PENETRATION AND ANTIBACTERIAL ABILITY IN DENTINAL TUBULES OF NEEM PASTE FOR CARIES TREATMENT (*IN VITRO*)

BY

KAMONCHANOK TANGSAPSATHIT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MAJOR IN PEDIATRIC DENTISTRY,

FACULTY OF DENTISTRY

THAMMASAT UNIVERSITY

ACADEMIC YEAR 2023

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ENTITLED

PENETRATION AND ANTIBACTERIAL ABILITY IN DENTINAL TUBULES OF NEEM PASTE FOR CARIES TREATMENT *(IN VITRO)*

was approved as partial fulfillment of the requirements for the degree of Master of science Program in Dentistry (Major in Pediatric Dentistry)

on October .....19 th..... 2023

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Academic Year	2023

บทคัดย่อ

ฟันผุเป็นปัญหาสุขภาพช่องปากที่พบมากในหลายประเทศ ฟันผุที่ไม่ได้รับการรักษาจะทำให้เนื้อเยื่อในฟันเกิดการติดเชื้อและอักเสบและลุกลามไปสู่เนื้อเยื่อรอบๆฟันจนนำไปสู่การสูญเสียฟันในที่สุด กระบวนการป้องกันเนื้อเยื่อในฟัน ด้วยการใช้วัสดุปิดโพรงฟัน (protective pulp liner) เพื่อรักษาความมีชีวิตของเนื้อเยื่อในฟันเป็นหนึ่งในวิธีจัดการฟันผุ การที่แบคทีเรียสามารถเจริญเติบโตและแทรกซึมเข้าไปในท่อเนื้อฟันได้ลึกกว่าวัสดุปิดโพรงฟัน ส่งผลให้เกิดการลุกลามของฟันผุนำไปสู่ความล้มเหลวในการรักษา โดยแบคทีเรียที่พบมากในรอยโรคฟันผุระดับลึกที่รักษาด้วยวัสดุปิดโพรงฟัน คือ แลคโตบาซิลลัส รามโนซัส (*Lactobacillus rhamnosus*) สะเดา ชื่อวิทยาศาสตร์ คือ *Azadirachta indica* var. *siamensis* valeton มีคุณสมบัติทางชีวภาพที่หลากหลาย โดยเฉพาะมีคุณสมบัติในการต้านเชื้อแบคทีเรียที่ก่อให้เกิดโรคฟันผุในช่องปาก การศึกษานี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพการฆ่าเชื้อ *L. rhamnosus* ในท่อเนื้อฟันของสารสกัดสะเดาในรูปแบบเพสต์ วิธีการวิเคราะห์สารประกอบสำคัญในสารสกัดสะเดาโดยการใช้เครื่องแก๊สโครมาโตกราฟี-แมสสเปกโทรเมทรี (gas chromatography mass spectrometry, GC-MS) และเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง (high performance liquid chromatography, HPLC) การทดสอบฤทธิ์ในการต้านเชื้อแบคทีเรีย *L. rhamnousus* โดยวิธี agar disc diffusion และคุณสมบัติในการต้านเชื้อแบคทีเรียในท่อเนื้อฟันโดยวิธีการย้อมด้วย LIVE/DEAD® BacLight และยืนยันผลการทดลองด้วย confocal laser scanning microscope (CLSM) ผลการทดลอง ตรวจสารประกอบหลักในสะเดาด้วย GS-MS พบว่าในสารสกัดจากสะเดามี phytol เป็นองค์ประกอบหลัก ร้อยละ 65.97 และจากการวิเคราะห์ด้วย HPLC พบสารประกอบสำคัญได้แก่ azadirachtin, querecetin, nimbin และ nimbidin จากการเตรียมสารสกัดสะเดาในรูปแบบเพสต์และนำมาทดสอบคุณสมบัติทางการแพร่ผ่านท่อเนื้อฟันพบว่า สะเดาเพสต์สามารถแพร่ผ่านท่อเนื้อฟันได้ลึกถึง 1,030 ± 39.61 μm และมีความสามารถในการยับยั้งเชื้อ *L. rhamnosus* ในท่อเนื้อฟันได้ ดังนั้นสารสกัดสะเดาในรูปแบบเพสต์ จึงอาจเป็นทางเลือกใหม่ที่สามารถนำมาใช้เป็นวัสดุปกปิดเนื้อเยื่อโพรงประสาทฟันในการรักษาโรคฟันผุ

ABSTRACT

Dental caries is the most common oral health issue worldwide. Untreated caries causes deterioration to the pulp and periapical tissues, eventually leading to tooth loss. Foreign organisms, like the bacteria *Lactobacillus rhamnosus*, can penetrate to dentinal tubules and develop under pulp liner material, resulting in caries progression and pulp inflammation. *Azadirachta indica* var. *siamensis* valeton (neem) leaf extract has been reported to possess antibacterial properties effective against oral acidogenic bacteria.

Objectives: This study proposes to assess neem paste's antibacterial efficacy against *L. rhamnosus* in dentinal tubules.

Methods: The bioactive constituents of neem extract were investigated using gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). The physicochemical properties of neem paste were investigated using visual, pH and viscosity measurements. The antimicrobial activity of neem pastes on *L. rhamnousus*, was also investigated, using an agar disc diffusion assay while the antimicrobial properties in dentin were examined using LIVE/DEAD BacLight® staining and confirmed by confocal laser scanning microscope (CLSM).

Results: Phytol was determined to be the primary constituent compound (65.97 percent). Others active compounds, including azadirachtin, querecetin, nimbin, and nimbidin were detected. Neem paste penetrated the dentinal tubules by 1,030 ± 39.61 µm. Neem paste inhibited *L. rhamnosus* growth in an agar disc diffusion assay and eliminated *L. rhamnosus* in dentinal tubules at all experiment dentin depths.

Conclusion: Neem paste has satisfactory antibacterial activity and highly effective penetration through infected dentin. Therefore, neem paste might be developed to be an alternative material for indirect pulp treatment.

Key words: *Azadirachta indica*, neem, antibacterial, *L. rhamnosus*, penetration

ACKNOWLEDGEMENTS

First and foremost, I would like to express sincere appreciation to my thesis advisor, Associate Professor Dr. Parinda Tasanarong, and my co-advisor, Associate Professor Dr. Visakha Aupaphong, for their invaluable advice, patience and strong encouragements, excellent supervision, and comments on my thesis. Dr. Chiravoot Pechyen was thoughtful enough to provide me with the HPLC and GC-MS analysis parts for the experiment.

Thank you to Ms. Chayada Teanchai and Mr. Sarut Thairat for helpful suggestions and assistance with the SEM and confocal fluorescence microscopy sections of the experiment, respectively.

I would definitely also wish to thank Faculty of Dentistry of Thammasat University for their financial support. (Grant number 3/2565).

I cannot leave without thanking my family and friends for their ongoing encouragement, unending support, and unconditional love throughout my time in this course.

I would like to dedicate all the best of my thesis to my beloved for my family for their love and understanding during my study. Finally, I would like to express my heartfelt appreciation to all of my teachers at all levels; my accomplishment would not have been possible without them.

Kamonchanok Tangsapsathit

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Abbreviations	Description
AAPD	American Academy of Pediatric Dentistry
$Ca(OH)_2$	Calcium hydroxide
CHX	CHX
CLSM	Confocal laser scanning microscope
CO_2	Carbon dioxide
cm	Centimeter
DMBA	Dimethylbenzanthracene
dw	Dry weight
EDTA	Ethylene diamine tetra-acetic acid
g	Gram
GC-MS	Gas chromatography -mass spectrometry
GMP	Good manufacturing practices
h	Hour
HBP	Hamster buccal pouch
HPLC	High performance liquid chromatography
HSV	Herpes simplex virus
ICH Q6A	The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
ISO	International Organization for Standardization
L.acidophilus	*Lactobacillus aciodophilus*
L.casie	*Lactobacillus casei*
L.rhamnosus	*Lactobacillus rhamnosus*
M	Molar
m	Meter
mg	Milligram
mol	Molecule
MID	Minimal intervention dentistry
min	Minute
ml	Milliliter
mm	Millimeter
MRS	De Man Rosoga and Sharpe
MTA	Mineral trioxide aggregate
NaOCl	Sodium hypochlorite

NE	Neem nanoemulsion
nm	Nanometer
PBS	Phosphate buffered saline
s	Second
S.mutans	*Streptococcus mutans*
TIMP	Tissue inhibitor of metalloproteinases
WHO	World Health Organization
ZnO	Zinc oxide
µg	Microgram
µm	Micrometer
µl	Microliter
^{o}C	Degree Celsius

CHAPTER 1

INTRODUCTION

Dental caries has been a long global health burden according to the World Health Organization (WHO) statistics in 2016, which reports that 60-90 percent of children are affected (1). This major issue is one Thailand shares with the rest of the world. The Thai Department of Health's 8th National Oral Health surveyor, Thailand in 2017 examined children aged 3 and 5 years old. They represent as the primary dentition and have the prevalence percentage of dental caries disease of 52.9 and 75.6, respectively. Those children aged 5 reported that they have early loss of primary teeth in percentage of 6.5. While children aged 12 and 15 years old represent as the permanent dentition and have prevalence percentage of dental caries disease at 52 and 62.7, respectively. Those children aged 12 and 15 reports that they have not received any treatment highly to 31.5 and 40.1 percent lead to an early loss of teeth in the percentage of 3 and 7.1, respectively (2).

Dental caries has been described as a chronic, invasive, and infectious process caused by multiple factors; from to susceptible teeth, pathogenic organisms to the frequent intake of fermentable carbohydrates (3). Nishikawara *et al*.'s hypothesis discusses dental caries as a dynamic process in which the demineralization process occurs in enamel when the pH in dental plaque is less than 5.5 (4). In dentin, which has a larger organic component, the progression of disease is involved in both the mechanisms of demineralization and proteolysis (5). Furthermore, the organism species in dentin carious lesion have a more complex in ecosystem due to their ecological conditions, such as oxygen tension and types of nutrients available (6). More recent studies on the deep areas of dentin carious lesions report that there are strictly anaerobic conditions where only obligately anaerobic bacteria and gram-positive rods, such as *Propionibacterium* spp., *Eubacterium* spp., and *Bifidobacterium* spp., predominant (7). In the deepest layers, the most prevalent species are *Atopobium genomospecies C1, Fusobacterium nucleatum* and Lactobacillus which are actively contributing to disease progression (8-10).

The treatment of dental caries depends on the severity of lesions as grounded in the concept of minimal intervention dentistry (MID) which focuses on preserving the natural tooth structure and pulp vitality (11, 12). According to the guidelines of the American Academy of Pediatric Dentistry (AAPD), MID is also known as indirect pulp treatment and is recommended for the treatment of teeth with deep carious lesions that do not display signs or symptoms of

pulpal inflammation (13). The main goal is to modify the microenvironment which has been left under the restoration by arresting the caries progression, stimulating tertiary dentin formation, and allowing remineralization of the lesions by the restoration (11, 12). AAPD has recommended materials used as a liner and placed over the remaining carious dentin lesion. These materials, including dentin bonding agents, resin modified glass ionomers, calcium hydroxide ($Ca(OH)_2$), mineral trioxide aggregate (MTA), or any other biocompatible materials, should be able to stimulate healing. However, the current evidence does not support a recommendation for any one of the materials over the other (13). $Ca(OH)_2$ is still the material of choice in practices because of its excellent therapeutic options (14). Indirect pulp treatment with $Ca(OH)_2$ has an overall success rate of 86.9 percent. Therefore, the failure to recover is around 13 percent and can develop into pulpitis (15, 16).

$Ca(OH)_2$ is the most used protective pulp liner due to its alkalinity as a strong base (approximately 12.5−12.8), its biocompatibility as the ability to induce dentin bridges formation, and antibacterial ability (17). The mechanical of action of $Ca(OH)_2$ is related to the ionic dissociation of the Ca^{2+} and OH^- ions in an aqueous environment, which effects the vital tissues (17, 18). According to the study of Cwikla *et al*., 2005, the antibacterial activity of $Ca(OH)_2$ increased effectiveness after 3 days as the more hydroxyl ions can diffuse into the dentinal tubules and $Ca(OH)_2$ alone showed a penetration depth of not more than 300 µm (19), whereas the bacteria can penetrate into dentinal tubules at least 500 µm from the cavity floors (20). Moreover, their antibacterial properties may deteriorate over time due to this material's tendency to dissolve within 1−2 years (21). As a result, the antibacterial activity of $Ca(OH)_2$ for the purpose of pulp protection, the elimination of residual bacteria underneath restorations, and to arrest caries progression, has been questioned.

Recent studies have identified bacteria species after using $Ca(OH)_2$ as a liner in the indirect caries treatments. *L. rhamnosus* is the most common anaerobic remaining bacteria found underneath restorations (22-24). It has an ability to survive in environments with a pH as low as 2.2. It uses the glycoprotein-rich dentinal fluid that diffuses from the pulp through dentinal tubules as a growth factor and its toxic products can diffuse, enter, and damage pulpal tissues. This may cause caries progression and pulp inflammation (6, 7, 9, 10, 22). Therefore, the development of a pulp lining material with antibacterial activity against *L. rhamnosus* is proposed.

Azadirachta indica or neem is a tropical tree native to India, Pakistan, Sri Lanka, Bangladesh, Thailand, Malaysia, Burma and widely spread in more than 30 countries (25, 26).

It has been utilized for treatment of various diseases since ancient times due to its therapeutic properties (27). In 1992, The United State National Academy of Science recognized it with an "Neem: A Tree For Solving Global Problems" (28). Recent studies have showed that neem possesses antiinflammatory, antipyretic, antibacterial, hepatoprotective and wound healing effects. It also exhibits anticancerous, antioxidant, antiviral, and antifungal behaviors. While there are more than 250 active ingredients that have established a relationship to therapeutic management, quercetin and ß−sitosterol are the first polyphenolic flavonoids isolated from the fresh leaves of neem. It is these flavonoids which influence antifungal and antibacterial activities (29, 30). Recent studies on neem leaf extract have showed that it has antibacterial properties effective against oral acidogenic bacteria such as *Lactobacillus* spp. (29-31). As neem has various therapeutic properties and grows easily in Thailand, the exceptional qualifications of these plants have influenced the development of an application for MID treatment. In this study, we investigated the penetrative and antibacterial abilities of an innovative paste containing neem extract against *L.rhamnosus* in the dentinal tubules.

1.1 Research objectives

1.1.1 To investigate the penetrative ability of neem paste through dentinal tubules.

1.1.2 To investigate the antibacterial ability of neem paste on *L.rhamnosus* in dentinal tubules.

1.2 Research hypotheses

1.2.1 General hypothesis:

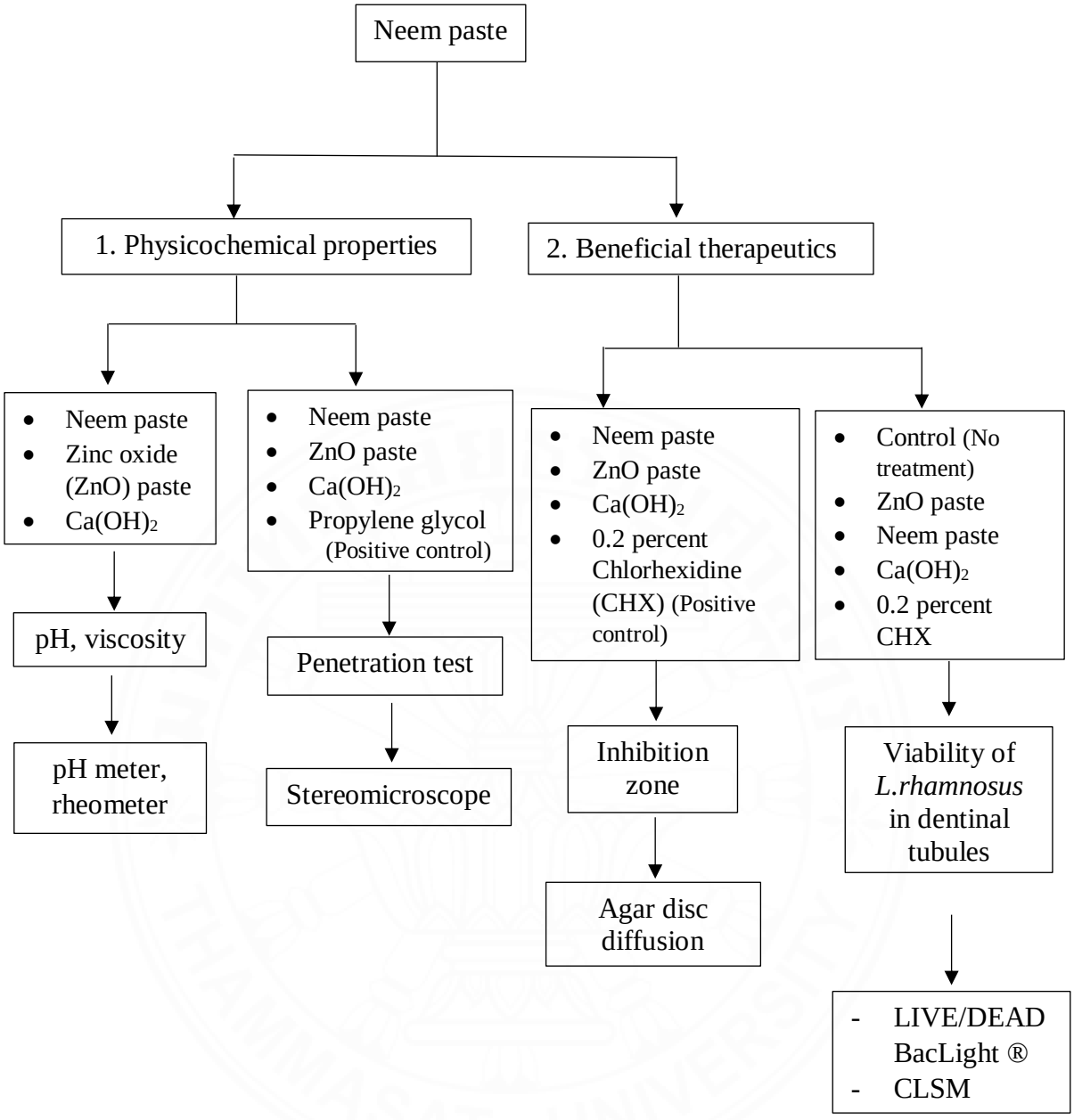
Neem paste is properly penetrating through dentinal tubules and inhibiting growth of *L.rhamnosus* in those dentinal tubules.

