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ANTIMICROBIAL AND ANTIBIOFILM PROPERTIES OF MALAYSIAN STINGLESS BEE PROPOLIS AGAINST *ENTEROCOCCUS FAECALIS*: AN *IN VITRO* STUDY FOR ENDODONTIC INTRACANAL APPLICATION

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CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on February 2026 to conduct the final examination of Ahmad Iman Ammer Bin Azman on his Master of Science thesis entitled "Antimicrobial and Antibiofilm Properties of Malaysian Stingless Bee Propolis against *Enterococcus faecalis*: An *In Vitro* Study for Endodontic Intracanal Application" in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiners recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

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ABSTRACT

Enterococcus faecalis is a persistent pathogen associated with endodontic treatment failures due to its resistance to most common intracanal medicament, non-setting calcium hydroxide [NS-$Ca(OH)_2$]. The limitations of current treatment have led to interest in researching alternative compounds, with stingless bee propolis emerging as a possible option. Hence, this study aimed to evaluate the antimicrobial efficacy of its ethanolic extracts from three Malaysian stingless bee species (*Heterotrigona itama, Geniotrigona thoracica, Tetrigona apicalis*) against both *E. faecalis* ATCC 29212 and ATCC 4082 via agar-well diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), antibiofilm, anti-adherence assays, time-kill study (TKS), and scanning electron microscope (SEM) analysis. Further evaluation was made on potential synergistic effects compared to positive controls, 45 mg/mL NS-$Ca(OH)_2$ and 2% (20 mg/mL) chlorhexidine (CHX) gel. Statistical analyses were conducted using two-way ANOVA (for agar-well diffusion, antibiofilm, and anti-adherence) and repeated-measure ANOVA (for TKS). For non-normally distributed data, the Kruskal-Wallis test (for MIC and MBC) was used. CHX gel demonstrated the strongest antimicrobial effect for most of the microbiological tests (agar-well diffusion; ATCC 29212 = 16 ± 0.6 mm, antibiofilm effect; ATCC 29212 = 92.40 ± 1.70% and ATCC 4082 = 91.31 ± 1.18%, anti-adherence effect; ATCC 29212 = 85.54 ± 0.65% and ATCC 4082 = 90.07 ± 0.70%). Notably, propolis obtained from the same varied in its antimicrobial properties as *H. itama* propolis demonstrated the highest efficacy among the propolis extracts, with low MIC values (ATCC 29212 = 1.56 mg/mL (IQR: 1.56), ATCC 4082 = 0.78 mg/mL (IQR: 0.78)) and complete bactericidal activity (0 CFU/mL) within 24 hours. Its anti-adherence activity (ATCC 29212 = 68.50 ± 4.11% and ATCC 4082 = 76.54 ± 2.03%) was also substantially higher than NS-$Ca(OH)_2$. In contrast, other propolis extracts showed comparatively weaker and inconsistent effects. Synergistic effect was observed in extract mixture against ATCC 4082 (ΣFIC = 0.364), while additive effect for ATCC 29212 (ΣFIC = 0.752). SEM revealed cellular disruption in both treated *E. faecalis* strains. Regarding bacterial strains, *E. faecalis* ATCC 4082 was observed to be more susceptible to NS-$Ca(OH)_2$, CHX gel, and all three propolis extracts compared to ATCC 29212 based on all microbiological test. *H. itama* propolis exhibits strong antimicrobial potential comparable to conventional intracanal medicaments, supporting its potential application as an alternative agent in endodontic medicament.

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LIST OF SYMBOLS

Symbols

°	Degree
µ	Micro
®	Registered Trademark
C	Celcius
G	Gram
H	Hour
L	Litre
M	Milli
Min	Minutes
Rpm	Revolutions Per Minute
w/v	Ratio Weight Per Volume
α	Alpha
β	Beta
γ	Gamma

LIST OF ABBREVIATIONS

Abbreviations

3D	Three-Dimension
ANOVA	Analysis of Variance
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
BHI	Brain-heart Infusion Media
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DCNA	Dental Clinics of North America
ECM	Extracellular Matrix
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HDPSC	Human Dental Pulp Stem Cells
IBM	International Business Machines Corporation
ICC	Intraclass Correlation Coefficient
IQR	Interquartile Range
LAB	Lactic Acid Bacteria
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MRS	DeMan, Rogosa, and Sharpe Broth
PCR	Polymerase Chain Reaction

PEM	Propolis Extract Mixture
PYR	Pyrrolidonyl Arylamidase Test
SD	Standard Deviation
SEM	Scanning Electron Microscope
SPSS	Statistical Package for the Social Sciences
TEM	Transmission Electron Microscope
TKS	Time-kill Study
USA	United States of America
WGS	Whole Genome Sequencing
ZOI	Zone of Inhibition
ΣFIC	Total Fractional Inhibitory Concentration

LIST OF NOMENCLATURES

Nomenclatures

Ace	Adhesin of Collagen from *E. faecalis*
Agg	Aggregation Substances
Ca^{2+}	Calcium Ion
$CaCl_2$	Calcium Chloride
CHX	Chlorhexidine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
eDNA	Extracellular Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ESP	Enterococcal Surface Protein
GelE	Gelatinase
H_2S	Hydrogen Sulfide
H_2SO_4	Sulfuric Acid
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IL	Interleukins
KH_2PO_4	Potassium Dihydrogen Phosphate
LAP	Leucine Aminopeptidase

MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry
MTAD	Mixture of Tetracycline, Citric Acid, and Detergent
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaCl	Sodium Chloride
NaOCl	Sodium Hypochlorite
NaOH	Sodium Hydroxide
NO	Nitric Oxide
NS-$Ca(OH)_2$	Non-setting Calcium Hydroxide
OH^-	Hydroxyl Ion
PBS	Phosphate-buffered Saline
PLA2	Phospholipase A2
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
Spr	Serine Protease
TNF	Tumor Necrosis Factor
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER 1
INTRODUCTION

1.1 Research Background

Endodontic also known as root canal treatment, aims to treat and preserve teeth with microbial-infected or inflamed pulp, while preventing reinfection by microorganisms (Abbott, 2012). Despite adhering to the appropriate treatment regime, root canal therapy is not always successful. The most common reason for treatment failure is persistent bacterial infection, with *Enterococcus faecalis* identified as the predominant pathogen, found in 67-77% of failed cases (Vidana et al., 2011).

Intracanal medication plays a vital role in endodontics, especially in cases where treatment requires follow-up with multiple sessions, during which, it disinfect the surviving bacteria, promotes periapical tissue healing and reduces inflammation of surrounding tissues (Hargreaves et al., 2016). With increased anatomical complexity of the root canal system and resistance of certain oral pathogens, an ideal intracanal medicament must provide prolonged antimicrobial effect without interfering with the patient's immune response and tissue repair (Hargreaves et al., 2016; A. Kumar et al., 2019).

Calcium hydroxide ($Ca(OH)_2$) is widely used in dentistry in two forms namely non-setting calcium hydroxide (NS-$Ca(OH)_2$) and calcium hydroxide (as cavity liners). NS-$Ca(OH)_2$ is the most extensively used intracanal medicament (Alsubait et al., 2020), but it is notably ineffective against *E. faecalis*, a resilient facultative anaerobe, frequently implicated in persistent endodontic re-infections. This is due to NS-$Ca(OH)_2$ limited ability to penetrate dentinal tubules and exert sustained antimicrobial activity significantly, thus reduces its efficacy in eliminating deeply embedded microorganisms. (Abbaszadegan et al., 2016; John et al., 2015; Mohammadi & Dummer, 2011).

Occasionally, chlorhexidine (CHX), is also used. It is a synthetic cationic bis-biguanide, a versatile substance that has been utilised in endodontics as both irrigant and intracanal medicament (in 2% gel form) as it offers superior antimicrobial potency and substantivity within the root canal system (Gomes et al., 2013). Nevertheless, CHX gel as intracanal medicaments lacks tissue-dissolving properties and toxic to human

tissue, which limits its function as a standalone medicament (Mohammadi, 2008). Therefore, ongoing research has explored combining or replacing conventional agents with nutraceutical alternatives, such as propolis, to enhance antimicrobial effectiveness while minimising cytotoxic effects and antimicrobial resistance (Mohammadi, 2008).

E. faecalis is a facultative anaerobic Gram-positive, coccus-shaped bacterium that has been found to be predominantly present in failed root filling (Hargreaves et al., 2016). Its clinical relevance in endodontics is highlighted by its resistance to the conventional disinfection methods due to its ability to invade dentinal tubules, forming single-species resilient biofilm, and survive harsh environments (Yang et al., 2024; Zheng et al., 2017). Out of 69 strains of *E. faecalis*, the list of strains studied can be shown in Table 2.3. Most studies focused on other periodontal pathogens and relied heavily on *E. faecalis* ATCC 29212, isolated from urine, due to its being readily available. Strains such as ATCC 4082 and ATCC 4083, isolated from the pulpless teeth is also studied but less so (Zancan et al., 2018).

With this ultimate challenge, natural products such as propolis (Figure 3.1) have gradually gained attention as potential alternative intracanal medicaments as they possess the criteria of ideal intracanal medicament (John et al., 2015) due to its complex chemical composition and long-standing use in traditional medicine (Hossain et al., 2022). Recent studies have highlighted its potential as a nutraceutical alternative for intracanal medicaments in endodontic therapy, owing to its rich pharmacological profile including antimicrobial, anti-inflammatory, antioxidant, and antiseptic properties (Abdullah et al., 2019; Endo et al., 2017; Shabbir et al., 2020; Wagh, 2013). The therapeutic efficacy of propolis is largely due to the synergistic interactions among its complex constituents, including flavonoids, phenolic acids, and terpenoids (Bak-Badowska et al., 2019; Braakhuis, 2019).

Stinging bee (Figure 2.3 and 2.4) and stingless bee (Figure 2.6) propolis have been documented, their efficacy against *E. faecalis,* particularly in oral study remain insufficiently explored. Therefore, systematic evaluation using established microbiological assays, including agar-well diffusion, broth microdilution, fractional inhibitory concentration (ΣFIC), antibiofilm and anti-adherence assays, scanning electron microscopy (SEM), and time-kill study (TKS), is essential to determine its suitability as a nutraceutical alternative intracanal medicament.

The ΣFIC test approximates the interaction between antimicrobial agents intended for combined use (Shafiei et al., 2016). Antibiofilm and anti-adherence test

were conducted on *E. faecalis* because it is able to adhere to dentine, forming calcified biofilm. These biofilms contribute to the tenacity and endurance of *E. faecalis* against accepted treatments, to completely disinfecting the treated area during root canal therapy (Wahjuningrum et al., 2017). Nutraceuticals containing polyphenols, flavonoids, and D-amino acids have been shown to hinder pathogenic biofilm formation (Elgamoudi & Korolik, 2023; Wahjuningrum et al., 2017). TKS is a procedure used to evaluate the bactericidal effect against tested bacteria at different time intervals (Alshareef, 2021).

1.2 Motivation for This Work

Persistent issue in efficiently eliminating *E. faecalis*, a prevalent pathogen linked to most of the failed endodontic cases and recurrent root canal infection has been the primary reason for this research. Even with the widespread application of conventional intracanal medicaments including the 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel, their limitation in disinfecting pathogens and preventing biofilm formation, especially by *E. faecalis,* has induced the need to seek alternative intracanal medicaments that help in improving the endodontic retreatment, thus enhancing the quality of oral healthcare. The inadequacy of current medication in overcoming this problem not only compromises the success rate of endodontic treatment but also poses a concern for long-term oral health outcomes.

The growing interest in natural products with antibacterial, antibiofilm, and anti-adherence properties has spotlighted propolis, particularly those produced by stingless bees, as a promising candidate for novel antimicrobial agents in dentistry. Although the application of propolis as medication has widely been acknowledged, the antibacterial and antibiofilm effect of propolis obtained from stingless bees has not been thoroughly investigated, especially from species originated in Malaysia.

1.3 Problem Statement

Chemo-mechanical preparation in endodontic treatment remains insufficient for complete microbial elimination from the root canal system, necessitating the use of

intracanal medicaments. NS-$Ca(OH)_2$ is the gold standard of intracanal medicament, but possess limitation as *E. faecalis* is able to survive the alkaline environment. Although CHX gel demonstrates superior antimicrobial activity, its clinical application is associated with several drawbacks, including cytotoxicity, tooth discolouration, and precipitate formation when sodium hypochlorite used as irrigant. These limitations highlight the need for safer and more effective alternative intracanal medicaments.

E. faecalis has been a primary concern in endodontic treatment due to its ability to form biofilms, penetrate dentinal tubules, and survive under harsh conditions including nutrient deprivation and alkaline environments. Although endodontic infections are typically polymicrobial, *E. faecalis* has the capacity to persist as a dominant species and form single-species biofilms under certain conditions, particularly in secondary or persistent infections. While most studies focus on a single reference strain, the comparison observation between *E. faecalis* strains, ATCC 29212 (source-urine) and ATCC 4082 (source–pulpless teeth) remains scarce, since the strain ATCC 4082 may serve as a more appropriate model for evaluating antimicrobial strategies. This gap limits the understanding of strain-dependent variability in antimicrobial susceptibility.

Stingless bee propolis has emerged as a promising natural antimicrobial agent. However, current studies are largely focused on honeybee propolis, with limited comparative data on Malaysian stingless bee species such as *Heterotrigona itama* (*H. itama*), *Geniotrigona thoracica* (*G. thoracica*) and *Tetrigona apicalis* (*T. apicalis*). Although stingless bee propolis have been widely documented, its efficacy against *E. faecalis,* especially in oral study, remain insufficiently explored. Furthermore, existing studies on propolis primarily evaluate antibacterial activity, with insufficient investigation into antibiofilm, anti-adherence, and morphological effects against *E. faecalis*, whch are critical factors in endodontic infections.

In addition, there is a lack of comprehensive *in vitro* studies comparing the antimicrobial efficacy of Malaysian stingless bee propolis with established intracanal medicaments, such as 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel, across multiple microbiological assays. Evidence on the potential synergistic interactions between different propolis extracts, as well as their time-dependent bactericidal activity, also remains scarce.

1.4 Research Hypotheses

a) There are significant differences in the antimicrobial activity between the three Malaysian stingless bee propolis, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel against *E. faecalis* ATCC 29212 and ATCC 4082.

b) The combination of three stingless bee propolis ethanolic extracts exhibits a synergistic antimicrobial effect against *E. faecalis* strains.

c) There are significant differences between the three Malaysian stingless bee propolis, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel in altering *E. faecalis* biofilm formation and morphology.

d) At minimum bactericidal concentration (MBC), there are significant differences in bactericidal activity rates against the most susceptible *E. faecalis* strain (against propolis and intracanal medicaments) between the three Malaysian stingless bee propolis extracts, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel.

1.5 Research Objectives

This study aims to evaluate the antimicrobial activity of propolis extracted from three different stingless bee species, which are *H. itama*, *G. thoracica,* and *T. apicalis* against two strains of *E. faecalis* (ATCC 29212 and ATCC 4082) and to compare their effectiveness with 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel. The objectives of this research are outlined below:

a) To determine and compare the antimicrobial activity of Malaysian stingless bee propolis, NS-$Ca(OH)_2$ and CHX gel against *E. faecalis* ATCC 29212 and ATCC 4082.

b) To evaluate the synergistic antimicrobial effect of propolis ethanolic extract mixture against *E. faecalis* strains.

c) To assess antibiofilm, anti-adherence activities, and morphological changes of *E. faecalis* after treatment with Malaysian stingless bee propolis, NS-$Ca(OH)_2$ and CHX gel.

d) To determine the time-dependent bactericidal activity at MBC value of Malaysian propolis extracts, NS-$Ca(OH)_2$, and CHX gel against the most susceptible *E. faecalis*.

1.6 Research Questions

i) Among the stingless bee species (*H. itama*, *G. thoracica,* and *T. apicalis*) from the same farm, do they exhibit different antimicrobial activities against *E. faecalis* strains (ATCC 29212 and ATCC 4082) compared to 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel?

ii) Is there any notable increase in antimicrobial effect due to synergistic interaction when a mixture of propolis is used?

iii) Between the propolis extracts, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel; how do they influence the biofilm formation, bacterial adherence, and morphology of both *E. faecalis* strains?

iv) What are the microbial killing rates between propolis extracts, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel against the most susceptible *E. faecalis* strain within 24 hours?

1.7 Significance of Study

This research bridges the knowledge gap regarding the antimicrobial, antibiofilm, and anti-adherence properties of Malaysian stingless bee propolis, focusing on *H. itama*, *G. thoracica,* and *T. apicalis*. As Malaysia is situated in a region rich in biodiversity, propolis produced by different types of bees may vary in terms of their biological and therapeutic effects (Abdullah et al., 2019; Nafi et al., 2019; Salleh et al., 2021). Therefore, this study aims to compare the antimicrobial efficacy of Malaysian stingless bee propolis against *E. faecalis* and to identify its potential utilization in

medication.

Given the flaws of conventional intracanal medicaments, propolis has emerged as a promising alternative, as it is proven to possess antibacterial, antioxidant, and anti-inflammatory properties, and has shown high efficacy against *E. faecalis* (Elsayed et al., 2021; Ismail et al., 2020; Shabbir et al., 2020). While previous studies have established the antimicrobial properties of propolis, this study further explores its antibiofilm and anti-adherence effects in comparison with conventional intracanal medicaments, providing more clinically relevant evidence for its application in endodontics. Besides from its therapeutic properties, propolis also a compound with fewer side effects, affordable, and sustainable. The development of a alternative intracanal medicament may improve the success rate of root canal treatment and ultimately enhance patients' quality of life.

Considering that different strains of the same bacterial species may exhibit distinct phenotypic and genotypic characteristics, the inclusion of clinically relevant bacterial strains in this study enhances the translational value of the findings by better reflecting real-world endodontic conditions. This approach strengthens the applicability of the data to clinical practice and supports more meaningful evaluation of antimicrobial efficacy.

The synergistic interaction among diverse classes of bioactive compounds within propolis extracts may potentiate their antimicrobial efficacy, particularly when used in combination. This possible synergistic interaction may reduce the required concentration of propolis in the formulation, maximise its therapeutic action as an intracanal medicament, minimise potential side effects, and help prevent the development of resistance among oral pathogens (Eslami et al., 2016). Exploring the synergistic effects of propolis mixtures can contribute to the development of optimised antimicrobial formulations.

CHAPTER 2
LITERATURE REVIEW

2.1 Anatomy of the Tooth

Understanding dental anatomy and the biological dynamics of the oral environment provides valuable insight into disease progression, particularly in the pathogenesis of endodontic infections. The oral cavity is a complex environment that includes the teeth, buccal mucosa, tongue, soft and hard palate (Lu et al., 2019). Among these structures, teeth are essential component of human mouth as it plays crucial role in mastication, speech, and overall facial aesthetics. Human teeth are categorised into incisors, canines, premolars, and molars. Structurally, a tooth is composed of enamel, dentine and pulp. Enamel is the outermost layer of the tooth that are highly mineralised which act as protectitive barrier against mechanical damage and microbial invasion. Dentine, which is a prorus, tubular structure that facilitates communication between the external environment and the pulp, lies beneath the enamel and at the core of the tooth is the dental pulp. The pulp is composed of blood vessels, nerves, and connective tissues that are essential for nourishment, sensation, and tooth development. The pulp is enclosed within the pulp chamber and root canal system, which extends apically to the periapical tissues (Robert, 2023).

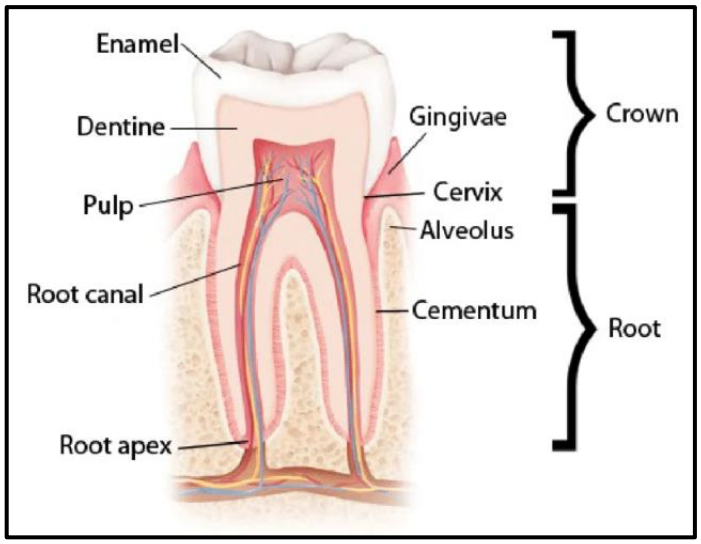


Figure 2.1 General Tooth Anatomy

Sources: Stress, life history and dental development : a histological study of mandrills (*Mandrillus sphinx*).(Lemmers, 2017, p. 5)

2.2 Oral Microbiome

Oral microbiome, also known as oral microbiota or oral microflora is the diverse community of symbiotic, commensal and pathogenic microorganisms that inhabit the human mouth cavity. Human oral microbiota is one of the human body's most intricate microbial populations as it is made up of roughly 700 different types of microbial species (Deo & Deshmukh, 2019). The oral cavity is a complex ecosystem that includes several different ecological niches which form a species-rich heterogeneous ecological system. These oral surfaces offer an ideal habitat for the microbes to live and proliferate in environments that are nutrient-rich, moist, normal temperature (37°C), and neutral pH (6.5 – 7.0).

The mouth is inhabited by a variety of microorganisms (as shown in figure 2.2) including bacteria, such as, *Firmicutes, Bacillus, Proteobacteria*, *Actinomycetes*, fungi, such as *Candida*, and viruses, such as mumps virus and human immunodeficiency virus (HIV) (Dewhirst et al., 2010; Lu et al., 2019). These microorganisms co-exist in a symbiotic relationship with the human body of healthy normal humans, but a dysbiosis event can occur when these microorganisms invade and proliferate in the oral cavity, interrupt the symbiosis, and cause oral infection (Thomas et al., 2021).

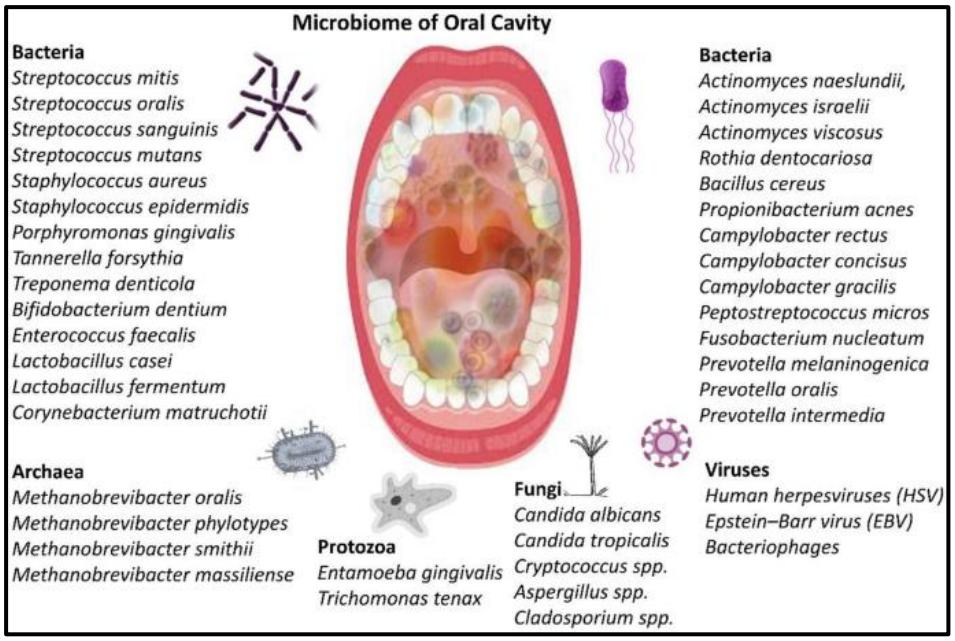


Figure 2.2 Composition of Human Oral Microbiome

Sources: A critical review on the roles of natural products in shaping oral microbiota and preventing chronic diseases. (Kumar & Xu, 2026, p. 4)

Symbiosis refers to a balanced, mutually beneficial relationship between the host and the resident oral microbiota, in which a diverse and functionally stable microbial community coexists with the host without inducing disease (Lin et al., 2024). In this state, commensal in oral cavity such as *Staphylococci*, *Lactobacilli*, and *Corynebacterium*, and *Streptococcus* species contribute to colonization resistance, immune modulation, and maintenance of tissue homeostasis, thereby limiting the overgrowth of pathogenic species (Nandhinipriya et al., 2024). In contrast, dysbiosis in oral ecosystem describes a disruption of this host-oral microbiome interaction (Lin et al., 2024) characterised by a shift in microbial composition and function that favours the proliferation of oral opportunistic and pathogenic organisms such as *Streptococcus mutans, Staphylococcus aureus, Fusobacterium nucleatum, Porphyromonas gingivalis, Enterococcus faecalis,* and *Candida* sp. (Fernandes et al., 2023; Krishnamoorthy et al., 2020).Numerous oral infections, including tonsillitis, dental caries, periodontitis, pulpitis, and loss of alveolar bone are some of the diseases caused by these oral microbiota (Sudhakara et al., 2018).

2.3 Pathogenesis of Root Canal Infection

The root canal system is made up of dental pulp that consists of vascular tissues, nerves, and protected with strong dental components like as dentine, enamel, and cementum. Under normal conditions, the dental pulp is physiologically sterile connective tissue where the presence of microorganisms is indicative of pathological sign. When enamel is compromised, such as through dental caries, cracks, trauma, restorative defects and iatrogenic causes, microorganisms can penetrate the underlying dentine and progress toward the pulp via the dentinal tubules (Hargreaves et al., 2016). Due to the confined environment of the pulp chamber, infection can rapidly lead to inflammation that can compromise vascular supply, ultimately leading to pulp necrosis. Once the pulp becomes necrotic, the root canal system provides a favourable environment for microbial colonisation and biofilm formation, ultimately resulting in root canal infection and periapical disease (Hargreaves et al., 2016; Wong et al., 2021).

2.4 Endodontic Treatment

Endodontic comes from the Greek word where “endo” means inside and “odont” means tooth. Endodontic treatment, also known as root canal, is a dental procedure that removes damaged pulpal tissue, infected debris, and pathogens inside the root canal using mechanical instrumentation coupled with irrigation (Swetah & Ranjan, 2017). The fundamental principles of endodontic treatment encompass accurate diagnosis, access cavity preparation, meticulous cleaning and debridement, canal shaping, obturation, and complete three-dimensional filling of the root canal system. Endodontic treatment can be performed with two plans: completing the whole treatment regime in a single visit or multiple-visit treatment (Łucja et al., 2021).

Between these two approaches of root canal treatment, there are no significant advantages in terms of efficacy and success rate (Łucja et al., 2021; Manfredi et al., 2016). The decision between these strategies is based on case selection, infection severity, and specific to the patient’s factors. A single-visit approach is generally selected when the risk of infection is minimal and no anatomic or procedural difficulties. In contrast, multiple-visit treatment is performed over two or more appointments to lessen the technical error, post-operative infection, and pain. A distinct

step in multiple-visit endodontic treatment is the application of intracanal medicament to disinfect and avoid regrowth of bacteria (Manfredi et al., 2016; Rathi et al., 2022).

Chemo-mechanical preparation cannot fully eradicate microbes in the dentinal tubules (Rathi et al., 2022). After the mechanical instrumentation, the routinely applied intracanal medicament in endodontic treatment is to eradicate any surviving bacteria and tissue debris. This is to minimise inflamed tissue, shaping the root canal, drying the canal, to serve as a barrier by preventing leaks from the temporary inter appointment filling (Chong & Ford, 1992). Current intracanal medicaments can be categorised into phenolic derivatives (eugenol, camphorated para-monochlorophenol, camphorated phenol, metacresyl acetate, beechwood creosote), aldehydes (formocresol), halides (iodine-potassium iodide), NS-$Ca(OH)_2$, antibiotics, or other combinations (Manfredi et al., 2016).

During endodontic treatment, creating a sterile field around the patient, surgical equipment, and the surgical site is essential to avoid cross-contamination and infections by pathogens. Additionally, the root canal system must be cleared of all viable pathogens, pulpal tissues, and dentinal debris, where improper eradication of these agents during endodontic treatment may result in ongoing inflammation, impaired wound healing, and re-infections. However, *E. faecalis* which is the predominant pathogen in oral post-treatment infection, is more resistant and cannot be eradicated completely even with the current endodontic medicaments (Alghamdi & Shakir, 2020). Compared to primary endodontic infections, *E. faecalis* is approximately nine times more likely to be present in failed root canal treatment cases. It has been reported to be the most often encountered microorganism in all documented cases of post-endodontic therapy discomfort and infection, with prevalence values up to 90% (Mayer & Pinheiro, 2015). Recent research further proved that the prevalence of *E. faecalis* in failed endodontic cases was between 67% to 77% by polymerase chain reaction as a detection method (Vidana et al., 2011) and it can withstand and endure within the root canal for extended periods of time even after utilising intracanal medicament (Jain et al., 2016).

2.5 *Enterococcus faecalis* (*E. faecalis*)

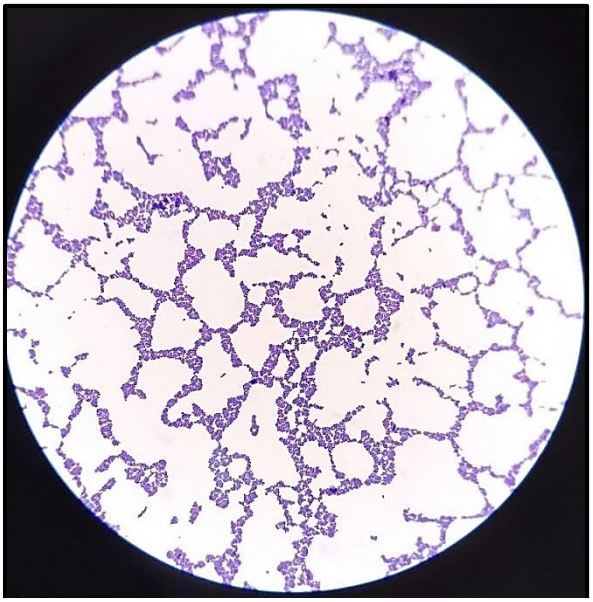


Plate 2.1 Gram Staining of *E. faecalis* Observed Through Oil Emulsion Light Microscope (100X Magnification)

E. faecalis is a Gram-positive bacterium (shown in Plate 2.1) that is classified initially as a group D *Streptococci*. However, *enterococci* species were brought out from the genus *Streptococcus* and are now classified into a new genus, which is *Enterococcus*. The classification of *E. faecalis* is shown in Table 2.1. This new classification is due to the genetic differences and notable features such as Gram-positive, catalase-negative, non-spore-forming, and facultative anaerobic bacteria. In addition, it is categorised in the lactic acid bacteria (LAB) group, which can be recognised by low G+C content and is capable of growing in a wide range of temperatures (Gijo et al., 2015). A common microorganism that can be found in numerous ecosystems such as soil, water, plants, and animals, and is abundantly found in faecal-contaminated environments. This opportunistic bacterium frequently found in the gastrointestinal tract, genitourinary tract and oral cavity of human and other mammals which are normally harmless to host (Lee et al., 2019), but may harm the host with weakened immune systems or under certain conditions that can lead to several infections such as urinary tract infections, bacteraemia, wound infection, and oral infection (endocarditis, marginal periodontitis, and periradicular abscesses) (Stuart et al., 2006). *E. faecalis* is not considered an oral microbiota, but frequently isolated from oral diseases including periodontitis and dental caries (Zaatout, 2021). *E. faecalis* is

recognised as a transient inhabitant of the oral cavity, often introduced through external sources such as contaminated food and water, direct contact with animals, or oral exposure to various objects including chewing items and fomites. (Zaatout, 2021). *E. faecalis* infections can be very difficult to treat due to their resistance to several antimicrobials, including vancomycin, which is an antibiotic of last resort for infections (Kau et al., 2005).

Table 2.1
Classification of *E. faecalis*

E. faecalis Classification	
Domain	Bacteria
Phylum	Firmicutes (Previously known as Bacillota)
Class	Bacilli
Order	Lactobacillales
Family	Enterococcaceae
Genus	*Enterococcus*
Species	*E. faecalis*

2.5.1 Physiological and Biochemical Features

E. faecalis is a Gram-positive bacterium with a thick cell wall and characteristic surface structures. A single cell contains cytoplasm and non-membrane-bound organelles, enclosed within the cell wall, and often displays pili and flagella on its surface (Kayser et al., 2005). The cell wall is primarily composed of peptidoglycan (a cross-linked peptide-sugar structure), wall teichoic acids, and lipoteichoic acids. The intercellular glycerol teichoic acid associated with the cytoplasmic membrane forms the group D carbohydrate cell wall surface antigen, also known as the Lancefield antigen, while no presence of proteins or lipids in its cell wall (Růžičková et al., 2020; Yang et

al., 2017). Observation under a high magnification microscopy shows that *E. faecalis* appears slightly oval in shape with a diameter of 0.5 to 2.5 μm. In terms of orientation, *E. faecalis* can be seen as single cells, in pairs or arranged in short chains (Růžičková et al., 2020).

E. faecalis is able to catabolise various energy sources, including glycerol, carbohydrates, malate, lactate, agmatine, arginine, citrate, and multiple α-keto acids. It has the ability to ferment glucose without producing gas and does not exhibit a catalase reaction with hydrogen peroxide (John et al., 2015). *E. faecalis* can endure harsh environments as it is capable of withstanding strong alkaline conditions (up to pH 11.5) and high salt concentrations (6.5% sodium chloride). It can grow across a wide temperature range (10°C to 45°C), survive extreme heat at 60°C for up to 30 minutes, and has the potential to form biofilms. In addition, it demonstrates resistance to several antimicrobial substances, including bile salts, detergents, heavy metals, ethanol, azide, and desiccation. *E. faecalis* is classified as a facultative anaerobic bacterium, meaning it prefer to grow in an oxygen-absent environment but can also survive under aerobic conditions (Alghamdi & Shakir, 2020; John et al., 2015; Stuart et al., 2006).

2.5.2 Isolation and Identification

Isolation of *E. faecalis* can be performed from various sources, including clinical specimens (e.g. human root canal infections, urine, blood, and exudates), food samples, fecal samples, hospital environments, and others. The isolates can then be cultured on different types of media depending on the purpose (Shanmukhappa et al., 2015). In general, *E. faecalis* can grow on standard enrichment media such as nutrient agar and brain-heart infusion agar, where it typically forms small, spherical, smooth, opaque, creamy colonies. For primary isolation, characterisation and differentiation in diagnostic laboratories, blood agar is commonly used. On the medium, *E. faecalis* produces colonies that are small, smooth, grey to greyish-white, with non-haemolytic (γ- haemolytic) characteristics. Some strains may occasionally display a narrow zone of β- or α- haemolysis around the colonies. *E. faecalis* is bile-tolerant and can grow in the presence of up to 40% bile salts. On bile esculin agar, it produces tiny, convex, slightly brownish colonies with a characteristic brown-black halo surrounding them (Maza et al., 1997).

Following the culturing and isolation, *E. faecalis* can be identified through various biochemical tests, including general biochemical assays, carbohydrate fermentation tests, and enzymatic hydrolysis tests. The reactions and results of these identification methods are summarised in the table below.

Table 2.2
List of Biochemical Tests for *E. faecalis* Identification

Biochemical tests	**Result**	**Reaction**
General biochemical tests		
Arginine dihydrolase	+	Breakdown of arginine into ornithine, with ammonia as a byproduct. Indication by media colour changes from purple to yellow and then back to purple.
40% Bile-salt tolerance	+	Visible growth of bacteria in media
Bile solubility	Insoluble	Does not undergo lysis in the presence of bile
Capsule stain	-	Absence of a clear halo zone
Catalase	-	No bubbles appear
Citrate	-	No colour changes
Coagulase	-	No clot formation
Deoxyribonuclease (DNase)	-	Absence of a clear area or cloudy precipitate
Esculin hydrolysis	+	Dark brown or black colour halo surrounding colonies
Gram-stain	Gram-positive cocci	Appear purple or blue
Gas production (in De Man, Rogosa and Sharpe (MRS) broth)	-	No gas production in Durham tube

Haemolysis	γ- haemolytic (rarely happens, but some strain produces α-and β-haemolytic)	No discolouration on the blood agar
Hydrogen sulfide (H_2S)	-	Media does not turn to black colour
Leucine aminopeptidase (LAP) test	+	Development of cherry red or reddish-purple colour
Motility	Non-motile	Growth only along inoculation line
6.5% NaCl tolerance	+	Broth becomes turbid
Nitrate reduction	+	Development of red colouration
Oxidase	-	No colour changes
Oxidative fermentation	Facultative anaerobes	Changing colour to yellow in both aerobic and anaerobic tubes
Pyrrolidonyl aryl amidase (PYR) test	+	Visible red colour development
Pyruvate broth test	+	Broth colour changes from greenish blue to yellow
Schaeffer-Fulton endospore staining	-	Cell appears entirely red with no green spores.
Temperature i) Growth at 4°C ii) Growth at 10°C iii) Growth at 40°C	 - + +	Grow in a wide range of temperatures between 10°C to 45°C
Carbohydrate fermentation tests		
N-Acetylglucosamine	+	Media colour changes to yellow, but no gas production

Adonitol	-	No colour changes
L-Arabinose	-	No colour changes
D-Arabitol	-	No colour changes
L-Arabitol	-	No colour changes
Arbutin	-	No colour changes
Cellobiose	+	Media colour changes to yellow, but no gas production
Dulcitol	-	No colour changes
D-Fructose	+	Media colour changes to yellow, but no gas production
D- and L-Fucose	-	No colour changes
Galactose	+	Media colour changes to yellow, but no gas production
D-Glucose	+	Media colour changes to yellow, but no gas production
Glycerol	+	Media colour changes to yellow, but no gas production
Glycogen	-	No colour changes
Inulin	-	No colour changes
Lactose	+	Media colour changes to yellow, but no gas production
Maltose	+	Media colour changes to yellow, but no gas production
Mannitol	+	Media colour changes to yellow, but no gas production
D-Mannose	+	Media colour changes to yellow, but no gas production

D-Raffinose	-	No colour changes
Rhamnose	Variable (Most studies show *E. faecalis* can ferment rhamnose)	Media colour changes to yellow, but no gas production
Ribose	+	Media colour changes to yellow, but no gas production
Sorbitol	+	Media colour changes to yellow, but no gas production
Sorbose	-	No colour changes
Sucrose	+	Media colour changes to yellow, but no gas production
D-Tagatose	+	Media colour changes to yellow, but no gas production
Trehalose	+	Media colour changes to yellow, but no gas production
Tartrate	-	No colour changes
D-Xylose	-	No colour changes
L-Xylose	Variable (Most studies show *E. faecalis* can ferment rhamnose)	Media colour changes to yellow, but no gas production
Enzymatic hydrolysis tests		
Caseinase	-	No clear zone of hydrolysis around the colonies
α-Galactosidase	-	No colour changes
Gelatinase	+	total liquefaction of gelatine (tube test) and clear zone around the colonies' growth (plate test)
Lipase	+	Clear zones around bacterial colonies
Lysine decarboxylase	-	No colour changes

Ornithine decarboxylase	-	No colour changes
Phenylalanine deaminase	-	No green colour formation
Tryptophanase	+	Red colour layer develops at the top of the tube

Note: Sign " + " indicates positive result while " - " indicates negative result

In addition to biochemical testing, modern molecular diagnostic methods can be applied to achieve more rapid and accurate identification. Polymerase chain reaction (PCR) and sequencing are widely used for species-specific detection, targeting genes such as *16S rRNA*, *ddlE. faecalis*, or *EF3314*. Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS), which analyses bacterial protein spectra, offers a highly accurate and efficient identification approach. Furthermore, Whole Genome Sequencing (WGS) provides comprehensive genetic information, including the identification of genes associated with antibiotic resistance (Maza et al., 1997).

2.5.3 Virulence Factors

The pathogenic potential of *E. faecalis* is largely attributed to its virulence factors, which enhance its ability to survive in harsh environments and evade the host's immune system. These factors facilitate adherence to host cells, modulate host responses through protein production, and enable the transfer of virulence traits between strains, thereby supporting persistence and dissemination (Stuart et al., 2006).

E. faecalis possesses various adhesins, surface proteins or structures that promote attachment to host tissues, biofilm development, colonisation, and protection against host defences. Prominent examples include pili or fimbriae, enterococcal surface proteins (ESPs), aggregation substances (Agg), and collagen-binding adhesins. ESPs are a large surface antigen of *E. faecalis* that support cell adherence, biofilm formation, and immune evasion, while also providing resistance against mechanical clearance in the urinary tract (John et al., 2015; Paganelli et al., 2012).

One notable virulence factor is aggregation substances, a plasmid-encoded adhesin that promotes bacterial aggregation and aids plasmid transmission. The transfer

of plasmid facilitates *E. faecalis* to share its antimicrobial resistance and virulence traits (Hirt et al., 2018). Moreover, aggregation substances also promote the binding to host extracellular matrix (ECM) proteins, which may be particularly significant in endodontic infections since ECM proteins such as collagen type I, are the primary organic component of dentine. Additionally, these substances have been shown to facilitate the direct binding of *E. faecalis* to human neutrophils and macrophages, thereby increasing its survival and resistance to host immune (Kayaoglu & Ørstavik, 2004).

Furthermore, *E. faecalis* also produces extracellular proteins such as Enterococcal-surface-proteins, which promote bacterial cell adhesion to the host cells. This contributes to biofilm formation of *E. faecalis* within dentine, enabling the microbes to withstand the bactericidal effects of NS-$Ca(OH)_2$ during endodontic treatment of infected root canals. (Gijo et al., 2015). It also produces gelatinase, a metalloproteinase (protease enzyme) that can hydrolyse gelatin, collagen, fibrinogen, casein, haemoglobin, insulin, and several other bioactive peptides. This enzyme facilitates attachment to dental surfaces and accelerates the breakdown of collagen and fibrin in the oral cavity. Consequently, gelatinase plays a significant role in the pathophysiology of periapical inflammation (Kayaoglu & Ørstavik, 2004).

2.5.4 Biofilm Formation

Bacterial biofilm is a community of bacteria encased in a self-synthesised, slimy substance known as extracellular polymeric substance (EPS) and adheres to a surface (Yang et al., 2024; Zhao et al., 2023). Biofilm can be produced by a single microbial species or a mixture of microorganisms, including bacteria, archaea, fungi, protozoa, and yeast. Biofilm formation provides significant advantages to microbial communities as its facilitate microbial growth and reproduction, promote viability, maximising resource utilisation, enables adaption, and is categorised as one of the microbial virulence factors since it protect against the host immune system, antibiotic action, and harsh environment (e.g. pH, temperature, pressure, and salinity) (Foley & Gilbert, 1997; Karatan & Watnick, 2009; Kristich et al., 2004).

The biofilm formed by *E. faecalis* can be divided into four phases as shown in figure 2.3: initial attachment, microcolony formation, maturation, and dispersal (Ch'ng

et al., 2019; Yang et al., 2024). Biofilm formation begins with the irreversible attachment of *E. faecalis* planktonic cells to a surface. During the early stage of attachment, proteases, glycolipids, and several surface proteins, such as ESP, Agg, and others are involved in mediating the process (Ch'ng et al., 2019). After the attachment, *E. faecalis* begins to proliferate and produce the biofilm matrix, forming microcolonies. At this stage, the biofilm reaches the early phase of maturation. The maturation process continues with the development of complex, three-dimensional structure (Ch'ng et al., 2019; Ramakrishnan et al., 2022). At full maturation, the biofilm achieves maximum cell density through active growth, modification of the biofilm matrix, and production of extracellular components such as extracellular DNA (eDNA), polysaccharides, lipoteichoic acid, and extracellular proteases (Ch'ng et al., 2019; Zhao et al., 2023). eDNA is the key component of the biofilm matrix, critical for maintaining structural integrity, facilitating genetic exchange within the biofilm, and providing protection against antimicrobial agents (Serrage et al., 2022). In addition, bacterial aggregation and stability within the biofilm are strengthened by cytolysin, which lyses other bacteria and releases eDNA. Gelatinase (GelE), a virulence factor encoded by the *gelE* gene, degrades collagen, other proteins, while also promoting eDNA release through activation of the major autolysin AtlA (Șchiopu et al., 2023; Yang et al., 2024).

As the biofilm becomes more complex with higher metabolic rates, the bacterial communities optimise survivability and communication through quorum sensing and the formation of water channels for nutrient transfer and waste removal. The *E. faecalis* quorum-sensing system plays a key role in biofilm formation, virulence regulation, and antibiotic resistance. One of the notable quorum sensing systems is the *fsr* operon, which regulates the transcription of gelatinase and serine protease, thereby influencing biofilm development. Mature biofilms eventually disperse microcolonies of cells, which can migrate to other surfaces and spread the infection (Ch'ng et al., 2019; Ramakrishnan et al., 2022; Șchiopu et al., 2023; Yang et al., 2024; Zhao et al., 2023).

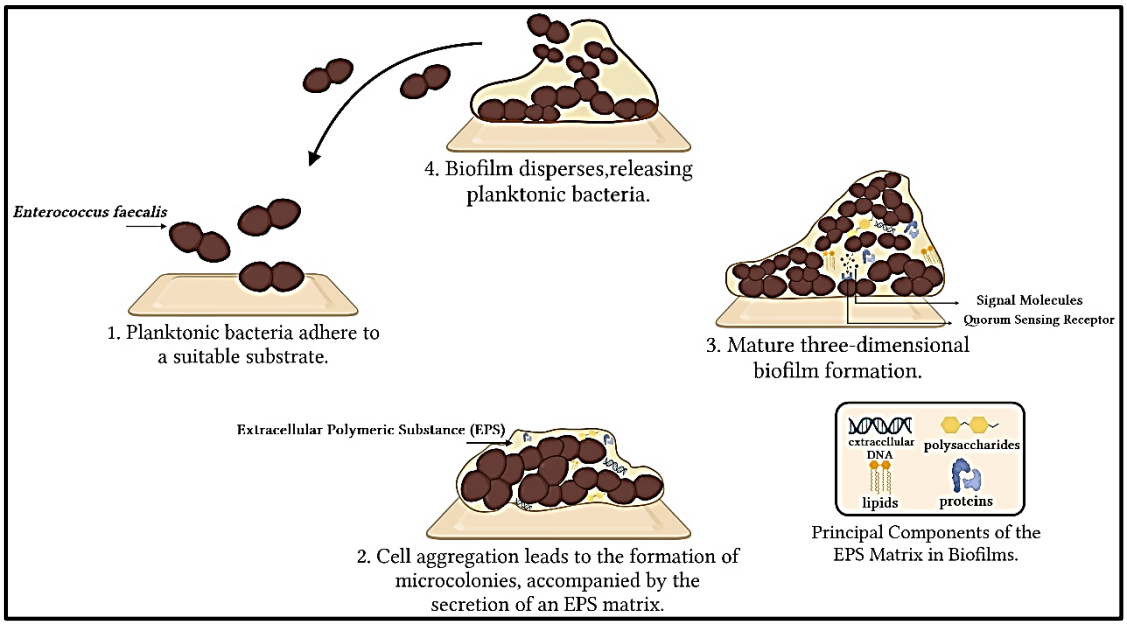


Figure 2.3 Stages of *E. faecalis* Biofilm Formation

Source: Strategies and mechanisms targeting *Enterococcus faecalis* biofilms associated with endodontic infections: a comprehensive review. (Yang et al., 2024, p. 3)

2.5.5 Strains

Since *E. faecalis* is a well-known pathogen causing various types of infections, numerous studies have been conducted on multiple strains to observe their genotype, phenotype, behaviour, and resistance to current drugs. Notably, the ease of culturing *E. faecalis* to form biofilms *in-vitro,* and its significant role as a pathogen have been attracted a great deal in research attention, particularly to evaluate its susceptibility to various antibacterial substances (Newberry et al., 2007; Zancan et al., 2018). To date, a total of 69 commercially available strains of *E. faecalis* have been identified under the American Type Culture Collection (ATCC) (Zancan et al., 2018).

Endodontic re-infections caused by surviving pathogens inside the root canal system remain a serious issue in dentistry. *E. faecalis*, the most persistent bacterium in failed endodontic treatment, is often implicated and is more resistant to existing therapies and drugs, making it a prominent target in endodontic research (Hargreaves et al., 2016; Pinheiro & Mayer, 2015; Vidana et al., 2011). Since *E. faecalis* ATCC 29212 has been frequently used in oral studies but is mainly isolated from urine, it is

more sensible to study other strains isolated from the oral region. Strains such as ATCC 4082 and ATCC 4083 may be suitable for further investigation since both strains were isolated from the root canal of pulpless teeth (Zancan et al., 2018). The table below highlights the application of several *E. faecalis* strains used in oral research.

Table 2.3
Various *E. faecalis* Strains Used in Previous Research

***E. faecalis* Strains**	**Isolation Source**	**Research Outcome**	**Reference**
E. faecalis ATCC 29212	Urine	The addition of 5% propolis nanoparticles to commercial bio-ceramic and epoxy resin sealers can eliminate *E. faecalis* ATCC 29212 in the root canals	(Wahyuni et al., 2023)
		Propolis nanoparticles were equally as effective as 6% NaOCl and 2% CHX in reducing the *E. faecalis* ATCC 29212 biofilms.	(Parolia et al., 2020)
		Antibacterial activity of propolis against *E. faecalis* ATCC 29212 was comparable with that of NS-$Ca(OH)_2$ at different time intervals.	(Ahangari et al., 2018)
		Susceptibility of *E. faecalis* ATCC 29212 and ATCC 4083 against different endodontic pastes were compared. It was found that *E. faecalis* ATCC 4083 was more susceptible to all intracanal medicaments used.	(Zancan et al., 2018)
		The results showed that propolis was effective in inhibiting Gram-positive bacteria (including *E. faecalis*) and it was suggested that propolis could be as effective as CHX.	(Akca et al., 2016)
		Brazilian brown propolis effectively inhibited *E. faecalis* ATCC 29212 in an *in*	(Pimenta et al., 2015)

		vitro study, suggesting its potential as an intracanal medication.	
		Turkish propolis samples were effective against *E. faecalis* ATCC 29212, but their activity did not exceed CHX.	(Kayaoglu & Ørstavik, 2004)
E. faecalis ATCC 4083	Root canal of pulpless tooth	*E. faecalis* ATCC 4083 was more susceptible to all tested intracanal medicaments compared to *E. faecalis* ATCC 29212. The medications used are listed below: • NS-$Ca(OH)_2$ • NS-$Ca(OH)_2$ and polyethylene glycol • NS-$Ca(OH)_2$, polyethylene glycol and camphorated paramonochlorophenol • NS-$Ca(OH)_2$, CHX, and propylene glycol • NS-$Ca(OH)_2$, and double antibiotic paste • Triple antibiotic paste	(Zancan et al., 2018)
E. faecalis ATCC 4082	Root canal of pulpless tooth	An amount of 2 mL of BioPure Mixture of tetracycline, citric acid, and detergent (MTAD) was more effective than 3% NaOCl and propolis against *E. faecalis* ATCC 4082.	(Nara et al., 2010)
E. faecalis ATCC 4082 and *E. faecalis* ATCC 4083	Root canal of pulpless tooth	Treatment with 4 to 5 mL of BioPure MTAD was effective in eliminating the growth of *E. faecalis* ATCC 4082 and *E. faecalis* ATCC 4083.	(Newberry et al., 2007)

E. faecalis ATCC 51299	Peritoneal fluid	NS-$Ca(OH)_2$ significantly reduced fibroblast viability, more than Iranian propolis.	(Jahromi et al., 2012)
E. faecalis ATCC 29532	Blood	Treatment regimen (BioPure MTAD as a final irrigant in conjunction with 1.3% NaOCl) was effective in completely eliminating growth of *E. faecalis* ATCC 4082, ATCC 4083, ATCC 49532, ATCC 49383, ATCC 49452, ATCC 49477, ATCC 10541, and ATCC 19433.	(Newberry et al., 2007)
E. faecalis ATCC 49383	Blood		
E. faecalis ATCC 49452	Not Disclosed		
E. faecalis ATCC 49477	Clinical isolate		
E. faecalis ATCC 10541	Not Disclosed		
E. faecalis ATCC 19433	Not disclosed		

2.5.6 Role of *E. faecalis* in Failed Endodontic Treatment

Even when adhering to proper procedure and medication, endodontic treatment may not always be successful, as recurrence of clinical symptoms such as discomfort, swelling, bleeding, tooth discolouration, and abscesses with pus around the treated tooth together with the presence of a periapical radiolucency, may occur (Tabassum & Khan, 2016). The approximate failure rates of endodontic treatment across all cases is range

between 7 to 18 % (Jang et al., 2024; Ng et al., 2008, 2010). One of the most common reasons for endodontic failure is persistent bacterial infection due to poorly treated canals and the ability of some bacterial species to survive the chemo-mechanical preparation and disinfection process (Tabassum & Khan, 2016). Several factors likely contribute to its enhanced survival in treated root canals, including the tolerance to alkaline stress via proton pump mechanism to maintain homoeostasis, biofilm formation abilities, expression of specific adhesins to dentine, and its capacity to thrive as single-species without metabolic contingency on other bacteria (Parga et al., 2025).

