Antibacterial and antibiofilm activities of Sarawak stingless bee honey against *Pseudomonas aeruginosa*

by

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Abstract

Pseudomonas aeruginosa causes nosocomial infections among immuno-compromised patients. *P. aeruginosa* is known for its ability to form biofilm and increasingly resistant to many antibiotics, making this infection more difficult to treat. This led to the search for new nature-based antibiotics or antibacterial agents. This study aims to compare the antibacterial and antibiofilm effect of fourteen local stingless bee honey (SBH) samples and an Australian manuka honey against *P. aeruginosa*. The broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) of honey samples against *P. aeruginosa* growth. The inhibition of the bacterium's biofilm formation and the eradication of pre-formed biofilm cultured in different concentrations of honey samples were evaluated with the crystal violet assay. All honey samples, particularly SBHs, are naturally acidic, with a pH range of 2.5 to 3.8, as permitted by the Malaysian Standard for Kelulut (Stingless bee) honey. The effect of acidic components in the honey on the antibacterial and antibiofilm was evaluated by comparing the honey samples in their original pH (no pH modification) and in neutralised pH (pH7).

It was found that all honey samples showed 100% bacterial growth inhibition at 25% (v/v) concentrations. The MIC values for the SBH at original pH ranges between 3% to 5% while the MIC value of the manuka honey was slightly higher at 6%. For the MIC values for the raw SBH, SBHR 1,3,6 and 11 showed the lowest MIC at 3% (v/v) %. The antibiofilm effect is less extensive with only two of the samples (SBHR1 and SBHR11) showing up to 80% antibiofilm inhibition effect and only one of the samples (SBHR6) showing up to 40% biofilm degradation effect at 25% concentration. All SBH samples above (SBHR1, SBHR6 and SBHR6) are raw stingless bee honeys from Sarawak. Comparisons between the original pH and neutralised pH samples showed varying results. Most of the SBH samples showed that pH neutralization decreased their antibacterial effect by up to 4-folds while the manuka honey sample showed a 4.2-fold reduction in antibacterial effect. The SBH samples with the lowest MIC values (SBHR 1,3,6 and 11) were also observed to have a reduction in antibacterial activity by 4-folds. Similar effects were observed in the antibiofilm effects as well. This strongly suggests the important role of the acidic components in the honey samples in the antibacterial effect of these samples.

These results suggest that all SBH samples, especially those from Sarawak, are better antimicrobial and antibiofilm agents compared to the manuka honey analysed in this study. These findings warrant further investigation into the specific chemical compounds and mechanism of actions involved in these bioactive effects before they could be fully utilised in the health and medical industries.

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Declaration

I, Luke Shakti Weissmann declare that this thesis is my own original work and does not contain material that has been accepted for the award of any other degree or diploma. I have disclosed the relative contributions of the respective creators or authors using the Authorship Declaration Form. In addition, I warrant that I have obtained, where necessary, permission from the copyright owners to use any third-party copyright material reproduced in the thesis (such as artwork, images, and unpublished documents) or any of my own published work (such as journal articles) in which the copyright is held by another party (such as publisher, co- author).

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Table of Contents

List of Tables

List of figures

List of Abbreviations

ACE2-spike Angiotensin-converting enzyme 2 AU Absorbance unit

CFU colony forming unit

CSSI Chronic subclinical systemic inflammation DNA Deoxyribonucleic acid

IC inhibitory concentration

IC50 Half-maximal inhibitory concentration LB Luria broth

LPS lipopolysaccharide

MBC Minimum Bactericidal Concentration MDA multiple discriminant analysis

MIC Minimum inhibitory concentration

MRSA Methicillin-resistant Streptococcus aureus

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide NaCl Sodium Chloride

NaOH Sodium Hydroxide

OD Optical density

OHdG 8-hydroxy-2' -deoxyguanosine

qRT-PCR Real-Time Quantitative Reverse Transcription Real-Time Quantitative Reverse Transcription PCR

QS Quorum sensing

RNA Ribonucleic acid

RsmA Regulator of Secondary Metabolites SagS Super Antigen

SARS-Cov-2 severe acute respiratory syndrome coronavirus 2 UMFunique manuka factor

Thesis outline

This thesis comprised of the following chapters,

Chapter 1 provides a brief introduction to research backgrounds and objectives.

Chapter 2 gives an introduction of the honey samples used in this study, and summaries the critical reviews of antibacterial effects of common (Apis), Manuka and stingless bee honeys.

Chapter 3 describes the methodologies used to achieve the two objectives above.

Chapter 4 demonstrates the antimicrobial and antibiofilm activities of the honey samples against *P. aeruginosa*; and

Chapter 5 summaries the conclusions of the studies and discusses the future perspectives of this research.

Chapter 1: Introduction

1.1 Background

Meliponini also known as stingless bees are a tribe of bees. The other commonly found bee tribes are Apini (honeybees), Bombini (bumble bees) and Euglossini (orchid bees). The global honey market was worth an estimated USD 9.21 billion as of 2020 and much of the honey available commercially comes from the Apis tribe of bees (Grand View Research 2021). This is mainly because of their unparalleled ability to produce large quantities of honey. The other bee tribes (Euglossini and Bombini) do not produce enough honey to be commercially viable. The honey produced from Apini is viscous because it undergoes dehydration. This is achieved when the bees fan their wings at their nest entrances to improve ventilation (Ramli et al 2017). This dehydration allows the honey to be naturally preserved. In comparison, the honey from stingless bees is naturally preserved by a combination of dehydration and fermentation. The dehydration process in stingless bees is like that of Apini bee tribes, but it is limited due to the smaller wing size of stingless bees. Additionally, since stingless bees are commonly found in tropical areas with high humidity, their honey typically has higher moisture content (Ramli et al 2017). This higher water content gives microbes a more favorable growth environment thus promoting fermentation. As a result, stingless bee honey is typically less viscous or more watery, with a sour taste due to the presence of organic acids from fermentation (Basharat et al 2023). These factors make stingless bee honey unique from other honeys.

The rapid emergence of bacteria with antibiotic resistance is occurring worldwide. The antibiotic resistance of bacteria has been linked to the over usage and misuse of antibiotics when treating bacterial infections. There is a need to combat antibiotic resistant bacteria through the discovery of new substances. This is to ease the threats and burdens placed on the healthcare system from emerging antibiotic resistant bacteria. One such substance is honey which has been used since ancient times. Its management of microbial infections has been well documented in ancient cultures such as in Greece, Egypt and in China (Sengupta et al 2013). The National Surveillance of Antibiotic Resistance (NSAR) reported on an increasing trend of antimicrobial resistance (AMR) bacteria in the last decade in Malaysia (Ministry of Health Malaysia 2017). It was reported that bacteria such as *Enterococcus faecium*'s resistant to vancomycin prevalence increased from 8.7% in 2012 to 14.9% in 2016. *Acinetobacter baumanii* was also reported by NSAR, showing an increased

prevalence in bacteria resistance to meropenem where it was 49% in 2008 and 61% in 2016. This uptrend in AMR was also noted in the report for a variety of other bacteria. This highlights that AMR bacteria are a problem that will get worse over time (Ministry of Health Malaysia 2017).

Malaysia is ranked among the world's most biodiverse countries having more than 15,000 species of plants, trees, and flowers (Tong et al 2020). This is one of the contributing factors making honey sourced from stingless bees unique to Sarawak. Stingless bee honey varies from western honeybee honey in many factors such as moisture content, higher acidity, lower enzyme activity, and lower sugar content. Besides that, stingless bee honey composition also varies between stingless bee species (Nordin et al 2018). The composition and antimicrobial activity of honey is very dependent on factors such as botanical sources, geographical location, environmental factors, and the processing it undergoes (Siok et al 2017). These factors make the stingless bee honey found in Sarawak unique.

1.2 Problem Statement

Honey is a well-known substance which has been used in home remedies for a long time (Hegazi et al 2022). Of all the different types of honey, the most expensive and well researched honey is manuka honey. It costs on average USD $22-42 for 250 grams (Fuller 2017) which is 5 times more when compared to normal bee honey. Manuka honey is expensive because of a combination of different factors. The first factor is its uniquely high concentration of methylglyoxal (MGO). This compound gives manuka honey good antibacterial properties and is not found in as high concentrations in other honey. The next factor is that manuka honey is only produced in New Zealand and parts of Australia due to the location of the manuka trees. This is the primary source of nectar for the bees that produce manuka honey. Moreover, manuka trees exhibit a brief blooming period, typically spanning from two to six weeks within a given year. This limited flowering window significantly reduces the availability of manuka honey, amplifying its value and demand in the market. The last major factor is that the global demand for manuka is much higher than the supply (Perelmutter 2023). Unlike manuka honey, Sarawak stingless bee honey is the lesser-known type of honey. While it is gaining interest as potential functional food product or ingredient, studies on its nutritional as well as pharmaceutical properties are still very limited.

The most common use for honey is in the food industry as a flavoring or an alternative sweetener. Honey can also be used as a topical treatment to kill certain

bacteria and fungi. It is mostly used in the treatment of minor wounds and burns but also for treatment of skin diseases such as dermatitis and eczema (Alangari et al 2017). These uses are all associated with honeybee honey while stingless bee honey is less utilized.

Over the last few decades there have been observations in increasing frequencies of antibiotic resistant bacteria. These antibiotic resistant bacteria cause many challenges such as economic impacts, morbidity, and increased morality rates. As such, there is a need to develop new means of fighting against antibiotic resistant bacteria. Honey is known to have broad-spectrum antibacterial activity. This is due to honey's high sugar content, low moisture content, low pH, and hydrogen peroxide. Besides that, honey is known to contain antioxidants, vitamins, and minerals.

Pseudomonas aeruginosa is a bacterium that is becoming increasingly difficult due to its remarkable antibiotic resistance. One of the mechanisms that this bacterium uses for its antibiotic resistance is the development of biofilms. The formation of a bacterial biofilm allows the conference of antibiotic resistance through chromosomal resistant gene expression. The biofilm also allows the bacteria to confer other processes such as antibiotic restriction, countering the hosts immune system as well as alteration of the bacterial growth rate (Macia et al 2014). There is an increasing need to develop alternative therapeutic treatments that target the bacteria as well as its biofilm production.

Stingless bee honey is far less researched in terms of published articles and journals when compared to honeybee honey. This is in terms of practical applications as well as research done on studying the effects of their honeys. Honey is unique for different bees because of many factors such as environmental factors, geographical location, botanical sources as well as bee species. Therefore, stingless bee honey sourced in Sarawak is unique to not only honeybee honey, but it is unique to different species of stingless bees.

Sarawak has a unique ecosystem which contains a wide variety of botanical resources. When comparing tree species, Sarawak and Sabah were found to have tree endemism of 42% while Peninsular Malaysia had about half of that, 26.4% (Saw et al 2010). As such, the stingless bee honey that are found in Sarawak could be also quite diverse and could be unique within the region, due to their diverse and unique source of food or nectar.

1.3 Aim of study

In this study 12 Sarawak stingless bee honeys, two West Malaysian stingless bee honeys, and one Australian manuka honey were analysed. This research aims to improve the knowledge of antimicrobial and antibiofilm activity of Sarawak stingless bee honey. This work will form the foundation for future research exploring the medical applications of Sarawak stingless bee honey.

1.4 Objectives

1. To determine antimicrobial activity of Sarawak stingless honey against *Pseudomonas aeruginosa* through microdilution assays.
2. To investigate the antibiofilm activity of Sarawak stingless bee honey against *Pseudomonas aeruginosa* by determining their biofilm formation inhibition, biofilm degradation and pyocyanin production inhibition activities.

Chapter 2: Literature review

2.1 Honey

There are roughly 20,000 species of bees that are part of seven different families. These seven families are *Andrenidae, Apidae, Colletidae, Jalictidae, Megachilidae, Melittidae* and *Stenotritidae*. Of these families, the biggest tribe is the Apidae tribe. The Apidae tribe includes bees such as honeybees, bumblebees, and stingless bees. Bees have an important ecological role as pollinators to flowering plants. Bees are the most dominant pollinators in nature. They are important to crop pollination. This highlights the need to improve the conservation of wild bees and their habitats (Patel et al 2021). Of the world's top 107 crops it is reported that 90% of them are visited by bees (Klein et al 2007).

Honey is a naturally sweet product. It is produced by bees mainly from plant secretions and plant nectars. Nectars are aqueous sugary compounds which are turned into honey by utilizing enzymes secreted by glands on the bees. The honey is then deposited by the bees into cells in the beehives where water evaporates off. The honey is then sealed in beeswax. The composition of honey is very dependent on factors such as botanical sources, geographical location, environmental factors, and the processing it undergoes (Siok et al 2017).

There are many studies which classify honey depending to factors such as carbohydrate composition, botanical sources, and species of bees.

2.1.1 Apis honey

The honeybee better known as the European honeybee (*Apis* mellifera) is the bee species that is used the most widely in crop pollination and honey production. An example of European honeybees can be seen in Figure 1. This is due to its ability to produce large amounts of honey compared to other species (Alfredo et al 2019). Honeybees were estimated to provide pollination services to United States crops worth $14.6 billion (USD) in 2000 (Greenleaf et al 2006). Honeybees forage plant products which they use as food sources and as building materials.

Honeybees can live in many different habitats. They manipulate their environment to maintain their colony health, this is done by producing wax and honey which reduce the spread of parasites and diseases (Easton-Calabria et al 2019). Since honeybees live in colonies and are in close contact with one another, there is always the risk of

diseases. This risk is reduced by the implementation of colony-level defences. One such defence is known as social immunity where the honeybees have cooperative and altruistic behaviour between individuals (Easton-Calabria et al 2019).

Honeybees differ from stingless bees because of the presence of stingers and venom sacs. The venom of honeybees is not only used as a defensive mechanism against predators, but it is also used to sterilize the comb of their nests (Baracchi et al 2011). Honeybee venom is made of primarily of the enzyme phospholipase A2 and the polypeptide melittin. Melittin works by enhancing the activity of phospholipase A2. Melittin is known to have antiseptic properties while phospholipase A2 has antibacterial activity which causes inhabitation of Gram-negative bacterial (Samy et al 2006). It has been found that honeybees spread venom on their bodies as well as on their nest.

Honeybees found in nature have a large range of local and regional adaptations to their environments. These adaptations arise from specific climate patterns as well as the availability of different resources at different times of the year. One of these adaptations was observed in *A. melllifera* in southwest France, the annual brood cycle of the bee populations was linked to the cycle of a local flower (De la Rua et al 2009). These adaptations make the conservation of honeybee populations a pressing issue because their survival is closely linked to their local environments. It was found that if the honeybees were removed from their local environment, it reduced their productivity of honey as well as lowered their survival rate (Büchler et al 2015). Büchler et al (2015) studied the effects of Varroa mite infestations in honeybee colonies. It was discovered that honeybee colonies, which had shown resistance to mites in the past, became sensitive to them after being removed from their environment. This shows that the mechanism of the mite resistance was dependent on the genotype- environment interactions.

Figure 1: European honeybees, *Apis mellifera* (Mortensen et al. 2013)

Honeybee honey is made by honeybees when they visit flowering plants and collect their nectar. The collected nectar is held within a honeybee's honey stomach. When their honey stomach is full, the honeybees fly back to their hive. After returning to their hive the honeybees pass the collected nectar from one honeybee to another. As the nectar is passed, it is continually chewed by worker honeybees. This process goes on for about half an hour. After half an hour, the nectar turns into honey at which point it is deposited by the honeybee into a honeycomb cell (Figure 1). These cells are small jars made from wax. After being deposited into the cell, the honeybees fan the honey with their wings. This wing fanning causes water to evaporate causing the honey to become thicker and stickier. After the honey is dried off, the honeycomb cell is sealed off. This sealing keeps the honey within clean (The Guardian 2022). Honeybee honey is known to contain 17% water, 82% sugars, 0.1%-3.3% proteins, 0.57% organic acids, minerals 0.04%-0.2% and vitamins (Missio da Silva et al 2015).

Some Apis honey are further categorised based on their botanical source. For example, manuka honey is a mono-floral honey obtained from the manuka tea tree (*Leptospermum scoparium*) found in New Zealand and Eastern Australia. The manuka tree is part of the Myrtaceae family which grows into a small tree or shrub. manuka honey was only recently brought about by the introduction of European honeybees in the 19th century. Manuka honey typically carries a higher price tag compared to other varieties, averaging between USD $22-42 for a 250-gram (Fuller 2017). Manuka honey derives its antimicrobial potency from its methylglyoxal (MGO) content. Beyond its antimicrobial properties, manuka honey manuka honey is rich in sugars, free amino acids, enzymes, proteins, essential minerals, flavonoids, phenolic acids, and vitamins. All these characteristics make manuka honey a highly sought after product. Manuka honey is produced at a limited quantity because it is only produced in New Zealand and parts of Australia. This causes the price of manuka honey to be higher than that of other honeys (Perelmutter, 2023).

Many of manuka honey's chemical compositions are products of secondary plant metabolism (El-Senduny et al 2021). These compounds are integrated into the honey through nectar, pollen, and honeydew. These unique compounds in manuka honey are directly linked to its sought-after health benefits. These benefits include anticancer, antioxidant as well as wound healing properties (El-Senduny et al 2021). These properties allow manuka to be widely used in medical applications as well as being used in the food industry.

2.1.2 Stingless bee honey

The stingless bee naturally exists on almost every continent. Stingless bees are the largest group of eusocial bees on the Earth, having more than 500 different species (Hrncir et al 2016). Stingless bees are mainly found in tropical regions where they are primary pollinators. They are easily distinguishable from other bees by having 3 unique characteristics. Firstly, their wings have less venation compared to other bees, the presence of a setae located on the hind tibia, and lastly the absence of a sting. This can be better visualized in Figure 2. It is also understood that stingless bees have a lower flight range of less than 1km compared to honeybees (more than 3km) (Wille 1983). Much less is known about stingless bees compared to honeybees. This is because of the difficulty of access to their natural habitats and lastly their inability to maintain colonies outside of the tropics (Jalil et al. 2017).

Stingless bees are known to have the ability to pollinate small-medium sized flowers which cannot be achieved by the bigger honeybee. This makes the sources of pollen collection unique to stingless bees. A study by Oethe et al. (2020) investigated the food source selection of stingless bees and honeybees (*P. flavocincta, M. subnitida,* and *A. mellifera*). The study reported that all the three bee species reacted differently towards colour, scent, markers, and food source locations. *A. mellifera* choose its food sources according to both colour and scent, *P. flavocincta* mainly used scent to choose its food source, and *M. subnitida* choose its food sources primarily on location and colour. These variations make the honey produced from different bee species unique from one another.

Besides that, stingless bees have the added advantage where they do not sting, this makes the collection of honey relatively easier. Furthermore, stingless bees are not choosey when building a colony hive, this makes it easier to manipulate the colony locations by building artificial hives (Jalil et al 2017). Like other bees', stingless bees store their honey in small resins pots within their hive. The honey pots are made of a mixture of beeswax and resins.

A review by Bath et al (2008) highlighted that there is a lot of evidence which shows that stingless bees give out chemical and mechanical signals when communicating about food sources. One such example would be that *Melipona* are known the give out vibratory sound pulses. These signals were found to correlate positively with the distance of its food source. The sound pulses are created by thoracic vibrations and linked to being an important carrier of information for stingless bees.