1.2.2 Working hypotheses:

1) Neem paste is more penetrable through dentinal tubules than $Ca(OH)_2$.

2) Neem paste is stronger at inhibiting growth of *L.rhamnosus* than $Ca(OH)_2$

1.3 Conceptual framework



CHAPTER 2
REVIEW OF LITERATURE

2.1 Prevalence of dental caries

Dental caries is a destructive process causing decalcification and proteolysis of teeth. This leads to destruction of enamel and dentin, which has been a long been health burden (32). According to the WHO statistics in 2016, while dental caries affects all age groups, it has a higher prevalence in children than in adults. It has been reported that 60-90 percent of children are affected by dental caries (1). The Global Burden of Diseases, Injuries, and Risk Factors Study, surveyed in 195 countries and territories from 1990 to 2017, estimated that the incidence of worldwide oral diseases affected close to 3.6 billion people. Approximately 2.5 billion people have suffered from caries of permanent teeth, and more than 1 billion children have suffered from caries of primary teeth. The prevalence of caries in deciduous teeth is about 5.3 million children, or 80.9 percent of all children (33). Dental caries is also one of the major problems for children in Thailand, as reported by the 8th National Oral Health Conditions of Thailand in 2017, a survey conducted every five years. The report showed that children aged 3 and 5 years old have the predominant percentage of dental caries at 52.9 and 75.6, respectively. Behavioral is also important to caries progression. Without behavior modification, caries will progress to permanent dentition, which is represented in children aged 12 and 15 years old. The prevalence of dental caries in children aged 12 and 15 was reported at 52 and 62.7 percent, respectively. Those who had not received any treatment had a higher rate of 31.5 and 40.1 percent, which contributed to early tooth loss at a rate of 3 and 7.1 percent, respectively. (2).

2.2 The etiology and caries hypotheses

Etiology of dental caries is still a challenge as this disease has been described as a chronic, invasive, and infectious process caused by complex factors resulting in a highly dynamic series of the disease's development. This can be characterized by inorganic demineralization and organic substance destruction of teeth, leading to cavitation (3). The necessary factors for caries's development include:

Firstly, a susceptible tooth represents a morphology position that gives microbials a chance to accumulate, such as in deep anatomical grooves, partial eruptions, gingival margin areas, areas under tooth contact, and fillings with inappropriate margins. These are considered

as caries risk factors because they are favorable areas for the retention and accumulation of plaque while presenting difficulties in self-cleaning and tooth brushing (34).

Secondly, while oral flora, pathogenic-organism composition, and thickness of the biofilm play roles in caries development, there is an early colonizer in the dynamic stability stage. The non-mutans bacteria (primarily non-mutans streptococci and Actinomyces) are the key microorganisms that presenting in early microbial ecosystems. Once the thickness of the biofilms is compromised and the acidic environment has been established, mutans streptococci and other aciduric bacteria may increase and promote lesion development. This leads to the aciduric stage, characterized as a net mineral loss environment (demineralization). The high proportions of mutans streptococci and/or other aciduric bacteria may be considered biomarkers of sites of particularly rapid caries progression (3).

Lastly, diet/food are key to caries development. The more frequent intake of fermentable carbohydrates in the diet, especially sucrose, causes the dental plaque to be more frequently exposed to low pH conditions. Oral bacteria such as *S. mutans* can rapidly metabolize dietary sugars into acids and be able to survive under these acidic conditions which promotes other cariogenic bacteria. Therefore, microbial homeostasis could contribute to greater risk of cavities development (3).

The current caries hypotheses theories attempt to explain the involvement of bacteria plaques and environmental changes in the development of dental caries:

- The Ecological Plaque Hypothesis:

Up to now, this has been an acceptable hypothesis to explain the initial stages of cariogenicity i.e., the enamel demineralization due to the disequilibrium in ecology. As a result of an increased fermentable carbohydrate intake, the environment in the plaque will change, spending more time below the critical pH level (approximately pH 5.5). At this point, the microbial ecology in the plaque promotes the proliferation of aciduric and acidogenic bacteria, which favors low pH. As the pH of the plaque environment becomes more acidic, the imbalance will lead to net mineral loss (Figure 2.1) (4, 35, 36).

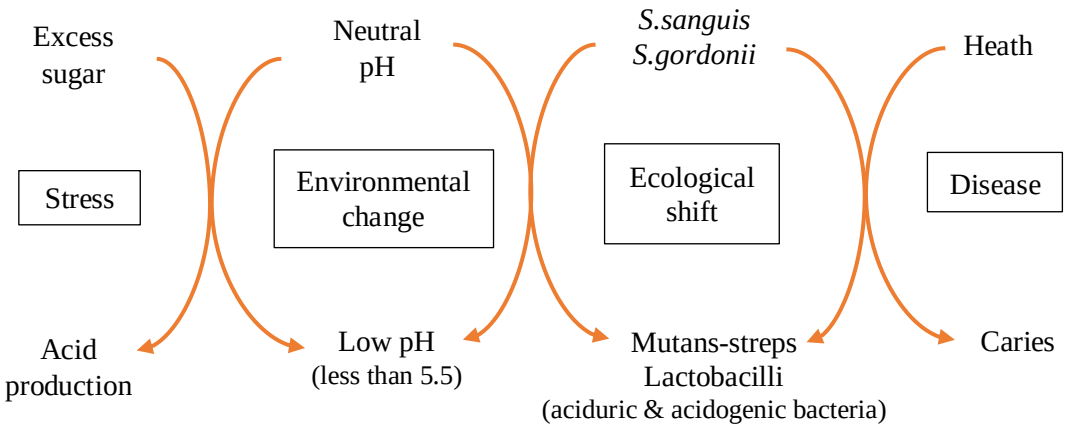


Figure 2.1 The ecological plaque hypothesis and etiology of dental caries: This diagram represents a dynamic relationship of environmental change in dental plaque which in result in caries due to increased acid production from higher sugar intake which leads to low pH. As more aciduric situations occur, the ecology will shift to acidogenic and acid tolerating species such as Mutans streptococci and Lactobacilli and finally to the development of disease. Modified from Marsh in 2006 (36).

- The Extended Ecological Plaque Hypothesis:

The concept of ecological plaque hypothesis is focused on the dynamic processes of mineral loss and gain (de- and remineralization) in dental biofilm related to sugar supply and pH change. Moreover, this microflora shift should be extended and strengthened by detailing the microbial acid adaptation, acid selection processes and including clinical manifestations of caries lesion processes. The concept of extended ecological plaque is supported by the mixed-bacteria ecological approach, which holds that dental caries is an infectious disease and that the proportion of acid producing bacteria is at the major of caries progression. It also states that to prevent and control the disease, the limiting of specific groups of microorganisms, such as *S. mutans* is necessary. That function will be provided by non-mutans bacteria. The non-mutans streptococci and Actinomyces will perform this function to transition the environment to a dynamic stability stage. (Figure 2.2) (4).

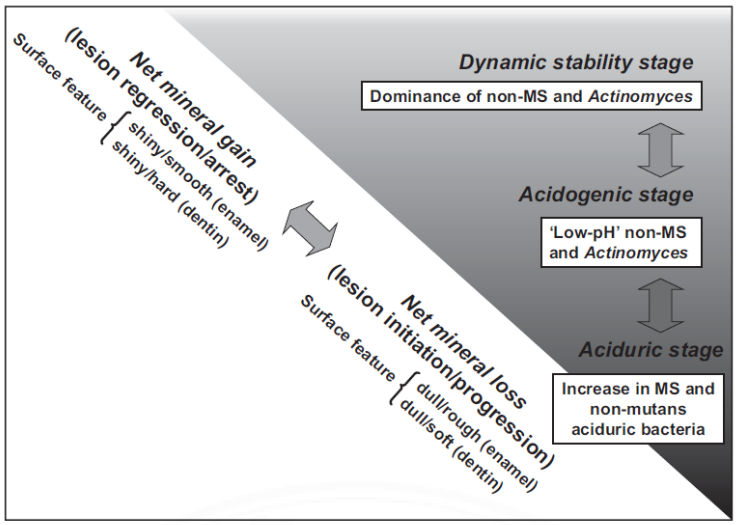


Figure 2.2 The extended caries ecological plaque hypothesis considering in changes of dental hard tissues 'mineral balancing related to the acidogenic and aciduric shift of the microorganisms in dental biofilm. Moreover, as this event is a reversible process, the dental hard tissues' surface could be reflected at any stage of lesion formation (4).

These two hypotheses focus on phenomena occurring in initial caries, which may differ from advanced caries due to lesions reaching dentin. Since dentin's composition is different from enamel, about 30 percent of it is composed of organic materials; primarily collagen type I. Therefore, the proteolysis hypothesis includes the destruction of organic component in protein elements by proteolytic enzymes in its explanation of dentin caries (5).

Dentin caries involves both demineralization and collagen degradation. These occur in 2 stages. The early stage is characterized by acidic conditions. Minerals are dissolved and retained in the cross banding of collagen fibers which serve as a scaffold for colonizing bacteria. In the advanced stage, once collagen is exposed, it can be broken down. This causes collagen fibers to lose their structural characteristics as host derived collagenase proteolytic enzymes are activated in the acidic environment. The dentin organic components contain proteases such as matrix metalloproteinases- 2, 3, 8, 9, and 20 and cysteine cathepsins B and K. These are produced during dentin formation and then become embedded in the dentin matrix (inactive forms), along with protease inhibitors such as tissue inhibitor of metalloproteinases (TIMP)-1 and -2. Once dentin is demineralized or exposed to the aqueous phase, these proteases activate and start to degrade the dentin organic matrix. Cysteine cathepsin B is reported to inactivate TIMP-1 and -2, and promote dentin caries formation (Figure 2.3). Once the superficial dentin

has been severely damaged, the pathologic bacteria will invade into the deeper dentinal tubules in the pulpal direction (37).

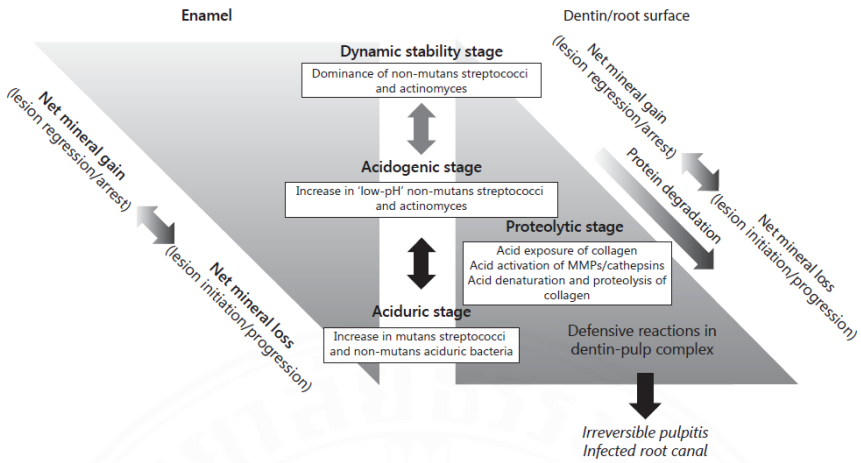


Figure 2.3 The expanded ecological plaque hypothesis considering which a proteolytic stage has been added for the development of dentin caries (37).

2.3 The microbiology of dental caries

The oral cavity is a host to a variety of microorganisms which can survive in a wide range of environmental conditions. When the balance of the oral environment shifts toward to low pH (acidic stage), the growth of acid producing and acid tolerant organisms will be supported and kill off acid susceptible bacteria (35).

S. mutans has been widely recognized as a key etiological agent of dental caries. It is an important constituent of the dental plaque biofilm in the oral cavity. It is also a predominant microflora of caries lesions and major virulent pathogens because *S. mutans* can synthesize extracellular polysaccharide glucan from sucrose which promotes bacterial adhesion to the tooth surface and to other bacteria. *S. mutans* also can produce intracellular polysaccharides as glycogen which act as a food store when dietary carbohydrates are low. Moreover, *S. mutans* are highly acidogenicity and acid tolerant. Their higher acid production gives an enrichment to aciduric species in the biofilm because others flora cannot survive under acidic conditions, leading to the caries progression (4, 35, 38). Compared to enamel caries, species composition may present divergently with dentin caries. This is due to its different ecological conditions, such as oxygen tension and types of nutrients available. Bacteria in dentin caries may have more complex ecosystem. In dentin caries, their locations can be divided into two parts. The

first part is superficial, lesion is soft, necrotic due to the collagen denatured, heavily infected with bacteria and is a highly demineralized zone (often referred as the decomposed zone and the infected zone). The other one, lesion is deeper and less infected. Even though dentin is demineralized, the collagens are still intact, so tissues are reversibly demineralized (often referred as the affected zone) (6).

In 1973, Loesche and Syed isolated organisms from human dentin carious lesion and report that *Streptococcus* spp., *Lactobacillus* spp., *Actinomyces* spp., and gram positive rod are strongly associated with the initiation or progression of dental caries (39). Other studies have showed that the deep areas of dentin carious lesion have strictly anaerobic conditions. There, obligately anaerobic bacteria and gram positive rods, such as *Propionibacterium* spp., *Eubacterium* spp., and *Bifidobacterium* spp., are predominant (7).

More recent studies have used molecular techniques for clonal analysis of the 16S rRNA bacterial gene. They have revealed that bacteria in dentin caries are more complex. The results show *S. mutans*, non-mutans streptococci, Actinomyces, Lactobacillus, Bifidobacterium, Propionibacterium, Veillonella, Selenomonas and Atopobium all present. In the deepest layers, the most prevalent species actively contributing to disease progression are *Atopobium genomospecies C1, Fusobacerium nucleatum* and Lactobacillus (8-10). The identification of bacteria in dentin carious lesions are showed in Table 2.1 (6, 7, 9, 10).

Table 2.1 Identification of bacteria species in dentin carious lesions

Bacterial genera or species	**Isolation frequency in carious lesion**	
	Superficial	**Deep**
Actinomyces *A. odontolyticus* (7) *A. gerencseriae* (7) *A. israelii* (7)	High	High
Atopobium genomospecies C1 (9)	High	High
Eubacterium *E. alactolyticum* (7) *E. aerofaciens* (7) *E. saburreum* (7)	High	High
Fusobacterium nucleatum (6)	High	High

Lactobacillus *L. fermentum* (6, 10) *L. casei* (6, 10) *L. rhamnosus* (10) *L. salivarius* (10)	High	High
Veillonella sp. (6)	High	Moderate-High
Streptococcus *S. mutans* (6, 9, 10) *S. sobrinus* (6) *S. sanguinis* (9, 10) *S. gorgonii* (9, 10) *S. salivarius* (9)	High	Moderate
Propionibacterium *P. acnes* (7) *P. avidum* (7) *P. lymphophilum* (7) *P. granulosum* (7) *P. jensenii* (7)	Moderate-High	High
Bifidobacterium spp. (6, 7, 10)	Moderate	Moderate
Prevotella spp. (9, 10)	Low	High
Olsenella uli (6)	Low	Low
Porphyromonas endodontalis (6)	Low	Low

Modified from Hoshino in 1985, Chhour *et al*., 2005, Aas *et al*., 2008 and Lima *et al*., 2011

2.4 Deep dental caries management

The traditional management of dental caries, known as complete caries removal, has involved the removal of all demineralized dentin before the filling is placed. This is a concept of "extension for prevention" which has been considered a gold standard for more than one hundred and fifteen years since it was proposed by Greene V. Black in 1908 (40). However, this concept has been questioned for possible adverse effects of removing all dentin carious lesions, since there are risks of pulp exposure and weakening of the tooth structure (41).

In 1992, Dawson presented the new ideology in conservative dentistry as MID (42) which considers on preserving the tooth structure and pulp vitality. The main goal of MID is to modify the microenvironment of the contaminated dentin that has been left under the

restoration by arresting the progression, stimulating tertiary dentin formation and allowing remineralization of the lesion under the concept of removal all superficial necrotic decomposed and infected dentin and leaving demineralized or affected dentin underneath after that restore with a good sealing restoration that has optimum biological and physical properties (11, 12). According to the guidelines of the AAPD, MID is also known as indirect pulp treatment which is recommended for treatment teeth with deep carious lesions without signs or symptoms of pulp inflammation (13).

2.4.1 The minimal intervention techniques

The minimal intervention approach can be classified into 4 techniques based on the level of lesions: shallow and moderate lesions are determined as there is no risk of pulp exposure and the radiograph examination, the lesion involves the outer-middle one-thirds of dentin. While deep lesions are determined that there is a risk of pulpal exposure and the radiograph examination, the lesion involves the inner one-third of dentin. The classification of MID has been showed as follows: (43)

2.4.1.1 Atraumatic restorative treatment:

This technique requires the removal of carious tissues using hand instruments only. The pulpal floor is excavated to firm dentin in shallow lesions and to soft dentin in deep lesions. Then, they are filled the cavity with adhesive dental material, usually a high viscosity glass ionomer cement.

- Indications for primary and permanent teeth: shallow and moderate dentin carious lesions

2.4.1.2 Selective removal to soft dentin: (partial, incomplete caries removal):

This technique, the peripheral of the carious cavity is removed to hard dentin, but the carious tissues on the pulpal floor are removed until soft dentin, to avoid pulp exposure. Then, they are filled the cavity with adhesive dental material, usually a high viscosity glass ionomer cement.

- Indications for primary and permanent teeth: deep carious lesions

2.4.1.3 Selective removal to firm dentin: (partial, incomplete caries removal):

This technique is like the second one except on the pulpal floor, the carious tissues are removed until leathery or firm dentin (resistant to hand excavator). Then, they are filled the cavity with adhesive dental material, usually a high viscosity glass ionomer cement.

- Indications for primary and permanent teeth: shallow and moderate dentin carious lesions

2.4.1.4 Stepwise caries removal: (stepwise excavation, 2-step caries removal):

Regrading technique, carious lesion is a selective removal to soft dentin. After 6-12 months, the provisional restoration is removed and reentered to the pulpal floor of the lesion, selective caries removal is continued to firm dentin, and then the final restoration is placed.