E. faecalis has been widely discovered in root canal re-infection, showing its crucial role in failed endodontic treatment (Almelan et al., 2023; Hoque et al., 2023; John et al., 2015). Aside from its capability to withstand the root canal environment, biofilm formation is one of its strategies to persist in the canal. Adhesins such as the collagen-binding protein (Ace) and serine protease (Spr) play crucial roles in binding to collagen in the root canal. The mechanism of *E. faecalis* penetration into dentinal tubules remains unknown. However, it is hypothesized that penetration may result from intratubular cell growth (John et al., 2015). After *E. faecalis* penetrates the dentinal tubules, it can form single-species biofilm independent of other bacterial species. This renders it more resistant to standard disinfection procedures and capable of developing mono-infections (Almelan et al., 2023).

Despite the utilisation of intracanal medicaments like NS-$Ca(OH)_2$ during treatment, the ability of *E. faecalis* to endure the NS-$Ca(OH)_2$ has been explained by several possible mechanisms. Although NS-$Ca(OH)_2$ induces alkalinity, *E. faecalis* can regulate the pH homeostasis within its cell and the environment through the cytoplasm's buffering capacity and proton pumps that translocate hydrogen ions into the cells, thereby maintaining internal pH (John et al., 2015; Ran et al., 2013). The growth of *E. faecalis* can be inhibited by increasing the pH to at least 11.5, and NS-$Ca(OH)_2$ appears to be suitable due to its alkaline pH range of 12.5 to 12.8 (Dohyun & Euiseong, 2014). However, this desired pH in the root canal is unlikely to be achieved due to dentine's buffering capacity (John et al., 2015; Ismail et al., 2021). Dentine consists of hydroxyapatite, a mineral that helps maintain balanced pH by buffering acidic surroundings. NS-$Ca(OH)_2$ induces an alkaline environment, releasing calcium and phosphate ions that reduce its antimicrobial effectiveness (Carvalho et al., 2015; Slutzky-Goldberg et al., 2013). In addition, *E. faecalis* has mechanisms for regulating its internal pH even in acidic conditions. The proton pump in *E. faecalis* promotes active

transport of protons out of the cell, thereby preserving cellular pH homeostasis. This characteristic enables *E. faecalis* to thrive in harsh and acidic conditions found in treated root canals (John et al., 2015; Ran et al., 2013).

2.6 Intracanal Medicaments

Certain oral pathogens are difficult to disinfect from infected root canals. During the intervals between endodontic appointments, bacteria that survive the instrumentation and irrigation have been shown to proliferate rapidly, causing secondary infection (Byström & Sundqvist, 1981). Hence, an intracanal medicament is a temporary medication placed inside root canals between appointments to aid endodontic treatment by disinfecting pathogens that cause persistent infections and pain, reducing inflammation, acting as a barrier to prevent leakage, and drying the wet canal (Chong & Ford, 1992). The intracanal medicament must possess certain qualities to be eligible for clinical use in human. Some properties of an ideal intracanal medicaments include having powerful and prolonged antimicrobial effects against a broad range of oral pathogens, not causing irritation to the surrounding tissue, maintaining stability in solution, having low surface tension, not inducing an immune response, and not obstructing the ability of periradicular tissues to heal (A. Kumar et al., 2019; Pal et al., 2019; Rathi et al., 2022). NS-$Ca(OH)_2$ is the most widely utilised intracanal medicaments, alongside other alternatives such as CHX, phenol, formaldehyde, and polyantibiotic paste. These substances have demonstrated strong antimicrobial efficacy, effectively eliminating pathogens and minimising inter-appointment flare-ups. According to Grossman Dental Clinics of North America (DCNA), intracanal medicaments are classified according to their chemical nature, as shown in the table below.

Table 2.4
Classification of Intracanal Medicaments Based on Grossman DCNA

Classification	Example
Essential oils	• Eugenol
Phenolic compounds	• Phenol

	• Parachlorophenol • Camphorated parachlorophenol • Cresol • Formocresol • Creosote • Cresatin • Cresanol
Nitrogen (N_2)	• N_2
Salt of heavy metals	• Metaphen • Merthiolate • Mercurophen
Halogens	• Sodium hypochlorite • Iodides • **Chlorhexidine**
Quaternary ammonium compounds	• 9-aminoacidine
Fatty acids	• Propionic acid • Caproic acid • Cuprylic acid
Sulphonamides	• Sulphonamides
Antibiotics	• Triple antibiotic paste (Ciprofloxacin, Metronidazole, Minocycline) • Ledermix (demeclocycline + triamcinolone)
Calcium Hydroxide [$Ca(OH_2]$	• Setting $Ca(OH)_2$ • **NS-$Ca(OH)_2$**

Source: Application of intracanal medicaments: A review. (Pal et al., 2019, p. 14-21)

2.6.1 Non-setting Calcium Hydroxide [NS-$Ca(OH)_2$]

NS-$Ca(OH)_2$ began to be utilised in endodontics in 1920 by Herman as an intracanal medicament (Mohammadi et al., 2012). Due to its ability to promote a temporary physical covering of the root canal, neutralise debris in the canal, exhibit antimicrobial properties and provide osteogenic potential, NS-$Ca(OH)_2$ has become the gold standard intracanal medicament (Lima et al., 2012; Pimenta et al., 2015). NS-

$Ca(OH)_2$ is one of the commonly used intracanal medications, along with other alternatives such as phenol, formaldehyde, CHX, and triple antibiotic paste, all of which are highly effective in disinfecting pathogens and reducing flare-ups between appointments. It is commonly known for its characteristics: an odourless white powder with a molecular weight of 74.08, a high pH ranging from 12.5 to 12.8, low solubility in water, and insolubility in alcohol. NS-$Ca(OH)_2$ is considered to possess good clinical qualities due to its low solubility in tissue fluids, antimicrobial properties attributed to its high alkalinity that can inhibit pathogens, and its slow release when dissociating into calcium ions (Ca^{2+}) and hydroxyl ions (OH^-) (Ba-Hattab et al., 2016; Dohyun & Euiseong, 2014).

2.6.1.1 Antimicrobial Properties

NS-$Ca(OH)_2$ exerts its antimicrobial effect through its strong alkaline properties and the ionic dissociation of Ca^{2+} and OH^- ions. When NS-$Ca(OH)_2$ dissolves in body fluids, it slowly releases both Ca^{2+} and a high concentration of OH^- ions (Mohammadi et al., 2012). The OH^- ions are highly reactive free radicals that interact with many biomolecules. They degrade the phospholipids and proteins, altering the pH gradient and the integrity of the cytoplasmic cell membrane. These ions also cause irreversible chemical injury by denaturing the bacterial intracellular and extracellular enzymes, leading to metabolic dysfunction and cell death. In addition, OH^- ions damage the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), thereby preventing bacterial proliferation and transcription (Estrela et al., 1999; Mohammadi et al., 2012; Mohammadi & Dummer, 2011; Rathi et al., 2022).

Furthermore, NS-$Ca(OH)_2$ also inactivates the toxin effects of endotoxin. Also referred to as lipopolysaccharides, endotoxins are composed of polysaccharides, lipids, and proteins, with their toxicity located in the lipid A region (Mohammadi et al., 2012). Endotoxins are released when bacteria proliferate or undergo lysis after the endodontic treatment, causing an inflammatory reaction and periapical bone resorption. NS-$Ca(OH)_2$ can hydrolyse the toxic lipid A into fatty acids and amino sugars, which are non-toxic to oral tissues (Mohammadi et al., 2012; Safavi & Nichols, 1994).

Although still debated, some studies show that NS-$Ca(OH)_2$ exhibits antibiofilm properties. Root canal pathogens, particularly *E. faecalis,* can form biofilms that

facilitate microbial growth and provide protection against the host immune system and antimicrobial agents. NS-$Ca(OH)_2$ possesses the ability to degrade EPS and weaken the microbial adhesion within the biofilm, rendering pathogens more vulnerable to subsequent antimicrobial treatments (Momenijavid et al., 2022).

2.6.1.2 Cytotoxicity and Proliferation ability of NS-$Ca(OH)_2$

Reports regarding the cytotoxicity of NS-$Ca(OH)_2$ towards human cells are debatable, as studies appear equivocal. The cytotoxicity of NS-$Ca(OH)_2$ on human oral cells is attributed to OH^- ions, which can cause lipid peroxidation, protein denaturation, and DNA interference (Widjiastuti et al., 2020). An *in vitro* study on cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) by Paramitta et al. (2015) reported cell death of Vero fibroblast when treated with 62.5 µg/mL of NS-$Ca(OH)_2$. Compared with natural substances, NS-$Ca(OH)_2$ demonstrated higher cytotoxicity effects on fibroblast cells. This was shown by Jahromi et al. (2014) where dental pulp fibroblasts treated with 1 mg/mL of propolis and NS-$Ca(OH)_2$, exhibited cell viability of 75.20% and 11.34%, respectively. Similarly, another study demonstrated that propolis treatment resulted in greater proliferation and viability of human dental pulp stem cells (HDPSC) compared to NS-$Ca(OH)_2$ after 24 and 48 hours of *in vitro* testing (Ahangari et al., 2018).

Nevertheless, NS-$Ca(OH)_2$ has also been reported to be among the least irritating and cytotoxic intracanal medicaments compared with others (Mohammadi & Dummer, 2011). This was supported by Bhandi et al. (2022), who found that NS-$Ca(OH)_2$ was least likely to adversely affect the viability and survival of HDPSC compared with other intracanal medicaments including doxycycline, potassium iodide, triamcinolone, and glutaraldehyde, after 6, 24, and 48 hours of treatment. In addition, NS-$Ca(OH)_2$ outperformed several antibiotic paste mixtures (i.e. double antibiotic paste and triple antibiotic paste) in terms of cytotoxicity and proliferation effects on stem cells from the apical papilla (Rahul et al., 2022).

2.6.1.3 Significant Flaws

Besides its ambiguous reports on the cytotoxicity, NS-$Ca(OH)_2$ possesses significant limitations, as certain pathogens in infected root canals are able to resist its antimicrobial effect. While it demonstrates antimicrobial activity against a wide spectrum of microorganisms, it has been found to be less effective against specific oral pathogens such as *E. faecalis* and *Candida albicans* (*C. albicans*) (Dohyun & Euiseong, 2014). Even with prolonged NS-$Ca(OH)_2$ treatment, *E. faecalis* can retain its viability and survive within dentinal tubules, living independently of other bacterial species. Moreover, intracanal medications containing NS-$Ca(OH)_2$ show significant antibacterial activity during the first two days of use, but their efficacy rapidly decreases and becomes ineffective after 10 days (Awawdeh et al., 2009).

Although NS-$Ca(OH)_2$ is considered the gold standard of intracanal medicament, it is difficult to completely remove from the root canal. Residual traces can hamper the penetration of sealers into the dentinal tubules and negatively affect the quality of the root canal filling (Lambrianidis et al., 1999; Rathi et al., 2022). Research by Maalouf et al. (2013) demonstrated that most current irrigation methods (e.g. physiological saline solution coupled with syringe irrigation, ultrasonic systems, and the RinsEndo® system) are unable to completely remove the NS-$Ca(OH)_2$ from the canal. Similarly, different irrigant solutions such as NaOCl, ethylenediaminetetraacetic acid (EDTA), citric acid, and chitosan have also been shown to fail completely in removing NS-$Ca(OH)_2$ from the root canal (Özlek & Kadı, 2021; Raghu et al., 2017).

2.6.2 Chlorhexidine (CHX)

CHX gel is rarely used in endodontics as intracanal medicament, but it is used in this study as positive control due to the flaws of NS-$Ca(OH)_2$ to eradicate *E. faecalis*. CHX is a colourless to opalescent, odourless substance that has been widely utilised in dentistry, medicine, veterinary, and food manufacturing. First manufactured in 1947, it is known for its strong antimicrobial properties and has been used as an antiseptic and disinfectant agent in medicine, including in ophthalmology, urology, gynaecology, otorhinolaryngology, and dentistry (Löue et al., 1976; Tomás et al., 2011). Generally, CHX in oral application contributes to the multifunction mouth care, periodontal gum

health, prolonged fresh breath, and dental plaque control. In endodontic treatment, CHX is used as an irrigant (liquid-based) and intracanal medicament (gel-based) in both vital and non-vital teeth due to its antimicrobial activity, substantivity, and biocompatibility. CHX gel for intracanal medicament is usually composed CHX gluconate, gel base and distilled water, and is typically preferred because of its remarkable advantages (Ferraz, 2001; Vivacqua-Gomes et al., 2002). CHX gel (2% or equivalent to 20 mg/mL) used in endodontics possesses a broader diffusion capability and a prolonged residual effect due to its strong affinity for dentine and oral tissues. These characteristics enable it to disrupt the bacterial cell membrane and effectively eradicate *E. faecalis*, even in complex root canal anatomies where other agents may fail (Gomes et al., 2013).

2.6.2.1 Antimicrobial Properties

The antimicrobial activity of CHX gel is pH-dependent, with the optimal range being 5.5-7.0, which corresponds to the pH of body surfaces and tissues. The bactericidal effect of CHX gel is attributed to its cationic molecules, which attach to extracellular complexes and negatively charged microbial cell walls, thereby disrupting the osmotic balance of the cells. At low concentration, CHX gel causes the release of low-molecular-weight components, such as potassium and phosphorus, resulting in bacteriostatic effects. In contrast, high concentrations of CHX gel act on microorganisms through precipitation and coagulation of the microbial cytoplasm, leading to cell death (Gomes et al., 2013).

CHX gel is proven to be bactericidal against a wide spectrum of Gram-negative and Gram-positive bacteria (Gomes et al., 2013). Wendt et al. (2007) demonstrated that CHX gel is able to inhibit the growth of antibiotic-resistant *Staphylococcus aureus*. CHX gel has also been shown to overcome the limitations of NS-$Ca(OH)_2$ as it is effective against *E. faecalis*, the most persistent bacterium in failed root canal treatment (Aviv et al., 2024; Ferraz, 2001; Karpiński & Szkaradkiewicz, 2015). Other periopathogens and harmful oral bacteria, including *Streptococcus mutans (S. mutans), Actinomyces israelii, P. endodontalis, Porphyromonas gingivalis* (*P. gingivalis*), *Prevotella intermedia, Escherichia coli (E. coli)*, and *Fusobacterium nucleatum,* have also been confirmed to be susceptible to CHX gel (Gomes et al., 2013; Karpiński & Szkaradkiewicz, 2015). Additionally, the bactericidal potential of CHX gel extends to

lipophilic viruses such as HIV and cytomegalovirus, as well as bacterial spores (Gomes et al., 2005; Karpiński & Szkaradkiewicz, 2015).

The documented antifungal activity of CHX gel against *C. albicans* and other yeast species supports its use as an adjuvant in the treatment of oral candidiasis (Chavarría-Bolaños et al., 2022). Exposure of *C. albicans* and other fungi isolates to low doses of CHX gel can reduce germ tube production and potentially inhibit pathogenicity (Ellepola et al., 2012; Shino et al., 2016).

Recurrent root canal infections often occur because certain pathogens form biofilms that colonise dentinal tubules and root canal spaces, requiring more targeted treatment (Flemming et al., 2016). This ability poses a major challenge in endodontics, as most intracanal medicaments fail to disrupt or eradicate biofilm structure. Recently, Sebbane et al. (2024) demonstrated that CHX effectively inhibits the synthesis of EPS, compromises biofilm structural integrity, and reduces biofilm mass and bacterial viability. The antibiofilm properties of CHX gel have also been demonstrated at the molecular level, where genes such as *fsrB, esp*, and *efbA,* which are vital for quorum sensing, are significantly downregulated, thereby altering the bacterial communication systems (Ran et al., 2013).

2.6.2.2 Cytotoxicity

Despite its advantages, CHX gel is toxic to various human cells, including vital oral cells, with its cytotoxicity being non-specific to cell type and proportional to its concentration (Abbaszadegan et al., 2016). Its cytotoxic effects have been shown to compromise the viability of human gingival fibroblasts (HGF), periodontal ligament cells, alveolar bone cells, and osteoblastic cells (Gomes et al., 2013; Lee et al., 2010; Li et al., 2014). In addition, toxic effects have also been observed in osteoblastic, endothelial, and fibroblastic cell lines, as CHX gel triggers apoptotic and autophagic cell deaths, mitochondrial dysfunction, increased intracellular Ca^{2+} levels, and oxidative stress (Giannelli et al., 2008).

2.6.2.3 Significant Flaws

Although CHX gel is widely recognised as an effective antimicrobial agent, its use has been associated with tooth and oral mucosal discolouration, and in some cases, dysgeusia (Chavarría-Bolaños et al., 2022). Furthermore, Sharma et al. (2022) recommend careful consideration when using CHX gel as an intracanal medicament due to reported cases of hypersensitivity and allergic reactions. Beyond cytotoxicity, studies have shown that combining NaOCl with CHX gel during treatment results in noticeable dark-brown discolouration and precipitate formation due to chemical interactions between the two agents (Basrani et al., 2007; Vivacqua-Gomes et al., 2002). Upon initial administration, temporary taste disturbances and a burning sensation on the tongue also occur (Gomes et al., 2013).

2.7 Alternative to Intracanal Medicaments

With the increasing emergence of bacterial resistance to antibiotics and the limitations of NS-$Ca(OH)_2$ and CHX gel as intracanal medications, it has become necessary to consider alternate options (Abbaszadegan et al., 2016). Recent studies have explored natural products as promising alternatives due to their therapeutic effectiveness, availability, and biocompatibility, offering solutions to overcome the shortcomings of current intracanal medicaments (John et al., 2015). Numerous plant-derived compounds, such as *Curcuma longa* (turmeric), *Allium sativum* (garlic), miswak extracts, green tea, aloe vera, *Glycyrrhiza glabra* (liquorice), grape seed extracts, and triphala have been recognised as suitable options for intracanal use (Almadi & Almohaimede, 2018; Ayub et al., 2024; John et al., 2015; Kishan et al., 2020). Among these, propolis produced by stingless bees, has been highlighted as the most promising due to its potent biological properties (Ayub et al., 2024; Shabbir et al., 2020).

2.8 Bees and Their Types

Bees are among the most diverse species on Earth, found on every continent except Antarctica, inhabiting both natural and agricultural ecosystems. With more than 20,000 known species worldwide, bees are highly significant to humans and the ecosystem due to their vital role as plant pollinators and their contribution to agricultural productivity (Almeida et al., 2023; Orr et al., 2022). The most well-known types are honeybees and stingless bees, both of which are social insects that live in colonies consisting of a queen who lays eggs, numerous sterile female workers, and male drones, depending on the season (Orr et al., 2022).

Bee anatomy can generally be divided into three parts, the head, thorax, and abdomen as shown in figure 2.4. The head houses the most vital organs, including the eyes, sensory organs called ocelli, antennae, and jaws. The length of the antennae can serve as a distinguishing factor between male and female bees, as males usually have longer, more curved antennae. The thorax, the central section of the bee's body, has two pairs of wings and six legs. The abdomen is the longest part and it contains the digestive, respiratory, and reproductive systems. In some species, a stinger is also located at the tip of the abdomen (Halvaci et al., 2023; Saifullizan, Azmi, & Omar, 2021). Examining features such as wing structure, colour, and size can help identify different bee species (Saifullizan, Azmi, & Omar, 2021; Veen, 2014).

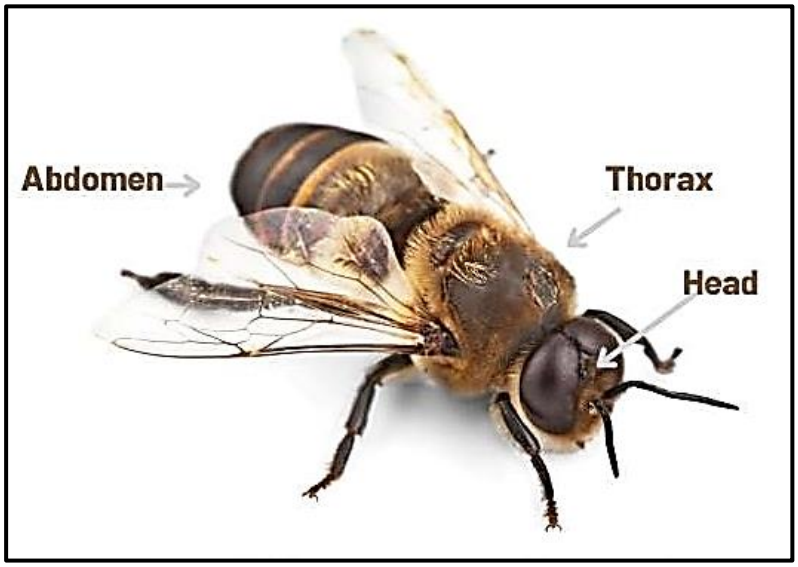


Figure 2.4 General Anatomy of a Honey or Stinging Bee

Source: Discovering the Secrets of Honeybee Anatomy and Physiology: A Comprehensive Guide for Beekeepers. (https://honeyhub.ir/Honeybee-Anatomy-Physiology.htm)

The honeybees, or Apini are a group of bees found worldwide and consist of only one genus, *Apis*. Their distinctive features include hairy, golden-brown bodies marked with black stripes, transparent wings, and a barbed stinger as shown in figure 2.5. In addition, they possess specialised structures called corbiculae, or pollen baskets, on their hind legs for efficient pollen collection. (Faux, 2021). Geographically, *Apis cerana, Apis dorsata*, and *Apis florea* are species found throughout tropical Asia, while *Apis mellifera* is native to Europe and Africa but has been introduced to many other regions of the world due to its importance in honey production and agricultural pollination (Veen, 2014). Of the eight honeybee species worldwide, five (e.g. *Apis dorsata, Apis andreniformis, Apis cerana, Apis koschevnikovi*, and *Apis nuluensis*) inhabit Malaysia's tropical rainforests. The abundance of flora and fauna interactions in this ecosystem provides suitable habitats for their survival (Ismail, 2014).

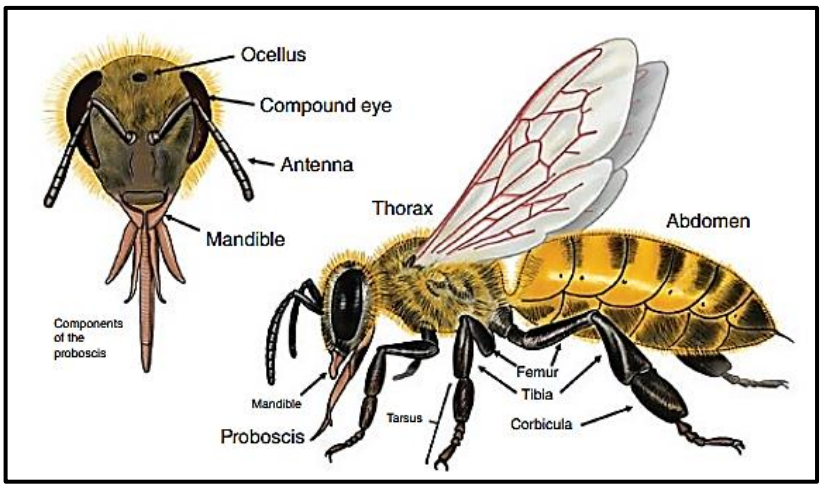


Figure 2.5 Morphology of a Honeybee

Source: Honey bee anatomy. (Faux, 2021)

Meliponines or stingless bees, locally known in Malaysia as “lebah kelulut” are the largest group of eusocial bees, with about 600 species identified under this category (Hrncir et al., 2016; Saifullizan, Azmi, & Wan Omar, 2021). Apart from lacking a sting

and venom, stingless bees can be distinguished from honeybees by their smaller size, distinct morphology (as shown in figure 2.6), and the construction of unique storage pots made of propolis for honey collection, instead of the typical hexagonal-shaped beeswax combs (Omar et al., 2019). They are distributed worldwide, particularly in tropical and subtropical regions, and can be found in South America, Central America, South of North America, Africa, Southeast Asia, and Northern Oceania (Heah et al., 2013; Lavinas et al., 2019). Known as "lebah kelulut" in Malaysia, approximately 50 species of stingless bees have been reported in the country, with *H. itama*, *G. thoracica, Lepidotrigona terminata, Tetragonula laeviceps* (Kelly et al., 2014; Omar et al., 2019) and *T. apicalis,* are among the most common species used in pollination and bee product commercialisation (Jaapar et al., 2016; Samsudin et al., 2018). Figure 2.7 shows the abundance of stingless bee species in Peninsular Malaysia.

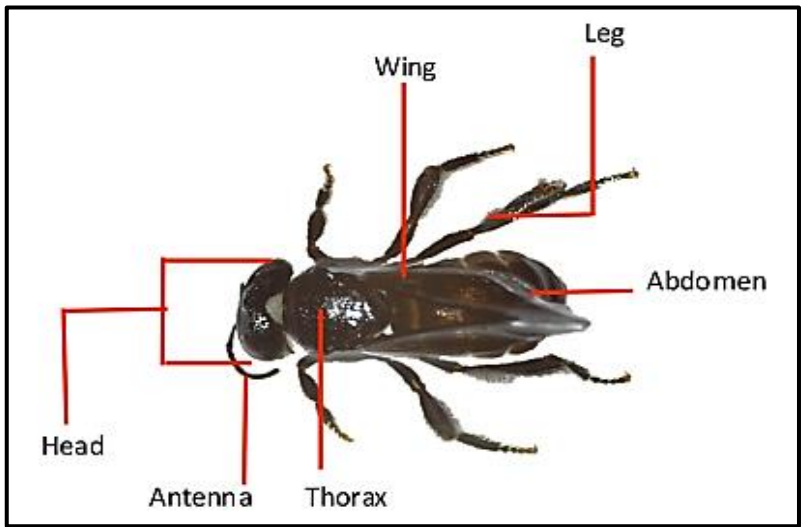


Figure 2.6 Morphology of *Heterotrigona itama*, One of The Stingless Bee Species

Source: Study on morphological characteristic of stingless bee (*Heterotrigona itama*) in Terengganu. (Saifullizan, Azmi, & Omar, 2021, p. 122)

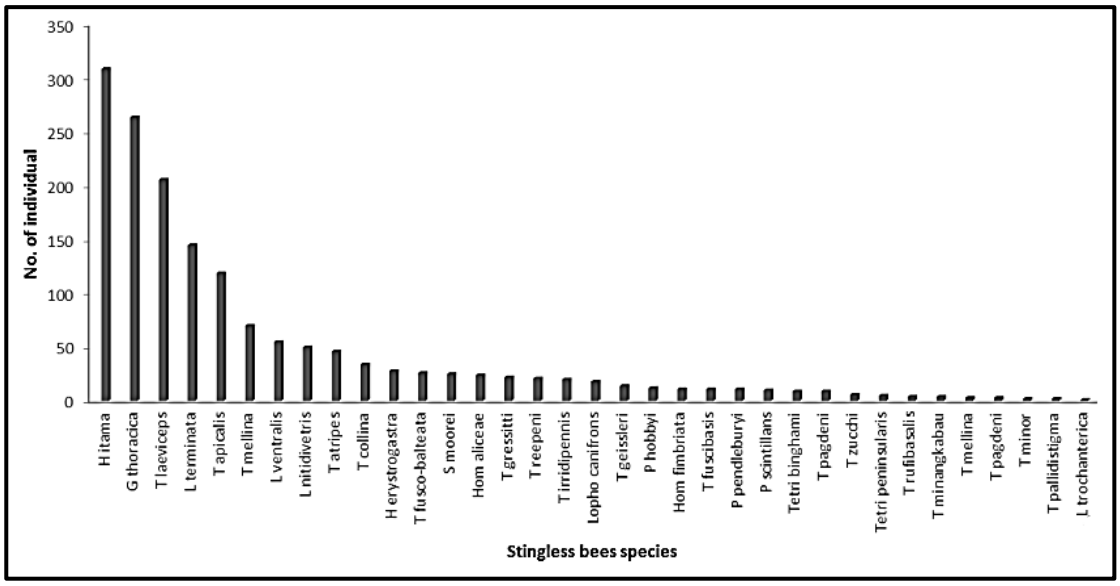


Figure 2.7 Abundance of Stingless Bee Species in Peninsular Malaysia

Source: The diversity and abundance of stingless bee (Hymenoptera: Meliponini) in peninsular Malaysia (Jaapar et al., 2016, p. 3)

2.9 Magnificent Product from Bees

In Islam, the extraordinary product produced by bees are mentioned in the Quran in Surah Al-Nahl (16: 68 & 69), where it was revealed: "*And your Lord inspired the bees to make their homes in the mountains, the trees, and in what people construct (16:68). And feed from the flower of any fruit you please and follow the ways your Lord has made easy for you. From their bellies comes forth liquid of varying colours, in which there is healing for people. Surely in this is a sign for those who reflect (16:69)*." These verses clearly state that bees produce honey, propolis, royal jelly, and beeswax, all of which contain therapeutics benefits for mankind (Al-Hariri, 2011; Zulkarnain & Suprapto, 2022).

Bee products have long been recognised for their therapeutic potential and are increasingly explored in alternative medicine due to their natural bioactive compounds. Apitherapy is a branch of alternative medicine that utilises bee products, such as honey, propolis, pollen, royal jelly, beeswax, bee bread, and bee venom to treat and prevent diseases as well as boost the human immune system (Al-Bayaty et al., 2017; Al Naggar et al., 2021; El-Didamony et al., 2024).

Honey is a natural substance synthesized by bees through the combination of enzymes in their saliva with nectar and plant sap, which is subsequently processed and

undergoes ripening within the hives (Nainu et al., 2021; Zulkarnain & Suprapto, 2022). It primarily comprises simple sugars, mainly glucose and fructose, along with water, and other bioactive components such as amino acids, vitamins, enzymes, organic acids, flavonoids, volatile compounds, and minerals. This complex nutritional profile not only contributes to honey's dietary value but also underpins its role as functional food or "superfood" for humans. Research has revealed numerous therapeutic effects of honey, including antimicrobial, anticancer, antioxidant, antidiabetic, anti-inflammatory, wound healing, and cardioprotective properties (Al Naggar et al., 2021; Asma et al., 2022; El-Didamony et al., 2024; Giampieri et al., 2022).

Propolis, derived from the Greek words "*pro*" (means at the entrance of) and "*polis*" (means city) is a viscous substance produced by bees that serves multiple critical functions within the hive, such as building materials, sealant, and disinfectant. It is formed through a combination of collected plant resins, salivary enzymes, and beeswax from the abdominal glands (Heah et al., 2023; Okhale et al., 2021). Beyond its ecological role, propolis has been widely used in modern medicine due to its therapeutic effects. Historically, it was utilised by the Greeks, Arabs, and Balkan communities to treat wounds, burns, and ulcers, as well as for managing eczema, muscle pain and rheumatism (Eroğlu & Yuksel, 2020).

Bee pollen is a staple food produced by bees to nourish their larvae, formed through the agglutination of flower pollen, nectar, and salivary secretions. Its physical characteristics vary, appearing as single grains of different colours (e.g. yellow, brown, and black) and shapes (e.g. triangular, cylindrical, and bell-shaped), depending on the bee species, food sources, and geographical origin (Nainu et al., 2021). Nutritionally, bee pollen contains essential macronutrients, with high levels of carbohydrates (13–55%), followed by proteins (10–40%), lipids (1–13%), crude fibre (0.3–20%) and ash content (2–6%) (Asma et al., 2022; Campos et al., 2008; Thakur & Nanda, 2020). Today, bee pollen is widely used in cosmetic formulations and skincare supplements to prevent dermatological conditions, as well as in dietary supplements to improve cholesterol level, blood circulation, treat allergic conditions (e.g. rhinitis and asthma), and act as an energy and immune booster due to its antioxidant, antimicrobial, anticancer, anti-allergenic, hepatoprotective, and immunomodulatory properties (El-Didamony et al., 2024; Giampieri et al., 2022; Nainu et al., 2021).

Royal jelly, also known as bee's milk, is a viscous, creamy, acidic substance (pH of 3.1 – 3.9), secreted by the mandibular and hypopharyngeal glands of young

worker bees. Within the beehive, it plays a crucial biological role as the nourishment for the larvae during the maturation phase and as the lifelong diet of the queen bee. The major components of royal jelly are water (50 – 70%, carbohydrates (30%), proteins (27 - 41%), lipids (3 – 19%) and other trace elements such as vitamins, amino acids, polyphenols, and enzymes (Asma et al., 2022; Oršolić & Maja, 2024). Although it has a pungent taste and odour, royal jelly has garnered considerable scientific interest due to its pharmacological properties including antimicrobial, anticancer, antidiabetic, anti-inflammatory, antiallergic, immunomodulatory, hepato- and neuroprotective effects (Giampieri et al., 2022; Nainu et al., 2021; Oršolić & Maja, 2024).

Beeswax is produced by young bees aged between 12 – 18 days through their wax glands or ceriferous glands, and serves as a fundamental building material for honeycomb by honeybees or honeypots by the stingless bees. With more than 300 identified compounds, beeswax is mainly composed of 15-27% esters, 12-16% hydrocarbons, 35-45% hydroxyl-monoester, <1% fatty alcohol, exogenous molecules as a result of residues of propolis and pollen, minerals, and vitamins (Asma et al., 2022; El-Didamony et al., 2024; Fratini et al., 2016). The medicinal value of beeswax has been recognised since ancient Egyptian times, where it was utilised as an ointment to treat burns, inflammation, and joint pain (Fratini et al., 2016). Today, beeswax is widely incorporated in the food, pharmaceutical, and cosmetic industries (Asma et al., 2022).

Bee bread, also known as ambrosia or perga, is a fermented mixture of bee pollen, bee saliva, and flower nectar that serves as an important source of nutrients in the bee colony. Unlike bee pollen, bee bread undergoes an anaerobic lactic acid fermentation process performed by bacteria such as *Lactobacillus* spp., yeast, and other microorganisms within the beehive that enhances its bioavailability and preserves its nutritional value (Bakour et al., 2022; El-Didamony et al., 2024; Giampieri et al., 2022). Scientific interest in bee bread is largely driven by its reported antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory properties (Ćirić et al., 2022).

Bee venom, also known as apitoxin, is a yellowish, bitter, pungent, and acidic liquid secreted from two venom glands in the bee's abdominal cavity and associated with the sting apparatus. Traditionally used in various forms of complementary and alternative medicine, particularly apitherapy, bee venom has attracted increasing scientific attention for its therapeutic potential. The pharmacologically active constituents of bee venom include melittin, apamin, adolapin, phospholipase A2 (PLA2), hyaluronidase, and a range of other enzymes, peptides, and amines. These

components contribute to its diverse biological effects, including anti-inflammatory, antimicrobial, analgesic, and anticancer activities (Al Naggar et al., 2021; El-Didamony et al., 2024; Stela et al., 2024; Wehbe et al., 2019).

2.10 Propolis

Propolis, also referred to as bee glue, is a sticky resinous substance produced by bees through a combination of beeswax, saliva, and several plant resources including bud exudates, flowers, and leaves (Almuhayawi, 2020; Braakhuis, 2019). Its primary function within the beehive is to seal cracks, reduce microbial invasion, and maintain internal hive stability. It is composed of approximately 50% resins (including flavonoids and related phenolic acids), 30% waxes, 10% essential oils, 5% pollen, and 5% other organic compounds (Avinash et al., 2017; Pietta et al., 2002). Propolis contains both hydrophobic and hydrophilic compounds (Silva et al., 2011). Its hydrophobic nature, due to the presence of resins and waxes, poses a biopharmaceutical challenge as it is not easily absorbed into the body (Javed et al., 2022). The hydrophilic properties of propolis are derived from its chemical constituents, such as phenolic acids, flavonoids, and amino acids (Hossain et al., 2022). Over the past decades, propolis has garnered increasing scientific interest due to its complex composition and broad spectrum of bioactive properties, particularly antimicrobial, antioxidant, anti-inflammatory, and wound-healing activities (Heah et al., 2023; Ismail et al., 2025).

2.10.1 Physical Characteristics

The physical characteristics of propolis vary significantly depending on its botanical origin, geographical location, and the bee species involved in its collection. It can be described as sticky, pliable, waterproof, and having a pungent aromatic odour. Its colour varies, ranging from green to dark red or black depending on the surrounding plant resources and its age (Avinash et al., 2017; Eroğlu & Yuksel, 2020). Malaysian stingless bees produce two distinct types of propolis: sticky and hard. The most commonly domesticated species in Malaysia, *H. itama* and *G thoracica*, are known to produce sticky propolis. In contrast, species such as *Homotrigona aliceae* (*H. aliceae*),

Homotrigona fimbriata (*H. fimbriata*), *T. apicalis*, *Tetrigona vidua* (*T. vidua*), *Tetrigona peninsularis* (*T. peninsularis*), *Lophotrigona canifrons* (*L. canifrons*), *Tetrigona melanoleuca* (*T. melanoleuca*), and *Tetrigona binghami* (*T. binghami*) predominantly produce hard propolis (Awang et al., 2018). Besides that, propolis is insoluble in water but shows high solubility in ethanol and other organic solvents, a property that facilitates its extraction and analysis for pharmacological studies.

2.10.2 Chemical Characteristics

With more than 300 chemical compounds present in propolis, the synergistic interaction among these elements contributes to its numerous biological activities including antimicrobial, anti-inflammatory, antioxidant, anti-obesity, anti-cancer, and antiseptic effect (Endo et al., 2017; Salleh et al., 2022). Furthermore, recent research has discovered additional biological effects of propolis, such as antifungal, antihepatotoxic, anti-diabetic, antiangiogenic, antiproliferative, antiviral, local anaesthesia, and anti-quorum sensing (Al-Hariri, 2011; Ismail et al., 2020; Okhale et al., 2021). The essential medicinal effects of propolis are mainly associated with phenolic compounds, such as flavonoids and hydroxycinnamic acid derivatives (Eroğlu & Yuksel, 2020). Recent research further elaborated on the pharmacological properties of propolis, showing that they are largely attributed to the presence of carboxylic acids (20.4%), terpenoids (15.0%), steroids (11.5%), hydrocarbons (9.6%), sugars (6.4%), alkaloids (6.4%), flavonoids (4.3%), phenols (3.2%), ketones (2.1%), amino acid (2.1%), vitamins (2.1%) and other compounds (15.0%) (Salleh et al., 2021). The table below further characterises these compounds and their contributions to the biological effects of propolis:

Table 2.5
Overview on Several Active Constituents of Propolis and Its Biological Effect

No	Biological activity	Compound class	Constituent	References
1	Antimicrobial	Flavonoids	• Luteolin • Pinocembrin (antifungal, antibacterial, anti-mould) • Apigenin • Chrysin (antibacterial)	(Bogdanov, 2012; Okhale et al., 2021; Toreti et al., 2013)
		Prenylated flavanones	• 7-O-prenylpinocembrin (destroys cell wall and membrane)	

		Phenylpropanoids	• Para-methoxycinnamic acid (weak antiviral)	
		Prenylated Phenylpropanoids	• 3-Prenyl-4-hydroxycinnamic acid (antiviral and antibacterial against E. coli and B. subtilis)	
		Stilbenes	• 4-Prenylresveratrol (antifungal)	
		Lignans	• 6-Methoxydiphyllin (antiviral)	
		Coumarins	• Prenylated coumarin suberosin (antibacterial and antiretroviral)	
		Prenylated cinnamic acid	• Artepillin C	
		Phenolics	• Caffeic acid (antiviral and antibacterial) • Protocatechuic acid (antibacterial, antifungal, anti-mould) • ρ-coumaric acid (antibacterial)	
		Pentacyclic triterpenoid	• Moronic acid (anti-HIV)	
2	Anti-inflammatory	Flavonoids	• Luteolin • Acacetin • Apigenin • Chrysin • Galangin	(Okhale et al., 2021; Toreti et al., 2013)
		Neo-flavonoids	• Cearoin	
		Phenolics	• Caffeic acid phenyl • ester • Caffeic acid • Ferulic acid • Isoferulic acid • Protocatechuic acid	
		Triterpenes	• Lupeol acetate	
		Cinnamic acids	• Cinnamic acid	
		Polyphenolics	• Gallic acid	
3	Antioxidant	Flavonoids	• Luteolin	
		Terpenoids	• Linalool and Abietic Acid (Hepatoprotection)	(Okhale et al., 2021; Toreti et al., 2013)
		Triterpenes	• Lupeol acetate	
		Prenylated Phenylpropanoids	• 3-Prenyl-4-hydroxycinnamic acid	
		Prenylated cinnamic acid	• Artepillin C	
		Phenolics	• Propofol	
4	Anti-diabetic	Phenylpropanoids	• Para-methoxycinnamic acid	(Okhale et al., 2021)
5	Antitumor	Lignans	• 6-Methoxydiphyllin	(Okhale et al., 2021; Toreti et al., 2013)
		Prenylated cinnamic acid	• Artepillin C	
		Phenolics	• Caffeic acid phenyl ester	
6	Anticoagulant	Coumarins	• Prenylated coumarin suberosin	(Okhale et al., 2021)
7	Local Anaesthesia	Flavonoids	• Pinostrobin • Pinocembrin	(Okhale et al., 2021; Toreti et al., 2013)
8	Antineoplastic	Flavonoids	• Luteolin (inhibit angiogenesis)	(Okhale et al., 2021)

2.11 Outstanding Biological Properties of Stingless Bee Propolis

Similar to the common honeybee, stingless bees also produce beneficial products such as honey, propolis, bee pollen, and wax (Popova et al., 2021; Rozman et al., 2022). Research has found that the propolis produced by stingless bee (*lebah kelulut*) species is more potent compared to propolis from other honeybee species (Ibrahim et al., 2016; Salleh et al., 2022). According to Salleh et al. (2022), due to a lack of a self-defence mechanism, stingless bees produced a higher quality and quantity of propolis, which serves as an alternative defence mechanism to protect the colony from physical harm and microbial infection. Unlike the more extensively studied *Apis mellifera* propolis, stingless bee propolis has only recently garnered substantial scientific attention, particularly in tropical and subtropical countries such as Brazil, China, United States of America (USA), India, Turkey, Japan, Italy, South Korea, Iran, and Poland (Ayad et al., 2025). The growing interest is attributed to its unique chemical profile and broad range of potent biological activities, including antimicrobial, anti-inflammatory, and healing properties, making it a promising candidate in natural product research and biomedical applications (Ayad et al., 2025; Woźniak et al., 2023; Zullkiflee et al., 2022).

2.11.1 Antimicrobial Effect

One of the most prominent biological properties of stingless bee propolis is its antimicrobial activity. Numerous studies have demonstrated its effectiveness against a wide array of pathogenic microorganisms, including Gram-positive and Gram-negative bacteria such as *S. aureus*, *E. coli*, *C. albicans*, and *E. faecalis*, fungi, and even some viruses such as Herpes Simplex Virus (HSV-1), influenza virus, HIV-1, and SARS-CoV-2) (Al-Ani et al., 2018; Przybyłek & Karpiński, 2019). Studies by Pimenta et al. (2015) and Koru et al. (2007) showed that Brazilian stingless bee propolis is capable of inhibiting oral pathogens at significantly low minimum inhibitory concentration (MIC), including *Peptostreptococcus anaerobius P. gingivalis*, *Fusobacterium nucleatum*, *Prevotella melaninogenica*, and *E. faecalis*. Similarly, stingless bee propolis obtained from Southeast Asia, including Malaysia, Brunei, and Indonesia reported good antimicrobial effect against *Pseudomonas aeruginosa* (*P. aeruginosa*)*, S. aureus*, and

Bacillus subtilis (*B. subtilis*) (Abdullah et al., 2019; Akhir et al., 2017; Salleh et al., 2022).

The antimicrobial properties of propolis are primarily attributed to the presence of polyphenolic compounds, such as flavonoids, phenolic acids, and diterpenes (Burdock, 1998). Recent findings suggest that this inhibitory effect on bacteria depends on synergistic interactions among these compounds rather than the action of individual components alone (Koru et al., 2007). Propolis exerts its antibacterial effects through various mechanisms, such as impairing the microbial cell wall synthesis, protein function, and nucleic acid replication (Rosli et al., 2023). Polyphenols can bind to microbial proteins via hydrogen and ionic bonds, resulting in alterations to the three-dimensional (3D) structure of proteins and consequently diminishing their functionality. These mechanisms position stingless bee propolis as a potential natural antimicrobial agent, particularly in an era where antimicrobial resistance is a growing concern (Al-Ani et al., 2018).

Previous studies have reported a wide range of propolis concentrations with varying antimicrobial efficacy, as reflected by differences in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Therefore, identifying the optimum concentration is essential to ensure effective antimicrobial activity while maintaining clinical applicability. Table 2.6 provides a comparative analysis of previous studies, highlighting the concentrations of propolis used and their corresponding antimicrobial outcomes to identify the optimum concentration.

Table 2.6
Review on Propolis Concentration Used for Antimicrobial Test

Name of Propolis (concentration)	MIC	MBC	Reference
Iranian Propolis extracts (**20 mg/mL)** against *S. mutans, Streptococcus salivarius (S. salivarius), S. aureus, E. faecalis* and *Lactobacillus casei (L. casei)*	250-500 µg/mL (equivalent to **0.5 mg/mL**).	250-500 µg/mL (equivalent to **0.5 mg/mL**).	(Kashi et al., 2011)

Thai Propolis extract (**100 mg/mL)** against *E. faecalis*	-	**100 mg/mL**	(Pengchum et al., 2018)
Brazilian brown propolis (**20, 50, 100, and 200 mg/mL** in DMSO**)** against *E. faecalis*	**1000 mg/mL**	-	(Pimenta et al., 2015)
Propolis from Esfahan, Iran (**150 mg/mL)** against *E. faecalis*	**340 mg/mL**	-	(Jahromi et al., 2012)
Azerbaijan propolis (working concentration is not mentioned) against *E. faecalis*	**64 mg/mL**	**128 mg/mL**	(Kousedghi et al., 2012)
Azerbaijan propolis (**25 mg/mL)** against *E. faecalis, S. aureus,* and *Escherichia coli (E. coli)*	**0.58 mg/mL** (±0.11)	**1.56 mg/mL**	(Eslami et al., 2016)

2.11.2 Anti-Inflammatory

Another significant pharmacological property of stingless bee propolis is its anti-inflammatory activity, which has been validated through both *in vitro* and *in vivo* experimental models. The bioactive compounds found in stingless bee propolis such as flavonoids (pinocembrin, galangin, and pinobanksin) regulate and reduce inflammatory responses, while phenolic compounds and terpenoids have been shown to inhibit pro-inflammatory mediators such as nitric oxide (NO), prostaglandins, and cytokines including TNF-α, IL-1β, IL-2, IL-5, and IL-6 (Ahangari et al., 2018; Bueno-Silva et al., 2015; Manginstar et al., 2025). Emerging evidence indicates that propolis helps reduce inflammation and promotes wound healing through several mechanisms, including suppression of oxidative stress, modulation of immune function, and inhibition of microbial proliferation (Bhargava et al., 2021). By modulating these inflammatory

pathways, propolis exerts a protective effect in conditions characterised by excessive inflammation, thereby offering therapeutic potential in managing a range of inflammation-associated disorders, including chronic inflammatory conditions such as arthritis, wound healing complications, and certain metabolic diseases (Calimag et al., 2021).

2.11.3 Healing Properties

Stingless bee propolis has emerged as a potential natural active ingredient in endodontic medicament as it has been proven to promote periapical healing and oral tissue regeneration after the root canal therapy (Alghutaimel et al., 2024). It facilitates wound healing by enhancing the expression and deposition of type I collagen, upregulating the expression of growth factors and promoting cell proliferation (Ceylan, 2021; Ibrahim et al., 2022; Yang et al., 2023). Its antimicrobial and anti-inflammatory effects, combined with the ability to stimulate collagen synthesis and angiogenesis, are key properties that contribute to accelerated healing of periapical tissue and the regeneration of periodontal structures (Pribadi et al., 2020). Propolis revealed a considerable HGF proliferation, while cisplatin did not. Several studies also reported that propolis did not have the same potentially fatal side effects as cisplatin, including ototoxicity, gastrotoxicity, myelosuppression, and nephrotoxicity (Ismail et al., 2025).

2.12 Propolis Extraction Methods

Raw propolis cannot be directly applied in research or therapeutic settings due to the presence of inert materials and waxes that hinder the release of its bioactive constituents. According to the literature on propolis extractions, the yield of active ingredients varies with the extraction methods employed (Gómez-Caravaca et al., 2006). Selecting an appropriate extraction technique and solvents is therefore a critical preliminary step to isolate the pharmacologically active compounds with optimum yield and biological efficacy (Pobiega et al., 2019).

The extraction process transforms raw propolis from a solid into a liquid form, enabling the release of bioactive constituents while separating out inert components

such as waxes and resins (Heah et al., 2023). A wide range of solvents has been employed, including ethanol, methanol, water, hexane, acetone, dichloromethane, and chloroform. These solvents differ in polarity, which directly influences both the yield and the chemical profile of the extracted compounds (Pobiega et al., 2019; Przybyłek & Karpiński, 2019; Gómez-Caravaca et al., 2006). For applications in food and health-related fields, ethanol and water are most commonly used (Kubiliene et al., 2015; Bankova et al., 2016). Ethanolic extraction is particularly effective for producing deparaffinised extracts that are rich in polyphenols, whereas pure water solvent favors the recovery of water-soluble phenolic acids. Although methanol and acetone offer high extraction efficiency, their associated toxicity limits their suitability, especially for biomedical or consumable products (Pobiega et al., 2019; Przybyłek & Karpiński, 2019; Gómez-Caravaca et al., 2006).

Traditionally, propolis extraction has relied on techniques such as room-temperature maceration and hot reflux extraction (HRE) (Trusheva et al., 2007). However, more recent approaches have introduced advanced methods, including microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and supercritical carbon dioxide extraction (Heah et al., 2023; Al-Masoodi et al., 2022). These modern techniques typically enhance extraction efficiency, reduce processing time, and minimize solvent usage, offering a more optimized and environmentally considerate alternative to conventional methods (Zhang et al. 2011; Zhou et al. 2009).

2.13 Antimicrobial Susceptibility Testing (AST)

AST is a laboratory method that serves as a fundamental tool in determining the effectiveness of synthetic or natural antimicrobial agents against specific bacterial strains (Salam et al., 2023). Besides screening and confirming the antimicrobial capability of selected compounds, it can detect resistance in particular bacterial isolates (Jorgensen & Ferraro, 2009). With the emerging threats of antimicrobial resistance among pathogenic microorganisms, AST plays a pivotal role in discovering and developing novel antimicrobial agents from natural products by providing crucial insight into their efficacy, mechanism of action, and comparative effectiveness, thereby validating their potential as alternatives to conventional agents (Hossain, 2024). The Clinical and Laboratory Standards Institute (CLSI) and European Committee on

Antimicrobial Susceptibility Testing (EUCAST) guidelines are often recognised as references for AST procedures, ensuring standardised methodologies to ensure reliability and accuracy of the results obtained (Salam et al., 2023).

Common *in vitro* AST methods can be categorised into phenotypic and genotypic approaches. Phenotypic methods observe the actual effect of microorganisms against antimicrobial agents, and can be further divided into qualitative techniques (e.g. disc diffusion method) and quantitative techniques (e.g. broth and agar dilution method). Examples of conventional *in vitro* phenotypic AST include disc diffusion, agar-well diffusion, and broth and agar dilution assays (Baumgartner & Xia, 2003). On the other hand, genotypic AST methods such as PCR, DNA microarray and DNA chips detect genetic markers associated with resistance (Balouiri et al., 2016; Baumgartner & Xia, 2003; Khan et al., 2019). Although genotypic AST provide rapid detection and highly sensitive result, phenotypic AST remains the gold standard in most research as it offers both qualitative and quantitative outcomes such as the growth inhibition, metabolic activity, cell viability, antimicrobial's impact on cell physiology, and the antimicrobial effectiveness of the tested compound (Bubonja-Šonje et al., 2020; Salam et al., 2023).

In the context of improving endodontic therapy, AST provides essential data on the sensitivity or resistance of *E. faecalis* to various intracanal medicaments (Manoil et al., 2024) and also evaluate the antimicrobial potential of the tested compound (Bubonja-Šonje et al., 2020). Testing natural compounds, such as propolis, through AST is crucial for validating their antimicrobial properties compared to conventional medications like NS-$Ca(OH)_2$ and CHX gel (John et al., 2015). The integration of multiple *in vitro* methodologies provides a comprehensive framework for evaluating the antimicrobial potential of Malaysian stingless bee propolis (Tiang et al., 2025). Malaysian propolis can be evaluated into a chip and used to treat patients with periodontal disease (Balouiri et al., 2016).

2.13.1 Preliminary Antimicrobial Screening Test

Agar diffusion assay, including both agar-well diffusion and disc diffusion method are widely used techniques in preliminary screening the antimicrobial properties of a compound (Hossain, 2024). These techniques rely on the diffusion of

antimicrobial agents through the agar medium inoculated with a test microorganism, resulting in the formation of inhibition zone that reflects antimicrobial activity. (Hossain, 2024).

