BIOACTIVITIES AND BOTANICAL ORIGIN DIFFERENTIATION OF MALAYSIAN STINGLESS BEE HONEY PRODUCED BY *Heterotrigona itama* AND *Geniotrigona thoracica*

NG WEN JIE

DOCTOR OF PHILOSOPHY (SCIENCE)

FACULTY OF SCIENCE
UNIVERSITI TUNKU ABDUL RAHMAN
APRIL 2022

BIOACTIVITIES AND BOTANICAL ORIGIN DIFFERENTIATION OF MALAYSIAN STINGLESS BEE HONEY PRODUCED BY *HETEROTRIGONA ITAMA* AND *GENIOTRIGONA THORACICA*

By

NG WEN JIE

A thesis submitted to the Faculty of Science,
Universiti Tunku Abdul Rahman,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Science
April 2022

ABSTRACT

BIOACTIVITIES AND BOTANICAL ORIGIN DIFFERENTIATION OF MALAYSIAN STINGLESS BEE HONEY PRODUCED BY *Heterotrigona itama* AND *Geniotrigona thoracica*

Ng Wen Jie

Stingless bee honey is generally valued for its distinguished flavor and health benefits but scientific studies about the differentiation of stingless bee honey based on honeydew and blossom origins are very limited. Such differentiation is crucial for the authenticity of stingless bee honey. In this study, 23 raw stingless bee honey samples produced by *Heterotrigona itama* and *Geniotrigona thoracica* were analysed. ^{13}C NMR spectroscopy was employed to quantify the seven major sugar tautomers in stingless bee honey samples, the major sugar compositions of honeydew and blossom types were found not to be significantly different. Although physicochemical parameters including moisture content, free acidity, electrical conductivity, ash content, acetic acid, diastase, hydrogen peroxide and mineral elements levels of honeydew honey were found to be significantly higher; total soluble solid, proline and hydroxymethylfurfural were significantly lower than blossom honey. Greater antioxidant capacity in honeydew honey was proven with higher total phenolic compounds, ABTS, DPPH, superoxide radical scavenging activities, peroxy-radical inhibition, iron chelation and ferric reducing power. Principal component analysis (PCA) revealed that differentiation between honeydew and blossom origin of stingless bee honey is possible with certain physicochemical

and antioxidant parameters. Chemometrics are suggested to be useful to determine the authenticity and botanical origin of stingless bee honey. Greater antibacterial effects of honeydew honey were observed in inhibiting *Staphylococcus aureus* and *Escherichia coli*. In addition, higher bactericidal effects were observed on *E. coli* that led to morphological alteration and destruction. The combination of this honey with antibiotics also showed synergistic inhibitory effects on *E. coli* clinical isolates, including antibiotic resistant strains. Lastly, a sugar compound *n*-butyl β-D-glucopyranoside ($C_{10}H_{20}O_6$) was isolated and characterized from butanolic honeydew honey extract. Although this is the first report about the identification of butyl-glucoside in honey samples, the presence of this compound is most probably an artifact from the extraction process.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisors, Assoc. Prof. Dr. Lim Tuck Meng, Assoc, Prof. Dr. Sit Nam Weng and Prof. Dr. Ooi Aun Chuan for their insightful suggestions, motivation and patience during my PhD study. Their immense knowledge, vast experience and technical skills have guided me all the time of my academic research.

I thank profusely all the laboratory staff in the Faculty of Science who have assisted me in this study. Without the facilities and their support, it would not have been possible to complete this research. I am also grateful to Universiti Tunku Abdul Rahman (UTAR) for the opportunity and scholarship to support my study. Next, I would like to dedicate my warmest thanks to my friends, colleagues and students for all the encouragement and moral support throughout the journey.

Last but not the least, my utmost gratitude goes to my parents, my brothers, my sister and my late grandmother, for their tremendous understanding and constant belief in me. Without their unwavering support over the past few years, it would have been impossible for me to complete this journey.

I can't thank you enough.

APPROVAL SHEET

This thesis entitled "**BIOACTIVITIES AND BOTANICAL ORIGIN DIFFERENTIATION OF MALAYSIAN STINGLESS BEE HONEY PRODUCED BY *HETEROTRIGONA ITAMA* AND *GENIOTRIGONA THORACICA*"** was prepared by NG WEN JIE and submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Science at Universiti Tunku Abdul Rahman.