- Indications for only permanent teeth: deep carious lesions

According to AAPD, materials which have been recommended for being a liner that can be placed over the remaining carious dentin lesion and be able to stimulate healing include dentin bonding agent, resin modified glass ionomer, $Ca(OH)_2$, MTA or any other biocompatible materials. However, the current evidence does not support a recommendation for any one of the materials over the other (12, 13). In 2014, Petrou *et al.*, has been studied on comparison of the clinical and microbiological outcomes of MTA, medical Portland cement, and $Ca(OH)_2$ as a liner for pulp protection of permanent and primary teeth with indirect pulp treatment. Among the different materials, they do not have a difference in significance. The overall success rate of the indirect pulp treatment is 90.3 percent, while $Ca(OH)_2$ results are 86.9 percent (15). However, even though the successes of indirect pulp treatment show no difference among materials, $Ca(OH)_2$ is still preferred as the appropriate material. It is a material of choice in practices because of its excellent therapeutic option (14, 16).

2.4.2 Calcium hydroxide

$Ca(OH)_2$ has been used as a cavity liner in the deep dental caries treatment for a long time. The purpose of using a liner is to reduce the remaining infected bacteria that are left over the pulpal floor and to protect pulp vitality by promoting the development of reactionary dentin and capability to induce the formation of a mineralized bridge over pulpal tissue, a process known as dentin bridge formation (44). $Ca(OH)_2$ is the most used as a protective pulp liner due to its alkalinity as a strong base with a high pH (approximately 12.5–12.8), biocompatibility as

the ability to induce pulp-dentin remineralization, and a wide range of antimicrobial action to decrease bacterial dentin infection (17).

2.4.2.1 Mechanical of action of calcium hydroxide

Mechanisms of antimicrobial activity of $Ca(OH)_2$ is related to the ionic dissociation of calcium ion (Ca^{2+}) and hydroxyl ion (OH^-) ions in an aqueous environment which effecting the vital tissues. OH^- are highly oxidant free radicals that show extreme reactions with several biomolecules. It is portable that the lethal effects on bacterial cells are probably due to the following mechanisms (Figure 2.4) (17, 18).

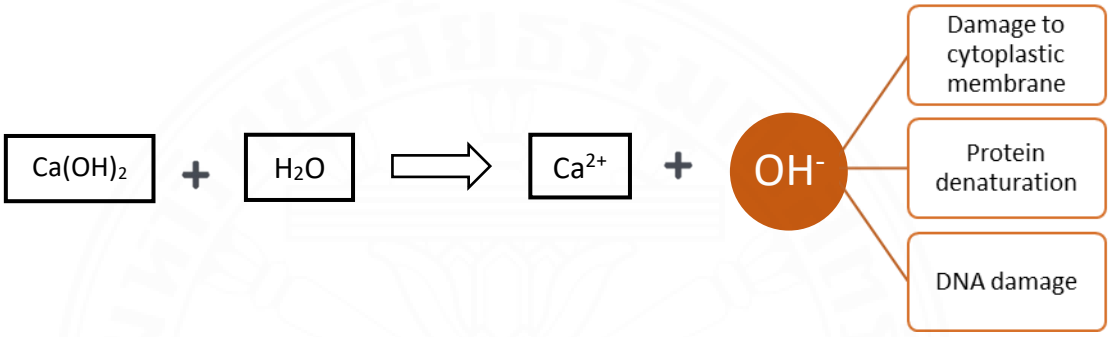


Figure 2.4 The mechanism of $Ca(OH)_2$ in antimicrobial activity

- Damage the bacterial cytoplasmic membrane

The bacterial cytoplasmic membrane is a vital component that acts as a selective permeability membrane, transporting an electron and other important substances related to cell survival. Hydroxyl free radical ions of $Ca(OH)_2$ will induce lipid peroxidation, resulting in the destruction of phospholipids, structural components of the cellular membrane and finally causing an extensive membrane damage (18).

- Protein denaturation

Cellular metabolism is dependent on enzymatic activities which can function normally in a narrow range of pH. However, the high pH (alkalinization) provided by $Ca(OH)_2$ will induce the breakdown of ionic bonds that maintain the tertiary structure of proteins. These changes frequently result in the loss of the biological activity of the enzyme and the disruption of the cellular metabolism leading to structural protein denaturation (17).

- Damage the DNA

Since $Ca(OH)_2$ can release OH^- as highly oxidant free radicals in the aqueous environment, these ions will react with the bacterial DNA and induce the splitting of the strands. Genes are misplaced. Therefore, DNA replication is inhibited, leading to lethal mutations (17, 18).

Although $Ca(OH)_2$ applied as a cavity liner can cause dentin bridges formation over pulpal tissues while its contact, their antimicrobial properties may deteriorate over time due to this material is tendency to dissolve within 1-2 years. Furthermore, the majority of dentin bridges formation appear to have tunnels defects which may affect pulpal tissues becoming infected or necrotic because of microleakages (21). Moreover, the antimicrobial activity of $Ca(OH)_2$ is related to the alkalinizing action. According to the study of Cwikla *et al.*, 2005, the antimicrobial activity of $Ca(OH)_2$ increased effectiveness after 3 days since the more OH^- can diffuse into the dentinal tubules and $Ca(OH)_2$ alone showed a penetration depth not more than 300 µm (19), whereas the bacterial can penetrate into dentinal tubules at least 500 µm from the cavity floors (20). As a result, the reliance on the antimicrobial activity of $Ca(OH)_2$ for the purpose of pulp protection and the elimination of residual bacteria underneath restorations in order to arrest caries progression has been questioned.

In 2018, Luciana Bitello-Firmino *et al.*, had a comparison of microbial load between selective caries removal by using $Ca(OH)_2$ as a protective pulp liner and complete caries removal. Three months after sealing, there is no difference between the microorganism counts in the complete caries removal before and after sealing, whereas the selective group, after sealing, showed a reduction of total microorganisms (*Streptococcus* spp. and *Lactobacillus* spp.) compared to the complete group. However, in the selective caries removal, *S. mutans* is no longer visible whereas *Lactobacillus* spp. is still detected (45).

Lactobacillus spp. is represented as the dominating genus before sealing on permanent teeth, as reported in 45 percent of the total colony forming units. The most frequently isolated and predominant species is *Lactobacillus casei* subsp. *rhamnosus* followed by *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus casei* subsp. *casei* in the study of Bjorndal and Larsen in 2000 (24). Furthermore, a study on the prevalence of *Lactobacilli* sp. in different stages in primary molar by Kneist *et al.*, 2010 found that after caries removal, the *Lactobacilli* sp. decreases to 34 percent, while after 8 weeks and 11 months as $Ca(OH)_2$ being a liner, the *Lactobacilli* sp. remains at 11 and 9 percent, respectively. Even though this study reveals a decrease in *Lactobacilli* sp., some species, such as *L. rhamnosus*

and *L. paracasei* subsp. *paracasei*, are still found in all stages of caries progression (22). Moreover, Callaway *et al*., 2013 also studied on *Lactobacilli* sp. in primary teeth with modern techniques for identification of microorganisms like matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. After 8 weeks of using $Ca(OH)_2$ as a liner, there are 5 species are detected: *L. gasseri, L. parabuchneri, L. paracasei, L. paracasei* subsp. *tolerans and L. rhamnosus.* However, after 11 months, the diversity among the species of lactobacilli has decreased significantly, and only *L. paracasei* and *L. rhamnosus* remain, with *L. rhamnosus* being the most prevalent (23). The identification of bacteria species after using $Ca(OH)_2$ as a liner in either selective or stepwise caries treatment is showed in Table 2.2.

Table 2.2 Identification of bacterial species after selective or step wise caries treatment

	Bjorndal and Larsen., 2010 (24)	**Kneist *et al*., 2010** (22)	**Callaway *et al*., 2013** (23)
Times of dentin collection	4-6 months after step wise caries treatment in permanent teeth	8 weeks and 11 months after step wise caries treatment in primary teeth	8 weeks and 11 months after step wise caries treatment in primary teeth
Identification technique	Species determination among the colony formation units	Species determination among the colony formation units and MALDI-TOF mass spectrometry	Species-specific PCR and MALDI-TOF mass spectrometry
Results	Detected species after 4-6 months: - *L. brevis,* - *L. plantarum,* - *L. acidophilus* - *L. casei* subsp. *casei* - *L. casei* subsp. *rhamnosus* (The most prevalent)	Detected species after 8 weeks: - *L. alimentarius* - *L. paracasei* subsp. *paracasei* - *L. rhamnosus* Detected species after 11 months: - *L. paracasei* subsp. *paracasei* - *L. rhamnosus* (The most prevalent)	Detected species after 8 weeks: - *L. gasseri* - *L. parabuchneri* - *L. paracasei* - *L. paracasei* subsp. *tolerans* - *L. rhamnosus* Detected species after 11 months: - *L. paracasei* - *L. rhamnosus* (The most prevalent)

2.5 *Lactobacillus rhamnosus*

L.rhamnosus is a gram positive, acidogenic, aciduric, and proteolytic anaerobic bacteria that can exist without oxygen and survive on their fermentative metabolism by producing lactic acid from carbohydrate fermentation. Moreover, it is not affected by the presence of oxygen as an obligatory anaerobe. Their morphological features can be either elongated or short nonmotile rods, which are usually seen in chains and can be bent (Figure 2.5) (46).

In deep carious lesions, *L.rhamnosus* still persists and can be a major pathogen causing pulp inflammation. In the previous reviews, it is the most prevalent anaerobic bacteria remaining beneath restorations may cause by its ability to survive with a pH as low as 2.2. Moreover, it also has a type I collagen binding activity and the ability to survive and grow by utilizing the glycoprotein-rich dentinal fluid that diffuses from the pulp through dentinal tubules. As, its ability to proliferate near the pulp, that may favor the diffusion of its toxic products into the pulp tissues and cause tissues damage. Furthermore, a reduction in dentin permeability due to dentinal tubular sclerosis leads to some bacteria being unable to survive after sealing, components of those dead bacteria may act as a source of nutrients for *L. rhamnosus* to survive beneath the restorations and cause caries progression and pulp inflammation (6, 7, 9, 10, 22). As a result, the developing a pulp lining material with antibacterial activity against *L. rhamnosus* is critical.

In the laboratory, they can growth at 37°C in an atmosphere of 5 percent of CO_2 or aerobically for 24-48 h in the De Man, Rogosa and Sharpe (MRS) agar/broth (47).

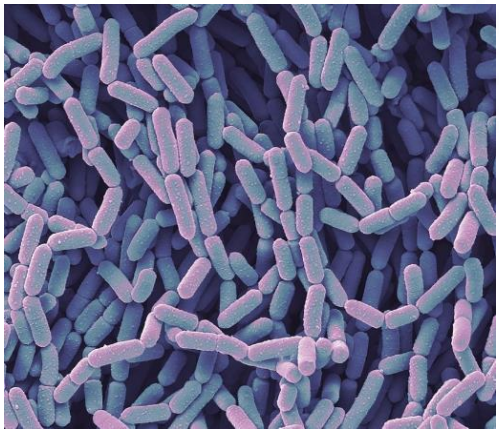


Figure 2.5 *L. rhamnosus*, colored scanning electron micrograph (SEM) (48).

2.6 Neem

2.6.1 Botanical description

Neem is a member of the Meliaceae family, which is also known by the botanical name *Azadirachta indica*. Neem is mainly abundant in India and widely found in more than 30 countries; Asia, Africa, and America. The Taxonomic position of neem is displayed in Table 2.3 (25).

Table 2.3 Taxonomic position of neem

Scientific classification	
Kingdom	Plantae
Division	Magnoliophyte
Order	Rutales
Suborder	Rutinae
Family	Meliaceae
Subfamily	Meliodeae
Tribe	Melieae
Genus	*Azadirachta*
Species	*Indica* *Indica* var. *siamensis* *Indica excelxa*

Neem is fast growing, evergreen and often found in tropical and semitropical areas. The average height is approximately 16 m. The bark is thick, dark gray in color with longitudinal grooves, while the heartwood is reddish brown. The leaves are dark green, alternate compound leaves, and lanceolate leaflets are 4-8 cm long. The off-white-colored flowers have a light scent and also have 5 petals and 5 sepals. The Neem fruit resembles a grape, has smooth skin and there are 1-2 seeds inside (26).

2.6.2 Therapeutic properties

Neem has plenty of different active compounds to play in many therapeutic management roles. More than 250 active ingredients have been established. The main constituent is azadirachtin. Others are numbin, nimbidin, nimbolinin, nimbidol, sodium nimbinate, salannin, gedunin, quercetin, and polyphenolic flavonoids. The leaves, blossoms, seeds, fruits, roots, and bark of the neem tree have all been traditionally used to treat a variety of diseases (29).

1) Anticancerous activity

Cancer is a group of diseases in which uncontrolled growth of cells develops by gene or DNA mutation with/without spreads into other parts of body. Cancer is a big problem in the worldwide medical industry because it is usually in remission and the current treatments introduce side effects to normal cells. Thus, researcher is continuing pursued for new treatment to stop the remission and completely cure the diseases. The experiment was carried out to evaluate the chemopreventive potential of the neem limonoids azadirachtin and nimbolide based on *in vivo* inhibitory effects on 7,12-dimethylbenzanthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis. The results revealed azadirachtin and nimbolide can suppress DMBA-induced HBP carcinomas by multiple pathways, including prevention of procarcinogen activation and oxidative DNA damage, upregulation of antioxidant and carcinogen detoxification enzymes, and inhibition of tumor invasion and angiogenesis (49). Furthermore, induction of apoptosis and anti-proliferative activity by ethanolic extraction of neem leaves could be the reason cancer cells significantly decrease protein expression, including vascular endothelial growth factor signaling molecules (50).

2) Hepatoprotective effect

The experiment was conducted to investigate the neem active constituent nimbolide for its hepatoprotective properties against carbon tetrachloride induced liver toxicity in rats compared with Silymarin, the hepatoprotective standard treatment. The study results showed nimbolide has dose dependent hepatoprotective with an efficiency like that of Silymarin (51).

3) Wound healing effect

A study was conducted to determine the wound healing effect of the aqueous leaf extracts of the neem tree. The results showed the extract promotes wound healing activity through increases mediator response and neovascularization (52).

4) Antioxidant activity

Free radicals, unstable molecules, are the main cause of cell/DNA/cell membrane damage. They are important criminals who can develop many diseases in the medical industry. Interruption of the free radical chain is a priority for management of diseases. Antioxidants, substances that can scavenge free radicals, come from many medicinal plants, including neem (29). Leaves, flowers, fruits, and bark extracts from the Siamese neem tree have been studied for their antioxidant activity. The results of the studies showed that leaf aqueous extract, flower and bark ethanol extracts have strong free radical scavenging activity (53). A study was performed to assess the antiradical scavenging activity and reductive potential of azadirachtin and nimbolide. The results showed that both azadirachtin and nimbolide effectively play a role as free radical scavenging activities. Azadirachtin is reported to have higher radical scavenging activity than nimbolide (49).

5) Antiviral activity

A study of antiviral effect of herpes simplex visus-1 (HSV) infection of naturally susceptible cells, such as HeLa and retinal pigment epithelial cells, could be inhibited by an aqueous extract of neem. Since glycoprotein D expression which found in neem extract was significantly reduced X-gal stained HSV cells known as blue cell (54).

6) Antifungal activity

The antifungal activities of neem powder added to denture base material against *Candida albicans* were studied by Hamid *et al*., in 2019. Neem and acrylic powder were mixed with an electric mixer at a rotating speed of 400 rpm to produce a homogenous powder. The specimens were prepared using heat cured and self cured acrylic resin before being examined for *C. albicans* adhesion. They submerged the specimens in $2x10^6$ *C. albicans* cells in artificial saliva. The antifungal activity of heat cured acrylic resin containing neem powder was remarkable (55).

7) Antiinflammatory effect

A variety of immunological responses to intrusive stimuli, such as infections, injuries, and poisons are referred to as inflammatory reactions. Inflammation is characterized by pain, swelling, redness, and heat. Many chronic diseases such as diabetes, arthritis, asthma, atherosclerosis and cancer can be triggered by inflammation. The study has been reported to investigated the antiinflammatory effects of a methanolic neem leaf extract mediated by regulation of the nuclear factor-kB pathway. They treated human chronic myeloid leukemia cells with pure compounds present in neem leaves, including quercetin, nimbidin, and salannin. The result found that quercetin had a strong NF-kB inhibitory effect as measured by a luciferase assay system as a dosage of 240 μg/ml of neem leaf extract, transcription factor concentration was suppressed by more than 80 percent (56). A study was undertaken to evaluate the antiinflammatory effect of aqueous extract of neem leaves compared with dexamethasone. The finding showed neem leaf extract has a significant antiinflammatory effect but less than dexamethasone (57). Another result of the study concluded that seed oil of neem producing nimbidin has the potential to suppress the functions of macrophages and neutrophils related to inflammation (58).

8) Antipyretic effect

A study was conducted to prove the efficacy of neem leaf extract by using the brewer's yeast induced pyrexia model in rats. The results reported the dose of neem leaf extract of 125 mg/kg bodyweight showed a significant antipyretic effect (27). Another finding based on 75 percent of methanolic extract of neem leaves was performed to evaluate the activity against fever and the results showed the extract also has a good antipyretic effect (59). Nimbidin is neem's active constituents for antipyretic effect can be found in aqueous extract bark, methanolic extract leaves (60).

9) Antibacterial effect

Bacteria are microorganisms that have a variety of functions that are beneficial to human beings, and some are also harmful and cause infections. Neem and its active ingredients have been reported to play a key role in bacterial growth inhibition. An experiment was performed to assess the antimicrobial activity of neem extract against 21 strains of foodborne pathogens. The finding confirmed that neem leaf extract has the potential to control foodborne pathogens (61). In dental application, neem also works against periodontal pathogens as well as oral acidogenic bacteria that can cause dental diseases. Therefore, the following literature

review will focus on different components of neem extract to an oral acidogenic bacteria, and primarily to *S. mutans* and *Lactobacillus* sp. (62).

(1) *Streptococcus mutans*

Jain *et al*., in 2015 evaluated aqueous extracts, crude drug powder, and organic solvent based extracts (chloroform, acetone, ethanol) of neem leaves for their ability to inhibit the growth of *S. mutans* by the agar well diffusion method. The result showed that aqueous neem leaf extract (powder 10 g to final volume one-fourth of the original volume) inhibited *S. mutans* more effectively than organic solvent based extracts (2 ml of stored plant extracts were dissolved in 1:l of dimethyl sulfoxide) while the crude drug powder does not show an inhibition zone (63).