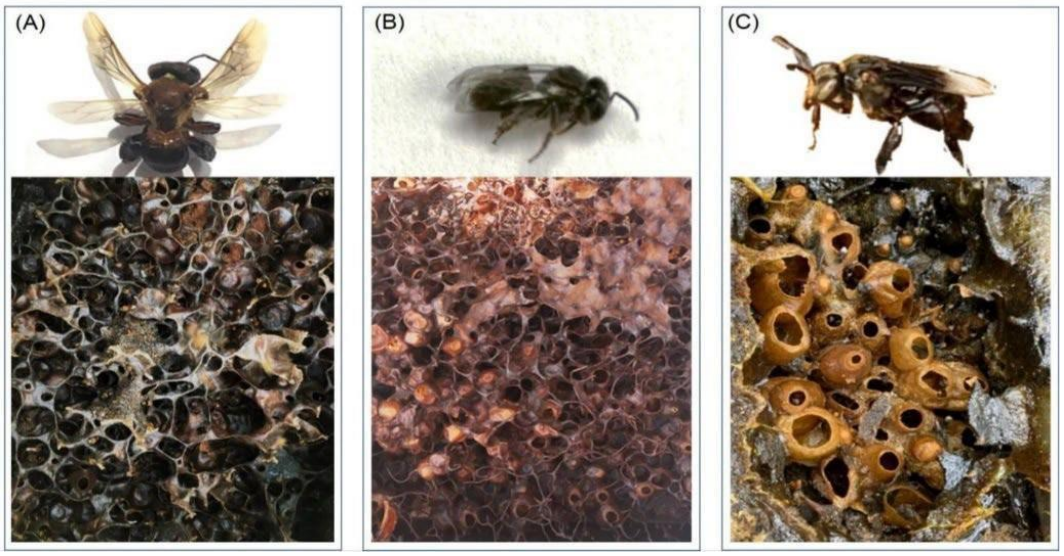


Figure 2: Different species of stingless bee (A) *G. thoracic*a, (B) *H. itama*, and (C) *T. binghami* with their propolis (Abdullah et al 2020)

The most common genus of stingless bees is *Trigona* and *Melipona*. The honey of stingless bees is noted to have higher nutritional and medicinal properties compared to western honeybee products (Zuluaga-Dominguez et al 2012). As such it is seen that stingless bee honey products can be a promising source of biologically active compounds. Stingless bees are smaller and more agile compared to Apis bees, allowing them to access a wider variety of flowers, including those with narrow openings or unusual shapes. Their smaller size enables them to navigate through intricate floral structures that may be inaccessible to larger bees. Despite this, stingless bee produce less honey per hive (1-5 kg) compared to regular honeybees who produce on average 20 kg of honey (Chuttong et al 2016). Stingless bees collect and process nectar into honey similarly as honeybees where the nectar is chewed and passed on from one bee to the next until it turns into honey. The main difference between stingless bees and honeybees is that stingless bees store their honey in cerumen pots (Figure 2) instead of the usual honeycomb pots (Fletcher et al 2020). This causes the harvesting of stingless bee honey to be much more difficult. Stingless bee honey has a higher moisture content compared to honeybee honey. The moisture content of stingless bee honey can range between 28.4%- 42% (Lubertus et al 2006).

Stingless bee honey is known to have a distinct flavour accompanied by a sour taste compared to mainly sweet Apis honey. It is noted that stingless bee farming is increasing among rural residents to meet the growing demand for stingless bee honey. Stingless bee honey is shown to have therapeutic effects as it has antioxidative, anti-inflammatory and antibacterial properties. These properties give

stingless bee honey potential in commercialisation. Stingless bee honey is known to have a high number of polyphenol compounds which are good at promoting cell proliferation, reduce free radicals at wound areas as well as protecting the cellular structure of the cells (Jalil et al 2017).

Table 1: Summary of research on stingless bee honey

Reference	Country	Purpose	Summary
Tuksitha et al 2018	Taiwan	Antioxidant and antibacterial activity of Sarawak stingless bee honey.	A comparison of honeys from three different stingless bee species, namely *Geniotrigona thoracica*, *Heterotrigona itama*, and *Heterotrigona erythrogastra*, all from the same farm, revealed that *G. thoracica* honey had the highest phenolic content (99.04 ± 5.14 mg/ml). The antimicrobial activity, tested using the zone of inhibition method, showed that *G. thoracica* honey exhibited the strongest activity.
Ramlan et al 2021	Malaysia	Test the effects of heating on the antioxidant and antibacterial activity of Malaysian (Selangor) and Australian (Brisbane) stingless bee honey	The study found that heat treatment at 45°C, 55°C, and 65°C for 60 minutes did not affect the total phenolic content. However, the heating did cause a decrease in the antibacterial activity of stingless bee honeys from both countries.
Chuah et al 2023	Malaysia	Antioxidant detection in monofloral stingless bee honeys using mass spectrometry and metabolomics	The study found that each type of stingless bee honey contains a unique blend of antioxidant metabolites, derived from the diverse botanical sources near the bees' hives. Among the tested honeys, acacia honey exhibited the highest antioxidant properties.
Rosli et al 2020	Malaysia	Antibacterial activity and bacterial diversity of Selangor stingless bee honey	*Homotrigonia fimbriata* honey was shown to inhibit 4 out of 5 tested bacterial species. The study, which utilized 16S sequencing, identified eight phyla, 71 families, 155 genera, and 70 species within the honey.

The information in Table 1 shows summaries of some of the recent studies conducted on stingless bee honeys.

2.2 *Pseudomonas aeruginosa*

2.2.1 General background

P. aeruginosa causes is a major contributor to nosocomial associated infections where exposure to it can cause illnesses such as infections of the bloodstream, urinary tract infections and skin infections. The bacterium causes an estimated 51,000 infections in the United States, where an approximate 13% showed multidrug resistance (Sader et al 2017). *P. aeruginosa* is a well-equipped pathogen that produces a big variety of virulence factors and is known to be able to develop resistances to antibacterial drugs such as pepercallin, ceftazidimine, imipenem, ciprofloxacin, and aminoglycosides (Christian van Delden 2007). The development of these antimicrobial resistances is associated with patients who have been previously exposed to antimicrobials. This highlights a need for alternative types of therapeutical treatments for *P. aeruginosa.*

2.2.2 Biofilms

The commonly accepted terminology which identifies a biofilm is ‘a structured community of self-developed polymeric matrix and adherent to a living or inert surface’ (Costerton et al 1987). Biofilms are bacterial aggregates that are formed by bacteria, they are formed in the extracellular matrices of its enzymes, proteins, polysaccharides as well as nucleic acids. This aggregation of bacteria facilitates their anchorage to most surfaces. This anchorage is irreversible (Dumaru et al 2019). The formation of a bacterial biofilm allows the conference of antibiotic resistance through chromosomal resistant gene expression. The biofilm also allows the bacteria to confer other processes such as antibiotic restriction, countering the hosts immune system as well as alteration of the bacterial growth rate (Macia et al 2014). These characteristics of biofilms contribute to the development of persistent infections in patients. Costerton et al (1987), indicates that in nature up to 99% of microbes are living in biofilm micro-ecosystems.

The formation of biofilms in microorganisms is known to be a survival mechanism to defend against stressors in the environment such as UV-radiation, temperature changes, drying, cleaning agents such as disinfectants as well as immune response from the hosts immune system. Because of the biofilms ability to defend against such a wide variety of stressors, biofilm associated bacteria are very difficult to treat (Costerton et al 1987).

Biofilm's ability to adhere to surfaces has many negative impacts on a variety of different industrial processes such as paper production, oil drilling, food processing, as well as in the medical field. The processes of biofilm formation are well studied and understood but the effective removal or prevention of biofilm formation is not (Stoodley et al 2002).

When bacteria are in a biofilm their behaviour is different from when they are planktonic, this is especially the case when they undergo antibiotic treatment. When in a biofilm the bacteria become highly resistant to antibiotics. This resistance is known to be caused by the extracellular matrix which acts as a physical barrier preventing the antibiotics from reaching the bacteria. Another reason for their antibiotic resistance is that in a biofilm the bacteria behave less actively because of accumulation of waste as well as depletion of nutrients. This inactivity causes antibiotics to be less effective towards the bacteria compared to active bacteria. (Otto 2008).

Stages of a biofilms

The formation of biofilms begins with free floating planktonic bacterial cells**.** These cells undergo a profound change which causes the transition from planktonic bacteria into biofilm producing bacteria. This change is a highly complex and regulated process that is known to be caused during the development of bacteria (Sauer et al 2022). It was not well understood whether the formation of biofilms is caused by the accumulation of cells because of cell division or if it is caused by external factors. In Sauer et al (2002), to better understand biofilm formation multiple observations (biofilm morphology, quorum sensing genes, matrix polymer and protein abundance) were analysed during the formation of biofilms in *P. aeruginosa*. This led to understanding that during the formation of biofilms the bacteria shows multiple phenotypes and different physiological characteristics. These phenotypes can be classified into different stages of the bacteria's production of the biofilm. Also, the bacteria produce unique protein patterns as well as gene expression during the different stages of the biofilm. The stages of biofilms are known as reversable attachment, irreversible attachment, maturation, and dispersal. The reversible attachment stage in biofilms is characterized when bacteria attach only by a single pole. This single pole attachment is very unstable which usually causes the cell to return to the planktonic phase. If the bacteria were to instead attach with its longitudinal axis, then the biofilm stage would be in the irreversible attachment stage. Davies et al (1993) has shown that if the bacteria were to go into the irreversible

attachment stage the bacteria will begin a cascade of changes. One such a change would be that the bacteria will stop flagella motility. The other changes are noted to be genetic expression of genes linked to antibiotic resistance such as SagS, Br1R and phenazine as well as gene expression of matrix polymer production (Sauer et al 2022). This shows that surface attachment causes the production of biofilm complexes as well as increases the antimicrobial tolerances of the bacteria.

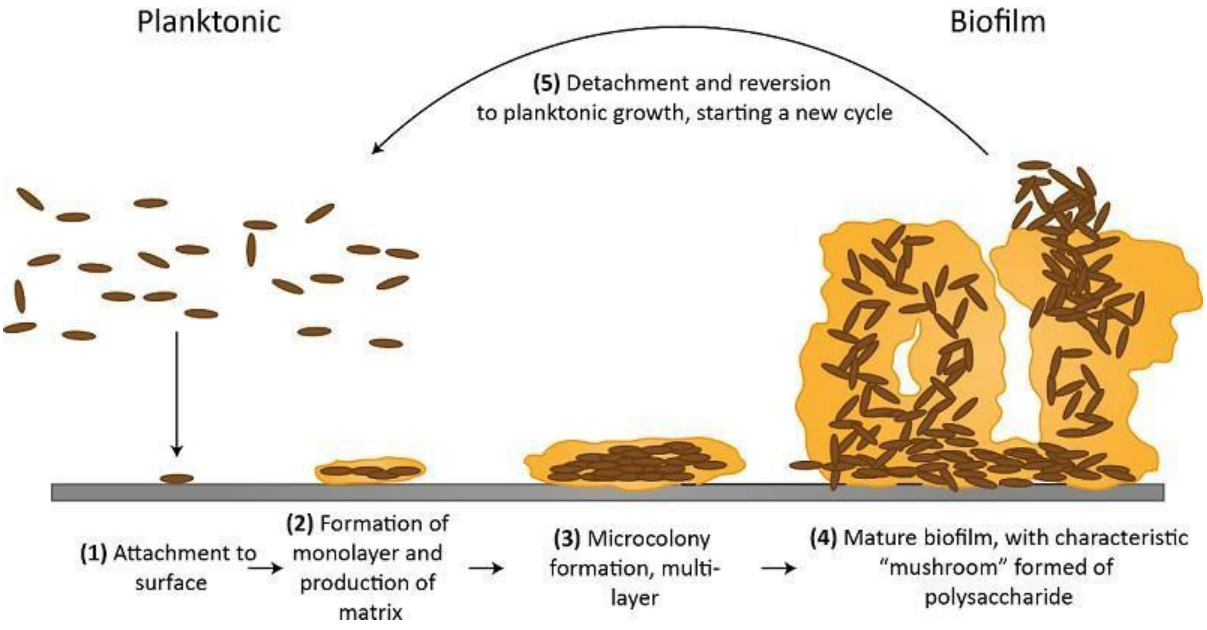


Figure 3: Stages of biofilm formation (image from Vasudevan 2014 with permission).

After the bacteria are attached the cells begin to grow into a more complex and mature form Figure 3 – (3). In some bacteria like *P. aeruginosa* this maturation can be observed by pillar- like microcolonies that have fluid filled channels Figure 3 – (4). These fluid channels in biofilms are associated with bacterial communication in the biofilm. It has also been noted in Purevdorj et al (2002), that if the genes in bacteria responsible for signalling have been knocked out, the bacteria will still develop these fluid channels for communication. This shows that these channels are produced by bacterial regulation as well as environmental factors. As the biofilm further matures the bacteria who are closer to the base are separated from the liquid interface which cuts it off from nutrients. As such bacteria in a biofilm are experiencing changes in their microenvironment. These changes are caused by nutrient competition, overcrowding as well as chemical gradients. Because of this there is stratification of bacteria within the biofilm causing there to be subpopulations (Stewart et al 2008). It is common for bacteria within the same biofilm to experience different gradients of oxygen, waste products and nutrients. This is further highlighted by bacteria in a biofilm expressing

genes that are linked to nutrient stress, slow growth as well as oxygen deprivation (Haussler et al 2012).

Bacterial cells can leave formed biofilms by a process called dispersion Figure 3 – (5). During dispersion the matrix encased bacterial cell escapes the biofilm. This dispersion is the next stage of a biofilm. During dispersal the bacteria disseminates and colonizes new areas (Purevdorj-Gage et al 2005).

Biofilms in healthcare

Biofilms have high tolerances against desiccation which means they can survive in dry environments which would normally kill planktonic bacteria. Dry surface biofilms are known to be able to survive more than 12 months within a sterile container as well as on a bench without the availability of nutrients (Hu et al 2015). Biofilms have reportedly been detected on 90% of dry hospital surfaces in four different countries (Australia, Brazil, Saudi Arabia, and the UK). Infections related to biofilms cause a significant increase in morbidity and mortality. The patients who are the most affected by biofilms are those who have implanted medical devices and those with an immune system that is weakened. A high number of hospitals acquired bacterial infections are associated to the use of medical devices and prosthetics which were contaminated which biofilms. Biofilms significantly increase the virulence of bacteria, but they are rarely thought off during the management of infections. As most clinicians base their treatment plan according to planktonic in vitro bacteria instead of towards biofilm associated infections (Vazquez et al 2020).

As of now the most common ways to counter the formation of biofilms is by prevention. Clinics currently use methods such as silver coating of implants and filling of implants which antibiotics. These methods are effective but are only used in implants (Polivache et al 2020).

The other commonly used methods of biofilm prevention are focused on the matrix formed by the biofilms. These techniques are using lasers, heat, or electrical currents to break-up the biofilm matrix so that they can be targeted by antibiotics. These methods are time consuming and difficult to use (Polivache et al 2020).

A newer developed method is using anti-persister molecules which directly target the bacteria in the biofilm. These anti-persister molecules are newly discovered and are still undergoing clinical trials (Lin et al 2022). Besides that, another newly developed method would be the use of bacteriophages. The bacteriophages are used to produce enzymes which can breakdown the biofilm matrices. However, there are many risks in using bacteriophages as they can interact with the human immune response

(Polivache et al 2020). Hence, there is a real need to develop a safe and effective treatment towards antibiofilm activity of bacteria.

Antibiofilm compounds

Biofilms as mentioned are difficult to treat. There has been research on finding compounds which provide effective treatments against biofilm formation as well as the bacteria producing these biofilms. Gowrishankar et al (2016), observed and reported on biofilm forming methicillin-resistant *Streptococcus aureus* (MRSA) isolated from pharyngitis patients. In this study it was noted that MRSA was identified along with another *Streptococcus spp.* to be the cause for pharyngitis. This identification focuses on the need for broad-spectrum antibiotics for treatment in such infections. This shows that there is a need for therapeutic treatments in the medical industry. Biofilms are also associated with food spoilage bacteria. The main difficulties faced by the food industry from biofilms is the formation of biofilms on processing equipment. This biofilm formation on the equipment causes food contamination during the cooking or packaging of foods. Currently, the food industry carries out disinfection of its equipment using sanitizers. A study Cincarova et al (2017) tested the effects of sublethal concentrations of disinfectants on *S. aureus* biofilms in a meat processing factory. The study showed that the optimization of sanitizers and duration of disinfection is vital when sterilizing equipment. Suboptimal sanitiser concentrations can trigger a defence mechanism in the bacteria which leads to the bacteria resisting cleaning. This may cause food contamination further down the line.

An antibiofilm compound was studied by Mohanta et al (2020). In this study silver nanoparticles were combined with plant extracts to determine their ability to inhibit the biofilm production of *S. aureus, P. aeruginosa* and *E. coli*. The study found that for the treatment of *P. aeruginosa* for 24 hours with silver nanoparticles using the plant extract of *G. lanceolarim* plant extract at 100 µg/ml resulted in a reduction of biofilm activity of >99%. While using other extracts of *S. anacardium* and *B. retusa* with silver nanoparticles in concentrations of 50 and 60 µg/ml showed >99% biofilm inhibition. This study showed that the extracts showed strong antibiofilm activity when in combination with silver nanoparticles.

Another study by Alam et al (2020) tested plant derived extracts against *P. aeruginosa* biofilms. In this study several plants were extracted using a large variety of solvents (ethanol, methanol, acetone, ethyl acetate, hexane, and chloroform). These extracts were then tested for the ability to inhibit biofilms. The species of plants which were extracted were *B. ciliate, C. grata* and *C. viticella*. The plants were thoroughly

washed and then airdried. After which they were ground into powder from. The powders then underwent solvent extraction using the forementioned solvents. After extraction the extracts were concentrated using a rotary evaporator. The results showed that the different plants as well as the different extracts used played a major difference in the effective inhibition of the biofilms formed. The best combination of plant and solvent was *B. ciliata* using methanol extraction which showed 80% inhibition of *P. aeruginosa* biofilms. Other notable extract and solvent combinations were ethyl acetate with all plants which had 50% inhibition. While the other solvents had a range of 0-40% inhibition. This study shows that different solvent extractions can extract different bioactive molecules who have different antibiofilm activity. The extracts of methanol had the best activity.

In a study by Costa et al (2016), a peptide P34 was tested against *S. aureus* and *Eenterococcus faecalis* for its antibiofilm activity. In this study the P34 peptide was produced and purified from *Bacillus* sp. The bacteria were grown and then underwent specific purification and extraction to obtain the P34 peptide. Solutions equivalent to 1600 AU were prepared of the P34 peptide. From which 100 microliters were added into 96-well plates. The selected test cultures were then added to the wells and incubated for 24 hours. Then the absorbance was determined using crystal violet staining. The results for the antibiofilm activity assay showed that the P34 had antibiofilm activity towards both against *S. aureus* and *E. faecalis.* The best activity was observed at 46.9% reduction of cell adhesion when tested against *S. aureus*. The results of this study varied as there were multiple strains of each bacteria used. For *S. aureus* the inhibition of cell adhesion ranged between 8. 8%-46.9%. For *E. faecalis* the inhibitory activity ranged between 2 . 4 % -31.4%. This study highlights that the antibiofilm activity of compounds can vary greatly depending on different strains of the same species of bacteria.

Honeys are known to contain polyphenols. A study by Matilla-Cuenca et al (2020) tested flavonoids on the biofilms of *S. aureus*. The study used a collection of polyphenolic compounds which included flavonoids, phenolic acids as well as stilbenoids. *S. aureus* were incubated in the presence of these polyphenols and then were stained using crystal violet. The study determined that myricetin 20 µg/ml, baicalein 20 µg/ml and scutellarein 10 µg/ml all showed strong biofilm formation inhibition. They had inhibited biofilm formation by 94% ± 1%, 91% ± 3%, and 92% ± 3%, respectively. The study concludes that the likelihood of *S. aureus* biofilm inhibition is due to the targeting of Bap expression. This is due to the interaction of polyphenols with oligomers. This impedes the polymerization of fibres that are vital in the

production of biofilms. This shows that antibiofilm compounds target different aspects of the formation of biofilms.