Approved by:

_____
(Assoc. Prof. Dr. Lim Tuck Meng)
Date: 14/4/2022
Supervisor
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman

_____
(Assoc. Prof. Dr. Sit Nam Weng)
Date: 14/4/2022
Co-supervisor
Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: __14/4/2022____

SUBMISSION OF THESIS

It is hereby certified that _____**Ng Wen Jie**____ (ID No: __**1300880**___) has completed this ~~final year project/ dissertation/~~ thesis* entitled "BIOACTIVITIES AND BOTANICAL ORIGIN DIFFERENTIATION OF MALAYSIAN STINGLESS BEE HONEY PRODUCED BY HETEROTRIGONA ITAMA AND GENIOTRIGONA THORACICA" under the supervision of Assoc. Prof. Dr. Lim Tuck Meng (Supervisor) from the Department of Chemical Science, and Assoc. Prof. Dr. Sit Nam Weng (Co-Supervisor) from the Department of Allied Health Sciences, Faculty of Science.

I understand that University will upload softcopy of my ~~final year project / dissertation/~~ thesis* in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(Ng Wen Jie)

*Delete whichever not applicable

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Name Ng Wen Jie

Date 14/4/2022

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS & NOTATIONS

ABS450	Absorbance at 450nm
AU	Absorbance Unit
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
^{13}C NMR	Carbon-13 NMR
COSY	Correlated Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
RM	Malaysian ringgit
mEq/kg	milliequivalents per kilogram
m/z	Mass-to-charge ratio
M	Molarity
N	Normality
Pa	Pascal
^{1}H NMR	Proton NMR
rpm	Revolutions per minute
sp. and spp.	Species and several species
TNF-α, IL-1β, and IL-6	Tumor necrosis factor alpha, Interleukin 1 beta and interleukin 6
v/v	Volume/volume
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

1.1 Honey

As defined by the Codex Alimentarius Commission (2001), “honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects”. In short, the formation of honey originates within the upper aero-digestive tract of bees as a by-product of nectar. It is then concentrated within the bee nest via dehydration and enzymatic processes (Eteraf-Oskouei and Najafi, 2013). This is when the nectar is converted into honey and stored in honeycombs or honey pots, a process known as the ripening of honey (Eyer *et al.*, 2016). The bees will then cap the honeycombs or pots with wax to prevent the hygroscopic honey from absorbing any moisture from the air (Bogdanov *et al.*, 1999).

1.2 Honey Producing Bees

Bees, a type of flying insects, are best known for their involvement in pollination. There are roughly 20 thousand species of bee that are split into seven families, but only one family is involved in honey production, the Apidae (Figure 1.1). Subfamilies of Apinae: Apini, Bombini, Euglossini and Meliponini are known as corbiculate bees that possess corbicula or pollen baskets, on their hind

legs (Figure 1.2). However, only Apini, Bombini and Meliponini show eusocial behavior, living socially in colonies; while Euglossini, commonly known as orchid bees, are mostly solitary (Michener, 2000). These bees feed on nectar and pollen of plants, which are the source of their energy and nutrients. Although Bombini bees, which are known as bumble bees do collect nectar, the stored nectar is not considered as honey because it is not dehydrated. Among the known bee species, only Apini bees, known as honey bees and Meliponini bees, best known as stingless bees, produce honey (Michener, 2000).