In the disc diffusion method, sterile filter paper discs impregnated with a fixed volume and concentration of the antimicrobial agent and placed on the agar surface. This method is widely applied in testing antimicrobial of conventional antibiotics, but its application limited when evaluating viscous, poorly soluble, or complex natural products, as absorption and release of the agent from the disc can be inconsistent (Hossain, 2024; Balouiri et al., 2016)

On the other hand, agar well diffusion method placed the test substance directly into wells created in the inoculated agar. It is a favoured method for antimicrobial of natural and synthetic compounds (Azman et al., 2025; Balouiri et al., 2016). This method is well-known for its simplicity, cost-effectiveness, and relatively rapid results. It allows multiple substances to be simultaneously evaluated against a single microorganism, and it is highly versatile as it can be utilised to test a wide range of substances, such as synthetic compounds, conventional antibiotics, plant extracts, and microbial extracts (Balouiri et al., 2016; Hossain, 2024). Despite its practicality, it is only suitable for a qualitative assessment, as the concentration of the compound that diffuses and inhibits microbial growth cannot be quantified (Hossain, 2024).

2.13.2 Minimum Inhibitory Concentration (MIC)

Besides qualitative approaches, the determination of MIC provides a more precise and quantitative evaluation of a compound's antimicrobial effectiveness. MIC represents the lowest concentration of an antimicrobial agent that visibly inhibits bacterial growth under standardised *in vitro* conditions (Kowalska-Krochmal & Dudek-Wicher, 2021). Dilution methods are the most suitable techniques for determining MIC values in either agar or liquid media, as they can precisely estimate the concentration of the compound needed to inhibit microbial proliferation. These methods incorporate the test substance in a series of concentrations achieved through two-fold dilution, followed by the addition of a standardised microbial suspension (Hossain, 2024).

The broth microdilution test conducted in a 96-well microtiter plate, is a commonly applied method for evaluating the antimicrobial potential of natural product

extracts, as it offers flexibility, rapid results, cost-effectiveness, and reproducibility (Khezripour et al., 2019). However, despite these advantages, a notable limitation is the potential interference of coloured or viscous extracts such as propolis, which can hinder the visual determination of MIC or affect the turbidity of the solution (Balouiri et al., 2016; Hossain, 2024).

2.13.3 Minimum Bactericidal Concentration (MBC)

Complementary to MIC, the MBC determines the lowest concentration of a compound required to completely eradicate the test microorganism, rather than simply inhibit its growth. Typically, the MBC is determined by sub-culturing samples from the MIC tubes or wells that show no visible growth after incubation (Hulankova, 2024). Value of MBC higher than the MIC is commonly observed, as inhibition of bacterial growth requires a lower antimicrobial concentration compared to bactericidal activity. This difference reflects the greater concentration needed to achieve irreversible cellular damage and complete bacterial eradication (Ishak et al., 2024). Although the MBC value provides deeper insight into the antimicrobial potential of novel compounds, the test is more time-consuming and labour-intensive, as it requires subculturing onto fresh media to confirm bacterial killing (Balouiri et al., 2016).

2.13.4 Antibiofilm Assay

In addition to directly killing pathogenic cells, another crucial aspect of intracanal medicaments is their ability to disrupt biofilm formation, which provides substantial resistance to standard antimicrobial agents (Pourhajibagher et al., 2016). *E. faecalis* has a remarkable ability to adhere to dentinal surfaces, forming calcified biofilm within the harsh environment of root canals. These biofilms significantly contribute to the persistence and resistance of *E. faecalis* against standard treatments and intracanal medicaments, posing major challenges to completely disinfecting the treated area during endodontic therapy (Wahjuningrum et al., 2017). Natural products containing bioactive compounds such as polyphenols, flavonoids, and D-amino acids

have previously been shown to inhibit pathogenic biofilm (Elgamoudi & Korolik, 2023; Wahjuningrum et al., 2017).

Various methods have been developed for *in vitro* analysis of biofilm formation and disruption. Spectrophotometric approaches are commonly used, as they do not require specialised equipment, reagents, or high level of expertise. Typically, the biofilm viability assays involve culturing planktonic pathogens on a flat surface plate, such as 96-well plates, and allowing biofilm formation through incubation. The formed biofilms are then treated with antimicrobial compounds, and the remaining biofilm is quantified through staining techniques such as crystal violet, syto9, resazurin, and XTT assays. Viable biofilm levels are subsequently compared with non-treated biofilm controls (Elgamoudi & Korolik, 2023; Peeters et al., 2008).

2.13.5 Anti-adherence assay

In addition to disrupting biofilm formation, a complementary antimicrobial strategy involves inhibiting the initial attachment of bacteria to surfaces. This effect is different from antibiofilm as anti-adherence focused on early-stage intervention by preventing the attachment between microorganisms and a surface. It is an important property of an intracanal medicament as it helps to stop pathogen attachment to the treated root canal and prevent re-infection. This anti-adherence mechanism has been attributed to phytochemicals such as alkaloids, tannins, flavonoids, and essential oils found in natural products. These compounds not only reduce microbial colonisation and subsequent biofilm development but also exhibit minimal cytotoxicity, highlighting their potential as safe and effective agents for incorporation into oral healthcare formulations (Bhadoria et al., 2019).

Various in vitro techniques have been developed to assess the anti-adherence ability of a compound, with spectrophotometric analysis being the most widely used. In this method, the test surface, often a microtiter plate well, is pre-treated with the candidate compound at the desired concentrations. Following pre-treatment, the bacterial suspension is added and incubated for a fixed duration to allow initial attachment. The adhered cells are then quantified spectrophotometrically. This approach offers a relatively quick and reproducible means of screening potential anti-

adherence agents, particularly those derived from natural products, for application in oral care and biomedical fields (Wang et al., 2021).

2.13.6 Scanning Electron Microscope (SEM) Analysis

SEM provides 3D visual confirmation of bacterial morphology following treatment with antimicrobial agents. The process involves several steps, such as chemical fixation to preserve cell structure, followed by dehydration using a graded series of ethanol concentrations. Once dried, the specimens are mounted on conductive stubs and coated with a thin layer of metal, such as gold or platinum, to enhance conductivity and image clarity. The samples are then imaged under high vacuum with an electron beam to produce detailed surface images. SEM is often chosen for its simplicity. However, it does not provide as high a resolution as other microscopy methods such as Transmission Electron Microscope (TEM) (Kaláb et al., 2008).

2.13.7 Time-kill Study

Time-kill study, or time-kill method, is a procedure used to evaluate the bactericidal effect of a compound against tested bacteria at different time intervals to observe its antibacterial activity over time (Alshareef, 2021). It provides valuable insight into microbial inhibition by evaluating time-dependent or concentration-dependent kinetics, aiding in the understanding of dynamic interactions between the antimicrobial compounds and microorganisms. This method is commonly used to assess novel antimicrobial agents, investigate synergistic effects when several compounds are tested in combination and compare the killing rate of conventional drugs with test compounds (Garcia, 2010; Hossain, 2024).

In practice, a standardised microbial suspension is treated with a specific concentration of an antimicrobial compound in test tubes or wells of a microtiter plate containing appropriate growth media. The samples are incubated, and aliquots are taken at predetermined time intervals and plated onto fresh agar media. Colony-forming units (CFUs) are enumerated to determine viable microorganisms at each time point.

Sequential sampling provides quantitative data on antimicrobial efficacy over time, enabling the microbial killing rates (Doern, 2014; Hossain, 2024).

Unlike other AST methods that assess antimicrobial activity at a single static time point, the time-kill study continuously monitors microbial growth over time after treatment. This enables simultaneous observation of both the rate and extent of microbial growth inhibition. However, it is labour and time-consuming, requiring frequent sampling, and is generally considered a costly method (Hossain, 2024).

2.14 Synergistic Effect of Antimicrobial Agents

Besides discovering novel antimicrobial compounds, certain agents may exhibit stronger efficacy when combined with others. With the emerging threat of antimicrobial-resistant organisms, this strategy provides an avenue for identifying more effective antimicrobial therapies (Doern, 2014). According to the EUCAST guidelines, the simplest evaluation of synergistic effect can be performed using the fractional inhibitory concentration (ΣFIC) index. The ΣFIC test estimates the interaction between two or more antimicrobial agents intended for combined use. Combining multiple antimicrobial agents may result in a synergistic effect, where the antimicrobial activity is greater than the sum of each constituent's activity, or it may produce an addictive effect, where the overall activity is simply the cumulative effect of the antimicrobial agents (Doern, 2014). For example, a study by Ismail et al. (2021) showed that Malaysian propolis (MP) with aloe vera (AV) has a higher antimicrobial activity when compared to MP or AV individually.

2.15 Research Gap and Rationale for Study

In summary, propolis warrants greater attention elucidating its therapeutic benefits, especially since many beekeepers overlook its high value and discard it in favour of the more marketable honey. Despite the growing interest in propolis for endodontic treatment, several gaps remain in the literature regarding its use against *E. faecalis*. Existing studies have primarily focused on *in vitro* antimicrobial analyses, however, more comprehensive investigations are required to understand its effects on

E. faecalis biofilm formation and viability (Elsayed et al., 2021). Furthermore, although findings from previous research suggest promising antimicrobial properties, propolis cannot yet be considered a reliable alternative to conventional intracanal medicaments without further validation through rigorous *in vitro* and *in vivo* studies (Elsayed et al., 2021; Kashi et al., 2011). Another limitation lies in the frequent use of *E. faecalis* ATCC 29212, a urinary isolate, which may not accurately represent endodontic infections. In contrast, strain ATCC 4082, derived from pulpless teeth, is more clinically relevant for dental-related research (Elsayed et al., 2021; Zancan et al., 2018). A further research gap is the limited exploration of antimicrobial, antibiofilm, and anti-adherence variations among different species of stingless bee propolis within the same geographical region, which may significantly influence therapeutic potential (Kashi et al., 2011). Moreover, few comparative studies have directly evaluated the efficacy of propolis alongside commonly used medicaments such as NS-$Ca(OH)_2$ and CHX gel. The potential synergistic interactions within propolis extracts, and whether they contribute to enhanced antimicrobial effects, remain poorly understood and warrant further exploration (Kashi et al., 2011). A study by (Azman et al., 2025) indicated a longer observation period, time-kill study, anti-adherence, and anti-biofilm of *E. faecalis* ATCC 4082 acted upon by *H. itama* propolis may be useful parameters to consider before formulating a therapeutic design.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Study Design

3.1.1 Pilot Study on Propolis Antimicrobial Ability and Determination of Intraclass Correlation Coefficient (ICC))

This was conducted prior to the main investigation on the *H. itama* propolis extract against *E. faecalis* ATCC 4082 to ensure the feasibility, reproducibility, and consistency to support the main study. The pilot study involved the AST via measurement of ZOI in the agar-well diffusion test to determine the ICC (Koo & Li, 2016) measuring ZOI in the agar-well diffusion technique (Refer to Appendix 1). The ICC was determined using the IBM SPSS Statistical version 27. The ICC was used to assess the level of agreement and reliability between measurements. In this study, ICC was applied to determine the degree of agreement between repeated assessments of a same rater in order to minimise intra-operator memory bias.

3.1.2 Main Study

The main *in vitro* experimental study was conducted (see Figure 3.1) to determine the antibacterial properties of three ethanolic propolis extracts from different Malaysian stingless bee species against two *E. faecalis* strains (ATCC 29212 and ATCC 4082). The effectiveness of these propolis extracts were then compared with commonly used intracanal medicaments; 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel (both served as positive controls).

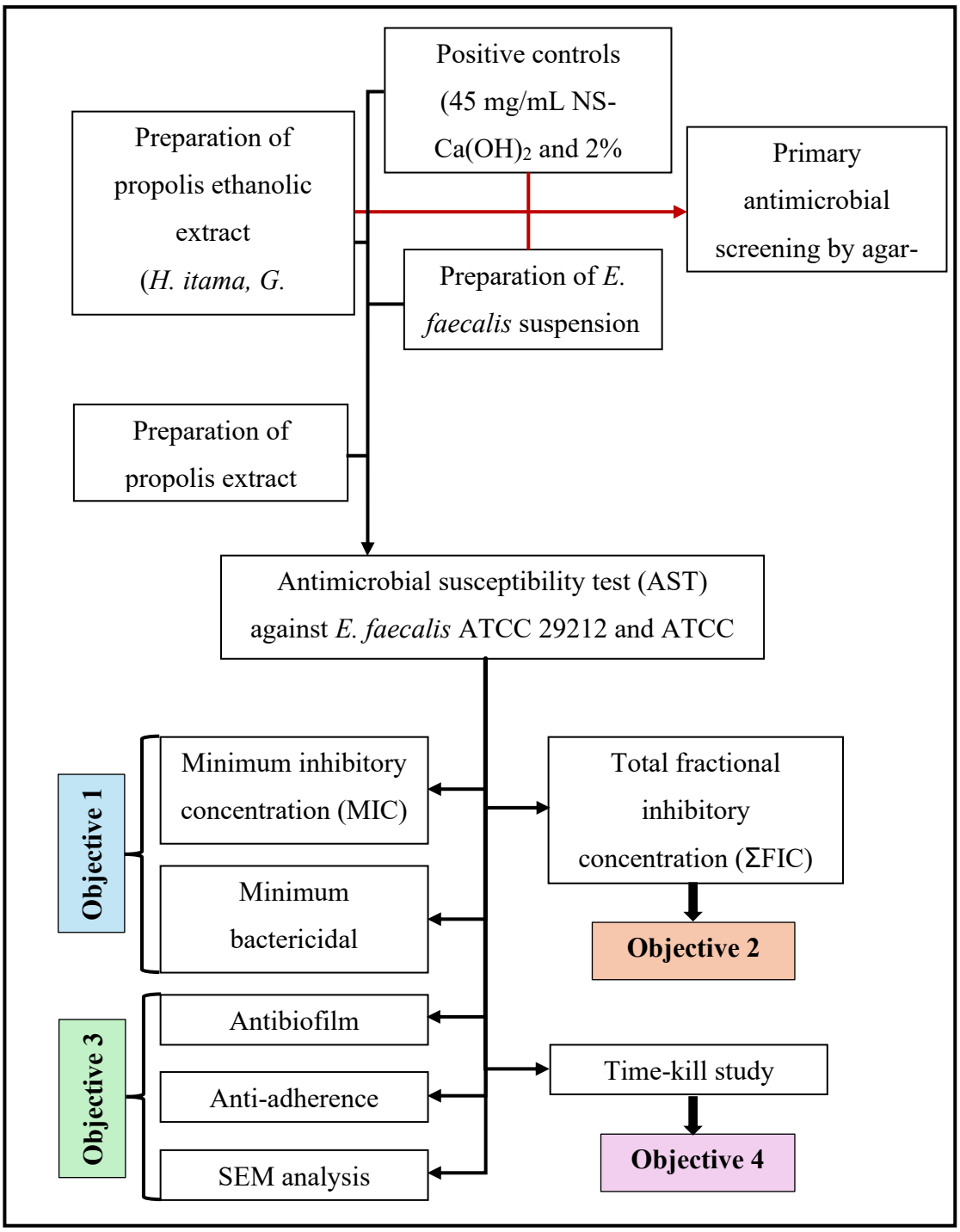


Figure 3.1 Flowchart of Research Methodology

The table below shows the equipment and materials used in the study.

Table 3.1
List of Materials and Equipment

Category	**Items**
Chemicals and Clinicals Items	>99% industrial grade ethanol, Brain heart infusion (BHI) agar, BHI broth, 99% Glycerol, Crystal violet stain, Iodine solution, Acetone, Safranin, and Dimethyl sulfoxide (DMSO), Immersion oil, Distilled water, Deionised water, Urea, Sodium chloride (NaCl), Calcium chloride ($CaCl_2$), Potassium dihydrogen phosphate (KH_2PO_4), Sulfuric acid (H_2SO_4), Sodium hydroxide (NaOH), Glutaraldehyde, Phosphate buffer saline (PBS), Waldent AlChem 2% CHX gel and Calcicur® NS-$Ca(OH)_2$ paste (syringe form, 45 mg/mL)
Glassware	Universal bottles, 250 mL Conical flask, 250 mL Beaker, 500 mL Laboratory bottle, 500 mL Graduated cylinder, Filter funnel
Items and Consumables	Plastic spatula, Cork borer, Tweezer, Petri dish, Parafilm, Whatman filter paper no.1, 15 mL and 50 mL Conical tube, Tube rack, weighing boat, Aluminium foil, Cotton swab, 2 mL cryovial tube, Cryobox, disposable inoculation loop, hockey stick, Micropipette (10, 200, and 1000 µL), Micropipette tips (10, 200, and 1000 µL), Microscope slide, Wash bottle, Cuvette, Bunsen burner, 96-well microtiter plate, 1.5 mL Microcentrifuge tube, pH test strips
Equipment	Autoclave, Heavy duty dry blender, Rotary evaporator (Rotavapor R-210), Freezer, Chiller, Orbital shaker (SK-600), Ultrasonic bath, Vacuum concentrator (Genevac™ miVAC), Freeze dryer (FreeZone), Vortex mixer, Biosafety cabinet, Microscope (Olympus CX23), Spectrophotometer, Incubator, Microplate reader, pH meter, Centrifuge, Sputter coater, Scanning electron microscope (Hitachi TM3000), Colony counter, Analytical balance
Raw Samples	*H. itama* raw propolis, *G. thoracica* raw propolis, *T. apicalis* raw propolis, *E. faecalis* ATCC 29212, *E. faecalis* ATCC 4082

3.2 Preparation of Propolis Extract

3.2.1 Collection and Handling of Raw Propolis Sample

The handling of raw propolis samples follows the method described by Al-Masoodi et al. (2022). Raw propolis produced by *H. itama*, *G. thoracica,* and *T. apicalis* (Plate 3.1) were purchased from Aasta Stingless Bee Honey Farm, Raub, Pahang (Coordinates: 3.8210721, 101.8407539). These three stingless bee species were selected because they are among the most commonly found and commercially available in Malaysia, as shown in Figure 2.6. Plus, these three species, specifically from Raub were selected due to convenience and dependable supply. All three species were obtained from the same farm, allowing direct comparison of the properties of propolis derived from different species under the same environmental conditions. All these raw materials were packed separately to avoid cross-contamination and stored in a -20°C prior to further analysis.

Plate 3.1 Raw Propolis Obtained from *H. itama, G. thoracica*, and *T. apicalis*

3.2.2 Preparation of Propolis Ethanolic Extract

The extraction process followed the previously described methods (Al-Masoodi et al., 2022; Al-Qurashi & Awad, 2018; Pobiega et al., 2019) with slight modification. Figure 3.2 illustrates the extraction steps. Small pieces of raw propolis from each sample were grinded using a blender and 10 g of each pulverised sample was diluted with 100 mL of 70% diluted ethanol (1:10 w/v ratio) in a separate 200 mL conical flask, to facilitate the extraction of bioactive compounds. Each conical flask was properly labelled, sealed with parafilm, and shaken at room temperature on an orbital shaker (SK-600 shaker, Jeiotech, Korea) at 200 rpm for 7 days. The mixture was then sonicated in an ultrasonic bath (Ultrasonic, Jeiotech, Korea) for 30 minutes at 27°C at a moderate setting. The mixture was filtered overnight on the bench using Whatman filter paper No.1 to remove wax and resin. The filtrate was then concentrated for 2 hours until an appropriate viscosity was obtained using a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) to remove the solvents. The remaining extract in the flask was subsequently transferred into a centrifuge tube to separate it from the wax adhering to the walls of the flask and further concentrated using a vacuum concentrator (Genevac™ miVAC, United Kingdom) for 2 hours at 40°C. The extract was stored overnight inside a -80°C freezer before it was lyophilised using a freeze dryer (FreeZone, Labconco, US) for 3 days. The powdered propolis extract was measured to calculate the percentage yield using the formula below. Finally, the lyophilised samples were transferred into universal bottles and stored in a -25°C for further use.

$$\text{Percentage Yield (\%)} = \frac{Final\ weight\ of\ extract}{Initial\ weight\ of\ raw\ product} \times 100 \qquad (3.1)$$

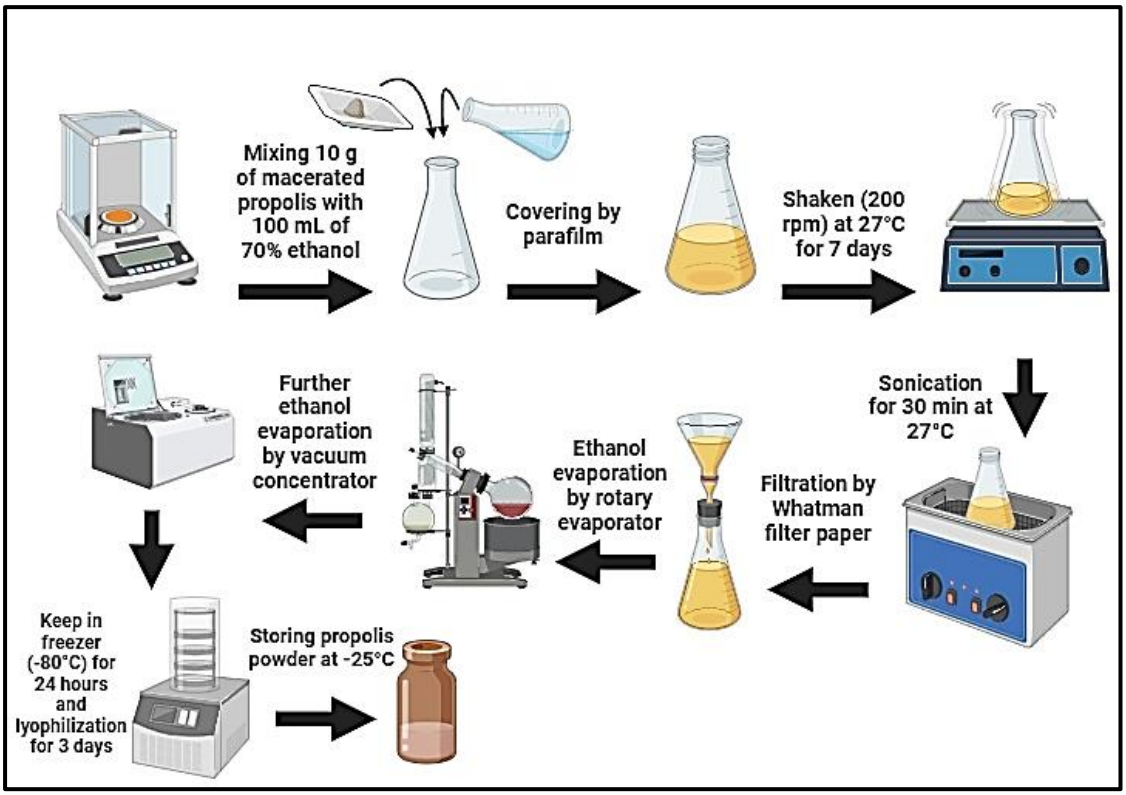


Figure 3.2 Schematic Diagram for Preparation of Propolis Ethanolic Extracts Using Shaking-assisted Extraction Methods

3.2.3 Preparation of Propolis Extract Stock and Working Solution

Propolis extract stock of 200 mg/mL and 50 mg/mL working solutions of each *H. itama*, *G. thoracica*, and *T. apicalis* propolis extracts were prepared by reconstituting the extract powder with 5% (v/v) DMSO. The working solution concentration in this study was based on the average concentration reported in previous research (Table 3.2).

For the stock solution, 1000 mg of each lyophilised propolis extract powder was weighed and 5 mL of 5% (v/v) DMSO was added. The extracts were mixed thoroughly, and the solutions were stored at 4°C for further use. Subsequently, the pH of the propolis extracts, 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel was determined using a pH test strip. The working solution was prepared by performing a four-fold dilution of the stock, calculated using the formula 3.2. Specifically, 10 mL of working solution (50 mg/mL) was prepared by adding 2.5 mL of stock solution into a universal bottle, followed by 7.5 mL of 5% (v/v) DMSO. All solutions were stored at 4°C until further use.

$$C1V1 = C2V2 \quad (3.2)$$

Note: C1 = Initial concentration of propolis extract, C2 = Final concentration of propolis extract, V1 = Initial volume of propolis extract used, V2 = Final volume of solution

The 5 mL of 5% (v/v) DMSO solution was prepared by mixing 250 µL of 100% DMSO with 4.75 mL of distilled water in a 15 mL conical tube. A concentration of 5% DMSO was used as it is considered safe and does not interfere with bacterial cells during the antimicrobial test. Further clarification on the appropriate DMSO concentration for microbiology tests is provided in Table 3.2

Table 3.2
Effect of DMSO on Bacterial Cells

DMSO Concentration	Bacteria indicator	Effect	Reference
Range from 0 to 20%, at 1% increments.	*E. coli*, *P. aeruginosa*, and *Bacillus megaterium* (*B. megaterium*)	• The bacteriostatic concentration towards the tested bacteria cultured in BHI broth ranged from 5% to 10% • Bactericidal effect starts from a concentration of more than 70% • 5% DMSO caused no effect to *E. coli* cells, as it only binds and forms precipitate on the bacterial cell wall • Cell wall of *E. coli* treated with 10% DMSO was still intact but had started to alter the bacterial cytoplasm and plasma membrane.	(Ansel et al., 1969)
DMSO with several concentration diluted with 0.1% (w/v) peptone saline (100% DMSO, 10-fold, 100-fold, 1000-fold, and 10,000-fold	*Mycobacterium abscessus* (*M. abscessus*)	• Pure DMSO completely disinfect all isolates • 10-fold diluted DMSO inhibited half of the bacterial growth • 10,000-fold dilution has shown no significant effect.	(Kirkwood et al., 2018)
DMSO with ranging concentrations	*E. faecalis*	• 6.25% of DMSO neither inhibits the growth nor kills the bacteria • 12.5% DMSO started to show an inhibitory	(Al-Masoodi et al.,

that were continually diluted with a two-fold dilution (100, 50, 25, 12.50, 6.25, 3.14, 1.56, 0.78, 0.39, 0.20)	effect towards the bacterial growth	2022)

3.3 Preparation of *E. faecalis* Suspension

3.3.1 Rehydration of *E. faecalis*

The method for rehydrating and reviving the cryopreserved *E. faecalis* followed the guidelines by CLSI (2012) with slight modification. The glycerol stocks of *E. faecalis* ATCC 29212 and ATCC 4082 were previously stored in a -80°C freezer at the Faculty of Dentistry, UiTM. The stock was thawed at room temperature and rehydrated in a cryovial tube containing 0.5 mL of brain-heart infusion broth (BHIB). The suspension was incubated at 37°C for 24 hours. The inoculated cryovial tube was then transferred into 5 mL of BHIB in a universal bottle and the bacterial culture was incubated at 37°C for 24 hours.

3.3.2 Confirmation of *E. faecalis* by Gram Staining

E. faecalis ATCC 4082 and ATCC 29212 suspensions were streaked onto BHI agar to obtain a pure single colony. The plates were then incubated at 37°C for 24 hours. The Gram staining was method followed Smith & Hussey, (2019). Gram staining was performed on both bacterial strains to confirm purity of the bacterial stock and to avoid contamination by other bacterial species.

A loopful of sterile distilled water was placed on a microscope slide. A single colony of *E. faecalis* was then transferred onto the slide and evenly spread within a 15 mm diameter circle using an inoculating loop. The slide was air-dried for approximately

15 minutes, and heat-fixed by passing it over a gentle flame several times. Heat fixation ensured adhesion of cells to the slide and minimised sample loss during rinsing. The slide was stained with crystal violet for one minute, then, gently rinsed with distilled water and treated with iodine solution for another one minute. After rinsing with distilled water, the slide was decolourised with acetone for about 10 seconds. Finally, it was counterstained with safranin solution for one minute, and gently rinsed again. Excess water was removed by blotting with clean tissue paper, and the slide was left to air-dry for approximately 15 minutes. All prepared slides were observed under a light microscope (Olympus CX23) at 100X magnification. The images obtained were examined and recorded.

3.3.3 Preparation of Bacterial Glycerol Stock

The preparation of *E. faecalis* glycerol stock followed the method described by Ibrahim et al. (2024). A 50% (v/v) glycerol solution was used for the storage and preservation of each *E. faecalis* strain. From the inoculated BHI broth, 0.5 mL was transferred into a cryovial, followed by the addition of 0.5 mL of sterilised glycerol solution. The resulting bacterial-glycerol mixture was carefully vortex and stored at -80°C.

3.3.4 Standardisation of Bacterial Suspension

The standardisation of *E. faecalis* suspension follows CLSI (2012) guidelines with slight modifications. The bacterial suspension used for antimicrobial susceptibility testing was freshly prepared one day prior to each experiment.

A single colony of *E. faecalis* was inoculated into 10 mL of BHIB and incubated until the exponential phase (6-8 h) was reached. The working suspension was prepared by adding 15 mL of BHIB to 1 mL of bacterial culture in a universal bottle. The suspension was labelled and incubated at 37°C for 24 hours. The bacterial cell density was then standardised to an optical density (OD) range of 0.08 to 0.13 at OD_{600} nm (equivalent to $1x10^8$ CFU/mL) using a spectrophotometer (CLSI, 2012). A blank control consisting of BHI broth was used to correct for background absorbance during

microplate reader analysis. The OD values of bacterial suspensions were obtained by subtracting the blank OD, which allowed for accurate measurement of turbidity and bacterial growth.

3.4 Intracanal Medicaments

Two types of intracanal medicaments, as shown in Plate 3.2 and Plate 3.3 were included in this study as positive control, which were Calcicur® NS-$Ca(OH)_2$ paste (ready-to-use syringe form, 45 mg/mL) and Waldent AlChem 2% (w/v) CHX gel (equivalent to 20 mg/mL), where both were clinically used directly without dilution. NS-$Ca(OH)_2$ and CHX gel were used as a positive controls and 5% DMSO as the negative control.

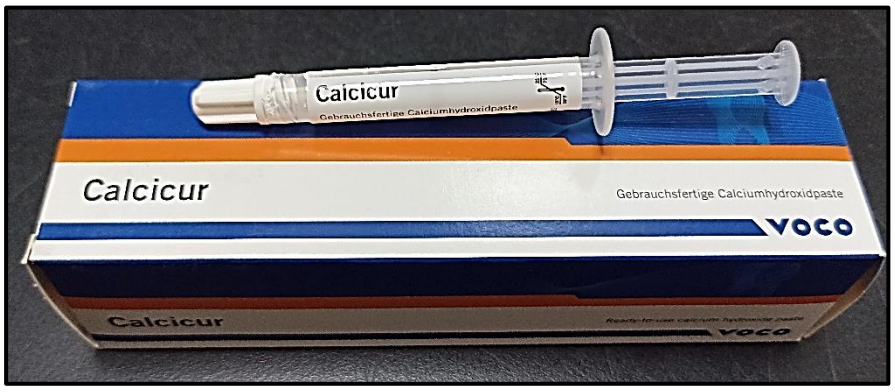


Plate 3.2 Calcicur® 45 mg/mL NS-$Ca(OH)_2$ Paste in Syringe Form

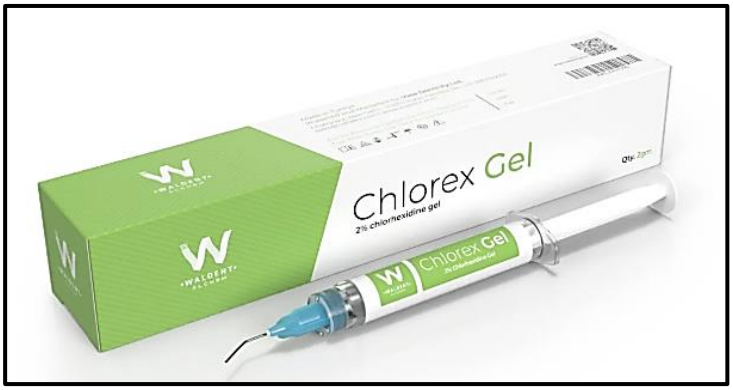


Plate 3.3 Waldent® AlChem 2% (w/v) CHX Gel, Equivalent to 20 mg/mL

3.5 *In vitro* AST

3.5.1 Agar-Well Diffusion Technique

The agar-well diffusion method, as shown in Figure 3.3, was performed following Al-Masoodi et al. (2022) and Azman et al. (2025). Standardised *E. faecalis* suspension (equivalent to a cell count of 1×10^8 CFU/mL) was uniformly swabbed onto the agar surface using a sterile cotton swab. Wells with a 6 mm diameter were made on the BHI agar plate using cork borers. A volume of 100 µL from each propolis extract, prepared at a concentration of 50 mg/mL, was dispensed into individual wells for antimicrobial testing. Additionally, 100 µL of both 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel were used as positive controls, while 100 µL of 5% DMSO served as a negative control. The plates were left at room temperature for 2 hours to allow compound diffusion across the agar, followed by incubation at 37°C for 24 hours. The zone of inhibition was measured, and the procedure was repeated three times to ensure accuracy (Gebreyohannes et al., 2013).

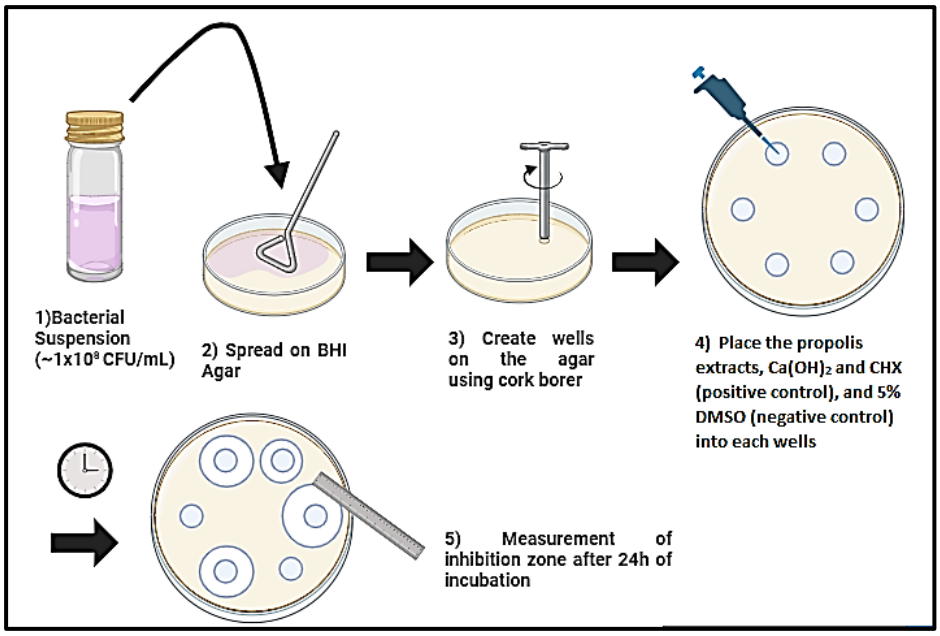


Figure 3.3 Primary Antimicrobial Screening Test by Agar-well Diffusion Method

Source: Antimicrobial activity of Malaysian *Heterotrigona itama* propolis against *Enterococcus faecalis* ATCC 4082: A study in endodontic applications (Azman et al., 2025, p. 476)

3.5.2 Preparation of Propolis Extract Mixture (PEM)

Equal amounts of propolis extracts from *H. itama*, *G. thoracica*, and *T. apicalis* (33.33 mg each; 1:1:1 weight ratio) were combined to obtain a total mass of approximately 100 mg. The pooled extract was dissolved in 1 mL of 5% (v/v) DMSO to produce a stock solution with a final concentration of 100 mg/mL.

3.5.3 Determination of MIC

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial substance including antifungal, antibiotic or bacteriostatic that can inhibit microbial growth after overnight incubation. MIC value provides essential information on the concentration required to inhibit microbial growth and indicates the susceptibility or resistance of specific bacterial strains to tested antimicrobial substances. MIC determination will be carried out using the broth microdilution method following the CLSI (2012) guidelines and the steps described by Zothanpuia et al., (2018) with slight modification. Figure 3.4 illustrates the method on determination of MIC and MBC.

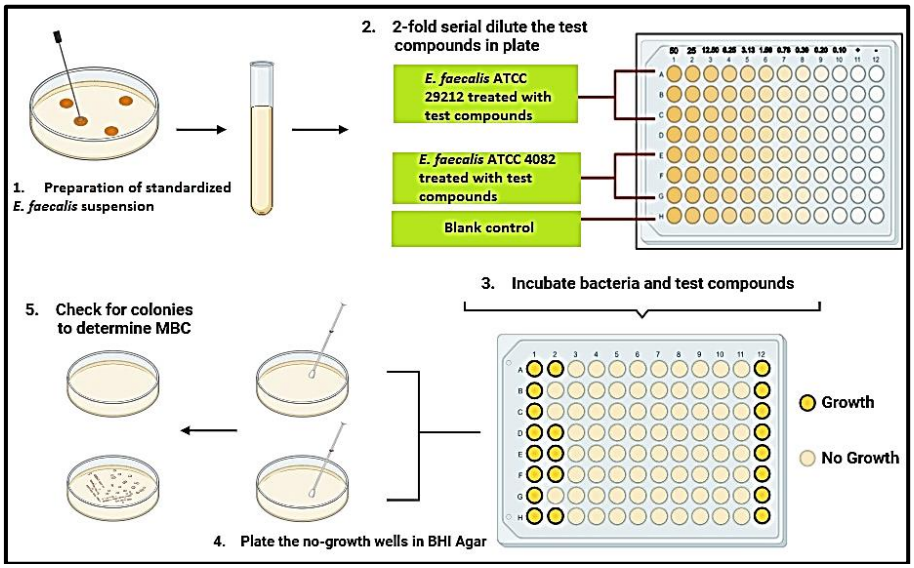


Figure 3.4 Determination of MIC and MBC

Source: Antimicrobial activity of Malaysian *Heterotrigona itama* propolis against *Enterococcus faecalis* ATCC 4082: A study in endodontic applications (Azman et al.,

2025, p. 477)

The MIC method was performed in a 96-well microtiter plate, consisting of 12 columns and 8 rows. A volume of 100 µL of BHI broth was dispensed into well 1 to 11, and 110 µL into wells 12 (negative control). A two-fold dilution of the propolis stock solution (200 mg/mL) for each species was prepared by adding 1 mL of 5% DMSO to 1 mL of the extract, yielding a final concentration of 100 mg/mL in a total volume of 2 mL. Then, 100 µL of the propolis extract (100 mg/mL) was added to well 1 and gently mixed using a micropipette. Serial two-fold dilutions were carried out by transferring 100 µL from well 1 to well 2, and continued sequentially up to well 10, producing a final concentration range of 50 – 0.1 mg/mL. A standardised *E. faecalis* culture (1 x 10^8 CFU/mL) was diluted to 5 x 10^6 CFU/mL and 10 µL of this diluted culture was added into wells 1 to 10 (CLSI, 2012), and well 11 (positive control with 2% CHX gel) as well as the wells containing 45 mg/mL NS-$Ca(OH)_2$ (second positive control). The final *E. faecalis* concentration in each well was approximately equivalent to 4.5x10^5 CFU/mL, with the total volume in each well was 110 µL (preferable range based on CLSI guideline = 2 to 8 × 10^5 CFU/mL). The plates were incubated at 37℃ overnight.

On another 96-well microplate, 110 µL of two-fold serial dilutions in brain-heart infusion broth (BHIB) of all propolis extracts were prepared to serve as blank control to minus the effect of compound's colour. The procedure was performed in triplicate for both bacterial strains. Bacterial growth in each was first observed visually, followed by measuring absorbance at OD_{600} nm using a microplate reader. The similar experiment was repeated three times, each with three technical replicates.

3.5.4 Total Fractional Inhibitory Concentration (ΣFIC)

The calculation of ΣFIC followed Shafiei et al. (2016). To determine the ΣFIC, the MIC value of the propolis extract mixture (PEM) was first determined using the same MIC procedure. The ΣFIC was then calculated using formula in (3.3) and the FICs values of each individual constituent were determined using formula (3.4), (3.5), and (3.6).

$$\Sigma FIC = \frac{FIC1 + FIC2 + FIC3}{3} \quad (3.3)$$

$$FIC1\ (FIC\ of\ H.itama) = \frac{MIC\ (PEM)}{MIC\ (H.itama)} \quad (3.4)$$

$$FIC2\ (FIC\ of\ G.thoracica) = \frac{MIC\ (PEM)}{MIC\ (G.thoracica)} \quad (3.5)$$

$$FIC3\ (FIC\ of\ T.apicalis) = \frac{MIC\ (PEM)}{MIC\ (T.apicalis)} \quad (3.6)$$

The ΣFIC index determines the interaction between the propolis extracts in the mixture, and the interaction is interpreted based on the range of values shown below in table 3.3

Table 3.3
Evaluation of Interaction Based on ΣFIC Value

Value	**Interaction**
< 0.5	Synergistic
0.5 – 1.0	Additive
1.0 – 4.0	Indifferent
> 4.0	Antagonistic

Source: Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium sp., Mangifera sp.,* and *Mentha sp.*) on single- and dual-species biofilms. (Shafiei et al., 2016, p. 5)

3.5.5 Determination of MBC

From the MIC determination method, the well that showed no bacterial growth was spread over the surface of BHI agar plates using a sterile a cotton swab and incubated at 37°C overnight, as shown in Figure 3.4. Bacterial growth was observed for each section on the agar, and the MBC value was determined (Gebreyohannes et al., 2013).

3.5.6 Determination of Antibiofilm and Anti-adherence Effect of Propolis Extracts and Intracanal Medicaments

3.5.6.1 Preparation of Artificial Saliva

Artificial saliva solution was prepared using 500 mL of deionised water, 500g urea, 0.276g sodium chloride (NaCl), 0.3g calcium chloride ($CaCl_2$), and 0.13g potassium dihydrogen phosphate (KH_2PO_4). The pH of the artificial saliva solution was measured using a pH meter and adjusted to approximately pH 6.7 by adding a few drops of H_2SO_4 or NaOH (Diamantopoulou et al., 2020).

3.5.6.2 Antibiofilm Effect

The methods for culturing 24-hour *E. faecalis* biofilm followed Dudek-Wicher et al. (2022) and Kwasny & Opperman (2010), methods with slight modification, as shown in Figure 3.5. The wells of a sterile 96-well microtiter plate were filled with 100 µL of artificial saliva solution and incubated at 37°C for 30 minutes. Incubation following the addition of artificial saliva was performed to allow formation of a conditioning pellicle on the well surface, thereby simulating the initial stages of oral biofilm development and providing a biologically relevant substrate for subsequent microbial adhesion and antibiofilm assessment. The washing step was then performed as outlined in subchapter 3.5.6.4. From the standardised *E. faecalis* culture of each strain, a suspension was diluted to 1×10^5 CFU/mL in BHIB. Then, 100 µL of the

diluted suspension was added to the wells and incubated for 24 hours to allow formation of 24-hour biofilm. After incubation, the bacterial suspension was removed, and the plate was rinsed once. The antibiofilm activity of propolis extracts were evaluated by treating the biofilm-coated well with 100 µL of each compound at the MBC value. The plate was incubated at 37°C for 30 minutes, followed by the removal of the solution and the washing step. The treatment with each compound was performed across an entire column of the plate (9 wells) with 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel serving as positive controls, and an untreated well as a negative control. The experiment was repeated three times, with each three technical replicates to ensure reproducibility and statistical reliability.

Biofilm biomass in each well was quantified using the crystal violet assay. The biofilm was stained by adding 100 µL of 0.06% (v/v) crystal violet and incubating for 15 minutes. Excess stain was then removed, and the washing step was performed. The retained stain, which is proportional to the total biofilm biomass was solubilised by adding 200 µL of 30% (v/v) acetic acid into each well.

Quantification of biofilm biomass was performed by measuring the optical density at 600 nm (OD_{600} nm) using a microplate reader. The OD_{600} values were used as an indirect measure of biofilm biomass, whereby higher absorbance indicates greater biofilm biomass and lower antibiofilm activity of the tested compounds (Peeters et al., 2008; Wilson et al., 2017). For comparative analysis, the biofilm biomass of the untreated negative control was considered as 100%. The percentage of antibiofilm activity was calculated as formula (3.7) below:

$$\text{Antibiofilm activity (\%)} = 100 - \left(\frac{OD_{600}\text{ nm (compound)}}{OD_{600}\text{ nm (negative control)}}\right) \text{x } 100 \qquad (3.7)$$

Source: Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium sp., Mangifera sp., and Mentha sp.*) on single- and dual-species biofilms. (Shafiei et al., 2016, p. 7)

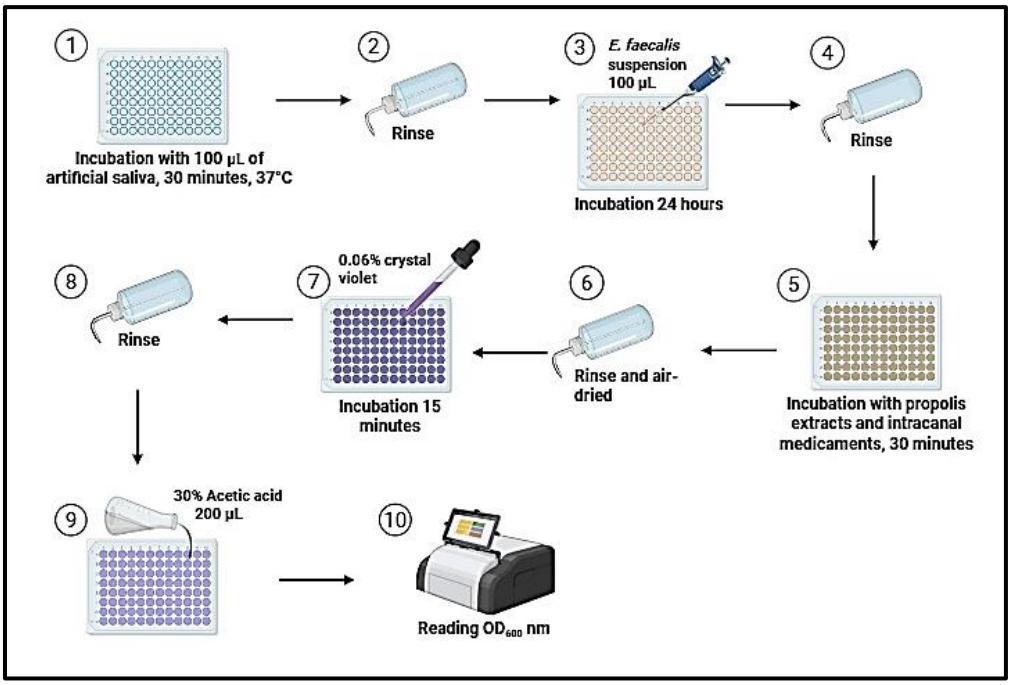


Figure 3.5 Biofilm Formation, Treatment and Quantification of Biofilm Biomass Using the Crystal Violet Assay

3.5.6.3 Anti-adherence Effect

The method follows Dudek-Wicher *et al*. (2022) and Kwasny & Opperman, (2010) with slight modifications, as shown in Figure 3.6. The wells of the sterile 96-well plate were filled with 100 µL of artificial saliva solution and incubated for 30 minutes to simulate oral surface conditions. The saliva was then removed, and the plate was rinsed with sterile phosphate buffer saline (PBS) solution. The anti-adherence ability of propolis extracts were evaluated by pre-coating the well with 100 µL of each extract at their respective MBC values and incubating for 30 minutes. After incubation, the solution was removed, and the plate was rinsed once. A standardised *E. faecalis* suspension (1×10^5 CFU/mL) was then prepared, and 100 µL of this suspension was added to each well, followed by 24 hours of incubation to allow 24-hour biofilm formation. After the incubation, the bacterial suspension was removed, and the plate was rinsed. The treatment with each compound was performed across an entire column of the plate (9 wells) with 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel serving as positive control, and an untreated well as negative control. Each experiment was repeated three times, with three technical replicates per run.

The adhered biofilm in each treated well were quantified using the crystal violet assay. Briefly, the wells were stained with 100 µL of 0.06% (v/v) crystal violet and incubated for 15 minutes. Following the washing step to remove excess dye, the retained stain was solubilised using 200 µL of 30% (v/v) acetic acid to facilitate quantification. The extent of bacterial adherence was quantified by measuring the optical density OD600 nm using a microplate reader. The OD_{600} values were used as an indirect measure of adhered biofilm biomass, where higher absorbance indicates greater bacterial adherence and lower anti-adherence efficacy of the tested compounds (Peeters et al., 2008; Wilson et al., 2017). The adhered biomass of the negative control was considered as 100%, and the percentage of anti-adherence was calculated as formula (3.8) below:

$$\text{Anti-adherence activity (\%)} = 100 - \left(\frac{OD_{600}\text{ nm (compound)}}{OD_{600}\text{ nm (negative control)}}\right) \times 100 \qquad (3.8)$$

Source: Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium sp., Mangifera sp., and Mentha sp.*) on single- and dual-species biofilms. (Shafiei et al., 2016, p. 7)

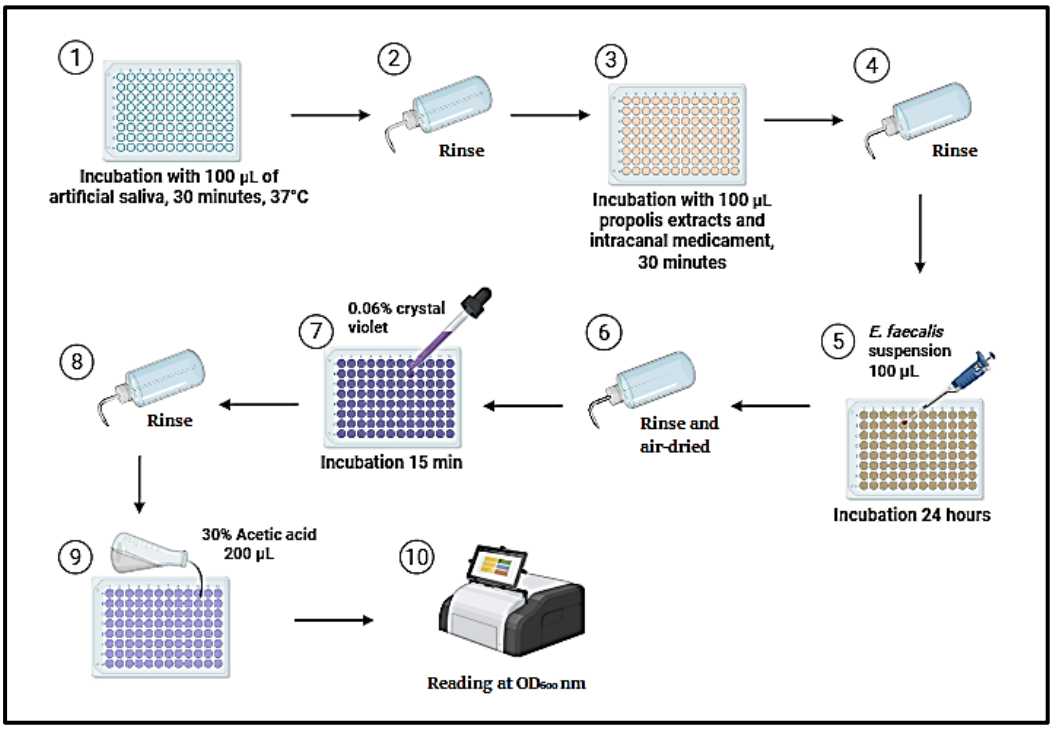


Figure 3.6 Determination of Anti-adherence Effect

3.5.6.4 Plate Washing Step

The washing step during antibiofilm and anti-adherence evaluation involves liquid removal and rinsing with sterile PBS solution. Rinsing steps were performed to remove non-adherent or planktonic cells and residual media, ensuring that only firmly attached or established biofilm remained for subsequent treatment and quantification. This step is critical to minimise background interference and to improve the accuracy and reproducibility of biofilm measurements (Dudek-Wicher et al., 2022; Kwasny & Opperman, 2010). The solution inside each well was first discarded by gently inverting the plate with a flick of the wrist and decanting into a waste container. Next, 200 µL of sterile PBS solution was added to each well. Care was taken to avoid disrupting the biofilm by tilting the plate and slowly dispensing the PBS along the wall of the well. The PBS solution was left for 1 minute, after which the plate was tilted again to remove the liquid. The process was repeated three times.

