spearment) Leaf Oil, O-cymen-5-ol, Mentha Arvensis (Mint) Leaf Oil, Eucalyptus Globulus (Eucalyptus) Leaf Oil, Aloe Barbadensis Leaf Juice Powder, Eugenia Caryophyllus (Clove) Bud Oil
A013 Toothpaste Wisut Pure Extra Gum Protection & Gentle Powerful Whitening Toothpaste	Water, Calcium Carbonate, Sorbitol, Hydrated Silica, Sodium Cocoyl Glutamate, Glycerin, Menthol, Borneol, Propanediol, Rebaudioside A (Stevia Extract), Sodium Bicarbonate, Cellulose Gum, Xanthan Gum, Mentha Viridis (Spearment) Leaf Oil, Mentha Piperita (Peppermint) Oil, Ethyl Mentane Carboxamide, Sasa Kurilensis Water, Punica Granatum Fruit Extract, Cetylpyridinium Chloride, Mentha Arvensis (Mint) Leaf Oil, <i>Eucalyptus Globulus</i> (Eucalyptus) Leaf Oil, O-cymen-5-ol, Ubiquinone, Aloe Barbadensis Leaf Juice Powder, Eugenia Caryophyllus (Clove) Bud Oil, Polysorbate 20, Caprylic/Capric Triglyceride, Ascorbic Acid
A014 MYSEPTIC MYBACIN Toothpaste with Andrographis paniculata formula	Water, Hydrated silica, Sorbitol, Glycerine, Polyethylene Glycol 400, Sodium lauroyl sarcosinate, Sodium lauryl sulfate, Menthol, Xylitol, <i>Mentha arvensis</i> leaf oil, Peppermint oil, Spearment oil, Aloe vera gel, <i>Psidium</i> <i>guajava</i> leaf extract, Flavour, Sodium saccharin, Cellulose gum, Titanium dioxide, Sodium benzoate, Xanthan gum, Methylparaben, Chlorophyllin-copper complex, Citric acid, anhydrous, Menthyl succinate, <i>Andrographis paniculata</i>

	extract, Cetylpyridinium chloride, Green tea extract, Acid blue
A015 Denticon Q10 total care toothpaste	Sorbital, Calcium Carbonate, Water, Glycerin, Silica, Potassium Nitrate, Sodium Lauryl Sulfate, Dicalcium Phosphate, Tricalcium Phosphate, Mentha Piperita (Peppermint) Oil, Cellulose Gum, Ubiquinone, PEG-32, Menthol, Squalane, Xylitol, Sodium Saccharin, Sodium Benzoate, Alcloxa, Hydroxyapatite, Tocopheryl Acetate, Propolis Extract, Ascorbic Acid, <i>Camellia Sinensis</i> Leaf Extract, Potassium Citrate
A016 Denticon Q10 rose refresh toothpaste	Sorbitol, Calcium Carbonate, Water, Silica, Glycerin, PEG-32, Peppermint Oil, Xylitol, Menthol, Cellulose Gum, Xanthan Gum, Sodium Cocoyl Glutamate, Sodium Benzoate, Sodium Saccharin, Tocopheryl Acetate, <i>Rosa Damascena</i> Flower Extract, Rosa Hybrid Flower Water, Camellia Sinensis Leaf Extract, Ubiquinone, <i>Sapindus Trifoliatus</i> Fruit Extract

A017 Blooms Nature Love Natural Fresh & Gum Care Tooth Paste	Aqua, Sorbitol, Hydrated Silica, Glycerin, Sodium Coco-Sulfate, Cellulose Gum, Aroma, Titanium Dioxide, Menthol, Mentha Piperita Oil, Stevia Rebaudiana Leaf Extract, Xylitol, Menthyl Lactate, Zinc Lactate, O - Cymen-5-ol, Eugenia Caryophyllus Bud Oil, Propanediol, Centella Asiatica Extract, Propolis Extract, Polygonum Cuspidatum Root Extract, Scutellaria Baicalensis Root Extract, Camellia Sinensis Leaf Extract, Glycyrrhiza Glabra Root Extract, Vaccinium Macrocarpon Fruit Extract, Chamomilla Recutita Flower Extract, Rosmarinus Officinalis Leaf Extract, Salvia Officinalis Extract, Sodium Benzoate Potassium Sorbate
A018 Herbal Den Plus Neem Toothpaste	Azadirachta Indica (Neem) Extract, Guava Leaf Extract, Clove Oil, <i>Aloe Vera</i> Leaf Powder, Peppermint Oil, Menthol, Borneol and Camphor
A019 Veldent Extreme Awake Tooth Paste	Aqua, Sorbitol, silica, Sodium Lauryl Sulfate, Flavour, Cellulose Gum, Titanium Dioxide, Menthol. Sodium Benzoate, Potassium Sorbate, Menthodextrin, Tocopheryl Acetate, Cyclodextrin, Calcium Lactate, Zinc Lactate , Propylene Glycol, Malpighia Glabra Fruit Extract, Ubiquinone, Pyrus Malus Fruit Extract, Arvensis Extract, Ascorbic Acid, Sodium Citrate, Citric Acid, Phenoxyethanol, Sodium Hydroxide, Ethylparaben Methylparaben. Cyanocobalamin, Cetyl Palmitate, C12-15 Alkyl Benzoate, Glycerin, Lauryl Glucoside, Xanthan Gum
A020 Thep Thai Herbal Toothpaste	Calcium Carbonate, Water, Sorbitol, Sodium Lauryl Sulfate, Sodium Carboxymethyl Cellulose, Borneol, Menthol, Camphor, Streblus Asper Branch Powder,