The other studies of neem and babool sticks (*Acacia nilotica*) aqueous extract with 5,10 and 50 percent (the power weight 5 g, 10 g, 50 g with 10 ml distilled water) compared with 0.2 percent CHX for testing on antibacterial efficacy against *S. mutans*. Antibacterial activity was evaluated using an agar ditch plate method. Neem extract had a highest inhibitory effect compared with babool extract and CHX at all concentrations. However, only 5 percent of neem extracts showed no difference than babool extract (64). Similar studies of neem and babool sticks aqueous extract have examined their effects on *S. mutans*. They have had similar results, as neem aqueous extract has a significantly higher level of antimicrobial activity than babool aqueous extract, while maximum antimicrobial effect was exhibited by 50 percent of neem extract with a mean zone of inhibition of 4.0 mm (65).

Bhuva and Dixit in 2015 had a study on antibacterial effects on *S. mutans* by comparing different solvent of neem leaf extracts (ethanol, methanol and distilled water) by 1 g of powdered plant material of neem is added to 10 ml of methanol, ethanol and distilled water on an agar diffusion technique. The results showed neem leaf extracted by methanol extract recorded higher activity against *S. mutans* than ethanol or aqueous extracts (66).

Siswomihardjo *et al*., 2017 had a study on the antibacterial activity of neem leaf and stick extracts against *S. mutans* as agar disc diffusion technique at 10 and 20 percent concentrations of leaf and stick extracts using ethanol. The antibacterial properties of neem stick extract were higher than those of the leaf extract at all concentrations (67).

Pai *et al*., 2004 had a study on neem leaf extracted by ethanol in formulation as gel with final concentration of 25 mg/g against plaque formation compared to 0.2 percent CHX mouthwash at 6 weeks. The result showed as dental gel containing neem extract has

significantly reduced the plaque index and bacterial count than that of the control group due to the reduction on *S. mutans* in saliva (68).

The study of Mittal *et al*., in 2011 had a study on the effective of neem leaf extracted by methanol (power of neem: 50 g) against *S.mutan* compares with 0.2 percent CHX on the agar ditch plate method. The result showed antimicrobial activity of neem leaf extract on *S.mutan* is comparable to that of 0.2 percent CHX (69).

The other study of antibacterial activity of neem leaf extracts against pathogens causing dental plaque in fixed orthodontic appliances was examined as the result found that neem ethanolic leaf extracts at concentration of 500 µg/ml and 1,5 and 10 mg/ml have antibacterial activity over against *S. mutans* (70).

(2) *Lactobacillus* sp.

Antiplaque activity of neem leaf extract gel in a clinical study was performed by Pai *et al*., in 2004 as the effectiveness of 25 mg/g neem extract gel formulation is tested by administering neem gel to subjects for a period of 6 weeks. The result showed that treatment with neem extract gel significantly reduced plaque index, and the *Lactobacillus* sp. count in saliva after 6 weeks (68).

Yerima *et al*., in 2012 performed an agar diffusion study on the antibacterial activity of neem bark, leaf, seed, and fruit aqueous extracts on bacteria isolated from adult mouth. All of the bacteria tested showed antibacterial activity against the bark and leaf extracts. The zone of inhibition expanded as extract concentrations increased. The antibacterial property of 30 and 60 mg/ml stem extracts and 90 mg/ml leaf extracts were detected over *Lactobacillus* sp. and the concentration of 90 mg/ml leaf extracts resulted in the most sensitivity (28).

(2.1) *Lactobacillus acidophilus*

Mittal *et al*., in 2011 compared the antimicrobial activity of neem leaf extracted by methanol (powder of neem: 50 g) on *L. acidophilus* to that of 0.2 percent CHX. The results showed that the antimicrobial activity of neem leaf extract is closed to that of 0.2 percent CHX (69). Alqahtani discovered a similar result when he prepared a 2 percent mouthwash with neem leaf extract by alcoholic solvent (sodium benzoate and benzoic acid) to compare its antimicrobial property against *L. acidophilus* to a 0.2 percent CHX mouthwash. *L. acidophilus* is sensitive to neem extract at concentrations as low as 1.6 µg/ml (31).

The antimicrobial activity of neem twig extracts was investigated by Shah *et al*. They used 10 and 25 percentage concentrations of neem extract to test antibacterial activity against *L. acidophilus* with 0.2 percent CHX as a positive control. The disc diffusion method was included in this study. They discovered that neem extract was less effective than 0.2 percent CHX (71).

(2.2) *Lactobacillus rhamnosus and casei*

Gupta *et al*., in 2016 conducted a study on the antibiotic activity of neem in the form of aqueous extracts of the leaves. The results showed that the aqueous extract of neem leaf has effective bactericidal activity against *L. rhamnosus*, which is greater than the inhibition zone found in the aqueous extract of neem seed, although there was no antimicrobial activity against *L. casei* (30).

2.6.3 Biocompatibility of neem extract

Jerobin *et al*., in 2015 investigated the toxicity of neem nanoemulsion (NE) on human lymphocytes as well as its antibacterial activity. The toxicity of human lymphocytes is determined by measuring the levels of lactate dehydrogenase, reactive oxygen species, and lipid peroxidation. Gel electrophoresis have been used to examine DNA damage after exposure to NE at various concentrations (0.7, 1.0, 1.2, 1.5, 1.7, and 2 mg/ml). The results showed that the ability of the NE at lower concentrations (<1 mg/ml) can induce significant toxicity to bacteria without any damage to lymphocytes whereas at high concentrations of 1.2–2 mg/ml found to be toxic to human lymphocytes (72).

Verma *et al*., in 2018 determined the cytotoxicity of neem extract of neem leaf and bark extracted by 0.5 percent ethyl acetate on neem leaves and 15 percent ethanol on neem barks in on human gingival fibroblasts using fluorescence activated cell sorting analysis to determine cell cycle stages with 0.2 percent of CHX mouthwash. The neem extract treated group does not adversely affect the fibroblasts even up to 50 percent concentration showing a less toxic effect in comparison with the experimental group (73).

2.7 Pharmaceuticals

2.7.1 Topical medications

Topical pharmaceuticals are drugs that are applied to the skin or mucous membrane and can be administered by a variety of routes, including dermatological, ophthalmic, vaginal, oral, nasal and, rectal (74, 75).

2.7.1.1 Formulation of topical medications

To formulate topical medication, there are 4 important components, which are power, water, ointment, and emulsifier. The difference in the ratio of these components will relate to the physical properties and dosage forms of medications. In the universe of topical medications, each part and dosage forms was presented in Figure 2.6 (74-76).

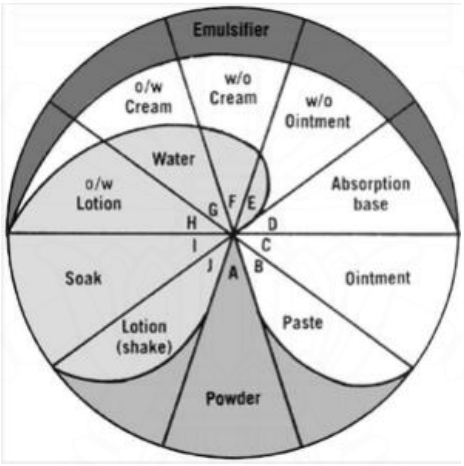


Figure 2.6 Universe of topical medications (76)

According to the universe of topical medications (Figure 6), the difference in 4 main components could form to 10 forms of dosage. However, there are only 5 forms that are used regularly (74-76) including:

(1) Powder

A powder is a solid (or a mixture of solids) that has been finely ground. Topical powder should be nonirritation, uniform, free-flowing and easy to skin adherence. The characteristics of powder determine by size and surface area of particle. Powder can be used as a moisture adsorption drying agent, a dermatological protective agent, or a lubricant in many applications.

(2) Paste

Paste, a semisolid preparation, is formed from powder and oleaginous components which are wax or fat, such as paraffin, petrolatum, or mineral oil. Stiffness, porousness, greasiness, and nonocclusiveness are characteristics of the paste which are beneficial since they aid in skin adherence, and absorption.

(3) Ointment

An ointment is a semisolid mixture of medical substances and ointment bases or oleaginous materials. Lubrication and emollient properties are beneficial for dry, scaly, and cracked regions. In which oleaginous attributes produce a long lasting effect. Ointment bases are used as drug carriers which transport medicines or substances to the skin. The United State Pharmacopoeia (USP) classified ointment bases into 4 categories: hydrocarbon/oleaginous bases, absorption bases, water removal bases, and water soluble bases. Appropriate base selection could result in excellent drug release, absorption, therapeutic effect, removability and stability of the product.

(4) Cream

Cream is either viscous liquid or semisolid that is made from 3 portions, including: emulsifier, oleaginous ingredients, and water in various proportions. The emulsifier plays a significant role in maintaining the lipid and water phase singularly. Emulsifying agents are involved in nonionic surfactants, detergents, and soaps. Creams are frequently used in a variety of means because they are easy to apply to the affected area, have an occlusive effect, are washable rapidly and are gentle to the touch.

(5) Lotion

Lotion is a type of fluid emulsion dosage form that contains a lot of water, as well as an oily phase and an emulsifying agent. The affected region receives protection and moisture through lotion. Lotion should be easy to apply, have a smooth texture, be redispersible, dry quickly, be washable, and maintain an elastic protective layer after water evaporation.

2.7.2 Pastes

According to the USP, pastes are semisolid dosage forms containing one or more drug ingredients intended for topical application. Pastes are classified into 4 categories according to the type of base involved in its formulation (74, 77).

(1) Hydrocarbon or Oleaginous base

A hydrocarbon base, commonly known as an oleaginous base, is a base that contains a chain of hydrocarbons. The main characteristics of this base are its greasy, anhydrous, and water insoluble. They use an emollient effect to keep moisture for the skin, an occlusive effect to maintain a protective layer on the affected area, and as an excellent base nomination for medical substances that are water insoluble. The hydrocarbon bases are stable and emollient, although they have a severe weakness such as greasy. This greasy or oily substance is difficult to remove and can stain skin or clothing. Petrolatum, paraffin, vegetable oil, animal wax, white ointment, yellow ointment, and synthetic ester are examples of hydrocarbon bases.

(2) Absorption base

Absorption bases, as a hydrocarbon base, provide excellent emollient and occlusive properties because of their difficult removal from the skin with water. The absorption base is divided into 2 categories:

(2.1) Anhydrous base

Anhydrous base, such as anhydrous lanolin and hydrophilic petrolatum USP, is a type of base that comes with the capability of absorbing aqueous solution. The anhydrous base can become hydrous once it absorbs aqueous solution, generating a water in oil emulsion. This base is a useful incorporation aid for water soluble drugs.

(2.2) Hydrous base

Hydrous base is in the form of a water in oil emulsion. The aqueous absorption performance of the hydrous base persists, although with less effect than the anhydrous base. Modified lanolin is an example of hydrous base that is processed to reduce irritation.

(3) Emulsion base

Emulsion bases are 2 types of oil in water emulsions and water in oil emulsions. Cold cream is a sample defined as water in oil emulsion because the outer phase being oil. The water in oil emulsion acts as protective film on the surface, resulting in an occlusive effect. On the

other hand, oil in water emulsion, also known as water removable base, is widely used, and easily removed by water.

(4) Water soluble base

The water soluble base is nonoleaginous, nonocclusive, water soluble, completely water washable and nonabsorption base. This base is typically used to integrate solid materials and are ineffective when used with aqueous solutions.

Polyethylene glycol (PEG) is the prototype example of a water soluble base. The characteristics of PEG are inert, nonvolatile, water soluble and appear in both liquid and wax depending on the molecular weight. PEG is a clear, colorless, and viscous liquid with a low molecular weight of 200–600 g/mol. While PEG is an opaque, silky, white semisolid that exists between molecular weight of 1000 and 1500 g/mol. Moreover, PEG is a waxy white substance when it has a molecular weight of more than 1,500 g/mol.

2.7.2.1 Paste formulations

Pastes can be made by 2 different methods including fusion and inclusion. The distinction between the 2 procedures is mostly determined by the type of the ingredients as follow. The excipients and roles for paste formulation are represented in Table 2.4 (74, 77).

(1) Fusion method is mainly used to prepare oleaginous or water soluble bases. Substances are melted together in a porcelain dish and stirred continuously until they congeal by 3 different techniques, including:

Technique 1: Produce a melted substance, the maximum melting point of a substance is heated to the lowest required temperature, then keep stirring and other components will be added.

Technique 2: The lowest melting point of a substance is beginning to heat. Other materials will be topped up by order of mixing.

Technique 3: All of the components are melted together as the temperature rises.

(2) Inclusion method by levigating: prepared by using a spatula and rubbing components on a slab (a large glass or porcelain plate). The incorporation method is suitable for paste that has powder or liquid as active ingredients.

Technique 1: Incorporation of powder with base. Initially, powders are sieved into tiny particles or ground into extremely minute particles. Powders are levigated together with small

amounts of base using geometric dilution. In addition, a levigating agent was added to the paste as a homogenizing agent. The geometric dilution process is repeated until the powder and base are uniformly mixed.

Technique 2: Incorporation of liquid with base. Liquid, active ingredients, must be composed to formulate. First, liquid is combined with a small amount of absorption base, then blended into an oleaginous base. The incorporation of excessive liquid active ingredients into the base might impact the physical characteristics of the base. It may cause the base to become liquid or lose its properties.

Table 2.4 Excipients and roles for paste formulation (74, 77)

Excipients	**Example**	**Roles**
Levigating agent	Mineral oil, glycerin	Homogenizing ointment and/or paste
Surfactant	Sodium lauryl sulfate, sodium dioctylsulfosuccinate, polysorbate	Reduce surface or interfacial tension
Solubilizing agent	Alcohol, glycerin, propylene glycol, water	Improve solubility of ingredient
Emoillient	Paraffin, mineral oil, acetyl alcohol, stearyl alcohol, olive oil, lanolin	Provide softness and smoothness to the skin and to adjust the consistency of preparation
Emulsifying agent/emulsifier	Glyceryl monostearate, stearic acid, poloxamer, oleic acid, diethanolamine, polysorbate, wax	Stabilize the 2 phases system that is at least 2 immiscible liquids or droplets/subdivided particles dispersed in other liquids
Humectant	Glycerin, propylene glycol, sorbital	Prevent moisture leaking out of the skin
Thickening agent	Paraffin, stearyl alcohol	Increases the viscosity of formulation
Stabilizing agent	Citric acid, sodium citrate	Improve product stability

Excipients	Example	Roles
Preservative	Parabens, benzyl alcohol, CHX gluconate, sodium benzoate	Antimicrobial growth

2.7.2.2 Ideal properties of paste (78)

(1) Physical properties: nonirritation, nongreasiness and nonstaining, smooth, and good appearance
(2) Application properties: deliver drug to affected area completely, simple applicable, and easy water washable
(3) Storage properties: stable under various conditions and storage should be at room temperature

2.7.2.3 General product quality tests (79)

The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH Q6A) recommends specifications that are universally applied to ensure safety and efficacy as showed in Table 2.5.

Table 2.5 Specific recommendation of ICH Q6A for safety & efficacy of products (79)

Tests	Parameter	Acceptant guidance
Description	Size, shape, color, etc.	Conform
Identification	Identification of active ingredient by infrared absorption spectrum, chromatographic retention time or other analytical method	Identity of active ingredient presents in product
Assay	Active ingredient content, percentage labeled amount	Meet specific content requirements
Impurities	Impurities content, residual solvent, heavy metals	Meet the requirements
Physicochemical Properties	pH, viscosity, specific gravity	Meet the requirements

Uniformity of Dosage Units	Dose uniformity	Meet the requirements
Water content	Water content	Meet the requirements
Microbial limits	Microbiological examination of nonsterile product, tests for specified microorganisms	Meet the requirements
Antimicrobial Preservative Content	Preservative content	Meet the requirements
Other tests	Rheological properties, functionality testing of delivery systems	Meet the requirements

2.7.2.4 Quality Assurance (80)

Quality assurance for the manufacture of finished products should follow WHO guidelines for good manufacturing practices (GMP) to ensure the quality, efficacy and safety of products that are designed and developed through the optimal process.

GMP requirements including parts of personnel, premises including personnel facilities, maintenance of buildings and equipment, storage of starting materials and finished products, equipment, production and in process controls, quality control, documentation, sanitation and hygiene, validation and revalidation program, calibration of instruments or measurement systems, recall procedures, complaint management and label control.

2.7.2.5 Packaging and storage (77)

Topical dermatological preparations are packed in well closed collapsible tubes, syringes or jars. Pastes are suitable for jars due to the high viscosity of materials. Pastes should be stored in a cool place at room temperature in a well closed container.

CHAPTER 3

RESEARCH METHODOLOGY

The methods of these experiment were approved by the Research Ethics Committee of Thammasat university, Pathum Thani, Thailand (Reference No. 137/2564) and the biosafety committee of Thammasat university, Pathum Thani, Thailand (Reference No. 077/2564).

