DEVELOPMENT AND CHARACTERIZATION OF HONEY-PLGA MICROPARTICLES WITH ANTIBACTERIAL PROPERTIES AGAINST *Streptococcus mutans* IN ORAL DISEASE

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by

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LIST OF SYMBOLS

ºC	degree Celsius
ºC/min	degree Celsius per minute
%	percent
±	plus-minus
<	lesser than
µL	microlitre
µm	micrometre
CFU/mL	colony-forming units per millilitre
eV	electronvolt
g	gram
h	hours
kDa	kilodalton
kV	kilovolt
m	meter
m/z	mass-to-charge ratio
min	minutes
mg	milligram
mg/mL	milligram per millilitre
mL	millilitre
mL/min	millilitre per minute
mm	millimetre
mM	millimolar
mV	millivolt
nm	nanometre

psi	pound per square inch
rpm	revolutions per minute
w/v	weight/volume

LIST OF ABBREVIATIONS

ATR	acid tolerance response
DCM	dichloromethane
eDNA	extracellular DNA
EI	electron ionisation
EPS	extracellular polysaccharides
EKH	extracted Kelulut honey
EMH	extracted Manuka honey
ETH	extracted Tualang honey
EKHMP	extracted Kelulut honey microparticles
EMHMP	extracted Manuka honey microparticles
ETHMP	extracted Tualang honey microparticles
FDA	food and drug administration
FESEM	field emission scanning electron microscopy
GC	gas chromatography
GC-MS	gas chromatography–mass spectrometry
HMF	hydroxymethylfurfural
HMP	honey-PLGA microparticles
H_2O_2	hydrogen peroxide
KHMP	Kelulut honey microparticles
LDH	lactate dehydrogenase
LTA	lipoteichoic acid
MBC	minimum bactericidal concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth

MHMP	Manuka honey microparticles
MIC	minimum inhibitory concentration
MP	microparticles
MRSA	methicillin-resistant *Staphylococcus aureus*
NaCl	sodium chloride
OD	optical density
OHE	oral hygience education
PBS	phosphate-buffered saline
PCL	poly (ε-caprolactone)
PGA	poly (glycolic acid)
pH	potential of hydrogen
PHA	poly (hydroxy alkanoates)
PLA	poly (lactic acid)
PLGA	poly (lactic-co-glycolic acid)
PVA	poly (vinyl alcohol)
RKH	raw Kelulut honey
RMH	raw Manuka honey
ROS	reactive oxygen species
RTH	raw Tualang honey
RKHMP	raw Kelulut honey microparticles
RMHMP	raw Manuka honey microparticles
RTHMP	raw Tualang honey microparticles
SD	standard deviation
SE	secondary electrons
SEM	scanning electron microscopy
SIM	selected ion monitoring
THMP	Tualang honey microparticles

XHR-FESEM	extreme high resolution field emission scanning electron microscopy

LIST OF APPENDICES

PENGHASILAN DAN PENCIRIAN MIKROPARTIKEL MADU-PLGA DENGAN AKTIVITI ANTIBAKTERIA TERHADAP *Streptococcus mutans* DALAM PENYAKIT ORAL

ABSTRAK

Penggunaan madu pada setengah tempat pada tubuh manusia seperti di dalam mulut mungkin menjadi masalah kerana kecairannya. Oleh itu, madu telah diterokai untuk digunakan dalam bentuk lain untuk kesesuaian penggunaan. Salah satu bentuk sistem penyampaian ubat adalah melalui penggabungan ubat ke dalam mikropartikel. Oleh itu, tujuan kajian ini adalah untuk mengkaji potensi madu untuk dimasukkan ke dalam mikropartikel untuk meningkatkan keberkesanan aktiviti antibakteria. Tiga jenis madu digunakan; Kelulut, Tualang dan Manuka. Mikropartikel madu-PLGA disediakan menggunakan kaedah penyejatan pelarut emulsi berganda. Kemudian mikropartikel madu-PLGA menjalani pelbagai analisis dan ujian, termasuk analisis GC-MS, FESEM, analisis zetasizer, pelepasan *in vitro* dan ujian antibakteria. *Streptococcus mutans* digunakan dalam ujian antibakteria kerana bakteria ini menyebabkan karies dan radang periodontium. Hasil kajian menunjukkan bahawa semua jenis madu yang digunakan dalam kajian ini boleh digabungkan ke dalam mikropartikel menjadi serbuk putih. Berdasarkan analisis FESEM, mikropartikel madu-PLGA berbentuk sfera, mempunyai permukaan yang licin, dan berdiameter antara 1 hingga 10 mikrometer. Analisis GC-MS menunjukkan mikropartikel madu Kelulut mentah (RKHMP), mikropartikel madu Manuka mentah (RMHMP) dan mikropartikel madu Tualang mentah (RTHMP) mempunyai 1, 11 dan 5 sebatian yang dikenalpasti, mengikut jujukan. Manakala mikropartikel madu Kelulut yang diekstrak (EKHMP), mikropartikel madu Manuka yang diekstrak

(EMHMP) dan mikropartikel madu Tualang yang diekstrak (ETHMP) masing-masing mempunyai 15, 12 dan 26 sebatian yang dikenalpasti. Oleh itu, mikropartikel madu yang diekstrak mengandungi lebih banyak sebatian yang dikenal pasti berbanding mikropartikel madu mentah. Analisis potensi zeta menunjukkan mikropartikel mempunyai potensi zeta yang rendah sehingga cenderung untuk menjadi mendakan dalam ampaian. Mikropartikel menunjukkan ciri pelepasan perlahan tetapi tiada aktiviti antibakteria terhadap *Streptococcus mutans*. Dalam kekangan kajian ini, dapat disimpulkan bahawa ketiga-tiga jenis madu boleh digabungkan dengan bahan lain untuk menghasilkan mikropartikel madu-PLGA, namun kajian lanjut diperlukan dari segi menambahbaik formulasi untuk menghasilkan mikropartikel yang lebih berkesan terhadap mikroorganisma sasaran.