Concentrated Formula Spearmint Flavor	Acmella Oleracea Extract, Guava Extract, Clove Oil, Aloe Vera Callus Extract, Spearmint Oil
A021 Thep Thai herbal toothpaste, concentrated formula, mixed fruit flavor	Calcium Carbonate, Water, Sorbitol, Sodium Lauryl Sulfate, Sodium Carboxymethyl Cellulose, Borneol, Menthol, Camphor, Streblus Asper Leaf Extract, Caesalpinia Sappan Extract, Aloe Vera Callus Extract, Cinnamon Extract, Clove Oil, Flavor
A022 Thep Thai Herbal Toothpaste, concentrated formula, original flavor	Water, Calcium Carbonate, Sodium Lauryl Sulfate, Cellulose Gum, Sorbitol, Camphor, Borneol, Menthol, Peppermint Oil, Licorice Extract, Eclipta Prostrata Leaf Extract, <i>Psidium Guajava</i> Leaf Extract, Streblus Asper Leaf Extract
A023 Thep Thai Herbal Toothpaste, concentrated formula, salt flavor	Water, Calcium Carbonate, Sodium Chloride, Sodium Lauryl Sulfate, Sorbitol, Sodium Carboxymethyl Cellulose, Borneol, Camphor, Menthol, Clove Bud Oil, Peppermint Oil, Phenoxyethanol, Eclipta Prostrata Extract, Streblus Asper Leaf Extract, Guava Extract
A024 Dentiste toothpaste original formula	Sorbitol, Silicon Dioxide, Deionized Water, Xylitol, Glycerin, Green Tea Extract, Sodium Lauryl Sulfate, PVP, Hydroxyapatite, Sodium Tripolyphosphate, Cellulose Gum, Titanium Dioxide, Sodium Benzoate, Sodium Saccharin. Menthol, Ascorbic Acid, Mica, <i>Mentha Piperita</i> Oil, <i>Eucalyptus Globulus</i> Leaf Oil, <i>Eugenia Caryophyllus</i> Flower Oil, <i>Salvia Officinalis</i> Extract, <i>Anithemis Nobilis</i> Extract, <i>Foeniculum Vulgare</i> Extract, <i>Glycyrrhiza Glabra</i> Extract, <i>Cinnamomum Cassia</i> Bark Extract

A025 Dentamate Herbals Toothpaste	Aqua, Sorbitol, Hydrated silica, Glycerin, Calcium carbonate, Propylene glycol, Cocamidopropyl betaine, Camphor, Sodium bicarbonate, Menthol, Borneol, Sodium lauryl sarcosinate, Sodium chloride, Magnesium aluminum silicate, Cellulose gum, <i>Mentha piperita</i> oil, <i>Potassium sorbate</i> , <i>Eugenia caryophyllus</i> bud oil, Silica, Titanium dioxide, Zinc citrate, Aloe barbadensis leaf juice, Maltodextrin, Sodium benzoate
A026 Colgate Total Advanced Fresh Toothpaste	Glycerin, Water, Hydrated Silica, Sodium Lauryl Sulfate, Flavor, Arginine, Zinc Oxide, Cellulose Gum, Poloxamer 407, Tetrasodium Pyrophosphate, Zinc Citrate, Benzyl Alcohol, Xanthan Gum, Cocamidopropyl Betaine, Sodium Fluoride, Sodium Saccharin, Phosphoric Acid, Sucralose
A027 Colgate Total 12 Professional Clean Toothpaste	Glycerin, Water, Hydrated Silica, Sodium Lauryl Sulfate, Flavor, Arginine, Zinc Oxide, Cellulose Gum, Poloxamer 407, Zinc Citrate, Tetrasodium Pyrophosphate, Xanthan Gum, Benzyl Alcohol, Cocamidopropyl Betaine, Sodium Fluoride, Sodium Saccharin, Mica, Sucralose

Toothpaste Preparation

Toothpastes were prepared diluted with 2% Dimethyl sulfoxide (DMSO) by mixing 1 g of toothpaste and 1 ml of DMSO in a ratio of 1:1. The mixture was then diluted by two-fold serial dilution to obtain four concentrations: 1:1, 1:2, 1:4, and 1:8, respectively, to maintain the complete composition of the toothpaste. The mixture was mixed until homogeneous using a Vortex Mixer for 5 min⁷¹.

Antimicrobial sensitivity testing of each toothpaste type

The test used Colgate Total Advanced Fresh Toothpaste and Colgate Total 12 Professional Clean Toothpaste with antimicrobial properties as controls and distilled water as negative control.

Firstly, prepare BHI agar culture media. Then, adjust the pH of this medium to be equal to 7.3 and mix with each toothpaste formula at a concentration of 1:1 to obtain a volume of 25 ml in a 10 cm Petri dish to obtain a thickness of approximately 4 mm. Next, prepare the culture by adjusting the turbidity to 0.5 McFarland turbidity standard and drop *S. mutans* and *C. albicans* into the agar culture media, 3 positions for each toothpaste formula. There are Petri dishes for adding positive control and negative control, doing the same as in each test set⁷². Then, culture in an incubator with 5% carbon dioxide gas at 37°C for 24 hours. The experiment was repeated 3 times⁷⁵⁻⁷⁶. The diameter of the sterile area was measured and recorded using a ruler with a precision of mm. The measurements and records were made by a single researcher throughout the study. The precision of the measurements was tested by repeating the measurements 3 times.

Minimum Inhibitory Concentration (MIC)

The first step is to transfer 100 µl of culture medium into the wells containing 100 µl of 1:1 toothpaste in a 96-well plate. Sterile distilled water is used as a negative control. Chlorhexidine is used as a positive control. The toothpaste is mixed with the culture medium as a control for each toothpaste because each toothpaste has a different color. Then, mix well and incubate in an incubator at 37°C for 24 hours⁷⁷. Add 30 µl of 0.015% Resazurin. The result is read after 2-4 hours of incubation by observing the change in the color of resazurin. The concentration indicated is the MIC value. The color of resazurin will not change from the original color (blue). However, if the color changes from the original color, it indicates that the organism has grown. The result will not be read as the MIC value repeated 3 times⁷⁸.

Minimum Bactericidal Concentration (MBC) Test

After obtaining the MIC value, spread the wells with the minimum and maximum concentrations that inhibit *S. mutans* onto BHI agar, and spread the wells with the minimum and maximum concentrations that inhibit *C. albicans* onto SDA. After that, incubate in an incubator at 37°C for 48 hours. Then, count the number of colonies growing on the medium and calculate in CFU/ml. The number of bacteria that decreases more than or equal to 3 log (99.9%) is indicated as the MBC value compared to the control⁷⁹.

Testing the effectiveness of toothpaste in killing bacteria

Saliva samples were collected from patients and mixed with 1 g of toothpaste in test tubes containing 900 µl of culture and incubated at 37°C with 5% CO₂. Bacterial growth was then assessed for 2 min by aspirating 100 µl of culture and quantifying the bacterial count by serial dilution. In the control group, phosphate buffered saline solution was used instead of toothpaste and the number of colonies formed in the plates was counted. The experiments were repeated three times⁸⁰. Bacterial growth was evaluated based on the percentage of colony coverage on the agar plates: plates with 100% colony coverage were interpreted as ‘+++’ (Heavy growth), approximately 75% coverage as ‘++’ (Moderate growth), 25–50% coverage as ‘+’ (Mild growth), and 0% colony formation as ‘-’ (No growth).