In a study by Nassar et al (2023), Germany store bought honey (Apis Honey) was tested for its effect on *Streptococcus mutans* growth as well as biofilm formation. Bacteriaformationsand biofilm formation were tested using either the German honeybee honey or an artificially made honey. The results of this testing showed that when comparing both types of honey for bacterial growth showed that the natural honey showed significantly less bacterial growth. For the biofilm testing the study showed that the natural honey was able to inhibit biofilm formation at concentrations between 50% – 12.5%. The artificial honey did not have similar inhibition. This study shows that honey can inhibit other bacteria species other than *P. aeruginosa*. It also shows that the artificial honey (40.5% fructose, 33.5% glucose, 7.5% maltose, and 1.5% sucrose in deionised water) was not as effective as the natural honey against both inhibiting bacteria growth as well as inhibiting biofilm formation. This highlights that other compounds in the honey not only sugars give honey its antibiofilm properties.

Another study by Iseppi et al (2023), investigated the antibiofilm properties of two different essential oils. In this study tea-tree oil and eucalyptus oil were tested against both biofilms undergoing formation as well as mature biofilms. In this study the oils were tested against a variety of bacterial biofilms (*S. aeurus, Enterococci spp.* and *E. coil)*. The study was conducted using microdilution and the antibiofilm activity was measured using 96-well microplates. The results of this study showed that both essential oils showed inhibitory activity against mature and biofilms undergoing formation. The essential oils showed better results against biofilm formation. When both oils were used together it was found that there were synergistic effects against the formation of biofilms.

Heather honey and its effects on biofilms was studied by Shirlaw et al (2020). In this study the effects of heather honey on biofilms of *S. aureus, P. aeruginosa, E. coli, K. pneumoniae. E. faecalis, S. enteriditis* and *A. baumaniil* were studied and compared to the effects of manuka honey. It was noted that at 0.25 mg/ mL, heather honey inhibited the biofilm formation of *P. aeruginosa, E. coli, K. pneumoniae. E. faecalis, S. enteriditis* and *A. baumaniil* but it promoted the growth of *S. aureus* biofilms. While the manuka honey was noted to decrease the biofilms in *P. aeruginosa, E. coli, E. faecalis* and *A. baumanii*. The study used crystal violet assay to quantify the effects of the honey on bacteria biofilms. It was determined that when testing the honeys at sub-inhibitory concentrations they cause an increase in the growth of the biofilms. It

was concluded that the presence of benzoic acid in manuka and heather honey as well as other small molecules such as MGO in manuka honey can target the virulence in some of the bacteria tested and aid in the inhibition of their biofilms.

2.2.3 Pyocyanin

General background

Pyocyanin was first noted in 1860 by Fordos, where it was observed as a bluish sample from an infection that was caused by *P. aeruginosa* (Kurachi, 1958). The name pyocyanin comes from a combination of Greek words used to describe pus and the colour blue. Fordos also described pyocyanin's properties such as its solubility as well as its changes colour according to different pH levels (Kurachi, 1958). Pyocyanin was first isolated in 1924, becoming the first natural phenazine to be purified in a lab (Gaby et al 1946). Later, in 1942 pyocyanin was classified as an antibiotic because of its therapeutic effects against infectious diseases (Waksman 1973).

Pyocyanin is a chloroform and water-soluble compound. The phenol group in pyocyanin is attributed to its acidic characteristics. Pyocyanin behaves in three different states according to the pH of its environment, ionized at physiological pH (blue colour), protonated in an acidic environment (red colour) and neutral (blue colour). This allows pyocyanin to move across cell membranes. It is noted by Mavrodi et al (2010), that pyocyanin producing *P. aeruginosa* strains are dominant compared to the non-producing strains. It was noted that 95% of clinical and 100% of wild *P. aeruginosa* strains produce pyocyanin (Nowroozi et al 2012).

The production of pyocyanin is a requirement in the *P. aeruginosa* found in the environment as an ecological competence. The synthesis of pyocyanin is known to be controlled by quorum-sensing in *P. aeruginosa*. This QS mechanism is dependent on small diffusible molecules called autoinducers. These are produced by each individual bacterium. Environmental conditions are also known to increase the production of pyocyanin. Such environmental changes include pH, oxidative stress, and temperature (Goncalves et al 2021).

Pyocyanin is an important metabolite to *P. aeruginosa.* It increases the assimilation of oxygen by the bacterial cells as well as acting as a physiological signal for the upregulation of quorum sensing genes. This makes pyocyanin vital to the bacteria for tuning the cells to different physiological states.

Pyocyanin is known to inhibit the growth of other organisms. It does this by causing oxidative stress in eukaryotes and prokaryote cells. It does this through the flow of

electrons causing a build-up of reactive oxygen species such as hydrogen peroxide. It was noted by Goncalves et al (2021) that pyocyanin affects fungi, yeasts, protozoa, algae, bacteria as well as small animals. It was evaluated that lethal concentration of pyocyanin ranged from small amounts up to 2000 µg/mL against the organisms.

Inhibition of pyocyanin production

P. aeruginosa is an opportunistic pathogen that causes infections in immunocompromised individuals (Silby et al 2011). Pyocyanin is considered a virulence factor and plays a significant role in the pathogenesis of *Pseudomonas* infections. The biosynthesis of pyocyanin involves a complex pathway with multiple enzymes (Rada & Leto 2013). Pyocyanin's antimicrobial activity and role in microbial interactions have been extensively studied (Dietrich et al 2013). It helps *P. aeruginosa* establish dominance by inhibiting the growth of competing microorganisms (Lau et al 2004). Pyocyanin also contributes to the formation and dispersal of biofilms, which protect bacteria from antibiotics and the immune system (Hassett et al., 2009). In terms of host-pathogen interactions, pyocyanin induces oxidative stress and damages host cells and tissues (Hall and Ji, 2016). This contributes to the pathogenicity of *Pseudomonas* infections. Pyocyanin is particularly associated with chronic infections in individuals with cystic fibrosis (Mulcahy et al 2014). It has been implicated in tissue damage and inflammation in various host environments (Rada & Leto 2013). Researchers have explored potential applications of pyocyanin in biotechnology and medicine. Its redox activity and stability make it a promising candidate for use in bioelectrochemical systems and biocatalysis (El-Gebali et al 2020). Pyocyanin has also been investigated for its antimicrobial properties and immunomodulatory effects (Fothergill et al 2007).

Elshaer et al (2021), tested the ability of biosynthesized gold and selenium nanoparticles in their ability to inhibit quorum sensing as well as virulence factors in *P. aeruginosa*. In this study one of these factors was pyocyanin. The pyocyanin levels were determined in this study by growing 5 ml of tested bacterial culture and using chloroform extraction to quantify the concentrations of pyocyanin. The non-metal-treated cultures were tested to the control cultures. Results showed that both selenium and gold nanoparticles inhibited pyocyanin production in the range of 43%–90% and 20%–88%. This reduction in pyocyanin can be one of the factors to explain the anti-quorum sensing activity shown by the nanoparticles.

In a study by O'Loughlin et al (2013), *P. aeruginosa* quorum receptor inhibitors were tested to determine the effects on the bacteria's virulence and biofilm formation.

Chloroacetone was tested on *P. aeruginosa* and was found to not inhibit pyocyanin production in vivo. While another tested molecule meta-bromo-thioacetone was found to inhibit the production of pyocyanin. Although, it did not affect the cell growth of *P. aeruginosa.* The meta-bromo- thioacetone was reported to have an IC50 of 8 μM. The study concludes that the molecules tested showed anti–quorum-sensing capabilities which can influence *P. aeruginosa* virulence in tissue cultures as well as in animal models. This demonstrates the potential for small molecules as moderators in quorum sensing.

Jiang et al (2023), studied the effects of low concentration ethanol on *P. aeruginosa* and its ability to synthesis pyocyanin under these conditions. In this study the effects of ethanol were determined by using qRT-PCR and Western blotting. The research found that the low concentration ethanol greatly decreased the production of pyocyanin without reducing the growth rate of the bacteria. When the concentration of ethanol increases it causes more inhibition. It was found that ethanol inhibits the production of the gene which is involved in the production of pyocyanin. The study showed that the inhibition was mostly observed at protein level. It was also determined that when exposed to low concentrations of ethanol the bacteria began expressing the post transcriptional regulator RsmA, known for inhibiting pyocyanin production.

Hajardhini et al (2021) tested the enhancement of pyocyanin production by *P. aeruginosa* using sub inhibitory concentrations of royal jelly. This study noted that high concentrations of royal jelly have an antibacterial activity. It was stated that in some cases an antibiotic tolerance can occur when exposed to low concentrations of the antibacterial substance. The study aimed to determine the effects of low royal jelly concentrations on pyocyanin production of *P. aeruginosa*. The results showed that at a concentration of 25% the royal jelly caused the inhibition of the *P. aeruginosa* such that it was no longer viable. This caused there to be no pyocyanin production. At 6.25% royal jelly it was noted that the pyocyanin production rate was the highest. When the concentration of royal jelly was further decreased, it was noted that the pyocyanin production rate also decreased. This study shows that sub inhibitory concentration of compounds can increase the pyocyanin production rate in *P. aeruginosa*.

A study by Kamer et al (2023) tested the effects of pyocyanin on methicillin-resistant MRSA. The study highlighted that MRSA is a major public health problem and there are few treatments available against it. The study was conducted in vitro and in vivo. The important defence mechanism for MRSA is the formation of biofilms which improve its antibiotic resistance. This study tested the effects of pyocyanin on MRSA as well as in MRSA biofilms. The study noted that in MRSA virulence factors such as

hemolysin, protease and motility are directly controlled by Agr-mediated quorum sensing. The study chose to use pyocyanin because it is ample to diffuse and permeate cell membranes. This permeation and production of oxygen species give pyocyanin the ability to kill other microorganisms by oxidative stress. The study concluded that the MIC of pyocyanin against MRSA was 8 µg/ml. The MIC was obtained by using the broth dilution method. It also found that 88% of MRSA biofilms were eradicated when pyocyanin treatment was used. The biofilm quantification was done through crystal violet staining. The study obtained its MRSA samples by isolation from patients who were admitted to Tanta University Hospital in Egypt. The bacteria were isolated from blood (23 isolates), wounds (78 isolates), sputum (26 isolates), and abscess (33 isolates). The study noted the disruption and formation of microcolonies in MRSA when treated with pyocyanin using an electron microscope. The study also noted that the production of quorum dependant virulence factors was decreased as it was found that the agrA gene (responsible for the production of virulence factors) was decreased after the pyocyanin treatment. In silico analysis by the study confirmed that pyocyanin was binding to the agrA protein active sites, blocking its actions. This study shows that pyocyanin has the potential to being an effective compound to treat MRSA infections.

These studies show that there is ongoing research on anti-pyocyanin activity and that it plays an important role in the reduction of biofilm formation as well as its virulence factor.

2.2.4 Apis honey

Honey is prized for its therapeutic effects as an alternative medicine. There are 320 known varieties of honey originating from different floral sources. The colour and flavour of the honey depends on the sources of flowers and plants that the bees visit. It also depends on the climate, season, rainfall, and temperature of when the honey is produced (Meo et al 2017).

The use of honey has been traced to as early as Stone age paintings 8000 years ago. It has been recorded that the traditional use of honey was widespread where civilizations such as the ancient Egyptians, Assyrians, Chinese, Greeks and Romans used honey for the treatment of wounds as well as intestine diseases. Honey was a very popular substance in ancient Egypt as it was mentioned in 500 of the 900 recorded remedies. While in ancient Greece was prescribed for gout and other diseases. It was even favoured by Hippocrates for the treatment of wound healing

(Eteraf-Oskouei & Najafi 2013). The popularity of honey shows its significance to history and its importance.

Antibacterial properties

Honey as a substance has been studied for a long time. Its antibacterial properties were noted as early as 1892 (Dustmann 1979). The effects of raw unheated honey have been noted to have broad-spectrum antibacterial activity where it was tested against food spoilage bacteria, oral bacteria as well as pathogenic bacteria (Mundo et al 2004; Mohapatra et al 2011).

The mechanisms of the antibacterial activity of the honey are known to be bactericidal and bacteriostatic which is useful when used against bacteria who have developed resistances to common antibiotics (Patton et al 2006).

Badaway et al (2004) showed that the Egyptian clover honey has better inhibitory effects against *E. coli* compared to *Salmonella typhimurium*. It was observed that the pH, water content and hydrogen peroxide content all contributed to the antimicrobial activity of the honey. This study also conducted in vivo testing using mice as test subjects and it was reported that the honey in high concentrations was able to be an effective antibacterial agent. It was also found that honey stored for a long time had reduced antibacterial activity.

Honey is even known to show antibacterial activity towards MRSA (methicillin resistant *S. aureus*) (Albaridi 2019) with bactericidal activity ranging between 63%-73% when tested with manuka honey (Alandejani et al 2009). These effects are due to the honey's high sugar content, low moisture content, low pH, and hydrogen peroxide. Hydrogen peroxide in honey is naturally occurring from glucose oxidase which oxidizes glucose into gluconic acid and hydrogen peroxide (Mohapatra et al 2011). The hydrogen peroxide found in the honey is one of the major contributors to its antibacterial activity (Molan 1992). Honey is known to have high concentrations of sugars (80%) which contributes to its antibacterial activity. The high sugar content creates a high osmotic pressure environment. This in turn inhibits the development of bacteria (Szweda 2017). While honey's low pH values are due to it having a high concentration of organic acids. The pH range of honey is typically between pH 3.4 – pH 6.1.

Honey contains a peptide called bee defensin 1. This peptide is a proponent of royal jelly and honey, and its concentrations vary in different types of honey. Bee defensin 1 is effective against Gram-positive bacteria as observed by Bachanova et al 2002, it

also shows antimicrobial activity against *P. aeruginosa* and *Salmonella enterica* (Tseng et al 2011). Bee defensin 1 reduces the viability of bacteria and reduces the formation of biofilms. It was reported by Sojka et al (2016) that bee defensin 1 was more effective against Gram- positive bacteria than Gram-negative bacteria. In this study gram-negative *E. faecalis* and *Streptococcus agactiae* showed little effect towards bee defensin 1. However, the biofilm formation of both bacteria was still significantly inhibited.

Studies have shown (Almasaudi et al 2017, Al-Nahari et al 2015) that not only manuka honey but other bee honeys have inhibitory effects towards bacteria when incubated at 10% to 50% honey concentrations. The effect varied according to the honey used; Manuka UMF-20 had bactericidal effects while Sidr honey and *Nigella sativa* oil samples were shown to have bacteriostatic activity. In another study (Jenkins et al 2012) it was shown that when manuka honey and tetracycline were used together against *S. aureus* and *P.* aeruginosa there was better antibacterial activity rather than when both compounds were applied on their own. It was also reported that honey modulates antibiotic resistance in MRSA. In this testing the honey was applied in subinhibitory concentrations and caused the MRSA to become susceptible to oxacillin. Other synergistic effects were noted by (Campeau et al 2014) where manuka honey in combination with vancomycin had positive effects against *S. aureus* biofilms.

In a study done by Irish et al (2011), the antibacterial activity of honey derived from Australian honey was determined. It was recorded that the antibacterial activity of honeys is highly variable. It was noted that even honeys collected in the same area but from different beehives had an antibacterial activity ranging from 11.4% to 19.2%. The variability of the antimicrobial effect was seen to be more of entomological factors rather than floral sources. Bee colony health, the age of foraging workers and the different secretions of enzymes are examples of such entomological factors. In treating skin infection, the application of honey was found to be the most effective (Wahdan 1998) when applied to the site of infection in as little dilution as possible. This was noted when application was done to septic wounds, skin diseases by bacteria and in eye infections. The same application was noted for antifungal activity, where undiluted honey showed the best activity when used to treat fungal skin infections such as ring worm. Certain honeys even showed better ability to clear up septic wounds when compared to Salvon antiseptic.

Another use of honey was in the treatment of burns. The addition of honey to the burn dressing caused reduced rates of infection as well as a reduction of swelling at the

wound sites (Zbuchea et al 2014). It has also been noted that honey increases the rates of wound healing for not just burns but other types of wounds (Medhi et al 2008). Besides honey itself the contents of honey were used in cosmetics. Methylglyoxal, which is the major antibacterial factor found in manuka honey was tested in cosmetics and was found to be a good alternative to other antibacterial compounds (Juliano et al 2019).

Other applications or therapeutic properties

Anti-inflammatory: Honey is known to be a good anti-inflammatory compound. The anti- inflammatory process is normally triggered by chemicals and biologicals such as pro- inflammatory enzymes, cytokines, and small molecules such as eicosanoids (Dao et al 2004). The anti-inflammatory compounds in honey have been identified to be flavonoids which inhibit the development of inflammation (Ali et al 1991). Of the flavonoids galangin and chrysin are both shown to have high anti-inflammatory activity.

Antioxidant: Honey is known to pose good antioxidant activity due to the presence of polyphenols in it (Jalil et al 2017). Polyphenols are believed to originate from plant nectar. Phenolic compounds are classified into two groups, flavonoids, and non-flavonoids. The quality of the polyphenols is dependent on factors such as bee type, climate, floral sources, and geographical location. The biggest factor that affects the phenolic content of honey is its floral origin, as it allows for the characterization and authentication of honeys. In food preservation, honey can replace sodium tripolyphosphate commonly used to prevent lipid oxidation if foods (Johnston et al 2005). Due to its antioxidant content, honey may also have potential to be used as a preventative against cardiovascular diseases, cancer, inflammation, and neurological disorders (Kishore et al 2011).

Antiviral: Honey is not only effective against bacteria. Honey had been shown to be an effective agent against skin infections caused by dermatophytes (Anand et al 2019); against Rubella virus (Zenia et al 2007); herpes simplex virus (Viuda-Maros et al 2008); and influenza virus (H1N1) (Watanabe et al 2014). These studies show that honey in general has potential as an antiviral medicine.

Food additives: Honey is also known to be able to inhibit the enzymatic browning of fruits and vegetables. Enzymatic browning of fruit impacts the quality and the shelf life of foods. Enzymatic browning in fruits and vegetables is caused by polyphenol oxidase (Viuda-Martos et al 2008). Foods are usually treated with chemicals such as sulfites, ascorbic acid, and citric acid. But this process can be costly and comes with

potential health hazards such as sulfites which may induce asthma or anaphylactic reactions to it. Honey has a wide variety of compounds such as small peptides, alpha tocopherol, flavonoids, glucose oxidase, catalase, and peroxidase that prevent the process of enzymatic browning (Jeon & Zhao 2005). Other than that, honey is also commonly used as a natural preservative in milk, effectively inhibiting the growth of pathogenic microorganisms (Krushna et al 2005).

Nutrients: Honey contains a large variety of compounds. Honey consists mainly of carbohydrates that are present as both mono and disaccharides, these are what contribute to the characteristic sweetness of honey. Honey also contains oligosaccharides such as panose and anderose. It contains enzymes such as acid phosphorylase, oxidase peroxide and amylase. Besides those honey also contains Vitamin C, Vitamin B, niacin, amino acids, folic acids, minerals, and many other compounds (Ball 2007).