DEVELOPMENT AND CHARACTERIZATION OF HONEY-PLGA MICROPARTICLES WITH ANTIBACTERIAL PROPERTIES AGAINST *Streptococcus mutans* IN ORAL DISEASE

ABSTRACT

Application of honey in a certain area of the human body like in the oral cavity might pose a problem due to its liquidity. Thus, honey also has been explored to be used in other forms for suitable application. One form of drug delivery system is through the incorporation of drugs into microparticles. Therefore, the aim of this study is to investigate the potential of honey to be incorporated into microparticles to enhance antibacterial activity. Three types of honey were used; Kelulut, Tualang and Manuka. Honey-PLGA microparticles were prepared using the double emulsion solvent evaporation method. Then the honey-PLGA microparticles were subjected to a variety of analyses and tests, including GC-MS analysis, FESEM, zetasizer analysis, *in vitro* release and an antibacterial test. *Streptococcus mutans* was used for antibacterial testing because this bacterium causes caries and periodontitis. The results showed that all types of honey used in this study could be incorporated into microparticles as a white powder. Based on FESEM analysis, honey-PLGA microparticles were spherical, and had a smooth surface, with a diameter ranging from 1 to 10 micrometer. GC-MS analysis showed the identified compounds found in raw Kelulut honey microparticles (RKHMP), raw Manuka honey microparticles (RMHMP) and raw Tualang honey microparticles (RTHMP) were 1, 11 and 5 compounds, respectively. The identified compounds found in extracted Kelulut honey microparticles (EKHMP), extracted Manuka honey microparticles (EMHMP) and extracted Tualang honey microparticles (ETHMP) were 15, 12 and 26

compounds, respectively. Extracted honey microparticles contain more identified compounds compared to raw honey microparticles. Zetasizer analysis showed the microparticles had low zeta potential and thus tended to precipitate in suspension. The microparticles showed slow-release character but no antibacterial activity against *Streptococcus mutans*. Within the limitation of the study, it can be concluded that, the three types of honey can be incorporated with other materials to produce honey-PLGA microparticles, however further study is needed in term of improving the formulation to produce microparticles that are highly effective against target microorganisms.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Caries and periodontitis are common types of oral diseases. One of the bacteria that causes caries and is involved in the progression of periodontitis is *Streptococcus mutans*. Caries are induced by acids generated by microorganisms in dental plaque. These acidic conditions lead to enamel demineralisation and subsequently cavity formation (Rathee & Sapra, 2019). Periodontitis is an inflammatory condition that affects the tissues that encircle teeth, including the gingiva (gum), alveolar bone, cementum, and periodontal ligaments. This disease is caused by toxin releases from polymicrobial infections. Oral hygiene education (OHE) is the paramount management for both caries and periodontitis cases to reduce the bacterial load. Apart from OHE, the basic treatment of caries is the removal of the decayed material on the carious tooth followed by filling with suitable material. In extensive caries, calcium hydroxide was placed before the restorative material to kill the remaining bacteria. Whereas for periodontitis, treatment is by removing the plaque and calculus (source of bacteria toxin) around the teeth through scaling and root debridement. It can be followed by adjunctive treatment such as local delivery antimicrobials, systemic antibiotics, and host modulation therapy to combat the bacteria.

Honey was reported to be useful for the treatment of many oral diseases, including gum problems (Thomas, 2014). Honey consists of carbohydrates, water, proteins, ashes, and trace amounts of amino acids, phenols, pigments, and vitamins (Bogdanov, 2008; Miguel et al., 2017). Raw honey is honey in its purest form, straight from the beehive, as nature intended. However, extracted honey is honey that

has undergone a processing step and is extracted using a solvent like ethanol or methanol. Honey was shown to exert antimicrobial activity against anaerobic bacteria and prevent gum disease (Eick et al., 2014). It produces hydrogen peroxide when diluted because the enzyme glucose oxidase is activated, oxidising glucose to produce gluconic acid and hydrogen peroxide. The antibacterial properties of honey are primarily attributed to hydrogen peroxide and methylglyoxal. However, honey poses a problem to be used in the dental area as it is easily diluted and washed away by the gingival crevicular fluid (the fluid that oozes from inside the gum) and saliva. Therefore, a good carrier that can retain and slow-release honey is needed to ensure the optimum usage of honey as adjunct in periodontal treatment and caries management.

Microparticles, which typically range in size from 1 to 1000 micrometres, are common components of multiparticulate drug delivery systems, offering both therapeutic and technological benefits. Depending on the composition, they can be incorporated into a variety of pharmaceutical shapes, including solids, semisolids, and liquids (Lengyel et al., 2019). Microparticles can deliver medications to specific regions, resulting in increased drug concentrations on-the-spot and lower systemic exposure (Vlachopoulos et al., 2022). Poly-(lactic-co-glycolic acid) (PLGA) is one of the successful drug delivery polymeric microparticles which is biocompatible, biodegradable and proven safe (Kapoor et al., 2015).