Anti-biofilm formation activity of toothpaste (Biofilm formation assay)

Firstly, 100 µl of culture was transferred to wells containing 1/4MIC, 1/2MIC, and 100 µl of MIC toothpaste in a 96-well plate. Then, sterile distilled water was used as a negative control and chlorhexidine was used as a positive control. Next, the cultures were mixed and incubated in an incubator at 37°C with 5% CO₂ for 24 hours. After that, all the cultures in the wells were aspirated, taking care not to touch the

biofilm. The cultures were washed twice with distilled water, 150 μ l per well, and 125 μ l of 0.1 crystal violet was added to each well. The cultures were left for 15 minutes. Finally, the cultures were aspirated again, washed twice with distilled water, 150 μ l per well, and 250 μ l of 90% ethanol was added. The cultures were measured for OD at 560 nm. The experiment was repeated three times.

Inhibition of acid production test of toothpaste

First, the cultures were centrifuged at 8000 rpm at 4°C for 30 min. Then, the cells were washed twice with potassium chloride and homogenized using a vortex mixer. Next, 40 mg/ml potassium chloride was added, and the cultures were incubated at 37°C and 5% CO₂ for 3 hours.

After that, prepared toothpaste with a concentration of 1:1 was adjusted pH with potassium chloride (concentration of 0.02 mg/ml). Next, 500 μ l of 10% glucose was added and incubated at 37°C, 5% carbon dioxide for 0, 30, 60, 90, 120 and 150 min. Lastly, the pH was measured at the specified incubation times, repeated 3 times⁸¹.

Statistical analysis

The data from all experimental results were analyzed and presented using inferential statistics, expressed as mean $\pm$ standard deviation (Mean $\pm$ SD). Differences between groups were evaluated using One-way ANOVA, followed by post-hoc comparisons with Tukey's test to determine statistically significant differences among the tested groups.

Table 3 showing the types of culture media used in the experiment

Bacteria strains

Organisms	broth	agar
<i>Streptococcus mutans</i> (ATCC 25175)	BHIb	BHIa

Brain heart infusion broth = BHIb, Brain heart infusion agar = BHIa

Fungi strains

Organisms	broth	agar
<i>Candida albicans</i> (ATCC 90028)	BHIb	BHIa

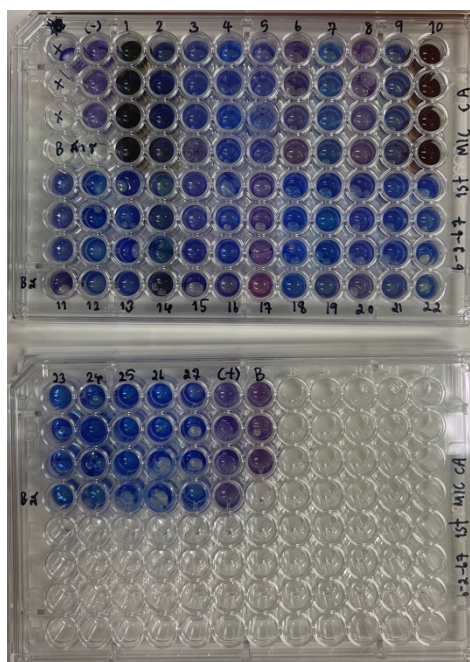
Sabouraud's dextrose broth = SDb, Sabouraud's dextrose broth-agar = SDa

CHAPTER 4

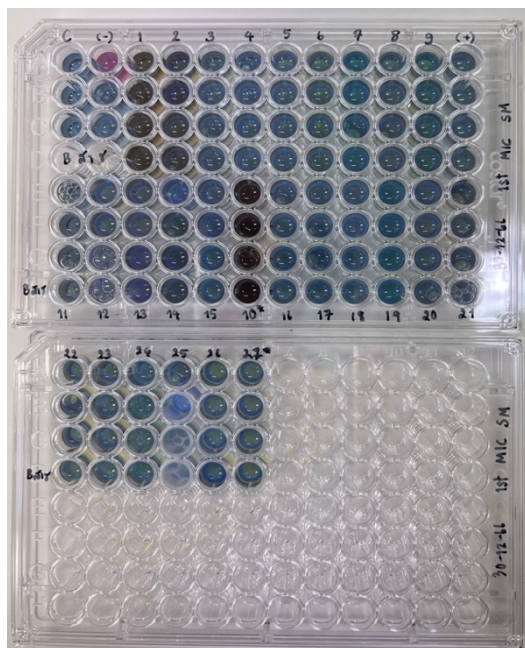
RESULT

Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Test

The experiment to determine the MIC value using a 96-well plate with 0.015% resazurin solution revealed that none of the toothpaste formulas exhibited a color change from the blank for their respective types, indicating that all formulas were able to inhibit *C. albicans*. Similarly, the experiment to determine the MBC value using the drop plate method showed no microbial growth in any of the herbal toothpaste formulas, demonstrating that all formulas were capable of killing *C. albicans*. Thus, it can be concluded that all 27 herbal toothpaste formulas tested at a concentration of 1 g/ml were effective in inhibiting and killing *C. albicans*.



Figures 4 MIC testing after the addition of resazurin, showing color changes that can be detected to inhibitory bacteria growth in a 96-well plate.



Figures 5 MIC testing after the addition of resazurin, showing color changes that can be detected to inhibitory fungi growth in a 96-well plate.

All formulas were able to inhibit *S. mutans*. Similarly, the experiment to determine the MBC value using the drop plate method showed no microbial growth in any of the herbal toothpaste formulas, indicating that all formulas were capable of killing *S. mutans*. Therefore, it can be concluded that all 27 herbal toothpaste formulas, tested at a concentration of 1 g/mL, were effective in both inhibiting and killing *S. mutans*.

Microbial sensitivity testing of each toothpaste type

Table 4 shows the inhibitory effectiveness of 27 types of toothpaste against *Streptococcus mutans* and *Candida albicans*.