3.1 Materials and instruments

3.1.1 Materials and Equipment

- 2 percent CHX, Sigma Aldrich, Thailand
- 17 percent Ethylene Diamine Tetra-acetic Acid (EDTA), CU dent, Thailand
- Aluminum foil
- Carbide burs (012,014,016,021,023 mm), Dentsply-Maillefer, Ballaigues, Switzerland
- $Ca(OH)_2$ (UltraCal® XS), Ultradent Products Inc, USA
- Centrifuge tube 1 ml, Costar®, Corning, USA
- Chloroform, BDH®, UK
- Culture black plate 96 well, Nunc™, Thermo, USA
- MRS agar, Difco™, USA
- MRS broth, Difco™, USA
- Diamond bur no. 330, Dentsply-Maillefer, Ballaigues, Switzerland
- Di-Sodium Hydrogen Phosphate Anhydrous, Merck®, Germany
- Disposable Polystyrene pipette tip 10, 200, 1000 µl, Costar®, Corning, USA
- Distilled water
- Dried neem leaves, Thailand
- Ethanol, BDH®, UK
- Ethyl Acetated, BDH®, UK
- Filter paper no.1, 25 mm. Whatman®, UK
- Glycerol, Ajax Finechem pty Ltd., Australia
- Hexane, BDH®, UK
- *L.rhamnosus* (ATCC 10863), ATCC, Manassas, USA
- Methanol, BDH®, UK
- Microcentrifuge Tube, Rnase & Dnase Free 1 ml, Costar®, Corning, USA

- Normal Saline, GHP®, Thailand
- Plastic tube 15 ml, 50 ml, Costar®, Corning, USA
- Plate culture size 100 mm, Fisher Scientific, USA
- Polyethylene glycol 400 (PEG 400), KemAus™, Australia
- Propylene glycol, KemAus™, Australia
- Safranin O, Acros Organics™, Belgium
- Sodium Chloride (NaCl), Merck®, Germany
- Sodium Dihydrogen Phosphate Monohydrate, Merck®, Germany
- Sodium hypochlorite (NaOCl), CU dent, Thailand
- Sodium Lauryl Sulfate, Ajax Finechem pty Ltd., Australia
- Staining kit (LIVE/DEAD BacLight ®), Invitrogen, USA
- Stearyl Alcohol, KemAus™, Australia
- Tuberculin Syringe 1 ml, Nipro, Thailand
- ZnO powder, KemAus™, Australia

3.1.2 Instruments

- Auto Sample Injector SIL-20A, Shimadzu Co, Ltd., Japan
- Autoclave, Sanyo autoclave C, USA
- Biosafety cabinet class II, Heal Force®, China
- Centrifuge 5810R machine, Eppendorf, Germany
- Column oven 37°C CTO-20A, Shimadzu Co, Ltd., Japan
- CLSM, Leica Stellaris 5 inverse (USZ)-1, USA
- Coupled Perkin Elmer GC-MS, Model Perkin Elmer Clarus 500, USA
- Haake™ Mars™ Rheometers, Thermo Scientific, USA
- Hot Air Oven, Memert, Germany
- HPLC machine system, CBM-20A, Shimadzu Co, Ltd., Japan
- Incubator Shaker series, Innova® 40, Thermo Scientific, USA
- Mass spectrometer, Thermo Scientific, USA
- Micro-pipette, 20, 100, 200, 1000, Gilson, France
- Microplate reader, (DTX 880 Multimode Detector), Beckman Culture, USA
- Microplate shaker, Monoshake, Variomag®, USA
- Perkin Elmer Clarus 600C MS, Thermo Scientific, USA
- pH meter & electrode, Orion 2 Star, Thermo Scientific, USA
- Refrigerator, Sanyo, Japan

- Rotary evaporator, BÜCHI Rotavary R-205, Flawil, Switzerland
- Scanning electron microscope, JEOL, JSM-6610/LV/A/LA, USA
- Shaking incubator, SI 500 Stuart, Thermo Scientific, USA
- Spectrophotometer, Bio-Rad SmartSpec 3000 UV/Vis, USA
- Stereoscope, Carl Zeizz 426126, Thermo Scientific, USA
- Thermometer, Labworld, India
- Vortex, Vorter-2 Genie, Thermo Scientific, USA
- Water bath, Heto heat master, Green Science Tech Co., Ltd, Thailand

3.2 Methods

3.2.1 Specimen preparation

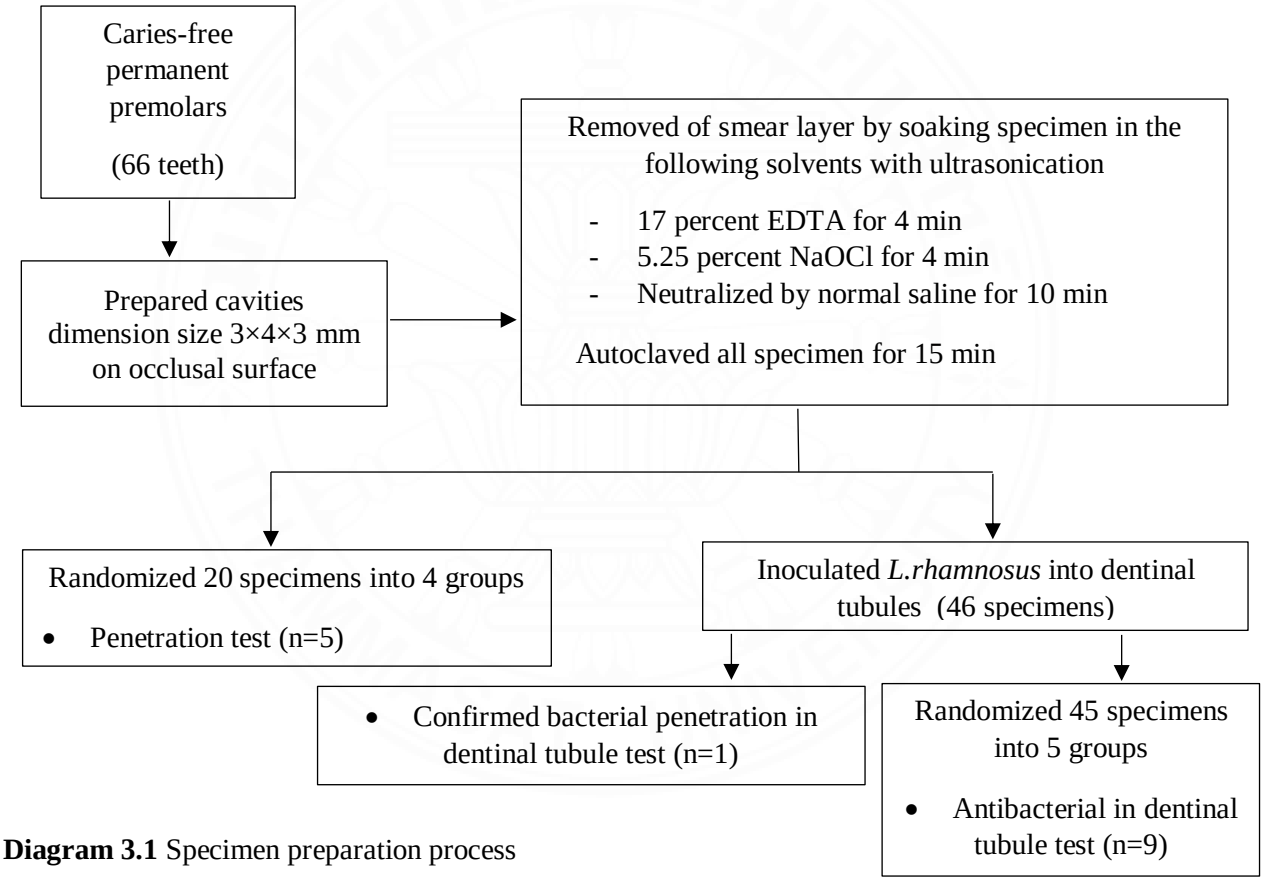


Diagram 3.1 Specimen preparation process

Caries free human permanent premolars which were qualified, were stored in phosphate buffered saline (pH 7.4, PBS) at 4°C. The experimental cavities (3×4×3 mm, width × length × depth) were prepared on occlusal surfaces towards the dentinoenamel junction using a diamond fissure bur with a high speed air turbine hand piece. Then all specimen cavities were reevaluated under radiographic examination (Figure 3.1). The inorganic and organic components of smear layer of specimens' experimental cavities were removed by immerging

in 17 percent EDTA, pH 7.8 for 4 min, follow by 5.25 percent NaOCl for 4 min with ultrasonication. Then the cavity were immerging with normal saline for 10 min to neutralize the NaOCl and then autoclaved at 121°C at 15 lbs psi for 15 min (Diagram 3.1) (20, 81-82).

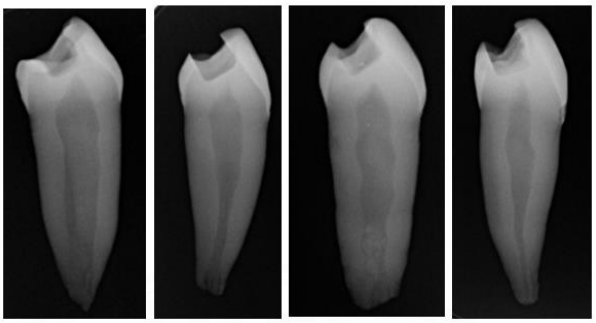


Figure 3.1 Radiographic confirmation of specimen cavities

3.2.2 Preparation of neem paste and determination on its properties

1) Ethanolic neem leaf extract

Neem leaves were initially cleaned with tap water and dried under shade and hot oven at 50°C for an hour. Then, they were crushed up. The 30 g of pulverized neem leaves were macerated in 500 ml of 95 percent ethanol. The mixtures were incubated in a shaker incubator for 24 h. Then, there were put 70°C water bath for 30 min and further incubated in a shaker incubator for 24 h. The crude extract was then produced by vacuum filtering the mixtures through Whatman No. 1 filter paper and evaporating the solutions using a Buchi Rotavapor R-200.

Figure 3.2 Neem leaf extract procedures

2) Phytochemical properties of neem leaf extract

2.1) Gas chromatography mass spectrometry analysis

The ethanolic neem leaf extract was redissolved in ethyl acetate, chloroform, hexane, butanol, and methanol. The mixtures were diluted by 1/100 v/v and filtered. A coupled Perkin Elmer GC-MS with a fused silica capillary column (30 m 0.25 i.d., film thickness 0.25 m) and a Perkin Elmer Clarus 600C MS were used to analyze the sample. Helium, an inert gas, was used as a mobile phase with a 1 ml/min constant flow rate. The 1 µl sample was initially placed into the GC by a split mode auto-sampler with a 1:120 split ratios. Then the sample was heated starting from 50°C to 150°C at 3°C/min, held for 10 min, and finally raised to 300°C at 10°C/min into vapor. The sample components were identified and characterized by their differential retention times, and the percentages of each composition were measured by peak area. After being separated, the eluted analyte was fragmented by 70 electron volts of electron ionization in a mass spectrometer. The mass transfer line and injector temperature were heated to 220 and 300°C, respectively. The charged components were observed, the subsequent spectra were recorded and used to interpret the molecule by matching them with standards databases in a computer library (Mainlab, Replib, and Tutorial data of GC–MS systems).

2.2) High performance liquid chromatography analysis

The ethanolic neem leaf extract was redissolved in a 1:1 mixture of 80 percent methanol and hexane. These mixtures were filtered before quantitative analysis was performed. To determine querecetin, nimbin, and nimbidin in ethanolic extracts. The HPLC system used two gradient pump systems (LC-20AT, Shimadzu), an auto sample injector, a UV detector and a column oven were utilized for analysis. The mobile phases were water (A) and acetonitrile (B) in gradient mode. The mixtures were delivered to separate components using a C18 column (Synergi 4u MA-RP 80A, 150 × 4.6 mm, 4 µm Phenomenex®) as the stationary phase and the UV detector at 217 nm, 1.0 ml/min flow rate. A gradient elution was carried out with the following concentrations: 0–10 min, 30–40 percent B; 10–15 min, 40–45 percent B; 15–20 min, 45–50 percent B; 20–25 min, 50–60 percent B; and 25–35 min, 60–70 percent B. Ten µl of each mixture was injected into the C18 column, and querecetin, nimbin, nimbidin were separated, then identified and quantified. Retention times (RT) and spectra were collected to identify and quantify each compound, respectively. A reverse phase Phenomenex® Luna 5µm C18 (2) 100A (250 × 4.60 mm i.d) column was applied to identify azadirachtin. Acetonitrile, methanol, and 1 percent triethylamine were the components of the mobile phase (60:40:1)

which was adjusted to pH 4 and filtered through a filter using a value-stage vacuum pump. The mobile phase flow rate was 1 ml/min, and UV 210 nm was the detecting wavelength.

3) Zinc oxide paste preparation

The ZnO paste was prepared from:

Polyethylene Glycol 4000 (PEG)	20.0	g
Glycerin	30.0	g
Stearyl Alcohol	37.0	g
Sodium Lauryl Sulfate	1.0	g
Distilled Water	12.0	g
ZnO Powder	1:10	g

After measuring all the above ingredients. Firstly, the PEG base was prepared by mixing the polyethylene glycol 4000, stearyl alcohol, and glycerin together by heating in a water bath to 75°C (oil phase). The sodium lauryl sulfate was dissolved in water and heated to 75°C (aqueous phase) on a water bath. The oil phase was then added to the aqueous phase and stirred until the base congealed (Figure 3.3 A.). Second, ZnO paste was prepared by adding ZnO powder in a 1:10 ratio to the PEG base. In a 1:10 ratio, ZnO powder was added to the PEG base then, mixed until it was a homogeneous paste.

4) Neem paste preparation

Neem paste preparation was prepared: a combination of ethanolic neem leaf extract with ZnO paste carrier to the final concentration of 20 mg/g of neem leaf extract. The neem paste was then stored in a tuberculin syringe in 4°C for physicochemical testing (Figure 3.3 C.).

Figure 3.3 Neem paste preparation. **A.** PEG base, the preparation was smooth paste liked substance with opaque white in color. **B.** ZnO paste, a homogenous paste liked and matt white in color, and neem extract. **C.** The final concentration of neem paste (20 mg/g) with homogenous paste liked and matt green in color.

3.2.3 Physicochemical property of neem paste

1) Appearances

The appearances of the ZnO and neem paste were evaluated based on texture, color and smoothness using visual inspection.

2) Viscosity

The viscosity of the pastes was evaluated by using the rheometer. A 20 mm diameter stainless steel parallel plate rotor was filled with 20 mg/g of neem paste, ZnO paste, or $Ca(OH)_2$. A dynamic or oscillatory assay was used to analyze the viscoelastic properties of the pastes. Oscillatory measurements were performed at 1 Hz with a constant strain of 0.1 percent, at 37°C, and the data were collected every 5 s for 5 min. The time dependent analysis determined the material's flowability under force. The storage modulus (G'), loss modulus (G"), and loss tangent (tan δ) were also measured. G' represents the energy retained within a sample's elastic structure and G" represents the energy dissipated during each vibration cycle. The material is less elastic when G" is higher than G'. Tan δ reflects the proportional amount of energy loss as a ratio of G"/G'. When tan $\delta > 1$, the material is more flowable than elastic. In contrast, when Tan $\delta < 1$, the material is more elastic. When a material is in the range of $0 <$ Tan $\delta < 1$, its fluidity or elasticity is uncertain. However, when Tan δ is close to 1, the material has a fluid liked characteristic and when Tan δ is close to 0, the material is gel liked (85,86). The pH of 1 M ZnO paste, and 20 mg/g neem paste solutions were tested using a pH meter (Thermo Scientific Orion Versastar pro). The measurement was performed in triplicate. The properties were reevaluated in 6 months (Figure 3.4).

Figure 3.4 Sample preparation for viscosity testing.

3) Penetration test

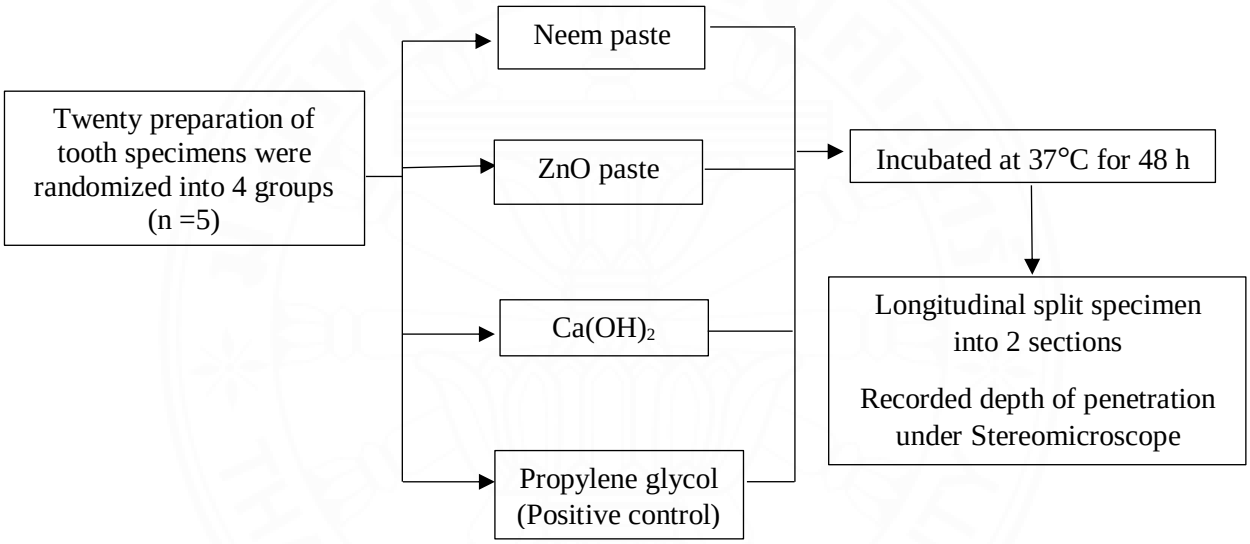


Diagram 3.2 Penetration test process

The method of this experiment were modified from Tasanarong *et al.*, (20). Total of 20 preparation specimens were randomized into 4 groups (n = 5 in each group). The testing materials: neem paste, ZnO paste, $Ca(OH)_2$ and propylene glycol were placed into prepared cavity specimen. Since ZnO paste and propylene glycol are clear in color, they were mixed with 0.1 M safranin O solution at the concentration of 1 g percent before testing for easy detection (83). The specimens were be sealed with occlusal indicator wax to avoid moisture contamination. Each specimen were placed into a closed container with wet paper towels to maintain a humid atmosphere and incubated at 37°C for 48 h. After that the specimens were longitudinal split into 2 sections by carborundum disc. Each section was viewed under a

stereomicroscope at 15× magnification, the distance of penetration was measured in micrometer from cavity floors to the pulp chamber direction as a penetration depth (20, 83) (Diagram 3.2).

4) pH

The pH of the ZnO and neem paste were evaluated by using a pH meter. Those pastes were prepared by dissolving in water then adjusted to concentration of 1 M. The measurement was performed in triplicate. The properties were reevaluated in 6 months.

3.2.4 Antibacterial abilities

1) Antibacterial ability on agar disc diffusion

The agar disc diffusion assay was used to evaluate the antibacterial effect of the tested materials. *L. rhamnosus* (ATCC 10863), representing deep carious microorganisms, was cultured in MRS broth at 37°C for 24 h. The number of bacteria was adjusted to McFarland standard No. 0.5 (10^8 CFU/ml) by a spectrophotometer at 600 nm (OD: 0.2-0.3). A 200 µl bacteria suspension was spread on MRS agar. Six mm discs were immersed in 30 µl solutions of 2 percent CHX, which served as a positive control, or $Ca(OH)_2$ paste, which served as the standard treatment, 20 mg/g, 100 mg/g neem paste, and ZnO paste in the amount of 8 mg. The discs were placed on the agar surface and incubated at 37°C for 48 h. The diameter of the inhibition zone was measured and recorded in millimeters. The experiment was performed in three independent experiments, and each experiment was done in triplicate. The most effective neem concentration was used in subsequent experiments.