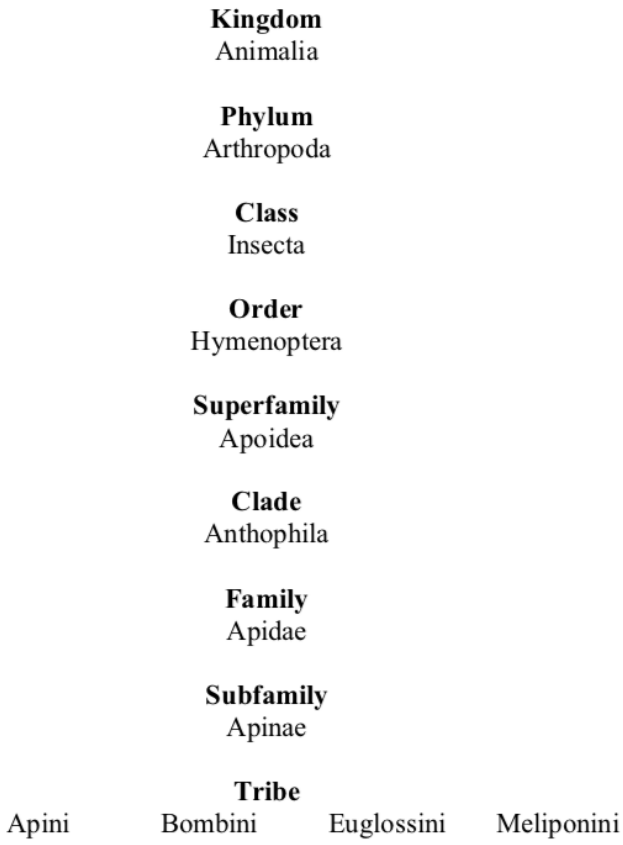


Figure 1.1: Taxonomy of bees.

Figure 1.2: Examples of corbiculate bee species (clockwise from top left): *Apis dorsata*, a member of the honey bee tribe Apini; *Bombus pauloensis*, of the bumble bee tribe Bombini, *Melipona quadrifasciata*, of the stingless bee tribe Meliponini, and *Exaerete smaragdina*, of the orchid bee tribe Euglossini. Only the tribes Apini and Meliponini are involved in honey production.
Copyright: Alemeida E.

Honey bees, the most well-known honey producers in the genus *Apis*, are broken down into three sub-groups: the giant honey bees, the dwarf honey bees and the cavity-nesting honey bees. Honey bees live on every continent except Antarctica (Figure 1.3). The giant bees comprise of *Apis dorsata* and *Apis laboriosa*, which can be found in South and Southeast Asia and the Himalayas, respectively. These bees are large and fiercely defensive, they can be deadly if provoked. *Apis florea* and *Apis andreniformis* are the dwarf honey bees of South and Southeast Asia. Although their tiny stings are barely able to penetrate human skin, they are not domesticated by beekeepers for honey production because the production rate is very low. The commonly found cavity-nesting honey bees include *Apis mellifera*, known as Western or European honey bees and other three Asian honey bees including *Apis cerana*, *Apis koschevnikovi* and *Apis nigrocincta* (Michener, 2000). However, *A. mellifera* has been introduced to the

Asian continent due to its high honey productivity and ease in breeding, making it a globally found honey bee species (Zhang *et al.*, 2019). On the other hand, *A. cerana*, which is known to be a local honey bee species in southern and eastern Asia has its own distinct behavioral traits than other *Apis* spp. such as the ability to adapt to extreme weather conditions and long flying duration (Xu *et al.*, 2009). Other than that, the honey produced is high in quality and potentially better than that from *A. mellifera* (Wang *et al.*, 2012).

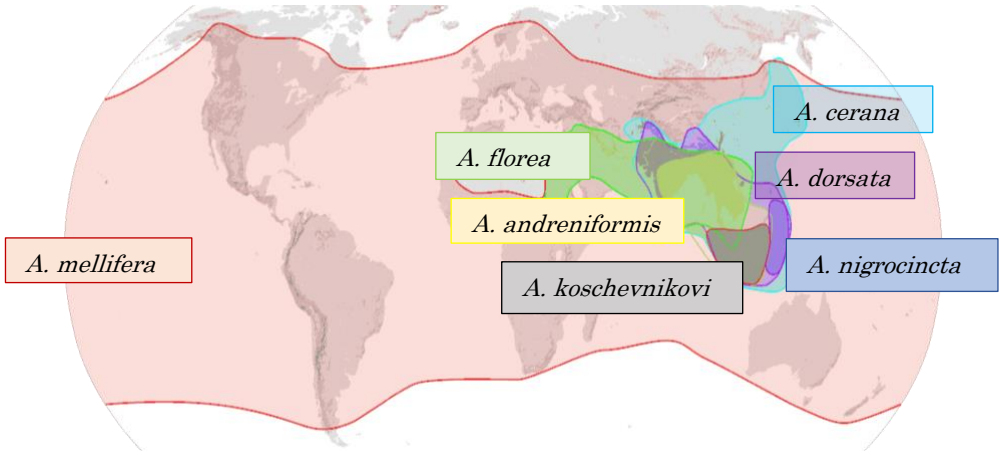


Figure 1.3: The distribution of honey bee in the world.