Herbal toothpaste	Median CA	Median SM
A001 Twin Lotus Herbal Toothpaste Original Formula	15.5	35.5
A002 Twin Lotus Herbal Toothpaste Everfresh Formula	15.25	35.75
A003 Goodgarnic Herbal Toothpaste	11.5	32.0
A004 Threeherb herbal toothpaste	18	33.5
A005 Sparkle Triple White Toothpaste	26	0.00
A006 Sparkle Double White Toothpaste Lemon Soda	13.25	26.5
A007 Sparkle White Toothpaste	14.25	29.5
A008 Sparkle Naturals Himalaya Pink Salt Toothpaste	12	29.5
A009 Sparkle Natural Complete Care Toothpaste	17.25	0.00
A010 Twin Lotus Herbalist Active Charcoal Toothpaste	0	33.25
A011 Doctor V Whitening System Toothpaste - Diavee 01	15.5	25.50
A012 I-Vish Gentle Powerful Whitening and Anti-Cavity Toothpaste	0	20.25
A013 Toothpaste Wisut Pure Extra Gum Protection & Gentle Powerful Whitening Toothpaste	0	33.25
A014 MYSEPTIC MYBACIN Toothpaste with Andrographis paniculata formula	11.5	33.25
A015 Denticon Q10 total care toothpaste	0	31.75
A016 Denticon Q10 rose refresh toothpaste	16	33.00
A017 Blooms Nature Love Natural Fresh & Gum Care Tooth Paste	16.5	37.00
A018 Herbal Den Plus Neem Toothpaste	23.75	32.00
A019 Veldent Extreme Awake Tooth Paste	17.75	35.50

A020 Thep Thai Herbal Toothpaste Concentrated Formula Spearmint Flavor	16.25	37.25
A021 Thep Thai herbal toothpaste, concentrated formula, mixed fruit flavor	12.5	37.00
A022 Thep Thai Herbal Toothpaste, concentrated formula, original flavor	13	40.50
A023 Thep Thai Herbal Toothpaste, concentrated formula, salt flavor	15.25	29.75
A024 Dentiste toothpaste original formula	12.5	30.00
A025 Dentamate Herbals Toothpaste	22.5	33.50
A026 Colgate Total Advanced Fresh Toothpaste	0	36.50
A027 Colgate Total 12 Professional Clean Toothpaste	0	33.75

Table 5 highlights 5 toothpaste formulations with notable inhibitory effects against *Candida albicans*.

No.	Toothpaste	Median
1	A005	26.00
2	A018	23.75
3	A025	22.50
4	A004	18.00
5	A023	15.25

From Table 4, 5 toothpaste formulations that showed notable inhibitory effects against *Candida albicans* were A005, A018, A025, A004, and A023, respectively. There were statistically significant differences among these five formulations as follows: Toothpastes A005, A018, and A025 were significantly different from A004 and A023; likewise, toothpastes A004 and A023 were significantly different from A005, A018, and A025.

Table 6 highlights 5 toothpaste formulations with notable inhibitory effects against *Streptococcus mutans*.

No.	Toothpaste	Median
1	A023	40.50a
2	A021	37.25a
3	A018	37.00a
4	A027	36.50a
5	A002	35.75a

From Table 6, 5 toothpaste formulations that showed notable inhibitory effects against *Streptococcus mutans* were A023, A018, A021, A027, and A002, respectively. However, there was no statistically significant difference among these five formulations.

Testing the effectiveness of toothpaste in killing bacteria

The effectiveness of the toothpaste samples in inhibiting the growth of *S. mutans* and *C. albicans* in patient saliva sample was evaluated. The results demonstrated that, overall, the herbal toothpaste without fluoride were mostly effective in inhibiting the growth of *S. mutans*, with no growth observed in several toothpaste formulas (indicated by "-"). However, some formulas, such as A005, A015, A016, and A024, exhibited moderate growth ("++"), while others, like A007 and A023, showed heavy growth ("+++"), indicating reduced effectiveness in inhibiting this bacterium.

Regarding *C. albicans*, several toothpaste samples effectively inhibited fungal growth, with most showing no growth (indicated by "-"). However, some formulas, including A005, A006, A015, and A024, exhibited mild to moderate growth ("+" or

"+++"), while only one formula, A023, demonstrated heavy fungal growth ("+++"), suggesting a decreased effectiveness in inhibiting *C. albicans*.

Overall, the results indicate that while most toothpaste samples were effective at inhibiting *S. mutans*, some exhibited reduced inhibition of *C. albicans*. Nonetheless, samples A005, A007, A015, A023, and A024 showed relatively low efficacy in inhibiting both organisms.

Table 7 Results of Testing the Effectiveness of Toothpaste in Killing Bacteria in Patient Saliva

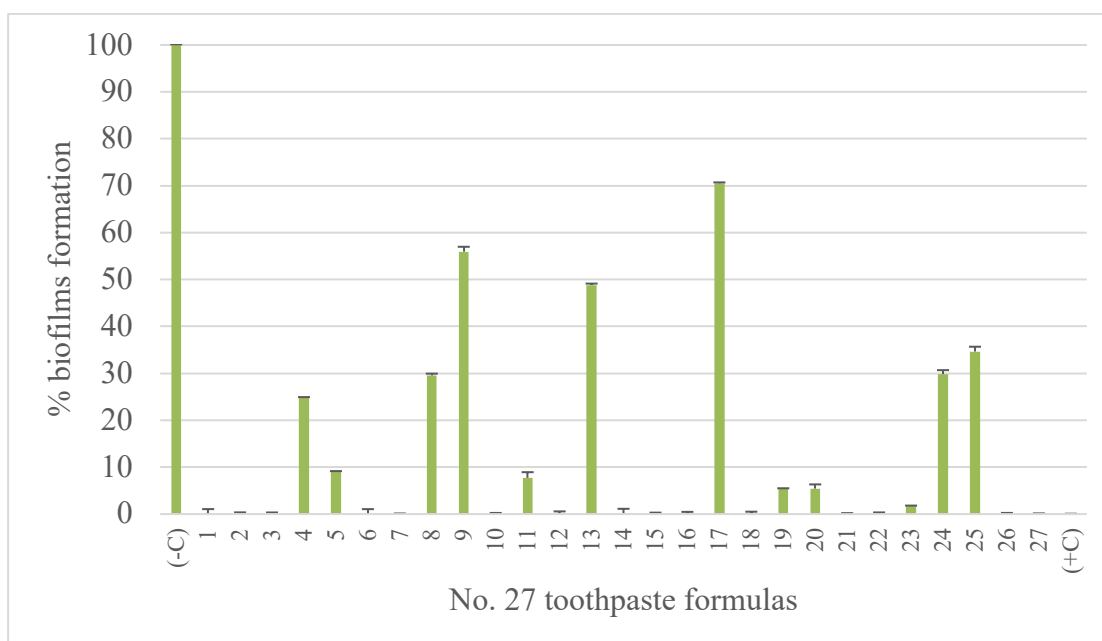
No. sample	Pure saliva on BHI	Pure saliva on SDA	Toothpaste	Saliva + Toothpaste on BHI	Saliva + Toothpaste on SDA
1	+++	+++	A001	-	-
2	+++	+++	A002	-	-
3	+++	+++	A003	-	-
4	+++	+++	A004	-	-
5	+++	+++	A005	++	+
6	+++	+++	A006	-	++
7	+++	+++	A007	+++	+++
8	+++	+++	A008	-	-
9	+++	+++	A009	-	-
10	+++	+++	A010	-	-
11	+++	+++	A011	-	-
12	+++	+++	A012	-	-