2) Bacterial inoculation in dentinal tubules

In this experiment, 46 specimens will be used. For the dentin infection, the specimens were kept in MRS broth containing 10^8 CFU/ml *L.rhamnosus* at the of ratio 5:1 and changed every 3 days for a period of 30 days. Strict asepsis technique was required to avoid contamination (20, 84-85). A specimen was subject to confirm the bacterial penetration under a SEM (Figure 3.5).

Figure 3.5 Sample preparation for SEM. **A.** The sample was dried in storage at room temperature for 24 h and had been notched as a crack method. **B., C.** The sample was cracked and split into two halves. **D., E.** Then, mounted the sample on SEM specimen holder and put carbon painted on the holder. After that, the specimen was coated with 20 nm of thick gold palladium layer. **F.** The SEM was used to evaluated bacterial penetration.

3) Antibacterial activity in dentinal tubules

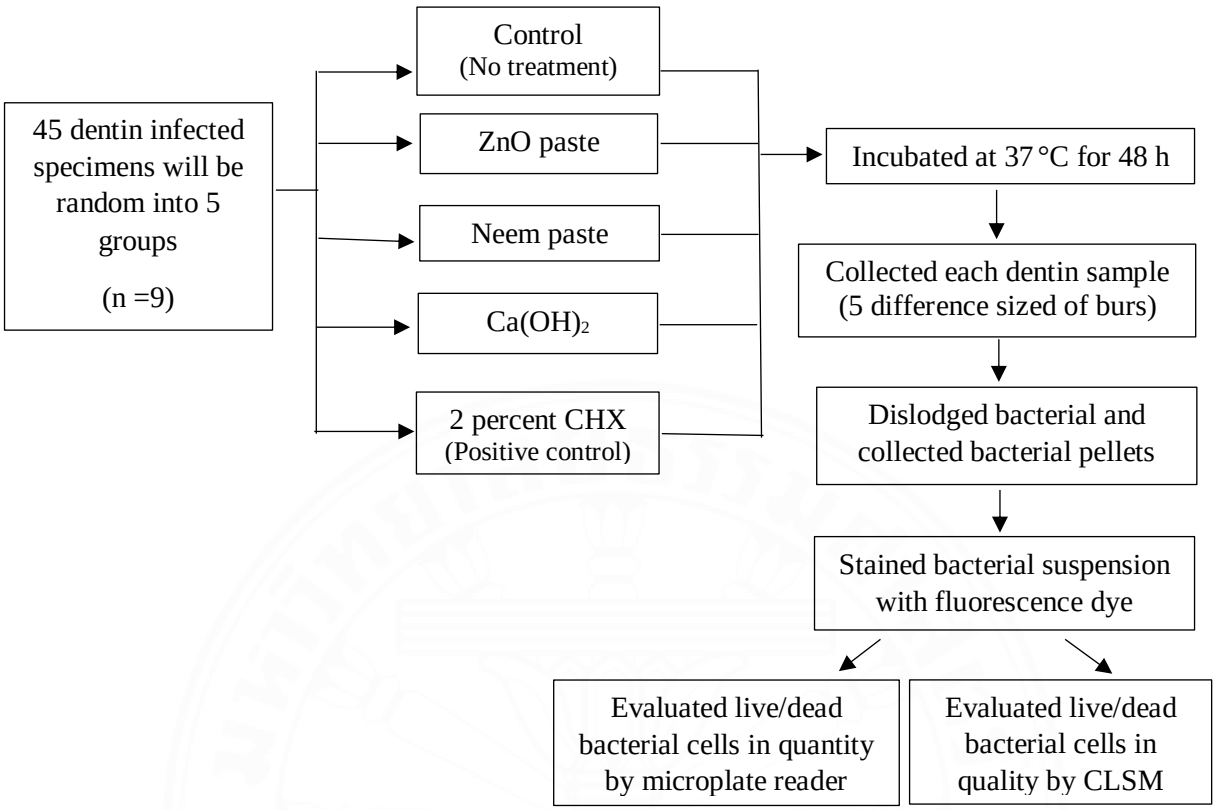


Diagram 3.3 Design of antibacterial activity in dentinal tubules

Forty-five infected dentin specimens were divided randomly into 5 groups (n = 9 in each group): group I: no treatment (control), group II: ZnO paste group III: neem paste, group IV: $Ca(OH)_2$ and group V: 2 percent CHX (positive control) then, all specimens were sealed with occlusal indicator wax to avoid moisture contamination. Each specimen was placed into a closed container and incubated at 37ºC for 48 h. After that, the medications were removed from the cavity by flushing 3 times with PBS and were dried with sterile gauze. Dentin samples of the pulpal floor were collected by using 5 different diameters of sterile steel round burs which the sizes of burs will be increased as ISO 012 to 021, corresponding to 100 through 500 µm (81). In order to calibrate the depth of the high speed hand piece burs, dentin samples were taken from 3 additional, sterile teeth. Then, the cavity size was measured in 5 different diameters. The collected dentin samples with the bur were placed in 1 ml PBS and dislodged from the bur by vortex. The dentin powder was removed by centrifugation for 2 min at a low speed of 1,200 rpm. Then the supernatants were collected and placed in 1.5 ml PBS and centrifuged at high speed for a min at 12,000 rpm to give the pellet bacterial cells (84). The

bacterial pellets were resuspended in 300 µl of the staining kit (LIVE/DEAD BacLight®) in a dark room and incubated for 40 min. According to the manufacturer's instructions of the staining kit, there are 2 types of stains (SYTO 9 and propidium iodide) that stain the nucleic acids of bacteria. However, SYTO 9 was stain living bacterial cells as it had the ability to penetrate intact bacterial cell walls and represented as a green color, while propidium iodide was stain the nucleic acids of dead bacterial cells when their membrane was damaged and represented as a red color. After that, a volume of 100 µl aliquots from stained bacterial suspensions were pipetted into a microtiter plate in triplicated. The fluorescence intensity was measured with a microtiter plate reader with excitation at 485 nm and detection at 535 nm (green) and 642 nm (red), for SYTO 9 and propidium iodide, respectively. The ratio of live and dead bacteria (green/red fluorescence) were expressed. The 20 µl of remaining stained suspensions were placed on glass slides and covered by slips. Bacterial viability was identified as survival and dead bacteria as visualized under CLSM at 63× magnification (Diagram 3.3) (20, 85).

3.3 Statistical analysis

The data were expressed in mean ± SE. Before comparison testing, the normality distribution data were tested using Kolmogorov-Smirnov test at the significant level of 95 percent ($\alpha = 0.05$). According to the data follows normal distribution, parametric test was used as a one-way ANOVA and the difference in groups were tested with post hoc comparison by using Tukey's test (*** $p<0.001$, $^{\#}$ $p<0.05$).

CHAPTER 4

RESULTS

4.1 Phytochemical properties of neem paste

4.1.1 Gas chromatography mass spectrometry

The organic chemical components of ethanolic neem leaf extracts were identified and described in various solvents based on GC retention times. The results revealed the following differences in chemical compounds. In the analysis of ethyl acetate solvent, which presented 9 chemical compounds, the diterpene alcohol group (60.67 percent phytol and 5.30 percent 3,7,11,15-Tetramethyl-2-hexadecen-1-ol) ranked as the highest percentage compound. (Table 4.1) The number of chemical compounds found in hexane and butanol solvents was 15 and 8, respectively. The alkane groups (2,6,10,14-tetramethyl heptadecane (2.68 percent), nonadecane (3.75 percent), heptacosane (8.1 percent), eicosane, 7-hexyl (10.01 percent), heptacosane, 7-hexyl (6.77 percent), octacosane (7.09 percent) in hexane, and nonacosane (12.82 percent), hentriacontane (13.91 percent) in butanol) were the most prevalent (Table 4.2 and 4.3). There were 6 and 4 chemical compounds presented in the chloroform and methanol solvents, respectively. The fatty acid group; methyl 14-methylpentadecanoate (31.83 percent) and methyl isoheptadecanoate (11.62 percent) in chloroform and methyl 14-methylpentadecanoate (37.10 percent) were the most detected. (Table 4.4 and 4.5).

Table 4.1 Results of chemical compositions in ethanolic neem leaf extract in ethyl acetate solvent

No.	Name of compounds	Retention time	Leave (percent)	MW	Activities
	Sesquiterpenes				
1	(Z,E)-α-Farnesene	28.53	2.33	204.4	Antioxidant (86)
2	Gamma.-elemene	29.25	4.12	204.4	Antifungal, antioxidant, and biocidal (87)
3	Hexahydrofarnesyl acetone	39.37	2.65	268.5	Anticancer (88), antibacterial (89)
	Diterpene alcohol				

4	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	39.17	5.30	296	Antitubercular, insecticidal, antiinflammatory, antioxidant, antimicrobial (90)
5	Phytol	47.71	60.67	296	Antioxidant, antimicrobial, anticancer, antidiuretic (90), immunestimulatory (91), antiinflammatory, anxiolytic (92)
	Fatty acid				
6	Methyl 14-methylpentadecanoate	41.82	6.66	270.5	Antibacterial (93)
7	9,12,15-Octadecatrienoic acid, methyl	47.34	6.20	292.5	Anticancer (94)
	Alkane				
8	1-Tridecene	52.87	2.78	182.4	N/A
	Fatty alcohol				
9	(9E,12E,15E)-9,12,15-Octadecatrien-1-ol	57.62	4.55	264.4	Antiinflammatory, anxiolytic (92), antioxidant, antibacterial (95)

Table 4.2 Results of chemical compositions in ethanolic neem leaf extract in hexane solvent

No.	Name of compounds	Retention time	Leave (percent)	MW	Activities
1	**Sesquiterpenes** Gamma-elemene	24.373	1.01	204.4	Antifungal, antioxidant, and biocidal (87)
	Diterpene alcohol				
2	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	39.134	2.97	296	Antitubercular, insecticidal, antiinflammatory, antioxidant, antimicrobial (90)
3	Phytol	47.677	2.65	296	Antioxidant, antimicrobial, anticancer, antidiuretic (90), immunestimulatory activity (91), antiinflammatory, anxiolytic (92)

	Fatty acid				
4	Methyl petroselinate	47.307	11.22	296.5	Antioxidant (96)
5	Methyl 15-methylhexadecanoate (Methyl isoheptadecanoate)	48.338	2.19	284.5	Antiviral (97)
6	Butyl palmitate (Hexadecanoic acid, butyl ester)	49.803	6.69	312.5	Antiinflammatory and hepatoprotective (98)
	Alkane				
7	2,6,10,14-Tetramethyl heptadecane	50.133	2.68		N/A
8	Nonadecane	52.905	3.75	268	Cytotoxic, antimicrobial (99)
9	Heptacosane	58.102	8.10	380	Antioxidant (100), antibacterial (89)
10	Eicosane, 7-hexyl	60.558	10.01	366.7	Antimicrobial (101)
11	Heptacosane, 7-hexyl	62.919	6.77	380.7	
12	Octacosane	65.205	7.09	394.8	
	Organosiloxane				
13	Hexadecamethyl cyclooctasiloxane	48.338	7.46	593.2	N/A
	Ester of isobutyl alcohol				
14	Isobutyl stearate (2-Methylpropyl octadecanoate)	55.261	4.25	340.6	N/A
	Others				
15	Oxalic acid, 2-ethylhexyl tetradecyl ester	55.556	13.70		N/A

Table 4.3 Results of chemical compositions in ethanolic neem leaf extract in butanol solvent

No.	Name of compounds	Retention time	Leave (percent)	MW	Activities
	Diterpene alcohol				
1	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	39.17	7.25	296	Antitubercular, antiinflammatory, antioxidant, antimicrobial (90)
	Fatty acid				
2	Methyl 14-methylpentadecanoate	41.82	13.45	270.5	Antibacterial (93)
	Alkane				
3	Nonacosane	66.42	12.82	408.8	Antibacterial (102)
4	Hentriacontane	70.91	13.91	436.8	Antiinflammatory, anticancer (103)
	Acyl chloride				
5	Linoleoyl chloride	46.78	13.61	298.9	Antisecretory, antispermatogenic and antitubercular (104)
	Ketone				
6	Levoglucosenone	11.58	7.11	126.11	Antineoplastic (105)
	Benzaldehydes				
7	Benzaldehyde, 2-methyl (o-Toluylaldehyde)	15.75	11.88	120.2	N/A
	Others				
8	2-Methyl-5-ethylfuran	31.65	4.90		N/A

Table 4.4 Results of chemical compositions in ethanolic neem leaf extract in chloroform solvent

No.	Name of compounds	Retention time	Leave (percent)	MW	Activities
	Diterpene alcohol				
1	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	39.17	14.46	296	Antitubercular, antiinflammatory, antioxidant, antimicrobial (90)

2	Phytol	47.7	10.04	296	Antioxidant, antimicrobial, anticancer (90), immunestimulatory (91), antiinflammatory, anxiolytic (92)
	Fatty acid				
3	Methyl 14-methylpentadecanoate	41.82	31.83	270.5	Antibacterial (93)
4	Methyl 15-methylhexadecanoate (Methyl isoheptadecanoate)	47.99	11.62	284.5	Antiviral (97)
	Alkane				
5	Nonacosane	67.43	20.22	408.8	Antibacterial (89, 102)
	Acyl chloride				
6	Linoleoyl chloride	46.78	11.31	298.9	Antisecretory, antispermatogenic and antitubercular (104)

Table 4.5 Results of chemical compositions in ethanolic neem leaf extract in methanol solvent

No.	Name of compounds	Retention time	Leave (percent)	MW	Activities
	Fatty acid				
1	Methyl 14-methylpentadecanoate	41.82	37.10	270.5	Antibacterial (93)
2	Methyl 15-methylhexadecanoate (Methyl isoheptadecanoate)	47.99	11.12	284.5	Antiviral (97)
	Acyl chloride				
3	Linoleoyl chloride	46.78	28.46	298.9	Antisecretory, antispermatogenic and antitubercular (104)
	Benzaldehydes				
4	m-Toluylaldehyde (Benzaldehyde, 3-methyl-)	15.56	21.55	120.2	

4.1.2 High performance liquid chromatography

The quantitative analysis of querecetin, nimbin and nimbidin compounds in different extracts performed using HPLC was given in Table 4.6. These compounds were identified in solvents used by matching their RT and spectra with those of the standards and the quantitative data were calculated based on the peak area of each compound. The result revealed that the concentration of ethanolic leaf neem extract which redissolved in methanol and hexane at the ratio of 1:1; quercetin was the highest constitutes (240.15 µg/g dw), followed by nimbin and nimbidin, respectively (Figure 4.1A). Azadirachtin was additionally found in the neem leaf extract (Figure 4.1B).

Table 4.6 The concentration of querecetin, nimbin and nimbidin in the ethanolic neem extract

Samples	Querecetin (µg/g dw)	Nimbin (µg/g dw)	Nimbidin (µg/g dw)
Neem crude (80 percent Methanol): (Hexane), 1:1	240.15	55.22	1.85

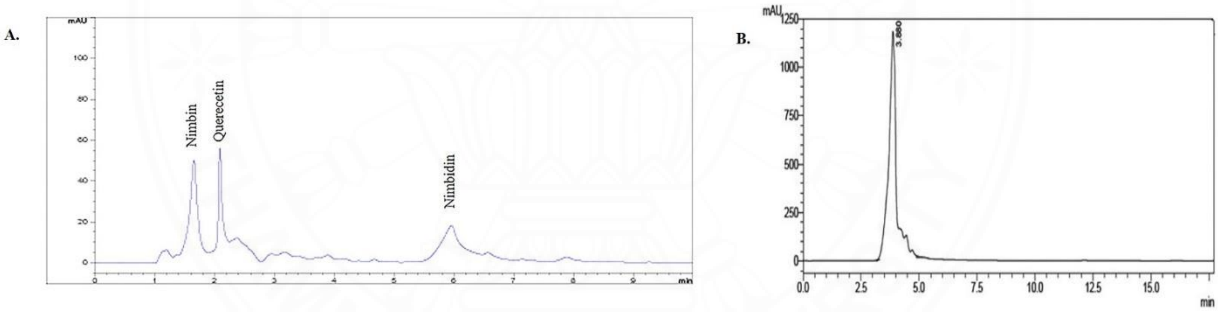


Figure 4.1 HPLC chromatogram of neem paste. **A.** HPLC chromatogram of nimbin, querecetin, and nimbidin compounds **B.** HPLC chromatogram of azadirachtin (RT=3.88 min).

4.2 Physicochemical properties

4.2.1 Texture, color, and pH

All the pastes' physicochemical properties were evaluated. The ZnO paste was a paste liked matt white, whereas the neem paste was a matt green (Table 4.7). Both pastes were miscible with water and had a similar smoothness. The pH of fresh neem paste preparation was 7.09, indicating that it was neutral to slightly alkaline. The pH of the ZnO paste was 7.69. The

pH of the neem paste was gradually increased over the subsequent 6 months while pH of the ZnO paste, on the other hand, was unremarkable. (Table 4.8).