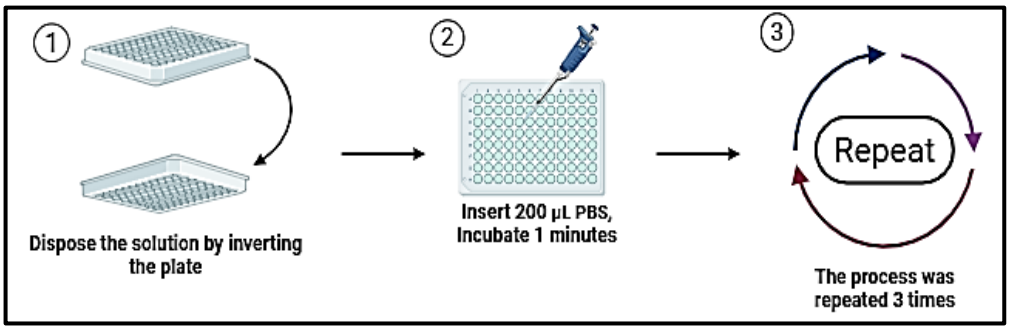


Figure 3.7 Plate Washing Steps

3.5.7 SEM Analysis

SEM analysis was performed following the method by Yenugu et al. (2004), with modifications, to evaluate the antimicrobial effect of the tested agents on *E. faecalis*. As shown in Figure 3.8, each propolis extract and PEM was diluted to its MBC with BHIB to a final volume of 1 mL (45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel as positive control). Then, 1 µL of standardised *E. faecalis* suspension was added to each mixture and incubated for 24 hours. After incubation, the suspension was centrifuged at 10,000 x g for 10 minutes, and the supernatant was discarded to obtain the bacterial pellet. The pellet was fixed with 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.2)

for 1 hour. Subsequently, the pellet was dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%) at 15-minute intervals to preserve cellular integrity (Navarro-Pérez *et al*., 2021). After air-drying for 30 minutes at 37°C, the pellet was gold-coated under low pressure using a sputter coater prior to SEM observation. Cell morphology was examined at 15,000X magnification using a scanning electron microscope (Hitachi TM3000, Tokyo, Japan).

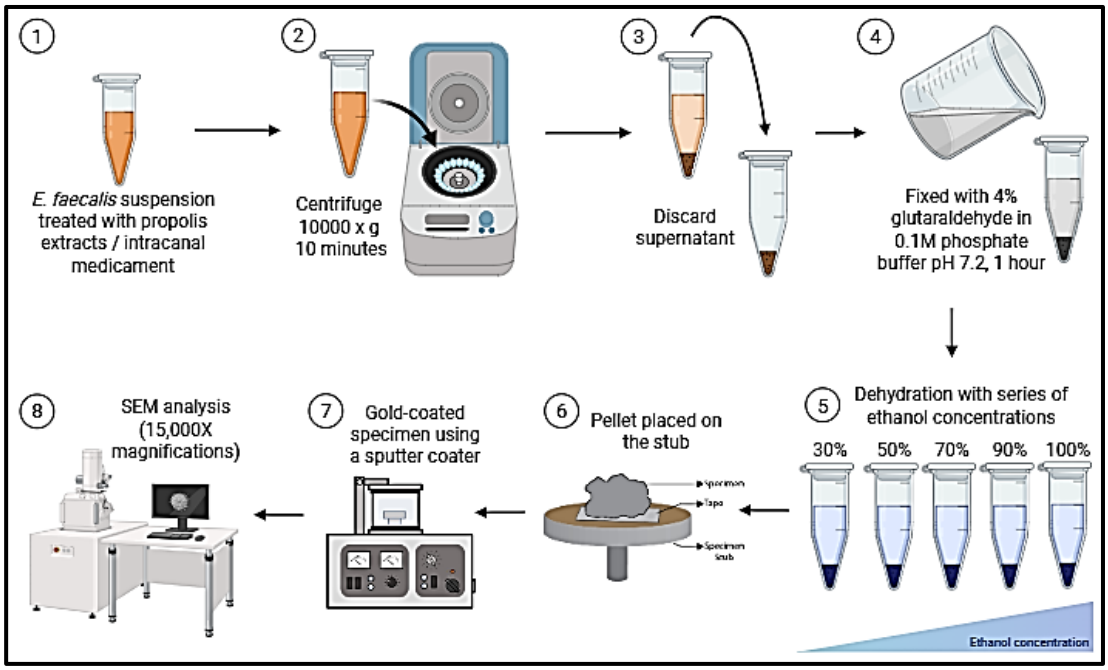


Figure 3.8 Sample Preparation and Morphology Analysis Using SEM

3.5.8 Time-kill Study

The procedure was carried out according to CLSI (M26-A), with modifications based on the methods by Al-Ani et al. (2018) and Sy et al. (2021), as shown in Figure 3.9. From the working solution, propolis extracts were mixed with BHIB in microcentrifuge tubes until the MBC was reached, with a final volume of 900 µL (45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel were used as positive control). A volume of 100 µL of bacteria inoculum (standardised to $1x10^6$ CFU/mL) was then added to each tube and incubated at 37°C, resulting in a final concentration of $1x10^5$ CFU/mL. At predetermined time intervals of 0, 1, 2, 4, 6, and 24 hours, 100 µL aliquots were withdrawn from the tubes. Each aliquot was serially diluted ten-fold (10^{-1} to 10^{-6}) using

PBS by mixing 100µL of the aliquot with 900 µL PBS. A volume of 10 µL from each diluted sample was streaked onto a BHI agar plate, which was incubated at 37°C for 24 hours. Colony count (CFU/mL) was determined, with only plates containing 25 - 250 colonies considered valid. Plates with >250 colonies were deemed too numerous to count (TNTC), while plates with <25 colonies were considered not significant. A time-kill curve was plotted by graphing log10 CFU/mL (y-axis) against time (x-axis). The bacterial suspension platted was 100 µL, and was calculated using formula (3.9):

$$\text{Total CFU/mL} = \frac{\text{Number of colonies x Dilution Factor}}{\text{Aliquot of bacterial suspension plated}} \quad (3.9)$$

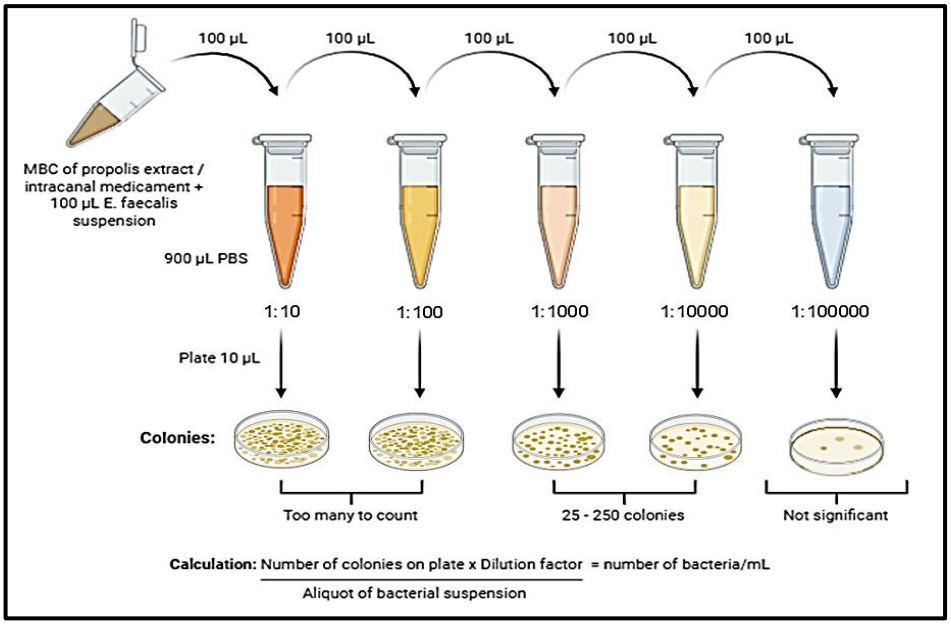


Figure 3.9 Procedure for Time-kill Study

3.6 Data Analysis

3.6.1 Descriptive Analysis

Descriptive analysis was used to summarise the characteristics of the data set, including distribution, central tendency, and variability. Normality testing for each dataset was performed using the Shapiro-Wilk test (for $n < 100$), Kolmogorov-Smirnov test (for $n > 100$), as well as skewness, and kurtosis analysis. The significance level was

set at $\alpha = 0.05$. A dataset was considered normally distributed (parametric) if the *p*-value was greater than 0.05. For skewness and kurtosis, values within ± 1.0 were considered indicative of normal distribution.

Normally distributed data were expressed as mean ± standard deviation (SD), while non-normally distributed data sets were presented as median with interquartile range (IQR).

3.6.2 Statistical Analysis

For normally distributed data, statistical comparisons were made using two-way and repeated-measure analysis of variance (ANOVA). For non-normally distributed data, the Kruskal-Wallis test was used. A *p*-value of < 0.05 was considered statistically significant. Post hoc tests were performed using Tukey's HSD (for normally distributed data) and Dunn's test (for non-normally distributed data) to evaluate multiple group comparisons. All statistical analyses were performed using IBM SPSS Statistical version 27.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents and interprets the findings from the *in vitro* study on the antimicrobial effects of various stingless bee propolis, 45 mg/mL NS-$Ca(OH)_2$ and 2% (equivalent to 20 mg/mL) CHX gel against *E. faecalis* ATCC 29212 and ATCC 4082. The results are analysed to determine the efficacy of propolis extracts in inhibiting the growth of both strains of *E. faecalis* and compare their effectiveness with current intracanal medicament, providing insight into the potential application of stingless bee propolis as a natural antimicrobial agent.

Key findings are discussed in the context of existing literature, with a focus on antimicrobial properties of propolis, exploring the specific bioactive compounds likely contributing to the observed effects, comparing the susceptibility patterns of each *E. faecalis* strain, and considering the broader implications of these results for developing alternative medication in endodontics. This section highlights the study's strengths and possible limitations while linking the experimental results to the research objectives.

4.2 Result of ICC

Intra-rater reliability was assessed to ensure consistency in the measurement of ZOI using the agar-well diffusion technique by the same evaluator. The Intraclass Correlation Coefficient (ICC) was calculated using IBM SPSS Statistical version 27 via a two-way mixed-effects model with absolute agreement and single-measure type, as it reflects the consistency of individual ratings made by the same rater. The ICC value obtained was 0.89 (95% CI: 0.62 – 0.97), indicating good reliability based on commonly accepted thresholds (ICC; 0.75 - 0.90) (Refer to Appendix 1).

4.3 Determination of Propolis Extract Yield and pH

The yield of propolis extract from different stingless bee species was compared to observe variation in composition and to ensure standardisation and reproducibility of the propolis preparation process. The ethanolic extract yield for *H. itama*, *G. thoracica* and *T. apicalis* propolis was 42.67%, 45.67% and 37.57% respectively. Additionally, the pH of the propolis extracts, 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel were evaluated to assess their acidity or alkalinity and their possible implications towards therapeutic properties. Table 4.1 shows the pH of each compound, with NS-$Ca(OH)_2$ showing the highest pH-alkalinity at 12.3, while the others were slightly acidic; *H. itama* and PEM (pH 6.6), *G. thoracica* and *T. apicalis* (pH 6.2), and CHX gel (pH 6.1).

Table 4.1
The pH Value and Extract Yield of All Samples

Sample	pH	Extract weight (Mean ± SD, g)	Extract yield (%)	*p*-value
H. itama propolis extract	6.6	4.26 ± 0.28	42.67	
G. thoracica propolis extract	6.2	4.56 ± 0.44	45.67	0.061
T. apicalis propolis extract	6.2	3.75 ± 0.22	37.57	
PEM	6.6			
45 mg/mL NS-$Ca(OH)_2$	12.3			
2% CHX gel	6.1			

Note: Extract weight results are shown as the mean of three biological replicates (SD = standard deviations). One-way ANOVA was performed on the various propolis extract weight with significance at $p < 0.05$

A one-way ANOVA was performed to compare the effect of different stingless bee species on the yield of propolis extract. As shown in Table 4.1, no significant difference ($p = 0.061$) was observed between the average extract yields of the three propolis species.

4.4 Result of AST

4.4.1 Agar-well Diffusion Method – Primary Antimicrobial Screening Test

In the AST, agar-well diffusion method was used to primarily screen the antibacterial effect of *H. itama, G. thoracica*, and *T. apicalis* propolis extract with 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel as positive controls and 5% DMSO as a negative control, in relation to their efficacy against *E. faecalis* strains (Plate 4.1). This method provides an initial evaluation of antimicrobial action but is not included in the overall antimicrobial properties analysis as it produces qualitative results by indicating the presence or absence of antimicrobial activity via inhibition zone formation. These zones serve only as a relative measure of antimicrobial activity, not as precise quantitative values.

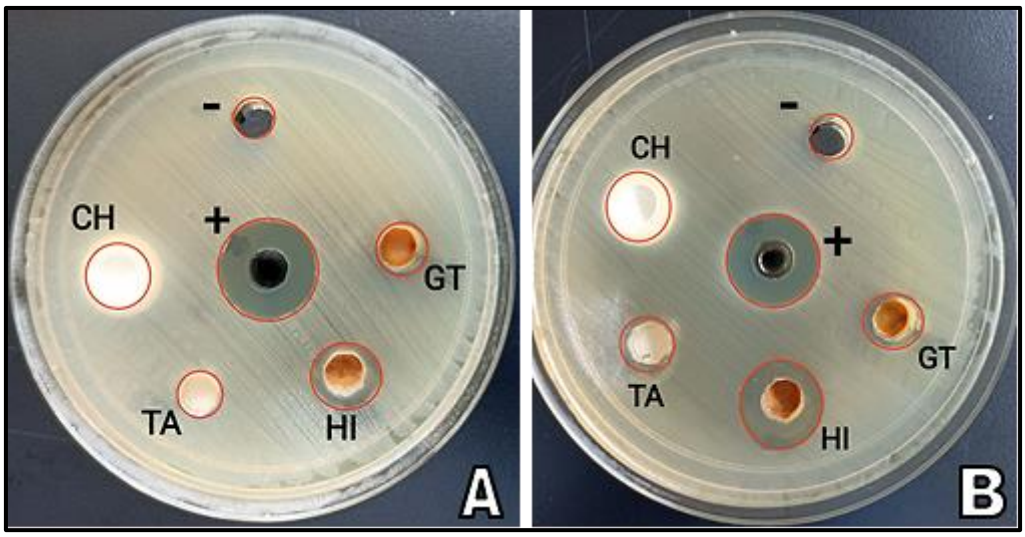


Plate 4.1 The Zone of Inhibition (ZOI) of (HI) 50 mg/mL *H. itama* Extract, (GT) 50 mg/mL *G. thoracica* Extract, (TA) 50 mg/mL *T. apicalis* Extract, (CH) 45 mg/mL NS-$Ca(OH)_2$, (+) 2% CHX Gel, and (-) 5% DMSO in Agar-well Diffusion Method for (A) *E. faecalis* ATCC 29212 and (B) *E. faecalis* ATCC 4082

Based on the agar-well diffusion results in Table 4.2, 2% (equivalent to 20 mg/mL) CHX gel shows the largest mean ZOI for both *E. faecalis* strains (*E. faecalis* ATCC 29212 was 15.7 ± 0.6 mm and *E. faecalis* ATCC 4082 was 17.7 ± 0.6 mm) compared to all tested samples. *H. itama* propolis extract yielded the largest mean ZOI

among the propolis extracts (*E. faecalis* ATCC 29212 was 11.7 ± 1.2 mm and *E. faecalis* ATCC 4082 was 12.7 ± 2.1 mm) and was even larger than that of NS-$Ca(OH)_2$ (*E. faecalis* ATCC 29212 was 11.0 ± 1.0 mm and *E. faecalis* ATCC 4082 was 12.0 ± 1.7 mm). Meanwhile, *G. thoracica* propolis extract produced slightly larger ZOI (*E. faecalis* ATCC 29212 was 7.3 ± 1.5 mm and *E. faecalis* ATCC 4082 was 8.3 ± 1.2 mm) compared to *T. apicalis* propolis extract (*E. faecalis* ATCC 29212 was 6.7 ± 0.6 mm and *E. faecalis* ATCC 4082 was 6.7 ± 0.6 mm).

All agar-well diffusion measurements (n = 36) were assessed for normality using skewness and kurtosis statistics to evaluate the distribution of the data. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test to compare mean ZOI among the tested compounds. The analysis (Table 4.2) revealed a statistically significant difference in ZOI between at least two groups ($p < 0.001$).

Table 4.2
Zone of Inhibition for Agar-well Diffusion Method

Sample	**Zone of Inhibition (ZOI) (Mean ± SD, mm)**		***p*-value**
	***E. faecalis* ATCC 29212**	***E. faecalis* ATCC 4082**	
50 mg/mL *H. itama* propolis	11.7 ± 1.2	12.7 ± 2.1	< 0.001
50 mg/mL *G. thoracica* propolis	7.3 ± 1.5	8.3 ± 1.2	
50 mg/mL *T. apicalis* propolis	6.7 ± 0.6	6.7 ± 0.6	
45 mg/mL NS-$Ca(OH)_2$	11.0 ± 1.0	12.0 ± 1.7	
2% CHX gel	15.7 ± 0.6	17.7 ± 0.6	
5% DMSO	6.0 ± 0.0	6.0 ± 0.0	

Note: ZOI were measured in centimetres (cm) and converted to millimetres (mm). The results were shown in mean ± standard deviation (SD) from three biological replicates, with one-way ANOVA performed on the ZOI and the significant level at $p < 0.05$

Based on Table 4.3, Tukey's HSD Test for multiple comparisons revealed that the mean ZOI was significantly different from that of 2% CHX gel and all other tested compounds ($p < 0.001$), highlighting its outstanding antimicrobial properties. Amongst the propolis extract, the mean ZOI of *H. itama* was significantly different from both *G.*

thoracica ($p < 0.001$) and *T. apicalis* ($p < 0.001$). However, no statistically significant difference was found between *H. itama* and 45 mg/mL NS-$Ca(OH)_2$ ($p = 0.914$). On the other hand, 45 mg/mL NS-$Ca(OH)_2$ exhibits significantly larger ZOI compared to *G. thoracica* ($p < 0.001$) and *T. apicalis* ($p < 0.001$).

Table 4.3
Multiple Comparisons Between ZOI of Samples Using Tukey Post-hoc Test

Sample_1	Sample_2	Mean Difference	*p*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
H. itama	*G. thoracica*	4.333*	< 0.001	2.3098	6.3568
	T. apicalis	5.500*	< 0.001	3.4765	7.5235
	45 mg/mL NS-$Ca(OH)_2$	0.667	0.914	- 1.3568	2.6902
	2% CHX gel	- 4.500*	< 0.001	- 6.5235	- 2.4765
	5% DMSO	6.167*	< 0.001	4.1432	8.1902
G. thoracica	*T. apicalis*	1.167	0.509	- 0.8568	3.1902
	45 mg/mL NS-$Ca(OH)_2$	- 3.667*	< 0.001	- 5.6902	- 1.6432
	2% CHX gel	- 8.833*	< 0.001	- 10.8568	- 6.8098
	5% DMSO	1.833	0.093	- 0.1902	3.8568
T. apicalis	45 mg/mL NS-$Ca(OH)_2$	- 4.833*	< 0.001	- 6.8568	- 2.8098
	2% CHX gel	- 10.000*	< 0.001	- 12.0235	- 7.9765
	5% DMSO	0.667	0.914	- 1.3568	2.6902
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	- 5.167*	< 0.001	- 7.1902	- 3.1432
	5% DMSO	5.500*	< 0.001	3.4765	7.5235
2% CHX gel	5% DMSO	10.667*	< 0.001	8.6432	12.6902

Note: The mean difference is significant at $p < 0.05$

4.4.2 Result of MIC

Table 4.4 shows the MIC for each propolis extract, and PEM against two strains of *E. faecalis*. A lower MIC value reflects a stronger inhibitory effect on bacterial growth. When determining the MIC using a spectrophotometer, a high absorbance reading indicates high bacterial growth and therefore, weak antibacterial activity. Normality testing through skewness and kurtosis values revealed that the MIC dataset (n = 108) was not normally distributed; thus, non-parametric statistical tests were used. A Kruskal-Wallis test was performed to compare MIC differences among compounds. Further statistical evaluation using Dunn's pairwise comparison test (Table 4.5) was conducted to observe significant differences between samples. Overall, all propolis extracts possessed lower MIC value for both strains compared to 45 mg/mL NS-$Ca(OH)_2$, while *H. itama* and PEM (for ATCC 29212) recorded lower MIC value than 2% CHX gel.

Based on the result, *H. itama* recorded the lowest MIC values (ATCC 29212: 1.56 (IQR: 1.56) mg/mL; ATCC 4082: 0.78 (IQR: 0.78) mg/mL) and this value is lower compared to both 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel, indicating its potent antibacterial activity. This was followed by PEM (ATCC 29212: 3.13 (IQR: 1.96) mg/mL; ATCC 4082: 0.78 (IQR: 0.78) mg/mL) and *G. thoracica* propolis extract, with MIC values of 25.00 (IQR: 15.63) mg/mL for ATCC 29212 and 12.50 (IQR: 6.25) mg/mL for ATCC 4082. In contrast, *T. apicalis* propolis extract recorded the highest MIC values for both bacterial strains (*E. faecalis* ATCC 29212 and ATCC 4082: 25.00 (IQR: 12.50) mg/mL), indicating the weakest antibacterial activity. Both well with positive control (2% CHX gel and 45 mg/mL $Ca(OH)_2$) remained clear.

Table 4.4
MIC for Propolis Extracts, NS-$Ca(OH)_2$, and CHX Against Two *E. faecalis* Strains

Sample	**MIC [Median (IQR)] (mg/mL)**		***p*-value**
	***E. faecalis* ATCC 29212**	***E. faecalis* ATCC 4082**	

H. itama propolis	1.56 (1.56)	0.78 (0.78)	< 0.001
G. thoracica propolis	25.00 (15.63)	12.50 (6.25)	
T. apicalis propolis	25.00 (12.50)	25.00 (12.50)	
PEM	3.13 (1.96)	0.78 (0.78)	
45 mg/mL NS-$Ca(OH)_2$ (positive control)	45.00 (0.00)	45.00 (0.00)	
2% CHX gel (positive control)	2.00 (0.00)	2.00 (0.00)	

Note: The results are shown as median (interquartile range, IQR) of three biological and technical replicates, with a Kruskal-Wallis test performed to compare the effect of different tested compounds on the MIC and the significance level at $p < 0.05$

The statistical analysis (Table 4.4) revealed a significant difference in MIC values among the compounds ($p < 0.001$). Dunn's pairwise comparison (Table 4.5) further indicated the potent antimicrobial activity of *H. itama,* as it had significantly lower MIC values than *G. thoracica* ($p < 0.001$), *T. apicalis* ($p < 0.001$), and 45 mg/mL NS-$Ca(OH)_2$ ($p < 0.001$), but was not significantly different from PEM ($p = 1.000$) and 2% CHX gel ($p = 0.746$). The PEM's MIC values were significantly lower MIC than those of *G. thoracica* ($p < 0.001$), *T. apicalis* ($p < 0.001$), and 45 mg/mL NS-$Ca(OH)_2$ ($p < 0.001$). Additionally, 2% CHX gel had significantly lower MIC values than *T. apicalis* ($p = 0.002$), and 45 mg/mL NS-$Ca(OH)_2$ ($p < 0.001$), but was not having significantly higher MIC from *H. itama*, and PEM ($p = 1.000$). However, no significant differences were observed between *G. thoracica* than those of *T. apicalis* ($p = 1.000$), 45 mg/mL NS-$Ca(OH)_2$ ($p = 0.061$), and 2% CHX gel ($p = 0.092$).

Table 4.5
Pairwise Comparisons Between Samples Using Dunn's Post-hoc Test for MIC Test

Sample 1	Sample 2	*p*-value	Adjusted *p*-value
H. itama	*G. thoracica*	< 0.001	< 0.001*
	T. apicalis	< 0.001	< 0.001*
	PEM	0.659	1.000

	45 mg/mL NS-$Ca(OH)_2$	< 0.001	< 0.001*
	2% CHX gel	0.050	0.746
G. thoracica	*T. apicalis*	0.273	1.000
	PEM	< 0.001	< 0.001
	45 mg/mL NS-$Ca(OH)_2$	0.004	0.061
	2% CHX gel	0.006	0.092
T. apicalis	PEM	< 0.001	0.001*
	45 mg/mL NS-$Ca(OH)_2$	0.076	1.000
	2% CHX gel	< 0.001	0.002*
PEM	45 mg/mL NS-$Ca(OH)_2$	< 0.001	< 0.001*
	2% CHX gel	0.128	1.000
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	< 0.001	< 0.001*

Note: Pairwise comparisons were conducted using Dunn's test with Bonferroni adjustment for multiple comparisons. $p < 0.05$ were considered statistically significant (*).

4.4.3 Outcome of Propolis Total Fractional Inhibitory Concentration (ΣFIC)

Table 4.6 presents the FIC index for three different Malaysian stingless bee propolis extracts (*H. itama*, *G. thoracica*, and *T. apicalis*) against two strains of *E. faecalis* (ATCC 29212 and ATCC 4082). The FIC index for each propolis extract was determined individually, and the ΣFIC value was calculated to assess the interaction type (e.g., synergistic, additive, indifferent, or antagonistic).

For *E. faecalis* ATCC 29212, the FIC values for *T. apicalis* and *G. thoracica* propolis extract were 0.125, indicating relatively weak antibacterial activity. In contrast, *H. itama* propolis extract exhibited a substantially higher FIC value of 2.006, indicating

a stronger effect. The calculated ΣFIC was 0.752, which falls within the additive interaction range ($0.5 < \Sigma FIC \leq 1.0$), suggesting that the combination of these extracts provides a mild enhancement in antimicrobial efficacy.

For *E. faecalis* ATCC 4082, all three propolis extracts exhibited moderate antibacterial effects, with *T. apicalis* (FIC = 0.031) and *G. thoracica* (FIC = 0.062) showing particularly low FIC values. *H. itama* demonstrated potent antibacterial activity (FIC = 1.000). The resulting ΣFIC value was 0.364, placing it within the synergistic interaction range ($\Sigma FIC \leq 0.5$), which indicates a significant enhancement in antibacterial activity when these propolis extracts were combined.

Overall, these findings suggest that Malaysian stingless bee propolis extracts can exert either synergistic or additive effects against *E. faecalis*, with more pronounced synergy observed against *E. faecalis* ATCC 4082. These results support the potential of propolis extracts as a natural alternative or adjunct in endodontic antimicrobial treatment.

Table 4.6
FIC Value of Each Propolis Extract and Determination of ΣFIC and Its Interaction

E. faecalis Strain	Fractional Inhibitory Concentration (FIC) Index				Interaction
	FIC1 (*H. itama*)	FIC2 (*G. thoracica*)	FIC3 (*T. apicalis*)	ΣFIC = 1/3 (FIC1 + FIC2 + FIC3)	
ATCC 29212	2.006	0.125	0.125	0.752	Additive
ATCC 4082	1.000	0.062	0.031	0.364	Synergistic

Note: The ΣFIC index was interpreted as synergistic interactions (if < 0.5), additive (if in the range of > 0.5–1), indifferent (if in the range > 1–4), or as an antagonist (if > 4).

4.4.4 Result of MBC

Samples previously tested for MIC were subsequently subjected to MBC determination. Similar to MIC, MBC provides insight into the antimicrobial efficacy of a compound, where a lower MBC value indicates stronger bactericidal activity. *H. itama* demonstrated the lowest MBC among the propolis samples, with MBC of 6.25 mg/mL (IQR: 7.81) for *E. faecalis* ATCC 29212 and 6.25 mg/mL (IQR: 3.13) for ATCC 4082.

However, the MBC value is four-fold higher than MIC. In contrast, PEM recorded a MBC of 12.50 (IQR: 18.75) mg/mL for ATCC 29212 and 12.50 (IQR: 12.50) mg/mL for ATCC 4082, which were lower compared to *G. thoracica* and *T. apicalis* propolis extract, both of which recorded the highest MBC value for both strains (50.00 (IQR: 0) mg/mL). The positive controls (45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel) showed no observable colony growth.

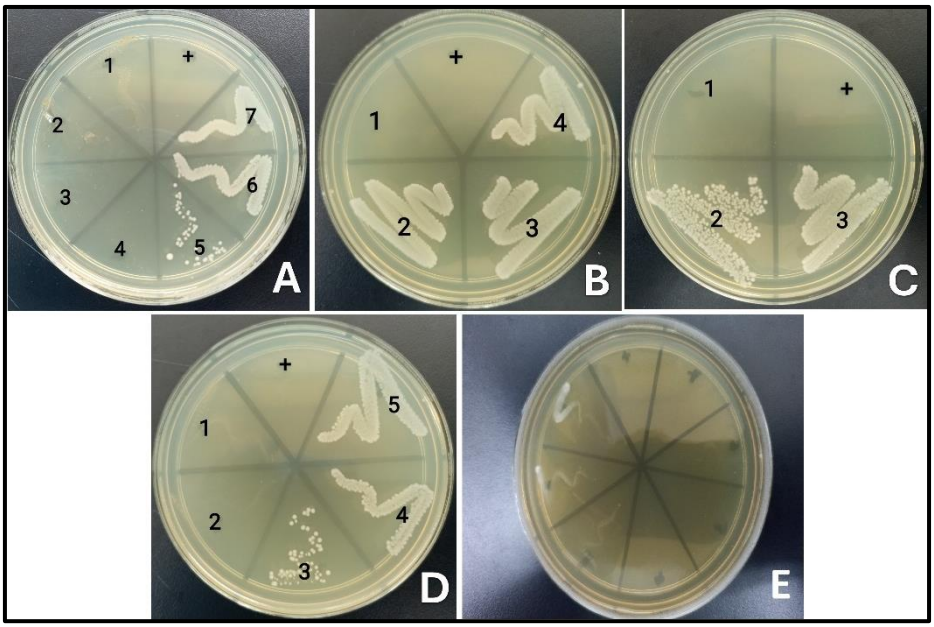


Plate 4.2 The MBC Results of (A) 50 mg/mL *H. itama* Extract, (B) 50 mg/mL *G. thoracica* Extract, (C) 50 mg/mL *T. apicalis* Extract, (D), and 50 mg/mL PEM, With (E) 45 mg/mL NS-$Ca(OH)_2$ and (+) 2% CHX Gel as Positive Controls for *E. faecalis* ATCC 29212

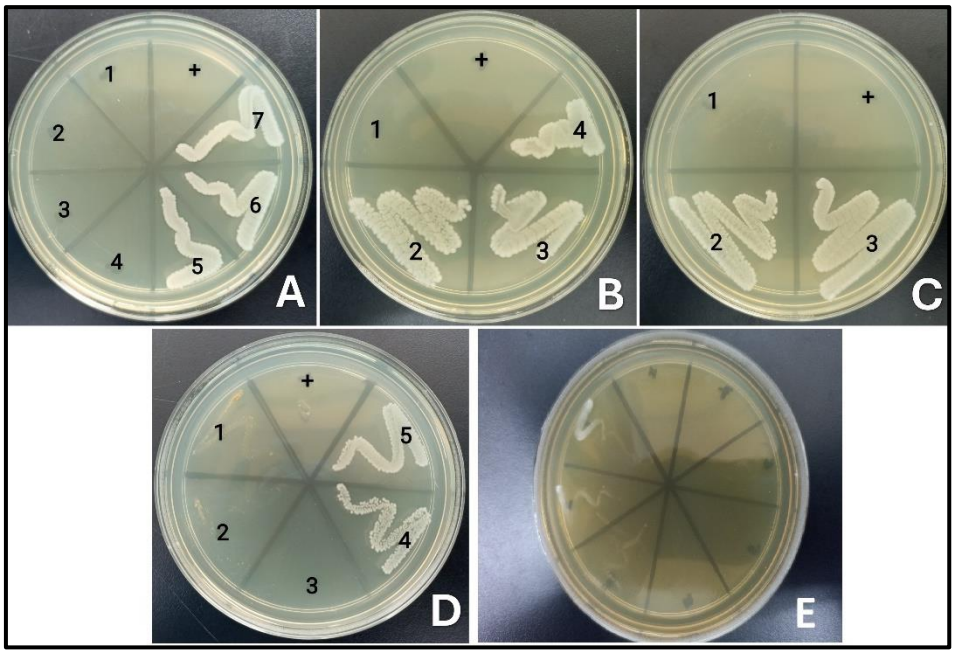


Plate 4.3 The MBC Results of (A) 50 mg/mL *H. itama* Extract, (B) 50 mg/mL *G. thoracica* Extract, (C) 50 mg/mL *T. apicalis* Extract, and (D) 50 mg/mL PEM, with (E) 45 mg/mL NS-$Ca(OH)_2$ and (+)2% CHX Gel as Positive Controls for *E. faecalis* ATCC 4082

Table 4.7
Results of MBC

Sample	MBC (mg/mL) [Median (IQR)]		
	***E. faecalis* ATCC 29212**	***E. faecalis* ATCC 4082**	***p*-value**
H. itama propolis	6.25 (7.81) MIC: 1.56	6.25 (3.13) MIC: 0.78	< 0.001
G. thoracica propolis	50.00 (0.00) MIC: 25.00	50.00 (0.00) MIC: 12.50	
T. apicalis propolis	50.00 (0.00) MIC: 25.00	50.00 (0.00) MIC: 25.00	
PEM	12.50 (18.75) MIC: 3.13	12.50 (12.50) MIC: 0.78	
45 mg/mL NS-$Ca(OH)_2$	45.00 (0.00) MIC: 45.00	45.00 (0.00) MIC: 45.00	

2% (20 mg/mL) CHX gel	20.00 (0.00) MIC: 20.00	20.00 (0.00) MIC: 45.00

Note: The results are shown as median (interquartile range, IQR) of three biological replicates, with a Kruskal-Wallis test performed to compare the effect of different tested compounds on the MBC and the significance level at $p < 0.05$

Normality testing using skewness and kurtosis revealed that the MBC dataset (n = 108) were not normally distributed; therefore, a non-parametric test was used. The Kruskal-Wallis test showed a statistically significant difference in MBC values between the compounds ($p < 0.001$). Subsequent pairwise comparisons using Dunn's post hoc test (Table 4.8), indicated that the MBC values of 2% CHX gel were significantly different from those of *G. thoracica* ($p < 0.001$), *T. apicalis* ($p < 0.001$), PEM ($p = 0.032$), and 45 mg/ mL NS-$Ca(OH)_2$ ($p < 0.001$), but not significantly different from *H. itama* ($p = 0.357$). Within the propolis group, *H. itama* had significantly lower MBC value compared to *G. thoracica*, *T. apicalis* ($p = 0.001$), and 45 mg/mL NS-$Ca(OH)_2$ ($p = 0.019$). However, no significant differences were observed between *H. itama* with PEM (p = 1.000). This highlights the potential of *H. itama* propolis as an alternative antimicrobial in endodontic applications, particularly against *E. faecalis*. Additionally, MBC value of PEM was significantly differed from those of *G. thoracica* ($p = 0.001$), and *T. apicalis* ($p = 0.001$), but not significantly differed from 45 mg/mL NS-$Ca(OH)_2$ ($p = 0.234$).

Table 4.8
Pairwise Comparisons Between Samples Using Dunn's Post-hoc Test for MBC Test

Sample 1	**Sample 2**	***p*-value**	**Adjusted *p*-value**
H. itama	*G. thoracica*	< 0.001	< 0.001*
	T. apicalis	< 0.001	< 0.001*
	PEM	0.417	1.000
	45 mg/mL NS-$Ca(OH)_2$	0.001	0.019*
	2% CHX gel	0.024	0.357

G. thoracica	*T. apicalis*	1.000	1.000
	PEM	< 0.001	< 0.001*
	45 mg/mL NS-$Ca(OH)_2$	0.020	0.294
	2% CHX gel	< 0.001	< 0.001*
T. apicalis	PEM	< 0.001	< 0.001*
	45 mg/mL NS-$Ca(OH)_2$	0.020	0.294
	2% CHX gel	< 0.001	< 0.001*
PEM	45 mg/mL NS-$Ca(OH)_2$	0.016	0.234
	2% CHX gel	0.002	0.032*
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	< 0.001	< 0.001*

Note: Pairwise comparison using the Dunn test was performed to further explore the differences between each group, with the difference being significant (*) at $p < 0.05$

4.4.5 Result of Antibiofilm Properties

Eradicating biofilm formed by *E. faecalis* is a critical determinant for the effectiveness of an intracanal medicament, as biofilms are highly resistant to antimicrobial agents and enhance bacterial survival. Hence, the antibiofilm activities of *H. itama*, *G. thoracica*, *T. apicalis*, PEM, as well as intracanal medicaments (45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel) as positive control, were evaluated by assessing their ability to disrupt 24-hour preformed biofilm. Biofilm biomass was quantified by measuring absorbance at OD_{600} nm, where lower optical density indicates reduced biofilm biomass and greater antibiofilm activity. Then, the antibiofilm effect of each sample was calculated.

Figure 4.1 illustrates the biofilm biomass of *E. faecalis* after treatment with the respective compounds. The 2% CHX gel-treated well showed the strongest antibiofilm effect, showing the lowest biofilm biomass for both *E. faecalis* ATCC 29212 (0.1863 ±

0.0418) and ATCC 4082 (0.2060 ± 0.0280). In contrast, 45 mg/mL NS-$Ca(OH)_2$ showed slightly higher biofilm biomass (*E. faecalis* ATCC 29212: 0.5757 ± 0.0523; ATCC 4082: 0.5123 ± 0.0661). Among the tested propolis extracts, *H. itama*-treated well exhibited the greatest antibiofilm activity (*E. faecalis* ATCC 29212: 0.9394 ± 0.0874; ATCC 4082: 0.7146 ± 0.0716), followed by PEM (*E. faecalis* ATCC 29212: 1.0871 ± 0.1089; ATCC 4082: 1.0223 ± 0.0916). Conversely, *T. apicalis* propolis extract displayed the weakest antibiofilm activity (*E. faecalis* ATCC 29212: 2.3176 ± 0.2423; ATCC 4082: 2.1206 ± 0.1397).

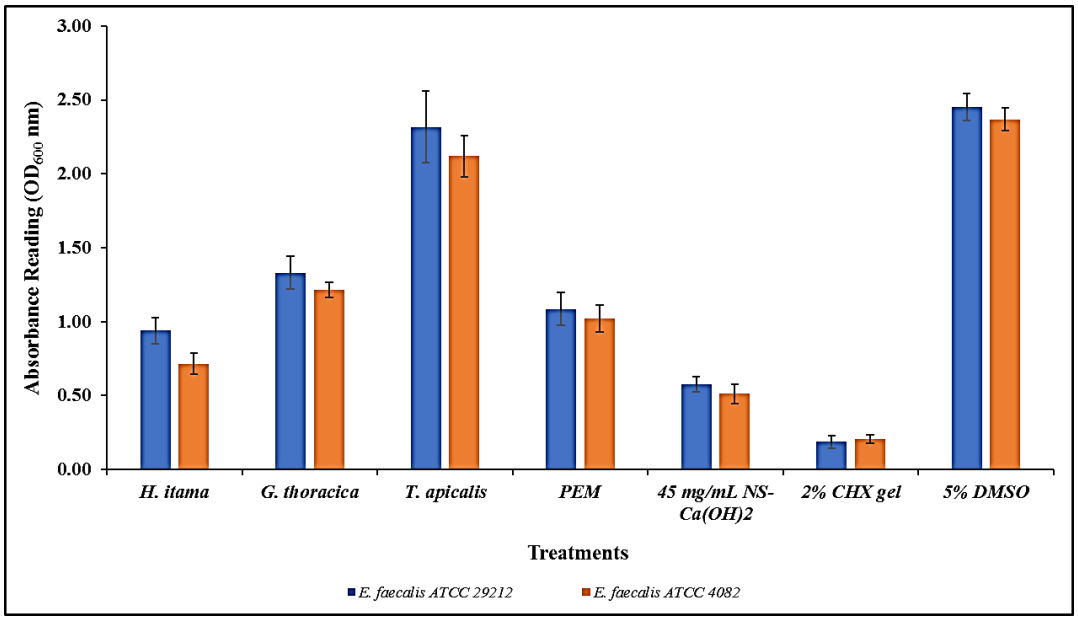


Figure 4.1 Quantification of Biofilm Biomass Using The Crystal Violet Assay Based on Absorbance Readings at OD_{600} Following Treatment with Test Samples

Table 4.9 shows the antibiofilm activity of each sample against both strains. Results showed that 2% CHX gel possessed the strongest antibiofilm effect against both *E. faecalis* ATCC 29212 (92.40 ± 1.70%) and ATCC 4082 (91.31 ± 1.18%). In contrast, 45 mg/mL NS-$Ca(OH)_2$ showed moderate antibiofilm activity (*E. faecalis* ATCC 29212: 76.53 ± 2.13%; ATCC 4082: 78.33 ± 2.79%). Among the tested propolis extracts, *H. itama* exhibited the strongest antibiofilm effect (*E. faecalis* ATCC 29212: 61.71 ± 3.56%; ATCC 4082: 69.84 ± 3.02%), followed by PEM (*E. faecalis* ATCC 29212: 55.69 ± 4.44%; ATCC 4082: 56.86 ± 3.87%). Conversely, *T. apicalis* propolis

extract displayed the weakest antibiofilm effect (*E. faecalis* ATCC 29212: 5.53 ± 9.88%; ATCC 4082: 10.51 ± 5.89%).

Table 4.9
The Percentage of Antibiofilm Effect for Each Sample Against Both Bacterial Strains

Sample	**Antibiofilm activity (mean ± SD) (%)**	
	***E. faecalis* ATCC 29212**	***E. faecalis* ATCC 4082**
H. itama propolis	61.71 ± 3.56	69.84 ± 3.02
G. thoracica propolis	45.74 ± 4.54	48.73 ± 2.20
T. apicalis propolis	5.53 ± 9.88	10.51 ± 5.89
PEM	55.69 ± 4.44	56.86 ± 3.87
45 mg/mL NS-$Ca(OH)_2$	76.53 ± 2.13	78.33 ± 2.79
2% CHX gel	92.40 ± 1.70	91.31 ± 1.18

Note: The results are shown as average values of three biological and technical replicates (n = 84), and are expressed as mean ± standard deviation (SD).

Normality testing through skewness and kurtosis revealed that the absorbance data at OD_{600} nm for antibiofilm properties (n = 84) were normally distributed. A two-way ANOVA was performed to assess the effects of different tested compounds and *E. faecalis* strains for antibiofilm activity through absorbance readings (OD_{600} nm). The analysis, as shown in Table 4.10 revealed a statistically significant interaction between compound type and *E. faecalis* strains ($p = 0.041$). Simple main effects analysis showed that both compound type ($p < 0.001$) and strain ($p < 0.001$) were statistically significant, demonstrating that both variables independently influenced biofilm disruption.

Table 4.10
Two-way ANOVA Tested on Biofilm Results In Antibiofilm Assay

Source	**Df**	**Mean Square**	**F**	***p*-value**
Sample	5	15121.94	786.31	< 0.001*
Bacterial Strain	1	242.31	12.60	< 0.001*

Sample * Bacterial Strain	5	46.64	2.42	0.041*

Note: The two-way ANOVA on antibiofilm results to observe the effect of sample and bacterial strain, as well as the interaction between these two groups on the anti-adherence effects, where the significant level (*) is at $p < 0.05$

Based on Table 4.11, Tukey's HSD multiple comparison test found that the mean absorbance for biofilm biomass after treatment was significantly different between 2% CHX gel and all other tested compounds ($p < 0.001$), highlighting its superior ability to disrupt *E. faecalis* biofilm. The outcome of 45 mg/mL NS-$Ca(OH)_2$ also exhibited a notable antibiofilm effect, with results significantly different from all tested compounds ($p < 0.001$), though slightly less effective than 2% CHX gel. Among the propolis extracts, *H. itama* differed significantly from *G. thoracica*, *T. apicalis* and PEM ($p < 0.001$), confirming its comparatively higher biofilm disruption capacity.

Table 4.11
Multiple Comparisons Between Samples Using Tukey's HSD Post-hoc Test for Antibiofilm Properties

Sample_1	**Sample_2**	**Mean Difference**	***p*-value**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
H. itama	*G. thoracica*	18.543*	< 0.001	14.292	22.794
	T. apicalis	57.754*	< 0.001	53.503	62.005
	PEM	9.502*	< 0.001	5.251	13.753
	45 mg/mL NS-$Ca(OH)_2$	- 11.656*	< 0.001	- 15.907	- 7.405
	2% CHX gel	- 26.078*	< 0.001	- 30.329	- 21.827
G. thoracica	*T. apicalis*	39.211*	< 0.001	34.960	43.462
	PEM	- 9.042*	< 0.001	- 13.293	- 4.791
	45mg/mL NS-$Ca(OH)_2$	- 30.199*	< 0.001	- 34.450	- 25.948
	2% CHX gel	- 44.622*	< 0.001	- 48.873	- 40.371

T. apicalis	PEM	- 48.252*	< 0.001	- 52.503	- 44.001
	45 mg/mL NS-$Ca(OH)_2$	- 69.409*	< 0.001	- 73.660	- 65.158
	2% CHX gel	- 83.832*	< 0.001	- 88.083	- 79.581
PEM	45 mg/mL NS-$Ca(OH)_2$	- 21.157*	< 0.001	- 25.408	- 16.906
	2% CHX gel	- 35.580*	< 0.001	- 39.831	- 31.329
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	- 14.423*	< 0.001	- 18.674	- 10.172

Note: Pairwise comparison using Tukey's HSD post hoc test was performed to further explore the differences between each sample, with the difference is significant (*) at $p < 0.05$

4.4.6 Result of Anti-adherence Properties

In contrast to the antibiofilm test, the anti-adherence assay evaluated the ability of test samples to inhibit the initial attachment of *E. faecalis* to surfaces, which is the critical first step in biofilm formation. Anti-adherence properties were quantified by measuring biofilm biomass retained on the surface following treatment with test compounds. Absorbance reading (OD_{600}) was used as an indirect measure of adhered biomass, where higher OD_{600} values indicate greater bacterial attachment and lower anti-adherence activity.

Figure 4.2 shows the adhered biofilm biomass of *E. faecalis* on surfaces pre-treated with test compounds, expressed as OD_{600} values. Results revealed that 2% CHX gel has the strongest anti-adherence effect against both *E. faecalis* ATCC 29212 (0.3148 ± 0.0142) and *E. faecalis* ATCC 4082 (0.2436 ± 0.0171), outperforming all other samples. Among the propolis extracts, *H. itama* shows the highest anti-adherence activity against both strains (0.6858 ± 0.0894 for ATCC 29212 and 0.5757 ± 0.0498 for ATCC 4082). These values were significantly lower than those for PEM (*E. faecalis* ATCC 29212 was 1.5892 ± 0.2985 and *E. faecalis* ATCC 4082 was 1.2975 ± 0.2129), *G. thoracica* propolis extract (*E. faecalis* ATCC 29212 was 1.4173 ± 0.2322 and *E. faecalis* ATCC 4082 was 1.6224 ± 0.0907), and *T. apicalis* propolis extract (*E. faecalis* ATCC 29212 was 2.6684 ± 0.2296 and *E. faecalis* ATCC 4082 was 2.8832 ± 0.1723).

Interestingly, the anti-adherence activity of *H. itama, G. thoracica,* and PEM was also greater than that of 45 mg/mL NS-$Ca(OH)_2$ (*E. faecalis* ATCC 29212 was 1.7730 ± 0.1534 and *E. faecalis* ATCC 4082 was 1.7237 ± 0.1426).

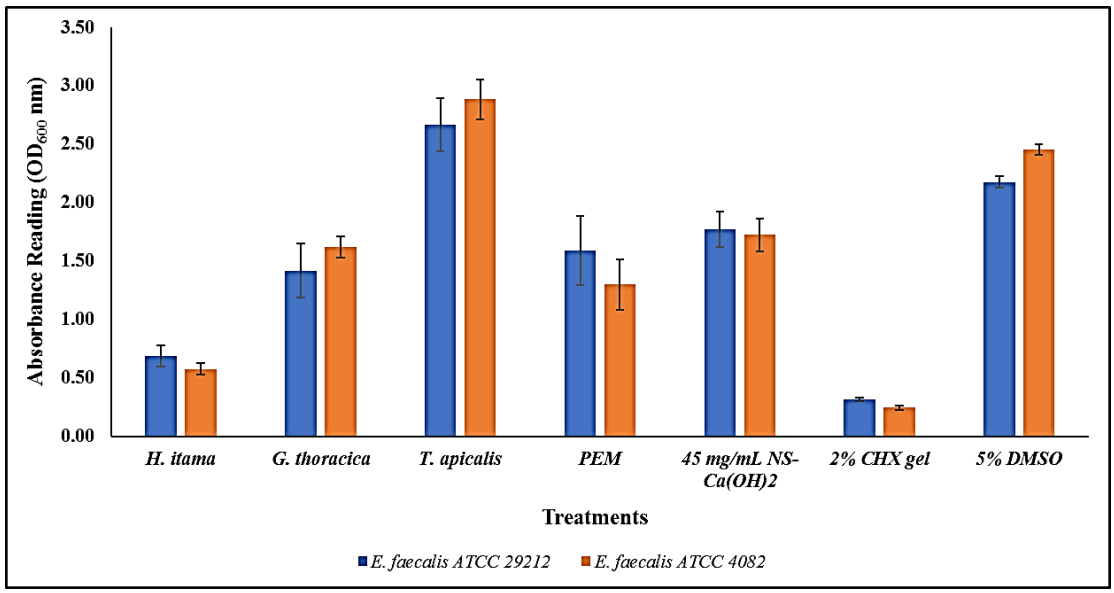


Figure 4.2 Adhered Biofilm Biomass of *E. faecalis* on Surfaces Pre-treated With Test Compounds, Expressed as OD_{600} Values

Table 4.12 shows the anti-adherence activity of each samples against both strains. Results revealed that 2% CHX gel has the strongest anti-adherence effect against both *E. faecalis* ATCC 29212 (85.54 ± 0.65%) and *E. faecalis* ATCC 4082 (90.07 ± 0.70%), outperforming all other samples. Among the propolis extracts, *H. itama* shows the highest anti-adherence activity against both strains (68.50 ± 4.11% for ATCC 29212 and 76.54 ± 2.03% for ATCC 4082). These values were significantly higher than those for PEM (*E. faecalis* ATCC 29212 was 27.01 ± 13.71% and *E. faecalis* ATCC 4082 was 47.12 ± 8.68%), *G. thoracica* propolis extract (*E. faecalis* ATCC 29212 was 34.91 ± 10.66% and *E. faecalis* ATCC 4082 was 33.87 ± 3.70%), and *T. apicalis* propolis extract (*E. faecalis* ATCC 29212 was -22.55 ± 10.55% and *E. faecalis* ATCC 4082 was -17.51 ± 7.02%). Interestingly, the anti-adherence activity of *H. itama, G. thoracica,* and PEM was also greater than that of 45 mg/mL NS-$Ca(OH)_2$ (*E. faecalis* ATCC 29212 was 18.57 ± 7.05% and *E. faecalis* ATCC 4082 was 29.75 ± 5.81%).

Table 4.12
The Percentage of Anti-adherence Effect for Each Sample Against Both Bacterial Strains

Sample	**Anti-adherence activity (mean ± SD) (%)**	
	***E. faecalis* ATCC 29212**	***E. faecalis* ATCC 4082**
H. itama propolis	68.50 ± 4.11	76.54 ± 2.03
G. thoracica propolis	34.91 ± 10.66	33.87 ± 3.70
T. apicalis propolis	-22.55 ± 10.55	-17.51 ± 7.02
PEM	27.01 ± 13.71	47.12 ± 8.68
45 mg/mL NS-$Ca(OH)_2$	18.57 ± 7.05	29.75 ± 5.81
2% CHX gel	85.54 ± 0.65	90.07 ± 0.70

Note: The results are shown as average values of triplicate (n = 84), and are expressed as mean ± standard deviation (SD).

Normality testing using skewness and kurtosis confirmed that the OD_{600} nm absorbance data for anti-adherence properties (n = 84) were normally distributed. A two-way ANOVA was used to examine the effects of different compounds and *E. faecalis* strains on the anti-adherence activity. The analysis, shown in Table 4.13, indicated a statistically significant interaction between test compounds and bacterial strains ($p = 0.002$). Simple main effects analysis revealed that tested compounds type significantly affected absorbance reading ($p < 0.001$), as well as strain type ($p < 0.001$).

Table 4.13
Two-way ANOVA of Biofilm Formation Based on Anti-adherence Assay

Source	**Df**	**Mean Square**	**F**	***p*-value**
Sample	5	26046.632	476.183	< 0.001*
Bacterial Strain	1	1716.579	31.382	< 0.001*
Sample and Bacterial Strain	5	233.137	4.262	0.002

Note: The two-way ANOVA on anti-adherence results to observe the effect of sample and bacterial strain, as well as the interaction between these two groups on the effects of anti-adherence, where the significant level (*) is at $p < 0.05$

Based on Table 4.14, Tukey's HSD test for multiple comparisons found that 2% CHX gel was significantly different from all other compounds ($p < 0.001$) in reducing the number of bacteria adhering to the surface. This observation is further supported by the mean absorbance readings for *H. itama*, which were significantly higher than those recorded for *G. thoracica*, *T. apicalis*, PEM, and NS-$Ca(OH)_2$, indicating a statistically distinct response ($p < 0.001$). The absorbance reading of 45 mg/mL NS-$Ca(OH)_2$ was also significantly different from *G. thoracica*, *T. apicalis*, and PEM ($p < 0.001$). However, no significant difference was observed between *G. thoracica* and PEM ($p = 0.886$).