13	+++	+++	A013	-	-
14	+++	+++	A014	-	-
15	+++	+++	A015	++	++
16	+++	+++	A016	++	-
17	+++	+++	A017	-	-
18	+++	+++	A018	-	-
19	+++	+++	A019	-	-
20	+++	+++	A020	-	-
21	+++	+++	A021	-	-
22	+++	+++	A022	-	-
23	+++	+++	A023	+++	+++
24	+++	+++	A024	++	++
25	+++	+++	A025	-	-
26	+++	+++	A026	-	-
27	+++	+++	A027	-	-

+++ Heavy growth, ++ Moderate growth, + Mild growth, - No growth

Biofilm formation assay

The results showed that all 27 toothpaste formulations exhibited biofilm formation compared to the negative control. Additionally, 15 toothpaste formulations demonstrated complete inhibition of biofilm formation. These findings suggest that all tested toothpaste formulations are capable of inhibiting the biofilm formation of *C. albicans*, as shown in figure 1.



Figures 6 The graph showing %biofilm formation compared to the negative control and positive control.

Similarly, all 27 toothpaste formulations also exhibited biofilm formation inhibition against *S. mutans* when compared to the negative control. Among them, 15 formulations showed complete inhibition of biofilm formation. These findings indicate that the tested toothpaste formulations are also effective in inhibiting the biofilm formation of *C. albicans*, as illustrated in Figure 6.

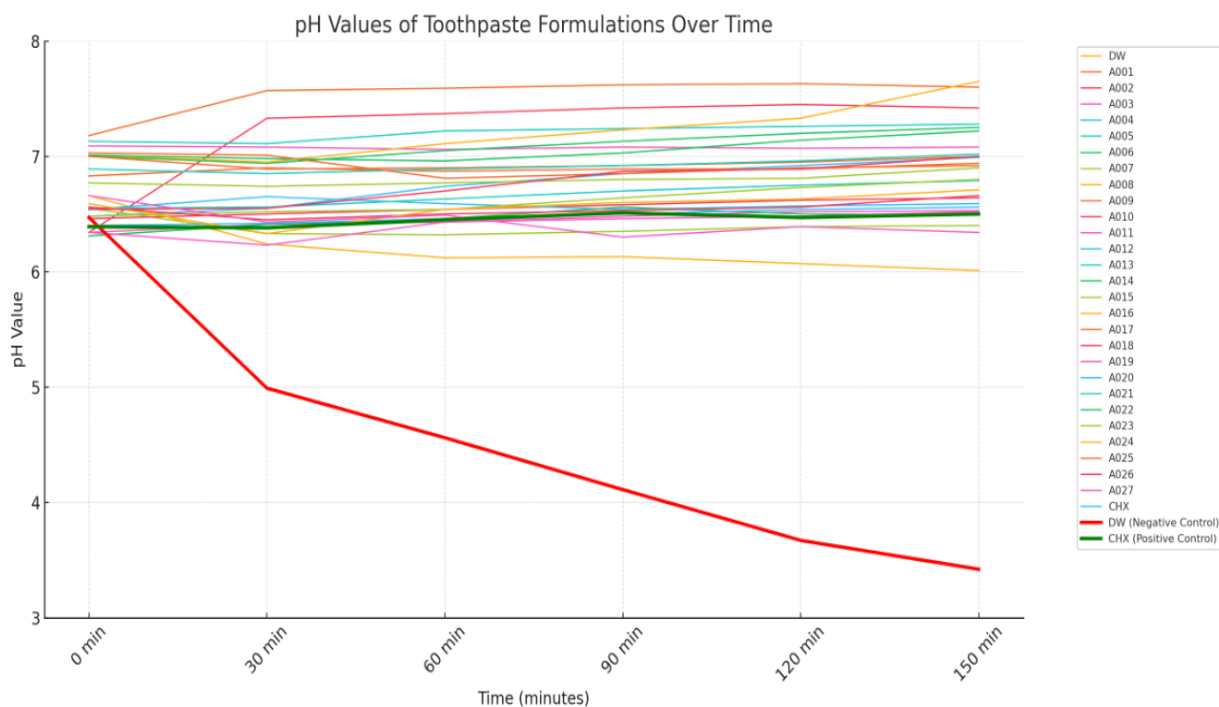
Inhibition of acid production test of toothpaste

From the acid production inhibition test of all 27 toothpaste formulations, the results indicated that, after incubation at 37°C, pH values were measured at 0, 30, 60, 90, 120, and 150 minutes. Throughout these time intervals, the pH values of the toothpaste-treated samples remained within a neutral range, suggesting that all formulations were able to inhibit acid production by *S. mutans* in every trial.

When compared with chlorhexidine (CHX), which was used as the positive control, it was found that the pH of the CHX-treated group remained within the range of 7.11–7.20. This demonstrates that CHX effectively inhibited acid production by *S. mutans*.

Table 8 the pH values at different time points during the acid production test of S. mutans in response to various toothpaste formulations.