Table 4.7 Physical appearance of ZnO and neem paste

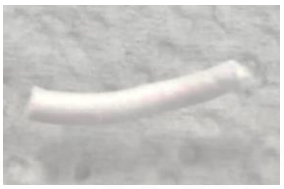
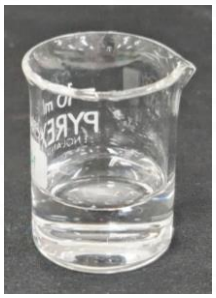
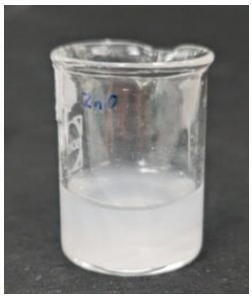
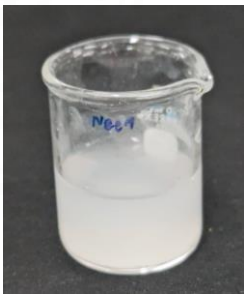
Preparation / **Characteristics**	**ZnO paste**	**Neem paste**
		
Color	Matt white	Matt green
Smoothness	+	+
Odor	None	None
Texture	Paste	Paste
Air bubbles	None	None

Table 4.8 pH results of ZnO and neem paste

Preparation / **Time**	**Control (Distilled water)**	**ZnO paste**	**Neem paste**
			
pH (fresh)	7.12	7.69	7.09
pH (6thmonth)	7.29	7.70	7.49
Miscible with water	-	Yes	Yes

4.2.2 Viscosity testing

Viscosity tests revealed that neem paste had the greatest in Tan δ value (0.8966 ± 0.014), followed by ZnO paste (0.8876 ± 0.0142) which closed to 1 indicating that both were fluid liked properties which were able to flow. On the other hand, Tan δ value of $Ca(OH)_2$ showed fluid gel liked property (0.5542 ± 0.0765). After 6 months, the Tan δ values of ZnO paste and neem paste had declined to the point of being a nearly "fluid gel liked". (Table 4.9, Figure 4.2)

Table 4.9 Time dependent of rheological results

Materials	Day 0 (mean ± SE)			
	G'	**G''**	**Tan δ**	**Flow ability level**
ZnO paste	0.0075 ± 7.021e-005	0.0067 ± 6.510e-005	0.8876 ± 0.0032	Fluid liked
Neem paste	0.0058 ± 4.314e-005	0.0052 ± 5.716e-005	0.8966 ± 0.0031	Fluid liked
$Ca(OH)_2$	0.0020 ± 0.0001	0.0013 ± 0.0001	0.5542 ± 0.0171	Fluid gel liked
	Day 180 (mean ± SE)			
	G'	**G''**	**Tan δ**	**Flow ability level**
ZnO paste	0.0099 ± 5.136e-005	0.0061 ± 4.041e-005	0.62 ± 0.0018	Fluid gel liked
Neem paste	0.0067 ± 6.416e-005	0.0044 ± 2.941e-005	0.66 ± 0.0035	Fluid gel liked
$Ca(OH)_2$	0.0034 ± 0.0002	0.0021 ± 0.0004	0.6176 ± 0.9012	Fluid gel liked

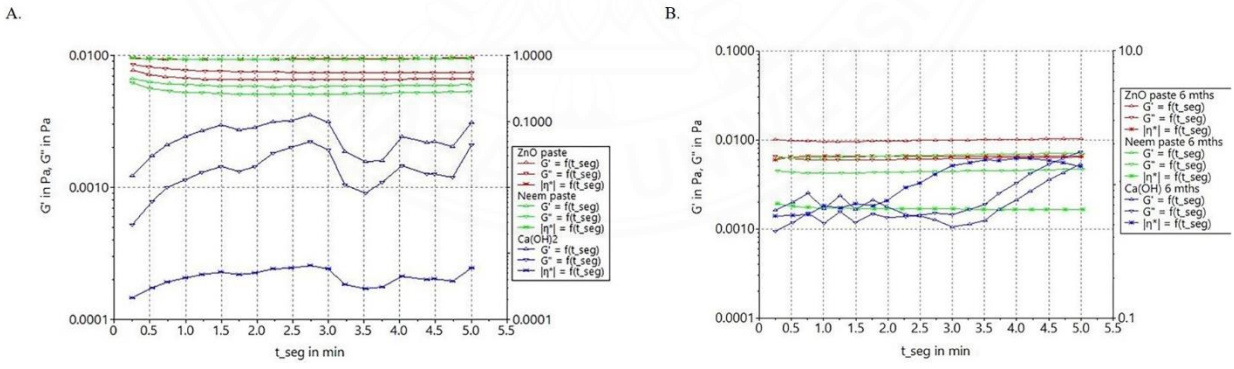


Figure 4.2 Graph of rheological testing. **A.** Day 0: The various flow capacities of materials in five minutes showed the flow ability of neem and ZnO paste appeared to be constant in all periods of time. While, $Ca(OH)_2$ started to increase in the first 3 min and then slightly decreased and fluctuated. However, the tan δ value of $Ca(OH)_2$ was less than value of neem and ZnO paste which implied it has higher in viscosity. **B.** Day 180: Neem and ZnO paste has decreased in viscosity, whereas $Ca(OH)_2$ has stabilized.

4.2.3 Penetration testing

Penetration of propylene glycol, ZnO paste, neem paste, and $Ca(OH)_2$ through the dentinal tubules were evaluated. Propylene glycol was regarded as a positive material which could effectively diffuse from the floor of experimental cavities into the pulp chamber (4,370 ± 1,565) Figure 4.3A. However, Neem paste and ZnO paste were able to effectively diffuse through the dentinal tubules (973.9 ± 158.1) and (384.5 ± 73.53), respectively. $Ca(OH)_2$, on the contrary, showed less permeable via the dentinal tubules (Figure 4.3). Neem paste exhibited the ability to penetrate pulp chambers, therefore the biocompatibility of dental pulp cells was examined.

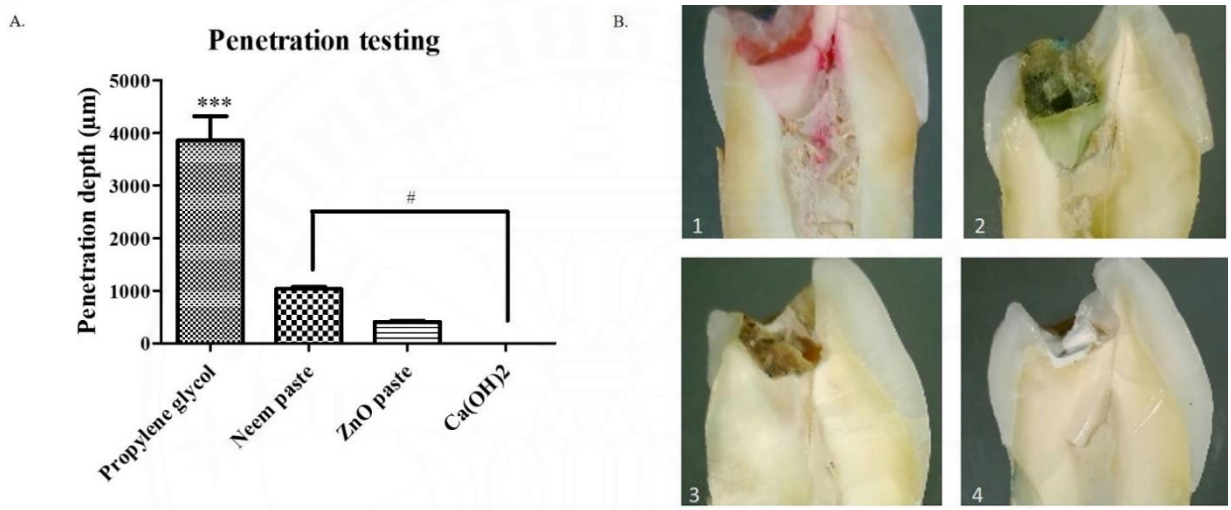


Figure 4.3 Penetration testing. **A.** In the graph of paste penetration, propylene glycol significantly penetrated through the dentinal tubules, followed by neem paste, ZnO pastes, and $Ca(OH)_2$, respectively (*** $p < 0.001$, $^{\#}p < 0.05$, n = 5). **B.** Stereomicroscopic photographs of the tested materials. Propylene glycol (B1) demonstrated the greatest penetration through the dentinal tubules followed by neem paste (B2), and ZnO paste (B3), respectively. $Ca(OH)_2$ (B4) demonstrated minimal penetration.

4.3 Antibacterial abilities

4.3.1 Agar disc diffusion

This experiment on disc diffusion on *L. rhamnosus* showed all testing materials could inhibited growth of test microorganisms. The 2 percent CHX had a maximum inhibition zone (14.83 ± 0.9014). However, there was no significant difference inhibiting zone between the ZnO paste (6.667 ± 0.2389), 100 mg/g of neem paste (6.889 ± 0.8580), 20 mg/g of neem paste (7.056 ± 0.9501) and $Ca(OH)_2$ (6.267 ± 0.1936) (Figure 4.4A). Nevertheless, both concentration of neem pastes had a better impact on bacterial growth than ZnO paste and $Ca(OH)_2$. Therefore, a further experiment the 20 mg/g of neem paste was conducted.

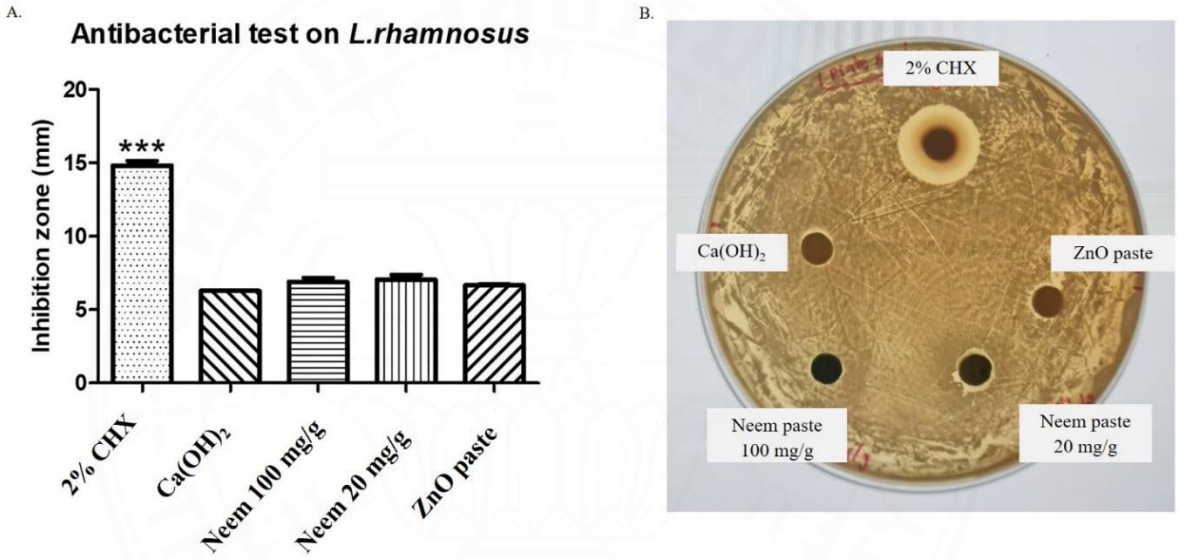


Figure 4.4 The graph of an inhibition zone of disc diffusion. **A.** There was revealed that 2 percent CHX had a statistically significant difference to inhibit *L.rhamnosus* (14.83 ± 0.9014) (***$p < 0.05$, n=3) compared to other groups. Neem paste had greater inhibition zone than the remaining materials. **B.** Disc diffusion tested on *L.rhamnosus.*

4.3.2 Bacterial inoculation in dentinal tubules

After incubating for 30 days, no bacteria were observed in the sterile dentinal tubules (Figure 4.5A). While *L. rhamnosus* was inoculated, bacteria penetrated the dentinal tubules at the deepest distance of 583.22 µm (Figure 4.5 B-D).

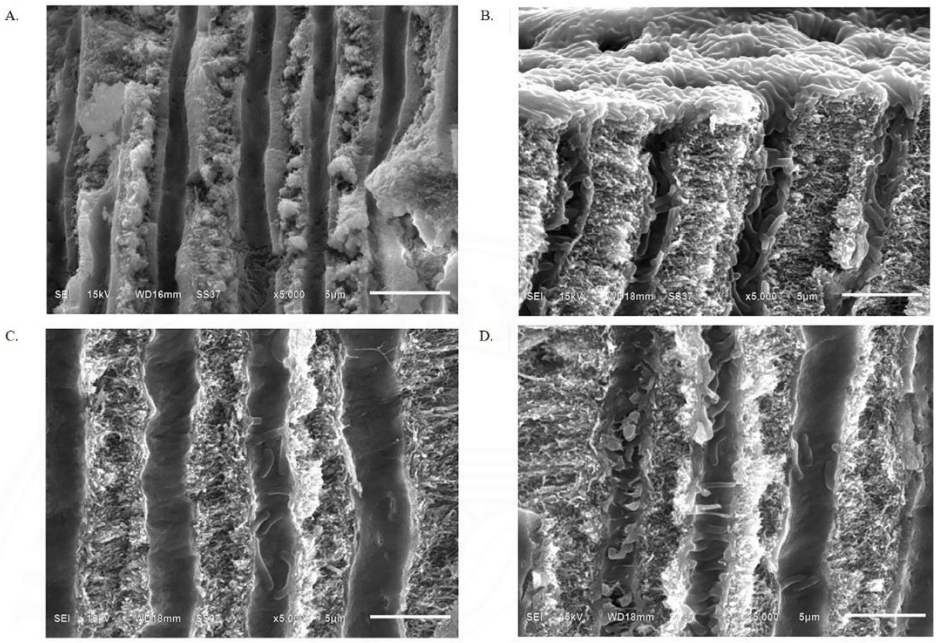


Figure 4.5 SEM images of inoculation dentinal tubules with *L. rhamnosus*. After inoculating for 30 days, **A.** Sterile dentin, no bacteria in dentinal tubules. **B., C., D.** Penetration of *L. rhamnosus* was detected at depth 175.07, 502.76, and 583.22 µm, respectively. Scale bar = 5 µm.

4.3.3 Antibacterial ability in dentinal tubules

L. rhamnosus in dentin at depth 100 through 500 µm below the cavity floor showed the number of viable cells in $Ca(OH)_2$ treatment was comparable to the no treatment group, while 2 percent CHX, neem paste, and ZnO paste showed potency against *L. rhamnosus* at all depths. Nevertheless, at a depth of 500 µm, neem paste demonstrated the greatest antibacterial efficacy (Table 4.10). The result was confirmed by CLSM (Figure 4.6).

Table 4.10 The live/dead percentage of *L. rhamnosus* after treatment (n = 9).

Live/dead ratio percentage	Dentin depth (µm) (mean ± SE)				
	100	**200**	**300**	**400**	**500**
No treatment	81.74 ± 3.67	77.15 ± 3.64	74.15 ± 4.76	69.87 ± 5.18	69.60 ± 3.94
$Ca(OH)_2$	73.44 ± 5.12	74.46 ± 5.21	71.96 ± 3.64	66.23 ± 4.38	71.20 ± 4.85
2 percent CHX	13.57 ± 0.92^{a}	14.09 ± 0.86^{b}	15.16 ± 1.16^{c}	17.69 ± 2.04^{d}	20.96 ± 2.96^{e}
Neem paste 20 mg/g	13.73 ± 0.74^{a}	14.06 ± 0.81^{b}	14.50 ± 0.54^{c}	15.05 ± 0.90^{d}	15.83 ± 1.00^{e}
ZnO paste	14.08 ± 0.79^{a}	15.00 ± 0.63^{b}	14.97 ± 0.69^{c}	16.56 ± 1.04^{d}	19.03 ± 1.57^{e}

$^{a,b,c,d,e}p < 0.001$ when compared with no treatment and $Ca(OH)_2$ group.

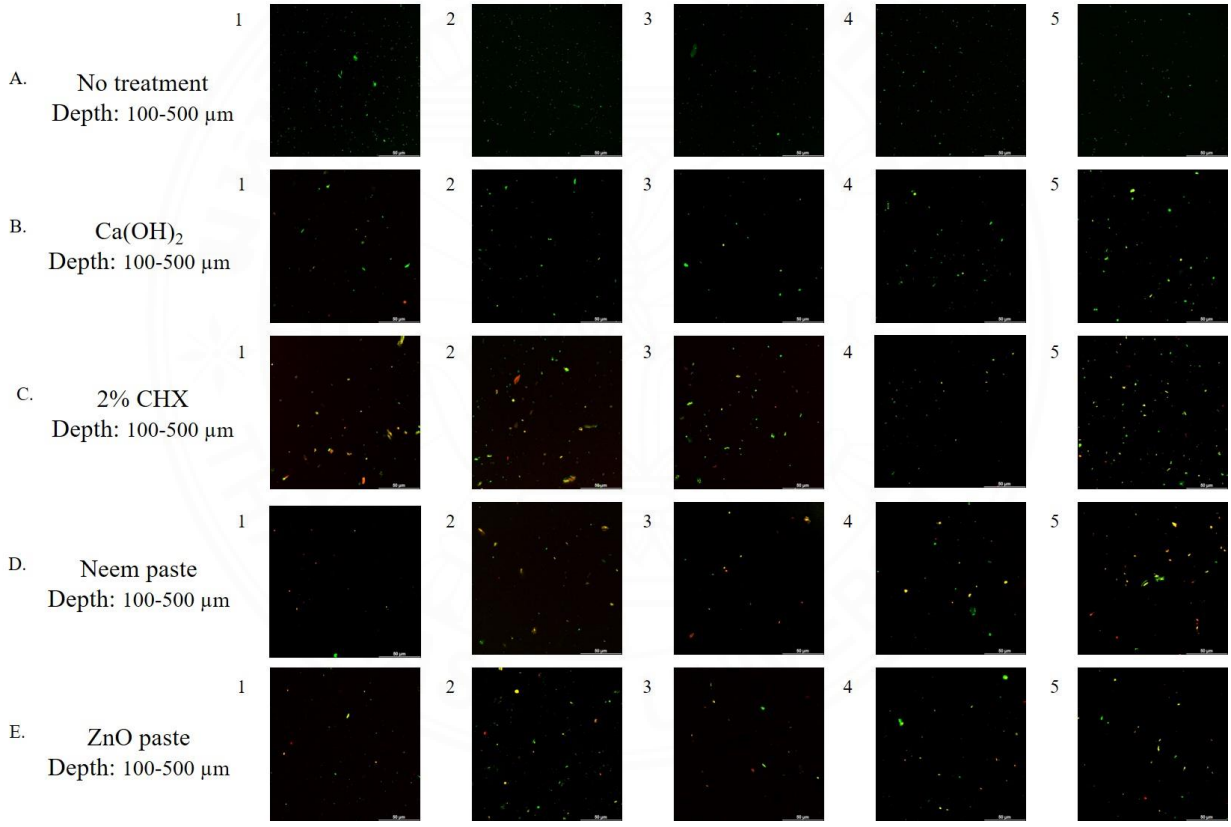


Figure 4.6 CLSM images. Images of viable (green) and dead (red) bacteria presented in dentin at depths of 100 to 500 µm (1-5) below the cavity floor. To those of 2 percent CHX (C), 20 mg/g neem paste (D), and ZnO paste (E), the viable *L. rhamnosus* cells (green) were reduced at all depths. Furthermore, the traditional $Ca(OH)_2$ treatment (B), resulted in fewer dead bacterial cells. Scale bar = 50 µm. The green cells exhibited as rod shape bacilli.