Table 4.14
Multiple Comparisons Between Samples Using Tukey Post-hoc Test for Antibiofilm Properties

Sample_1	Sample_2	Mean Difference	*p*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
H. itama	*G. thoracica*	38.129*	< 0.001	30.960	45.298
	T. apicalis	92.551*	< 0.001	85.382	99.720
	PEM	35.452*	< 0.001	28.283	42.621
	45 mg/mL NS-$Ca(OH)_2$	48.361*	< 0.001	41.191	55.530
	2% CHX gel	- 15.289*	< 0.001	- 22.458	- 8.120
G. thoracica	*T. apicalis*	54.422*	< 0.001	47.253	61.591
	PEM	- 2.677	0.886	- 9.846	4.493
	45 mg/mL NS-$Ca(OH)_2$	10.232*	< 0.001	3.062	17.401
	2% CHX gel	- 53.418*	< 0.001	-60.587	- 46.249
T. apicalis	PEM	- 57.099*	< 0.001	- 64.268	- 49.930
	45 mg/mL NS-$Ca(OH)_2$	- 44.191*	< 0.001	- 51.360	- 37.021

	2% CHX gel	- 101.840*	< 0.001	- 115.009	- 100.671
PEM	45 mg/mL NS-$Ca(OH)_2$	12.908*	< 0.001	5.739	20.078
	2% CHX gel	- 50.741*	< 0.001	- 57.910	- 43.572
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	- 63.649*	< 0.001	- 70.819	- 56.480

Note: Pairwise comparison using Tukey's HSD post hoc test was performed to further explore the differences between each sample, with the difference is significant (*) at $p < 0.05$

4.4.7 Observing the Effect of Various Treatments towards the Bacterial Morphology by SEM Analysis

The cell morphology of *E. faecalis* ATCC 29212 and ATCC 4082 after being treated with *H. itama* (6.25 mg/mL), *G. thoracica* (50.00 mg/mL), *T. apicalis* (50.00 mg/mL), and PEM (12.50 mg/mL), along with NS-$Ca(OH)_2$ (45.00 mg/mL), and CHX gel (2% or 20 mg/mL) as positive control and 5% DMSO as a negative control, was evaluated using SEM analysis with 15,000X magnification. In *E. faecalis* ATCC 29212, cells treated with 5% DMSO (Figure 4.3(G)) exhibited normal morphology, characterised by elongated oval shapes with smooth surfaces and an average diameter of 3–4 μm per cell. The cell wall structure remains intact, with some cells showing evidence of budding (highlighted in red). However, notable changes in morphology, size, and shape were observed when cells were treated with propolis extracts, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel.

After treatment with *H. itama* extracts, the cell surface appeared ruptured and rough, with a higher number of lysed cells and reduced bacterial populations. No budding stage or intact cell walls were observed (Figure 4.3 (A)). Similar disruption was observed with *G. thoracica* (Figure 4.3 (B)) and *T. apicalis* (Figure 4.3 (C)), although some budding was still seen with *G. thoracica*, and intact bacterial cells remained in *T. apicalis*-treated cultures.

2% CHX gel showed the strongest antimicrobial effect, as no bacterial cells were observed under SEM (Figure 4.3 (F)). 45 mg/mL NS-$Ca(OH)_2$ also caused cell damage (Figure 4.3 (E)), but a small number of intact cells were still present.

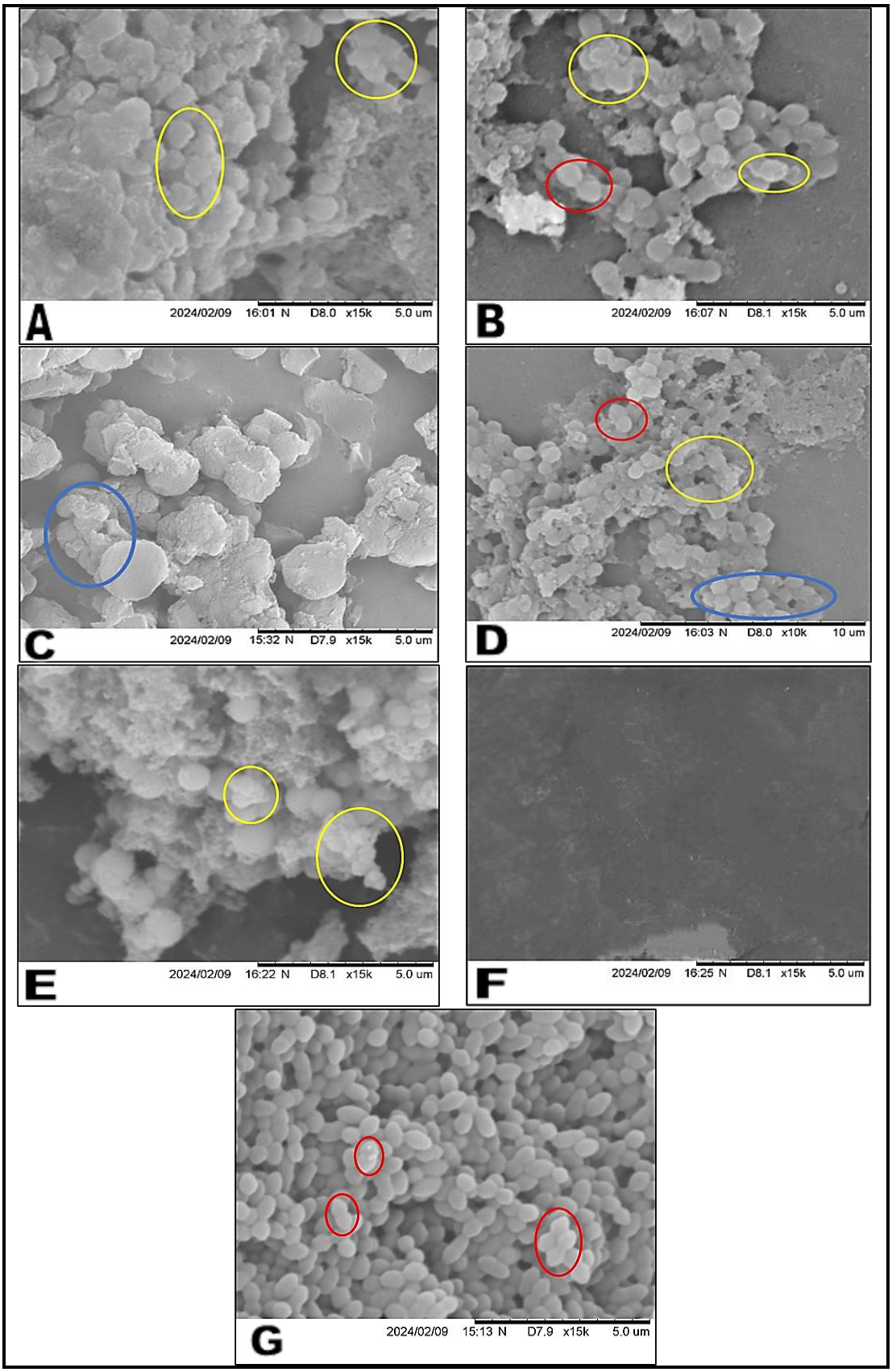


Figure 4.3 *E. faecalis* ATCC 29212 Cell Morphology Viewed Using SEM, Treatment with (A) *H. itama* Propolis, (B) *G. thoracica* Propolis, (C) *T. apicalis* Propolis, and (D) PEM at MBC Concentration, with (E) 45 mg/mL NS-$Ca(OH)_2$ and (F) 2% CHX Gel (Positive Controls), (G) 5%

DMSO (negative control). The Red Circle Indicates The Cell Budding Stage, The Blue Circle Indicates The Healthy Cell, While The Yellow Circle Indicates The Destruction of The Cell.

The cell morphology of *E. faecalis* ATCC 4082 after being treated with *H. itama* (6.25 mg/mL), *G. thoracica* (50.00 mg/mL), *T. apicalis* (50.00 mg/mL), and PEM (12.50 mg/mL) at their respective MBC value, along with the positive controls; NS-$Ca(OH)_2$ (45 mg/mL) and CHX gel (2% or 20 mg/mL) was evaluated by SEM analysis at 15,000X magnification. Based on the SEM images in Figure 4.4 (G), the normal cell morphology of *E. faecalis* ATCC 4082 can be observed after treatment with 5% DMSO (Figure 4.4(G)), showing a similar morphology to ATCC 29212. Similarly, morphology changes can also be observed after treatment with the tested sample.

The most significant damage was caused by the *H. itama* propolis extracts, where a distorted cell surfaces with a low bacterial density were observed. Additionally, the absence of budding stages and intact cell walls was noted after treatment (Figure 4.4 (A)). A weaker antibacterial effect was shown by *G. thoracica* (Figure 4.4 (B)) and *T. apicalis* (Figure 4.4 (C)) extracts, as healthy, intact cells with a high bacterial survival were still visible. On the other hand, a strong antimicrobial effect was observed with 45 mg/mL NS-$Ca(OH)_2$ (Figure 4.4 (E)) and 2% CHX gel (Figure 4.4 (F)).

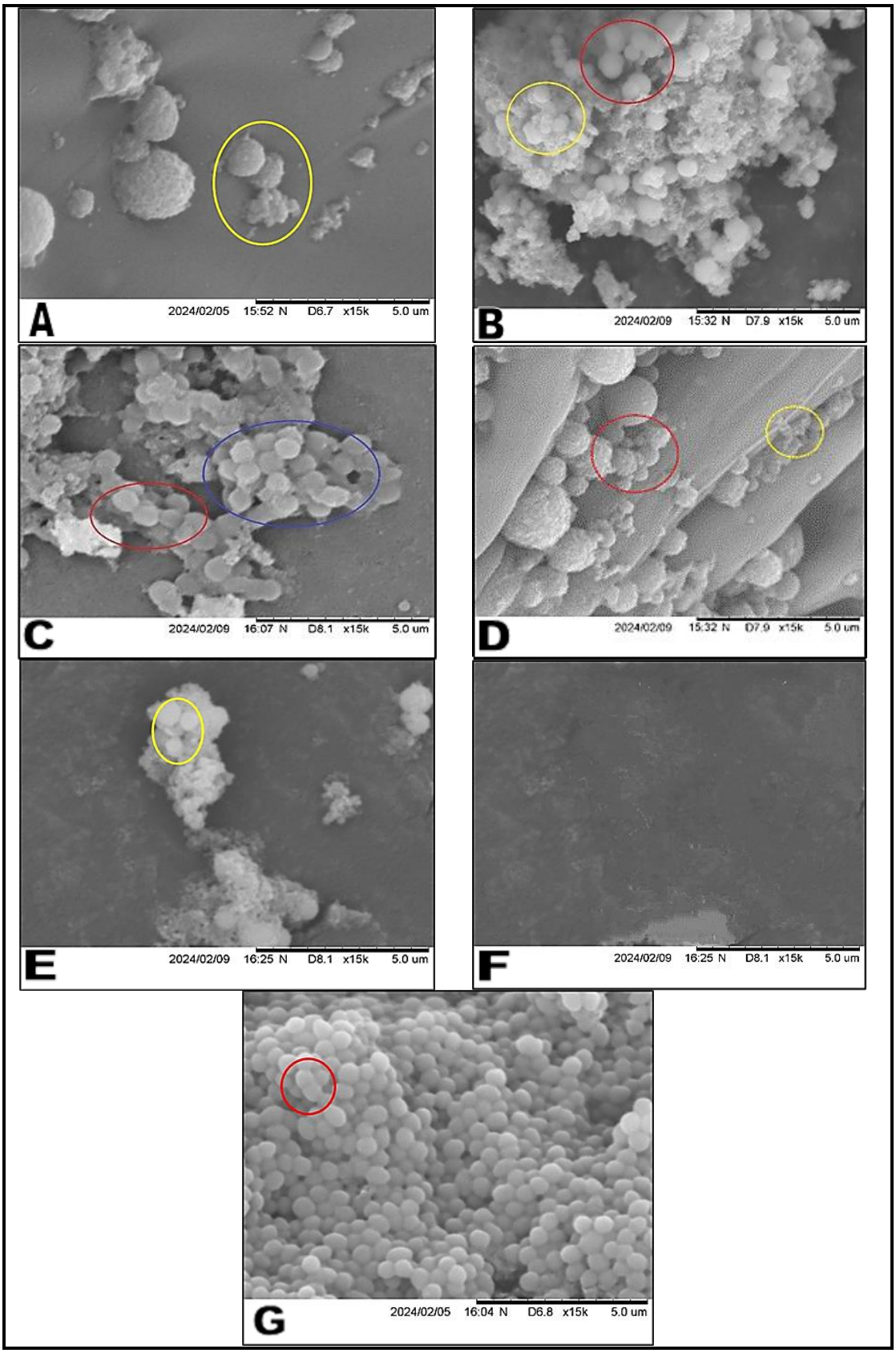


Figure 4.4 *E. faecalis* ATCC 4082 Cell Morphology Viewed Using SEM, Treatement with (A) *H. itama* Propolis, (B) *G. thoracica* Propolis, (C) *T. apicalis* Propolis, and (D) PEM at MBC Concentration, With (E) 45 mg/mL NS-$Ca(OH)_2$ and (F) 2% CHX Gel (Positive Controls), (G) 5% DMSO (Negative Control). The Red Circle Indicates The Cell Budding

Stage, Blue Circle Indicates a Healthy Cell, While The Yellow Circle Indicates The Destruction of The Cell.

4.4.8 Comparative Analysis of *E. faecalis* Strains Susceptibility based on AST Outcome

Table 4.15 compared *E. faecalis* (ATCC 29212 and ATCC 4082) based on agar-well diffusion, MIC, MBC, antibiofilm, and anti-adherence activities. The values reported are not specific to individual extracts, but represent the overall (cumulative) susceptibility response of each strain across all tested compounds within each respective assay. This approach enables a general comparison of strain-dependent susceptibility patterns rather than evaluating the effect of individual treatments.

Table 4.15
Comparison Between *E. faecalis* ATCC 29212 and ATCC 4082 Based on Previous AST Results

AST	*E. faecalis*		*p*-value
	ATCC 29212	ATCC 4082	
Agar-well diffusion (mean ± SD, mm)	10.00 ± 3.60	11.00 ± 4.30	0.033*
MIC (Median (IQR), mg/mL)	4.69 (23.00)	4.13 (23.44)	0.246
MBC (Median (IQR), mg/mL)	25.00 (43.75)	35.00 (43.75)	0.900
Antibiofilm (mean ± SD)	1.27 (0.80)	1.17 (0.76)	0.001*
Anti-adherence (mean ± SD)	1.52 (1.40)	1.54 (1.82)	0.372

Note: The values are presented as mean ± standard deviation (SD) for agar-well diffusion, antibiofilm, and anti-adherence assays, and as median (interquartile range, IQR) for MIC and MBC. Statistical analysis was conducted using two-way ANOVA for antibiofilm and anti-adherence comparisons, and the Mann-Whitnet U test test for MIC and MBC. A $p < 0.05$ was considered statistically significant (*).

The agar-well diffusion methods showed a significant difference in ZOI between strains ($p = 0.033$), with ATCC 4082 having a larger mean ZOI (11 ± 4.30 mm) compared to ATCC 29212 (10.00 ± 3.60 mm). Similarly, biofilm retention in the antibiofilm assay was significantly different ($p < 0.001$), with ATCC 4082 showing

lower biofilm formation (1.17 ± 0.76) than ATCC 29212 (1.27 ± 0.80). These results suggest that *E. faecalis* ATCC 4082 is more susceptible to propolis and intracanal medicaments.

For MIC and MBC results, no significant differences were observed between the *E. faecalis* strains, (p = 0.246 and p = 0.900, respectively). Nevertheless, the MIC recorded for *E. faecalis* ATCC 4082 (4.13 mg/mL, IQR = 23.44) was numerically lower than that of *E. faecalis* ATCC 29212 (4.69 mg/mL, IQR = 23.00). A similar result was obtained in the determination of MBC, where *E. faecalis* ATCC 4082 (35.00 µg/mL, IQR = 43.75) has a lower value compared to the MBC observed in ATCC 29212 (25.00 µg/mL, IQR = 43.75), though this difference were observed in anti-adherence activity between the two strains (p = 0.372), with the mean absorbance being nearly equal (1.54 ± 1.82 for *E. faecalis* ATCC 4082 and 1.52 ± 1.40 for *E. faecalis* ATCC 29212), suggesting that both strains exhibit similar initial attachment behaviours in response to antimicrobial exposure. Although these parameters were not significant, *E. faecalis* ATCC 4082 was shown to be more susceptible to both propolis extracts and intracanal medicaments.

4.4.9 Result of Time-Kill Study on *E. faecalis* ATCC 4082

This microbiological technique aids in assessing the activity of an antimicrobial agent by monitoring the microbial population over time after exposure. The time-kill study was conducted to compare each sample at its respective MBC against *E. faecalis* over 24 hours, with both 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel as positive controls. As shown in Table 4.16, the colony counts for all tested samples were comparable at baseline (0-hour), ranging from 10^4 to 10^5 CFU/mL. Statistical analysis using repeated measures ANOVA with Greenhouse–Geisser correction confirmed no significant difference at this time point (p = 0.305), indicating a uniform initial bacterial inoculum across all groups.

Table 4.16
Colony Count for Time-kill Study

Incubation time (hours)	Colony count (CFU/mL)						*p*-value
	***H. itama* propolis**	***G. thoracica* propolis**	***T. apicalis* propolis**	**PEM**	**45 mg/mL NS-$Ca(OH)_2$**	**2% CHX gel**	
0	1.19×10^5	7.27×10^4	8.90×10^4	8.43×10^4	6.07×10^4	5.53×10^4	0.305
1	1.01×10^6	3.30×10^5	8.93×10^4	1.10×10^5	1.35×10^7	3.40×10^4	0.002*
2	1.81×10^9	9.50×10^8	3.50×10^6	7.73×10^4	0.00	6.27×10^4	< 0.001*
3	5.87×10^5	1.15×10^6	1.54×10^5	4.87×10^6	0.00	2.83×10^4	< 0.001*
4	4.00×10^5	4.63×10^6	4.57×10^4	1.07×10^7	0.00	3.00×10^4	< 0.001*
24	0.00	1.66×10^9	9.60×10^8	3.63×10^4	0.00	0.00	< 0.001*

Note: The results are shown in mean colony count of three technical replicates, with a repeated measure ANOVA tested on each incubation period with a statistical significance at $p < 0.05$

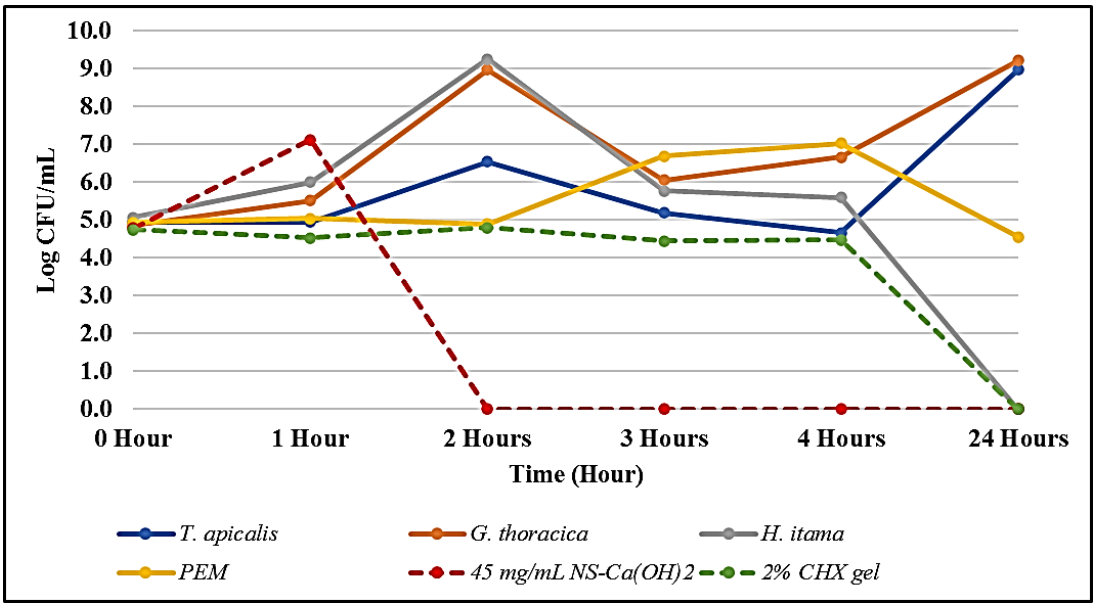


Figure 4.5 Time-kill Curve of *E. faecalis* ATCC 4082 After Being Treated With Different Samples

The antimicrobial effect of each compound over time is further illustrated in Figure 4.5. Although all groups began with similar bacterial loads, the rate and extent of bacterial reduction varied distinctly depending on the treatment applied. 45 mg/mL NS-$Ca(OH)_2$ (red line) demonstrated the most rapid and sustained bactericidal activity, achieving a complete bacterial eradication within 2 hours and maintaining this effect throughout the the 24 hours.

In contrast, *H. itama* propolis (grey line) exhibited a moderate antibacterial effect, with a noticeable reduction in bacterial counts beginning at 4 hours, followed by complete eradication at 24 hours. A similar trend was observed for the 2% CHX gel, suggesting a comparable time-dependent bactericidal effect, although with a slower onset compared to NS-$Ca(OH)_2$. Conversely, *T. apicalis* (blue line), *G. thoracica* (orange line) propolis, and PEM (yellow line) demonstrated fluctuating bacterial counts throughout the incubation period, indicating inconsistent or negligible antimicrobial activity. The persistence of viable bacteria suggests that these treatments were insufficient to exert sustained bactericidal effects at their tested concentrations.

After one hour of incubation, the colony count was approximately equal, ranging from 5 to 7- $\log_{10}$ CFU/mL. Statistical analysis with repeated measure ANOVA (Table 4.16) showed that the differences in bacterial colony count after treatment with different samples became significant (p = 0.002), due to the variation in antimicrobial effectiveness among the samples. Further analysis by Tukey's post hoc test was

performed to observe the differences between each sample at each incubation period, and the results are shown in Table 4.17. The analysis showed that 2% CHX gel had the lowest colony count (5.53 x 10^4 CFU/mL) and it was statistically significant when compared between *H. itama* (1.19 x 10^5 CFU/mL, $p = 0.009$), *G. thoracica* (7.27 x 10^4 CFU/mL, $p = 0.041$), and 45 mg/mL NS-$Ca(OH)_2$ (6.07 x 10^4 CFU/mL, $p = 0.023$). Unexpectedly, 45 mg/mL NS-$Ca(OH)_2$ recorded the highest colony count compared to other samples after one hour of incubation, but the difference was only significant with PEM ($p = 0.016$) and 2% CHX gel. Amongst the propolis extracts, *T. apicalis* propolis extract showed the lowest colony count (8.90 x 10^4 CFU/mL), but the comparison was only significant with *G. thoracica* ($p = 0.011$).

Table 4.17
Significant Difference Based on Multiple Comparisons Between Samples Using Tukey Post-hoc Test for Colony Count During Time-kill Study on 1-Hour Incubation Period

Sample 1	Sample 2	Mean Difference	*p*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
H. itama	2% CHX gel	1.462	0.009	0.831	2.094
G. thoracica	*T. apicalis*	0.567	0.011	0.298	0.835
	2% CHX gel	0.998	0.041	0.092	1.903
PEM	45 mg/mL NS-$Ca(OH)_2$	- 2.079	0.016	- 3.237	- 0.920
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	2.549	0.023	0.827	4.270

Note: Statistical difference was evaluated for each samples against other sample individually and only significant results are shown, with the significant level at $p < 0.05$

Starting from the 2-hour incubation period, differences in colony count between each treatment become more pronounced, and the difference was statistically analysed ($p < 0.001$) as shown in Table 4.18. The outcome for 45 mg/mL NS-$Ca(OH)_2$ showed the most potent bactericidal effect as complete bacterial elimination (0 CFU/mL) was achieved after 2 hours of treatment, and consistent results were observed until the end of the incubation period (24 hours). The difference in colony count compared between

45 mg/mL NS-$Ca(OH)_2$ and all other tested samples was significant, with $p = 0.001$. In contrast, *E. faecalis* was still able to proliferate after 2 hours of treatment with all propolis extracts and 2% CHX gel, with colony count ranging from 10^4 to 10^9 CFU/mL. An count of 1.81 x 10^9 CFU/mL was recorded after 2-hour of treatment with *H. itama* propolis extract, which was the significantly higher compared to *T. apicalis* (3.50 x 10^6 CFU/mL, $p = 0.002$), PEM (7.73 x 10^4 CFU/mL, $p = 0.002$), and 2% CHX gel (6.27 x 10^4 CFU/mL, $p = 0.002$). Amongst the propolis extracts, PEM showed the lowest colony count compared to *H. itama* ($p = 0.002$), *G. thoracica* ($p < 0.001$), and *T. apicalis* ($p = 0.003$).

Table 4.18
Multiple Comparisons Between Samples Using The Tukey Post-hoc Test For Colony Count During Time-kill Study on a 2-Hour Incubation Period

Sample 1	**Sample 2**	**Mean Difference**	***p*-value**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
H. itama	*T. apicalis*	2.704	0.002	2.163	3.246
	PEM	4.355	0.002	3.393	5.317
	45 mg/mL NS-$Ca(OH)_2$	9.240	0.002	7.643	10.837
	2% CHX gel	4.447	0.002	3.520	5.375
G. thoracica	*T. apicalis*	2.435	0.002	1.908	2.962
	PEM	4.086	< 0.001	3.717	4.455
	45 mg/mL NS-$Ca(OH)_2$	8.971	0.001	8.035	9.907
	2% CHX gel	4.178	< 0.001	3.903	4.453
T. apicalis	PEM	1.651	0.003	1.224	2.078
	45 mg/mL NS-$Ca(OH)_2$	6.536	0.001	5.479	7.593

	2% CHX gel	1.743	0.003	1.333	2.153
PEM	45 mg/mL NS-$Ca(OH)_2$	4.885	0.001	4.220	5.550
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	- 4.793	0.001	- 5.530	- 4.056

Note: Statistical difference was evaluated for each sample against other samples individually, and only significant results are shown, with the significance level at $p < 0.05$

At 3 hours of incubation, a slight bactericidal effect was observed as the colony count decreased to approximately 10^4 to 10^6 CFU/mL. Statistical analysis was then performed by comparing each sample individually, as shown in Table 4.19. The outcome for 45 mg/mL NS-$Ca(OH)_2$ retained its antimicrobial efficiency, as complete bacterial elimination (0 CFU/mL) was achieved at a 3-hour incubation period. Colony count decreased to 1.54 x 10^5 CFU/mL after treatment with *T. apicalis* propolis extract, which was the lowest colony count, compared to *H. itama* (5.87 x 10^5 CFU/mL), *G. thoracica* (1.15 x 10^6 CFU/mL), and PEM (4.87 x 10^6 CFU/mL). However, the difference in colony counts of *T. apicalis* was only significant when compared with *G. thoracica* ($p = 0.005$). Among the propolis extracts, the colony count with *H. itama* was significantly different compared to PEM ($p = 0.011$). The other intracanal medicament, 2% CHX gel, recorded a colony count of 2.83 x 10^4 CFU/mL after 3 hours of treatment, which was lower than the other propolis extracts. Nevertheless, the difference was only significant with *G. thoracica* ($p = 0.004$), *T. apicalis* ($p = 0.030$), and PEM ($p = 0.027$).

Table 4.19
Multiple Comparisons Between Samples Using Tukey Post-hoc Test for Colony Count During Time-kill Study on a 3-Hour Incubation Period

Sample 1	**Sample 2**	**Mean Difference**	***p*-value**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
H. itama	PEM	- 0.917	0.011	- 1.354	- 0.479
	45 mg/mL NS-$Ca(OH)_2$	5.736	0.006	3.676	7.796

	T. apicalis	0.871	0.005	0.606	1.135
G. thoracica	45 mg/mL NS-$Ca(OH)_2$	6.058	< 0.001	5.609	6.507
	2% CHX gel	1.610	0.004	1.172	2.048
T. apicalis	45 mg/mL NS-$Ca(OH)_2$	5.187	< 0.001	5.002	5.373
	2% CHX gel	0.739	0.030	0.164	1.314
PEM	45 mg/mL NS-$Ca(OH)_2$	6.653	0.005	4.471	8.835
	2% CHX gel	2.205	0.027	0.575	3.834
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	- 4.448	0.001	- 5.173	-3.723

Note: Statistical difference was evaluated for each sample against other samples individually, and only significant results are shown, with the significant level at $p < 0.05$

Table 4.20 shows the Tukey post hoc test for colony count during time-kill study at a 4-hour incubation period. After 4 hours of incubation, 45 mg/mL NS-$Ca(OH)_2$ still demonstrated the greatest bactericidal effect, as total bacterial elimination was achieved even after 4 hours of treatment, and the colony count was significantly lower compared to other tested samples ($p < 0.05$). The 2% CHX gel showed a lower colony count at 3.00 x 10^4 CFU/mL when compared to *H. itama* (4.00 x 10^5 CFU/mL), *G. thoracica* (4.63 x 10^6 CFU/mL), *T. apicalis* (4.57 x 10^4 CFU/mL), and PEM (1.07 x 10^7 CFU/mL). Post hoc comparisons showed that 2% CHX gel had significantly greater bacterial reductions compared to *H. itama* ($p = 0.02$), *G. thoracica* ($p = 0.007$), and PEM ($p = 0.021$). *T. apicalis* also showed a significant bactericidal effect after a 4-hour incubation period, as a low colony count was obtained compared to *H.itama* ($p = 0.008$), *G. thoracica* ($p = 0.002$), and PEM ($p = 0.014$). No significant difference was observed between *T. apicalis* and 2% CHX gel.

Table 4.20
Multiple Comparisons Between Samples Using Tukey Post-hoc Test for Colony Count During Time-kill Study on a 4-Hour Incubation Period

Sample_1	Sample_2	Mean Difference	*p*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
H. itama	*G. thoracica*	- 1.070	0.002	- 1.275	- 0.865
	T. apicalis	0.940	0.008	0.560	1.320
	PEM	- 1.453	0.045	- 2.835	- 0.071
	45 mg/mL NS-$Ca(OH)_2$	5.572	0.006	3.664	7.480
	2% CHX gel	1.136	0.020	0.418	1.854
G. thoracica	*T. apicalis*	2.010	0.002	1.612	2.409
	45 mg/mL NS-$Ca(OH)_2$	6.642	0.003	4.939	8.345
	2% CHX gel	2.206	0.007	1.382	3.029
T. apicalis	PEM	- 2.393	0.014	- 3.669	- 1.118
	45 mg/mL NS-$Ca(OH)_2$	4.632	0.008	2.772	6.491
PEM	45 mg/mL NS-$Ca(OH)_2$	7.025	< 0.001	6.414	7.636
	2% CHX gel	2.589	0.021	0.918	4.259
45 mg/mL NS-$Ca(OH)_2$	2% CHX gel	- 4.437	0.013	- 6.709	- 2.164

Note: Statistical difference was evaluated for each sample against other sample individually and only significant results are shown, with the significant level at $p < 0.05$

Total inhibition of *E. faecalis* growth by *H. itama* propolis extract, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel was observed after a 24-hour incubation. These results indicate that *H. itama* possesses strong antibacterial properties comparable to 2% CHX gel, as it was able to achieve the same bacterial reduction over time and could serve as

a promising natural alternative for intracanal disinfection. Conversely, other propolis extracts, including *G. thoracica* (1.66 x 10^9 CFU/mL), *T.apicalis* (9.60 x 10^8 CFU/mL), and PEM (3.63 x 10^4 CFU/mL), failed to completely inhibit the growth of *E. faecalis* within the study period. Instead, the *E. faecalis* colonies were increased.

Table 4.21
Multiple Comparisons Between Samples Using Tukey Post-hoc Test for Colony Count During Time-kill Study on a 24-Hour Incubation Period

Sample_1	**Sample_2**	**Mean Difference**	***p*-value**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
H. itama	*G. thoracica*	- 9.197	0.002	- 10.864	- 7.530
	T. apicalis	- 8.979	< 0.001	- 9.662	- 8.296
	PEM	- 4.558	0.001	- 5.142	- 3.974
G. thoracica	PEM	4.639	0.012	2.390	6.888
	45 mg/mL NS-$Ca(OH)_2$	9.197	0.002	7.530	10.864
	2% CHX gel	9.197	0.002	7.530	10.864
T. apicalis	PEM	4.421	0.004	3.176	5.666
	45 mg/mL NS-$Ca(OH)_2$	8.979	< 0.001	8.296	9.662
	2% CHX gel	8.979	< 0.001	8.296	9.662
PEM	45 mg/mL NS-$Ca(OH)_2$	4.558	0.001	3.974	5.142
	2% CHX gel	4.558	0.001	3.974	5.142

Note: Statistical difference was evaluated for each sample against other sample individually and only significant results are shown, with the significant level at $p < 0.05$

4.5 Discussion

The persistence of *E. faecalis* despite standard treatment highlights the limitations of current intracanal medicaments, thereby prompting interest in exploring natural alternative substances such as stingless bee propolis (John et al., 2015). This interest arises from propolis' remarkable biological properties, antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory properties, which make it a promising candidate for intracanal medicament use (Jahromi et al., 2012; Salleh et al., 2022; Zulhendri et al., 2021). However, the biological activity of propolis may vary depending on the bee species, geographical origin, and surrounding vegetation (Mohd-Yazid et al., 2018). Therefore, this study investigated the antimicrobial properties of propolis produced by three Malaysian stingless bee species, *H. itama, G. thoracica*, and *T. apicalis* against two strains of *E. faecalis* and compared their efficacy with 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel.

Combination of shaking-assisted extraction and ultrasonication, using 70% ethanol as the solvent, was employed as it able to produced propolis extracts with high concentrations of phenolic and flavonoid compounds, which are primarily responsible for the robust antimicrobial properties attributed to propolis (Azman et al., 2025; Heah et al., 2023; Al-Masoodi et al., 2022; Pobiega et al., 2019; Przybyłek & Karpiński, 2019).

In oral study, *E. faecalis* strain ATCC 29212 strain was widely used attributed to its lower cost compared to oral-associated strains. As different strains of the same bacterial species may exhibit variations in phenotypic and genotypic characteristics, the use of clinically relevant bacterial strains ensures that the outcomes more accurately reflect real-world clinical scenarios and enhance the relevance of the data for endodontic applications (Zancan et al., 2018). In this study, it was found that *E. faecalis* ATCC 4082 is more susceptible to propolis extracts, 45 mg/mL NS-$Ca(OH)_2$, and 2% CHX gel. No comparative study has been conducted to evaluate the resistant differences between *E. faecalis* ATCC 29212 (isolated from urine) and ATCC 4082 (isolated from pulpless teeth). Zancan et al. (2018) assessed the resistance of *E. faecalis* ATCC 29212 to various intracanal medicaments and compared it with *E. faecalis* ATCC 4083 (also isolated from urine), this study revealed that ATCC 4083 was more susceptible to NS-$Ca(OH)_2$, which aligns with the current findings.

This study demonstrated that *H. itama* propolis exhibited the most potent antimicrobial, antibiofilm, and anti-adherence effects among the tested propolis samples. However, 2% CHX gel showed the strongest and most consistent antimicrobial activity across all microbiological test, while 45 mg/mL NS-$Ca(OH)_2$ exhibited the most rapid bactericidal effect. In contrast, *G. thoracica* and *T. apicalis* propolis extracts showed relatively inconsistent or negligible antimicrobial activity, suggesting species-dependent variation in bioactive compound composition (Kashi et al., 2011). Collectively, these results suggest that *H. itama* propolis could serve as a viable adjunct or substitute for conventional intracanal medicaments in endodontic disinfection.

The agar-well diffusion method was selected for the initial antimicrobial screening, as it is suitable for compounds with high concentrations, high viscosity, poor surface adhesion, and large molecular weights, such as $Ca(OH)_2$ and propolis extracts (Azman et al., 2025; Hossain, 2024). A 2% CHX gel showed a larger ZOI compared to 45 mg/mL NS-$Ca(OH)_2$, which aligns with other studies reporting its superior ZOI relative to other intracanal medicaments (Sayed et al., 2020). Interestingly, *H. itama* propolis extract exhibited a larger ZOI than 45 mg/mL NS-$Ca(OH)_2$ and the other propolis extracts, demonstrating its potent antimicrobial effect. A similar result was reported by Kousedghi et al. (2012), where Iranian ethanolic propolis extract produced a wider ZOI than NS-$Ca(OH)_2$, despite its slow and weak diffusion ability. Although agar-well diffusion remains a valuable and widely applied method in microbiological testing, its results are primarily suitable for screening antimicrobial activity, as it is less accurate for comparative analysis than broth-based tests, such as MIC and MBC (Kousedghi et al., 2012; Rezende et al., 2008).

In terms of antimicrobial activity, *H. itama* propolis showed better inhibitory effect for both strains compared to other propolis and both intracanal medicaments. However, through determination of MBC, 2% CHX gel possessed significant bactericidal effect compared to all propolis extracts. Interestingly, all propolis extracts show significantly low MIC value, with *H. itama* and PEM having lower MBC compared to 45 mg/mL NS-$Ca(OH)_2$, indicating their potent antimicrobial. The findings of this study generally support the hypothesis (research hypothesis (a)) that *H. itama* propolis exhibits stronger antimicrobial effect compared to NS-$Ca(OH)_2$, and comparable effect with CHX. These findings are consistent with previous studies reporting comparable or even superior antimicrobial efficacy of propolis compared to

$Ca(OH)_2$ and CHX (Bazvand et al., 2014; Bhandari et al., 2014; Maekawa et al., 2013; Mejía, 2014). However, there are also several studies that have highlighted the potency of intracanal medicaments such as 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel compared to propolis, which reflect contrasting outcomes (Almadi et al., 2021; Kandaswamy et al., 2010; Kayaoglu et al., 2011; Vasudeva et al., 2017).

To date, no studies have explored the antimicrobial effects of combining multiple Malaysian propolis extracts in a single formulation. The ΣFIC analysis in this study revealed that combining different propolis extracts resulted in additive (for *E. faecalis* ATCC 29212) or synergistic (for *E. faecalis* ATCC 4082) interactions, thereby enhancing their antibacterial properties. The results of this study partially support the proposed hypothesis (research hypothesis (b)) as the synergistic interaction can only be observed in treatment of propolis extract mixture against *E. faecalis* ATCC 4082. Most available literature focuses on the synergistic effects of propolis when combined with other natural products or conventional antimicrobial agents. For instance, Al-Waili et al. (2012) reported enhanced antimicrobial activity when propolis was combined with honey against *E. coli*, *S. aureus*, and *C. albicans,* possibly due to increased concentrations of flavonoids and phenolic compounds. Similarly, studies have demonstrated synergistic interactions between propolis and antibiotics such as vancomycin (Al-Ani et al., 2018), tetracycline (Fernandes et al., 2005), trimethoprim + sulfamethoxazole and tobramycin (Wojtyczka et al., 2013) which enhanced antibacterial efficacy against various Gram-positive organisms.

In contrast, the present study explores the interaction between different propolis extracts, highlighting a gap in current knowledge. The observed enhanced antibacterial activity of the PEM against *E. faecalis* ATCC 4082 may be attributed to the combined effects of diverse phytochemical constituents, which could act on multiple bacterial targets simultaneously. This multi-component interaction may increase bacterial susceptibility compared to individual extracts (Al-Waili et al., 2012). Nonetheless, further studies incorporating additional confirmatory methods, such as time–kill assays or molecular analyses, are required to validate the presence and mechanism of true synergistic interactions. Harnessing this synergism could support the development of potent antimicrobial formulations with fewer side effects for use as intracanal medicaments.

In terms of antibiofilm and anti-adherence activity, *H. itama* propolis extract demonstrated significant antibiofilm and anti-adherence effect, which were stronger

compared to other propolis extracts and 45 mg/mL NS-$Ca(OH)_2$. Research by Rosli et al. (2023) found that Malaysian propolis was antibacterial and effective against *E. faecalis* and it was also more effective when compared to NS-$Ca(OH)_2$, which is commonly used as an intracanal medicament. Although studies on the antibiofilm effects of propolis remain limited, similar outcomes have also been reported, showing that propolis can inhibit biofilm formation by oral pathogens such as *E. faecalis* (Ong et al., 2017), *S. aureus* (Dogan et al., 2014), *S. epidermidis* (Wojtyczka et al., 2013), and *S. mutans* (Veloz et al., 2019). Both inhibition and disruption of *E. faecalis* biofilm by propolis have been attributed to its flavonoid content, where different components exert effects at various stages of biofilm development (Ong et al., 2017; Queiroga et al., 2023). For instance, apigenin, pinocembrin, and kaempferol-dimethyl-ether actively disrupt the biofilm structure without affecting the initial bacterial adherence (Queiroga et al., 2023). In contrast, flavonoids such as pinobanksin, chrysin, and acacetin contribute to the anti-adherence properties of propolis (Queiroga et al., 2023).

SEM analysis further supported the antimicrobial effects of propolis extracts, 45 mg/mL NS-$Ca(OH)_2$ and 2% CHX gel on both *E. faecalis* strains as evidenced by bacterial damage and cell death through distinct morphological alterations. All propolis extracts induced visible swelling of *E. faecalis* cells, resulting in a reduction in bacterial density, suggesting a membrane-compromising mechanism. This finding aligns with the study by Laleni et al. (2021), which reported similar swelling and increased cell size in propolis-treated *Escherichia coli*, likely due to osmotic imbalance caused by membrane disruption and water uptake. Similarly, Campos et al. (2020) found that propolis extract disrupted the *S. aureus* cell membrane, leading to leakage of intracellular contents and cell swelling caused by water influx, followed by cell lysis. Although direct SEM-based evidence of such effects on *E. faecalis* remains limited, the present study provides new visual confirmation of these morphological changes, highlighting propolis as a potential antimicrobial agent against this resilient pathogen.

While the current study did not observe morphological changes in *E. faecalis* following treatment with NS-$Ca(OH)_2$ via SEM, existing literature suggests that the release of hydroxyl ions from $Ca(OH)_2$ exerts its antibacterial effect by targeting the cytoplasmic membrane, promoting hydrolysis of structural components, and ultimately leading to cell lysis (Mohammadi et al., 2012). For 2% CHX gel, the SEM images revealed cell shrinkage, consistent with previous reports indicating that CHX disrupts bacterial membranes by forming pores, resulting in cytoplasmic leakage and cellular

collapse (Sebbane et al., 2024). These findings partially support the study hypothesis (research hypothesis (c)). Although *H. itama* propolis exhibited a significantly stronger anti-adherence effect compared to NS-$Ca(OH)_2$, its antibiofilm and morphological effects were comparable to those of NS-$Ca(OH)_2$ and CHX, indicating no superior effect in these aspects.

A 45 mg/mL NS-$Ca(OH)_2$ exhibited the most rapid and sustained antibacterial effects, completely eradicating *E. faecalis* growth after 2 hours of incubation. *H. itama* and 2% CHX gel displayed similar growth inhibition patterns, with both achieving complete bacterial eradication after incubating for 24 hours. The findings support hypothesis (research hypothesis (d)), as NS-$Ca(OH)_2$ demonstrated rapid antimicrobial activity, while *H. itama* propolis and CHX exhibited similar bacterial growth inhibition patterns. Jahromi et al. (2012) explained that NS-$Ca(OH)_2$ shows strong antimicrobial activity during the first 48 hours of use, but its efficacy gradually decreases after 240 hours. In contrast, propolis extract tends to exhibit a slow yet prolonged antimicrobial effect lasting more than 10 days. This is further supported by Awawdeh et al. (2009), and Madhubala et al. (2011), who found that while NS-$Ca(OH)_2$ demonstrated a stronger antimicrobial effect during the initial few days of application, propolis surpasses its efficacy after 7 days (168 hours) of treatment. This highlights a limitation of the present study, as the results were only observed within a 24 hours. Replenishment of the medium is generally not supported by previous studies, particularly in *in vitro* settings. However, Jahromi et al. (2012) demonstrated the ability to replenish the medium in an *ex vivo* study, although the detailed methodology was not disclosed. Nevertheless, different findings were reported by Marickar et al. (2014), who observed that CHX exhibited rapid antimicrobial activity against *E. faecalis* within just a few minutes of application.

A comparative study between propolis and non-setting NS-$Ca(OH)_2$ against *E. faecalis* was conducted by Awawdeh et al. (2009). Their study aims to compare the antibacterial efficacy of a propolis-based intracanal medicine with NS-$Ca(OH)_2$ paste when used as a short-term medication against *E. faecalis* using infected dentine models. The results showed that propolis was much more effective than NS-$Ca(OH)_2$ in eradicating *E. faecalis,* consistent with findings from other related studies. The study demonstrated that *E. faecalis* can remain viable in dentinal tubules even after prolonged treatment with NS-$Ca(OH)_2$ and is capable of surviving in a root canal independently, without assistance from other bacterial species. This highlights the potential of propolis

as a natural substitute for current medications. Similarly, Elsayed et al. (2021) also found that propolis was highly effective in inhibiting the growth of *E. faecalis* and suitable for use as an intracanal medicament.

In another study by Ahmed (2021), propolis showed better removal efficiency compared to NS-$Ca(OH)_2$, following irrigation with sodium hypochlorite. This is crucial, as complete removal of intracanal medicament is essential to achieve high-quality obturation without voids. Moreover, the use of NS-$Ca(OH)_2$ as an intracanal medicament can be harmful, as it may be toxic and cause cellular necrosis if it passes into the periapical area. Therefore, Ahmed (2021) concluded that NS-$Ca(OH)_2$ intracanal medication may be replaced with propolis. Likewise, research by Sirry et al. (2024) reported that the intracanal use of propolis was beneficial in reducing post-operative pain and may serve as a potential alternative to NS-$Ca(OH)_2$. Their research also noted that NS-$Ca(OH)_2$ was more toxic and less effective in eliminating *E. faecalis* compared to propolis.

Our findings show that 45 mg/mL NS-$Ca(OH)_2$ and *H. itama* propolis exhibit comparable antimicrobial properties. However, the key difference lies in their modes of action. $Ca(OH)_2$ relies on its high alkalinity to denature proteins and damage cell membranes. Its efficacy, however, is limited by the ability of *E. faecalis* to survive in highly alkaline environments through its proton pump mechanism (Saha et al., 2015). In contrast, propolis does not exert its antimicrobial effect via pH, but through its bioactive compounds such as phenolic acids, flavonoids, terpene esters, chalcones, dihydrochalcones, and terpenoids that directly inhibit *E. faecalis* growth (Ambi et al., 2017; Hossain et al., 2022). It exerts multifaceted antimicrobial actions, including disruption of cell walls and membranes (Silva et al., 2020), inhibition of nucleic acid synthesis (Almuhayawi, 2020), and interference with quorum sensing, mechanisms that likely contribute to its superior antibiofilm and anti-adherence properties (Borozan et al., 2023; Gopu et al., 2015). These findings highlighted propolis as a promising alternative antimicrobial agent for intracanal medicaments due to its potent and prolonged antimicrobial effects.

A 2% CHX gel demonstrated the most potent antimicrobial activity across most of the AST methods when compared to the other compounds evaluated in this study. It is a cationic bis-biguanide that binds to the negatively charged phosphate groups on bacterial cell walls, disrupting the bacterial cell membrane and leading to leakage of intracellular contents (Souza-Filho et al., 2008). However, despite its broad-spectrum

efficacy, CHX has notable drawbacks, including cytotoxic effects on oral tissues, potential for tooth discolouration, and the formation of orange-brown precipitates when used concurrently with NaOCl, which may interfere with the sealing ability of root canal fillings (Abbaszadegan et al., 2016; Gomes et al., 2013). Additionally CHX is not most commonly used intracanal medicament in endodontics due to the above drawbacks.

Although collected from the same farm in Malaysia, the antimicrobial properties of *H. itama, G. thoracica*, and *T. apicalis* propolis are notably different, thus confirming the influence of bee species on the biological profiles of propolis (Heah et al., 2023; Ibrahim et al., 2024). A recent study by Ibrahim et al. (2024) reported that *H. itama* propolis collected from two different farms exhibited distinct antimicrobial activities. Amongst the tested propolis extracts, *H. itama* propolis demonstrated the most potent antimicrobial activity against *E. faecalis*, as evidenced by its lower MIC and MBC, as well as significant antibiofilm, anti-adherence activity, and was able to eliminate *E. faecalis* growth at a 24-hour treatment. Similar outcomes were reported by Kustiawan et al. (2022), where *H. itama* propolis exhibited higher antimicrobial efficacy compared to *G. thoracica* propolis, despite a more complex and compositionally richer chemical profile of *G. thoracica* propolis. This superior effect may be attributed to its higher contents of flavonoids and phenolic compounds, which are known to disrupt bacterial cell walls, inhibit enzymatic activity, and interfere with biofilm formation (Rosli et al., 2023). The findings by Azman et al. (2025) are also similar with our outcome as it proved that *H. itama* propolis possess the most potent antibacterial properties compared to *G. thoracica* and *T. apicalis,* although it is collected from the same farm.

CHAPTER 5
CONCLUSION

5.1 Conclusion

In conclusion, the antimicrobial, antibiofilm, and anti-adherence efficacy of propolis from the same area varies according to the stingless bee species, with the *H. itama* propolis exhibited stronger antimicrobial properties against both *E. faecalis* strains (ATCC 29212 and ATCC 4082) compared to both to *G. thoracica* and *T. apicalis* propolis based on all microbiological tests. When compared to intracanal medicaments, *H. itama* propolis displayed substantial efficacy with lower MIC and MBC than 45 mg/mL NS-$Ca(OH)_2$ and comparable activity to 2% CHX gel, indicating its promising antimicrobial potency. Calculated ΣFIC values shows potential benefits of combining different propolis to enhance its antimicrobial efficacy as synergistic effect against ATCC 4082 can be observed, with additive effect towards the ATCC 29212. *H. itama* propolis indicates stronger anti-adherence properties compared to 45 mg/mL NS-$Ca(OH)_2$ and comparable antibiofilm effect to both intracanal medicaments. However, 2% CHX gel still demonstrated the highest overall antimicrobial effectiveness across most experimental assays (MBC, antibiofilm and anti-adherence effect). Furthermore, SEM analysis confirmed the ability of *H. itama* propolis to cause distinct morphological damage to bacterial cells, indicative of membrane disruption and cell lysis. Notably, based on all microbiological test, *E. faecalis* ATCC 4082 was more susceptible compared to ATCC 29212. Complete bacterial eradication was achieved within 24 hours of exposure to *H. itama* propolis, a rate equivalent to that of 2% CHX gel. This suggests that despite its relatively slower initial action, the sustained release profile of *H. itama* propolis provides prolonged antimicrobial effects, which are advantageous for endodontic applications, in the long run. These findings highlight the therapeutic potential of Malaysian stingless bee propolis, especially obtained from *H. itama* species as a natural and biocompatible alternative to conventional intracanal medicaments, with the potential to enhance the success rate of endodontic treatment.

5.2 Limitations and Future Recommendations

One notable limitation of this study is the limited number of sampling time points in the time-kill study. Incorporating additional intermediate time points, such as 12 and 18 hours, would provide a more comprehensive understanding of the antimicrobial killing dynamics over time. Furthermore, the observation period was restricted to 24 hours, which may be insufficient to fully capture the sustained antimicrobial activity or potential regrowth of *E. faecalis* following exposure to the tested agents. In clinical settings, intracanal medicaments are expected to maintain their antimicrobial efficacy over extended periods. Therefore, the results obtained within 24 hours may not accurately represent their sustained effectiveness. Future studies should include longer observation periods, such as 48 and 72 hours. to better evaluate the time-dependent antimicrobial properties of the tested compounds.

Optimization can be made on the extraction and purification process of propolis to improve the consistency and standardization of the final extract. This may include the removal of wax and resin to enhance purity by repeated filtration through Whatman filter paper, and the application of a homogenizer to ensure uniform dispersion of propolis constituents. Such improvements may help minimize variability between batches and strengthen the reliability of antimicrobial findings. A missing fixation step was observed in the antibiofilm and anti-adherence assays. Future studies should standardize this step by incorporating suitable fixatives such as methanol, ethanol, or formalin (paraformaldehyde) to preserve biofilm structure and enhance the reliability and reproducibility of analysis.

The absence of detailed cytotoxicity assessment limits the ability to determine the therapeutic window of propolis, where antimicrobial activity is achieved without compromising cell viability. Therefore, future studies should incorporate cytotoxicity evaluations using relevant human cell lines, such as periodontal ligament fibroblasts and osteoblasts, through established assays (e.g., live/dead assays). Additionally, investigating dose-dependent effects and comparing the biocompatibility profile of propolis with conventional intracanal medicaments, such as NS-$Ca(OH)_2$ and CHX, would provide a more comprehensive assessment of its clinical suitability.

The *H. itama* propolis collected in Malaysia exhibited promising antimicrobial effects, indicating its potential as a natural alternative to current intracanal

medicaments. To further elucidate the mechanism underlying its antimicrobial activity, future studies should include chemical profiling to identify the specific bioactive compounds responsible for its biological effects. The techniques such as Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy and gas chromatography–mass spectrometry (GC-MS) would provide a more comprehensive understanding of the antimicrobial properties of propolis against *E. faecalis*.