2.2.5 Manuka honey

Background

Manuka honey has been widely researched. It is known as the benchmark for antibacterial testing when using honeys. A study by Roberts et al (2015) reported on the mechanisms that manuka honey cause antimicrobial activity on *S. aureus* and *P. aeruginosa*. For *S. aureus* it was noted that the manuka honey inhibits the bacteria's ability to undergo cell division. In *P. aeruginosa* the manuka honey causes cells to lyse by inhibiting the production of structural proteins. These processes overall cause a reduction in both bacteria's virulence.

Manuka honey contains a unique compound such as methylglyoxal (MGO). The concentration of this MGO allows manuka honey's quality to be monitored by a measurement called unique manuka factor (UMF). A study by Girma et al (2019) tested whether there is a correlation between UMF and antimicrobial activity. The study tested manuka honeys with UMF values of 5+, 10+ and 15+. These honeys were all sourced from the same manufacturer. The antimicrobial activity was quantified through broth microdilution and minimum inhibitory concentration (MIC). The MIC of all the honeys were determined against 128 different wound cultures. These cultures included gram-negative, gram-positive, multi-drug resistant and drug-susceptible bacteria. The study determined that the UMF 5+ honey was noted to have lower MIC's compared to UMF 10+ and 15+ honeys when tested against *P. aeruginosa* and *S. aureus*. When testing Enterobacteriaceae, it was noted that UMF

5+ and 10+ had much lower MIC's when compared to +15 UMF. These results show that manuka honey exhibits antimicrobial activity against a wide range of bacteria. UMF values in manuka were seen to increase the antimicrobial activity. This shows that the UMF value may be a good indicator of antimicrobial activity in manuka honeys.

Applications

Manuka honey can be used in wound healing. In a study by Kapoor & Yadav (2021), the wound healing effects of manuka honey were studied. The study was conducted on 15 patients (nine males and six females) with an average age of 38.06 years old. The patients who were selected all complained of chronic non-healing wounds obtained from foreign bodies. These infections were caused by stains of antibiotic-resistant *S. aureus*. The study excluded patients suffering from systematic illness, diabetes, allergies to honey or honeybees, reactions to medication as well as pregnant patients. Before the study was done the patients were briefed in detail and consent was obtained. The study was conducted on the patients from January 2018 to January 2020. The method was that the wounds were irrigated with saline solution, Next, the manuka honey was applied directly to the wound. Then, the wound was covered by an absorbent material. The patients underwent antibiotic treatment in conjunction with the application of the manuka honey on the wounds. The dressings were changed daily until pus discharged ceased. After the cessation of pus discharge the dressings were changed weekly instead. The study assessed the wounds depending on depth of the wound as well as wound discharge. The results in this study showed that the topical application of the honey was able to stop pus discharge in the 1st week of application. No patients in this study reported allergies, pain, infection, swelling or inflammation after the treatment was completed. The study noted that the application of manuka honey on the wound sites stopped a prolonged inflammatory response. This was noted to be due to the stimulation of proinflammatory cytokines. These cytokines reduce inflammation which allows normal healing to occur. This study shows that manuka honey wound treatments work well together with regular antibiotic treatment.

Manuka honey was found to alter the size and the shape of some bacterial cells. This was reported by Henriques et al (2010). In this study transmission electron microscopy was used to visualize when *S. aureus* is treated with manuka honey. It was found that when the bacterial cells were treated with manuka honey they had more development of septa when compared to the bacteria being treated with artificial

honey. These results indicate that when the bacteria were treated with manuka honey most of them failed to complete cell division. The study by Lu et al (2013) conducted phase-contrast imaging after treating *Bacillus subtilis* and *S.aureus* were treated with a sub lethal dose of manuka honey. It was observed that the manuka treatment caused the DNA of the bacteria to be more condensed compared to untreated bacteria. The manuka treated cells were also observed to be much smaller in size. These results show that even sub-lethal concentrations of manuka honey affect the cell division as well as growth of bacteria cells. Lu et al (2013) also reported that *E. coli* and *P.* aeruginosa treated with manuka honey were observed to have abnormally longer as well as shorter bacterial cells.

A study by Brady et al (1996), tested the effects of manuka honey against dermatophytes. In this study, agar well diffusion was used to test the activity of the manuka honey. The manuka honey was tested with its natural hydrogen peroxide and with the hydrogen peroxide being removed with catalase. The contents of the agar wells were replaced in 24-hour intervals. The agar plates were incubated for three days. The results showed that at the lowest honey concentration 5% (v/v) of manuka honey with catalase treatment showed the inhibition of the fungi being 10% for *Epidermophyton floccosum,* 15% *for Microsporum canis*, 20% for *Microsporum gypseum,* 15% for *Trichophyton mentaprophytes*. When compared to the manuka honey without catalase treatment it was observed that the inhibitory effects towards the fungi were double. This study also tested a common Apis honey to compare its activity with the manuka honey. It was found that even at a concentration of 50% (v/v) the honey did not show any inhibitory activity against any of the fungi. The results of this study show that manuka honey has the potential to be used as a therapeutic treatment against dermatophytes. It also showed that besides hydrogen peroxide in the honey there are other compounds which contribute to its antimicrobial activity.

2.2.6 Stingless bee honey

Traditional applications

Kiprono et al (2022), conducted an ethnomedical survey in Kenya on the traditional medical uses of stingless bee honey. The survey was conducted on over 300 participants across 5 counties in Baringo, Kenya. The results showed that over 90% of the communities surveyed were aware of the medicinal properties of stingless bee honeys. The communities were also aware of its uses. These uses included treatment of respiratory disorders, infections, sore throat, gastrointestinal disorders, and wound

healing. While those surveyed perceived that stingless bee honey had only mild side effects such as nausea, throat irritation as well as loss of appetite. These side effects were associated with overconsumption. Over 50% of participants indicated that stingless bee honey was not available due to small supply. This survey shows that there is a huge demand of stingless bee honey in the traditional treatment of various health conditions. This shows that there is therapeutic potential for stingless bee honey.

Antibacterial properties

A study by Gopal et al (2021), showed the potential of using stingless bee honeys in cellulose hydrogels in treating wound infections. The study showed that the stingless bee hydrogels were able to improve the water absorption of the hydrogels. The inclusion of stingless bee honey to the hydrogels also enhanced the antibacterial activity, cell proliferation as well as inhibition of bacterial growth. The antibacterial properties of the stingless bee honeys were evaluated using zone of inhibition testing and colony counting. While the cytocompatibility was evaluated using MTT assay as well as cell scratch assay of human fibroblast cells. This shows that stingless bee honey has potential to be applied in the medical field.

Antiviral properties

Stingless bee honey was tested in a study by Arung et al (2022). In this study stingless bee honeys sourced from Indonesia were screen for ACE2-Spike protein-binding inhibition. This inhibition was linked to prevention of SARS-Cov-2 infections. The testing in this study directed the research towards the phytochemicals within the honey. These phytochemicals showed the best inhibitory effects towards the ACE2-spike protein-binding. These phytochemicals in the honey were known to be introduced to the honey from plant sources, especially nectars and pollen. The study concluded that 10 honeys samples were shown to have ACE2-spike protein binding inhibition. These findings give stingless bee honey potential to be used against SARS- CoV2.

Other therapeutic properties

A recent review by Zulkifli et al (2023) stated that stingless bee honey possesses beneficial properties such as anti-inflammatory, neurotherapeutic, neuroprotective, wound healing as well as sunburn healing properties. These benefits were attributed to its high contents of phenolic compounds such as phenolic acids and flavonoids. The study showed that stingless bee honeys also contain amino acids, organic acids, tocopherols, ascorbic acid. The contents were noted to be highly dependent on

geographical location as well as botanical sources. The study stated that stingless bee honey which contained high levels of flavonoids can reduce neuroinflammation by inhibiting the production of proinflammatory cytokines and reduce the number of free radicals produced. This property is linked to flavonoids having good antioxidant activity. The review noted that stingless bee honeys have much higher antioxidant activity compared to other Apis honey. Because of this stingless bee honey is more therapeutically helpful in terms of antioxidants. Stingless bee honey was found to containphytochemicals compounds such as luteolin and phenylalanine which are linked to aiding in neurological problems. While these properties show that stingless bee honey may have neuroprotective effects, extensive research in this field is minimal.

A study by Fletcher et al (2020) reported that stingless bee honey was a novel source of trehalulose, an isomer of sucrose with an unusual glycosidic linkage. Trehalulose is known to be acariogenic (not causing tooth decay) and has a low glycaemic index. The study tested stingless bee honeys from Australia, Brazil, and Malaysia. The study targeted the detection of trehalulose because before this study the compound has not been detected before from any food product. The study identified that all three stingless bee honeys had trehalulose present. Trehalulose can have beneficial effects to the small intestine where it reduces the rate of hydrolysis. It has potential to being used to control blood sugar levels in diabetics, those with glucose intolerances as well as obesity prevention. This is because trehalulose has a much slower rate of release of monosaccharides into the blood when compared to sucrose. It has potential in the food industry as it is 70% as sweet as sucrose and it is extremely water soluble. This gives it the ability to be used in jellies, jams, and juices. The presence of trehalulose gives stingless bee honey the potential to be used as an ingredient and achieve the same benefits as pure trehalulose.

Stingless bee honey was also tested by Ranneh et al (2019), for its protective effect against lipopolysaccharide (LPS)-induced CSSI, which is caused by the involvement of certain protein signalling. In this research the mice testing was used were CSSI was introduced to male mice by injection of lipopolysaccharides three times a week for 28 days. The mice then underwent treatment using stingless bee honey of 4.6 or 9 . 3 g/kg/day for 30 days. The results showed that mice which were injected with lipopolysaccharides showed significant leukocytosis as well as reduced levels of antioxidants. After treatment with stingless bee honey the mice howed increased levels of antioxidants as well as the presence of inflammatory markers MDA and 8-OHdG. The stingless bee honey also prevented LPS-induced functional and

histological changes in liver, kidney, and heart functions in the rats. It can be concluded from this research that stingless bee honey gives substantial protective effects against LPS- induced CSSI and oxidative stress against mice.

2.2.7 Antibacterial effects of honey on P. aeruginosa

In a study by Shenoy et al (2012), fifty strains of *P. aeruginosa* were isolated from infected wounds. The bacteria were tested against honey, antibiotics, and Dettol. The honey used was Agmark grade honey sourced from India. The study found that all strains of *P. aeruginosa* were inhibited by the honey. The MIC of honey was found to be 20% v/v while the Dettol had an MIC of 10% v/v. This testing was done using the agar well diffusion method. Besides that, bactericidal activity was also tested. The dilutions used in this experiment range between 20% to 100% honey concentration. The honey was tested against five different strains of *P. aeruginosa*; it was noted to kill all strains of the bacteria between 12 – 24 hours. This study concluded that honey is an effective alternative treatment to *P. aeruginosa* infections.

In a study by Lu et al (2019), manuka honey was tested against *P. aeruginosa* and its biofilm production. In this study New Zealand Medihoney (medical grade manuka honey) was used. In this study the minimum inhibitory concentration was determined using microdilution. The results showed that manuka honeys were effective in the elimination of *P. aeruginosa* biofilm formation and in the inhibition of growth. The MIC of the manuka honey tested against the bacteria was found to be 16% honey concentration. There was variation between the results of the experiment and prior studies which showed that the MIC of manuka honey ranged between 12%-50% (Irish et al 2011). The study concluded that the variation of MIC was due to different strains of *P. aeruginosa* being used. The study concludes that honey manuka honey is a good alternative to commonly used antibiotics as bacteria generally don't form resistances to honey. It also states that manuka honey is an effective compound against biofilm production of *P. aeruginosa*.

Bouzo et al (2020) studies the effects of manuka honey against *P. aeruginosa* using transcriptomics. In this study it was determined that manuka honey had a MIC of 10% w/v and an MBC of 12% w/v. The study tested using artificial honey in which they could test whether methyglyoxal levels affected the level of antimicrobial activity of the honey. Methyglyoxal is a unique compound found in manuka honey that contributes to its antimicrobial activity. The study altered the concentrations of the methyglyoxal in the artificial honey, this showed that not all the activity of manuka honey was determined by methyglyoxal. The best antimicrobial activity was observed

when the methyglyoxal was combined with the artificial honey.

In Roberts et al (2015) the effects of manuka honey on reduction of motility of *Pseudomonas aeruginosa* were studied. The testing included hydrophobicity assays, quantitative RT-PCR, and motility assay. It was noted that the exposure of *P. aeruginosa* to manuka honey caused a reduction in the swarming and swimming motility of the bacteria. This was linked to manuka honey causing the de-flagellation of the bacterial cells where there was a decrease in the expression of major structural flagellin proteins. In normal bacteria flagella play an important part in bacterial adhesion which is vital for infection and the formation of biofilms. Therefore, it can be inferred that manuka, which reduces the motility of *P. aeruginosa* impacts its virulence.

Antibacterial mechanisms

Pang et al (2019), discussed the antibiotic resistances of *P. aeruginosa*, its mechanisms as well as alternative therapeutic treatments for its infections. The review stated that *P. aeruginosa* utilizes acquired as well as intrinsic resistances to counter antibiotics. *P. aeruginosa* also has been noted to have adaptive resistances. One such resistance is biofilm- mediated resistance. This resistance causes relapse of *P. aeruginosa* infections. As stated in this review paper the combination of these resistances has led to there being a need to discover alternative therapeutic treatment to such infections. One such development is in the advances of antibiotic development. Three new antibiotics doripenem, plazomicin and POL7001 were shown to have better effect against *P. aeruginosa* when compared to traditional antibiotics. These new antibiotics are shown to also have a lower frequency of resistance development. Doripenem was noted to be resistant to hydrolysis by β-lactamases, it was also determined in a study by Chastre et al (2008), to have higher rates of curing patients compared to imipenem. Plazomicin a synthetic antibiotic is known to be resistant to aminoglycoside modifying enzymes. It also demonstrates potent in vitro activity against Gram- positive and Gram-negative pathogens. Lastly, POL7001 was noted to be effective against *P. aeruginosa*. A study by Cignana et al (2016), isolated *P. aeruginosa* from chronic pneumonia patients. The bacteria were then introduced to mice. The mice were subsequently treated with POL7001. It was found to significantly reduce the bacterial burden in the mice as well as reduce the inflammation in their lungs. These new antibiotics show that there is an increasing need for the development of new treatment options against *P. aeruginosa.*

The studies show that there is current and ongoing research on finding effective

therapeutical treatment against *P. aeruginosa*. The studies have highlighted that different honeys have a varied effect on the inhibition of the bacteria's growth. Therefore, there is a need to evaluate different types of honeys on *P. aeruginosa* as the effectiveness to inhibit the bacteria will vary.

2.3 Antibacterial assays

Antibacterial testing has a critical role in evaluating the effectiveness of antimicrobial agents and it is essential for the development of new antibacterial compounds. Methods used, including broth dilution assays, agar diffusion assays, and time-kill kinetics assays, are used to assess the inhibition and activity of antimicrobial agents (Andrews 2001). The tests provide both quantitative and qualitative analysis of antibacterial activity, facilitating comparisons between agents. Antibacterial testing is vital for detection of emergence and progression of antibiotic resistances in bacteria. It identifies mechanisms of resistance in bacteria, such as target site alterations, or enzymatic inactivation of drugs (Bush et al 2011).

By assessing bacterial susceptibility to different antimicrobial agents, testing allows for the identification of resistance patterns, guiding and speeding up the development of new therapeutic strategies.

When screening and identifying new compounds, antibacterial testing plays an important role. Techniques such as high-throughput screening enable the rapid assessment of vast compound libraries, facilitating the identification of compounds with potential antibacterial activity (Payne et al 2007). Understanding structural activity relationships and exploring new mechanisms of action are key aspects of antibacterial testing, contributing to the discovery and optimization of new antibacterial agents. During clinical development stages, antibacterial testing is critical for assessing the safety and efficacy of antimicrobial agents. One of the safety protocols is animal models. Animal models are used in preclinical studies to determine optimal dosage regimens, and overall effectiveness as well as safety (Spellberg et al 2008). Once animal models are completed, clinical trials further evaluate the agent's efficacy in humans, considering factors such as drug interactions and specific patient populations. Studies routinely test bacterial isolates to monitor resistance trends and identify new resistance mechanisms (World Health Organization, 2014). Rapid diagnostic tests are invaluable for tailoring specific antimicrobial therapy, facilitating the selection of appropriate agents, and stopping the development of further resistance.

An example of this testing was the study by Gonelimali et al (2018). This study tested the antimicrobial activity of ethanol extracts of roselle, clove, rosemary as well as thyme. They tested these extracts against food spoilage microorganisms. The study used the agar well diffusion method to test the inhibitory effects of the extracts on the selected bacteria. The bacteria used were *B. cereus, S, aureus, E, coli, S. enteritidis, V. parahaemolyticus and P. aeruginosa* as well as the fungus *C. albicans*. The results of the experiment showed that the roselle extract had significant antibacterial activity against all the bacteria tested. However, it showed no activity towards *C. albicans*. The clove and thyme extract did show inhibitory effects against the fungus. The study determined that there is a link between the changes of the internal pH and membrane of the bacteria when exposed to the extracts. The results indicated that the plant extracts primarily affect the cell membranes of the bacteria which was indicated by the decline of internal pH as well as the hyperpolarisation of the membrane. This study highlights the need to better develop an understanding of how crude plant extracts affect food spoilage bacteria. The method shows the effectiveness of antibacterial testing to identify new potential antimicrobial compounds.

Broth dilution method

For this testing the dilution is either micro or macro-dilution. It is regarded as the most basic of antimicrobial testing. In this method the antimicrobial compounds are diluted by two-fold dilution, for example 32, 16, 8, 4, 2, and 1 µg/mL. The samples are diluted with a liquid growth media, for macro-dilutions the tubes used will have volumes of more than 2 mL. For microdilutions the testing will be done using a 96-well microtiter plate. Each well is inoculated with the test microorganism which was adjusted to 0.5 McFarland. The test microorganism is mixed with the different antimicrobial compound dilutions and incubated under suitable conditions (Kreger et al 1980).

Broth microdilution was used in a study by van der Hejiden et al (2007). In this study polymyxins susceptibility of *P. aeruginosa* was tested. The study used twofold microdilution, etest as well as zone of inhibition testing. The results showed that there was good concordance between the etest as well as the broth microdilution. The results showed that of the 78 strains isolated and evaluated only 1 strain was resistant to polymyxin. This study shows that broth dilution is an effective technique that can be combined with other techniques such as zone of inhibition testing.

A study by Kohner et al (1997) compared broth dilution, disk diffusion and agar dilution methods. This study was done to determine the optimal testing for the susceptibility of 100 different isolates against vancomycin. These isolates included

Enterococcus spp. The study used Mueller-Hinton agar plates for the agar related testing and Mueller-Hinton broth for the broth dilution method. For all the methods mentioned the study incubated two sets of samples either at 24 hours or 48 hours. The results when comparing the incubation times of this testing showed that increasing the incubation time from 24 to 48 hours produced no difference in the results. Some 48 hour incubated samples even gave worse results when compared to incubation at 24 hours. The results showed that of all the tested methods disk diffusion had the highest rates of major error. It was also noted that the Mueller-Hinton media had 6 growth failures. This study showed that broth dilution is being used in studies and is known to have good reliability when it is being used in drug susceptibility testing.

2.3.1 Minimum inhibitory concentration (MIC)

The MIC is used to express the lowest concentration of an antibacterial agent that is required to completely prevent visible growth of the test organism. The MIC is determined using the dilution method either using agar or in a liquid medium. MICs are done to evaluate the antimicrobial efficacy of different antimicrobial agents. Various types of compounds can be inoculated with different cultures of bacteria at different concentrations (Clinical Microbiology and Infection 2003). MIC is used in diagnostic laboratories to confirm the resistances of microorganisms. It is an important research tool to determine the in vitro activity of new possible antimicrobial agents (Andrews 2001).