CHAPTER 5

DISCUSSION AND CONCLUSION

An innovative paste including neem extract has been investigated for antibacterial efficacy on *L. rhamnousus*. The results revealed that the 20 mg/g neem paste preparation had an effective penetration through the dentinal tubules, and showed eradication of *L. rhamnousus* in agar disc diffusion, and through dentinal tubules at all testing depths.

The GS-MS and HPLC analysis of the neem extract revealed that the 5 most important antibacterial compounds. Phytol generates its antibacterial effect through DNA damage and encouraging bacterial cell death via a mechanism known as oxidative stress (106). These results were confirmed with Enenche *et al*., which indicated the presence of phytol in the extract of neem which has an antibacterial property (107). Additionally, nimbidin, nimbin, and azadirachtin had also been detected in the neem extract which inhibited the proliferation of both gram positive and gram negative bacteria (108-110). These compounds have complicated molecular structures that prohibit oxidation reactions in cells (111). This study also determined that quercetin may be one of the beneficial constituents in neem extracts. Quercetin has been showed to inhibit the growth of several bacteria by affecting membrane permeability, disturbing mitochondrial function, and diminishing protein and nucleic acid synthesis. Quercetin also inhibits biofilm formation and downregulates the expression of virulence factors (112-114). Therefore, the bioactive compounds in neem extract might enhance the antibacterial activities of neem paste.

For the physicochemical testing, the pH of the neem paste gradually ascended from 7.09 to 7.49 over a period of 6 months. The pH results are like to those Hashim *et al*., who discovered that the pH of neem extract increased after 8 weeks (115). According to the normal pH range in human cells, as stated by Mackenzie *et al*., who found that the greatest growth of cultivated mammalian cells occurs at a pH range of 7.3-7.87 (116). As a result, the pH of neem paste should not influence the growth of human cells. Since the pH range for lactobacillus growth is 4.5-6.5 (117), and the pH of the neem paste in our findings was higher than the pH range for lactobacillus growth, this makes it inhibitive for *L. rhamnosus* growth. Despite its viscosity, neem paste maintained its physical characteristics throughout the 6 month period. Like the finding of Manca *et al*., neem oil viscosity rose after 14 days of storage (118). Fresh neem paste has a fluid liked consistency. However, after 6 months, the paste's viscosity was observed to have become marginally close to fluid gel liked. The increased viscosity of the

neem paste may have been caused by dehydration. Since neem paste became more viscous after 6 months of storage, this might impact its capacity to flow. Therefore, further research on neem paste storage should be conducted concerning the issue of effective penetration to determine neem paste shelf life.

The present study found that the neem paste penetrated through the dentinal tubules more than 1,000 µm which was more than the standard material $Ca(OH)_2$. $Ca(OH)_2$ entered the dentinal tubules to a depth of less than 150 µm, according to Zand *et al*., (119). Pursuant to the findings of Cwikla *et al*., $Ca(OH)_2$ was unable to sufficiently penetrate deeply into the dentin to inhibit the proliferation of bacterial cells (19). While the bacteria penetrated through the dentinal tubules at a distance of at least 500 µm from the cavity floor (120). As an outcome of neem paste's penetrating ability, neem paste could inhibit bacterial growth at deeper depths than $Ca(OH)_2$.

To evaluate the efficacy of material for disinfecting dentinal tubules. According to the findings of Tasanarong *et al*., (120) the SEM demonstrated that *L.rhamnosus* could penetrate the dentinal tubules for more than 500 µm. Because antibacterial activity is one of the conditions for the liners when placed in a cavity with residual caries, the materials should correctly penetrate the dentinal tubules.

This investigation revealed that $Ca(OH)_2$ has a negligible capacity to disinfect dentinal tubules at all depths of testing. This may be a consequence of its low penetrating capacity and high solubility, which can lead to dissolution and a diminished antibacterial effect. As showed by Zand *et al*., the present examination demonstrated that $Ca(OH)_2$ has a penetration ability of less than 200 µm into the dentinal tubules (119). As a result, *L. rhamnosus* is capable of enduring treatment with $Ca(OH)_2$, which is in accordance with prior research (22-24). This could be due to *L. rhamnosus*'s capacity to produce a capsule from extracellular polysaccharides, which allows them to defend themselves better against unfavorable changes in the environment (36). According Bhardwaj *et al*., 2 percent CHX has the greatest antibacterial activity in dentinal tubules at depths of 400 µm (121), which relates to our findings that CHX disinfects dentinal tubules at the fourth depth but reduces antibacterial effectiveness at 500 µm.

The antibacterial impact of ZnO paste was proven against *L. rhamnosus* at the fourth depth, when an increased number of live bacteria was observed in the deepest depth. The results indicated that ZnO paste had an effectiveness on antibacterial capacity comparable to the effect of CHX. Mina Zarei *et al*., demonstrated that ZnO nanoparticles have a penetrating depth of

approximately 300 µm (122), whereas our research shows that 384.5 ± 73.53 µm is the optimal penetrating depth for ZnO paste. This could be explained by the fact that the ZnO paste contains polyethylene glycol which is enhance penetration ability. Moreover, its solubilizing properties of ZnO promote the antibacterial efficacy of medicines by facilitating their diffusion to the dentinal tubules (120). ZnO is a component of neem paste that has showed a variety of effects on bacterial cell activities, including inhibiting glycolysis and glucosyltransferase production (123). Tabari *et al*., discovered that ZnO has substantial cytotoxicity to dental pulp stem cells, which distorts the attributes of pulp lining material (124).

Neem paste had potential antibacterial effects against *L. rhamnosus* in dentinal tubules at all tested depths. Therefore, neem paste was capable of penetrating at least 500 µm into the dentinal tubule. Neem paste's antibacterial effect is slightly more than ZnO paste's in the deepest layer of dentin. It is possible that the bioactive components found in neem and ZnO in neem paste may collaborate to create a more potent antibacterial effect than ZnO in ZnO paste alone. The study of Gupta *et al.,* showed that neem leaf extract is a potent bactericidal agent against *L. rhamnosus* (125). Alqahtani and Tasanarong *et al.,* reported that neem extract was capable of eradicating Lactobacillus (126, 127). As a result, neem extract in paste may have an additional effect in inhibiting bacteria proliferation. In addition, ZnO paste containing neem proved less cytotoxic than ZnO paste independently. Pati *et al*., and Raghavendra *et al*., demonstrated that ZnO nanoparticles containing neem leaf extracts are less toxic to macrophage cell lines than ZnO nanoparticles at equivalent concentrations (128, 129).

As the neem paste tended to penetrate through the pulp chamber, its biocompatibility with dental pulp cells should be examined in further study. The limitation of this study was including the tested organism. As carious lesions are caused by complex microorganisms known as biofilm, neem paste should be tested against biofilm in future research before determining its antibacterial benefits for caries treatment. Additionally, the inhibitory zone was not clearly demonstrated by the agar disc diffusion method since the neem paste was in paste form. As a consequence, the agar cup method may have effectively expressed not only antibacterial activity but also the paste's capacity to diffuse through the agar (130). The third was the LIVE/DEAD BacLight® bacterial viability staining technique, which is known to be prone to potentially producing false results (131). Therefore, the extensive optimization for *L. rhamnosus* was tested by CLSM for visualization purposes. However, in this research, the limitation of magnification is low; therefore, the CFU technique should be applied for better visualization in the subsequent investigation.

Conclusion neem paste has properly penetrating through dentinal tubules and inhibiting growth of *L.rhamnosus* in dentinal tubules. These findings indicated that the innovative neem paste may be an alternative material for vital pulp treatment.

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APPENDICES

APPENDIX A

RAW DATA

Viscosity testing

Table A1 Fresh preparation

	Neem 20 mg/g				ZnO paste				$Ca(OH)_2$		
TIME IN MIN	G' in Pa	G" in Pa	Tan δ		G" in Pa	G' in Pa	Tan δ		G' in Pa	G" in Pa	Tan δ
0.25	0.0066	0.0062	0.9292		0.0086	0.0078	0.9061		0.0012	0.0005	0.4279
0.5	0.0063	0.0056	0.8965		0.0081	0.0072	0.8826		0.0017	0.0008	0.4496
0.75	0.0061	0.0053	0.8807		0.0079	0.0069	0.8750		0.0021	0.0010	0.4718
1	0.0060	0.0052	0.8734		0.0077	0.0067	0.8694		0.0024	0.0011	0.4743
1.25	0.0060	0.0052	0.8688		0.0076	0.0066	0.8691		0.0027	0.0013	0.4825
1.5	0.0059	0.0051	0.8674		0.0076	0.0066	0.8700		0.0029	0.0014	0.4877
1.75	0.0059	0.0051	0.8662		0.0075	0.0065	0.8716		0.0027	0.0013	0.4844
2	0.0058	0.0051	0.8684		0.0075	0.0065	0.8743		0.0028	0.0014	0.5026
2.25	0.0058	0.0051	0.8699		0.0075	0.0065	0.8771		0.0032	0.0018	0.5792
2.5	0.0058	0.0051	0.8702		0.0074	0.0065	0.8804		0.0032	0.0020	0.6319
2.75	0.0058	0.0051	0.8725		0.0074	0.0066	0.8840		0.0035	0.0022	0.6331
3	0.0058	0.0051	0.8727		0.0074	0.0065	0.8869		0.0031	0.0019	0.6195
3.25	0.0058	0.0051	0.8742		0.0074	0.0066	0.8909		0.0019	0.0010	0.5631
3.5	0.0058	0.0051	0.8758		0.0074	0.0066	0.8928		0.0016	0.0009	0.5742
3.75	0.0059	0.0052	0.8774		0.0073	0.0066	0.8969		0.0016	0.0011	0.6846
4	0.0059	0.0052	0.8789		0.0074	0.0066	0.8997		0.0024	0.0015	0.6044
4.25	0.0059	0.0052	0.8805		0.0074	0.0066	0.9016		0.0022	0.0013	0.5805
4.5	0.0059	0.0052	0.8826		0.0074	0.0067	0.9050		0.0022	0.0013	0.5661
4.75	0.0060	0.0053	0.8838		0.0074	0.0067	0.9078		0.0020	0.0012	0.5915
5	0.0060	0.0053	0.8847		0.0074	0.0067	0.9107		0.0031	0.0021	0.6749

Table A2 Six months after preparation

	Neem 20 mg/g				ZnO paste				$Ca(OH)_2$		
TIME IN MIN	G' in Pa	G" in Pa	Tan δ		G" in Pa	G' in Pa	Tan δ		G' in Pa	G" in Pa	Tan δ
0.25	0.0063	0.0045	0.7129		0.0100	0.0065	0.6524		0.0016	0.0009	0.5762
0.5	0.0062	0.0043	0.6856		0.0098	0.0061	0.6244		0.0020	0.0012	0.5892
0.75	0.0063	0.0042	0.6737		0.0097	0.0060	0.6175		0.0025	0.0015	0.5936
1	0.0063	0.0042	0.6678		0.0096	0.0059	0.6158		0.0017	0.0012	0.6868
1.25	0.0064	0.0042	0.6629		0.0096	0.0059	0.6157		0.0024	0.0016	0.6648
1.5	0.0065	0.0043	0.6596		0.0096	0.0059	0.6159		0.0016	0.0012	0.7155
1.75	0.0065	0.0043	0.6577		0.0096	0.0059	0.6164		0.0021	0.0015	0.6910
2	0.0066	0.0043	0.6557		0.0097	0.0060	0.6167		0.0018	0.0013	0.7494
2.25	0.0066	0.0043	0.6553		0.0098	0.0060	0.6168		0.0014	0.0014	0.9499
2.5	0.0067	0.0044	0.6541		0.0098	0.0061	0.6170		0.0014	0.0014	1.0225
2.75	0.0067	0.0044	0.6532		0.0099	0.0061	0.6181		0.0013	0.0015	1.1843

3	0.0068	0.0044	0.6521		0.0099	0.0061	0.6182		0.0011	0.0015	1.3725
3.25	0.0068	0.0045	0.6519		0.0100	0.0062	0.6188		0.0011	0.0016	1.4423
3.5	0.0069	0.0045	0.6509		0.0100	0.0062	0.6190		0.0012	0.0019	1.5241
3.75	0.0069	0.0045	0.6514		0.0101	0.0062	0.6194		0.0017	0.0025	1.5000
4	0.0069	0.0045	0.6515		0.0101	0.0063	0.6195		0.0021	0.0033	1.5638
4.25	0.0070	0.0045	0.6511		0.0102	0.0063	0.6196		0.0027	0.0041	1.5613
4.5	0.0070	0.0046	0.6507		0.0102	0.0063	0.6196		0.0035	0.0053	1.4976
4.75	0.0071	0.0046	0.6512		0.0102	0.0063	0.6198		0.0043	0.0062	1.4521
5	0.0071	0.0046	0.6510		0.0103	0.0064	0.6196		0.0053	0.0073	1.3629

Table A3 Penetration testing **n=5**

Group of treatment	**Penetration depth in µm (n=5)**				
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Propylene glycol	3,178.67	4,863.00	4,817.33	3,939.00	2,477.67
Neem 20 mg/g	987.33	1,068.67	900.33	1,131.33	1,061.33
ZnO paste	402.33	321.67	398.67	488.67	415.00
$Ca(OH)_2$	0.11	0.21	0.01	0.02	0.11

Table A4 Agar disc diffusion testing **n=3**

	(Mean ± SE of inhibition zone in mm)
2 percent CHX	14.830 ± 0.3005
$Ca(OH)_2$	6.267 ± 0.0646
Neem 100 mg/g	6.889 ± 0.2860
Neem 20 mg/g	7.056 ± 0.3167
ZnO paste	6.667 ± 0.0799

APPENDIX B

MATERIAL PREPARATIONS

1. 1x Phosphate buffered saline (PBS) pH 7.4

Di-Sodium hydrogen phosphate anhydrous	1.42 g
Sodium dihydrogen phosphate monohydrate	1.76 g
Sodium chloride	8.76 g

2. De Man Rogosa and Sharpe Broth (MRS)

Enzymatic Digest of Animal Tissue	10 g
Beef Extract	10 g
Yeast Extract	5 g
Dextrose	20 g
Sodium Acetate	5 g
Polysorbate 80	1 g
Potassium Phosphate	2 g
Ammonium Citrate	2 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g

The broth was prepared by dissolving all of the above reagents in distilled H_2O final volume of one liter, and autoclaved.

3. De Man Rogosa and Sharpe Agar (MRS)

Enzymatic Digest of Animal Tissue	10 g
Beef Extract	10 g
Yeast Extract	5 g
Dextrose	20 g
Agar	12 g
Sodium Acetate	5 g

Polysorbate 80	1 g
Potassium Phosphate	2 g
Ammonium Citrate	2 g
Dipotassium hydrogen phosphate	2 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g

The agar was prepared by dissolving all of the above reagents in distilled H_2O final volume of one liter, and autoclaved.

APPENDIX C

DOCUMENTS

ScF 03_02 (Eng)

The Human Research Ethics Committee of Thammasat University (Science), (HREC-TUSc)
Room No. 110, Piyachart Building, 1st Floor, Thammasat University Rangsit Campus,
Prathumthani 12121, Thailand, Tel: 0-2986-9213 ext.7358 E-mail: ecsctu3@Staff.tu.ac.th

COE No. 022/2564

Certificate of Exemption

Project No. : 137/2564
Protocol Title : Penetration and antibacterial ability in dentinal tubules of NeemPaste for caries treatment *(In vitro study)*
Principle Investigator : Kamonchanok Tangsapsathit
Place of Proposed Study/Institution: Faculty of Dentistry, Thammasat University

The Human Research Ethics Committee of Thammasat University (Science), Thailand, has approved the above study project, in accordance with the compliance to the Declaration of Helsinki, the Belmont report, CIOMS guidelines and the International practice (ICH-GCP).

The Human Research Ethics Committee of Thammasat University (Science), decided to exempt the above study. These decision has been reported in 24/2564 meeting.

Signature:..... Jinda Wangboonskul
(Assoc. Prof. Jinda Wangboonskul, Ph.D.)
Chairman of The Human Research Ethics Committee of Thammasat University (Science)

Signature: ..... Laksana Laokiat
(Assoc. Prof.Laksana Laokiat, Ph.D.)
Secretary of The Human Research Ethics Committee of Thammasat University (Science)

Date of issue: December 17,2021

The approval documents including

1) Research proposal
2) Principal investigator's Curriculum Vitae

คณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยธรรมศาสตร์

หนังสือรับรองเลขที่ 002/2565

รหัสโครงการ 077/2564

ชื่อโครงการวิจัย ความสามารถในการแพร่และทำลายเชื้อแบคทีเรียก่อโรคฟันผุผ่านท่อเนื้อฟันของวัสดุชนิดใหม่นีมเพลต์

ชื่อผู้วิจัยหลัก นางสาวกมลชนก ตั้งทรัพย์สถิต (นักศึกษาปริญญาโท)

หน่วยงานที่รับผิดชอบ คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ที่ปรึกษาโครงการ อาจารย์ ทันตแพทย์หญิง ดร. ปริญดา ทัศณรงค์
คณะทันตแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ผลการพิจารณา

ประเภทของโครงการวิจัย ประเภทที่ 2

ระดับความปลอดภัยของห้องปฏิบัติการ ความปลอดภัยระดับที่ 1 (Biosafety Level 1) โดยต้องมี autoclave

คณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยธรรมศาสตร์ ได้พิจารณาประเภทของโครงการวิจัย และระดับความปลอดภัยของห้องปฏิบัติการ

ระยะเวลาที่อนุมัติ 1 ปี (เอกสารอนุมัติฉบับนี้มีผลตั้งแต่วันที่ 10 มกราคม 2565 ถึง วันที่ 9 มกราคม 2566)

กำหนดส่งรายงานความก้าวหน้า/ขยายเวลา/ปิดโครงการ 12 เดือน (9 มกราคม 2566)
ในกรณีเกิดอุบัติเหตุร้ายแรงหรือเกิดเหตุการณ์ไม่พึงประสงค์ให้แจ้งคณะกรรมการฯ ทราบภายใน 2 สัปดาห์

ลงชื่อ.....
(รองศาสตราจารย์ ดร. พจนีย์ ศรีมาโนชญ์)
ประธานกรรมการ

ลงชื่อ.....
(ผู้ช่วยศาสตราจารย์ ดร. ชาลินี รอนไพริน)
กรรมการและเลขานุการ

อนุมัติ ณ วันที่ 10 มกราคม 2565

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