Therefore, this study aims to explore the possibility of honey to be incorporated into poly-(lactic-co-glycolic acid) (PLGA) to formulate honey-PLGA microparticles drug carriers. To further examine the formulated honey-PLGA microparticles, field

emission scanning electron microscopy, zetasizer analysis, release assay, gas chromatography–mass analysis and antibacterial test were performed.

1.2 Problem statement

Caries and periodontitis are common dental diseases that can easily recur without proper plaque control. Therefore, it requires a lot of manpower and money in order to control the disease progression. In certain conditions both treatments for caries and periodontitis use local application of the antimicrobial agent to combat infection such as the placement of calcium hydroxide in the cavity and the local application of antibiotics, which are quite expensive and increase the risk of bacterial resistance. Therefore, the search for cheaper, safer and effective materials/drug replacement in the treatment of oral diseases will be very helpful in reducing the cost of managing patients in Malaysia. Honey will be an excellent candidate for an antimicrobial agent as it possesses antimicrobial properties and is readily available locally at a reasonable price. Therefore, Malaysian honey from sting and stingless bee sources could be tested for their potential to be used as an adjunct in treating common oral disease. Apart from investigating its potential, delivering honey using the appropriate delivery agent also needs to be addressed. Since, up to date, no study investigates the incorporation of honey into microparticles for delivering, this study was conducted to look into this matter which is intended for oral application. In this study, we formulated honey-PLGA microparticles, characterized it and evaluated its antibacterial potential against *Streptococcus mutans*.

1.3 Research questions

1. Can microparticles be formulated from PLGA and honey?
2. Do honey-PLGA microparticles have antibacterial properties?

1.4 Hypothesis

1. Honey can be formulated with PLGA to form microparticles.
2. The formulated honey-PLGA microparticles demonstrate antibacterial activity.

1.5 Objectives

General

This study aims to produce honey-PLGA microparticles with antibacterial properties against *Streptococcus mutans* for the treatment of oral disease.

Specific:

1. To analyse the phytochemicals of raw and crude-extract Kelulut, Tualang and Manuka honey and compare with the formulated honey-PLGA microparticles by GC-MS analysis.
2. To formulate the honey-PLGA microparticles using Kelulut, Tualang and Manuka honey.
3. To evaluate the physical and biological properties of formulated honey-PLGA microparticles by field emission scanning electron microscopy, zetasizer analysis and release assay.
4. To assess the antibacterial properties of raw, extracted and formulated honey-PLGA microparticles.

CHAPTER 2

LITERATURE REVIEW

2.1 Oral Diseases

Oral diseases are among the most prevalent non-communicable diseases globally affecting an estimated 3.5 billion people worldwide. Dental caries and periodontal disease are the most common oral diseases. Dental caries is dental decay caused by frequent exposure of teeth to low pH, whereas periodontitis is an inflammatory disease of the teeth's supporting tissues. Evidence suggests that dental caries and periodontal diseases are multifactorial in character, with a number of influencing variables that link them directly or tangentially (Marsh & Bradshaw, 1997).

2.1.1 Caries

Caries, often known as cavities, are dental decay resulting from acid production by cariogenic bacteria in the dental biofilm over time (Figure 2.1). Dental caries occur when the typically homeostatic biofilm microbiota transforms into an acidogenic, aciduric, and cariogenic community as a result of sugar intake (Schwendicke et al., 2016). The streptococci species, particularly *Streptococcus mutans (S. mutans)* and *Streptococcus sobrinus (S. sobrinus)*, and *lactobacilli* species are the most prevalent microorganisms linked to dental caries (Loesche, 1986).

Dental caries is a major healthcare issue because it is the most common disease globally (Schwendicke et al., 2015). Based on Malaysia's National Oral Health Survey 2010, 90% of adults have dental caries in Malaysia (NOSHA, 2010). An early sign of caries is the appearance of a white spot lesion with no symptoms. As it progresses, the cavity becomes noticeable, and symptoms develop from

hypersensitivity to pain worsening in response to a certain stimulus (Figure 2.1). Caries is treated by excavating the decayed tissue together with the associated bacteria; following that, the cavity is clean and lined with or without calcium hydroxide before it being filled by restorative dental material (Thompson et al., 2008).

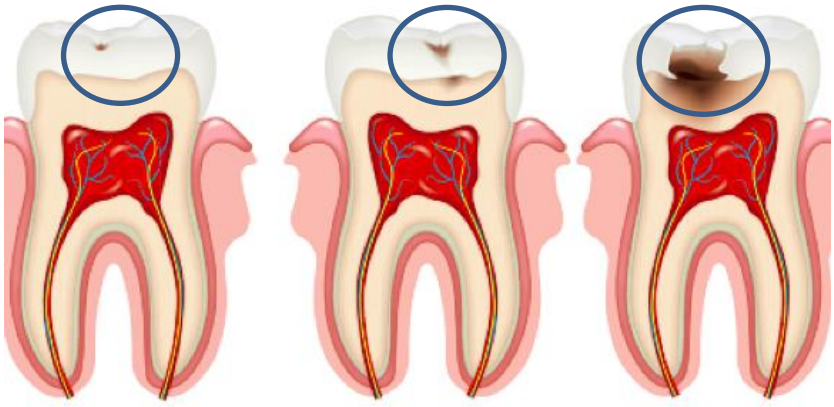

Figure 2.1 The illustration of caries development from initial lesion to a larger cavity (adapted from "New Caries Collection," 2022)

2.1.2 Periodontitis

The periodontium is composed of soft and hard tissues that surround and support the tooth root. The gingiva and periodontal ligament are soft tissue components, while the cementum and alveolar bone are hard tissue components. Periodontitis is an inflammatory disease that impacts the periodontium as a result of an imbalance in the normal balance of the oral bacteria and host resilience (dysbiosis), resulting in tooth loss (Figure 2.2). It is characterised by gingival bleeding, gum swelling, periodontal pocketing, and tooth mobility.