For minimum inhibitory concentration testing the standard protocol is to perform serial dilutions to test a range of concentrations. The test bacteria will be prepared overnight and adjusted to a standard turbidity. The bacteria are then added to microtiter plates with the different concentrations of the diluted honey. The plates are incubated at 37 ^{o}C for 24 hours, then the plates are read at a certain wavelength (Oses et al 2016).

A study by Oses et al (2015), used and compared different MIC's methods to obtain the antibacterial activity of honey against *S. aureus*. The study compared agar diffusion against microbroth dilution to obtain the MIC values of 56 different types of honeys. MIC values for the agar well diffusion method was determined as follows. Firstly, 10 mL of sterile nutrient brother were used to perform serial dilutions of the honey used. Next, 10 mL of the honey dilutions were added to 10 mL of liquid nutrient agar. The mixture was then vortexed and poured onto plates. The bacteria were then added to the plates in 5 µL spots. Controls were also made where no honey was added to observe the growth of the bacteria. The plates were then incubated for 24 hours. The plate with no visible growth was the MIC of that honey.

For the broth dilution method, 96 well sterile round bottom plates were used. To each well 100 µL of honey were added to 100 µL of bacteria, at different concentrations. Control wells consisted of 100 µL of the selected media with 100 µL of the bacteria. The plates were then incubated for 24 hours, and the absorbance of the plates were then read. The study determined that agar well diffusion assays are a good tool for screening samples for antimicrobial activity. It noted that the broth dilution used much less materials, it was easier to use and was found to be a good procedure to obtain the MIC values.

2.3.2 Half-maximal inhibitory concentration (IC50)

IC50 refers to the half-maximal inhibitory concentration, it is a term often used in biochemistry to determine the potency of a substance in habiting the process of a target (Swinney, 2011). The IC50 is widely used in drug discovery and development. It represents the concentration of a compound which inhibits the biological targets function by 50%. IC50 is important because it makes it easier to compare the potency of different drugs to one another.

The IC50 values are determined through different assays which involve the exposure of cells (tissue, bacteria, or fungi) to a range of concentrations of the selected drug or compound. From this exposure the compounds concentration is plotted against the response from this the IC50 is calculated. The lower the IC50 the greater potency the drug has, therefore a lower IC50 is desirable.It is noted that although IC50 values are important in drug discovery it does not paint the whole picture as other factors such as pharmacokinetics, drug-drug interactions as well as target selectivity play important roles in determining the effectiveness of different compounds.

2.4 Antibiofilm assays

Antibiofilm testing is done through a biofilm inhibition assay that is quantified through crystal violet staining. In antibiofilm testing, microdilutions of a potential antibiofilm compound are set up. The dilutions of the compound are then incubated along with the selected bacteria which produce biofilms. The cells of the bacteria are then stained and analysed using a specific wavelength.

In a study by Diaz et al (2015), a biofilm inhibition assay was used to determine the effect of five lipids, three terpenoids and a mixture of sterols on the formation of bacterial biofilms. The compounds were isolated from soft coral *Eunice sp*. The bacteria tested on composed of marine bacteria as well as *P. aeruginosa* as well as other bacteria associated with surface contamination. Of the compounds tested it was

determined that batyl alcohol and fuscoside E peracetate acted against four of the tested strains without effecting their bacterial growth. While up to 60% inhibition on the other strains. In this testing bacteria were grown overnight in trypticase soy broth until an OD of 600 was achieved. The bacteria were then inoculated in wells together with the selected compounds. The bacteria were then incubated for 24-48 hours. Next, the planktonic cells were washed off by distilled water. Then, the biofilms were stained with crystal violet. Lastly, ethanol-acetate was added to the biofilm's ethanol-acetate was added to remove the cell adhesion to the well walls. The absorbance was then read at OD600. This study is an example of a biofilm inhibition assay. It showed that by using this technique it is possible to determine the inhibitory effects of compounds on the formation of biofilms.

2.4.1 Antibiofilm formation assay

In this assay the targeted microorganisms were allowed to form biofilms under controlled conditions. This is done by inoculating the bacteria into the desired growth media. After, biofilms were allowed to grow antibiotics, enzymes, natural products, or synthetic products were introduced to the culture. Next, biofilms and microorganisms are incubated for a specific time depending on the experimental design. Then, after incubation the biofilms are evaluated to determine the inhibitory effect of the antimicrobial agent. For quantification biofilm biomasses are quantified using crystal violet staining. An example of this antibiofilm formation assay is given below.

In a study by Mombeshora et al (2021) an antibiofilm assay as mentioned above was used. In this study *P. aeruginosa* biofilms were set up where 1ml of bacterial cultures were inoculated into 24 well plates with the tested extract. The cultures were then incubated for 72 hours. Then, the non-adherent cells were washed off using sterile water. Next, the plates were dried. The cells were then stained with crystal violet. Then, the adherent cells were washed to remove the excess crystal violet. Lastly, to the wells 95% ethanol was added to the wells. The biofilms were then quantified by microplate reader at 590 nm wavelength. This study follows the foundations of antibiofilm testing.

2.4.2 Biofilm degradation assay

Biofilm degradation refers to the ability for compounds to be able to remove established biofilms. Compounds which are known to degrade biofilms are bisphenol compounds, heavy metals, and chlorinated compounds. These chemicals have been used in the past to disperse biofilms but due to their serious health hazards are no longer used. Therefore, there has been a discover compounds which effectively degrade biofilms that are safe to be used. In this assay, bacteria are inoculated without the test compounds and allowed to grow for a fixed period. After the biofilms are established the test compound is then applied to the biofilm and incubated for 24 hours. After incubation the degradation of the biofilms are quantified using crystal violet.

A study by Saggu et al (2019), tested the enzyme metalloprotease and its ability to degrade *S. aureus* biofilms. The study aimed to decrease the reliance on toxic chemicals in medical and industrial applications against biofilms. The study isolated and purified the enzyme metalloprotease from Mycobacterium sp. It was determined that the enzyme was able to degrade biofilms at a lower concentration when compared to other well-known enzymes such as papain, trypsin, and a-amylase. Metalloprotease was also found to be non-cytotoxic towards human carcinoma cells. From this it was determined that metalloprotease has potential to be an effect compound against biofilms. In the study biofilms were quantified using crystal violet staining. The study showed that the protease degraded S. aureus biofilms up to 61.923%, 73.732%, and 77.728% when treated at metalloprotease concentrations of 10, 100, and 1,000 µg/mL.

2.4.3 Crystal Violet assay

Crystal violet is also known as gentian violet or as methyl violet. It is a synthetic dye which belongs to the triarylmethane dye class. Crystal violet is known to be water soluble which makes it useful for staining. Crystal violet has a deep purple colour. One of the main uses of crystal violet is in biological staining. It is regularly used to stain cell nuclei, as it stains DNA as well as RNA allowing better visualization of these under microscopes. Besides this, it is also regularly used in microbiology to stain bacteria. In bacteria staining using crystal violet the bacteria are treated with a decolourising agent and then subsequently counterstained with crystal violet. From

these Gram-positive bacteria will retain the purple stain while Gram- negative bacteria will take on the counterstain colouring.

Crystal violet has been used in quantification of biofilms as well. Kamimura et al (2022), used crystal violet solution to quantify the biofilms produced. In this study *E. coli* biofilms were grown for 24 hours. After which 0.1% crystal violet stains were used on the samples and immersed for 30 minutes. Then the non-absorbed crystal violet was washed using sterile water. The 30-minute immersion time was used so that the bacterial samples were allowed to absorb crystal violet. While washing with sterile water, it is used to remove the non- absorbed crystal violet stains. The next step for this staining was quantitative analysis using absorbance of a specific wavelength.

In another study by Ball et al (2022), crystal violet staining was used to differentiate different biofilms produced by different strains of the same species of bacteria. This shows that crystal violet staining is still being used to this day as an effective method to quantify bacterial biofilms. In this study the bacteria were grown overnight in tryptic soy broth. Then, the bacteria were transferred to 96-well plates after undergoing dilutions. The bacteria were then incubated overnight again. Next, the bacteria were washed to remove non-adhered cells. The biofilms were then stained and quantified. This method is like the method by Kamimura et al (2022), which shows that multiple studies use crystal violet to quantify biofilms. This indicates that crystal violet quantification is a good indicator to quantify biofilms.

Chapter 3: Methodology

3.1 Materials

This chapter reports the methodologies that were used to achieve the objectives of this study,(1) To determine antimicrobial activity of Sarawak stingless honey against Pseudomonas aeruginosa through microdilution assays; and (2) To investigate the antibiofilm activity of Sarawak stingless bee honey against Pseudomonas aeruginosa by determining their biofilm formation inhibition, biofilm degradation and pyocyanin production inhibition activities.

3.1.1 Chemicals

All chemicals used in this study were of analytical grades. Luria agar (M557), Luria broth (M575), nutrient agar (M001), nutrient broth (M002), and plate count agar (MP001) were all purchased from Himedia Laboratories Pvt Ltd, India. Sodium hydroxide (NaOH) (S5881), acetic acid (A6283) and crystal violet (C0775) were obtained from Sigma Aldrich and 99% ethanol from DChemie.

3.1.2 Honey samples

All raw (SBHR1-SBHR11) and one processed (SBHP1) stingless bee honey samples were collected from local Sarawak honey producers, while two of the processed stingless bee honey samples were sourced from West Malaysia. The Australian Manuka honey (Manuka) was purchased from a local pharmacy. The honey samples are as labelled in Table 2 below.

Table 2: Honey samples used in this study.

Honey samples	Code	Supplier Name	Origin
Manuka Honey	Manuka	Nature's Way Manuka Honey (MG 100)	Australia
Processed stingless bee honey (SBHP*)	SBHP1	Melii	Sarawak, Malaysia
	SBHP2	Syamille	Perak, Malaysia
	SBHP3	H&B	Selangor, Malaysia
Raw stingless bee honey (SBHR**)	SBHR1	Ahmad	Sarawak, Malaysia
	SBHR2	Simpulan Emas	Sarawak, Malaysia
	SBHR3	Mireng	Sarawak, Malaysia
	SBHR4	Monday	Sarawak, Malaysia
	SBHR5	Stanley	Sarawak, Malaysia
	SBHR6	Sharon	Sarawak, Malaysia
	SBHR7	Lee	Sarawak, Malaysia
	SBHR8	Elvy	Sarawak, Malaysia
	SBHR9	Pojie	Sarawak, Malaysia
	SBHR10	Mundai	Sarawak, Malaysia
	SBHR11	Chen	Sarawak, Malaysia

**SBHR refers to stingless bee honey raw.*

***SBHP refers to stingless bee honey processed.*

All stingless bee honeys were produced by the *Heterotrigona itama* stingless bee species while the Manuka honey was produced by the Apis sp. bee.

3.1.3 Pseudomonas aeruginosa culture conditions

Gram-negative *P. aeruginosa* (PA01) strain was supplied by Swinburne University of Technology Sarawak campus (SUTS). The LB agar and broth were prepared as per manufacturer's instructions. For LB broth, 20 g of the LB broth powder was suspended in 1 L Milli-Q water. For LB agar, 35 g of the LB broth powder was suspended in 1 L Milli-Q water (Merck IQ-7000 Ultrapure Water System, Darmstadt, Germany). The mixture was heated or microwaved at low heat for 1 minute to dissolve the medium completely. The medium was sterilized by autoclaving at 15 psi (121 °C) for 15 minutes. Cool to 45-50 °C. The LB broth was poured into a 10 mL universal bottle and the LB agar onto sterile petri plates.

Frozen *P. aeruginosa* culture from -80 °C glycerol stock was revived in LB agar according to the methods outlined by Zainol et al (2013). Working aseptically under sterilized biological safety cabinet, LB agar plates and the vial containing frozen *P. aeruginosa* culture were surface sterilized with 70% ethanol. Next, while the bacteria were still frozen, a sterile loop was used to spread the frozen bacteria onto the LB agar plate. The bacteria were then covered and allowed to thaw shortly before being sealed with parafilm. The inoculated LB agar plate was then incubated in an incubator (Thermoline Laboratory Incubator TI-20F, New South Wales, Australia) for 24 hours at 37 °C. Next, a loopful of the revived bacterial colony was inoculated into 10 mL of LB broth. The inoculated LB broth was then incubated overnight at 37 °C. The overnight culture was then used for the preparation of working LB-culture broth (Section 3.2.1) and biofilm formation (Section 3.3.2).

3.2 Antibacterial assays

3.2.1 Preparation of working LB-culture broth

The antibacterial assay was adapted from Zainol et al (2013) with slight modification. The bacteria culture in LB broth was prepared according to previously mentioned (Section 3.1.3). Next the bacteria culture was adjusted to approximately 3 x 10^8 CFU by taking 2 mL of broth and measuring the absorbance at 625 nm to achieve an OD of 0.08-0.13 using a spectrophotometer (Thermofisher GENESYS 30, Massachusetts, USA). A culture broth turbidity that matches the 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension with a 1.5 x 10^8 colony forming units (CFU/ml). This cell density is especially required for

bacterial inocula for the antibacterial sensitivity test.

One (1) part of the adjusted LB-culture broth was further diluted with 199 parts of LB broth to make a working LB-culture broth. Next, 10 mL of the working LB-culture broth was pipetted into five sterile tubes and labelled accordingly.

3.2.2 Preparation of honey sample dilutions

Honey stock solutions were prepared to a concentration of 50% (w/v) in which 2 mL of LB broth were added to 1 g of honey. The 50% honey solution was then mixed well by vortexting for 30 s and filtered through a Corning® syringe filter (Sigma Aldrich CLS431229 polyethersulfone membrane, pore size 0.2 µm). Next, serial dilutions were carried out to obtain 25%, 12%, 10%, 8%, 6%, 5%, 4%, 3% 2% and 1% honey solution concentrations.

3.2.3 Preparation of neutralised and normal pH honey samples

Neutralised pH honey samples were prepared by first diluting the honey to 50% concentration (w/v). Then 1 M NaOH was filtered using Corning® syringe filter (Sigma Aldrich CLS431229 polyethersulfone membrane, pore size 0.2 µm) and was added in 10 µL increments to the honey until a pH of seven was reached. The pH was recorded using pH meter (Oakton pH 700 Benchtop meter, South Carolina, USA). All the solutions were vortexed for 30s to ensure uniform mixing. Next, serial dilutions were carried out to obtain 25%, 12%, 10%, 8%, 6%, 5%, 4%, 3% 2% and 1% honey solution concentrations.

3.2.4 Antibacterial screening at 5% honey concentration

In this testing the honey solutions were diluted to 5% honey concentration (w/v). Honey samples did not undergo neutralisation in this part of the testing. The diluted honey concentrations were added to 96-well plates in triplicates with three biological replicates. For each honey sample the samples were plated according to those shown in Table 3. This screening was replicated two more times to ensure reliable results.

3.2.5 Broth microdilution assay for determination of MIC and IC50

MIC refers to the minimum inhibition concentration which is the lowest concentration of honey sample that prevents visible in vitro growth of *P. aeruginosa.* Half maximal inhibitory concentration (IC50) refers to values and is a quantitative measure that indicates how much of the particular honey sample is needed to inhibit, in vitro, the

growth of *P. aeruginosa* by 50%.

Both were determined by the microdilution method where honey samples (0-25%) and bacterial culture are incubated in 96 well plates at 37 °C for 24 h. The contents of the well are listed in Table 3.

Table 3: List of well plate samples

Sample	Fresh LB broth (μL)	LB-Culture broth (μL)	Diluted honey sample (μL)
Sterile control	200	-	-
Growth control	-	200	-
Blank*	100	-	100
Test*		100	100

**To be prepared for every honey sample and every dilution.*

After the 24 hours incubation, the absorbance of the plates was measured at 590 nm using a well plate reader (Biotek Synergy HT Microplate Reader, Vermont, USA). From the absorbances reading, the percentage of growth inhibition of the bacteria was calculated according to the formula below.

$$Growth\ inhibition\ (\%) = 1 - \frac{(A_{Test} - A_{Control})}{(A_{Growth} - A_{Sterile})} \text{ x } 100$$

Where A_{Test} = Absorbance reading of the test (honey) well

A_{Blank} = Absorbance reading of the corresponding test (honey) blank control well A_{Growth} = Absorbance reading of the assay growth well

$A_{Sterile}$ = Absorbance reading of the sterile control well

Each concentration of honey is analysed in triplicates. The MIC is determined from the lowest honey concentration that resulted in 100% growth inhibition and is expressed in percentage (%), representing g honey/100 mL broth. The IC50 was determined by the plotting of a dose response curve (% growth inhibition vs honey concentration) using GraphPad Prism version 5.0. The IC50 values and the error values were calculated directly using the GraphPad Prism.

3.3 Antibiofilm Formation assay

3.3.1 Preparation of overnight P. aeruginosa culture

This method was adapted from Lu et al (2019). The overnight *P. aeruginosa* cultures (as prepared in Section 3.2.1) with modification where culture was diluted at 1:100 using LB broth. Next, 100 µL of the diluted culture was seeded into the desired well of a 96-well plate.

3.3.2 Incubation of overnight P. aeruginosa culture with honey

Serial dilutions using LB broth were carried out using the honey samples. To the wells which contained the culture 100 µL of the 2-fold serial dilution of honey samples was added (50%, 25%,12.5% and 0%). The final serial dilution concentrations were then obtained (25%, 12.5%, 6% and 0%). The 0% honey concentration dilution acted as the positive control. All samples were conducted in triplicates. The seeded 96-well plate was then incubated for 24 hours at 37 °C.

3.3.3 Crystal violet staining

The planktonic (free-living) bacteria from each well plate were removed by tipping the well over to discard the contents into a waste tray. The well plate was then submerged and vigorously shaken in a tray containing sterile water. Then, 200 µL of 0.1% crystal violet was added into each well. The mixture was allowed to stand for 10 minutes at room temperature. Next, the crystal violet was removed into the waste tray. The well plate was then rinsed by submerging it into a tray containing fresh distilled water and was shaken while submerged. This process was repeated once again. Next, the well plate was inverted and vigorously tapped on a paper towel to remove excess liquid. The plate was then allowed to air-dry at room temperature. Once dry, 200 µL acetic acid (33% w/w) was added into each well. The mixture was left to stand at room temperature for 15 minutes. Lastly, the contents of the wells were transferred to a new well plate and the absorbance of the content was measured at 595 nm. The experiments were performed in triplicate.

The absorbances collected were converted into % of biofilm inhibition by dividing the absorbance of the honey sample at the given concentration by the absorbance of the positive control. A graph of % biofilm formation inhibition against honey concentration was then plotted.

$$Biofilm\ inhibition\ (\%) = \frac{(A_{Test})}{(A_{Control})} \times 100$$

Where A_{Test} = Absorbance reading of the test (honey) well

$A_{Control}$ = Absorbance reading of the positive control

3.4 Biofilm elimination

3.4.1 Preparation of 1-week biofilm

This method was adapted from Lu et al (2019) with a slight variation. Firstly, an overnight culture of *P. aeruginosa was* made (as prepared in Section 3.1.3). Next, 200 µL of culture was added to 96-well wells plates and incubated for 24 hours at 37 °C. Next, the media (LB broth) was replaced every 24 hours for one week to all the biofilms to establish the biofilm maturity.