Most of the stingless bees are smaller as compared to the size of bumble bees and honey bees (Streinzer *et al.*, 2016). Despite the name, the parts of the sting of stingless bees are actually present, but much atrophied and not functional to sting. Stingless bees are found in the tropical and subtropical regions of Africa, America, Australia and Asia (Figure 1.4). More than 500 species from 32 existent genera of stingless bees have been identified and possibly 100 more species yet undescribed (Michener, 2000; Michener, 2013). As summarized by Rattanawannee and Duangphakdee (2019), the highest diversity of stingless bee species is found in the Neotropical region with about 391 species; whereas 50, 10 and 60 species have been reported in Africa, Australia and Asia, respectively.

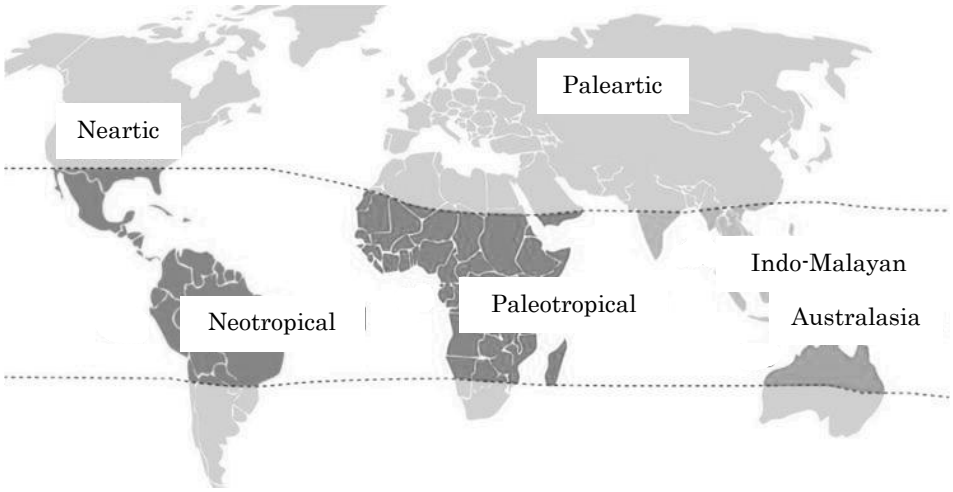


Figure 1.4: The distribution of stingless bees in the Tropical and Subtropical regions of the world.

1.3 Honey Production

Generally, only two types of bee namely honey bee and stingless bee are involved in honey production. Honey bee nests are commonly found in tree holes and on rock crevices. On the other hand, stingless bees build their nests in hollow branches or tree trunks, cavities under the ground and abandoned ant or termite nests (Roubik, 2006). The entrance to the nest is usually a small tubular structure extending towards the open air. Scientific literature distinguishes nests from hives. Nest refers to exposed colonies which house bees in natural or artificial cavities whereas hive is man-made or constructed artificially to house a bee nest (Roubik, 2006). Bees are kept in hives for honey production.

The production of honey begins with the collection of nectar, a sugary liquid from plants. Honey bees suck out nectar with their long, tube-shaped proboscis and store it in a specific nectar stomach instead of the usual stomach for food (Michener, 2000). The nectars are subsequently passed to the mouth of

other bees through regurgitation followed by deposition of partially digested nectar into hexagonal-shaped honeycombs (Figure 1.5). Then, the nectar is fanned by the wings of honey bees to promote the evaporation process. The dehydrated nectar thus becomes honey. Honey bees then seal the honeycomb by using wax secreted from their abdomens (Michener, 2000).

Figure 1.5: Honey bees and the hexagonal-shape of honeycombs.
Free of copyright.