Antimicrobial efficacy of propolis in this study was evaluated under simplified *in vitro* conditions using single-species cultures, which do not adequately replicate the complexity of polymicrobial biofilms in persistent infections. Microbial interactions and biofilm formation in oral environment may significantly alter antimicrobial susceptibility, meaning the observed efficacy may not fully reflect clinical performance. Additionally, the findings indicated that *E. faecalis* ATCC 4082 was more susceptible to NS-$Ca(OH)_2$, CHX, and propolis, highlighting the importance of selecting bacterial strains with clinically relevant origins, as susceptibility can vary across strains. Therefore, future studies should incorporate microorganism of multiple species and include clinically relevant oral strains, particularly those isolated directly from the oral cavity, to provide a more accurate and comprehensive evaluation of antimicrobial efficacy.

Additionally, molecular investigations using techniques such as quantitative PCR (qPCR) could provide deeper insight into the downregulation of virulence and resistance genes in response to propolis treatment. Finally, further *in vivo* and clinical studies are essential to validate the safety, biocompatibility, and therapeutic efficacy of propolis before its formulation into potential intracanal medicament designs.

REFERENCES

Abbaszadegan, A., Gholami, A., Ghahramani, Y., Ghareghan, R., Ghareghan, M., Kazemi, A., Iraji, A., & Ghasemi, Y. (2016). Antimicrobial and cytotoxic activity of *Cuminum cyminum* as an intracanal medicament compared to chlorhexidine gel. *Iranian Endodontic Journal*, *11*(1), 44–50. https://doi.org/10.7508/iej.2016.01.009

Abbott, P. V. (2012). Endodontics - Current and future. *Journal of Conservative Dentistry*, *15*(3), 202–205. https://doi.org/10.4103/0972-0707.97935

Abdullah, N. A., Ja'afar, F., Yasin, H. M., Taha, H., Petalcorin, M. I. R., Mamit, M. H., Kusrini, E., & Usman, A. (2019). Physicochemical analyses, antioxidant, antibacterial, and toxicity of propolis particles produced by stingless bee *Heterotrigona itama* found in Brunei Darussalam. *Heliyon*, *5*(9), 1–8. https://doi.org/10.1016/J.HELIYON.2019.E02476

Ahangari, Z., Naseri, M., & Vatandoost, F. (2018). Propolis: Chemical composition and its applications in endodontics. *Iranian Endodontic Journal*, *13*(3), 285–292. https://doi.org/10.22037/iej.v13i3.20994

Ahmed, M. A. (2021). Removal efficacy of propolis/calcium hydroxide medicaments from the root canal. *Pakistan Journal of Medical Sciences*, *37*(7), 1948–1952. https://doi.org/10.12669/pjms.37.7.4241

Akca, A. E., Akca, G., Topçu, F. T., Macit, E., Pikdöken, L., & Özgen, I. Ş. (2016). The comparative evaluation of the antimicrobial effect of propolis with chlorhexidine against oral pathogens: An *in vitro* study. BioMed Research International, 2016, 1–8. https://doi.org/10.1155/2016/3627463

Akhir, R. A. M., Bakar, M. F. A., & Sanusi, S. B. (2017). Antioxidant and antimicrobial activity of stingless bee bread and propolis extracts. *AIP Conference Proceedings*, 1891. https://doi.org/10.1063/1.5005423

Al-Naggar, Y., Giesy, J. P., Abdel-Daim, M. M., Ansari, M. J., Al-Kahtani, S. N., & Yahya, G. (2021). Fighting against the second wave of COVID-19: Can honeybee products help protect against the pandemic? *Saudi Journal of Biological Sciences*, *28*(3), 1519–1527. https://doi.org/10.1016/j.sjbs.2020.12.031

Al-Ani, I., Zimmermann, S., Reichling, J., & Wink, M. (2018). Antimicrobial activities of European propolis collected from various geographic origins alone and in

combination with antibiotics. *Medicines*, *5*(1), 1–17. https://doi.org/10.3390/medicines5010002

Alghamdi, F., & Shakir, M. (2020). The influence of *Enterococcus faecalis* as a dental root canal pathogen on endodontic treatment: A systematic review. *Cureus*, *12*(3), 1-10 (e7257). https://doi.org/10.7759/CUREUS.7257

Alghutaimel, H., Matoug-Elwerfelli, M., Alhaji, M., Albawardi, F., Nagendrababu, V., & Dummer, P. M. H. (2024). Propolis use in dentistry: A narrative review of its preventive and therapeutic applications. *International Dental Journal*, *74*(3), 365–386. https://doi.org/10.1016/j.identj.2024.01.018

Al-Hariri, M. (2011). Propolis and its direct and indirect hypoglycemic effects. *Journal of Family and Community Medicine*, *18*(3), 152–154. https://doi.org/10.4103/2230-8229.90015

Almadi, E. M., & Almohaimede, A. A. (2018). Natural products in endodontics. *Saudi Medical Journal*, *39*(2), 124–130. https://doi.org/10.15537/smj.2018.2.21038

Almadi, K. H., Ahmed, M. A., Ghazal, T., Jouhar, R., Alkahtany, M. F., Abduljabbar, T., & Vohra, F. (2021). Antimicrobial efficacy of propolis in comparison to chlorhexidine against *Enterococcus faecalis*: A systematic review and meta-analysis. *Applied Sciences (Switzerland)*, *11*(8), 1–10. https://doi.org/10.3390/app11083469

Al-Masoodi, O., Said Gulam Khan, H., Baharuddin, I., & Ismail, I. (2022). *In-vitro* comparison of antibacterial activities on stingless bee propolis using selected extraction methods. *Compendium of oral science*, *9*(2), 23–43. https://doi.org/10.24191/cos.v9i2.19230

Almeida, E. A. B., Bossert, S., Danforth, B. N., Porto, D. S., Freitas, F. V., Davis, C. C., Murray, E. A., Blaimer, B. B., Spasojevic, T., Ströher, P. R., Orr, M. C., Packer, L., Brady, S. G., Kuhlmann, M., Branstetter, M. G., & Pie, M. R. (2023). The evolutionary history of bees in time and space. *Current Biology*, *33*(16), 3409–3422. https://doi.org/10.1016/j.cub.2023.07.005

Almelan, M. F., Abbas, U. K., & Al-Zubidi, M. (2023). Isolation and characterization of bacteriophage targeting *Enterococcus faecalis* isolated from root canal infection (*in vitro* study). *Biomedicine (India)*, *43*(3), 977–982. https://doi.org/10.51248/.v43i3.2827

Almuhayawi, M. S. (2020). Propolis as a novel antibacterial agent. *Saudi Journal of Biological Sciences*, *27*(11), 3079–3086. https://doi.org/10.1016/j.sjbs.2020.09.016

Al-Qurashi, A. D., & Awad, M. A. (2018). Postharvest ethanolic extract of propolis treatment affects quality and biochemical changes of 'Hindi-Besennara' mangos during shelf life. *Scientia Horticulturae*, *233*, 520–525. https://doi.org/10.1016/j.scienta.2017.12.030

Alshareef, F. (2021). Protocol to evaluate antibacterial activity MIC, FIC and time kill method. *Acta Scientific Microbiology*, *4*(5), 2–6. https://doi.org/10.31080/asmi.2021.04.0825

Alsubait, S., Alsaad, N., Alahmari, S., Alfaraj, F., Alfawaz, H., & Alqedairi, A. (2020). The effect of intracanal medicaments used in Endodontics on the dislocation resistance of two calcium silicate-based filling materials. *BMC Oral Health*, *20*(1(57)), 1–7. https://doi.org/10.1186/s12903-020-1044-6

Al-Waili, N., Al-Ghamdi, A., Ansari, M. J., Al-Attal, Y., & Salom, K. (2012). Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* isolates in single and polymicrobial cultures. *International Journal of Medical Sciences*, *9*(9), 793–800. https://doi.org/10.7150/ijms.4722

Ambi, A., Bryan, J., Borbon, K., Centeno, D., Liu, T., Chen, T. P., Cattabiani, T., & Traba, C. (2017). Are Russian propolis ethanol extracts the future for the prevention of medical and biomedical implant contaminations? *Phytomedicine*, 30, 50. https://doi.org/10.1016/j.phymed.2017.03.006

Ansel, H. C., Norred, W. P., & Roth, I. L. (1969). Antimicrobial activity of dimethyl sulfoxide against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. *Journal of Pharmaceutical Sciences*, *58*(7), 836–839. https://doi.org/10.1002/jps.2600580708

Asma, S. T., Bobiş, O., Bonta, V., Acaroz, U., Shah, S. R. A., Istanbullugil, F. R., & Arslan-Acaroz, D. (2022). General nutritional profile of bee products and their potential antiviral properties against mammalian viruses. *Nutrients*, *14*(17), 1–23. https://doi.org/10.3390/nu14173579

Athanassiadis, B., Abbott, P. V., George, N., & Walsh, L. J. (2010). An *in vitro* study of the antimicrobial activity of some endodontic medicaments against

Enterococcus faecalis biofilms. *Australian Dental Journal*, *55*(2), 150–155. https://doi.org/10.1111/j.1834-7819.2010.01222.x

Avinash, A., Munot, H., Baranwal, R., Duggi, V., Dubey, A., & Pagaria, S. (2017). Propolis: A smart supplement for an intracanal medicament. *International Journal of Clinical Pediatric Dentistry*, *10*(4), 324–329. https://doi.org/10.5005/jp-journals-10005-1459

Aviv, S., Alin, Y., Neta, L., Yael, H., Lada, Z., Avia, F. N., Diana, R., Moti, M., & David, P. (2024). Elimination of *E. faecalis* with NaOCl versus chlorhexidine gluconate from primary molar root canal systems: an *ex vivo* model study. *Clinical Oral Investigations*, *28*(5), 1–7. https://doi.org/10.1007/s00784-024-05621-6

Awang, N., Ali, N., Majid, F. A. A., Hamzah, S., & Razak, S. B. A. (2018). Total flavonoids and phenolic contents of sticky and hard propolis from 10 species of Indo-Malayan stingless bees. *Malaysian Journal of Analytical Sciences*, *22*(5), 877–884. https://doi.org/10.17576/mjas-2018-2205-15

Awawdeh, L., Al-Beitawi, M., & Hammad, M. (2009). Effectiveness of propolis and calcium hydroxide as a short-term intracanal medicament against *Enterococcus faecalis*: A laboratory study. *Australian Endodontic Journal*, *35*(2), 52–58. https://doi.org/10.1111/j.1747-4477.2008.00125.x

Ayad, A. S., Benchaabane, S., Daas, T., Smagghe, G., & Loucif-Ayad, W. (2025). Propolis stands out as a multifaceted natural product: Meta-analysis on its sources, bioactivities, applications, and future perspectives. *Life*, *15*(5), 1–44. https://doi.org/10.3390/life15050764

Ayub, S., Gupta, S., Ojha, R., & Saket, A. (2024). Recent advancements in intracanal medicaments used in paediatric dentistry: A comprehensive review. *Journal of Health Science and Medical Research*, *e20241129*, 1–14. https://doi.org/10.31584/jhsmr.20241129

Azman, A. I. A., Hasirin, R. A., Khan, H. B. S. G., Shafiei, Z., & Ismail, I. H. (2025). Antimicrobial activity of Malaysian *Heterotrigona itama* propolis against *Enterococcus faecalis* ATCC 4082: A study in endodontic applications. *Malaysian Journal of Microbiology*, *21*(4), 472–483. https://doi.org/10.21161/mjm.240719

Badr, A. E., Omar, N., & Badria, F. A. (2011). A laboratory evaluation of the antibacterial and cytotoxic effect of Liquorice when used as root canal

medicament. *International Endodontic Journal*, *44*(1), 51–58. https://doi.org/10.1111/j.1365-2591.2010.01794.x

Ba-Hattab, R., Al-Jamie, M., Aldreib, H., Alessa, L., & Alonazi, M. (2016). Calcium hydroxide in endodontics: An overview. *Open Journal of Stomatology*, *6*(12), 274–289. https://doi.org/10.4236/ojst.2016.612033

Bak-Badowska, J., Zeber-Dzikowska, I., Gworek, B., Kacprzyk, W., & Chmielewski, J. (2019). The role and significance of stingless bees (*Hymenoptera: Apiformes: Meliponini*) in the natural environment. *Environmental Protection and Natural Resources*, *30*(2), 1–5. https://doi.org/10.2478/oszn-2019-0005

Bakour, M., Laaroussi, H., Ousaaid, D., El Ghouizi, A., Es-Safi, I., Mechchate, H., & Lyoussi, B. (2022). Bee bread as a promising source of bioactive molecules and functional properties: An up-to-date review. *Antibiotics*, *11*(2), 1–39. https://doi.org/10.3390/antibiotics11020203

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Basrani, B. R., Manek, S., Sodhi, R. N. S., Fillery, E., & Manzur, A. (2007). Interaction between sodium hypochlorite and chlorhexidine gluconate. *Journal of Endodontics*, *33*(8), 966–969. https://doi.org/10.1016/j.joen.2007.04.001

Baumgartner, J. C., & Xia, T. (2003). Antibiotic susceptibility of bacteria associated with endodontic abscesses. *Journal of Endodontics*, *29*(1), 44. https://doi.org/10.1097/00004770-200301000-00012

Bazvand, L., Aminozarbian, M. G., Farhad, A., Noormohammadi, H., Hasheminia, S. M., & Mobasherizadeh, S. (2014). Antibacterial effect of triantibiotic mixture, chlorhexidine gel, and two natural materials propolis and aloe vera against *Enterococcus faecalis*: An *ex vivo* study. *Dental Research Journal*, *11*(4), 469.

Bhadoria, N., Gunwal, M. K., Suryawanshi, H., & Sonarkar, S. (2019). Antiadherence and antimicrobial property of herbal extracts (*Glycyrrhiza glabra* and *Terminalia chebula*) on *Streptococcus mutans*: An *in vitro* experimental study. *Journal of Oral and Maxillofacial Pathology*, *23*(1), 73–77. https://doi.org/10.4103/jomfp.JOMFP_103_18

Bhandari, S., Ashwini, T. S., & Patil, C. R. (2014). An *in vitro* evaluation of antimicrobial efficacy of 2% chlorhexidine gel, propolis and calcium hydroxide against *Enterococcus faecalis* in human root dentin. *Journal of Clinical and*

Diagnostic Research, *8*(11), 60–63. https://doi.org/10.7860/JCDR/2014/10359.5144

Bhandi, S., Patil, S., Boreak, N., Chohan, H., Abumelha, A. S., Alkahtany, M. F., Almadi, K. H., Vinothkumar, T. S., Raj, A. T., & Testarelli, L. (2022). Effect of different intracanal medicaments on the viability and survival of dental pulp stem cells. *Journal of Personalized Medicine*, *12*(4), 1–10. https://doi.org/10.3390/jpm12040575

Bhargava, P., Mahanta, D., Kaul, A., Ishida, Y., Terao, K., Wadhwa, R., & Kaul, S. C. (2021). Experimental evidence for therapeutic potentials of propolis. *Nutrients*, *13*(8), 1–23. https://doi.org/10.3390/nu13082528

Bogdanov, S. (2012). Propolis : Biological properties and medical applications. In *The Propolis Book, Chapter 2* (Issue January, pp. 1–42).

Borozan, A. B., Popescu, S., Madosa, E., Ciulca, A., Moldovan, C., & Gergen, I. (2023). Comparative study on the antimicrobial activity of propolis, catechin, quercetin and gallic acid. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *51*(2), 1–21. https://doi.org/10.15835/nbha51212826

Braakhuis, A. (2019). Evidence on the health benefits of supplemental propolis. *Nutrients*, *11*(11), 2705. https://doi.org/10.3390/NU11112705

Bubonja-Šonje, M., Knezević, S., & Abram, M. (2020). Challenges to antimicrobial susceptibility testing of plant-derived polyphenolic compounds. *Arhiv za Higijenu Rada i Toksikologiju*, *71*(4), 300–311. https://doi.org/10.2478/aiht-2020-71-3396

Bueno-Silva, B., Kawamoto, D., Ando-Suguimoto, E. S., Alencar, S. M., Rosalen, P. L., & Mayer, M. P. A. (2015). Brazilian red propolis attenuates inflammatory signaling cascade in LPS-activated macrophages. *PLoS ONE, 10*(12), e0144954. https://doi.org/10.1371/journal.pone.0144954

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology*, *36*(4), 347–363. https://doi.org/10.1016/S0278-6915(97)00145-2

Byström, A., & Sundqvist, G. (1981). Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *European Journal of Oral Sciences*, *89*(4), 321–328. https://doi.org/10.1111/j.1600-0722.1981.tb01689.x

Calimag, K. P. D., Arbis, C. C. H., Collantes, T. M. A., Bariuan, J. V., Ang, M. J. C., Cervancia, C. A., Desamero, M. J. M., & Estacio, M. A. C. (2021). Attenuation of carrageenan-induced hind paw edema and plasma tnf-α level by Philippine stingless bee (*Tetragonula biroi friese*) propolis. *Experimental Animals*, *70*(2), 185–193. https://doi.org/10.1538/expanim.20-0118

Campos, J. V. de, Assis, O. B. G., & Bernardes-Filho, R. (2020). Atomic force microscopy evidences of bacterial cell damage caused by propolis extracts on *E. coli* and *S. aureus*. *Food Science and Technology (Brazil)*, *40*(1), 1–7. https://doi.org/10.1590/fst.32018

Campos, M. G. R., Bogdanov, S., de Almeida-Muradian, L. B., Szczesna, T., Mancebo, Y., Frigerio, C., & Ferreira, F. (2008). Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research, 47*(2), 156–163. https://doi.org/10.1080/00218839.2008.11101443

Carvalho, C. N., Freire, L. G., De Carvalho, A. P. L., Siqueira, E. L., Bauer, J., Gritti, G. C., Souza, J. P. D., & Gavini, G. (2015). The influence of dentine on the pH of calcium hydroxide, chlorhexidine gel, and experimental bioactive glass-based root canal medicament. *Scientific World Journal*, 2015. https://doi.org/10.1155/2015/686259

Ceylan, S. (2021). Propolis loaded and genipin-crosslinked PVA/chitosan membranes; characterization properties and cytocompatibility/genotoxicity response for wound dressing applications. *International Journal of Biological Macromolecules*, *181*, 1196–1206. https://doi.org/10.1016/j.ijbiomac.2021.05.069

Chavarría-Bolaños, D., Esparza-Villalpando, V., & Ramírez, K. (2022). Antibacterial and antifungal capacity of three commercially available mouthwashes with different concentrations of chlorhexidine. *Odovtos - International Journal of Dental Sciences*, *24*(2), 69–80. https://doi.org/10.15517/IJDS.2021.48079

Ch'ng, J. H., Chong, K. K. L., Lam, L. N., Wong, J. J., & Kline, K. A. (2019). Biofilm-associated infection by enterococci. *Nature Reviews Microbiology*, *17*(2). https://doi.org/10.1038/s41579-018-0107-z

Chong, B. S., & Ford, T. R. P. (1992). The role of intracanal medication in root canal treatment. *International Endodontic Journal*, *25*(2), 97–106. https://doi.org/10.1111/j.1365-2591.1992.tb00743.x

Ćirić, J., Haneklaus, N., Rajić, S., Baltić, T., Lazić, I. B., & Đorđević, V. (2022). Chemical composition of bee bread (perga), a functional food: A review. *Journal of Trace Elements and Minerals*, *2*, 1–9. https://doi.org/10.1016/j.jtemin.2022.100038

CLSI. (1999). M26-A: Methods for determining bactericidal activity of antimicrobial agents : Approved guideline. *National Committee for Clinical Laboratory Standards*. *19*(18), 1-50.

CLSI. (2012). M07-A9: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard—Ninth Edition. *CLSI Document M07-A9*, *32*(2), 1–68. www.clsi.org.

Da Mota, A. C. C., Gonçalves, M. L. L., Bortoletto, C., Olivan, S. R., Salgueiro, M., Godoy, C., Altavista, O. M., Pinto, M. M., Horliana, A. C., Motta, L. J., & Bussadori, S. K. (2015). Evaluation of the effectiveness of photodynamic therapy for the endodontic treatment of primary teeth: Study protocol for a randomized controlled clinical trial. *Trials*, *16:551*(1), 1–6. https://doi.org/10.1186/s13063-015-1086-2

De la Maza, L. M., Pezzlo, M. T., & Baron, E. J. (1997). *Color Atlas of Diagnostic Microbiology*. Mosby-Year Book, Inc.

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *Journal of Oral and Maxillofacial Pathology*, *23*(1), 122–128. https://doi.org/10.4103/jomfp.JOMFP_304_18

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R., Yu, W. H., Lakshmanan, A., & Wade, W. G. (2010). The human oral microbiome. *Journal of Bacteriology*, *192*(19), 5002–5017. https://doi.org/10.1128/JB.00542-10

Diamantopoulou, E. I., Plastiras, O. E., Mourouzis, P., & Samanidou, V. (2020). Validation of a simple HPLC-UV method for the determination of monomers released from dental resin composites in artificial saliva. *Methods and Protocols*, *3*(2). https://doi.org/10.3390/mps3020035

Doern, C. D. (2014). When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. *Journal of Clinical Microbiology*, *52*(12), 4124–4128. https://doi.org/10.1128/JCM.01121-14

Dogan, N., Doganlı, G., Ülger, G., Habesoglu, D., Güzel, S., Yasar, Y., Arar, D., Şensoy, T., & Bozbeyoglu, N. (2014). Antibiofilm effect of two propolis samples from Turkey. *Journal of Applied Biological Sciences*, *8*(2), 27–31.

Dohyun, K., & Euiseong, K. (2014). Antimicrobial effect of calcium hydroxide as an intracanal medicament in root canal treatment: A literature review - Part I. *In vitro* studies. *Restorative Dentistry & Endodontics*, *39*(4), 241–252. https://doi.org/10.5395/rde.2014.39.4.241

Dudek-Wicher, R., Junka, A. F., Migdał, P., Korzeniowska-Kowal, A., Wzorek, A., & Bartoszewicz, M. (2022). The antibiofilm activity of selected substances used in oral health prophylaxis. *BMC Oral Health*, *22*(1). https://doi.org/10.1186/s12903-022-02532-4

El-Didamony, S. E., Gouda, H. I. A., Zidan, M. M. M., & Amer, R. I. (2024). Bee products: An overview of sources, biological activities and advanced approaches used in apitherapy application. *Biotechnology Reports*, *44*, 1–13. https://doi.org/10.1016/j.btre.2024.e00862

Elgamoudi, B. A., & Korolik, V. (2023). A guideline for assessment and characterization of bacterial biofilm formation in the presence of inhibitory compounds. *Bio-Protocol, 13*(21), 1. https://doi.org/10.21769/BioProtoc.4866

Ellepola, A. N. B., Joseph, B. J., & Khan, Z. U. (2012). Effects of subtherapeutic concentrations of chlorhexidine gluconate on germ tube formation of oral Candida. *Medical Principles and Practice, 21*(2), 120–124. https://doi.org/10.1159/000332569

Elsayed, R. O., Yagoub, S. O., & Abubakr, N. H. (2021). Evaluation of propolis as intracanal medicament against *Enterococcus faecalis*. *Open Journal of Stomatology, 11*(5), 208–220. https://doi.org/10.4236/ojst.2021.115018

Endo, M. M., Estrela, C. R. A., Alencar, A. H. G., Decurcio, D. A., Silva, J. A., & Estrela, C. (2017). Antibacterial action of red and green propolis extract in infected root canal. *Revista Odonto Ciencia, 32*(2). https://doi.org/10.15448/1980-6523.2017.2.28930

Eroğlu, Ö., & Yuksel, S. (2020). Propolis from past to present. Journal of social *Humanities and Administrative Sciences*, *6*(26), 623–629. https://doi.org/10.31589/joshas.310

Eslami, H., Ariamanesh, N., Ariamanesh, A., & Kafil, H. S. (2016). Synergistic effect of honey and Azarian propolis on oral microorganisms: An *in vitro* study. *Journal of Advanced Oral Research, 7*(3), 31–36.

Estrela, C., Pimenta, F. C., Ito, I. Y., & Bammann, L. L. (1999). Antimicrobial evaluation of calcium hydroxide in infected dentinal tubules. *Journal of Endodontics, 25*(6), 416–418. https://doi.org/10.1016/S0099-2399(99)80269-6

Faux, C. M. (2021). Honey bee anatomy. *In Honey Bee Medicine for the Veterinary Practitioner*. https://doi.org/10.1002/9781119583417.ch3

Fernandes, A., Balestrin, E. C., Betoni, J. E. C., De Oliveira Orsi, R., De Souza Da Cunha, M. D. L. R., & Montelli, A. C. (2005). Propolis: Anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Memorias Do Instituto Oswaldo Cruz, 100*(5), 563–566. https://doi.org/10.1590/s0074-02762005000500018

Fernandes, M., Azevedo, M. J., Campos, C., Ferreira, A. F., Azevedo, Á., Falcão-Pires, I., Zaura, E., Ramalho, C., Campos, J., & Sampaio-Maia, B. (2023). Potential Pathogenic and Opportunistic Oral Bacteria in Early Life: The Role of Maternal Factors in a Portuguese Population. *Pathogens, 12(1),* 1–3. https://doi.org/10.3390/pathogens12010080

Ferraz, C. C. R. (2001). *In vitro* assessment of the antimicrobial action and the mechanical ability of chlorhexidine gel as an endodontic irrigant. *Journal of Endodontics, 27*(7), 452–455. https://doi.org/10.1097/00004770-200107000-00004

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews Microbiology, 14*(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

Foley, I., & Gilbert, P. (1997). *In vitro* studies of the activity of glycopeptide combinations against *Enterococcus faecalis* biofilms. *Journal of Antimicrobial Chemotherapy, 40*(5), 667–672. https://doi.org/10.1093/jac/40.5.667

Fratini, F., Cilia, G., Turchi, B., & Felicioli, A. (2016). Beeswax: A minireview of its antimicrobial activity and its application in medicine. *Asian Pacific Journal of Tropical Medicine, 9*(9), 839–843. https://doi.org/10.1016/j.apjtm.2016.07.003

Garcia, L. S. (2010). Clinical Microbiology Procedures Handbook. In *American Society for Microbiology (ASM)* (3rd Edition, Vols. 1–3). American Society for Microbiology Press.

Gebreyohannes, G., Moges, F., Sahile, S., & Raja, N. (2013). Isolation and characterization of potential antibiotic producing actinomycetes from water and

sediments of Lake Tana, Ethiopia. *Asian Pacific Journal of Tropical Biomedicine, 3*(6), 426–435. https://doi.org/10.1016/S2221-1691(13)60092-1

Giampieri, F., Quiles, J. L., Cianciosi, D., Forbes-Hernández, T. Y., Orantes-Bermejo, F. J., Alvarez-Suarez, J. M., & Battino, M. (2022). Bee products: An emblematic example of underutilized sources of bioactive compounds. *Journal of Agricultural and Food Chemistry, 70*(23), 6833–6848. https://doi.org/10.1021/acs.jafc.1c05822

Giannelli, M., Chellini, F., Margheri, M., Tonelli, P., & Tani, A. (2008). Effect of chlorhexidine digluconate on different cell types: A molecular and ultrastructural investigation. *Toxicology in vitro, 22*(2), 308–317. https://doi.org/10.1016/j.tiv.2007.09.012

Goh, L. P. W., Jawan, R., Faik, A. A. M., & Gansau, J. A. (2023). A review of stingless bees' bioactivity in different parts of the world. *Journal of Medicine and Life, 16*(1), 16-21. https://doi.org/10.25122/jml-2022-0160

Gomes, B. P. F. A., Vianna, M. E., Zaia, A. A., Almeida, J. F. A., Souza-Filho, F. J., & Ferraz, C. C. R. (2013). Chlorhexidine in endodontics. *Brazilian Dental Journal*, *24*(2), 89–102. https://doi.org/10.1590/0103-6440201302188

Gomes, B. P. F., Vianna, M. E., Matsumoto, C. U., Rossi, V. D. P., Zaia, A. A., Ferraz, C. C., & Filho, F. J. (2005). Disinfection of gutta-percha cones with chlorhexidine and sodium hypochlorite. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology, 100*(4), 512–517. https://doi.org/10.1016/j.tripleo.2004.10.002

Gopu, V., Meena, C. K., & Shetty, P. H. (2015). Quercetin influences quorum sensing in food borne bacteria: *In-vitro* and *in-silico* evidence. *PLoS ONE, 10*(8). https://doi.org/10.1371/journal.pone.0134684

Halvaci, E., Kozak, T., Gül, M., Kars, H., & Şen, F. (2023). Bee anatomy: A comprehensive overview of bee morphology and physiology. *Journal of Scientific Reports-B, 8*, 1–19.

Hargreaves, K. M., Berman, L. H., & Rotstein, I. (2016). *Cohen's Pathways of the Pulp* (11th Edition). Elsevier.

Heah, K. G., Yusof, E. M., Dhamotharan, S., & Ismail, I. H. (2023). Review of bioactive components property of Malaysian propolis: A review. *AsPac J. Mol. Biol. Biotechnol, 31*(3), 84–105.

Hirt, H., Greenwood-Quaintance, K. E., Karau, M. J., Till, L. M., Kashyap, P. C., Patel, R., & Dunny, G. M. (2018). *Enterococcus faecalis* sex pheromone cCF10 enhances conjugative plasmid transfer *in vivo*. *MBio, 9*(1), e00037-18. https://doi.org/10.1128/MBIO.00037-18/SUPPL_FILE/MBO001183729SF3.TIF

Hoque, T., Hossain, M., Mahmud, S., Saleh, A. A., & Moral, M. A. A. (2023). Rate of *Enterococcus faecalis* in saliva and failed root canal treated teeth - *In vivo* study. *European Journal of Dental and Oral Health, 4*(4), 15–18. https://doi.org/10.24018/ejdent.2023.4.4.288

Hossain, R., Quispe, C., Khan, R. A., Saikat, A. S. M., Ray, P., Ongalbek, D., Yeskaliyeva, B., Jain, D., Smeriglio, A., Trombetta, D., Kiani, R., Kobarfard, F., Mojgani, N., Saffarian, P., Ayatollahi, S. A., Sarkar, C., Islam, M. T., Keriman, D., Uçar, A., … Cho, W. C. (2022). Propolis: An update on its chemistry and pharmacological applications. *Chinese Medicine (United Kingdom), 17*(100), 1–60. https://doi.org/10.1186/s13020-022-00651-2

Hossain, T. J. (2024). Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. *European Journal of Microbiology and Immunology, 14*(2), 97–115. https://doi.org/10.1556/1886.2024.00035

Hrncir, M., Jarau, S., & Barth, F. G. (2016). Stingless bees (*Meliponini*): senses and behavior. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 202*(9–10), 597–601. https://doi.org/10.1007/s00359-016-1117-9

Hulankova, R. (2024). Methods for determination of antimicrobial activity of essential oils *in vitro* - A review. *Plants, 13*(19), 1–23. https://doi.org/10.3390/plants13192784

Ibrahim, H. I. M., Thangavelu, M., & Khalifa, A. (2022). Honey-propolis-engineered collagen peptides as promising wound-healing matrix in mouse model. *Molecules, 27*(20), 1–16. https://doi.org/10.3390/molecules27207090

Ibrahim, N. S. A., Ramli, M. R. H., Khan, H. B. S. G., Al-Bayaty, F. H. M. H., & Ismail, I. H. (2024). Comparing antimicrobial effects of *Heterotrigona itama* propolis from two regions of Malaysia against *Enterococcus faecalis. The Journal of Dentists, 12*, 10–26.

Ibrahim, N., Zakaria, A. J., Ismail, Z., & Mohd, K. S. (2016). Antibacterial and phenolic content of propolis produced by two Malaysian stingless bees, *Heterotrigona itama* and *Geniotrigona thoracica*. *International Journal of Pharmacognosy and Phytochemical Research, 8*(1).

Ismail, I. H., Al-Bayaty, F. H., Yusof, E. M., Khan, H. B. S. G., Hamka, F. A., & Azmi, N. A. (2020). Evaluation of antimicrobial effect of Malaysian geopropolis with Aloe vera against *Enterococcus faecalis* to be used as an intracanal medicament in endodontics. *Journal of Conservative Dentistry, 23*(5), 489–496. https://doi.org/10.4103/JCD.JCD_528_20

Ismail, I. H., Saharuzaman, S., Naqiyuddin, F. 'Afeefah A., Bismelah, A., Heah, K. G., & Ismail, W. I. W. (2025). *In vitro* cytotoxicity comparison of stingless bee propolis and cisplatin against ORL-48 and HGF. *Compendium of Oral Science, 12*(1), 65–81. https://doi.org/10.24191/cos.v12i1.5648

Ismail, M. M. (2014). Competitiveness of beekeeping industry in Malaysia. In *Book Section*. Universiti Putra Malaysia Press.

Jaapar, M. F., Halim, M., Mispan, M. R., Jajuli, R., Saranum, M. M., Zainuddin, M. Y., Ghazi, R., & Ghani, I. A. (2016). The diversity and abundance of stingless bee (*Hymenoptera: Meliponini*) in peninsular Malaysia. *Advances in Environmental Biology, 10*(9), 1–7. http://www.aensiweb.com/AEB/http://creativecommons.org/licenses/by/4.0/

Jahromi, M. Z., Ranjbarian, P., & Shiravi, S. (2014). Cytotoxicity evaluation of Iranian propolis and calcium hydroxide on dental pulp fibroblasts. *Journal of Dental Research, Dental Clinics, Dental Prospects, 8*(3), 130–133. https://doi.org/10.5681/joddd.2014.024

Jahromi, M. Z., Toubayani, H., & Rezaei, M. (2012). Propolis: A new alternative for root canal disinfection. *Iranian Endodontic Journal, 7*(3), 127–133.

Jain, H., Mulay, S., & Mullany, P. (2016). Persistence of endodontic infection and *Enterococcus faecalis*: Role of horizontal gene transfer. *Gene Reports, 5*, 112–116. https://doi.org/10.1016/j.genrep.2016.09.010

Jang, Y. E., Kim, Y., Kim, S. Y., & Kim, B. S. (2024). Predicting early endodontic treatment failure following primary root canal treatment. *BMC Oral Health, 24*(1). https://doi.org/10.1186/s12903-024-03974-8

Javed, S., Mangla, B., & Ahsan, W. (2022). From propolis to nanopropolis: An exemplary journey and a paradigm shift of a resinous substance produced by bees. *Phytotherapy Research, 36*(5). https://doi.org/10.1002/ptr.7435

John, G., Kumar, K. P., Gopal, S. S., Kumari, S., & Reddy, B. K. (2015*). Enterococcus faecalis*, a nightmare to endodontist: A systematic review. *African Journal of Microbiology Research, 9*(13), 898–908. https://doi.org/10.5897/ajmr2014.7122

Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: A review of general principles and contemporary practices. *Clinical Infectious Diseases, 49*(11), 1749–1755. https://doi.org/10.1086/647952/2/49-11-1749-FIG003.GIF

Kaláb, M., Yang, A.-F., & Chabot, D. (2008, June 6). Conventional scanning electron microscopy of bacteria. *Infocus Magazine, 10*, 42–61. https://doi.org/10.22443/rms.inf.1.33

Kandaswamy, D., Venkateshbabu, N., Gogulnath, D., & Kindo, A. J. (2010). Dentinal tubule disinfection with 2% chlorhexidine gel, propolis, *morinda citrifolia* juice, 2% povidone iodine, and calcium hydroxide. *International Endodontic Journal, 43*(5), 419–423. https://doi.org/10.1111/j.1365-2591.2010.01696.x

Karatan, E., & Watnick, P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiology and Molecular Biology Reviews, 73*(2), 310–347. https://doi.org/10.1128/mmbr.00041-08

Karpiński, T. M., & Szkaradkiewicz, A. K. (2015). Chlorhexidine - Pharmaco-biological activity and application. *European Review for Medical and Pharmacological Sciences, 19*(7), 1321–1326.

Kashi, T. S. J., Kermanshahi, R. K., Erfan, M., Dastjerdi, E. V., Rezaei, Y., & Tabatabaei, F. S. (2011). Evaluating the *in vitro* antibacterial effect of Iranian propolis on oral microorganisms. *Iranian Journal of Pharmaceutical Research, 10*(2), 363–368.

Kau, A. L., Martin, S. M., Lyon, W., Hayes, E., Caparon, M. G., & Hultgren, S. J. (2005). *Enterococcus faecalis* tropism for the kidneys in the urinary tract of C57BL/6J mice. *Infection and Immunity, 73*(4), 2461–2468. https://doi.org/10.1128/IAI.73.4.2461-2468.2005

Kayaoglu, G., Ömürlü, H., Akca, G., Gürel, M., Genay, Ö., Sorkun, K., & Salih, B. (2011). Antibacterial activity of propolis versus conventional endodontic

disinfectants against *Enterococcus faecalis* in infected dentinal tubules. *Journal of Endodontics, 37*(3), 376–381. https://doi.org/10.1016/j.joen.2010.11.024

Kayaoglu, G., & Ørstavik, D. (2004). Virulence factors of *Enterococcus faecalis* relationship to endodontic disease. *Critical Reviews in Oral Biology & Medicine, 15*(5), 308–320. https://doi.org/https://doi.org/10.1177/154411130401500506

Kayser, F. H., Bienz, K. A., Eckert, J., & Zinkernagel, R. M. (2005). *Medical Microbiology*. Thieme.

Kelly, N., Farisya, M. S. N., Kumara, T. K., & Marcela, P. (2014). Species diversity and external nest characteristics of stingless bees in meliponiculture. *Pertanika Journal of Tropical Agricultural Science, 37*(3), 293–298.

Khan, Z. A., Siddiqui, M. F., & Park, S. (2019). Current and emerging methods of antibiotic susceptibility testing. *Diagnostics, 9*(2), 1–17. https://doi.org/10.3390/diagnostics9020049

Khezripour, A. R., Souri, D., Tavafi, H., & Ghabooli, M. (2019). Serial dilution bioassay for the detection of antibacterial potential of ZnSe quantum dots and their Fourier transform infra-red spectroscopy. *Measurement: Journal of the International Measurement Confederation, 148*, 106939. https://doi.org/10.1016/j.measurement.2019.106939

Kirkwood, Z. I., Millar, B. C., Downey, D. G., & Moore, J. E. (2018). Antimicrobial effect of dimethyl sulfoxide and N, N-Dimethylformamide on *Mycobacterium abscessus*: Implications for antimicrobial susceptibility testing. *International Journal of Mycobacteriology, 7*(2), 134–136. https://doi.org/10.4103/ijmy.ijmy_35_18

Kishan, K. V., Shah, N., & Naveen, M. (2020). Use of natural substitutes as an intracanal medicament in the endodontics-an update for *in vivo* studies. *Indian Journal of Natural Products and Resources, 11*(3). https://doi.org/10.56042/ijnpr.v11i3.31613

Koo, T. K., & Li, M. Y. (2016). A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *Journal of Chiropractic Medicine, 15*(2), 155–163. https://doi.org/10.1016/j.jcm.2016.02.012

Koru, O., Toksoy, F., Acikel, C. H., Tunca, Y. M., Baysallar, M., Uskudar Guclu, A., Akca, E., Ozkok Tuylu, A., Sorkun, K., Tanyuksel, M., & Salih, B. (2007). *In vitro* antimicrobial activity of propolis samples from different geographical

origins against certain oral pathogens. *Anaerobe, 13*(3–4), 140–145. https://doi.org/10.1016/j.anaerobe.2007.02.001

Kousedghi, H., Ahangari, Z., Eslami, G., & Ayatolahi, A. (2012). Antibacterial activity of propolis and $Ca(OH)_2$ against L*actobacillus, Entrococus faecalis, Peptostreptococus* and *Candida albicans*. *African Journal of Microbiology Research, 6*(14), 3510–3515. https://doi.org/10.5897/ajmr12.029

Kowalska-Krochmal, B., & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens, 10*(2), 165. https://doi.org/10.3390/pathogens10020165

Kristich, C. J., Li, Y. H., Cvitkovitch, D. G., & Dunny, G. M. (2004). Esp-Independent biofilm formation by *Enterococcus faecalis*. *Journal of Bacteriology, 186*(1), 154–163. https://doi.org/10.1128/JB.186.1.154-163.2004

Krishnamoorthy, A. L., Lemus, A. A., Solomon, A. P., Valm, A. M., & Neelakantan, P. (2020). Interactions between *Candida albicans* and *Enterococcus faecalis* in an Organotypic Oral Epithelial Model. *Microorganisms, 8(11),* 1–13. https://doi.org/10.3390/microorganisms8111771

Kumar, A., Tamanna, S., & Iftekhar, H. (2019). Intracanal medicaments – Their use in modern endodontics: A narrative review. *Journal of Oral Research and Review, 11*(2), 94–99. https://doi.org/10.4103/JORR.JORR_3_19

Kumar, Y., & Xu, B. (2026). A critical review on the roles of natural products in shaping oral microbiota and preventing chronic diseases. *Natural Products and Bioprospecting, 16*, 1–20. https://doi.org/10.1007/s13659-026-00605-3

Kustiawan, P. M., Zulfa, A. F., Batistuta, M. A., Hanifa, D. N. C., & Setiawan, I. M. (2022). Comparative analysis of phytochemical, total phenolic content, antioxidant and antibacterial activity of two species stingless bee propolis from East Kalimantan. *Malaysian Journal of Medicine and Health Sciences, 18*(SUPP10), 50–55. https://doi.org/10.47836//mjmhs18.4.8

Kwasny, S. M., & Opperman, T. J. (2010). Static biofilm cultures of Gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. *Current Protocols in Pharmacology*, SUPPL. 50. https://doi.org/10.1002/0471141755.ph13a08s50

Laleni, N. C., Gomes, P. D. C., Gkatzionis, K., & Spyropoulos, F. (2021). Propolis particles incorporated in aqueous formulations with enhanced antibacterial

performance. *Food Hydrocolloids for Health, 1*. https://doi.org/10.1016/j.fhfh.2021.100040

Lambrianidis, T., Margelos, J., & Beltes, P. (1999). Removal efficiency of calcium hydroxide dressing from the root canal. *Journal of Endodontics, 25*(2), 85–88. https://doi.org/10.1016/S0099-2399(99)80002-8

Lavinas, F. C., Macedo, E. H. B. C., Sá, G. B. L., Amaral, A. C. F., Silva, J. R. A., Azevedo, M. M. B., Vieira, B. A., Domingos, T. F. S., Vermelho, A. B., Carneiro, C. S., & Rodrigues, I. A. (2019). Brazilian stingless bee propolis and geopropolis: promising sources of biologically active compounds. *Revista Brasileira de Farmacognosia, 29*(3), 389–399. https://doi.org/10.1016/j.bjp.2018.11.007

Lee, D., Im, J., Na, H., Ryu, S., Yun, C. H., & Han, S. H. (2019). The novel *Enterococcus* phage vB_EfaS_HEf13 has broad lytic activity against clinical isolates of *Enterococcus faecalis. Frontiers in Microbiology, 10*(2877), 1–14. https://doi.org/10.3389/fmicb.2019.02877

Lee, T. H., Hu, C. C., Lee, S. S., Chou, M. Y., & Chang, Y. C. (2010). Cytotoxicity of chlorhexidine on human osteoblastic cells is related to intracellular glutathione levels. *International Endodontic Journal, 43*(5), 430–435. https://doi.org/10.1111/j.1365-2591.2010.01700.x

Lemmers, S. A. M. (2017). Stress, life history and dental development : a histological study of mandrills (Mandrillus sphinx). In https://www.researchgate.net/publication/325929748 (Vol. 5, Number 2).

Li, Y. C., Kuan, Y. H., Lee, S. S., Huang, F. M., & Chang, Y. C. (2014). Cytotoxicity and genotoxicity of chlorhexidine on macrophages *in vitro. Environmental Toxicology, 29*(4), 452–458. https://doi.org/10.1002/tox.21771

Lima, R. K. P., Guerreiro-Tanomaru, J. M., Faria-Júnior, N. B., & Tanomaru-Filho, M. (2012). Effectiveness of calcium hydroxide-based intracanal medicaments against *Enterococcus faecalis. International Endodontic Journal, 45*(4), 311–316. https://doi.org/10.1111/j.1365-2591.2011.01976.x

Lin, Y., Liang, X., Li, Z., Gong, T., Ren, B., Li, Y., & Peng, X. (2024). Omics for deciphering oral microecology. *International Journal of Oral Science, 16(2),* 1–11. https://doi.org/10.1038/s41368-023-00264-x

Löue, H., Rindom Schiöutt, C., Glavind, L., & Karring, T. (1976). Two years oral use of chlorhexidine in man: I. General design and clinical effects. *Journal of*

Periodontal Research, 11(3), 135–144. https://doi.org/10.1111/j.1600-0765.1976.tb00061.x

Lu, M., Xuan, S., & Wang, Z. (2019). Oral microbiota: A new view of body health. *Food Science and Human Wellness, 8*(1), 8–15. https://doi.org/10.1016/j.fshw.2018.12.001

Łucja, K., Julita, K., & Aleksandra, K.-R. (2021). Endodontic treatment regimens and their application in practice - Survey and comparative study. *Journal of Education, Health and Sport, 11*(7), 30–43. https://doi.org/10.12775/jehs.2021.11.07.003

Maalouf, L., Zogheib, C., & Naaman, A. (2013). Removal efficiency of calcium hydroxide dressing from the root canal without chemically active adjuvant. *Journal of Contemporary Dental Practice, 14*(2), 188–192. https://doi.org/10.5005/jp-journals-10024-1298

Madhubala, M. M., Srinivasan, N., & Ahamed, S. (2011). Comparative evaluation of propolis and triantibiotic mixture as an intracanal medicament against *Enterococcus faecalis. Journal of Endodontics, 37*(9). https://doi.org/10.1016/j.joen.2011.05.028

Maekawa, L. E., Valera, M. C., de Oliveira, L. D., Carvalho, C. A. T., Camargo, C. H. R., & Jorge, A. O. C. (2013). Effect of *Zingiber officinale* and propolis on microorganisms and endotoxins in root canals. *Journal of Applied Oral Science, 21*(1), 25–31. https://doi.org/10.1590/1678-7757201302129

Manfredi, M., Figini, L., Gagliani, M., & Lodi, G. (2016). Single versus multiple visits for endodontic treatment of permanent teeth. *The Cochrane Database of Systematic Reviews, 2016*(12), CD005296. https://doi.org/10.1002/14651858.CD005296.PUB3

Manginstar, C. O., Tallei, T. E., Salaki, C. L., Niode, N. J., & Jaya, H. K. (2025). Dual anti-inflammatory and antimicrobial effects of stingless bee propolis on second-degree burns. *Narra J, 5*(1), e2359. https://doi.org/10.52225/narra.v5i1.2359

Manoil, D., Cerit, E. E., Fang, H., Durual, S., Brundin, M., & Belibasakis, G. N. (2024). Profiling antibiotic susceptibility among distinct *Enterococcus faecalis* isolates from dental root canals. *Antibiotics, 13*(1), 1–14. https://doi.org/10.3390/antibiotics13010018

Marickar, R. F., Geetha, R. V., & Neelakantan, P. (2014). Efficacy of contemporary and novel intracanal medicaments against *Enterococcus faecalis*. *Journal of*

Clinical Pediatric Dentistry, 39(1), 47–50. https://doi.org/10.17796/jcpd.39.1.wmw9768314h56666

McHugh, C. P., Zhang, P., Michalek, S., & Eleazer, P. D. (2004). pH required to kill *Enterococcus faecalis in vitro*. *Journal of Endodontics, 30*(4), 218–219. https://doi.org/10.1097/00004770-200404000-00008

Mejía, J. B. C. (2014). Antimicrobial effects of calcium hydroxide, chlorhexidine, and propolis on *Enterococcus faecalis* and *Candida albicans. Journal of Investigative and Clinical Dentistry, 5*(3), 194–200. https://doi.org/10.1111/jicd.12041

Mohamed, W. A. S., Ismail, N. Z., Omar, E. A., Abdul Samad, N., Adam, S. K., & Mohamad, S. (2020). GC-MS evaluation, antioxidant content, and cytotoxic activity of propolis extract from Peninsular Malaysian stingless bees, *Tetrigona apicalis*. *Evidence-Based Complementary and Alternative Medicine, 2020*(1), 1–9. https://doi.org/10.1155/2020/8895262

Mohammadi, Z. (2008). Chlorhexidine gluconate, its properties and applications in endodontics. *Iranian Endodontic Journal, 2*(4), 113–125.

Mohammadi, Z., & Dummer, P. M. H. (2011). Properties and applications of calcium hydroxide in endodontics and dental traumatology. *International Endodontic Journal, 44*(8), 697–730. https://doi.org/10.1111/j.1365-2591.2011.01886.x

Mohammadi, Z., Shalavi, S., & Yazdizadeh, M. (2012). Antimicrobial activity of calcium hydroxide in endodontics: A review. *Chonnam Medical Journal, 48*(3), 133–140. https://doi.org/10.4068/cmj.2012.48.3.133

Mohd-Yazid, N. A., Basyirah, N., Zin, M., Pauzi, N., & Mohd, K. S. (2018). Preliminary evaluation of antioxidant and cytotoxic activity of ethanolic extract of stingless bees propolis from different localities. *Journal of Agrobiotechnology (Journal of UniSZA-Universiti Sultan Zainal Abidin) 133/ J. Agrobiotech, 9*(1S), 132–141.

Momenijavid, M., Salimizand, H., Korani, A., Dianat, O., Nouri, B., Ramazanzadeh, R., Ahmadi, A., Rostamipour, J., & Khosravi, M. R. (2022). Effect of calcium hydroxide on morphology and physicochemical properties of *Enterococcus faecalis* biofilm. *Scientific Reports, 12*(1), 1–11. https://doi.org/10.1038/s41598-022-11780-x

Nafi, N. E. M., Zin, N. B. M., Pauzi, N., Khadar, A. S. A., Anisava, A. R., Badiazaman, A. A. M., & Mohd, K. S. (2019). Cytotoxicity, antioxidant and phytochemical

screening of propolis extracts from four different Malaysian stingless bee species. *Malaysian Journal of Fundamental and Applied Sciences, 15*(2–1), 307–312. https://doi.org/10.11113/mjfas.v15n2-1.1542

Nainu, F., Masyita, A., Bahar, M. A., Raihan, M., Prova, S. R., Mitra, S., Emran, T. Bin, & Simal-Gandara, J. (2021). Pharmaceutical prospects of bee products: Special focus on anticancer, antibacterial, antiviral, and antiparasitic properties. *Antibiotics, 10*(7) 1-37. https://doi.org/10.3390/antibiotics10070822

Nandhinipriya, B., Narayanarao, G., Sabarinath, T. R., Singh, B. R., Chandrasekaran, D., & Rakeeba, F. (2024). Oral Commensals in Healthy Individuals: A Clinicocytological Study. *Cureus, 16(7).* https://doi.org/10.7759/cureus.65317

Nara, A., Dhanu, Chandra, P., Anandakrishna, L., & Dhananjaya. (2010). Comparative evaluation of antimicrobial efficacy of MTAD, 3% NaOCl and propolis against *E. faecalis*. *International Journal of Clinical Pediatric Dentistry, 3*(1), 21–25. https://doi.org/10.5005/jp-journals-10005-1049

Newberry, B. M., Shabahang, S., Johnson, N., Aprecio, R. M., & Torabinejad, M. (2007). The antimicrobial effect of Biopure MTAD on eight strains of *Enterococcus faecalis*: An *in vitro* investigation. *Journal of Endodontics, 33*(11), 1352–1354. https://doi.org/10.1016/j.joen.2007.07.006

Ng, W. J., Sit, N. W., Ooi, P. A. C., Ee, K. Y., & Lim, T. M. (2021). Botanical origin differentiation of Malaysian stingless bee honey produced by *Heterotrigona itama* and *Geniotrigona thoracica* using chemometrics. *Molecules, 26*(24), 7628. https://doi.org/10.3390/molecules26247628

Ng, Y. L., Mann, V., & Gulabivala, K. (2010). Tooth survival following non-surgical root canal treatment: A systematic review of the literature. *International Endodontic Journal, 43*(3), 171–189. https://doi.org/10.1111/j.1365-2591.2009.01671.x

Ng, Y. L., Mann, V., Rahbaran, S., Lewsey, J., & Gulabivala, K. (2008). Outcome of primary root canal treatment: Systematic review of the literature - Part 2. Influence of clinical factors. *International Endodontic Journal, 41*(1), 6–31. https://doi.org/10.1111/j.1365-2591.2007.01323.x

Okhale, S. E., Nkwegu, C., Ugbabe, G. E., Ibrahim, J. A., Egharevba, H. O., Kunle, O. F., & Igoli, J. O. (2021). Bee propolis: Production optimization and applications in Nigeria. *Journal of Pharmacognosy and Phytotherapy, 13*(1), 33–45. https://doi.org/10.5897/jpp2019.0561

Omar, S., Enchang, F. K., Nazri, M. U. I. A., Ismail, M. M., & Ismail, W. I. W. (2019). Physicochemical profiles of honey harvested from four major species of stingless bee (Kelulut) in Northeast Peninsular of Malaysia. *Malaysian Applied Biology, 48*(1), 111–116.