3.4.1 Incubation of 1-week biofilm with honey samples

After 1 week, the well plates containing the established biofilm were washed three times with sterile water. Then, 200 µL diluted honey samples (32%, 16%, 8%, 4%, 2% and 1% (v/v) were added to the wells. The well plates were incubated at 24 hours at 37 °C. Next, the planktonic bacteria from each well plate were removed by washing.

Crystal violet staining

The crystal violet staining was performed as described earlier in Section 3.3.2. The collected absorbance values were converted to % inhibition by dividing the biofilm absorbance of that concentration of honey by the positive control. The experiment was carried out in triplicates. A graph of % biofilm degradation against honey concentration was plotted using GraphPad Prism version 5.0.

3.4.2 Determination of pyocyanin inhibition

The method was adapted from Hgurlu et al (2016). Overnight culture of *P. aeruginosa* grown in 5 mL of LB broth was used. The culture was incubated with honey samples at 32%, 16%, 8%, 4%, 2% and 1% (v/v) concentrations at 37 °C and shaken at 180 rpm for 30 seconds.

Next, 5 mL of culture was aliquoted into a centrifuge tube and centrifuged at 17000 g for 10 minutes. The supernatant was transferred into another tube, and 3 mL of chloromethane was added. The mixture was then vortexed for one minute. The bluish phase was then collected and transferred into another tube where 1 mL of HCl (0.2 M) was added. A reddish upper phase appeared, and the absorbance of this supernatant was measured at 520 nm. The extraction method is summarised in Figure 4 below.

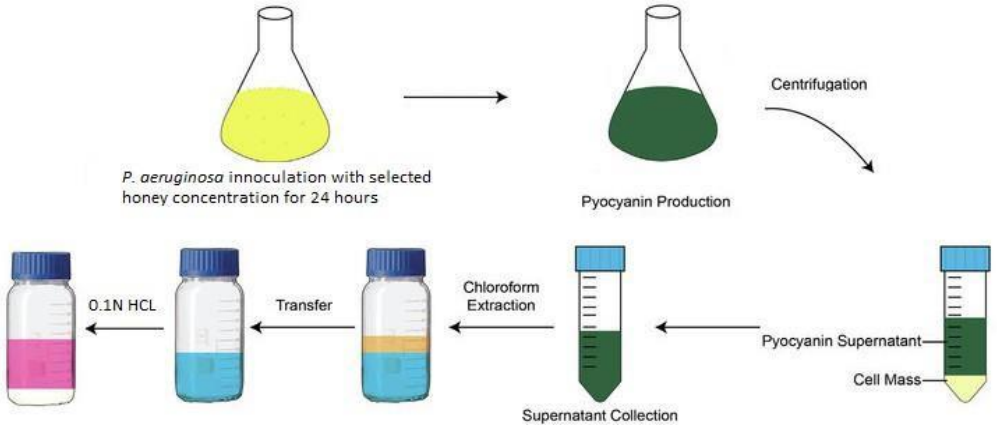


Figure 4: Pyocyanin extraction protocol (Saleem et al 2021)

The absorbance values were converted to % pyocyanin inhibition by dividing the absorbance of test sample (biofilm and honey) by the positive control (biofilm and LB broth) The positive control was the absorbance of 0% honey concentration (w/v). All determinations were performed in triplicates. Graph Prism version 5.0 was used to plot the pyocyanin inhibition (%) against honey concentration (%), the average activity and standard deviation values.

$$Pyocyanin\ inhibition\ (\%) = 100 - \left(\frac{(A_{Test})}{(A_{Control})} \times 100\right)$$

Where A_{Test} = Absorbance reading of the test (honey) well

$A_{Control}$ = Absorbance reading of the positive control

3.5 Statistical Analysis

The dose response plot and the statistical tests in this study were performed using GraphPad Prism (Version 5). The significance was set to $p < 0.05$. A two-way ANOVA was used to compare the concentration-response curve of different types of treatments (Neutralised pH and Normal pH) for the antimicrobial assays while single column t-test was performed to compare the concentration difference between different range of concentrations (biofilm degradation assay).

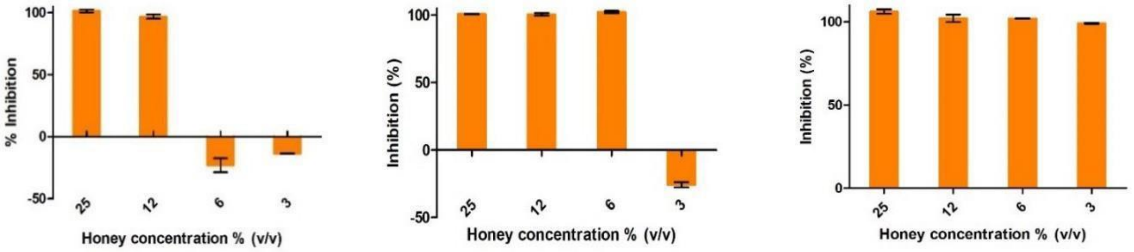
Chapter 4: Results & Discussion

4.1. Antibacterial assay

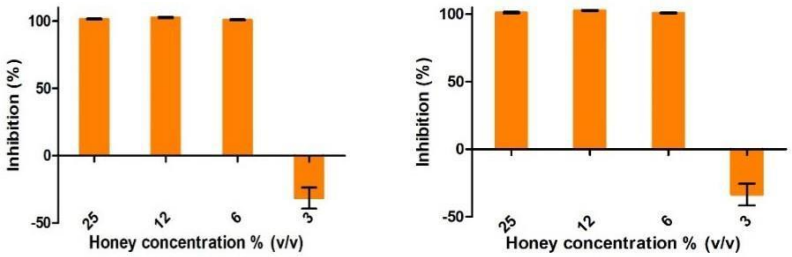
4.1.1 Screening of antibacterial activity

Determination of honey concentrations for initial antibacterial activity screening assays

A range of honey concentrations (3% - 25%) of five different types of honey (Manuka, SBHR1, SBHR4, SBHR10 and SBHR11) samples was tested against *P. aeruginosa* growth. Based on the results (Figures 5a - 5e), a stingless bee honey sample (SBHR4) was found to inhibit *P. aeruginosa* growth at all concentrations, while the rest of the stingless bee honey samples (SBHR1, SBHR10 and SBHR11) showed inhibition effect at concentrations above 3% (MIC = 6.25%). Manuka honey showed a weaker growth inhibition effect (MIC = 12.5%). Interestingly, at lower concentrations, the Manuka honey (≤6% concentration) as well as the SBHR1, SBHR10 and SBHR11 stingless bee honeys (≤3% concentration) had promoted the growth of the bacteria, as shown by the negative inhibition. Based on the results, a 5% (v/v) honey concentration was selected for the subsequent antimicrobial screening. The positive control was 200 µL LB broth with culture. The negative control was 200 µL of LB broth.



(a) Manuka honey (b) SBHR1 (c) SBHR4



(d) SBHR10 (e) SBHR11

Figure 5: Growth inhibition (%) effect of various honey samples at different concentrations against *P. aeruginosa* (n=3).

Screening of antibacterial activity against *P. aeruginosa* at 5% honey sample.

The inhibition assay was conducted at 5% honey concentration (v/v) in two trials, where the samples are analysed in triplicates in each trial. The results for the two trials are summarised in Table 4 below.

Table 4: The % of growth inhibition effect of 5% honey samples against *P. aeruginosa*.

No.	Code	Growth inhibition (%)		
		Trial 1*	Trial 2*	Average
1	Manuka	1.0 ± 0.5	1.1 ± 0.2	1.0 ± 0.4
2	SBHP1	99.6 ± 0.2	100.9 ± 1.0	100.2 ± 0.6
3	SBHP2	99.7 ± 2.1	100.1 ± 0.3	100.0 ± 1.2
4	SBHP3	100.0 ± 6.3	99.9 ± 0.1	99.9 ± 3.2
5	SBHR1	97.4 ± 3.1	99.5 ± 1.4	98.5 ± 2.2
6	SBHR2	100.0 ± 0.5	100.7 ± 0.2	100.4 ± 0.4
7	SBHR3	99.7 ± 2.7	99.2 ± 0.6	99.5 ± 1.6
8	SBHR4	98.2 ± 1.0	100.2 ± 2.1	99.2 ± 1.6
9	SBHR5	100.0 ± 1.7	100.7 ± 1.6	100.4 ± 1.6
10	SBHR6	100.4 ± 0.9	100.0 ± 2.4	100.2 ± 1.7
11	SBHR7	101.8 ± 1.1	102.5 ± 1.0	102.2 ± 1.6
12	SBHR8	100.0 ± 1.7	100.9 ± 0.9	100.5 ± 1.3
13	SBHR9	101.4 ± 2.1	100.7 ± 1.0	101.0 ± 1.6
14	SBHR10	100.6 ± 1.6	100.1 ± 0.8	100.3 ± 1.2
15	SBHR11	101.1 ± 1.0	100.3 ± 1.2	100.9 ± 1.0

*Values are expressed as Average ± Standard Deviation (n=3)

The results showed that at 5% concentration, Manuka honey did not have any inhibition towards the growth of *P. aeruginosa.* This is consistent with literature which shows that Manuka honey inhibited the bacteria growth at a higher range between 9.5%-15.3% (Henriques et al 2011, Camplin & Maddocks 2014). On the other hand, all stingless bee honey samples showed very high growth inhibition effect against *P. aeruginosa.* This was comparable to the result of a study conducted Brazilian stingless bee honey (Nishio et al 2016) which had low MIC values between 2.5% - 5.0% (v/v). Apart from the stingless bee honey samples used, the study was slightly different from the current study as the study tested the honey against different *P. aeruginosa* strains (ATCC27853 & ATCC9027). Current study used *P. aeruginosa* (PA01).

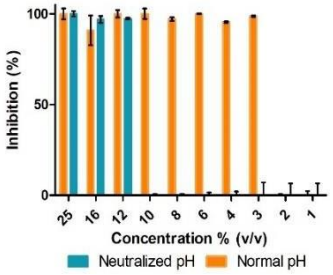
Mandal et al (2011), stated that the antibacterial activity can be attributed to compounds present in the honeys. These compounds include phytochemicals, phenolic compounds, flavonoids, peptides, methylglyoxal (present in manuka honey)

and antibiotic-like derivatives. Other factors which affect honey's antibacterial activity are pH, water content, sugar content and hydrogen peroxide content. The compounds present in different honeys are dependent on the plants in the environment. As many of the compounds in the honey are known to be plant derived. A study by Ulusoy et al (2010) also noted that the higher the antioxidant concentrations in the honey caused antimicrobial zone of inhibition to have a larger zone of inhibition. This indicates that higher antioxidant concentration improves the antimicrobial activity of the honeys tested. The results (Table 4) show that Sarawak's stingless bee honeys are shown to have comparable if not better antimicrobial activity when compared to the antimicrobial activity of the Australian Manuka honey sample. This antibacterial activity of stingless bee honey can be attributed to its content such as flavonoids and high sugar content. The potential of stingless bee honey as an antimicrobial agent is consistent with the study by Nishio et al (2016). In this study, two types of stingless bee honey were tested against a variety of gram-positive and gram-negative bacteria. This study also used broth microdilution method to determine the antibacterial activity of their stingless bee honeys. The study reported that stingless bee honeys are more effective against gram negative bacteria with MIC values ranging from 1.87% ± 0.39 to 2.50% ± 0.81. When tested against gram-negative bacteria, the MIC values ranged between 5.36% ± 0.78 to 6.07% ± 0.99. The study noted that the stingless bee honey treatment caused an enlargement in bacterial cells when compared to the control cells. This enlargement is linked to the occurrence of degradation of the bacterial cell wall which could lead to cell lysis and cytoplasm leakage.

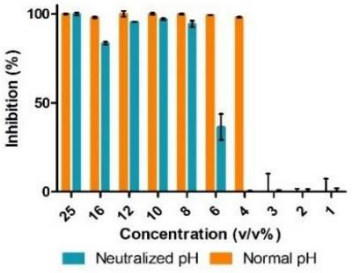
4.1.2 The effect of pH neutralisation on P. aeruginosa growth inhibition

This assay was used to determine the antimicrobial activity of stingless bee honey and Manuka honey against *P. aeruginosa*. The honeys were tested at their natural pH and at a neutralised pH to show how the antimicrobial activity is affected by the honey's pH.

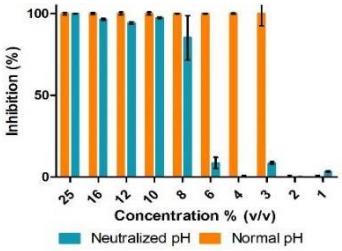
Figure 6 and Figure 7 were plotted using a dose response curve where the x-axis was $\log_{10}$ vs the y-axis. All stingless bee honey samples (Figure 6 and Figure 7) showed antimicrobial activity towards *P. aeruginosa,* with all reaching the 100% growth inhibition at the highest concentration of honey (25%). This result was in line with other literature where Brazilian stingless bee honey showed antimicrobial effects towards gram-positive and gram-negative bacteria (Nishio et al 2016). Another study by Boorn et al (2010), showed that Australian stingless bee honey had antimicrobial activity against both gram-positive and gram-negative bacteria.



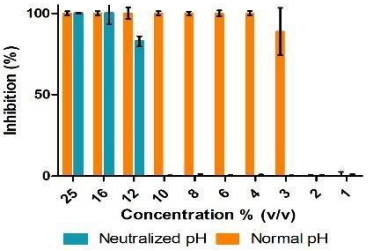
(a) SBHR1 Ahmad



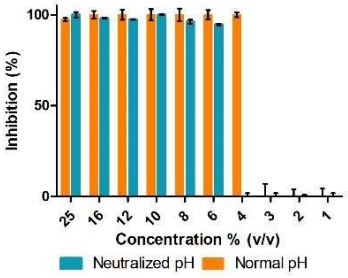
(b) SBHR2 Simpulan Emas



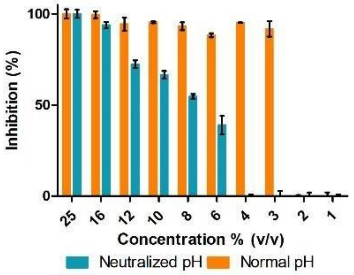
(c) SBHR3 Mireng



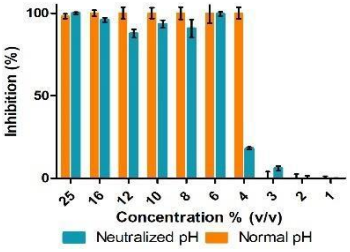
(d) SBHR4 Monday



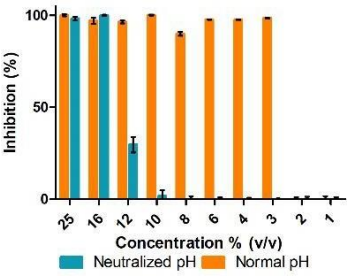
(e) SBHR5 Stanley



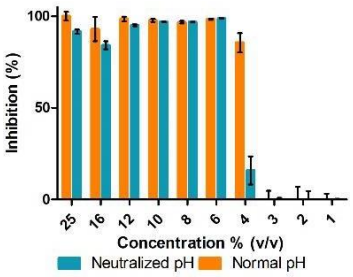
(f) SBHR6 Sharon



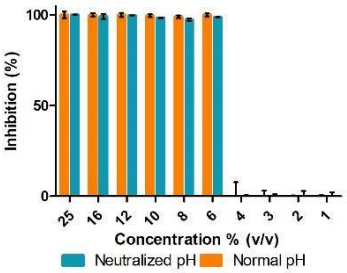
(g) SBHR7 Lee



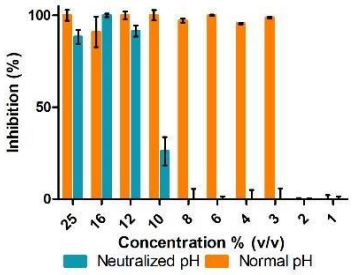
(h) SBHR8 Elvy



(i) SBHR9 Pojie

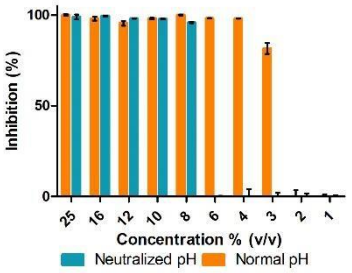


(j) SBHR10 Mundai

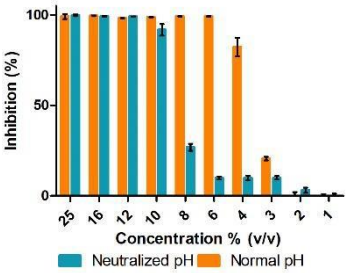


(k) SBHR11 Chen

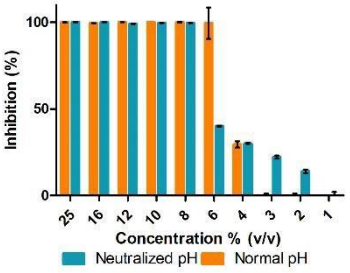
Figure 6: Growth inhibition (%) effect of raw stingless bee honey (a-k) at different concentrations and at different pH against *P. aeruginosa* (n=9).



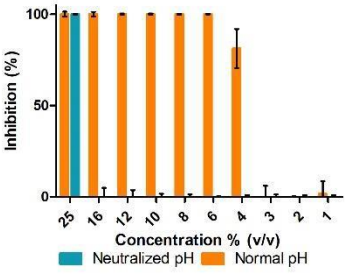
(a) SBHP1 Meli



(b) SBHP2 Syamille



(c) SBHP3 H&B



(d) Manuka

Figure 7: Growth inhibition (%) effect of processed stingless bee honey (a-c) and Manuka honey (d) at different concentrations and at different pH against *P. aeruginosa.* (n=9).

When comparing acidic pH vs neutralised pH, it was observed that the acidic pH had much better antimicrobial activity. In most cases, the acidic version of the honey had two to three- fold better antimicrobial activity than the neutralised honey samples. In a study by Sankaralingam et al (2014) it was noted that *P. aeruginosa* grows the best at pH 6-8. It was also noted that at lower pH ranges such as pH 2 - 4, *P. aeruginosa* has inhibited growth. It was noted that most honeys were greatly affected by the neutralization of the pH, which means that most of those honeys antimicrobial activity is contributed by its low pH.

The potency of the antimicrobial activity of the honey was also represented by their IC50 values. The lower the IC50 value is, the more potent the antimicrobial effect of the honey sample is. Table 5 summarised the IC50 values of each honey sample at acidic pH (not modified) and neutralised pH. Manuka was highly affected by the neutralization of its pH. Its IC50 value was raised to more than four times when it was neutralised, showing the reduction in antimicrobial effect. This suggests that the antimicrobial effect of Manuka honey is mainly acid-related. The difference in the IC50 values of the acidic and neutralised honey samples are significantly different ($p<0.05$), except for one sample. SBHR10 was the only honey sample that was not affected at all by the pH difference. It has an IC50 value of 5.0% at both normal (acidic pH) and at neutralised pH.

Table 5: The half maximal growth inhibition (IC50) value of different honey samples against *P. aeruginosa* at normal pH and normalised pH.