Similar to honey bees, stingless bees collect nectar for honey production, but they store the honey in honey pots instead of hexagonal honeycombs (Figure 1.6). The honey pots are made of cerumen, which is a mixture that is similar to propolis but with the addition of the mandibular secretion of the stingless bee during its construction (Santos *et al.*, 2009; Simone-Finstrom and Spivak, 2010). The propolis produced by honey bees is a mixture of beeswax and resins collected from plant parts. The functions of cerumen and propolis are slightly different. While the cerumen is used as a honey storage pot and to ensure sterility in the hive, propolis on the other hand is used as an internal layer and to seal the extra space surrounding the hexagonal-shaped honeycombs (Abd Jalil *et al.*, 2017).

In the cerumen honey pots, the nectar has to go through three

transformation processes before turning into honey. It starts with a physical transformation, when water is evaporated from the nectar. Unlike honey bees, stingless bees do not fan their nectar, thus making their honey less viscous with higher moisture content (Suntiparapop *et al.*, 2012). This is followed by a biological transformation due to a fermentation process by symbiont microorganisms (Menezes *et al.*, 2013). Lastly, a chemical transformation happens when the enzymes secreted by worker bees hydrolyse sucrose into fructose and glucose in the nectar (Menezes *et al.*, 2013). Due to the storage of honey in the cerumen pots, health benefits of the stingless bee honey are claimed due to the infiltration of phytochemicals from the cerumen (Abd Jalil *et al.*, 2017).

Figure 1.6: Stingless bees with the honey pots.
Free of copyright.

1.4 Health Benefits of Honey

As a natural food product, honey is the only sweetener that can be consumed by humans without processing (Kazalaki *et al.*, 2015). Besides its nutritional value, honey has been known to possess therapeutic properties due to the presence of bioactive compounds (Manyi-Loh *et al.*, 2011). The biologically

active compounds in honey are generally divided into antioxidant and antibacterial categories that contribute to the health-promoting properties of honey (Aggad and Guemour, 2014; Wieczorek *et al.*, 2014, Dzugan *et al.*, 2018).

Other than polyphenol compounds, which are mainly responsible for the antioxidant capacity of honey; organic acids, vitamins, enzymes, amino acids and trace elements are also involved in the antioxidant property (Flanjak *et al.*, 2016). Besides this, honey exhibits strong antibacterial activity. The bacteriostatic and bactericidal properties of honey were found to be effective against several human pathogens, including Gram-positive *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Boorn *et al.*, 2010). High sugar content or hyperosmolarity, low water activity, acidity, the presence of hydrogen peroxide, polyphenol compounds and other phytochemicals are proposed to be the basis of the unique antibacterial factors of honey (Kwakman and Zaat, 2012). However, bioactivities of honey are generally found to be different significantly in different honey types (Manyi-Loh *et al.*, 2011).

1.5 Types of Honey

There are two types of honey available on the market, namely raw honey and processed honey. Raw honey refers to pure honey that has been strained to remove impurities such as coarse debris, beeswax and dead bees. Without any heat treatment, the nutritional values of raw honey are greatly preserved

(Subramanian *et al.*, 2007). Processed honey refers to any raw honey that has undergone several more steps before it is bottled, such as pasteurization and filtration. Pasteurization with temperatures as high as 70 °C is commonly used to inactivate most of the microorganisms present in honey. Such heat treatment is also useful to reduce the water content in immature or non-ripened honey. Heating kills off the microorganisms as well as reducing the moisture level to prevent any unwanted microbial fermentation processes and to prolong the shelf-life of honey (Subramanian *et al.*, 2007). Filtration or ultrafiltration further removes small debris particles, pollens and air bubbles so that the honey looks more transparent, smooth and aesthetically appealing to consumers (Subramanian *et al.*, 2007). Other than that, some processed honey on the market might be adulterated. Adulteration or faking of honey can be achieved by either direct or indirect ways, by addition of sugar syrup into honey or feeding the bees with sugar solution, respectively. Excessive processing and adulteration can compromise the nutritional values and quality of honey significantly (Subramanian *et al.*, 2007). For example, a study conducted by Blasa *et al.* (2006) found that raw honey contained up to 4.3 times more antioxidants than processed honey.