Periodontal pathogens usually involve mixed bacteria species, most often anaerobic bacteria. The predominant bacteria in periodontitis are *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola and Fusobacterium nucleatum.* The bacteria produce a variety of toxic substances like hydrogen sulphide, ammonia, amines, toxins, enzymes, antigens and others that trigger an inflammatory reaction that is supposed to be protective but can also lead to the loss of periodontal tissue (Thompson et al., 2008).

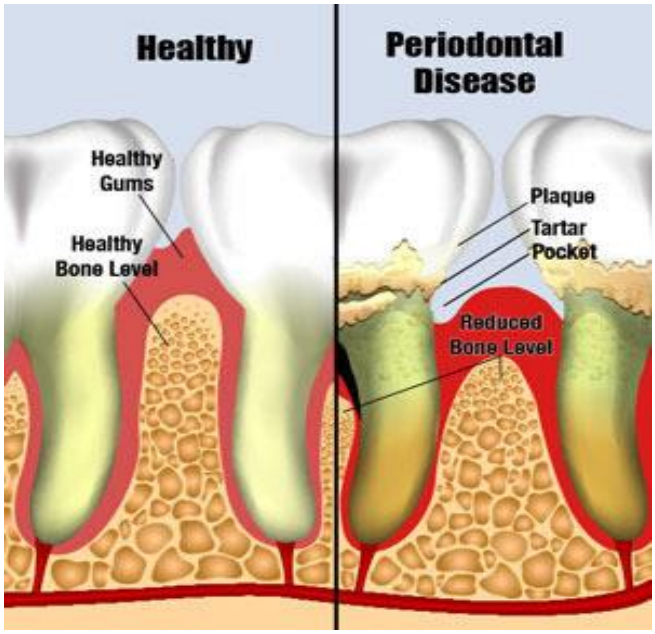

Figure 2.2 The illustration of healthy periodontium and periodontal disease (adapted from Mustapha, 2014)

The basis of periodontal treatment is the mechanical elimination of calculus and bacterial sediments from the supra and subgingival environment using either hand instruments or ultrasonic equipment. It can be done non-surgically or surgically, with a rigorous plaque control regimen (Shaddox & Walker, 2010). A combined scaling and root debridement with or without locally delivered antimicrobials may avoid the need for surgery. A locally delivered antimicrobial agent such as antibiotics can be

applied to the patient as an adjunct to scaling. When mechanical debridement was coupled with a local delivery antimicrobial agent, significant decreases in probing depth, gingival irritation, plaque scores, and bleeding indices were noted when compared to scaling and root planing alone (Kalsi et al., 2011). Various antibiotics and antiseptics have been used to help in arresting disease progression. However, the expense of care is quite costly for standard therapy, not to mention the chance of developing germs resistant to antibiotics.

2.1.3 Aetiology

Dental biofilm is the main etiology for both dental caries and periodontitis. It is the sticky, colourless film of bacteria that begins to build upon the tooth surface after dental cleaning as early as 2 hours. Initially, it is a soft, thin coating of bacteria, mucus, decomposed epithelial cells, and food fragments. After 72 hours, soft plaque eventually hardens into calculus (hard plaque or tartar), which is challenging to brush off with a toothbrush because it mineralises with calcium, phosphate, and other minerals. Dental biofilm is primarily made up of microbes that live within an intercellular matrix composed of organic and inorganic elements generated from saliva, gingival crevicular fluid, and bacterial products (Saini et al., 2011). Microorganisms attach to surfaces through flagella, pili, proteins, and polysaccharide adhesins mechanisms (Hall-Stoodley & Stoodley, 2002). Microbes can accumulate and create biofilms on both biotic and abiotic surfaces, making identification and isolation of a treatment target challenging (Morse et al., 2018). In the oral cavity, there are around 700–800 different kinds of bacteria species that fight it out for resources, binding sites, and the chance to survive in the biofilm. The most

extensively studied is the *streptococcus* species, which includes the *S. mutans* (Huang et al., 2011).

2.1.4 *Streptococcus mutans*

S. mutans live mainly in the mouth, throat, and gut (Loesche, 1986). *S. mutans* is known as the primary pathogen in the early phases of dental caries due to its ability to attach to tooth surfaces, produce sticky extracellular polysaccharides (EPS) from sucrose, and ferment sucrose and other carbohydrates into acids that demineralise tooth enamel. Caries frequently form 6-24 months after the appearance of *S. mutans* in tooth cavities (Balakrishnan et al., 2000). The capacity of *S. mutans* to generate significant quantities of EPSs from sucrose is an important factor in its cariogenicity (Shellis & Dibdin, 1988).

Apart from its involvement in caries initiation, it is also reported that periodontal disease develops due to the low pH environment produced by *S. mutans.* High *S. mutans* levels were also shown to be directly related to greater severity of periodontal disease at older ages in untreated individuals (Contardo et al., 2011). Both saliva and subgingival plaque samples from periodontitis patients showed an increase in *S. mutans* colonisation. Additionally, the periodontal parameters showed a positive correlation (Dani et al., 2016). The virulence factors of bacteria are substances that harm hosts at various stages of the organism's life cycle, increasing their effectiveness (Jia et al., 2019). The main virulence traits of *S. mutans* are acidogenicity, acid tolerance, EPS production, and biofilm formation (Li et al., 2020).