No.	Code		
		Normal pH	Neutralised pH
1	Manuka	3.6 ± 0.1	21.2 ± 3.2
2	SBHP1	2.5 ± 0.1	7.0 ± 0.1
3	SBHP2	3.4 ± 0.1	8.6 ± 0.1
4	SBHP3	4.4 ± 0.1	6.4 ± 0.1
5	SBHR1	2.4 ± 0.2	11.0 ± 0.3
6	SBHR2	3.5 ± 0.1	6.2 ± 0.1
7	SBHR3	2.4 ± 0.1	7.2 ± 0.1
8	SBHR4	2.5 ± 0.1	11.4 ± 0.3
9	SBHR5	3.5 ± 0.1	5.0 ± 0.1
10	SBHR6	2.4 ± 0.1	7.7 ± 0.3
11	SBHR7	3.5 ± 0.2	4.6 ± 0.1
12	SBHR8	2.4 ± 0.1	12.4 ± 0.1
13	SBHR9	3.6± 0.1	4.6 ± 0.2
14	SBHR10	5.0 ± 0.2	5.0 ± 0.1
15	SBHR11	2.4 ± 0.2	10.4 ± 0.2

*Values are expressed as Average ± Standard Deviation (n=9)

The results observed in Figure 6, Figure 7 and Table 5 show that pH is one of the main factors that affect the antimicrobial activity of the honey. Xiong et al (1996), studied the antimicrobial activity of aminoglycosides, amikacin and netilmicin at different pH, and reported that at neutral pH of 7.4 the bactericidal activity of the compounds was concentration-dependent rather than dependent on the pH. At pH 6.5 the study noted that the killing rates of both amikacin and netilmicin had no bactericidal activity whatsoever on the *P. aeruginosa*. This study highlights that compounds used to treat *P. aeruginosa* are concentration-dependent and it was possible that their activity can be reduced at acidic pH's.

Honey contains organic acids which may contribute to its antimicrobial activity. A study by Bushell et al (2019), tested the synergistic effects on organic acids and pH on the growth of *P. aeruginosa*. The study notes that weak organic acids have good potential to be used as topical treatments against opportunistic pathogens. This study used a variety of organic acids at a range of pH's to determine their effects on the growth of *P. aeruginosa*. It was determined that acetic, propionic, and butyric acids all had detrimental effects on the growth of the bacteria. Under the strongest conditions at high organic acid concentration and low pH it was noted that some organic acids were more effective than others in inhibiting the growth of the bacteria. It was noted that acetic acid at pH 5.5 was observed to be completely bacteriostatic while benzoic acid had no effects on the bacterial growth. The study ranked the organic acids from 'most active' to 'least active'; propionic acid > butyric > acetic > citric > sorbic > malic > benzoic acid. Stingless bee honey contains a variety of organic acids such as acetic, butanoic, formic, citric, and malic acid. Therefore, the higher antimicrobial activity observed at lower pH's in this study can be attributed to the organic acids having higher antimicrobial activity at lower pH's.

4.1.3 Minimum Inhibition Concentration (MIC) Assay

All honeys were tested under two different pH conditions which are at their natural pH as well as they were tested at neutralised pH. This testing was done to determine how much the pH of the honeys affect the antimicrobial activity of the honeys. The results (Table 5) for the raw stingless bee honey showed that out of the 11 honeys tested, the increased pH caused ten of the honeys to require a higher concentration of honey to reach the same MIC when at its natural pH. Only SBHR10 was shown to be unaffected by the neutralization of the pH.

As previously mentioned, the study by Bushell et al (2019) identified that organic acids which are found in stingless bee honeys have better antibacterial activity at lower pH .

Therefore, one of the reasons that the Normal pH had lower MIC values is because the lower pH gives the organic acids better antimicrobial activity. A study by Lin et al (2021), tested how an acidic environment affects the antibiotic susceptibility and biofilm formation of *P. aeruginosa*. The study changed the pH of the environment in which the bacteria grew in. The bacteria were found to be able to thrive in a lower pH (6.0). It was found that the HCl adjusted lower pH of 6.0 caused no difference in the rate of proliferation of the *P. aeruginosa* tested. The study also found that at lower pH's the bacteria had better resistances to the antibiotics. Table 6 shows that at the lower Normal pH the MIC values were lower. This lower MIC can be attributed to not only the pH effect directly on the bacteria but also the effects of the lower pH on the antibacterial activity of the compounds in the honeys.

Table 6: MIC values of antimicrobial assay. Showing both acidic pH and neutralised pH honey.

Honey	Code	Normal pH	Neutralised pH
		MIC (v/v) %	MIC (v/v) %
Meli	SBHF1	4	8
Syamile	SBHF2	5	12
H&B	SBHF3	5	8
Ahmad	SBHR1	3	12
Simpulan Emas	SBHR2	4	8
Mireng	SBHR3	3	10
Monday	SBHR4	4	12
Stanley	SBHR5	4	6
Sharon	SBHR6	3	12
Lee	SBHR7	4	6
Elvy	SBHR8	3	12
Pojie	SBHR9	5	6
Mundai	SBHR10	5	5
Chen	SBHR11	3	12
Manuka	MANUKA	6	25

Values are expressed as Average (n=3). All replicates gave the same MIC values in their respective sample, hence no standard deviation values stated.

The range of MIC values shown in Table 6 for raw stingless bee honey at normal pH was 4%- 5% (v/v) while at neutralised pH was 8%-12%. For processed stingless bee honey, the MIC at normal pH was 3-5% while at neutralised pH the MIC increased to 5%-16%. The results for stingless bee honey MIC were consistent when compared to literature where Brazilian stingless bee honey showed MIC of 5%-6% towards gram-negative bacteria (Nishio 2016). For the MIC of manuka honey at normal pH was 6% while at neutralised pH it was 20%. The literature shows that MIC of manuka honey

against *P. aeruginosa* ranges between 6%-20% depending on brand (Shenoy et al 2012, Roberts et al 2019, Mandal et al 2011).

The remaining antibacterial effects of the honey after neutralization can be attributed to the other antimicrobial characteristics of honey as the effects of the pH have been ruled out by the testing above. A study by Havasi et al 2008, tested the effect of NaCl on the growth of *P.aeruginosa*. It was determined that up to 2% NaCl (v/v) concentration had very little effect on the growth rate of the bacteria. While at 7% NaCl (v/v) concentration the growth of *P. aeruginosa* is impacted. As for this study it was not determined how much salt was produced when the acidity of honey was neutralised. Therefore, future work will have to investigate the amount of salt that is produced. Other characteristics that would have contributed to the antimicrobial activity of the stingless bee honeys are the hydrogen peroxide content of the honey (Shenoy et al 2012). Another factor is the high sugar content of the honey which in combination with low moisture content give honey antibacterial properties through high osmotic pressure (Mandal et al 2011).

4.2 Antibiofilm formation assay

4.2.1 Screening for antibiofilm activity

The antibiofilm assay was part of the initial screening to gauge whether the tested honeys have antibiofilm formation activity. It was also done to determine what range of concentrations to be used for the honey concentrations. Four honey samples were selected, namely SHBR3, SHBR4, SHBR6 and SHBR7. The result of this screening is summarised in Figure 8 below.

The antibiofilm formation assay was conducted in triplicates. The result seen in Figure 8 was a trial run to determine what a good range of honey concentrations would be. The results showed that at 6.25% (v/v) honey concentration, there was an inhibition of almost 50% across all stingless bee honeys tested. There was little increase of inhibition with increasing concentrations.

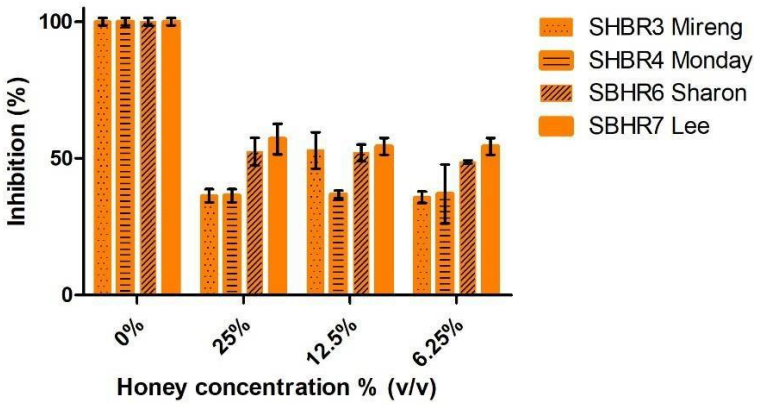


Figure 8: The % formation of *P. aeruginosa* biofilm at different honey concentrations (n = 3).

Lu et al (2019) tested honey as potential antibiofilm agents. The study noted that the formation of biofilms results in an increase of resistance against negative environmental influences as well as increasing resistance to antimicrobial agents. It was also noted that cell-to-cell communication is an important mechanism when bacteria develop biofilms. It also helps the bacteria in the biofilm better balance the environment when high bacterial density is reached. The study linked honey to compounds such as hydrogen peroxide, antimicrobial peptides, phenolics as well as MG. These are known to disrupt the cell-to-cell communication in bacterial cells as well as cause cell proliferation. The study tested four different honeys against *P. aeruginosa,* the MICs of the honeys ranged between 12% - 50%. The wide range of MICs in the honeys is due to the varied content of the honeys. This highlights the need to determine the key antibacterial compounds of the honeys to make comparison to one another easier between studies. As noted by the study, honey is difficult to characterize fully due to its complex nature of the individual compounds inside.

Another study by Alandejani et al (2009), used manuka honey to determine its effectiveness on *S. aureus* and *P. aeruginosa* biofilms. This study determined that the concentration of killing 100% of the bacteria 63% - 91% honey concentrations. This study showed that although high MICs were noted that honeys have antibiofilm activity.

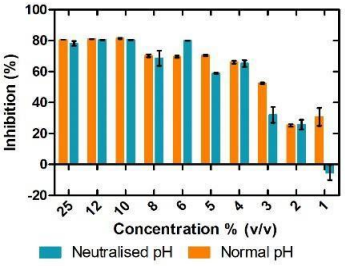
4.2.2 Antibiofilm activity

Figure 9 and Figure 10 below show the results for the antibiofilm formation assay of honey samples with concentrations ranging from 1% to 25%. They were also tested at their normal pH as well as at a neutralised pH.

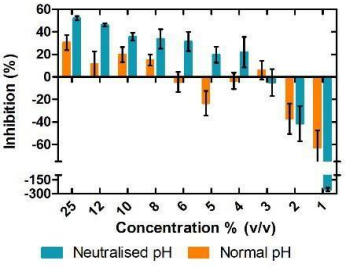
The results showed that all honeys had inhibitory effects towards the formation of *P. aeruginosa* biofilms. For the raw stingless honey samples, SBHR1 and SBHR 11 showed the highest antibiofilm formation activity of all honeys at 80% inhibition at 25% (v/v) concentration. The rest of the raw stingless bee honey samples showed between 20% to 70% inhibition at 25% concentration. When compared to the processed honey samples (Figure 10), none of the samples reached 80% inhibition at 25% honey concentration. Most showed around 45% inhibition. The low antibiofilm activity of honey samples at 25% concentration were also reported elsewhere where Heather honey had 29.9% and Manuka honey had 34.2% antibiofilm activity at 25% concentration, respectively (Shirlaw et al 2020). This study shows the potency of selected raw stingless bee honey (SBHR1 and SBHR11) from Sarawak as an

antibiofilm agent.

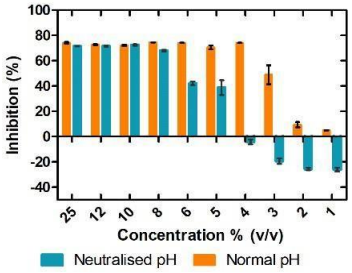
The effect of pH neutralization was also investigated, like those in the antimicrobial assays earlier. The decrease in antibiofilm effect because of neutralization is less prominent (5-10% lesser) in honey samples at 25% concentration. The neutralization effect became more obvious in honey samples with lower concentrations, as shown by the results of SBHR4, SHBR6, SHBR8, SBHP1, SBHP3 and Manuka honey samples. At concentration range between 1% to 3%, most honey samples even aided in the formation of biofilms (Figure 9 and Figure 10).



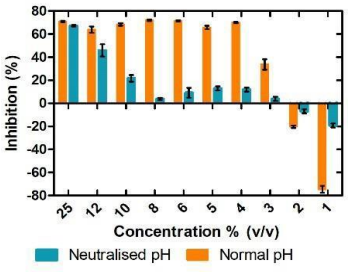
(a) SBHR1 Ahmad



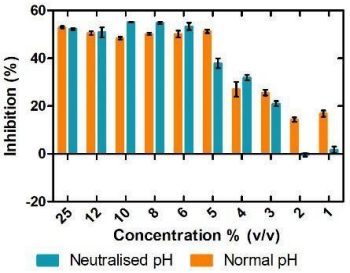
(b) SBHR2 Simpulan Emas



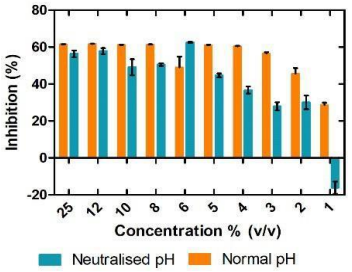
(c) SBHR3 Mireng



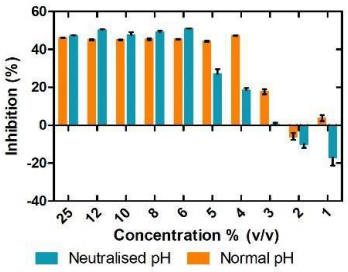
(d) SBHR4 Monday



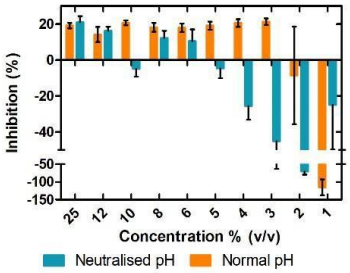
(e) SBHR5 Stanley



(f) SBHR6 Sharon

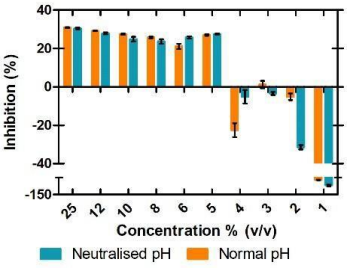


(g) SBHR7 Lee

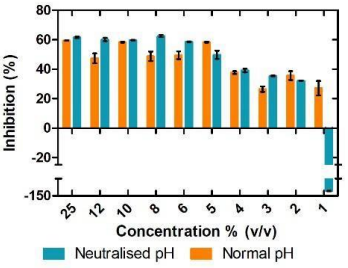


(h) SBHR8 Elvy

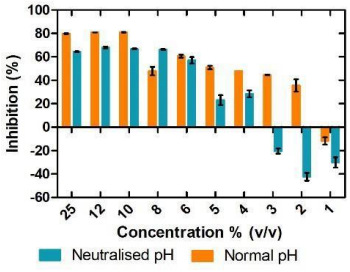
Figure 9: The inhibition effect of stingless bee honey samples (a-k) against *P. aeruginosa* biofilm formation (n=9).



(i) SBHR9 Pojie



(j) SBHR10 Mundai



(k) SBHR11 Chen

Figure 10: The inhibition effect of processed honey (a-c) and Manuka honey (d) against *P. aeruginosa* biofilm formation (n=9).

For the antibiofilm testing, a 2-way ANNOVA was conducted. In this analysis the significance of the concentration of honey dilution was analyzed. This analysis showed that the significance of different concentrations of honey had $P < 0.05$. This result means that the difference in inhibitory effects of the honeys tested were statistically significant. The second variable that was tested in this 2-way ANNOVA was the effects of the neutralization of the honeys. In this testing it was determined that mean differences of inhibition between the neutralised honey and normal pH

honey were $P < 0.05$. Only SBHP3 ($P = 0.0619$) showed $P > 0.05$ during the effects of pH on inhibition, this meant that the neutralization of the honey did not affect SBHP3.

The phenomenon of sub inhibitory concentrations increasing the biofilm formation was observed in a study by Bernardi et al (2021). In this study sub inhibitory concentrations of antibiotics were used to test its effects on the biofilm formation of *E. faecalis* isolates. The study used eight different *E. faecalis* isolates. The study tested its hypothesis using sub inhibitory concentrations of Penicillin G, Amoxicillin, Doxycycline, Fosfomycin, Tetracycline and Vanomycin. Firstly, the study tested the MICs for all the antibiotics against the selected isolates. Then, serial dilutions were made. Then, the treated bacteria were incubated overnight. The biofilms were then quantified using microtiter plate assay. The study determined that all the isolates were able to form biofilms in the absence of antibiotics. In the presence of sub inhibitory concentrations of antibiotics, it was found that there was a significant increase in the biofilm formation increasing up to 50% in some cases. Of the 8 isolates it was noted that three of the isolates had a significant increase in biofilm concentrations against five of the tested antibiotics when in sub inhibitory concentrations. The study noted that increases in biofilm formations were observed at the concentration ranges of ½ – 1/64 of the MIC values. This can be observed in the antibiofilm testing where the lowest honey concentrations also increase biofilm formation. The link was that sub inhibitory concentrations of antimicrobial agents cause stress on the bacteria. This stress influences the genotype and phenotype of the bacteria to increase its resistance against the antimicrobial agent by increasing biofilm formation. This was a possible explanation for the increases in biofilm formation observed at 1%-6% honey concentrations when biofilm inhibition was a negative value.

4.3 Biofilm Degradation Activity

Figure 11 show the results for the biofilm degradation activity assays of honey samples with concentrations ranging from 1% to 32%. The six honey samples were selected from the previous results. Samples SBHR1, SBHR3 and SBHR6 represents honey with high antibiofilm inhibition activity (60% - 80%) at 25% concentration (Figure 9) while samples SBHR7 and SBHR9 represent honey with minimal neutralisation effect on their antimicrobial (Table 6) and antibiofilm (Figure 9) activities. Manuka honey was selected to allow a comparison to be made between the two types of honeys.

The biofilm degradation assays show the effects of stingless bee honey and manuka honey on matured biofilms (one week old). This assay was conducted to determine

whether the tested honeys had the ability to cause a reduction in biofilms after they are established. The results showed that for all honeys at the highest concentration 32% (v/v) that there was a reduction in the biofilm of *P. aeruginosa* of 15% - 41%. The honey SBHR6 showed the highest reduction in biofilm at 32% honey concentration (v/v) at 41% reduction of biofilm. In most honeys as the concentration of honey decreases the biofilm degradation decreases. In all honeys at 4% honey concentration (v/v) and below the honey causes an increase in the biofilm formed. Literature values show that the biofilm degradation assay conducted by Farkas et al (2022), when using Chestnut honey at 45.5% (v/v) concentration there was a 68.5% degradation to the biofilm. However, for the study the biofilms were only allowed to form for 24 hours before they were washed away using sterile buffer. Therefore, the results cannot be properly compared as the results were obtained from biofilms which were grown for one week.