Other than the classification of honey type based on honey bee and stingless bee, honey also can be classified based on botanical sources, which are blossom honey and honeydew honey. Blossom honey, also known as floral honey, is the most common type of honey worldwide. Blossom honey is produced from the nectar of flowering plants collected by bees; whereas honeydew honey originates from the secretions of living parts of plants or

excretions of plant-sucking insects on plants (Pita-Calvo and Vázquez, 2017). Hence, the composition of honey is tightly associated to its botanical origin.

Different types of honey vary primarily by biological activities, as well as by their physical properties and chemical composition. Other than seasonal and environmental factors, the composition of honey depends primarily on its floral source (Manyi-Loh *et al.*, 2011). Therefore, different varieties of honey exhibit different health promoting properties (da Silva *et al.*, 2016). The high market value of honey for its therapeutic effect has always attracted honey fraudulence issues such as its substitution with low-valued honey, adulteration with sugars or mislabeling of its botanical source and entomological origin to gain a higher selling price. For this reason, it is very important to accurately determine the authenticity of different honey varieties (Moore *et al.*, 2012).

1.6 Authenticity of Honey

Although the melissopalynological method is a useful technique to authenticate honey samples based on the microscopic quantitative identification of pollen of plants present in different honey varieties, there are several drawbacks as it is time-consuming, requiring expertise and the availability of a comprehensive collection of pollen grains (Dzugan *et al.*, 2018). To overcome this issue, and to save time and money, physicochemical analyses have been proposed for honey authentication studies including determination of its botanical, geographical and entomological origins and detection of adulterated substances (Perna *et al.*, 2012; Flanjak *et al.*, 2016; Oroian *et al.*, 2015; Kek *et*

al., 2017b). Due to the complexity of honey composition, several physicochemical parameters are measured to evaluate the authenticity and quality of honey. The Codex Alimentarius Commission (2001) has published an international standard guideline for the physicochemical properties of honey bee honey.

The total antioxidant capacity of honey also has to be evaluated with a combination of methods based on different reaction mechanisms and experimental conditions. Considering many different parameters that are responsible for the physicochemical properties and antioxidant capacity, characterization of different honey types without losing some important factors is possible only from a combination of physicochemical analyses, antioxidant assays and chemometric evaluations of results (Duarte *et al.*, 2012; Moniruzzaman *et al.*, 2012; Perna *et al.*, 2012; da Silva *et al.*, 2013; Flanjak *et al.*, 2016; Kek *et al.*, 2017a; 2017b; Dzugan *et al.*, 2018). Although the quality parameters for honey have been defined by the Codex Alimentarius Commission (2001) and the Council Directive of the European Union (2002), they are not applicable for honey originating from tropical and subtropical regions, including Malaysia (Bergamo *et al.*, 2019).

1.7 The Honey Industry in Malaysia

There are approximately 100 species of bees in Malaysia, including *A. mellifera* (Western honey bee), *A. cerana* (local honey bee*), A. dorsata* (forest bee) and the meliponines (stingless bees) (Ismail, 2016). Beekeeping is

important to the agricultural sector, providing extra revenue to beekeepers and indirectly generating food for the population via pollination services. According to a record from the Ministry of Science Technology and Innovation (MOSTI), there are around 750 to 1,000 beekeepers in Malaysia, generating an estimated 30 metric tons honey per year (Saludin *et al.*, 2019).

Most of the Malaysian honey is produced in Sarawak, Sabah, Johor and Melaka (Saludin *et al.*, 2019). Both *A. mellifera* and *A. cerana* are the dominant domesticated honey bee species in apiculture for the production of honey (Figure 1.7 and Figure 1.8). Stingless bees, which are locally known as *Kelulut*, are domesticated for honey production as well. In Malaysia, stingless bee beekeeping or meliponiculture has been slowly rising since 2007 (Mustafa *et al.*, 2018). In 2012, the stingless bee honey industry was transformed into a sustainable source of income for local beekeepers via the production of good quality honey, together with bee conservative activities to ensure the sustainability of stingless bees for pollination (Mustafa *et al.*, 2018). Unlike honey bees, which are more vulnerable to disease, stingless bees do not migrate and abandon their hive (Abd Jalil *et al.*, 2017). According to research by the Malaysian Agricultural Research and Development Institute (MARDI), stingless bees are able to pollinate and collect nectar from small-sized flowers, due to their smaller size, which cannot be reached by the relatively larger honey bees (Abd Jalil *et al.*, 2017). Furthermore, stingless bees are not fussy about building a colony hive. Hence, it is easier for beekeepers to build an artificial hive to cultivate and manipulate the stingless bee colonies for the production of honey (Abd Jalil *et al.*, 2017).