Toothpaste	pH					
	0 min	30 min	60 min	90 min	120 min	150 min
1. DW	6.47	4.99	4.56	4.11	3.67	3.42
2. A001	7.18	7.57	7.59	7.62	7.63	7.6
3. A002	6.34	7.33	7.37	7.42	7.45	7.42
4. A003	7.09	7.08	7.06	7.08	7.07	7.08
5. A004	6.48	6.55	6.74	6.85	6.92	6.99
6. A005	7.13	7.11	7.22	7.24	7.26	7.28
7. A006	7	6.94	7.05	7.13	7.2	7.25
8. A007	6.55	6.33	6.32	6.35	6.39	6.4
9. A008	6.66	6.24	6.12	6.13	6.07	6.01
10. A009	6.83	6.9	6.87	6.89	6.89	6.94
11. A010	6.46	6.5	6.54	6.58	6.62	6.64
12. A011	6.34	6.41	6.43	6.48	6.52	6.53
13. A012	6.54	6.65	6.59	6.54	6.57	6.59
14. A013	6.55	6.56	6.63	6.7	6.75	6.79
15. A014	6.31	6.41	6.46	6.56	6.5	6.49
16. A015	6.48	6.52	6.54	6.64	6.73	6.8
17. A016	7.01	6.95	7.11	7.23	7.33	7.65
18. A017	7	6.89	6.9	6.92	6.95	7
19. A018	6.56	6.45	6.5	6.53	6.56	6.66
20. A019	6.34	6.23	6.43	6.46	6.48	6.52
21. A020	6.4	6.42	6.46	6.5	6.54	6.56
22. A021	6.89	6.85	6.89	6.92	6.96	7.02
23. A022	7.01	6.98	6.96	7.03	7.14	7.22
24. A023	6.77	6.74	6.77	6.8	6.81	6.9
25. A024	6.59	6.33	6.54	6.6	6.63	6.71
26. A025	7.03	7.01	6.81	6.85	6.9	6.92
27. A026	6.54	6.55	6.7	6.87	6.89	7
28. A027	6.66	6.43	6.49	6.3	6.39	6.34
29. CHX	6.39	6.38	6.45	6.51	6.47	6.50



Figures 7 The graph shows the reduction of acid production by *S. mutans* in response to 27 different toothpaste formulations.

CHAPTER 5

CONCLUSION AND DISCUSSION

Discussion

Dental caries is considered as one of the most common oral diseases that can occur in both children and adults, caused by the breakdown of food for energy, acid formation, tooth demineralization, and bacterial destruction of the tooth structure, which results in a hole or cavity on the tooth. If left untreated, it will spread, expand, and become deeper, causing pain and suffering, and eventually tooth loss, which may have to be extracted.

Oral candidiasis is a fungal infection of the oral cavity caused by *C. albicans* and is a major global public health concern as well as a significant economic burden worldwide. The primary cause of oral candidiasis is often a disruption of oral homeostasis, which can lead to tooth loss, significantly impacting an individual's quality of life, daily activities, and work performance while also contributing to substantial medical expenses. Several risk factors are associated with oral candidiasis, including the consumption of foods and beverages high in sugar, smoking, and poor oral hygiene practices, such as inadequate brushing and insufficient removal of dental plaque through scaling.

Experimental results demonstrated that all 27 toothpaste formulations tested were capable of inhibiting *C. albicans* and *S. mutans*, with significant variations in antifungal efficacy depending on the specific ingredients present in each formulation. These findings support the effectiveness of herbal toothpaste in preventing oral fungal infections and align with previous research on the topic.

For instance, a study conducted by Ozaki F *et al.* (2006) evaluated the efficacy of an herbal toothpaste in 48 patients with gingivitis. Participants were instructed to use the toothpaste three times daily for 28 days. A toothpaste containing triclosan and fluoride was used as a positive control. The study found that both the test and control groups experienced a significant reduction in dental plaque and gingival inflammation,

with no adverse effects reported. These findings further support the potential of herbal toothpaste formulations in maintaining oral health.

In this study, DMSO was used as a solvent to extract active compounds from toothpaste prior to antimicrobial testing. Due to its ability to dissolve both polar and non-polar substances, DMSO is considered suitable for preparing samples from complex formulations such as toothpaste. Preliminary testing confirmed that at a concentration of 2%, DMSO did not exhibit any antimicrobial activity, thereby validating its use as a neutral solvent in this experimental context.

The study found that five toothpaste formulations demonstrated remarkable efficacy in inhibiting *C. albicans*. These formulations include Sparkle Triple White Toothpaste (A005), Herbal Dentply Toothpaste (A018), Dentamate Herbal Toothpaste (A025) Threeherb Herbal Toothpaste (A004) Thepthai Concentrated Herbal Salt Toothpaste (A023) Statistical analysis revealed significant differences among these 5 toothpaste formulations. Specifically, formulas A005, A018, and A025 exhibited statistically significant differences compared to formulas A004 and A023. Upon analyzing the herbal compositions of these 5 toothpaste formulations, the following key ingredients were identified: A005: Contains *Mentha piperita* oil and *Eugenia caryophyllus* leaf oil. A018: Contains *Azadirachta indica* extract, *Psidium guajava* leaf extract, *Eugenia caryophyllus* oil, *Aloe barbadensis* leaf powder, and *Mentha piperita* oil. A025: Contains *Mentha piperita* oil, *Eugenia caryophyllus* bud oil, and *Aloe barbadensis* leaf juice. A004: Contains *Psidium guajava* leaf extract, *Eugenia caryophyllus* oil, and *Streblus asper* Lour. Formula A023: Contains *Mentha piperita* oil, *Psidium guajava* extract, *Streblus asper* leaf extract, and *Eclipta prostrata* extract.

Based on the findings, five toothpaste formulations that demonstrated notable inhibitory effects against *Streptococcus mutans* were: Thepthai Herbal Toothpaste Concentrated Salt Formula (A023) Herbal Dentply Herbal Toothpaste (A018) Thepthai Herbal Toothpaste Mixed Fruit Formula (A021) Colgate Total 12 Professional Clean (A027) Dok Bua Ku Herbal Toothpaste Air Fresh Formula (A002) There were no statistically significant differences among these five toothpaste formulations. An analysis of the ingredients in each revealed the following: Toothpaste A023 contains herbal extracts such as *Mentha piperita* Oil, *Psidium guajava* Extract,

Streblus asper Leaf Extract, and Eclipta prostrata Extract. Toothpaste A018 contains Azadirachta indica Extract, Psidium guajava Leaf Extract, Eugenia caryophyllus Oil, Aloe barbadensis Leaf Powder, and Mentha piperita Oil. Toothpaste A021 includes Caesalpinia sappan Extract, Aloe barbadensis Callus Extract, Cinnamon Extract, Eugenia caryophyllus Oil, and Streblus asper Leaf Extract. Toothpaste A027 does not contain any herbal ingredients. Toothpaste A002 contains Eucalyptus melliodora Oil, Acmella oleracea (Para Cress), Wintergreen Oil, Streblus asper (Toothbrush Tree), and *Clinacanthus nutans*.