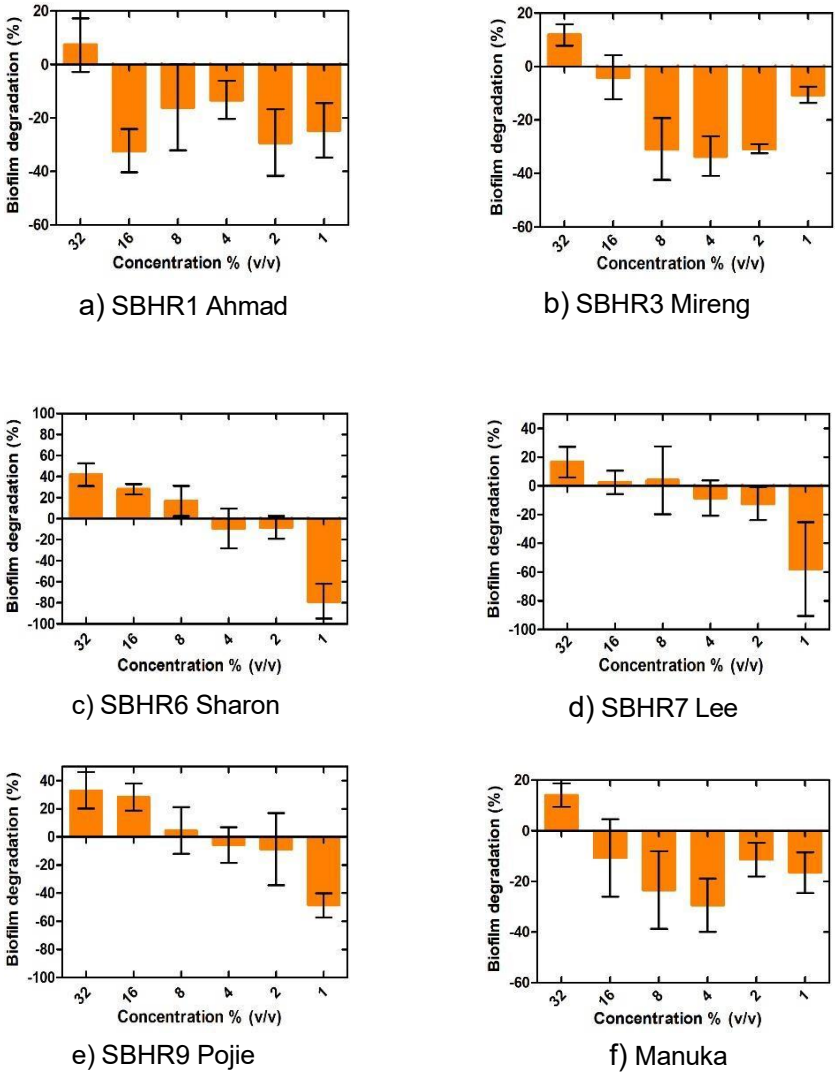


Figure 11: The biofilm degradation effect of raw stingless bee honey (a-e) and Manuka honey (f) (n=3).

In another study by Balazs et al (2023) a range of honey concentrations (20%, 40%, 60% and 90%) were used. The results showed at the lower concentrations 0-20% honey concentrations there was no activity against the biofilms while at 40% honey concentration there was activity ranging between 21% - 37% depending on the honey type. At 60% honey concentration there was activity between 32% - 44%, while at 90% where was 100% biofilm degradation. When comparing the results shown in the degradation assay, the highest honey concentration of 32% for SHBR6 had an inhibition value of 41% which is comparable to the literature values.

Roy et al (2018), reviewed effective strategies in combating bacterial biofilms. The study highlights that pre-mature biofilms can be treated more easily with antibiotic compounds. But mature biofilms are difficult to treat and are often the type of biofilm which was associated with clinical conditions. The key to treating established biofilms is causing the biofilms to disperse or degrade. The honeys in the degradation assay showed the highest rate of degradation for SBHR6 which reduced the biofilm by 41%.

The results in the degradation assay can be compared to the study by Lu et al (2019) which studied the effects of Australian and New Zealand manuka honey on the biofilms of *P. aeruginosa*. The research also tested the ability of the honey to eliminate established biofilms, similar to the work in this study. The results of the study showed that at higher concentrations of honey (16% and 32%), there was a significant eradication of *P. aeruginosa* biofilms. It was also noted that at sub inhibitory concentrations, the biofilm biomass had significant enhancements to their growth. This can also be seen in Figure 9 and Figure 10 where at lower concentrations of honey, the biofilm biomass was increased as indicated by a negative inhibitory value. Lu et al (2019) reported that at the highest concentration of honey 32% there was a <40% reduction to established biofilms. This result is comparable to the results of SBHR6 honey sample which at 32% honey concentration also reduced established biofilms by 40%.

A single column t-test was conducted to compare if the % inhibition values are different from each other within the same honey sample. While there is a trend of dose- or concentration- dependent relationship between the concentration of honey and the % of biofilm degradation, the difference in the values were not statistically different ($P>0.05$) in all honey samples, except for SBHR1 sample. This is attributed to the high variability (error bars) in the results of the replicates. To improve these results for future works, it would be recommended to increase the length of incubation of the honeys from 24 hours to 48 hours, increase the number of replicates, as well as increase the range of concentrations to better observe the inhibitory effects of the honey.

4.4.1 Pyocyanin Inhibition Assay

Figure 12 below show the results for the effect of honey samples at concentrations ranging from 1% to 32% on pyocyanin production. The effects of pH neutralisation on the inhibition of pyocyanin were also studied. The study was performed on the same honey samples that were analysed for biofilm degradation assay.

For Figure 12, 2-way ANNOVA was conducted. In this analysis the significance of the

concentration of honey dilution was analyzed. This analysis showed that the significance of different concentrations of honey had $P < 0.05$. This result means that the difference in inhibitory effects of the honeys tested were statistically significant. The second variable that was tested in this 2-way ANNOVA was the effects of the neutralization of the honeys. In this testing it was determined that mean differences of inhibition between the neutralised honey and normal pH honey were $P < 0.05$.

Figure 12 showed the pyocyanin production rate of the *P. aeruginosa* under different concentrations of stingless bee honeys and Manuka honey. The results showed that both SBHR3 as well as SBHR6 had near 0% production of pyocyanin at 8% honey concentration. This was the lowest concentration among the honeys tested to have near 0% pyocyanin production rate. When comparing both the best performing honeys (SBHR3 and SBHR6), SBHR6 performed marginally better as at 2% honey concentration, it had 40% pyocyanin production rate while at that concentration SBHR3 had 65% pyocyanin production rate.

Figure 12 also tested the pyocyanin inhibition observed at two different pH scenarios. This pH ranged between 2.5-5.0 pH depending on the honey. At the neutralised pH, 1M NaOH was added until the pH was noted to be at 7.0 pH. The results of this pH moderation when testing the honeys anti-pyocyanin activity showed that at the more acidic pH, the honeys had much higher anti-pyocyanin activity. At 32% honey concentration all honeys in Figure 12 showed close to 100% inhibition of pyocyanin production. SBHR3 showed the best anti- pyocyanin activity. At lower concentrations SBHR3 still showed relatively high anti-pyocyanin activity. At 4% honey concentration at natural pH (acidic) SBHR3 had 85% pyocyanin activity inhibition while the next best anti-pyocyanin activity at that honey concentration was seen by SBHR6 with 70% inhibition. While at 4% honey concentration the other 4 honeys had less than 50% anti-pyocyanin activity. At 1% honey concentrations both natural (acidic) pH and neutralised pH had close to no anti-pyocyanin activity. When the pH of the honeys was neutralised the anti-pyocyanin activity diminished to 1/3 of the activity when compared to the honeys natural pH (acidic). Kurashi et al (1958) noted that the optimum pH range to produce pyocyanin was 7.4-8.4 pH. Also, it stated that the pH should not be lower than 6.0 or higher than 9.0. The study noted that when the pH was greater than 9 it causes the growth media to become viscous causing the formation of pyocyanin to be obstructed. This could be one of the reasons that at lower pH there was a reduction in the pyocyanin activity.

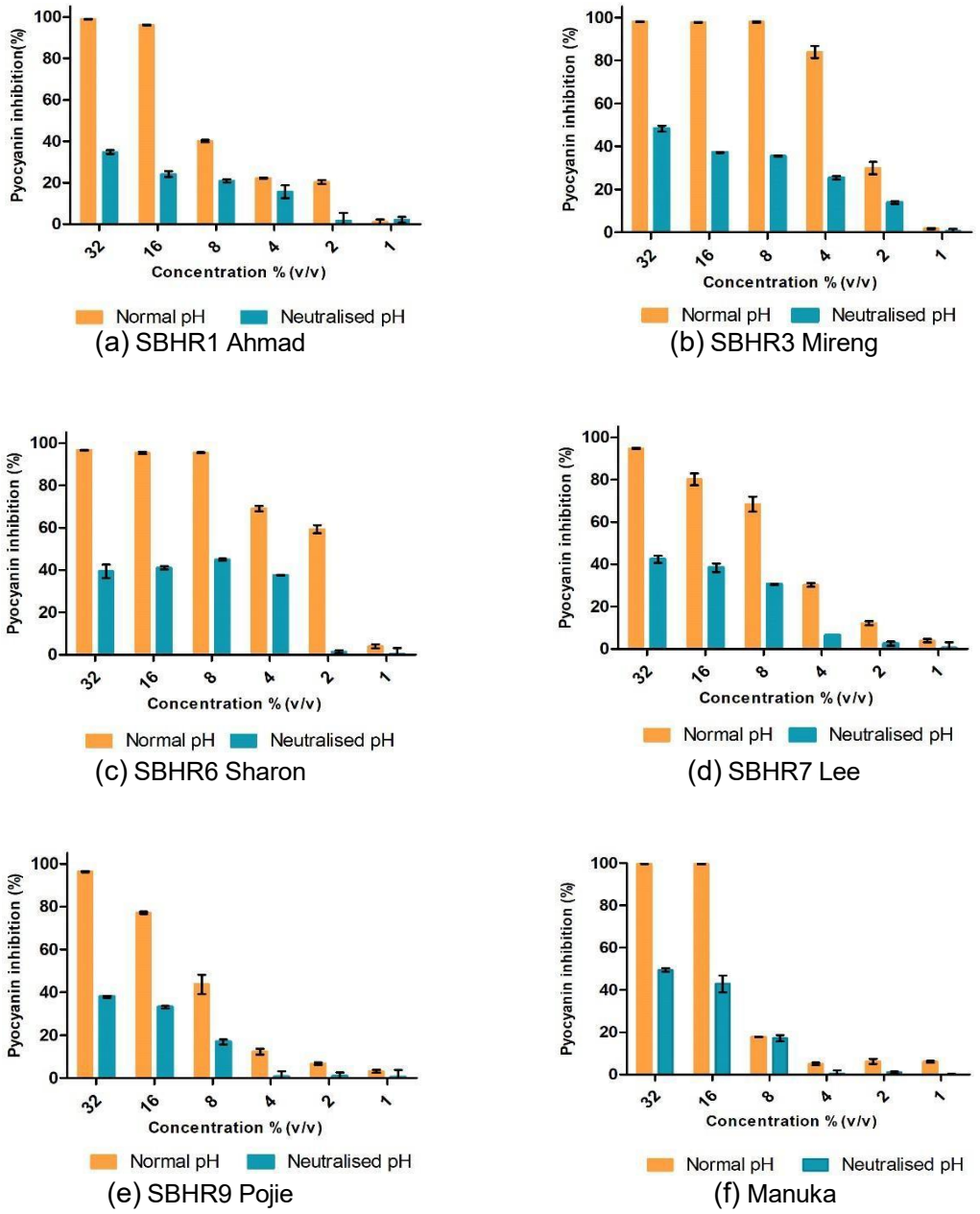


Figure 12: The pyocyanin formation inhibition effect of raw stingless bee honey (a-e) and Manuka honey (f). (n=9)

In a study by Wang et al (2012), the study tested reduction of pyocyanin production of *P. aeruginosa* when incubating the bacteria with 4% concentration of honey (Italian honeybee honey). The study determined that at 4% honey concentration there was an average reduction of 50% pyocyanin production. It was also noted that the reduction of pyocyanin production was independent of the bactericidal properties, as honeys which lacked bactericidal activity still reduced the pyocyanin production. The study also found that when honeys are heat- treated it did not have significant reduction of pyocyanin production inhibition. The article noted that at 4% honey concentration there was a reduction in pyocyanin production of 50%. This shows that both SBHR3

and SBHR6 performed better when compared to this literature honey. Whereas the other four honeys tested did not perform as well as the literature honey.

4.5 Summary

In this study 14 Sarawak stingless bee honeys (11 raw and three processed) and one Manuka honey were tested against *P. aeruginosa*. They were subjected to an antimicrobial assay, antibiofilm formation assay, biofilm degradation assay and pyocyanin assay. All Sarawak stingless bee honeys showed antimicrobial activity against *P. aeruginosa* that had a lower MIC compared to the Manuka honey tested.

Of the tested honeys, SBHR6 overall showed itself to be the most promising honey. The honey was noted to have a low MIC value of 3% (v/v). This was one of the lowest MICs of the honeys tested. SBHR6 also was noted to have good antibiofilm formation activity at 32% honey concentration and it inhibited biofilm production by 60%. This antibiofilm activity was among one of the highest of all the tested honeys. SBHR6's antibiofilm was noted to also not be affected by its neutralization as the antibiofilm activity at natural (acidic) and neutralised pH was very similar. SBHR6 was able to have significant antibiofilm activity even at lower concentrations. It was observed to still have 40% inhibition of biofilm formation. For biofilm degradation SBHR6 was found to have the best activity at 32% concentration it was able to reduce mature biofilms by 40%. Lastly, SBHR6 had a good inhibition of pyocyanin production. It performed the best among the honeys tested at low concentrations. At 2% concentration SBHR6 was still able to inhibit pyocyanin production by 60%. This was when compared to the pyocyanin inhibition at natural (acidic) pH.

A high anti-pyocyanin activity showcased by SBHR6 could be one of the factors which contributes to SBHR6's good antibiofilm activity as a low rate of pyocyanin production interferes with *P. aeruginosa's* biofilm development. Pyocyanin is needed by *P. aeruginosa* in biofilms for gene expression, cellular respiration, release of eDNA to the environment and other vital mechanisms. Therefore, inhibiting the production of pyocyanin in *P. aeruginosa* will affect its biofilm production. As such SBHR6 shows potential to be used as a therapeutic treatment against *P. aeruginosa* infections and biofilms.

Chapter 5: Conclusion

In this study 14 different Sarawak stingless bees and one Manuka honey were used. The first objective was to determine the antimicrobial activity of Sarawak stingless bee honey against *P. aeruginosa*. For this objective a microdilution assay was used. This assay was found to be the most suitable because it is relatively cheap, easy, and fast to use. The honeys first underwent screening at 5% honey concentration to see whether the honey had good antibacterial activity. After the screening, a range of honey concentrations 25%, 12%, 10%, 8%, 6%, 5%, 4%, 3%, 2%, 1% and 0% were used to better understand the effects of varied concentrations of honey on the bacteria. The microdilution allowed the IC50 and MIC to be determined. For the MICs of the honey, it was found that SBHR1, SBHR3, SBHR6 and SBHR11 had the lowest MICs at natural (acidic) pH of 3% honey concentration. While at the neutralised pH SBHR10 had the best MIC at 5% honey concentration. The result from this testing shows that the stingless bee honeys used in this study had antimicrobial activity against *P. aeruginosa*. The honey's pH was also neutralised to better understand the relationship of how the pH of the honey affects the antimicrobial activity of the honey. Honeys' pH does play an important role in its antimicrobial activity. Its antimicrobial activity decreased when the pH was increased. This shows that the antimicrobial compounds in honey are less effective at higher pH's. An explanation for this is that honey contains organic acids. A study by Bushell et al (2019), tested the antimicrobial activity of organic acids and pH on *P. aeruginosa*. The study found that the lower pH's the organic acids were determined to have better antimicrobial activity. Stingless bee honey contains a variety of organic acids such as acetic, butanoic, formic citric and malic acids. The increase of the pH during the neutralization of the honey may cause the organic acid antimicrobial activity to reduce.

The second objective was to determine the biofilm inhibition and degradation activity of Sarawak stingless bee honey against *P. aeruginosa* biofilms. The results of the antibiofilm formation assay show that Sarawak stingless bee honey has good inhibitory activity. At the highest honey concentration of 32% (v/v) SBHR1 was noted to inhibit biofilm formation by 80%. This was the highest among all the honeys tested. This shows that certain stingless bee honey has good potential to be used against *P. aeruginosa* biofilm formation. Some of the honeys tested had much lower antibiofilm formation activity. SBHR2, SBHR8, SBHR9 and SBHP2 did not have as good antibiofilm formation activity, at 32% honey concentration they were observed to only have 20%-30% inhibitory activity. This was lower than manuka honey which at 32%

concentration had 47% inhibitory activity.

This study successfully completed its last objective which was to determine the anti-pyocyanin production activity of Sarawak stingless bee honey in *P. aeruginosa*. At the highest honey concentration of 32% (v/v) all of the six tested honeys were noted to have near 100% inhibition of pyocyanin production in *P. aeruginosa*. The five stingless bee honeys were observed to have better inhibitory activity at lower concentrations when compared to manuka honey. The pyocyanin inhibition of manuka honey dropped off at lower concentrations. At 8% (v/v) honey concentration the manuka honey only had inhibitory activity of <20%. While stingless bee honeys at 8% (v/v) concentration had activity of >40%. This result shows the Sarawak stingless bee honey shows potential has an anti-pyocyanin compound.

5.1 Further Work

All the testing done was as in vitro assays. The use of animal testing in further work on the antimicrobial activity of Sarawak stingless bee honey can help to evaluate the safety, efficacy, and toxicity of the honeys in a more complex model. Chaundhary et al (2019) tested the wound healing efficacy of Jamun honey in diabetic mice. The study wanted to evaluate the wound healing effects of the honey on patients suffering from diabetic chronic wounds. This effect was tested on Swiss albino mice. This testing enabled the researchers to better understand the in vivo activity of their honey. It was found that the honey was able to successfully treat wounds induced on the mice. The results of the Jamun honey were comparable to medical grade manuka honey. This study shows that other honeys besides manuka honey have potential to be used as wound treatment. From the antimicrobial activity assay, it was determined that Sarawak stingless bee honey had comparable activity to manuka honey. This shows that the stingless bee honey has potential to be used in an in vivo study to make the results more translatable to humans.

The use of medical devices in healthcare is a growing industry. But it faces challenges from the colonisation of these medical devices by microorganisms. These colonisations are due to the formation of biofilms by infectious bacteria. These biofilms, especially on implanted medical devices, are difficult to treat as bacteria in biofilms develop antibiotic resistances. The antibiofilm formation and biofilm degradation results observed by Sarawak stingless bee honey gives it a potential solution to medical devise associated biofilms. The alternatives to a therapeutic treatment are the use of strong disinfectants which are not practical when used on patient implants or the excising of infected tissues.

The Sarawak stingless bee honeys were noted to have good anti-pyocyanin activity. This allows the stingless bee honey to be used as a potential inhibitory of pyocyanin in *P. aeruginosa*. The stingless bee honeys activity would be significant to identify other compounds with anti-virulence activity. The understanding of how stingless bee honey impacts the quorum sensing of *P. aeruginosa* needs to be further explored. As honey is acomplex compound further research is required to understand the contributing factors of these compounds to the inhibition of pyocyanin production.

Further studies will need to be done on the chemical composition of Sarawak stingless bee honey. Manuka honey is unique because of its methylglyoxal content which has been directly linked to increasing its antibacterial activity. In comparison Sarawak stingless bee honey is a complex compound which has been less studied and understood. Therefore, there is a need to identify whether Sarawak stingless bee honey has any unique compounds such as methylglyoxal. It is also important to determine its chemical composition as it will help us to fully understand how Sarawak stingless bee honey gets its antimicrobial activity from.

The sugar concentration will have to be considered for future testing. In this case a sugar solution will be mixed to mimic the sugar make-up of the stingless bee honeys. The sugar solution will be tested alongside the stingless bee honeys to determine the effects of sugar on the antimicrobial activity of the stingless bee honey.

Another factor to consider for future work would be to study the effects of a range of pH’s on the antimicrobial and antibiofilm activity of the stingless bee honey. This can be achieved by selecting a range of pH’s and the associated buffers. This will give a clear picture of the exact activity of the stingless bee honey at a specific pH.

To conclude, the study of stingless bee honey can benefit not only the food industry, but it can also benefit the medical industry. This shows the importance to further study Sarawak stingless bee honey and improve the knowledge on it so that its unique characteristics can be better utilised and commercialised.

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