There are at least 32 stingless bee species identified in Malaysia, and at least four of them, *Geniotrigona thoracica*, *Heterotrigona itama*, *Lepidotrigona terminata* and *Tetragonula leviceps* can be potentially domesticated for pollination in the agricultural sector (Mohd Norowi *et al.*, 2010). However, the most abundant species found in meliponiculture are *G. thoracica* and *H. itama* because they produce higher volumes of honey compared to the other stingless bee species (Figure 1.9 and Figure 1.10) (Kelly *et al.*, 2014; Saludin *et al.*, 2019).

Figure 1.7: Western honey bee, *Apis mellifera*.
Adopted from https://www.padil.gov.au

Figure 1.8: Eastern honey bee, *Apis cerana*.

Figure 1.9: Stingless bee, *Geniotrigona throracica*.
Copyright: Chui S.X.

Figure 1.10: Stingless bee, *Heterotrigona itama*.
Copyright: Chui S.X.

Ever since stingless bee beekeeping was launched by MARDI in 2004, the total number of beekeepers has increased tremendously. The trend appears to be towards high honey production and increased honey demand and consumption (Ismail and Ismail, 2018). However, Malaysia still imports honey from abroad to meet the local market demands. Although imported honeys from China and Iran are much cheaper than the local honey, Malaysians still prefer the local honey. This is reflected in the premium prices of the local honey sold in Malaysia at more than RM120 per kg (Ismail and Ismail, 2018; Abdul Hamid,

2019). To date, stingless bee honey contributes RM200 million annually to the economy in Malaysia, with Sarawak ranking the highest in production, followed by Sabah and Peninsular Malaysia (Abdul Hamid, 2019).

According to data provided by the Agriculture Department in 2017, the total stingless bee honey production in Malaysia was 134,244 kg which generated a total of RM19.3 million. However, total production was still small compared to the market demand of 802,962 kg. Despite the challenges to penetrate overseas markets, in 2018, Malaysia had managed to export 100 kg of stingless bee honey to Japan, 300 kg to Singapore and 500 kg to India, Taiwan and China respectively (Jmaludin, 2020). The market potential for stingless bee honey is RM67.2 million but the current total market volume of stingless bee honey industry stands at only RM33.6 million. Hence, the local stingless bee honey industry still has the potential to reap more revenue if the business is further developed (Jmaludin, 2020).

However, according to Lim and Baharun (2008), compared to other major honey producers in Southeast Asia, for example Thailand and Vietnam, honey production in Malaysia is still very low and underdeveloped. Generally, local apiaries are running on a small scale, scattered in suburban and rural areas throughout the West Coast of Malaysia. The low production of local honey which cannot meet the increasing market demand has led to continual imports of low-grade honey from China, Australia and the United States. Furthermore, the retail price for local honey is much higher than imported honey due to the high demand and short supply of the local honey.

Other than insufficient supply of local honey to meet the market demand, low stingless bee honey production due to lower honey yield generated by stingless bees is also an issue in the beekeeping sector in Malaysia (Ismail, 2016). Besides massive deforestation that destroys the natural habitat of bees, the weather is probably another crucial factor that affects bee productivity (Lim and Baharun, 2008). Honey production and rainfall are strongly correlated. Heavy rains drain away nectars, hence the bees are unable to secure sufficient food to produce honey. Moreover, high humidity weather makes nectar difficult to ripen which would promote the growth of yeasts causing a serious reduction in hive population; while yeast fermentation also can affect the quality of honey significantly (Lim and Baharun, 2008). Inconsistent quality of local honey is also a major concern since there were no guidelines to preserve honey at the highest quality without fermentation and no quality standard enforcement to evaluate the quality of honey. Due to inconsistency in quality, the price of honey, especially stingless bee honey sold in the local markets, is also not standardized and may not reflect the quality of the honey sold (Lim and Baharun, 2008).