Biofilms exhibit significantly greater resistance to antimicrobial agents compared to their planktonic counterparts due to the protective extracellular polymeric substance (EPS) matrix, altered metabolic activity, and intercellular communication mechanisms such as quorum sensing. These factors collectively impede the penetration and efficacy of active compounds, resulting in discrepancies between planktonic inhibition and biofilm control (Hall-Stoodley *et al.*, 2004; Flemming *et al.*, 2016). Understanding these complex biofilm defense strategies is crucial for developing effective oral care products targeting both microbial states (Mah & O'Toole, 2001; Koo *et al.*, 2017).

The findings of this study demonstrate that several herbal toothpaste formulations exhibit varying degrees of inhibitory effects against *C. albicans* and *S. mutans*, suggesting potential antifungal properties. However, these variations are likely influenced not only by the presence of herbal ingredients but also by other chemical constituents commonly found in toothpaste, such as fluoride, detergents (e.g., sodium lauryl sulfate), humectants, preservatives, and essential oils. These components may contribute synergistically or independently to the observed antifungal activity.

It is important to note that the complexity of each toothpaste formulation introduces challenges in identifying the specific active agents responsible for the inhibition of *C. albicans* and *S. mutans*. While herbal extracts are often cited for their antimicrobial properties, the antifungal effect observed may result from a combined action of multiple ingredients, both natural and synthetic. For instance, ingredients

like triclosan, zinc compounds, or certain alcohols (used as solvents or preservatives) have known antimicrobial effects and may have contributed to the outcomes.

This multifactorial nature of the formulations presents a key limitation of the current study. The absence of standardized concentrations and compositions across the 27 tested toothpaste products makes it difficult to draw definitive conclusions about which specific compounds are most effective. Additionally, potential interactions between ingredients, whether enhancing or antagonizing, remain unexplored in this study.

To better understand the mechanisms underlying the antifungal activity, future research should focus on fractionating the toothpaste formulations and testing individual components or combinations thereof. Advanced analytical techniques, such as GC-MS or LC-MS, may also be employed to identify and quantify active compounds. Investigating both herbal and non-herbal constituents in a controlled setting would provide more comprehensive insights into their respective contributions.

Ultimately, a deeper understanding of how various chemical components—both herbal and synthetic—interact to inhibit *C. albicans* and *S. mutans* could lead to the development of more effective oral care products, especially for populations susceptible to oral candidiasis and dental caries.

Conclusion

This study confirms the antifungal potential of various herbal toothpaste formulations against *C. albicans* and *S. mutans*. The findings emphasize that some formulations are more effective than others, highlighting the need for further investigation into the specific active ingredients and their mechanisms of action. The ability of some formulations to inhibit biofilm formation is particularly valuable, as biofilms contribute to persistent and hard-to-treat infections. These results support the use of fluoride-free herbal toothpaste as a preventive measure against *C. albicans*-related oral infections.

When comparing a variety of fluoride-free herbal toothpaste brands and formulations commonly found in the Thai market, it was found that their ability to inhibit *Streptococcus mutans* and *Candida albicans* varied. The differences were

observed both statistically significantly and non-significantly, depending on the type of toothpaste and the microorganism tested. The antimicrobial effectiveness of the toothpastes is influenced by several factors, including: The types and number of herbal ingredients used, the concentration and extraction methods of the herbal components, the formulation techniques and the presence of other additives

The study also acknowledges the presence of other chemical components in the toothpaste formulations, which may contribute to the antimicrobial activity alongside the herbal extracts. These include stabilizers, humectants, and certain flavoring agents, some of which have been reported to exhibit biological activity to varying degrees. Therefore, the observed antimicrobial effects cannot be solely attributed to herbal ingredients but rather result from the combined effects of the overall formulation. This highlights the complexity of finished products and underscores the need for further studies to isolate and evaluate the individual and synergistic effects of various active and auxiliary compounds.

Each toothpaste formulation is unique, and these variations may affect the bioactivity of the herbal extracts. Herbs contain a wide range of bioactive compounds, many of which have been well-documented for their antimicrobial properties through both domestic and international research.

Notably, international studies have also evaluated the antimicrobial properties of fluoride-free herbal toothpaste, as mentioned in the literature review. However, such studies have been limited in Thailand. Therefore, this research serves as an important foundation by providing relevant data on the antimicrobial effects of widely available Thai herbal toothpaste without fluoride formulations.

This study does not conclude which toothpaste is the most effective due to several limitations, including:

The experiments were conducted in vitro using single-species cultures, which do not fully represent the complex environment of the oral cavity (which includes mixed-species biofilms and the presence of saliva).

The conditions in a laboratory setting may not perfectly reflect real-life outcomes in the mouth.

Despite these limitations, the findings are expected to serve as valuable baseline data for consumers, healthcare professionals, and future researchers who are interested in the efficacy of fluoride-free herbal toothpaste commonly used in Thailand. Further research is encouraged to explore the long-term clinical effects and to expand the scope of microbial testing under more realistic oral conditions.

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APPENDIX

APPENDIX A

1. *Mitis salivarius* agar (MSA)

Pancreatic digest of casein	6.0	g
Proteose peptone No.3	9.0	g
Proteose peptone	5.0	g
Dextrose	1.0	g
Saccharose	50.0	g
Dipotassium phosphate	4.0	g
Trypan blue	0.075	g
Crystal violet	0.0008	g
Agar	15.0	g

Preparation method: Weigh 90.0 g of the medium and dissolve it in 1000 ml of distilled water. Stir the mixture until well combined using a magnetic stirrer. Sterilize by autoclaving at 15 psi pressure and 121°C for 15 minutes. Let the medium cool slightly, then add bacitracin 0.0003 g per 100 ml and mix thoroughly.

2. Brain heart infusion broth (BHI broth)

Brain heart infusion	2.7	g
Distill water	100	ml

Preparation method: Weigh the substance and dissolve it in distilled water. Place on a hot plate until dissolved. Cover and sterilize at 121°C for 15 min and store at 4°C.

3. Brain heart infusion agar (BHI agar)

Brain heart infusion	2.7	g
Agar technical	1.5	g
Distill water	100	ml

Preparation method: Weigh the substance and dissolve it in distilled water. Place on a hot plate until dissolved. Cover and sterilize at 121°C for 15 min. When the temperature drops to 70°C, pour it onto a culture plate and set at 37°C to solidify the agar. Store at 4°C.