Ong, T. H., Chitra, E., Ramamurthy, S., Siddalingam, R. P., Yuen, K. H., Ambu, S. P., & Davamani, F. (2017). Chitosan-propolis nanoparticle formulation demonstrates anti-bacterial activity against *Enterococcus faecalis* biofilms. *PLoS ONE, 12*(3), 1–22. https://doi.org/10.1371/journal.pone.0174888

Orr, M. C., Jakob, M., Harmon-Threatt, A., & Mupepele, A. C. (2022). A review of global trends in the study types used to investigate bee nesting biology. *Basic and Applied Ecology, 62*, 12–21. https://doi.org/10.1016/j.baae.2022.03.012

Oršolić, N., & Maja, J. J. (2024). Royal jelly: Biological action and health benefits. *International Journal of Molecular Sciences, 25*(11), 1–79. https://doi.org/10.3390/ijms25116023

Ørstavik, D. (2019). Essential endodontology: Prevention and treatment of apical periodontitis. In E*ssential Endodontology: Prevention and Treatment of Apical Periodontitis* (Third Edition). Wiley-Blackwell. https://doi.org/10.1002/9781119272014

Özlek, E., & Kadı, G. (2021). Evaluation of calcium hydroxide removal efficiency of different concentrations and forms of sodium hypochlorite. *Eastern Journal of Medicine, 26*(4), 543–549. https://doi.org/10.5505/ejm.2021.26986

Paganelli, F. L., Willems, R. J., & Leavis, H. L. (2012). Optimizing future treatment of enterococcal infections: Attacking the biofilm? *Trends in Microbiology, 20*(1), 40-49. https://doi.org/10.1016/j.tim.2011.11.001

Pal, H., Sarkar, A., Das, L., Saha, S., & Sarkar, S. (2019). Application of intracanal medicaments: A review. *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS) e-ISSN, 18*(1), 14–21.

Paramitta, V. A., Heni, T. H., & Susilowati, S. (2015). The effect of calcium hydroxide on fibroblast cells viability. *The Indonesian Journal of Dental Research, 1*(2), 105–108. https://doi.org/10.22146/theindjdentres.9996

Parolia, A., Kumar, H., Ramamurthy, S., Davamani, F., & Pau, A. (2020). Effectiveness of chitosan-propolis nanoparticle against *Enterococcus faecalis* biofilms in the root canal. *BMC Oral Health, 20*(339), 1–14. https://doi.org/10.1186/s12903-020-01330-0

Peeters, E., Nelis, H. J., & Coenye, T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiological Methods, 72*(2), 157–165. https://doi.org/10.1016/j.mimet.2007.11.010

Pengchum, S., Chailertvanitkul, P., Laopaiboon, M., Thaweesit, P., Abbott, P., & Krisanaprakornkit, S. (2018). Thai propolis extract as a root canal medication against *Enterococcus faecalis* Infection. *Khon Kaen Dental Journal, 21*(1), 30–38.

Pietta, P. G., Gardana, C., & Pietta, A. M. (2002). Analytical methods for quality control of propolis. *Fitoterapia, 73*(SUPPL. 1), S7–S20. https://doi.org/10.1016/S0367-326X(02)00186-7

Pimenta, H. C., Violante, I. M. P., de Musis, C. R., Borges, Á. H., & Aranha, A. M. F. (2015). *In vitro* effectiveness of Brazilian brown propolis against *Enterococcus faecalis*. *Brazilian Oral Research, 29*(1), 1–6. https://doi.org/10.1590/1807-3107BOR-2015.VOL29.0058

Pinheiro, E. T., & Mayer, M. P. A. (2015). *Enterococcus faecalis* in oral infections. *JBR Journal of Interdisciplinary Medicine and Dental Science, 3*(1), 1–5. https://doi.org/10.4172/2376-032x.1000160

Pobiega, K., Kraśniewska, K., Derewiaka, D., & Gniewosz, M. (2019). Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods. *Journal of Food Science and Technology, 56*(12), 5386–5395. https://doi.org/10.1007/s13197-019-04009-9

Popova, M., Trusheva, B., & Bankova, V. (2021). Propolis of stingless bees: A phytochemist's guide through the jungle of tropical biodiversity. *Phytomedicine, 86*, 1–12. https://doi.org/10.1016/j.phymed.2019.153098

Pourhajibagher, M., Chiniforush, N., Shahabi, S., Ghorbanzadeh, R., & Bahador, A. (2016). Sub-lethal doses of photodynamic therapy affect biofilm formation ability and metabolic activity of *Enterococcus faecalis*. *Photodiagnosis and Photodynamic Therapy, 15*. https://doi.org/10.1016/j.pdpdt.2016.06.003

Pribadi, N., Widjiastuti, I., & Nadia, A. (2020). Effect of calcium hydroxide-propolis combination on the number of fibroblast cells and angiogenesis in wistar rats pulp. *Conservative Dentistry Journal, 10*(1), 14–18. https://doi.org/10.20473/cdj.v10i1.2020.14-18

Przybyłek, I., & Karpiński, T. M. (2019). Antibacterial properties of propolis. *Molecules, 24*(11), 2047. https://doi.org/10.3390/molecules24112047

Queiroga, M. C., Laranjo, M., Andrade, N., Marques, M., Costa, A. R., & Antunes, C. M. (2023). Antimicrobial, antibiofilm and toxicological assessment of propolis. *Antibiotics, 12*(2), 1–10. https://doi.org/10.3390/antibiotics12020347

Raghu, R., Pradeep, G., Shetty, A., Gautham, P., Puneetha, P., & Reddy, T. (2017). Retrievability of calcium hydroxide intracanal medicament with three calcium chelators, ethylenediaminetetraacetic acid, citric acid, and chitosan from root canals: An *in vitro* cone beam computed tomography volumetric analysis. *Journal of Conservative Dentistry, 20*(1), 25–29. https://doi.org/10.4103/0972-0707.209068

Rahul, M., Tewari, N., Mathur, V., Atif, M., Bansal, K., Agrawal, S., & Mir, R. (2022). *In vitro* evaluation of effects of intracanal medicaments on viability, proliferation, and differentiation of stem cells from apical papilla - A systematic review. *European Endodontic Journal, 7*, 167–177. https://doi.org/10.14744/eej.2022.63835

Ramakrishnan, R., Singh, A. K., Singh, S., Chakravortty, D., & Das, D. (2022). Enzymatic dispersion of biofilms: An emerging biocatalytic avenue to combat biofilm-mediated microbial infections. *Journal of Biological Chemistry, 298*(9), 1–14. https://doi.org/10.1016/j.jbc.2022.102352

Ran, S., He, Z., & Liang, J. (2013). Survival of *Enterococcus faecalis* during alkaline stress: Changes in morphology, ultrastructure, physiochemical properties of the cell wall and specific gene transcripts. *Archives of Oral Biology, 58*(11), 1667–1676. https://doi.org/10.1016/j.archoralbio.2013.08.013

Rathi, H. P., Chandak, M., Chaudhari, P., & Ikhar, A. (2022). Intracanal medicaments and its recent advances: A review. *Journal of Research in Medical and Dental Science, 10*(11), 163–167. www.jrmds.in

Rezende, G. P. da S. R., Costa, L. R. de R. S., Pimenta, F. C., & Baroni, D. A. (2008). *In vitro* antimicrobial activity of endodontic pastes with propolis extracts and calcium hydroxide: A preliminary study. *Brazilian Dental Journal, 19*(4), 301–305. https://doi.org/10.1590/s0103-64402008000400003

Robert, J. (2023). Dental Anatomy Understanding the Structure and Function of Teeth. *JBR Journal of Interdisciplinary Medicine and Dental Sciences, 6(3),* 32–35. https://doi.org/10.37532/2376-Introduction

Rocha, V. M., Portela, R. D., dos Anjos, J. P., de Souza, C. O., & Umsza-Guez, M. A. (2023). Stingless bee propolis: composition, biological activities and its applications in the food industry. *Food Production, Processing and Nutrition, 5*(29), 1–13. https://doi.org/10.1186/s43014-023-00146-z

Rosli, N., Che Elliaziz, N. A., Al-Bayaty, F. H., & Ismail, I. H. (2023). The antimicrobial properties of Malaysian propolis as intracanal medicament in endodontics. *The Journal of Dentist, 11*, 16–22.

Rozman, A. S., Hashim, N., Maringgal, B., & Abdan, K. (2022). A comprehensive review of stingless bee products: Phytochemical composition and beneficial properties of honey, propolis, and pollen. *Applied Sciences (Switzerland), 12*(13), 6370. https://doi.org/10.3390/app12136370

Růžičková, M., Vítězová, M., & Kushkevych, I. (2020). The characterization of Enterococcus genus: Resistance mechanisms and inflammatory bowel disease. *Open Medicine (Poland), 15*(1). https://doi.org/10.1515/med-2020-0032

Safavi, K. E., & Nichols, F. C. (1994). Alteration of biological properties of bacterial lipopolysaccharide by calcium hydroxide treatment. *Journal of Endodontics, 20*(3), 127–129. https://doi.org/10.1016/S0099-2399(06)80057-9

Saha, S., Nair, R., & Asrani, H. (2015). Comparative evaluation of propolis, metronidazole with chlorhexidine, calcium hydroxide and *Curcuma longa* extract as intracanal medicament against *E. faecalis* - An *in vitro* study. *Journal of Clinical and Diagnostic Research, 9*(11), ZC19–ZC21. https://doi.org/10.7860/JCDR/2015/14093.6734

Saifullizan, S. N., Azmi, W. A., & Omar, W. B. W. (2021). Study on morphological characteristic of stingless bee (*Heterotrigona itama*) in Terengganu. *Universiti Malaysia Terengganu Journal of Undergraduate Research, 3*(4), 121–126. https://doi.org/https://dx.doi.org/10.46754/umtjur.v3i4.245

Salam, M. A., Al-Amin, M. Y., Pawar, J. S., Akhter, N., & Lucy, I. B. (2023). Conventional methods and future trends in antimicrobial susceptibility testing. *Saudi Journal of Biological Sciences, 30*(3), 103582. https://doi.org/10.1016/j.sjbs.2023.103582

Salleh, S. N. A. S., Hanapiah, N. A. M., Johari, W. L. W., Ahmad, H., & Osman, N. H. (2021). Analysis of bioactive compounds and chemical composition of Malaysian stingless bee propolis water extracts. *Saudi Journal of Biological Sciences, 28*(12), 6705–6710. https://doi.org/10.1016/j.sjbs.2021.07.049

Salleh, S. N. A. S., Wan Johari, W. L., & Mohd Hanapiah, N. A. (2022). A comprehensive review on chemical compounds, biological actions and potential health benefits of stingless bee propolis. *Sains Malaysiana, 51*(3), 733–745. https://doi.org/10.17576/jsm-2022-5103-08

Samsudin, S. F., Mamat, M. R., & Hazmi, I. R. (2018). Taxonomic study on selected species of stingless bee (*Hymenoptera: Apidae: Meliponini*) in Peninsular Malaysia. *Serangga, 23*(2 Special Issue), 203–258.

Santonocito, S., Giudice, A., Polizzi, A., Troiano, G., Merlo, E. M., Sclafani, R., Grosso, G., & Isola, G. (2022). A cross-Talk between diet and the oral microbiome: Balance of nutrition on inflammation and immune system's response during periodontitis. *Nutrients, 14*(12), 1–21. https://doi.org/10.3390/nu14122426

Sayed, M. El, Ghanerad, N., Rahimi, F., Shabanpoor, M., & Shabanpour, Z. (2020). Antibacterial activity of sodium hypochlorite gel versus different types of root canal medicaments using agar diffusion test: An *in vitro* comparative study. *International Journal of Dentistry, 2020*, 1–11. https://doi.org/10.1155/2020/6483026

Șchiopu, P., Toc, D. A., Colosi, I. A., Costache, C., Ruospo, G., Berar, G., Gălbău, Ștefan G., Ghilea, A. C., Botan, A., Pană, A. G., Neculicioiu, V. S., & Todea, D. A. (2023). An overview of the factors involved in biofilm production by the *Enterococcus* genus. *International Journal of Molecular Sciences, 24*(14), 1–19. https://doi.org/10.3390/ijms241411577

Sebbane, N., Abramovitz, I., Kot-Limon, N., & Steinberg, D. (2024). Mechanistic insight into the anti-bacterial/anti-biofilm effects of low chlorhexidine concentrations on *Enterococcus faecalis* - *In vitro* study. *Microorganisms, 12*(11), 1–23. https://doi.org/10.3390/microorganisms12112297

Serrage, H. J., FitzGibbon, L., Alibhai, D., Cross, S., Rostami, N., Jack, A. A., Lawler, C. R. E., Jakubovics, N. S., Jepson, M. A., & Nobbs, A. H. (2022). Quantification of extracellular DNA network abundance and architecture within *Streptococcus gordonii* biofilms reveals modulatory factors. *Applied and Environmental Microbiology, 88*(13), 1–18. https://doi.org/10.1128/aem.00698-22

Shabbir, J., Qazi, F., Farooqui, W., Ahmed, S., Zehra, T., & Khurshid, Z. (2020). Effect of Chinese propolis as an intracanal medicament on post-operative endodontic

pain: A double-blind randomized controlled trial. *International Journal of Environmental Research and Public Health, 17*(2), 445. https://doi.org/10.3390/ijerph17020445

Shafiei, Z., Rahim, Z. H. A., Philip, K., & Thurairajah, N. (2016). Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium sp., Mangifera sp., and Mentha sp*.) on single- and dual-species biofilms. *PeerJ, 2016*(4: e2519), 1–19. https://doi.org/10.7717/peerj.2519

Shanmukhappa, Venkatesha, D., Ajantha, G. S., Amrutha, K. B., & Koppad, M. (2015). Isolation, identification and speciation of enterococci by conventional method and their antibiogram. *National Journal of Laboratory Medicine, 4*(2), 1–6.

Sharma, A., Tewari, S., Tewari, S., Sharma, R. K., Narula, S. C., & Gaur, A. (2022). Efficacy of chlorhexidine intracanal medicament on periodontal healing in concomitant endodontic-periodontal disease with communication: A randomized clinical trial. *European Journal of Dental and Oral Health, 3*(2), 49–54. https://doi.org/10.24018/ejdent.2022.3.2.153

Shino, B., Peedikayil, F. C., Jaiprakash, S. R., Ahmed Bijapur, G., Kottayi, S., & Jose, D. (2016). Comparison of antimicrobial activity of chlorhexidine, coconut oil, probiotics, and ketoconazole on *Candida albicans* Isolated in children with early childhood caries: An *in vitro* study. *Scientifica, 2016*, 1–5. https://doi.org/10.1155/2016/7061587

Silva, F. C. Da, Favaro-Trindade, C. S., de Alencar, S. M., Thomazini, M., & Balieiro, J. C. C. (2011). Physicochemical properties, antioxidant activity and stability of spray-dried propolis. *Journal of ApiProduct and ApiMedical Science, 3*(2), 94–100. https://doi.org/10.3896/ibra.4.03.2.05

Silva, M. V. da, de Moura, N. G., Motoyama, A. B., & Ferreira, V. M. (2020). A review of the potential therapeutic and cosmetic use of propolis in topical formulations. *Journal of Applied Pharmaceutical Science, 10*(1), 131–141. https://doi.org/10.7324/JAPS.2020.101018

Sirry, S., Marzouk, A., Gawdat, S., & Omer, A. (2024). Evaluation of the effect of propolis versus calcium hydroxide, intracanal medicaments, on post-operative pain in patients with necrotic pulp: A Randomized clinical trial. *Advanced Dental Journal, 6*(1), 1–14. https://doi.org/10.21608/adjc.2023.178663.1216

Slutzky-Goldberg, I., Hanut, A., Matalon, S., Baev, V., & Slutzky, H. (2013). The effect of dentin on the pulp tissue dissolution capacity of sodium hypochlorite and calcium hydroxide. *Journal of Endodontics, 39*(8), 980–983. https://doi.org/10.1016/j.joen.2013.04.040

Smith, Ann. C., & Hussey, M. A. (2019). *Gram Stain Protocols*. American Society for Microbiology (ASM). www.asmscience.org

Souza-Filho, F. J. de, Soares, A. de J., Vianna, M. E., Zaia, A. A., Ferraz, C. C. R., & Gomes, B. P. F. de A. (2008). Antimicrobial effect and pH of chlorhexidine gel and calcium hydroxide alone and associated with other materials. *Brazilian Dental Journal, 19*(1), 28–33. https://doi.org/10.1590/s0103-64402008000100005

Stela, M., Cichon, N., Spławska, A., Szyposzynska, M., & Bijak, M. (2024). Therapeutic potential and mechanisms of bee venom therapy: A comprehensive review of apitoxin applications and safety enhancement strategies. *Pharmaceuticals, 17*(9), 1–20. https://doi.org/10.3390/ph17091211

Stuart, C. H., Schwartz, S. A., Beeson, T. J., & Owatz, C. B. (2006). *Enterococcus faecalis*: Its role in root canal treatment failure and current concepts in retreatment. *Journal of Endodontics, 32*(2), 93–98. https://doi.org/10.1016/j.joen.2005.10.049

Sudhakara, P., Gupta, A., Bhardwaj, A., & Wilson, A. (2018). Oral dysbiotic communities and their implications in systemic diseases. *Dentistry Journal, 6*(2:10), 1–14. https://doi.org/10.3390/dj6020010

Swetah, Csv., & Ranjan, M. (2017). Single visit vs. multiple visits for endodontic treatment: A review. *International Journal of Scientific Development and Research, 2*(10), 23–27. www.ijsdr.org

Sy, K., Agossa, K., Maton, M., Chijcheapaza-Flores, H., Martel, B., Siepmann, F., Deveaux, E., Blanchemain, N., & Neut, C. (2021). How adding chlorhexidine or metallic nanoparticles affects the antimicrobial performance of calcium hydroxide paste as an intracanal medication: An *in vitro* study. *Antibiotics, 10*(11). https://doi.org/10.3390/antibiotics10111352

Tabassum, S., & Khan, F. R. (2016). Failure of endodontic treatment: The usual suspects. *European Journal of Dentistry, 10*(1), 144–147. https://doi.org/10.4103/1305-7456.175682

Thakur, M., & Nanda, V. (2020). Composition and functionality of bee pollen: A review. *Trends in Food Science and Technology, 98*, 82–106. https://doi.org/10.1016/j.tifs.2020.02.001

Thomas, C., Minty, M., Vinel, A., Canceill, T., Loubières, P., Burcelin, R., Kaddech, M., Blasco-Baque, V., & Laurencin-Dalicieux, S. (2021). Oral microbiota: A major player in the diagnosis of systemic diseases. *Diagnostics, 11*(8: 1378), 1–29. https://doi.org/10.3390/diagnostics11081376

Tiang, E. R., Han, L., & Hu, F. (2025). Physicochemical characteristics, antioxidant capacity, and antimicrobial activity of stingless bee honey from Malaysia: *Heterotrigona itama*, *Lophotrigona canifrons*, and *Tetrigona binghami*. *Foods, 14*(6). https://doi.org/10.3390/foods14060995

Tomás, I. C., Rubido, S., & Donos, N. (2011). In situ antimicrobial activity of chlorhexidine in the oral cavity. In A. Méndez-Vilas (Ed.), *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances* (pp. 530–541). Formatex Research Center, 2011. https://doi.org/10.13140/2.1.4496.6404

Toreti, V. C., Sato, H. H., Pastore, G. M., & Park, Y. K. (2013). Recent progress of propolis for its biological and chemical compositions and its botanical origin. *Evidence-Based Complementary and Alternative Medicine, 697390*, 1–13. https://doi.org/10.1155/2013/697390

Vasudeva, A., Sinha, D. J., Tyagi, S. P., Singh, N. N., Garg, P., & Upadhyay, D. (2017). Disinfection of dentinal tubules with 2% chlorhexidine gel, calcium hydroxide and herbal intracanal medicaments against *Enterococcus faecalis*: An *in vitro* study. *Singapore Dental Journal, 38*, 39–44. https://doi.org/10.1016/j.sdj.2017.06.001

Veen, J. W. Van. (2014). Biology of honeybees and stingless bees. In *Beekeeping for Poverty Alleviation and Livelihood Security: Vol. 1: Technological Aspects of Beekeeping* (pp. 105–123). Springer Netherlands. https://doi.org/10.1007/978-94-017-9199-1_3

Veloz, J. J., Alvear, M., & Salazar, L. A. (2019). Evaluation of alternative methods to assess the biological properties of propolis on metabolic activity and biofilm formation in *Streptococcus mutans*. *Evidence-Based Complementary and Alternative Medicine, 2019*. https://doi.org/10.1155/2019/1524195

Vidana, R., Sullivan, A., Billström, H., Ahlquist, M., & Lund, B. (2011). *Enterococcus faecalis* infection in root canals – host-derived or exogenous source? *Letters in Applied Microbiology, 52*(2), 109–115. https://doi.org/10.1111/J.1472-765X.2010.02972.X

Vivacqua-Gomes, N., Ferraz, C. C. R., Gomes, B. P. F. A., Zaia, A. A., Teixeira, F. B., & Souza-Filho, F. J. (2002). Influence of irrigants on the coronal microleakage of laterally condensed gutta-percha root fillings. *International Endodontic Journal, 35*(9), 791–795. https://doi.org/10.1046/j.1365-2591.2002.00569.x

Wagh, V. D. (2013). Propolis: A wonder bees product and its pharmacological potentials. *Advances in Pharmacological Sciences, 2013*(308249), 1–11. https://doi.org/10.1155/2013/308249

Wahjuningrum, D. A., Saraswati, W., Widjiastuti, I., Putri, A., Subijanto, A., Setijanto, D., Setiowatie, E. M., Mooduto, L., & Puteri, F. H. (2017). Effect of extract propolis on the adherence of *Enterococcus faecalis* as a candidate root canal irrigation solution. Inte*rnational Medical Device and Technology Conference*, 94–96. https://doi.org/10.3402/jom.v8.31095

Wahyuni, N., Yanti, N., Abidin, T., Prasetia, W., & Suryanto, D. (2023). The effect of addition 5% propolis nanoparticles in epoxy resin and bioceramic sealers on the growth of *Enterococcus faecalis* ATCC 29212 and the dentinal tubular penetration: *In vitro*. *International Journal of Applied Pharmaceutics, 15*(4), 99–105. https://doi.org/10.22159/ijap.2023v15i4.47486

Wang, L., Qiao, X., Gao, L., Chen, C., & Wan, Y. (2021). A quantitative method to assess bacterial adhesion using recombinant bioluminescent *Pseudomonas aeruginosa*. *Biophysics Reports, 7*(1), 55–70. https://doi.org/10.52601/bpr.2021.200043

Weckwerth, P. H., Zapata, R. O., Vivan, R. R., Tanomaru-Filho, M., Maliza, A. G. A., & Duarte, M. A. H. (2013). *In Vitro* alkaline pH resistance of *Enterococcus faecalis*. *Brazilian Dental Journal, 24*(5), 474–476. https://doi.org/10.1590/0103-6440201301731

Wehbe, R., Frangieh, J., Rima, M., Obeid, D. El, Sabatier, J. M., & Fajloun, Z. (2019). Bee venom: Overview of main compounds and bioactivities for therapeutic interests. *Molecules, 24*(16). https://doi.org/10.3390/molecules24162997

Wendt, C., Schinke, S., Württemberger, M., Oberdorfer, K., Bock-Hensley, O., & von Baum, H. (2007). value of whole-body washing with chlorhexidine for the

eradication of methicillin-resistant *Staphylococcus aureus*: A randomized, placebo-controlled, double-blind clinical trial. *Infection Control & Hospital Epidemiology, 28*(9), 1036–1043. https://doi.org/10.1086/519929

Widjiastuti, I., Dewi, M., Prasetyo, E., Pribadi, N., & Moedjiono, M. (2020). The cytotoxicity test of calcium hydroxide, propolis, and calcium hydroxide-propolis combination in human pulp fibroblast. *Journal of Advanced Pharmaceutical Technology and Research, 11*(1), 20–24. https://doi.org/10.4103/japtr.JAPTR_88_19

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., Okuom, M., Huber, C., Brooks, T. D., Wilson, E., Clement, B., Wentworth, C. D., & Holmes, A. E. (2017). Quantitative and qualitative assessment methods for biofilm growth: A mini-review. *Research and Reviews: Journal of Engineering and Technology, 6*(4), 1–41.

Wojtyczka, R. D., Dziedzic, A., Idzik, D., Kepa, M., Kubina, R., Kabała-Dzik, A., Smoleń-Dzirba, J., Stojko, J., Sajewicz, M., & Wasik, T. J. (2013). Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs. *Molecules, 18*(8), 9623–9640. https://doi.org/10.3390/molecules18089623

Wong, J., Manoil, D., Näsman, P., Belibasakis, G. N., & Neelakantan, P. (2021). Microbiological Aspects of Root Canal Infections and Disinfection Strategies: An Update Review on the Current Knowledge and Challenges. *Frontiers in Oral Health, 2*, 1–19. https://doi.org/10.3389/froh.2021.672887

Woźniak, M., Sip, A., Mrówczyńska, L., Broniarczyk, J., Waśkiewicz, A., & Ratajczak, I. (2023). Biological activity and chemical composition of propolis from various regions of Poland. *Molecules, 28*(1), 1–19. https://doi.org/10.3390/molecules28010141

Yang, H., Singh, M., Kim, S. J., & Schaefer, J. (2017). Characterization of the tertiary structure of the peptidoglycan of *Enterococcus faecalis*. *Biochimica et Biophysica Acta - Biomembranes, 1859*(11), 2171–2180. https://doi.org/10.1016/j.bbamem.2017.08.003

Yang, J., He, Y., Nan, S., Li, J., Pi, A., Yan, L., Xu, J., & Hao, Y. (2023). Therapeutic effect of propolis nanoparticles on wound healing. *Journal of Drug Delivery Science and Technology, 82*, 1–13. https://doi.org/10.1016/j.jddst.2023.104284

Yang, S., Meng, X., Zhen, Y., Baima, Q., Wang, Y., Jiang, X., & Xu, Z. (2024). Strategies and mechanisms targeting *Enterococcus faecalis* biofilms associated with endodontic infections: a comprehensive review. *Frontiers in Cellular and Infection Microbiology, 14*(14133313), 1–15. https://doi.org/10.3389/FCIMB.2024.1433313/PDF

Yenugu, S., Hamil, K. G., French, F. S., & Hall, S. H. (2004). Antimicrobial actions of the human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and HE2beta2. *Reproductive Biology and Endocrinology, 2*(61). https://doi.org/10.1186/1477-7827-2-61

Zaatout, N. (2021). Presence of non-oral bacteria in the oral cavity. Archives of *Microbiology, 203*(6), 2747–2760. https://doi.org/10.1007/s00203-021-02300-y

Zancan, R. F., Canali, L. C. F., Tartari, T., de Andrade, F. B., Vivan, R. R., & Duarte, M. A. H. (2018). Do different strains of *E. faecalis* have the same behavior towards intracanal medications in *in vitro* research? Brazilian Oral Research, 32(e46), 1–8. https://doi.org/10.1590/1807-3107bor-2018.vol32.0046

Zhao, A., Sun, J., & Liu, Y. (2023). Understanding bacterial biofilms: From definition to treatment strategies. *Frontiers in Cellular and Infection Microbiology, 13*, 1–23. https://doi.org/10.3389/fcimb.2023.1137947

Zheng, J. X., Wu, Y., Lin, Z. W., Pu, Z. Y., Yao, W. M., Chen, Z., Li, D. Y., Deng, Q. W., Qu, D., & Yu, Z. J. (2017). Characteristics of and virulence factors associated with biofilm formation in clinical *Enterococcus faecalis* isolates in China. *Frontiers in Microbiology, 8*(NOV), 1–9. https://doi.org/10.3389/fmicb.2017.02338

Zothanpuia, Passari, A. K., Leo, V. V., Chandra, P., Kumar, B., Nayak, C., Hashem, A., Abd Allah, E. F., Alqarawi, A. A., & Singh, B. P. (2018). Bioprospection of actinobacteria derived from freshwater sediments for their potential to produce antimicrobial compounds. *Microbial Cell Factories, 17*(1), 68. https://doi.org/10.1186/s12934-018-0912-0

Zulhendri, F., Felitti, R., Fearnley, J., & Ravalia, M. (2021). The use of propolis in dentistry, oral health, and medicine: A review. *Journal of Oral Biosciences, 63*(1), 23–34. https://doi.org/10.1016/j.job.2021.01.001

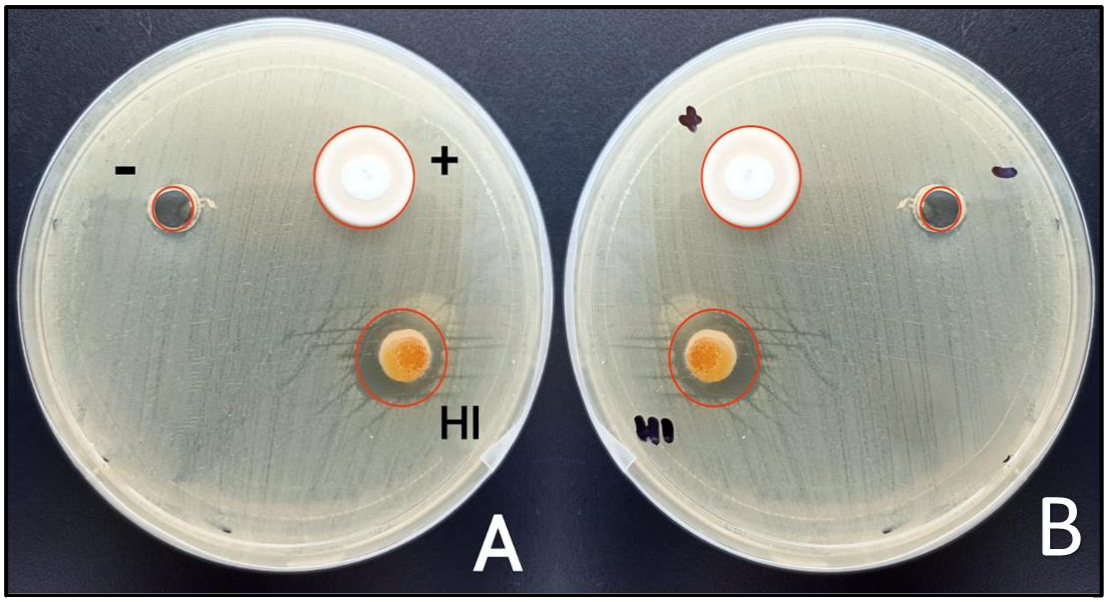
Zulkarnain, Z., & Suprapto, S. (2022). Chemical compounds of bees in Qur'an. Lantanida Journal, 10(2), 86–185. https://doi.org/10.22373/lj.v10i2.14900

Zullkiflee, N., Taha, H., Abdullah, N. A., Hashim, F., & Usman, A. (2022). Antibacterial and antioxidant activities of ethanolic and water extracts of stingless bees *Tetrigona binghami*, *Heterotrigona itama*, and *Geniotrigona thoracica* propolis found in Brunei. *Philippine Journal of Science, 151*(4), 1455–1462. https://doi.org/10.56899/151.04.13

APPENDICES

APPENDIX 1

Intraclass Correlation Coefficient (ICC)



Result of Agar-well Diffusion Methods for ICC Determination, Where (HI) Represents 50 mg/ml *H. Itama* Extract, (+) Represents Calcicur® 45 mg/ml NS-$Ca(OH)_2$ Paste, and (-) Represents 5% DMSO

Case Processing Summary

		N	%
Cases	Valid	12	100.0
	Excluded[a]	0	.0
	Total	12	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.890	2

Intraclass Correlation Coefficient

	Intraclass Correlation[b]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.802[a]	.447	.939	9.087	11	11	<.001
Average Measures	.890[c]	.618	.968	9.087	11	11	<.001

Two-way mixed effects model where people effects are random and measures effects are fixed.

a. The estimator is the same, whether the interaction effect is present or not.

b. Type C intraclass correlation coefficients using a consistency definition. The between-measure variance is excluded from the denominator variance.

c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

Result of ICC via SPSS

Dataset of ZOI Measurement for Agar-well Diffusion Method for Pilot Study

Replicates	Antimicrobial susceptibility test (AST)	
	***H. itama* Zone of Inhibition (mm) - Test on Day 1**	***H. itama* Zone of Inhibition (mm) - Test on Day 3**
Replicate 1 (first reading)	12.0	12.5
Replicate 2 (first reading)	11.5	11.0
Replicate 3 (first reading)	10.0	11.5
Replicate 4 (first reading)	9.0	9.0
Replicate 5 (first reading)	12.0	12.0
Replicate 6 (first reading)	9.5	10.0
Replicate 1 (second reading)	13.0	14.0
Replicate 2 (second reading)	10.0	10.0
Replicate 3 (second reading)	9.0	9.0
Replicate 4 (second reading)	8.5	9.0
Replicate 5 (second reading)	10.0	9.5
Replicate 6 (second reading)	9.0	12.0

APPENDIX 2

Results of AST of the tested samples

AST	*E. faecalis* Strain	Tested Sample					
		H. itama	***G. thoracica***	***T. apicalis***	**PEM**	**45 mg/mL NS-$Ca(OH)_2$**	**2% CHX gel**
Agar-well diffusion (mean ± SD, mm)	ATCC 29212	12 ± 1.2	7 ± 1.5	7 ± 0.6	-	11 ± 1.0	16 ± 0.6
	ATCC 4082	13 ± 2.1	8 ± 1.2	7 ± 0.6	-	12 ± 1.7	18 ± 0.6
MIC (median (IQR), mg/mL)	ATCC 29212	1.56 (1.56)	25.00 (15.63)	25.00 (12.50)	3.13 (1.96)	45.00 (0.00)	2.00 (0.00)
	ATCC 4082	0.78 (0.78)	12.50 (6.25)	25.00 (12.50)	0.78 (0.78)	45.00 (0.00)	2.00 (0.00)
MBC (median (IQR), mg/mL)	ATCC 29212	6.25 (7.81)	50.00 (0.00)	50.00 (0.00)	12.50 (18.75)	45.00 (0.00)	2.00 (0.00)
	ATCC 4082	6.25 (3.13)	50.00 (0.00)	50.00 (0.00)	12.50 (12.50)	45.00 (0.00)	2.00 (0.00)
Antibiofilm (mean ± SD, %)	ATCC 29212	61.71 (3.56)	45.74 (4.54)	5.53 (9.88)	55.69 (4.44)	76.53 (2.13)	92.40 (1.70)
	ATCC 4082	69.84 (3.02)	48.73 (2.20)	10.51 (5.89)	56.86 (3.87)	78.33 (2.79)	91.31 (1.18)
Anti-adherence (mean ± SD, %)	ATCC 29212	68.50 (4.11)	34.91 (10.66)	-22.55 (10.50)	27.01 (13.71)	18.57 (7.05)	85.54 (0.65)
	ATCC 4082	76.54 (2.03)	33.87 (3.70)	-17.51 (7.02)	47.12 (8.68)	29.75 (5.81)	90.07 (0.70)

APPENDIX 3

Malaysian Journal of Microbiology (MJM) – Journal Publication

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Antimicrobial activity of Malaysian *Heterotrigona itama* propolis against *Enterococcus faecalis* ATCC 4082: A study in endodontic applications

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ABSTRACT

Aims: The antimicrobial properties of Malaysian propolis towards *Enterococcus faecalis* (EF), the most prevalent pathogen in failed endodontics remained obscure. Moreover, methods in oral-propolis research, particularly in selecting the optimal antimicrobial susceptibility test, are still not standardised. This study was aimed to assess the *Heterotrigona itama* (HI) propolis antibacterial activity against EF ATCC 4082, isolated from pulpless teeth and compared it with calcium hydroxide [$Ca(OH)_2$].
Methodology and results: The antimicrobial activity of HI propolis collected from Aasta Stingless Bee Honey Farm, Raub, Pahang, Malaysia, was tested against EF ATCC 4082 using disc diffusion, agar-well diffusion, broth microdilution methods, and scanning electron microscope (SEM) analysis. Calcium hydroxide [$Ca(OH)_2$] and 5% dimethyl sulfoxide (DMSO) were positive and negative controls, respectively. Findings show that the agar-well diffusion method outperforms the disc diffusion method, with a statistically significant larger inhibition zone (Propolis: 10 ± 0.000 mm, $Ca(OH)_2$: 13.3 ± 0.577 mm). Propolis (MIC: 6.25 mg/mL and MBC: 6.25 mg/mL) and $Ca(OH)_2$ (MIC: 1.56 mg/mL and MBC: 1.56 mg/mL) effectively eliminated EF ATCC 4082 and their differences are not statistically significant. SEM analysis revealed the propolis at MBC value effectively lysed the EF ATCC 4082 cell wall compared to the $Ca(OH)_2$.
Conclusion, significance and impact of study: The agar-well diffusion method is a more appropriate method in oral-propolis study. Propolis displayed promising antimicrobial properties, especially against EF and may be a good option for use as an intracanal medication in endodontics. Further tests requiring a longer period to investigate the prolonged antimicrobial effects, antibiofilm and anti-adherence properties, and effects against other EF strains and oral pathogens are suggested.

Keywords: Heterotrigona itama propolis, antimicrobial, *Enterococcus faecalis* ATCC 4082, calcium hydroxide

INTRODUCTION

The diversified microbial population within the human oral cavity is known as the oral microbiome, sometimes called the oral microbiota or oral microflora. With around 700 distinct types of microbial species, the oral microbiota is one of the most complex microbial ecosystems in the human body with inhabitants such as bacteria, like Firmicutes, Bacillus, Proteobacteria, Actinomycetes, as well as fungi, like *Candida* sp. and viruses, like mumps virus and human immunodeficiency virus (HIV) (Lu *et al.*, 2019). These microorganisms co-exist in a symbiotic relationship with the human body in healthy individuals, but dysbiosis may possibly occur via invasion and proliferation within the oral cavity, consequently causing oral infections (Thomas *et al.*, 2021). Numerous oral infections, including tonsillitis, dental caries, periodontitis, and pulpitis, are some of the diseases they cause

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(Sudhakara *et al*., 2018).

In the dental practice, endodontic infections brought on by microbial colonisation within the root canal system pose a serious problem. Due to the microbe's persistent nature, there is a high occurrence of root canal treatment failures. EF, a Gram-positive bacterium, is commonly implicated and is more resilient against current treatment and medication, rendering it a major target in endodontic research (Vidana *et al*., 2011; Pinheiro and Mayer, 2015; Peters *et al*., 2016). Sixty-nine commercially available strains of EF were identified under the American Type Culture Collection (ATCC) (Zancan *et al*., 2018). Only three EF strains are widely studied, namely EF ATCC 29212, EF ATCC 4083, and EF ATCC 4082 (Table 1), with EF ATCC 4082 being scant in comparison.

Notably, one strain-comparative-study assessed the resistance of EF ATCC 29212 (from urine) to various intracanal medicaments and compared it with EF ATCC 4083 (from pulpless teeth), revealing that EF ATCC 4083 is more susceptible to $Ca(OH)_2$ (Zancan *et al*., 2018).

Common intracanal medicaments, such as non-setting calcium hydroxide [$Ca(OH)_2$] appear to be ideal substances, with a pH ranging from 12.5-12.8 (Dohyun and Euiseong, 2014). However, they are neutralised by the buffering abilities of both dentin and EF. Dentin contains hydroxyapatite, a mineral that helps maintain a stable pH by buffering acidic environments in $Ca(OH)_2$-induced-alkaline-environment, it will release calcium and phosphate ions which reduce $Ca(OH)_2$ antimicrobial effectiveness (Slutzky-Goldberg *et al*., 2013; Carvalho *et al*., 2015). This buffering effect contributes to its resistance to pH changes, which can influence microbial activity and survival. Furthermore, EF, a resilient microbe commonly associated with failed endodontic treatments, is equipped with mechanisms to regulate its intracellular pH even in acidic conditions. The proton pump in EF facilitates the active transport of protons out of the cell, thereby maintaining cellular pH homeostasis. This ability allows EF to survive in hostile and acidic environments found in infected root canals (John *et al*., 2015). This failure to eradicate microbes after primary-endodontic treatment has led to studies aimed at discovering alternative substances from natural products, with stingless bee (SLB) propolis being one of many options (John *et al*., 2015; Shabbir *et al*., 2020).

Propolis is a blend of beeswax, saliva, and various plant components (Almuhayawi, 2020) that exhibits biological activities such as antimicrobial, anti-inflammatory, anti-oxidant, anti-obesity, anti-cancer, and antiseptic properties (Al-Hariri, 2011; Salleh *et al*., 2022). In Malaysia, *Heterotrigona itama* (HI) is a prevalent SLB species renowned for its propolis, which possesses robust antimicrobial effects attributed to its high concentrations of flavonoids, phenolic compounds, cinnamic acid, and other bioactive constituents (Santos, 2012; Gyawali and Ibrahim, 2014; Ibrahim *et al*., 2016; Abdullah *et al*., 2020). Additionally, Malaysian HI propolis possess strong antioxidant and anti-radical activities (Awang *et al*., 2018). Due to its outstanding biological and medicinal activities, propolis from HI species in Malaysia is used in this study.

Indicative parameters via SEM, polymerase chain reaction (PCR), and proteomics elucidated the destructive effect of propolis on microorganisms by disclosing significant cell damage due to the membrane-rupturing and vesicle-forming effect on *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Candida albicans* (Navarro-Pérez *et al*., 2021; Stähli *et al*., 2021). SEM also reveals antibiofilm and anti-adherence effects of propolis, with a substantial recorded reduction in biofilm formation and live cells of the oral pathogens, including EF ATCC 29212, *Porphyromonas gingivalis* ATCC 33277, *Streptococcus mutans* ATCC 25175, *Fusobacterium nucleatum* ATCC 25586, *Treponema denticola* ATCC 35405, and *Candida albicans* ATCC 76615 (Ong *et al*., 2017; Stähli *et al*., 2021).

Furthermore, the methods for screening the antimicrobial properties of propolis in oral research are not standardised. Selecting the optimal primary antimicrobial screening tests among disc diffusion and agar-well diffusion techniques remain obscure. High viscosity of propolis and $Ca(OH)_2$ will directly affect its diffusion through the agar. Hence, choosing the appropriate method in the propolis study is crucial to obtain sensitive and precise results.

In order to investigate the extent of HI propolis in inhibiting the growth of oral endodontic post-treatment pathogens, like EF, it is vital to determine the range of therapeutic and biological effects due to its composition variation. As EF ATCC 29212 has been frequently used in the oral study, it is more sensible to study other strains isolated from the oral region because the strain of EF ATCC 29212 is mainly isolated from urine. EF ATCC 4082, a strain isolated from human pulpless teeth, was selected as the test bacterium in this study due to its close association with the intended target of intracanal medicaments and its ability to accurately represent clinically relevant scenarios.

This research aims to identify the optimal antimicrobial screening test (AST) between agar-well diffusion and disc diffusion methods in oral-propolis research and to assess the antibacterial properties of HI propolis and $Ca(OH)_2$ in relation to their efficacy against EF ATCC 4082 using broth microdilution methods and scanning electron microscope (SEM) analysis.

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Table 1: Different EF strains used in previous research.

EF strains	Isolation source	Research outcome	Reference
EF ATCC 29212	Urine	The addition of 5% propolis nanoparticles to commercial bio-ceramic and epoxy resin sealers can eliminate EF ATCC 29212 in the root canals.	(Wahyuni *et al.*, 2023)
		Propolis nanoparticles were equally as effective as 6% sodium hypochlorite (NaOCl) and 2% chlorhexidine (CHX) in reducing the EF ATCC 29212 biofilms.	(Parolia *et al.*, 2020)
		The antibacterial activity of propolis against EF ATCC 29212 was comparable with that of calcium hydroxide at different time intervals.	(Ahangari *et al.*, 2018)
		The susceptibility of EF ATCC 29212 and EF ATCC 4083 against different endodontic pastes were compared. It was found that EF ATCC 4083 was more susceptible to all intracanal medicaments used.	(Zancan *et al.*, 2018)
		The results showed that propolis was effective in inhibiting Gram-positive bacteria (including EF) and it was suggested that propolis could be as effective as CHX.	(Akca *et al.*, 2016)
		Brazilian brown propolis effectively inhibited EF ATCC 29212 *in vitro* study, suggesting its potential as an intracanal medication.	(Pimenta *et al.*, 2015)
		Turkish propolis samples were effective against EF ATCC 29212, but their activity did not exceed CHX.	(Kayaoglu, *et al.*, 2011)
EF ATCC 4083	Root canal of pulpless tooth	EF ATCC 4083 was more susceptible to all tested intracanal medicaments compared to EF ATCC 29212. Medication used are listed below: • $Ca(OH)_2$ • $Ca(OH)_2$ and polyethylene glycol • $Ca(OH)_2$, polyethylene glycol and camphorated paramonochlorophenol • $Ca(OH)_2$, CHX, and propylene glycol • $Ca(OH)_2$, and double antibiotic paste • Triple antibiotic paste	(Zancan *et al.*, 2018)
EF ATCC 4082	Root canal of pulpless tooth	2 mL of BioPure Mixture of tetracycline, citric acid, and detergent (MTAD) was more effective than 3% NaOCl and propolis against EF ATCC 4082.	(Nara *et al.*, 2010)
EF ATCC 4082 and EF ATCC 4083	Root canal of pulpless tooth	Treatment with 4 to 5 mL of BioPure MTAD was effective in eliminating the growth of EF ATCC 4082 and EF ATCC 4083	(Newberry *et al.*, 2007)
EF ATCC 51299	Peritoneal fluid	$Ca(OH)_2$ significantly reduced fibroblast viability, more than Iranian propolis	(Jahromi *et al.*, 2012)
EF ATCC 29532 EF ATCC 49383 EF ATCC 49452 EF ATCC 49477 EF ATCC 10541 EF ATCC 19433	Blood Blood Not Disclosed Clinical isolate Not Disclosed Not disclosed	Treatment regimen (BioPure MTAD as a final irrigant in conjunction with 1.3% NaOCl) was effective in completely eliminating growth of EF ATCC 4082, EF ATCC 4083, EF ATCC 49532, EF ATCC 49383, EF ATCC 49452, EF ATCC 49477, EF ATCC 10541, and EF ATCC 19433.	(Newberry *et al.*, 2007)

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MATERIALS AND METHODS

Propolis collection

For the extraction process, HI propolis was obtained from Aasta Stingless Bee Honey Farm, Raub, Pahang, Malaysia with a grid reference: 3.8210721, 101.8407539.

Preparations of dry ethanolic extracts of propolis

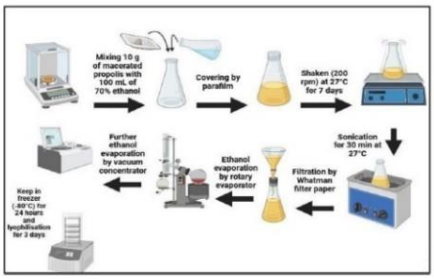

Figure 1: Schematic diagram of the preparation of dry ethanolic extracts of HI propolis using shaking-assisted extraction (SAE) method.

The HI propolis shaking-assisted extraction process was adapted from studies (Al-Qurashi and Awad, 2018; Pobiega ***et al.***, 2019; Al-Masoodi ***et al.***, 2022) with minor modification by incorporating the vacuum concentrator in the process. Ten grams of macerated raw HI propolis were diluted 1:10 (w/v) in 100 mL of 70% ethanolic solution with the mixture, shaken at 200 rpm at room temperature for 7 days (SK-600 shaker, Jeiotech, Korea), sonicated for 30 min at 27 °C at medium setting (Ultrasonic, Jeiotech, Korea) and finally filtered overnight using Whatman No. 1 filter paper. Further condensing processes at 40 °C under reduced pressure (Rotavapor R-210, Buchi, Switzerland) and concentrated using a vacuum concentrator (Genevac™ miVAC, United Kingdom) for 2 h at 40 °C were carried out. The evaporated filtrate was stored overnight at –80 °C before being lyophilised using FreeZone, Labconco, US for 3 days, as shown in Figure 1. The powdered extract yield was measured and stored at –25 °C. Subsequent reconstitution was carried out by dissolving in 5% dimethyl sulfoxide (DMSO). A concentration of 5% DMSO was used as it is safe and does not interrupt the bacterial cell during the antimicrobial test (Al-Masoodi ***et al.***, 2022). Subsequently, pH of the HI propolis extract was determined using a pH test strip.

Preparation of non-setting $Ca(OH)_2$

Ready-to-use Calcicur® non-setting $Ca(OH)_2$ paste (45% $Ca(OH)_2$ content) was purchased from NDent Sdn Bhd and directly used according to the manufacturer's instructions for clinical application, without dilution.

Preparation of EF ATCC 4082 suspension

The bacterial stock was prepared based on a previous study (Shakhatreh ***et al.***, 2017), for long-term preservation. EF ATCC 4082 from the previous stock culture stored at –80 °C was thawed and revived by streaking it onto the brain-heart infusion (BHI) agar plate which was subsequently incubated aerobically at 37 °C for 24 h. Further confirmation of EF ATCC 4082 colonies was performed using the Gram staining method, adapted from CLSI guidelines (Smith and Hussey, 2005) to determine the purity of the bacterial stock and observed using a microscope (Olympus CX23) at 100× magnification. Then, a single bacterial colony from the BHI agar plate was incubated in 10 mL of BHI broth at 37 °C until the late-log phase of growth (6-8 h). The EF ATCC 4082 suspension was centrifuged at 5000 rpm, 4 °C for 10 min. The supernatant was removed, and the pellet was resuspended with 5 mL fresh BHI broth and 5 mL of 50% glycerol solution (in a 1:1 ratio), mixed, and transferred 1 mL in a 1.5 mL cryovial tube, and stored at –80 °C for future use.

The preparation and standardisation of the bacterial suspension were carried out according to the CLSI (2012) guidelines. An amount of 1 mL of prepared EF ATCC 4082 stock solution was mixed into 10 mL BHI broth and incubated until the late-log phase was reached. Then, the turbidity was standardised by adjusting the absorbance at 600 nm to 0.08-0.13 (equivalent to 1.00×10^8 CFU/mL) using a spectrophotometer (SECOMAM Prim Light, France) (Al-Masoodi ***et al.***, 2022; Woitschach ***et al.***, 2022). The purity of the bacterial culture was checked using Gram-staining, prior to every test.

In vitro antimicrobial susceptibility test (AST)

The antimicrobial susceptibility test (AST) of HI propolis extract against EF ATCC 4082 was conducted using agar-well diffusion and disc diffusion methods for assessment the best method suitable for propolis, as well as the broth microdilution method. $Ca(OH)_2$ and 5% DMSO served as positive and negative controls, respectively. The experiment was carried out in three biological replicates, each with three technical replicates (n=18) for each sample, except for agar-well diffusion and disc diffusion method (only three biological replicates with n=9).

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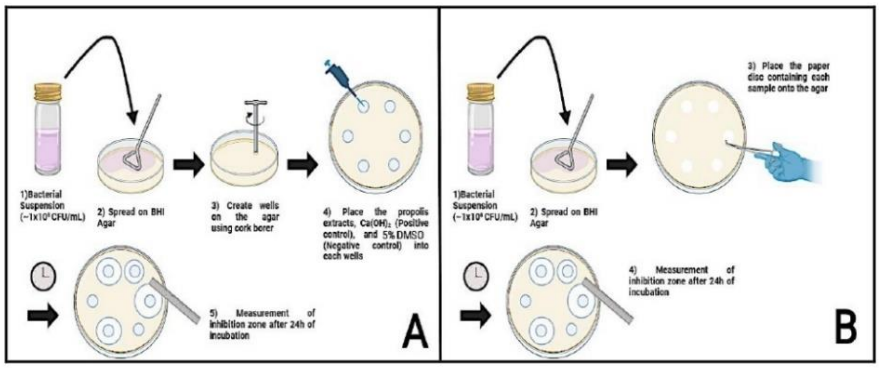

Figure 2: Antimicrobial susceptibility test (AST) of (A) agar-well diffusion method and (B) disc diffusion method.

Agar-well diffusion method

Agar-well diffusion method was run, according to the procedure described by researchers and guidelines (Gebreyohannes ***et al.***, 2013; Balouiri ***et al.***, 2016; EUCAST, 2023). The standardised suspension of EF ATCC 4082 (1.00 × 10^8 CFU/mL) was spread evenly onto the surface of BHI agar using a sterile cotton swab and wells were created on the agar plate using a sterile cork-borer (6 mm in diameter). A 100 µL of 50 mg/mL HI propolis extract was carefully dispensed into the wells, in triplicates and allowed to diffuse for 2 h before incubating at 37 °C for 24 h. Then, 100 µL of 5% DMSO and $Ca(OH)_2$ were used as negative and positive controls respectively. After 24 h of incubation, the zone of inhibition around each well was recorded.