2.1.4 (a) Acidogenicity

S. mutans, a lactic acid bacterium, exclusively produces energy through the process of glycolysis. This organism's capacity to digest a wide range of carbohydrates is one of its defining traits. *S. mutans* has a fully functional glycolytic pathway and can ferment lactate, formate, acetate, and ethanol (Ajdić et al., 2002). When glucose is abundant, the precise distribution of fermentation products is determined by growth circumstances, with lactate being the predominant product (Dashper & Reynolds, 1996). Lactate dehydrogenase (LDH) deficient strains exhibit decreased cariogenicity (Johnson et al., 1980; Fitzgerald et al., 1989), and the lack of LDH is lethal (Hillman et al., 1996). *S. mutans* produce more acid than other streptococci because it has a greater ATP-glucose phosphotransferase activity. In most instances, the rate at which *S. mutans* generate acid when evaluated at pH levels ranging from 7.0 to 5.0 exceeds that of other oral streptococci (de Soet et al., 2000). The percentage acidogenicity of *S. mutans* varies from one isolate to another, and precise relationships between acidogenicity and caries experience are lacking (Köhler et al., 1995). Nonetheless, it is widely assumed that *S. mutans*' acidogenicity affects the plaque flora ecologically, leading to a rise in *S. mutans* and other acidogenic and acid-tolerant species.

2.1.4 (b) Acid Tolerance

S. mutans' aciduricity or acid-tolerance is inextricably linked with its acidogenicity. *S. mutans* preserve glycolytic capabilities even at growth-inhibitory pH values (as low as pH 4.4) (Bender et al., 1985). *S. mutans*' acid tolerance is mostly mediated by an F1F0-ATPase proton pump, but it also requires adaptation and changes in gene and protein expression. They form the acid tolerance response (ATR) when

combined. The ATR was discovered *in vitro* to defend organisms from a sub-lethal pH challenge (Svensäter et al., 1997) and acid shock or acidic pH growth has been linked to changes in the expression of over 30 proteins (Hamilton & Svensäter, 1998; Wilkins et al., 2002). The creation of water-insoluble glucan and the establishment of a biofilm may enhance acid tolerance. *S. mutans* cells in a biofilm were more resistant to an acid assault than planktonic bacteria (McNeill & Hamilton, 2003). This might be connected to quorum sensing systems efficiently producing ATR and physical biofilm features. The speed of hydronium ion diffusion is related to the amount of water insoluble glucan generated by *S. mutans* (Hata & Mayanagi, 2003). These findings show the link between *S. mutans*' various virulence features and suggest that the significance of glucan extends beyond increasing adhesion. These changes might also explain why *S. mutans*' glucan-synthesizing capacity developed differently than other oral streptococci (Banas, 2004).

2.1.4 (c) Extracellular polysaccharide (EPS) synthesis

Exopolysaccharides (EPS) EPS generated from *S. mutans* enhance local microbe aggregation on the teeth while generating a spatially heterogeneous and diffusion-limiting matrix that preserves implanted bacteria. The EPS-rich matrix offers mechanical stability/cohesiveness and allows the formations of extremely acidic microenvironments, both of these factors are crucial in the development of dental caries. *S. mutans* also produce extracellular DNA (eDNA) and lipoteichoic acids (LTA), which can help in matrix growth. eDNA increases EPS (glucan) production locally, boosting *S. mutans* adherence to saliva-coated apatitic surfaces and the formation of extremely cohesive biofilms. eDNA and other extracellular molecules may influence the functional characteristics of the matrix and the pathogenicity of

cariogenic biofilms when combined with EPS (Klein et al., 2015). Given its ability to orchestrate changes in the plaque microbiome through the synthesis of EPS and acid, the evidence accumulated over many decades has amply demonstrated that *S. mutans* is a significant agent in dental caries, and currently was associated with periodontitis.

Thus, finding a safer and more reliable pharmacological agent could be helpful in preventing periodontitis and tooth decay. One of the potential resources could be honey which has been extensively utilised as medicine throughout human history in addition to its vast use as a common food and flavouring agent. This is due to the presence of a variety of bioactive chemicals as well as unique physicochemical features.

2.2 Honey

2.2.1 Bee products and honey

Bee products are bee pollen, beeswax, propolis, royal jelly, bee venom and honey. Honey is the most popular bee product. It is formed when honeybees naturally collect, modify, and store nectar and sweet deposits from plants in the honeycomb. Honey production starts when elder worker bees leave the hive to seek nectar-rich flowers or plant's saps. The elder bee sucks liquid nectar from flowers with its straw-like proboscis and stores it in a specific organ known as the honey stomach. When nectar reaches the honey stomach, enzymes begin to break down the nectar's complex carbohydrates into simpler sugars, a process is known as inversion. The older bee returns the hive with a full stomach and feeds a younger house bee the modified nectar. The house bees consume the sweet nectar from the elder bee, and its own enzymes further break down the sugars. House bees transmit nectar from mouth

to mouth within the hive until the water content in nectar is lowered to roughly 20%. At this time, the final house bee regurgitates the totally inverted nectar, which is now known as honey and stores it in a honeycomb cell (Scott, 2010).