4. Sabouraud's dextrose broth (SDB)

Sabouraud's dextrose	2	g
Distill water	100	ml

Preparation method: Weigh the substance and dissolve it in distilled water. Place on a hot plate until dissolved. Cover and sterilize at 121°C for 15 min and store at 4°C.

5. Sabouraud's dextrose agar (SDA)

Sabouraud's dextrose	2	g
Agar technical	1.5	g
Distill water	100	ml

Preparation method: Weigh the substance and dissolve it in distilled water. Place on a hot plate until dissolved. Cover and sterilize at 121°C for 15 min. When the temperature drops to 70°C, pour it onto a culture plate and set at 37°C to solidify the agar. Store at 4°C.

APPENDIX B

1. Crystal Violet 0.1% Solution

Crystal violet	0.1	g
Distilled water	100	ml

Preparation method: Dissolve all ingredients thoroughly and store in an amber bottle.

2. Sucrose 20%

Sucrose	20	g
Distilled water	100	ml

Preparation method: Dissolve all ingredients thoroughly. Then sterilize by filtering through a 0.45 m Millipore filter paper, and store in the refrigerator.

3. Glucose 20%

Glucose	20	g
Distilled water	100	ml

Preparation method: Dissolve all ingredients thoroughly. Then sterilize by filtering through a 0.45 m Millipore filter paper, and store in the refrigerator.

4. Sodium Fluoride 0.2%

Sodium fluoride	0.44	g
Distilled water	100	ml

Preparation method: Dissolve all ingredients thoroughly. Then sterilize by filtering through a 0.45 m Millipore filter paper.



Documentary Proof of Ethical Clearance
Human Research Ethics Committee (HREC)
Faculty of Dentistry, Prince of Songkla University

The Project Entitled Effect of herbal dentifrice without fluoride against oral microbial : an *in vitro* study
EC Project No. EC6702-008
Principal Investigator Assoc.Prof.Dr.Suwanna Jitpukdeebodintra
Affiliation Department of Oral Biology and Occlusion, Faculty of Dentistry, PSU
Co-Principal Investigator Assoc.Prof.Dr.Nattapon Rotpenpian and Miss Natrapee Akarathanakrit
Affiliation Department of Oral Biology and Occlusion, Faculty of Dentistry, PSU
Approved Documents :

- | | | |
|-------------------------------------|---|---------------------------------|
| ☒ | Submission Form | Version 2 Date 22 April 2024 |
| ☒ | Research Proposal | Version 1 Date 13 February 2024 |
| ☒ | Information Sheet | Version 1 Date 8 February 2024 |
| ☒ | Consent Form | Version 1 Date 8 February 2024 |
| ☒ | Informed Consent for the Collection of Biological Samples for Further Study | Version 1 Date 8 February 2024 |
| ☒ | Other ... | |
| ☒ | CVs and Certificate of participation in a workshop for ethics in human research | Version 1 Date 8 February 2024 |

Approved by Human Research Ethics Committee (HREC), Faculty of Dentistry, Prince of Songkla University.

This is to certify that HREC is in full compliance with International Guidelines for Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

This review is documented in the meeting minutes of the meeting 4/2025

Agenda 3.1.1 on 24 April 2025

Please submit the Progress Report every 12 months. (Renewal must be submitted at least 30 days prior to expired date.)

(Prof.Dr.Chairat Charoemratrote)
Chairman of Research Ethics Committee
Faculty of Dentistry, Prince of Songkla University

First Date of Approval: 25 April 2024
Last Date of Approval: 25 April 2025
Last Date of Expiration: 24 April 2026



หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัย เรื่อง
รหัสโครงการ
หัวหน้าโครงการ
สังกัด
ผู้ร่วมวิจัย
สังกัด

ผลของยาสปีนสมุนไพรมีผลต่อเชื้อในช่องปาก : การศึกษาในห้องปฏิบัติการ
EC6702-008

รองศาสตราจารย์ ดร.ทพญ.สุวรรณา จิตภักดิ์ดินทร์

สาขาวิชาชีววิทยาช่องปากและระบบการบดเคี้ยว คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

รองศาสตราจารย์ ดร.ทพ.ณัฐพล โรจน์เพ็ญเพียร และ นางสาวณัฐระพี อัครณกุล

สาขาวิชาชีววิทยาช่องปากและระบบการบดเคี้ยว คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

เอกสารที่รับรอง

- | | |
|---|-------------------------------------|
| ☒ แบบเสนอโครงการวิจัย | ฉบับที่ 2 วันที่ 22 เมษายน 2567 |
| ☒ โครงร่างการวิจัย | ฉบับที่ 1 วันที่ 13 กุมภาพันธ์ 2567 |
| ☒ เอกสารชี้แจงอาสาสมัคร | ฉบับที่ 1 วันที่ 8 กุมภาพันธ์ 2567 |
| ☒ เอกสารให้ความยินยอม | ฉบับที่ 1 วันที่ 8 กุมภาพันธ์ 2567 |
| ☒ เอกสารให้ความยินยอมเก็บตัวอย่างชีวภาพ | ฉบับที่ 1 วันที่ 8 กุมภาพันธ์ 2567 |
| เพื่อศึกษาวิจัยในอนาคต | |
| ☒ อื่น ๆ | |
| ☒ แบบประวัติผู้วิจัยและหลักฐานการอบรมจริยธรรมการวิจัยในมนุษย์ | ฉบับที่ 1 วันที่ 8 กุมภาพันธ์ 2567 |

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมการวิจัยในมนุษย์ (Human Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาการศึกษาการวิจัยในมนุษย์ของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในมนุษย์ที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

บรรจุในวาระการประชุมครั้งที่ 4/2567

วาระที่ 3.1.1

วันที่ 24 เมษายน 2568

ขอให้ผู้วิจัยรายงานความก้าวหน้าโครงการวิจัย ทุก ๆ 12 เดือน และยื่นต่ออายุก่อนถึงวันหมดอายุอย่างน้อย 30 วัน

(ศาสตราจารย์ ดร.ทพ.ไชยรัตน์ เฉลิมรัตนโรจน์)

ประธานกรรมการจริยธรรมการวิจัยในมนุษย์

คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

วันที่รับรอง (ครั้งแรก): ..25..เมษายน..2567..

วันที่รับรอง (ครั้งล่าสุด): ..25..เมษายน..2568..

วันหมดอายุ (ครั้งล่าสุด): ..24..เมษายน..2569..

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