Disc diffusion method

The disc diffusion method was performed with minor modification, as described by Gebreyohannes ***et al.*** (2013). A sterile cotton swab dipped into standardised EF ATCC 4082 suspension (1.00 × 10^8 CFU/mL) was spread uniformly onto the BHI agar surface. The inoculated plates were left at room temperature for 3-5 min to let absorption of surface moisture, before applying the extract. A volume of 10 µL of 50 mg/mL HI propolis extract was doused onto a filter 6 mm diameter paper disc and placed on the surface of BHI agar. The same procedure was repeated for $Ca(OH)_2$ (positive control) and 5% DMSO (negative control). A similar experiment was repeated three times, each with three technical replicates. The plates were then incubated at 37 °C for 24 h and the zone of inhibitions (in mm) were measured (Gebreyohannes ***et al.***, 2013; Balouiri ***et al.***, 2016; EUCAST, 2023).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC values were determined using the broth microdilution method adhering to the CLSI standard protocol (2012). Each MIC and MBC determination involved a total of 9 samples, where 3 each were of HI propolis extract, $Ca(OH)_2$, and 5% DMSO. In a 96-well microplate, 100 µL of BHI broth was dispensed into wells 1 to 11, while 200 µL of BHI broth was added to well 12 (media control). Subsequently, 100 µL of the HI propolis extract solution was added to well 1 and mixed using a micropipette. A 2-fold serial dilution was carried out by transferring 100 µL of the solution sequentially from well 1 to well 2, continuing this process up to well 10. From well 10, 100 µL was removed to achieve a range of final concentrations (50-0.1 mg/mL) in the respective well. Next, the standardised EF ATCC 4082 culture (1.00 × 10^8 CFU/mL) was diluted to a concentration of 1.00 × 10^6 CFU/mL and 10 µL of bacterial suspension was added to wells number 1 to 10, including 11 ($Ca(OH)_2$, positive control) and 12, giving a final concentration of EF ATCC 4082 in each well was 1.10 × 10^5 CFU/mL. On another microplate, 110 µL of the two-fold serially diluted HI propolis extract only was added which served as blank control. The microplate was incubated at 37 °C overnight, the visible bacterial growth was observed and measured using a microplate reader at 600 nm, for enhanced accuracy. Following the measurement, the turbidity was determined by comparing the absorbance of the suspension in wells of Hi propolis extract with the blank control. A similar experiment was repeated three times, each with three technical replicates.

Determination of the MBC was adapted from research by Gebreyohannes ***et al.*** (2013), see Figure 3. The well contents, showing no observable bacterial growth were spread on BHI agar plates using a sterile cotton swab and

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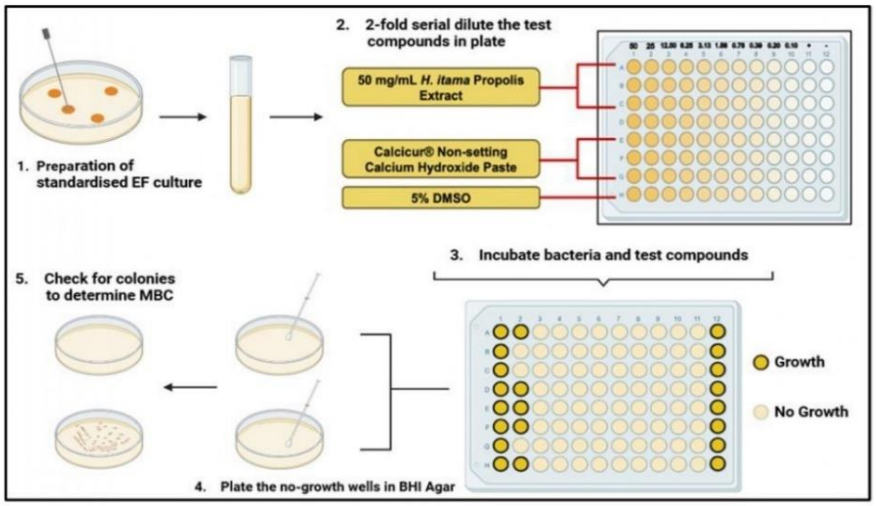

Figure 3: Determination of MIC and MBC.

growth in each agar section was examined. A well-containing concentration of HI propolis with no bacterial growth indicates the MBC value.

SEM analysis

The SEM analysis was conducted based on a method outlined with some modifications (Yenugu ***et al.***, 2004). The standardised EF ATCC 4082 suspension (equivalent to 1.00 × 10^8 CFU/mL) was prepared and mixed separately with the HI propolis extract and $Ca(OH)_2$ at MBC concentration accordingly, with 5% DMSO used as a negative control. Following a 24 h incubation period, the bacterial suspension underwent centrifugation at 10000 × *g* for 10 min at 4 °C, and the supernatant was removed to isolate the pellet which was then treated with 4% glutaraldehyde in 0.1M phosphate buffer pH 7.2 for 1 h. Subsequently, the pellet was dehydrated using a series of ethanol concentrations (30%, 50%, 70%, 90%, and 100%) at 15 min intervals to preserve the integrity of the cellular structure (Navarro-Pérez ***et al.***, 2021). After air drying for 30 min at 37 °C, the pellet was gold-coated under low pressure using a sputter coater prior to SEM observation. Cell morphology was examined at magnifications of 10,000× and 15,000× using a scanning electron microscope (Hitachi TM3000, Tokyo, Japan). The diameter of EF ATCC 4082 before and after being treated with respective samples was captured using ImageJ software.

Statistical analysis

The experiments were repeated and analysed using three biological replicates, each with three technical replicates and expressed as a mean ± standard deviation (SD) for AST (n=18) and median (IQR) for both MIC (n=9) and MBC (n=9). The data collected from the AST, MIC, and MBC were subjected to the Shapiro-Wilk test for normality testing and paired samples *t*-test ($p < 0.05$) was used to compare the level of significance between samples by using SPSS version 27.

RESULTS AND DISCUSSION

Extraction analysis

Propolis in its natural raw form cannot be utilised directly for research or therapy but must first be extracted to release its active compounds (Pobiega ***et al.***, 2019). Selecting an appropriate method for propolis extraction is vital to obtain optimum biological activity. The shaking-assisted method combined with ultrasonication as shown in Figure 1, was proven to produce higher yield, antimicrobial action, phenolic and flavonoid contents compared to other extraction processes (Pobiega ***et al.***, 2019). Another determining factor in acquiring propolis extract with ideal biological activity is the selection of extractant. Various solvents (ethanol, methanol, water, hexane, acetone, dichloromethane, and chloroform) result in an extract with different biological effects (Przybyłek and Karpiński, 2019). Most findings ascertained that

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Table 2: Yield and pH of ethanolic HI propolis extract.

Species	Sample weight (g)	Extract weight (g)	Extract yield (%)	pH
Heterotrigona itama	10	4.46	44.6	6.6
	10	3.94	39.4	6.6
	10	3.65	36.5	6.6
Mean ± SD	-	4.02 ± 0.41	40.17 ± 4.10	6.6 ± 0.0

ethanol as an extractants for propolis lead to high concentration of phenolic and flavonoid, inducing the antimicrobial properties (Przybyłek and Karpiński, 2019). In our study, the mean yield of ethanolic HI propolis extract was 40.17 ± 4.10% with a pH 6.6 (Table 2).

Purity Confirmation of EF ATCC 4082

Each freshly prepared stock and inoculum was subjected to Gram-staining method to determine its purity and avoid contamination. EF ATCC 4082 cells exhibit cocci-shape in clusters or long chain, and appeared to be purple in colour, indicating a Gram-positive bacterium, as shown in Figure 4.

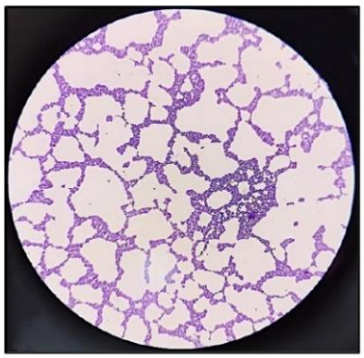

Figure 4: Gram-Staining of EF ATCC 4082 with oil emulsion light microscope (100× magnification).

Antimicrobial susceptibility test

Table 3 shows the mean of the inhibition zone, where the HI propolis extract (at 50 mg/mL) is slightly lower when compared with $Ca(OH)_2$ for both AST methods. Apart from that, it also shows that the agar-well diffusion method yields a larger and clearer inhibition zone compared to disc diffusion as shown in Figure 5. A paired samples *t*-test was performed to compare the mean of the inhibition zone

Table 3: Inhibitory zones of HI propolis extract and $Ca(OH)_2$ against EF ATCC 4082 using agar-well diffusion and disc diffusion methods.

Sample group	Zone of inhibition (mean ± SD) (mm)	
	Agar-well diffusion method	Disc diffusion method
HI propolis extract (50 mg/mL)	10.0 ± 0.000	9.3 ± 0.577
$Ca(OH)_2$	13.3 ± 0.577	8.5 ± 2.500
5% DMSO	6.00 ± 0.000	6.00 ± 0.000

Note: The results are shown in mean ± standard deviation (SD) from three biological replicates (n=9).

produced in agar-well diffusion and disc diffusion methods. There was a significant difference in the inhibition zone produced between the agar-well diffusion method (Mean = 12.3 mm, SD = 1.751) and disc diffusion method (Mean = 8.3 mm, SD = 2.425); $t(11) = 9.950$, $p < 0.001$).

In selecting the primary screening method for AST for propolis in oral studies, the results favoured the agar-well diffusion method over the disc diffusion method, producing significantly larger and clearer inhibition zones, as shown in Table 3. This is due to the disadvantages of the disc diffusion method where samples with water-insoluble substances will prevent the diffusion of antimicrobial substances into the media. In addition, the agar-well diffusion method allows a higher volume of test compound to diffuse across the media, allowing a broader inhibition zone. The difference in inhibition zone occurs as agar-well diffusion may be suitable with compound of high concentration, more viscous, poor surface adhesion, and larger molecular weights (Hossain, 2024). Currently, no research has been conducted to determine optimal antimicrobial screening techniques for propolis extract. One study pointed out that certain compounds, such as cationic substances, may adhere to the disc's surface, affecting result accuracy (Valgas *et al*., 2007).

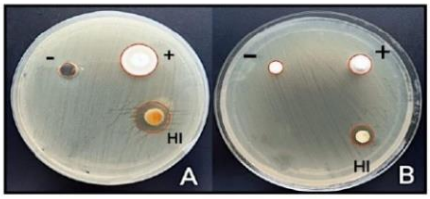

Figure 5: Inhibition zones of HI propolis extract towards EF ATCC 4082 using agar-well diffusion (A) and disc diffusion (B) methods, with $Ca(OH)_2$ (+) and 5% DMSO (-) controls.

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MIC of HI propolis extract and $Ca(OH)_2$ against EF ATCC 4082

The outcomes in Table 4 highlights the MIC values of $Ca(OH)_2$ (1.56 mg/mL) when tested against EF ATCC 4082, which are lower than the MIC values observed for HI propolis (6.25 mg/mL). This comparison implies that $Ca(OH)_2$ exhibits greater efficacy in inhibiting the growth of the EF ATCC 4082. Our study did not compare with EF ATCC 29219, and this may be interesting to note the differences, because one is sourced from urine while EF ATCC 4083 derived from pulpless teeth.

MBC of HI propolis extract and $Ca(OH)_2$ against EF ATCC 4082

Table 4 results show $Ca(OH)_2$, with an MBC value of 1.56 mg/mL (Figure 6), to be more effective in killing EF ATCC 4082 than the HI propolis extract, with an MBC value of 6.25 mg/mL. This highlights the comparative analysis that $Ca(OH)_2$ has lower MIC and MBC values compared to HI propolis extract. However, the difference is not statistically significant based on paired samples *t*-test ($p > 0.05$).

Table 4: Result of MIC and MBC of 50 mg/mL HI propolis extract and $Ca(OH)_2$ against EF ATCC 4082.

Sample Group	MIC Median (IQR) (mg/mL)	MBC Median (IQR) (mg/mL)
HI propolis extract (50 mg/mL)	6.25 (6.25)	6.25 (9.37)
$Ca(OH)_2$	1.56 (1.57)	1.56 (2.35)

Notes: The results are shown as median (IQR) of three biological replicates, each with three technical replicates (n=9).

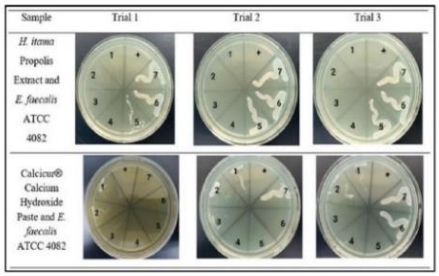

Figure 6: Results of MBC for HI propolis extract and $Ca(OH)_2$.

However, these findings are limited to a 24 h observation period compared to other research (Sjögren ***et al.***, 1991; Jahromi ***et al.***, 2012; Pimenta ***et al.***, 2015; Awawdeh ***et al.***, 2018). They were mostly conducted for a significantly longer period, ranging from 3 to 14 days. Nevertheless, research has found that stingless bee propolis is more potent in terms of both quantity and quality when compared to honeybee propolis (Salleh ***et al.***, 2022). Stingless bee propolis has drawn the attention of researchers due to its exceptional antimicrobial properties, which may have applications in the medical field. These include the treatment of oral infections such as dental cavities, gingivitis, periodontitis, and oral ulcers (Almuhayawi, 2020).

Despite extended treatment with $Ca(OH)_2$, EF may well survive in dentinal tubules by maintaining viability and surviving independently from other bacterial species (Awawdeh ***et al.***, 2009). Plausible explanations have been proposed to explain EF's ability to withstand the $Ca(OH)_2$ based on a study by John ***et al***. (2015) as EF is equipped with a proton pump that actively expels protons out of the cell, maintaining pH balance, which enables EF to survive in the harsh, acidic environment of treated root canals.

$Ca(OH)_2$ was shown to be more effective in inhibiting EF ATCC 4082 in our study. However, most research revealed that propolis has a higher antimicrobial capacity when compared to $Ca(OH)_2$. This is owing to the limitations of our study, which only observes antimicrobial activity for 24 h. While intracanal medicines containing $Ca(OH)_2$ showed heightened antibacterial activity in the first two days of usage, their efficiency rapidly diminished and became ineffective after 10 days. Propolis extract, on the other hand, revealed a slower but more sustained antibacterial action, lasting more than 10 days (Jahromi ***et al.***, 2012); moreover, EF may remain in dentinal tubules even after lengthy $Ca(OH)_2$ treatment. EF may well retain viability and survive independently of other bacterial species (Awawdeh ***et al.***, 2009). Additionally, when subjected to complete irrigation with sodium hypochlorite, propolis exhibited superior removal efficiency compared to $Ca(OH)_2$, emphasising its heightened effectiveness in endodontic applications (Ahmed, 2021). Showcasing its efficacy, propolis may possibly be an effective inhibitor of EF growth via its prolonged action, which may position it as a promising intracanal medicament replacement (Elsayed ***et al.***, 2021).

To our knowledge, most studies conducted in oral research were utilising EF ATCC 29212 (urine), instead of EF ATCC 4082 (pulpless teeth). The strain isolated from urine may develop higher resistance due to the urinary tract's extreme environment caused by diseases, diets, and medications (Zancan ***et al.***, 2018). Nonetheless, one study indicates that EF ATCC 4083, also a strain isolated from pulpless teeth, is more suitable for oral studies rather than ATCC 29212 as it is less resistant and has a relationship with the intended target, root intracanal medicament (Zancan ***et al.***, 2018). It is unclear regarding the difference between EF ATCC 4082 and 4083, where EF ATCC 4082 strain did not show resistance towards $Ca(OH)_2$ (Zancan ***et al.***, 2018).

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Cell morphology of EF ATCC 4082 by SEM analysis

The cell morphology of EF ATCC 4082 after being treated with HI propolis extract at MIC value (6.25 mg/mL), $Ca(OH)_2$, and 5% DMSO was evaluated by SEM analysis with 10,000× and 15,000× magnifications. Based on SEM images in Figure 7, normal cell morphology of EF ATCC 4082 can be observed after treatment with 5% DMSO (Figure 7A and Figure 7B), where elongated oval shape and smooth cell surface with an average diameter measurement of 3-4 µm per cell can be observed. The cell wall structure of EF ATCC 4082 remains intact, and some cells showed evidence of budding stages, as shown in the highlighted red circle. However, the EF ATCC 4082 cell morphology, size, and shape undergo notable changes when treated with HI propolis extract and $Ca(OH)_2$.

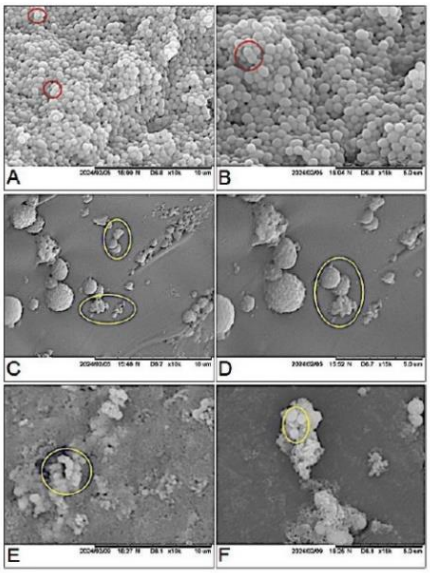

Figure 7: The EF ATCC 4082 cell morphology viewed using SEM, where it was treated with 5% DMSO [A (10,000× magnification) and B (15,000× magnification)], HI propolis extract (at MIC of 6.25 mg/mL) [C (10,000× magnification) and D (15,000× magnification)], and $Ca(OH)_2$ [E (10,000X magnification) and F (15,000× magnification)]. The red circle indicates the healthy, budding stage of the EF ATCC 4082, while the yellow circle indicates the destruction of the EF ATCC 4082 cell.

After treatment with HI propolis extract and $Ca(OH)_2$, major changes such as shrinkage, cell aggregation, and a decrease in cell numbers were observed in the EF ATCC 4082 strain. The cell shape became more rounded with a rough and irregular surface. No budding stage can be seen, and the integrity of the EF ATCC 4082 cell wall is lost, leading to rupture and losing its cell content. These alterations caused by the effect of $Ca(OH)_2$ and HI propolis extract's activity eventually cause the EF ATCC 4082 cell death. However, a higher number of healthy cells can be seen after incubation with $Ca(OH)_2$, but the shrinking effect is much more significant (Figure 7E and Figure 7F).

It is essential to comprehend how HI propolis extract affects EF ATCC 4082 proliferation and morphology to assess its potential as an alternative therapy in endodontic treatment. Through the SEM analysis, it is evident that HI propolis and $Ca(OH)_2$ effectively killed and prevented the growth of EF ATCC 4082 strain. Most of the EF ATCC 4082 cells treated with these substances exhibited shrinkage, irregular cell walls, a low number of live cells, and an inability to multiply, as no budding stages were observed. However, some healthy, normal EF ATCC 4082 cells were still seen in the $Ca(OH)_2$ treatment. No study has directly observed the effect of propolis on the EF ATCC 4082 morphology. However, similar findings have been reported in other research where the EF ATCC 29212 biofilm coverage and live cell counts significantly decreased after treatment with ethanolic extract of Malaysian bee propolis (Parolia ***et al.***, 2020). The antimicrobial effect of propolis is further supported by worldwide studies (using ethanolic extract of Brazilian red propolis, Brazilian green propolis, and Spanish propolis) showing its antibiofilm, anti-adherence and cell structural-altering effect on ***P. gingivalis***, ***S. mutans***, ***S. sanguinis***, and ***C. albicans*** (Yoshimasu ***et al.***, 2018; Navarro-Pérez ***et al.***, 2021; Stähli ***et al.***, 2021). These significant structural and functional damages are likely caused by the high concentration of flavonoids in propolis, which contributes to its antibacterial properties (Parolia ***et al.***, 2020; Nichitoi ***et al.***, 2021; Sa-Eed ***et al.***, 2023).

The normal, healthy EF ATCC 4082 cell was calculated to have an average diameter of 4.05 ± 0.405 µm, via Image J. The EF ATCC 4082 shrank after being treated with $Ca(OH)_2$ (3.12 ± 0.818 µm). Conversely, an increase in EF ATCC 4082 diameter was observed after being treated with HI propolis extract (8.63 ± 3.377 µm).

Table 5: EF ATCC 4082 diameter after each treatment observed with SEM analysis.

Treatment	EF ATCC 4082 diameter (Mean ± SD) (µm)
5% DMSO (negative control)	4.05 ± 0.405
HI propolis extract (6.25 mg/mL)	8.63 ± 3.377
$Ca(OH)_2$	3.12 ± 0.818

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A decrease in the diameter of EF ATCC 4082 cells was observed after being treated with $Ca(OH)_2$ (Table 5). The EF ATCC 4082 membrane's integrity is altered by the high pH of $Ca(OH)_2$ through the breakdown of phospholipids and chemical damage to the organic components. This led to the formation of wrinkles, shrinkage of EF cells, and eventually cell death (Mohammadi *et al*., 2012). As for EF ATCC 4082 treatment with HI propolis extract, the structure and fluidity of the membrane are compromised by this process, causing the cell to swell excessively, and ultimately results in cell lysis and the release of cellular contents (Almuhayawi, 2020; Otręba *et al*., 2022).

Based on microanalysis through SEM, EF ATCC 4082 cell disruption and death can be justified by several findings. The synergism among propolis bioactive compounds, including flavonoids (pinocembrin, pinostrobin, apigenin, galangin, and pinostrobin), phenolic acid, esters, and aromatic compounds, is believed to contribute to its antimicrobial action (Almuhayawi, 2020). Three possible pathways of propolis's antimicrobial action have been explained based on previous research. Propolis bioactive compounds attach to the bacterial cell wall, inducing the formation of transient pores or channels in the membrane, further compromising its integrity and function. Bioactive substances found in propolis, especially phenolic acids and flavonoids, integrate into the lipid bilayer of bacterial membranes. This process compromises the membrane structure and fluidity, which eventually leads to cell lysis and the release of cellular contents. Propolis bioactive components can block the function of membrane-bound enzymes necessary for bacterial survival and pathogenicity. For instance, certain flavonoids and phenolic acids inhibit ATPase enzymes involved in energy metabolism and ion transport across. By impairing these vital enzymatic activities, propolis disrupts membrane integrity and function, ultimately resulting in bacterial cell death (Przybyłek and Karpiński, 2019; Almuhayawi, 2020; Arief *et al*., 2022; Otręba *et al*., 2022).

CONCLUSION

This study concluded the agar-well diffusion method is an accurate screening method to evaluate the efficacy of HI propolis. $Ca(OH)_2$ possesses a slightly higher antimicrobial effect compared to HI propolis against EF ATCC 4082 over a 24 h observation period. It is indicated that HI propolis also exhibits decent antimicrobial properties due to the significant morphological changes observed in the EF ATCC 4082 cells via SEM analysis. Propolis may be a promising candidate for use as intracanal medicament in endodontic applications. Further research with a longer observation period, time-kill study, anti-adherence, and anti-biofilm of EF ATCC 4082 acted upon by HI propolis may be useful parameters to consider before formulating a therapeutic design.

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CONFLICT OF INTEREST STATEMENTS

None declared.

REFERENCES

Abdullah, N. A., Zulkiflee, N., Zaini, S. N. Z., Taha, H., Hashim, F. and Usman, A. (2020). Phytochemicals, mineral contents, antioxidants, and antimicrobial activities of propolis produced by Brunei stingless bees *Geniotrigona thoracica*, *Heterotrigona itama*, and *Tetrigona binghami*. *Saudi Journal of Biological Sciences* **27(11), 2902-2911.**

Ahangari, Z., Naseri, M. and Vatandoost, F. (2018). Propolis: Chemical composition and its applications in endodontics. *Iranian Endodontic Journal* **13(3), 285-292.**

Ahmed, M. A. (2021). Removal efficacy of propolis/calcium hydroxide medicaments from the root canal. *Pakistan Journal of Medical Sciences* **37(7), 1948-1952.**

Akca, A., Akca, G., Topçu, F., Macit, E., Pikdöken, L. and Özgen, I. (2016). The comparative evaluation of the antimicrobial effect of propolis with chlorhexidine against oral pathogens: An *in vitro* study. *BioMed Research International* **2016, Article ID 3627463.**

Al-Hariri, M. (2011). Propolis and its direct and indirect hypoglycemic effect. *Journal of Family and Community Medicine* **18(3), 152-154.**

Al-Masoodi, O., Said Gulam Khan, H., Baharuddin, I. and Ismail, I. (2022). *In-vitro* comparison of antibacterial activities on stingless bee propolis using selected extraction methods. *Compendium of Oral Science* **9(2), 23-43.**

Almuhayawi, M. S. (2020). Propolis as a novel antibacterial agent. *Saudi Journal of Biological Sciences* **27(11), 3079-3086.**

Al-Qurashi, A. D. and Awad, M. A. (2018). Postharvest ethanolic extract of propolis treatment affects quality and biochemical changes of 'Hindi-Besennara' mangos during shelf life. *Scientia Horticulturae* **233, 520-525.**

Andre Arief, I. I., Apriantini, A., Jayanegara, A. and Budiman, C. (2022). Antimicrobial activity of propolis extract and their application as a natural preservative in livestock products: A meta-analysis. *Food Science of Animal Resources* **42(2), 280-294.**

Awang, N., Ali, N., Abd Majid, F. A., Hamzah, S. and Abd Razak, S. B. (2018). Total flavonoids and phenolic contents of sticky and hard propolis from 10

species of indo-malayan stingless bees. *Malaysian Journal of Analytical Sciences* **22(5), 877-884**.

Awawdeh, L., Al-beitawi, M. and Hammad, M. (2009). Effectiveness of propolis and calcium hydroxide as a short-term intracanal medicament against *Enterococcus faecalis*: A laboratory study. *Australian Society of Endodontology* **35(2), 52-58.**

Awawdeh, L., Jamleh, A. and Al Beitawi, M. (2018). The antifungal effect of propolis endodontic irrigant with three other irrigation solutions in presence and absence of smear layer: An *in vitro* study. *Iranian Endodontic Journal* **13(2), 234-239.**

Balouiri, M., Sadiki, M. and Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* **6(2), 71-79.**

Carvalho, C. N., Freire, L. G., De Carvalho, A. P. L., Siqueira, E. L., Bauer, J., Gritti, G. C. *et al.* (2015). The influence of dentine on the pH of calcium hydroxide, chlorhexidine gel, and experimental bioactive glass-based root canal medicament. *The Scientific World Journal* **2015(1), 686259.**

CLSI, Clinical and Laboratory Standards Institute. (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. 9th ed. *CLSI Document M07-A9*. **pp. 1-68.**

Dohyun, K. and Euiseong, K. (2014). Antimicrobial effect of calcium hydroxide as an intracanal medicament in root canal treatment: A literature review - Part I. *In vitro* studies. *Restorative Dentistry & Endodontics* **39(4), 241-252.**

Elsayed, R. O., Yagoub, S. O. and Abubakr, N. H. (2021). Evaluation of propolis as intracanal medicament against *Enterococcus faecalis. Open Journal of Stomatology* **11(05), 208-220.**

EUCAST (2023). EUCAST disk diffusion method for antimicrobial susceptibility testing. *European Society of Clinical Microbiology and Infectious Diseases* **13, 1-22.**

Gebreyohannes, G., Moges, F., Sahile, S. and Raja, N. (2013). Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia. *Asian Pacific Journal of Tropical Biomedicine* **3(6), 426-435.**

Gyawali, R. and Ibrahim, S. A. (2014). Natural products as antimicrobial agents. *Food Control* **46, 412-429.**

Hossain, T. J. (2024). Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. *European Journal of Microbiology and Immunology* **14(2), 97-115.**

Ibrahim, N., Zakaria, A. J., Ismail, Z. and Mohd, K. S. (2016). Antibacterial and phenolic content of propolis produced by two Malaysian stingless bees, *Heterotrigona itama* and *Geniotrigona thoracica*. *International Journal of Pharmacognosy and Phytochemical Research* **8(1), 156-161.**

Jahromi, M. Z., Toubayani, H. and Rezaei, M. (2012). Propolis: A new alternative for root canal disinfection. *Iranian Endodontic Journal* **7(3), 127-133.**

John, G., Kumar, K. P., Gopal, S. S., Kumari, S. and Reddy, B. K. (2015). *Enterococcus faecalis*, a nightmare to endodontist: A systematic review. *African Journal of Microbiology Research* **9(13), 898-908.**

Kayaoglu, G., Ömürlü, H., Akca, G., Gürel, M., Genay, Ö., Sorkun, K. *et al.* (2011). Antibacterial activity of propolis versus conventional endodontic disinfectants against *Enterococcus faecalis* in infected dentinal tubules. *Journal of Endodontics* **37(3), 376-381.**

Lu, M., Xuan, S. and Wang, Z. (2019). Oral microbiota: A new view of body health. *Food Science and Human Wellness* **8(1), 8-15.**

Mohammadi, Z., Shalavi, S. and Yazdizadeh, M. (2012). Antimicrobial activity of calcium hydroxide in endodontics: A review. *Chonnam Medical Journal* **48(3), 133-140.**

Nara, A., Dhanu, Chandra, P., Anandakrishna, L. and Dhananjaya. (2010). Comparative evaluation of antimicrobial efficacy of MTAD, 3% NaOCl and propolis against *E. faecalis*. *International Journal of Clinical Pediatric Dentistry* **3(1), 21-25.**

Navarro-Pérez, M. L., Vadillo-Rodríguez, V., Fernández-Babiano, I., Pérez-Giraldo, C. and Fernández-Calderón, M. C. (2021). Antimicrobial activity of a novel Spanish propolis against planktonic and sessile oral *Streptococcus* spp. *Scientific Reports* **11(1), 1-10.**

Newberry, B. M., Shabahang, S., Johnson, N., Aprecio, R. M. and Torabinejad, M. (2007). The antimicrobial effect of biopure MTAD on eight strains of *Enterococcus faecalis*: An *in vitro* investigation. *Journal of Endodontics* **33(11), 1352-1354.**

Nichitoi, M. M., Josceanu, A. M., Isopescu, R. D., Isopencu, G. O., Geana, E. I., Ciucure, C. T. *et al.* (2021). Polyphenolics profile effects upon the antioxidant and antimicrobial activity of propolis extracts. *Scientific Reports* **11(1), 1-12.**

Ong, T. H., Chitra, E., Ramamurthy, S., Siddalingam, R. P., Yuen, K. H., Ambu, S. P. *et al.* (2017). Chitosan-propolis nanoparticle formulation demonstrates anti-bacterial activity against *Enterococcus faecalis* biofilms. *PLoS ONE* **12(3), 1-22.**

Otręba, M., Marek, Ł., Tyczyńska, N., Stojko, J., Kurek-Górecka, A., Górecki, M. *et al.* (2022). Propolis as natural product in the oral cavity bacterial infections treatment: A systematic review. *Applied Sciences (Switzerland)* **12(19), 10123.**

Parolia, A., Kumar, H., Ramamurthy, S., Davamani, F. and Pau, A. (2020). Effectiveness of chitosan-propolis nanoparticle against *Enterococcus faecalis* biofilms in the root canal. *BMC Oral Health* **20(339), 1-14.**

Peters, O. A., Peters, C. I. and Basrani, B. (2016). Cleaning and shaping the root canal system. *In*: Cohen's Pathways of the Pulp. Hargreaves, K. M., Berman, L. H., and Rotstein, I. (eds.). Edn.11th. Elsevier's Health Sciences Right Department, Philadelphia, PA, USA. **pp. 209-279.**

Pimenta, H. C., Violante, I. M. P., de Musis, C. R., Borges, Á. H. and Aranha, A. M. F. (2015). *In vitro*

effectiveness of Brazilian brown propolis against *Enterococcus faecalis*. *Brazilian Oral Research* **29(1), 1-6.**

Pinheiro, E. T. and Mayer, M. P. A. (2015). *Enterococcus faecalis* in oral infections. *JBR Journal of Interdisciplinary Medicine and Dental Science* **3(1), 1-5.**

Pobiega, K., Kraśniewska, K., Derewiaka, D. and Gniewosz, M. (2019). Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods. *Journal of Food Science and Technology* **56(12), 5386-5395.**

Przybylek, I. and Karpiński, T. M. (2019). Antibacterial properties of propolis. *Molecules* **24(11), 1-17.**

Sa-Eed, A., Donkor, E. S., Arhin, R. E., Tetteh-Quarcoo, P. B., Attah, S. K., Kabotso, D. E. K., *et al.* (2023). *In vitro* antimicrobial activity of crude propolis extracts and fractions. *FEMS Microbes* **4, 1-8.**

Salleh, S. N. A. S., Wan Johari, W. L. and Mohd Hanapiah, N. A. (2022). A comprehensive review on chemical compounds, biological actions and potential health benefits of stingless bee propolis. *Sains Malaysiana* **51(3), 733-745.**

Santos, V. R. (2012). Propolis: Alternative medicine for the treatment of oral microbial diseases. *In*: Alternative Medicine. Sakagami, H. (ed.). IntechOpen, Japan. **pp. 133-169.**

Shabbir, J., Qazi, F., Farooqui, W., Ahmed, S., Zehra, T. and Khurshid, Z. (2020). Effect of chinese propolis as an intracanal medicament on post-operative endodontic pain: A double-blind randomized controlled trial. *International Journal of Environmental Research and Public Health* **17(2), 445.**

Shakhatreh, M. A. K., Jacob, J. H., Hussein, E. I., Masadeh, M. M., Obeidat, S. M., Juhmani, A. *et al.* (2017). Microbiological analysis, antimicrobial activity, and heavy-metals content of Jordanian Ma'in hot-springs water. *Journal of Infection and Public Health* **10(6), 789-793.**

Sjögren, U., Figdor D. and Spangberg L. S. G. (1991). The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. *International Dental Journal* **24, 119-125.**

Slutzky-Goldberg, I., Hanut, A., Matalon, S., Baev, V. and Slutzky, H. (2013). The effect of dentin on the pulp tissue dissolution capacity of sodium hypochlorite and calcium hydroxide. *Journal of Endodontics* **39(8), 980-983.**

Smith, A. C. and Hussey, M. A. (2005). Gram stain protocols. *American Society for Microbiology (ASM)*, **1-9.**

Stähli, A., Schröter, H., Bullitta, S., Serralutzu, F., Dore, A., Nietzsche, S. *et al.* (2021). *In vitro* activity of propolis on oral microorganisms and biofilms. *Antibiotics* **10(9), 1045.**

Sudhakara, P., Gupta, A., Bhardwaj, A. and Wilson, A. (2018). Oral dysbiotic communities and their implications in systemic diseases. *Dentistry Journal* **6(2), 1-14.**

Thomas, C., Minty, M., Vinel, A., Canceill, T., Loubières, P., Burcelin, R. *et al.* (2021). Oral microbiota: A major player in the diagnosis of systemic diseases. *Diagnostics* **11(8), 1-29.**

Valgas, C., De Souza, S. M., Smânia, E. F. A. and Smânia, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology* **38(2), 369-380.**

Vidana, R., Sullivan, A., Billström, H., Ahlquist, M. and Lund, B. (2011). *Enterococcus faecalis* infection in root canals – host-derived or exogenous source? *Letters in Applied Microbiology* **52(2), 109-115.**

Wahyuni, N., Yanti, N., Abidin, T., Prasetia, W. and Suryanto, D. (2023). The effect of addition 5% propolis nanoparticles in epoxy resin and bioceramic sealers on the growth of *Enterococcus faecalis* ATCC 29212 and the dentinal tubular penetration: *In vitro*. *International Journal of Applied Pharmaceutics* **15(4), 99-105.**

Woitschach, F., Kloss, M., Schlodder, K., Borck, A., Grabow, N., Reisinger, E. *et al.* (2022). Bacterial adhesion and biofilm formation of *Enterococcus faecalis* on zwitterionic methylmethacrylat and polysulfones. *Frontiers in Cellular and Infection Microbiology* **12, 1-7.**

Yenugu, S., Hamil, K. G., French, F. S. and Hall, S. H. (2004). Antimicrobial actions of the human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and HE2beta2. *Reproductive Biology and Endocrinology* **2(61).**

Yoshimasu, Y., Ikeda, T., Sakai, N., Yagi, A., Hirayama, S., Morinaga, Y. *et al.* (2018). Rapid bactericidal action of propolis against *Porphyromonas gingivalis*. *Journal of Dental Research* **97(8), 928-936.**

Zancan, R. F., Canali, L. C. F., Tartari, T., de Andrade, F. B., Vivan, R. R. and Duarte, M. A. H. (2018). Do different strains of *E. faecalis* have the same behavior towards intracanal medications in *in vitro* research? *Brazilian Oral Research* **32(46), 1-8.**

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APPENDIX 3

Participation in the International Dental Collaboration of the Mekong River Region Conference

CERTIFICATE

OF ATTENDANCE

UNIVERSITI TEKNOLOGI MARA | Fakulti Pergigian

IDCMR International Dental Collaboration The Mekong River Region

This certificate is presented to:

Ahmad Iman Ammer Bin Azman

For attending the

17th IDCMR Conference 2023

16 CPD Points

on 14 & 15 October 2023 at Aloft Hotel, Langkawi

S. Kiattavorncharoen

CLINICAL PROF. DR. SIRICHAI KIATTAVORNCHAROEN
Dean, Faculty of Dentistry, Mahidol University
IDCMR Secretariat Office

PROF. DR. AIDA NUR ASHIKIN ABD RAHMAN
Chairperson of the 17th IDCMR Conference 2023

CERTIFICATE

OF PARTICIPATION

UNIVERSITI TEKNOLOGI MARA | Fakulti Pergigian

IDCMR International Dental Collaboration The Mekong River Region

This certificate is presented to:

Ahmad Iman Ammer Azman

As

PARTICIPANT FOR ORAL PRESENTATION

International Level

at the

17th IDCMR Conference 2023

on 14 & 15 October 2023 at Aloft Hotel, Langkawi

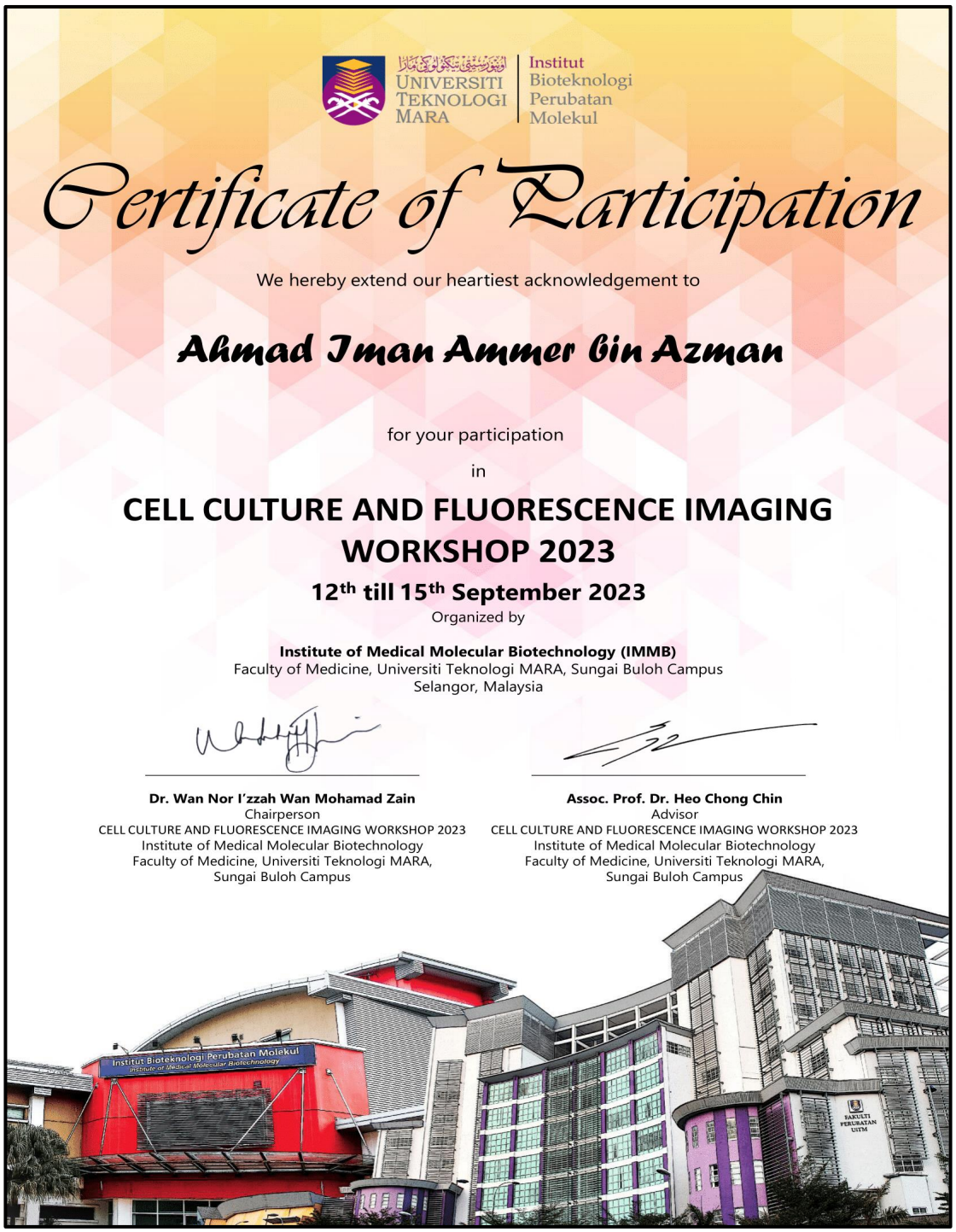
S. Kiattavorncharoen

CLINICAL PROF. DR. SIRICHAI KIATTAVORNCHAROEN
Dean, Faculty of Dentistry, Mahidol University
IDCMR Secretariat Office

PROF. DR. AIDA NUR ASHIKIN ABD RAHMAN
Chairperson of the 17th IDCMR Conference 2023

APPENDIX 4

Participation in Workshop / Courses



UNIVERSITI TEKNOLOGI MARA
Institut Bioteknologi Perubatan Molekul

Certificate of Participation

We hereby extend our heartiest acknowledgement to

Ahmad Iman Ammer Bin Azman

for your participation

in

CELL CULTURE AND FLUORESCENCE IMAGING WORKSHOP 2023

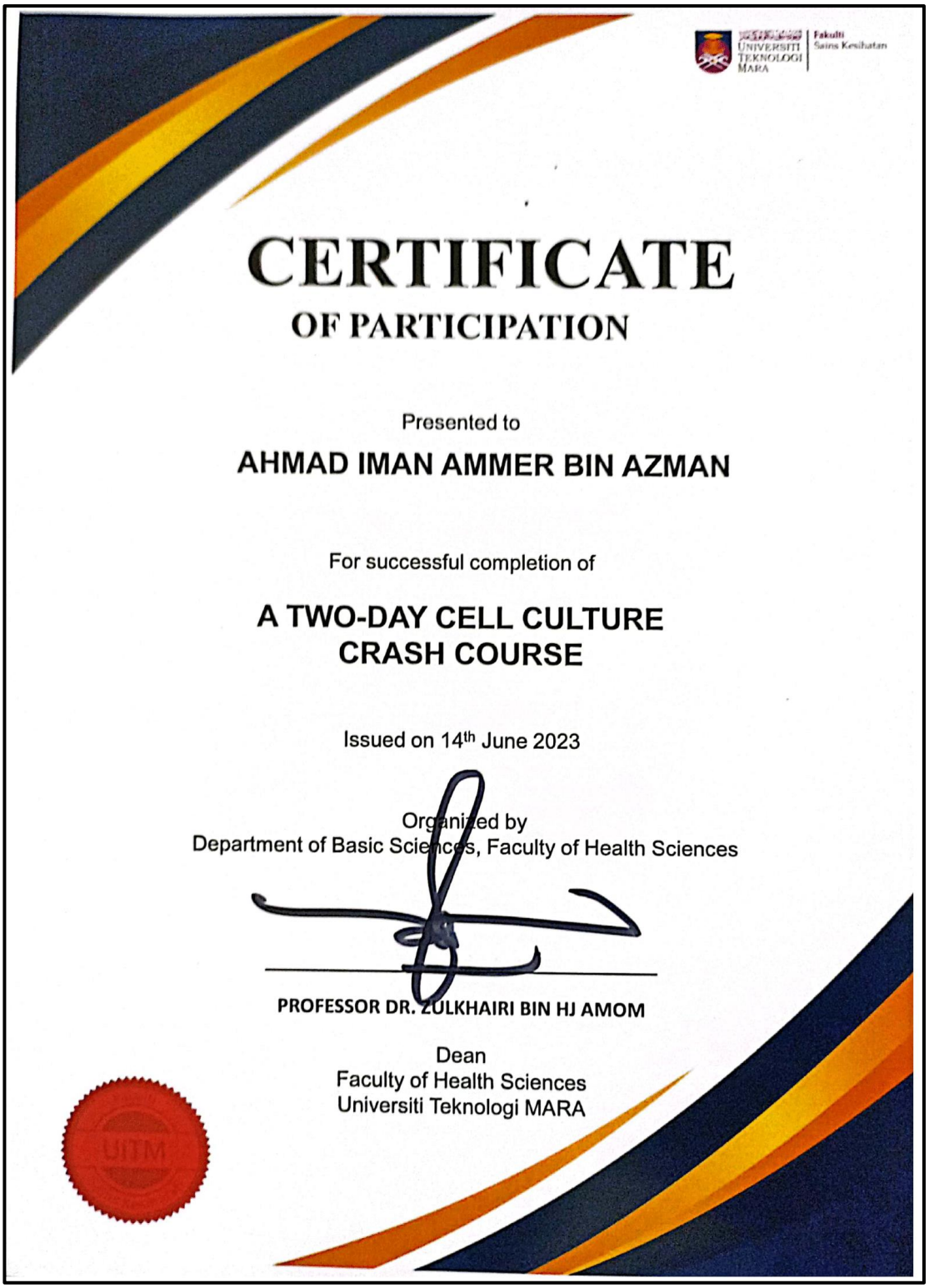
12th till 15th September 2023

Organized by

Institute of Medical Molecular Biotechnology (IMMB)
Faculty of Medicine, Universiti Teknologi MARA, Sungai Buloh Campus
Selangor, Malaysia

Dr. Wan Nor I'zzah Wan Mohamad Zain
Chairperson
CELL CULTURE AND FLUORESCENCE IMAGING WORKSHOP 2023
Institute of Medical Molecular Biotechnology
Faculty of Medicine, Universiti Teknologi MARA,
Sungai Buloh Campus

Assoc. Prof. Dr. Heo Chong Chin
Advisor
CELL CULTURE AND FLUORESCENCE IMAGING WORKSHOP 2023
Institute of Medical Molecular Biotechnology
Faculty of Medicine, Universiti Teknologi MARA,
Sungai Buloh Campus



Universiti Teknologi MARA | Fakulti Sains Kesihatan

CERTIFICATE
OF PARTICIPATION

Presented to

AHMAD IMAN AMMER BIN AZMAN

For successful completion of

A TWO-DAY CELL CULTURE CRASH COURSE

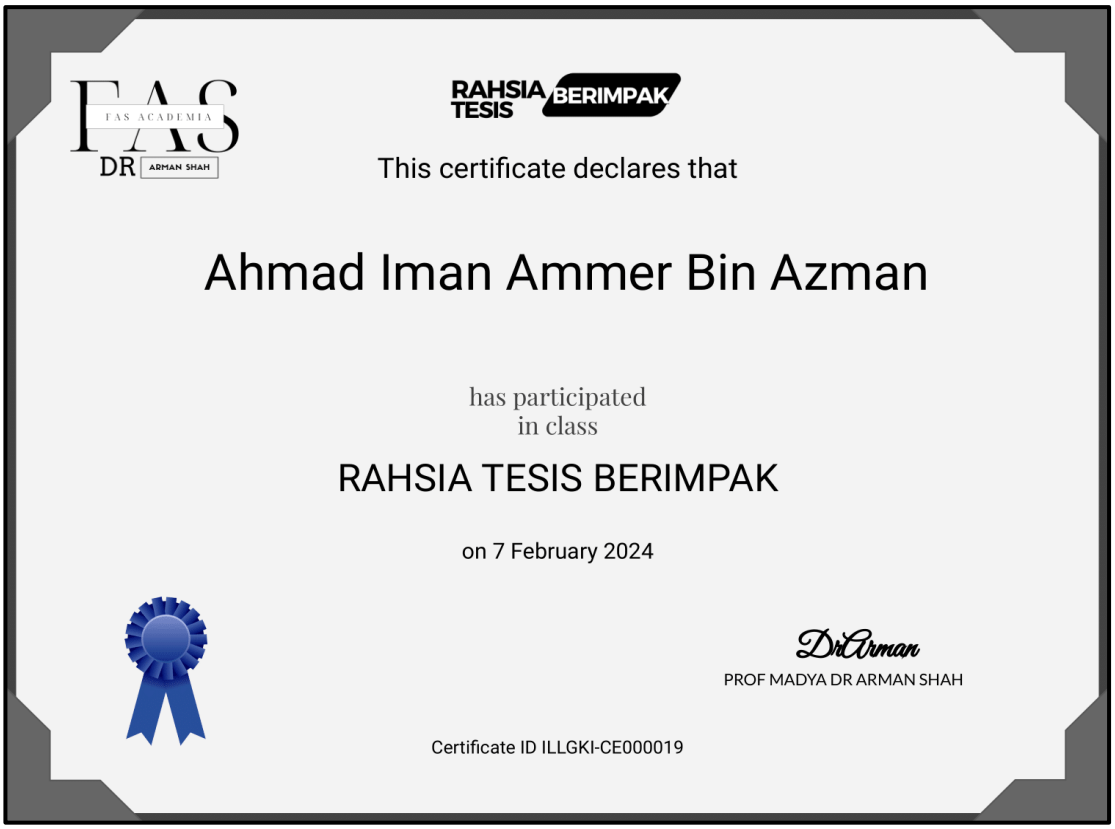
Issued on 14th June 2023

Organized by
Department of Basic Sciences, Faculty of Health Sciences

PROFESSOR DR. ZULKHAIRI BIN HJ AMOM

Dean
Faculty of Health Sciences
Universiti Teknologi MARA

UiTM


FAS ACADEMIA
DR ARMAN SHAH
RAHSIA TESIS BERIMPAK
This certificate declares that
Ahmad Iman Ammer Bin Azman
has participated
in class
RAHSIA TESIS BERIMPAK
on 7 February 2024
DrArman
PROF MADYA DR ARMAN SHAH
Certificate ID ILLGKI-CE000019

NIH INSTITUT KESIHATAN NEGARA
NATIONAL INSTITUTES OF HEALTH
KEMENTERIAN KESIHATAN MALAYSIA

Sijil Penyertaan

Setinggi-tinggi tahniah dan ucapan terima kasih kepada

AHMAD IMAN AMMER BIN AZMAN
001015140925

Kerana telah menyertai dengan jayanya

BENGKEL SCOPING & SYSTEMATIC REVIEW SIRI 1 (2025)

Anjuran

UNIT SYSTEMATIC REVIEW/SEKTOR EVIDENCE BASED HEALTHCARE/PEJABAT PENGURUS NIH

Pada

24/06/2025 hingga 25/06/2025

Bertempat di

BILIK MESYUARAT UTAMA (BMU)

DR MURIZAL BIN ZAINOL
PEJABAT PENGURUS NIH
Institut Kesihatan Negara (NIH)
Kementerian Kesihatan Malaysia

NIH
INSTITUT KESIHATAN NEGARA
NATIONAL INSTITUTES OF HEALTH
KEMENTERIAN KESIHATAN MALAYSIA

Sijil Penyertaan

Setinggi-tinggi tahniah dan ucapan terima kasih kepada

AHMAD IMAN AMMER BIN AZMAN
001015140925

Kerana telah menyertai dengan jayanya

MS EXCEL: MENUJU PAKAR PERINGKAT PERTENGAHAN

Anjuran

SEKTOR TEKNOLOGI MAKLUMAT/PEJABAT PENDAFTAR NIH

Pada

15/07/2025 hingga 16/07/2025

Bertempat di

Makmal Komputer 1 (D2-L5-24)

SAFRIAH BINTI MD ADZHAR
PEJABAT PENDAFTAR NIH
Institut Kesihatan Negara (NIH)
Kementerian Kesihatan Malaysia

AUTHOR'S PROFILE

Ahmad Iman Ammer Bin Azman obtained Bachelor of Science in Applied Microbiology (Hons.) in 2023 from Universiti Teknologi MARA (UiTM), Shah Alam, Selangor. His Master thesis involves in the exploration of antimicrobial properties of Malaysian stingless bee propolis, which includes several AST techniques such as agar-well diffusion method, broth microdilution, antibiofilm and anti-adherence assay, time-kill analysis, and scanning electron microscopy. Currently he is exploring several aspects to expend his current research on stingless bee propolis including cell culture, FTIR, and qPCR.

LIST OF PUBLICATIONS

Azman, A. I. A., Hasirin, R. A., Khan, H. B. S.G., Shafiei, Z., and Ismail, I. H. (2025). Antimicrobial activity of Malaysia *Heterotrigona itama* propolis against *Enterococcus faecalis*: A study in endodontic applications. *Malaysian Journal of Microbiology*. 21(4), pp. 472-483. eISSN 1823-8262

Azman, A. I. A., Rasol, R., and Jamil, N. M. (2023). Antimicrobial activity screening of bacteria isolated from Tasik Cermin. *Malaysian Journal of Medicine and Health Sciences*, 19(Supp.18). pp. 36-45. eISSN 2636-9346