Honey can be produced either by sting bees or stingless bees. Sting bees belong to the *Apis* genus, whereas stingless bees are divided into two genera namely, *Melipona* and *Trigona*. Both are essential for floral pollination. Stingless bee honey empirically has a similar therapeutic quality as sting bee honey (Amin et al., 2018). Therefore, stingless bee honey has great deal of potential to be developed for modern medicinal uses due to its therapeutic properties and availability. Kelulut honey is one of the most popular stingless bee honeys in Malaysia. On the other hand, sting bee honey such as Manuka honey, Jelly bush honey, and Tualang honey are the most well-researched natural honeys (Ahmed & Othman, 2013). Both sting and stingless bee honey exhibit significant antioxidant, anticancer, and antiatherogenic activities, which may be attributed partly to its phenolic content (Nosrati et al., 2018). Despite the fact that there are other varieties of honey in the world, this literature review will only be on Kelulut, Tualang, and Manuka honey.

2.2.2 Kelulut, Tualang and Manuka honey

Kelulut honey is produced by the stingless bees from the *Trigona* species. This species is domestical in Malaysia. In the Kelulut hive, the honey is stored naturally in the honey pot (cerumen), which can contribute to its beneficial properties (Figure 2.3A). Kelulut honey has the capability to inhibit the growth of Gram-positive bacteria like *S.mutans* (Choudhari et al., 2012) as well as Gram-negative bacteria like *Porphyromonas gingivalis* (Eick et al., 2014). It has been claimed that the

antimicrobial activity of the stingless bee honey may be a little stronger than the sting bee honey possibly influenced by the phytochemical content of the cerumen where the honey was stored (Guzman, 2014). The cerumen of the stingless bees is largely made of propolis and wax whereas the honeycomb of sting bees is made of beeswax. The cerumen has a different shape from the hexagonal cells in the honeycomb (Figure 2.3A & B). Stingless bee honey can be a promising source of biologically active compounds, which can be linked to the rich vegetation found in native environments (Abd Jalil et al., 2017). Furthermore, stingless bees have several distinguishing qualities that set them apart from honeybees, such as being less susceptible to disease, do not sting, and not fussy in building colony hives (Al-Hatamleh et al., 2020).

Tualang honey is produced by the wild rock bee name *Apis dorsata*. They fabricate hives high within the parts of Tualang tree (*Kompassia excelsa*). Tualang trees are typically found in tropical jungles. The colour of Tualang honey is dark brown, and its pH ranges from 3.55 to 4.00. It is slightly more acidic than other Malaysian honeys, like Kelulut Hitam, Kelulut Putih, and Gelam (Ghazali, 2009). Kamal et al. (2021) also reported that Tualang honey has a lower pH value compared to Kelulut and Gelam where the pH value of Tualang honey, Kelulut honey and Gelam honey were 3.14–3.80, 3.27–3.30 and 3.38–3.83 respectively. This characteristic could contribute to making Tualang honey more effective against several pathogenic microorganisms (Tan et al., 2009). Tualang honey contains more than half hydrocarbons, which include alcohols, ketones, aldehydes, furans, terpenes, flavonoids, and phenols. Tualang honey outperforms Manuka honey against some

Gram-negative bacterial strains, owing to higher levels of phenolics, flavonoids, and 5-(hydroxymethyl) furfural (HMF) (Ahmed & Othman, 2013).

Manuka honey is produced by the honeybees called *Apis mellifera* from the nectar of flowers of the Manuka tree (*Leptospermum scoparium*). Manuka honey is a dark monofloral honey that is high in phenolic content and well-known for its antimicrobial properties. It was found to be effective against a broad variety of pathogens. It is reported that Manuka honey is more resistant to Gram-negative bacteria than Gram-positive bacteria (Johnston et al., 2018). The compositions of Manuka honey include carbohydrates, minerals, proteins, fatty acids, phenolic and flavonoid compounds (Johnston et al., 2018). Manuka honey also has other unique features, like an extraordinarily high level of methylglyoxal (MGO) produced from dihydroxyacetone (DHA) which relates with its antibacterial activity (Montenegro & Mejias, 2013). Manuka honey is a well-researched honey, with 507 search results in PubMed, compared to Tualang at around 87 and 19 for Kelulut (on August 28, 2022).

A

B

Figure 2.3 Different type of bee hives A) Cerumen pots made by the stingless bees honey, and B) Honeycomb made by the sting bees

2.2.3 Composition of honey

Honey is primarily made up of 80-85% of sugar/carbohydrates, 15 to 20% of water, and minutes of additional components (Doner, 1977; White & Doner, 1980). Viscous liquids and sugar both contribute to honey's primary features, while the remaining ingredients influence how different types of honey differ from one another. Those components are vitamins, flavonoids, amino acids, enzymes, minerals, and phenolic acids. Honey composition varies depending on the plants that bees feed on, temperature, and environmental factors (Eteraf-Oskouei & Najafi, 2013). Honey is hygroscopic, meaning it may take moisture from the environment and dehydrate bacteria. Additionally, honey's high sugar content and low pH level can inhibit the growth of microorganisms (Mandal & Mandal, 2011).

2.2.3 (a) Chemical composition of honey

Carbohydrates include the monosaccharides fructose (41%) and glucose (34%), as well as the disaccharide sucrose (1-2%) (Tafere, 2021). The ratio of one form of sugar to another is determined by the source, such as floral pasture, and to some degree by the enzyme invertase, which breaks down sucrose into glucose and fructose. This enzyme is present in both the flower from which bees gather nectar and the bee's body (Di Pasquale et al., 2013).

Proteins are obtained from nectar and pollen, which are natural plant components. Proteins in honey can be made of basic compounds like amino acids or have very complex structures (Alvarez-Suarez et al., 2013). The amino acid and protein content is relatively low, at most 0.7%. Honey includes almost all of the essential amino

acids. Proline, the primary amino acid, is used to determine the ripeness of honey (Stefan Bogdanov, 2016).

Honey comprises trace levels of the B vitamins riboflavin, niacin, folic acid, pantothenic acid, and vitamin B6. It also contains calcium, iron, zinc, potassium, phosphorus, magnesium, selenium, chromium, and manganese, as well as ascorbic acid (vitamin C). Flavonoids are the main antioxidants in honey, and one of them, pinocembrin, is unique to honey and bee propolis. Antioxidants also include ascorbic acid, catalase, and selenium. In general, the darker the honey, the more antioxidising it is (Dżugan et al., 2018).

Additionally, honey contains a variety of aromatic acids as well as organic acids like acetic, butanoic, formic, citric, succinic, malic, lactic, pyroglutamic, and gluconic acids (De-Melo et al., 2017). The main acid present is gluconic acid, which is produced during the breakdown of glucose by glucose oxidase. These organic acids in honey contribute to its flavor complex. In addition, honey also contains hydroxymethylfurfural (HMF), a natural byproduct of simple sugar breakdown below pH 5 (Shapla et al., 2018).

HMF is the breakdown of fructose, one of the primary sugars in honey, in the presence of acid. It develops slowly in storage but quickly when heated, making it a good indicator of honey quality. HMF appears naturally in most honeys and tends to rise with age and heat treatment. The existence and accumulation of HMF in honey differs according to the type of honey. HMF per se, can be transformed into a non-excretable, genotoxic molecule called 5-sulfoxymethylfurfural, in previous studies,

HMF was reported to have harmful effects on human health, including cytotoxicity toward mucous membranes, the skin, and the upper respiratory tract; mutagenicity; chromosomal abnormalities; and carcinogenicity against both people and animals (Lee et al., 1995; Monien et al., 2012). Some of the effects of HMF on human health and its carcinogenic properties are still unknown, with many studies being conducted only at the preclinical level.

However, with more in-depth investigations, it has been demonstrated that HMF has a numerous benefits such as antioxidant properties, anti-allergen, anti-apoptotic agent and also exerted cardioprotective effects (Zhao et al., 2013; Wolkart et al., 2017; Shapla et al., 2018). Therefore, for honey usage, the Codex Alimentarius Standard commission has set the maximum limit for HMF in honey at 40 mg/kg with a higher limit of 80 mg/kg for tropical honeys to ensure that the product has not been overheated during processing and is safe for consumption (Alimentarius, 2001; Shapla et al., 2018). As raw honey contains a mixture of ingredients, the crude volatile bioactive compound in honey can be pooled by chemical extraction.

2.2.3 (b) Extraction of crude bioactive compound from honey

Honey typically contains small amounts of water and other non-bioactive ingredients. Therefore, honey extraction may help in pooling its entire bioactive component and eliminate the non-active one. Extraction is the first stage in extracting desired natural products from materials. There are several extraction methods, including solvent extraction, distillation, pressing, and sublimation, that follow the extraction principle. Solvent extraction is the most widely used method. The following stages are involved in the extraction of natural products. First, allowing the

solvent to permeate the solid matrix. Second, the solute is allowed to dissolve in the solvents. Third, the solute is allowed to diffuse out of the solid matrix, and finally, the extracted solutes are collected. Any component that enhances diffusivity and solubility in the previous phases will ease extraction. The extraction efficiency is influenced by the solvent's characteristics, the raw materials particle size, the solvent-to-solid ratio (Ping Li et al., 2014), the extraction temperature and the extraction time (Peng Li et al., 2008).

For solvent extraction, the choice of solvent is essential. The selectivity, solubility, expense, and safety of solvents should all be considered. According to the similarity and intermiscibility principle (like dissolves like), solvents with polarity values close to the solute's are likely to perform better, and vice versa. Alcohols (ethanol and methanol) are all-purpose solvents used in phytochemical study solvent extraction. The extraction is affected by particle size; small particle size increases extraction efficiency due to better solvent penetration and solute dispersion. On the other hand, if the particulate size is too tiny, the solid will absorb too much solute and subsequent filtration will be difficult.

With an increase in extraction time over a specific time, extraction efficiency increases. Adding more time will not affect extraction once the solute has achieved equilibrium both inside and outside of the solid substance. The extraction yield rises with a greater solvent to solid ratio; however, a solvent-to-solid ratio that is excessively high can result in excessive extraction solvent and prolonged concentration time (Zhang et al., 2018). In a study by Bakchiche et al. (2020), they extracted the honey using ethanol as the extraction solvent with constant agitation.

2.2.4 Properties of honey

Honey's components have been shown to have antibacterial, anti-inflammatory, antioxidant, and anticancer properties (Samarghandian et al., 2017). Honey has been known to have antimicrobial properties since the 19th century. Honey has the ability to inhibit the growth of a wide range of bacteria, fungi, protozoa and viruses (Tan et al., 2019). Honey is effective against both Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Jenkins & Cooper, 2012), *Shigella sonnei* (Lusby et al., 2005), *Helicobacter pylori* (Manyi-Loh et al., 2010), and yeasts like *Candida albicans* (Irish et al., 2006). The antibacterial efficacy of honey could be attributed to the production of hydrogen peroxide (H_2O_2), bee defensin-1, high osmolarity, and low pH. According to reports, phenolic compounds are also important components that contribute to honey's antibacterial qualities (Mandal & Mandal, 2011). Safii et al. (2017) demonstrated *in vitro* antimicrobial activity of Manuka honey towards dental plaque-associated bacteria which include *P. gingivalis* and *A. actinomycetemcomitans*. Both *P. gingivalis* and *A. actinomycetemcomitans*, which are linked to aggressive periodontitis and a variety of periodontal diseases, are susceptible to honey when grown as planktonic bacteria, but *P. gingivalis* is much more resistant when grown as a biofilm.

Apart from having the antibacterial property, honey's distinguishing feature that can be used in the treatment of oral disease is its anti-inflammatory activity, which also stimulates the growth of granulation tissue and epithelial cells (English et al., 2004). The role of honey in the treatment of periodontitis also lies within the stimulation of the expansion of granulation tissue and epithelial cells by its anti-inflammatory activity (Molan, 1992). This process helps in the healing of damaged tissues that

have been infected by microbes or free radicals as a result of the pro-inflammatory response. Honey's anti-inflammatory activity can protect dental connective tissues and bone from the pro-inflammatory response, resulting in less periodontal inflammation. It has also been suggested that antioxidants can be used to protect periodontal tissues from damaging free radicals generated by a pro-inflammatory process.

Honey has a high antioxidant activity which is attributed to phenolic acids and flavonoids. Antioxidants, either endogenously generated or externally supplied, are capable of scavenging reactive oxygen species (ROS) and reducing the oxidation of biological components, hence lowering oxidative stress (Gilgun-Sherki et al., 2001). ROS play a role in many important biological processes including gene transcription, signal transduction, and immune response. An overproduction of ROS can result in oxidative damage to biomolecules such as lipids, proteins, and DNA, which has been implicated in the development of ageing as well as various ailments including cancer, respiratory, cardiovascular, neurodegenerative, and digestive diseases. It is postulated that honey's antioxidant ability aids in the prevention of a variety of acute and chronic diseases such as inflammatory, allergic, thrombotic, diabetic, cardiovascular, cancer, and others (Nagai et al., 2001; Aljadi & Kamaruddin, 2004). Recent study showed that after consuming one month of honey, ROS were significantly reduced (Tsamesidis et al., 2022).

Honey was also reported to exert anticancer effects. It has been shown to induce apoptosis in several types of cancer cells (Jaganathan & Mandal, 2010; Fauzi et al., 2011). Apoptosis is a type of programmed cell death in which a cell dies as a result

of a series of molecular processes. This is one way the body gets rid of unwanted or aberrant cells. Honey's apoptotic characteristic is important since many cancer-treatment medicines promote apoptosis (Tomasin & Cintra Gomes-Marcondes, 2011).

Lastly, honey also boosts the immune system which helps in controlling disease. Honey's high antioxidant profile aids in the prevention of immune system damage and disease. Honey also stimulates the development of white blood cells, including T and B cells, as well as natural killer cells, which are all critical components of the immune system (Samarghandian et al., 2017). A study discovered that Manuka enhanced the release of several cytokines, which are important for the immunological response. Cytokines aid white blood cells in locating and eliminating diseased or damaged tissues (Minden-Birkenmaier et al., 2020).

2.2.5 Honey usage in dentistry

In dentistry, honey has been used to treat mouth ulcers and other oral health issues. Honey can remove toxins from the mucous membrane and precipitate proteins, pus and inflammatory exudates can be readily absorbed, saving the underlying tissues and promoting normal recovery and re-epithelialization. Natural honey's viscous properties assist in covering the ulcer. This procedure protects it from secondary infection and keeps the ulcer surface away from chemicals and bacteria. When used to treat sores, honey has no negative mucosal responses or negative effects (Ahmad et al., 2018).

Honey can help prevent caries by inhibiting the production of dextran, a substance that causes dangerous plaque to adhere to teeth (Albaridi, 2019). However, overeating honey, like other sweets, can lead to cavities and gum disease, especially if consumed before bed without cleaning the teeth afterwards. In addition, honey's anti-inflammatory properties shield dental connective tissues and bone from pro-inflammatory reactions, reducing periodontal inflammation (Ahmad et al., 2018). A study conducted by Patel et al. (2010) discovered that honey was a more effective antibacterial than some of the common antibiotics tested on bacterial isolates obtained from orthodontic patients, implying that honey may inhibit dental biofilm formation and aid in controlling gingivitis associated with orthodontic procedures (Steinberg et al., 1996; Nayak et al., 2010; Patel et al., 2010; Coutinho, 2012). Nevertheless, the relationship between honey and tooth caries is still debatable, and more research is required.

Stomatitis is an infection of the mucosal membranes of the mouth and lips, commonly known as "Mucositis". Honey is also used as a non-chemical mouthwash to protect cellular epithelial tissue in stomatitis situations, preventing intercellular rupture and wound infection. It is also used to inhibit bacterial growth (Ahmad et al., 2018). Honey's antibacterial effects have been shown to reduce biofilm development and bacterial adhesion at high doses (Saini et al., 2011). Manuka honey has also been demonstrated to successfully suppress the production of *Streptococcus pyogenes* biofilm (Grobler & Basson, 1997).

Honey's therapeutic characteristics, as demonstrated by its use in wound care elsewhere on the body, indicate that it may be beneficial for the prevention or