Although the International Honey Commission (IHC) has set quality guidelines for honey (Codex Alimentarius Commission, 2001), the guidelines are not implemented and enforced in Malaysia. This is because Malaysian honey is influenced by the tropical climate, which makes it different from other honeys originated from Europe (Moniruzzaman *et al.*, 2013b). Besides, the guidelines were set for honey produced by honey bees, not for stingless bee honey. This is due to stingless bees being limited to tropical countries such as in Malaysia. According to Moniruzzaman *et al.* (2013b) and certain physicochemical

properties, for example moisture content, sugar level and acidity, obtained from Malaysian honey bee and stingless bee honeys are different compared to the parameters set by the IHC. Hence, new quality guidelines and standards were needed for honey bee and stingless bee honeys originating from tropical regions. A specification standard for stingless bee honey was published by the Department of Standards Malaysia (2017) to control the quality of local honey. However, the standard is rather too generalized to differentiate between blossom honey and honeydew honey produced by stingless bees.

1.8 Problem Statement

In Malaysia, honey is harvested in two different ways; apiculture using honey bees and meliponiculture using stingless bees (Kek *et al.*, 2017a). Although the honey production yield of stingless bees is generally lower, this honey type fetches higher market prices than that from honey bees. The high market demand for stingless bee honey is in part due to its higher medicinal features (Vit *et al.*, 2013). With a growing popularity for stingless bee honey, more studies on it are needed to find suitable properties that can serve as parameters for determining honey origin in an effort to protect consumers against honey fraud. In addition, data of stingless bee honey properties can contribute to build up its international quality standardization as some physicochemical properties of stingless bee honey such as moisture content, sugar content, electrical conductivity, free acidity and enzyme activity are different compared to honey produced by *Apis mellifera* honey bees that are currently regulated in the Codex Standard for Honey (Codex Alimentarius

Commission, 2001; Vit *et al.*, 2013). Hence, compared with honey bee honey, systematic and in-depth studies on stingless bee honey are still rather limited.

As mentioned earlier, other than floral nectar, bees also harvest honeydew honey from plant secretions. Honeydew honey is highly valued by consumers because it is considered to possess more health benefits than floral or blossom honey (Gonzalez-Paramas *et al.*, 2007). Several studies have also demonstrated the antibacterial and antioxidant activities of honeydew honey are superior to those of blossom honey (Seraglio *et al.*, 2019). Despite a specification standard for stingless bee honey regulated by the Department of Standards Malaysia (2017) that serves to monitor the quality of locally produced stingless bee honey, the standard only focuses on blossom honey and not honeydew honey (Ng *et al.*, 2021).

Hence, honeydew honey produced by stingless bees is of particular interest. To our best knowledge, detailed investigations on honeydew honey produced by stingless bees is still very limited (Ng *et al.*, 2020; Ng *et al.*, 2021). Hence, the characterization of Malaysian honey, especially honeydew honey produced by stingless bees, based on physicochemical properties and bioactivities in this study will make it possible to evaluate its quality for local and international markets.

1.9 Objectives

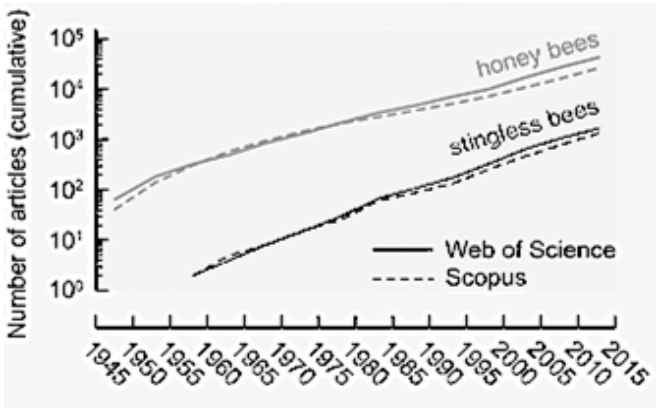
- To quantify the major sugar molecules in honey.
- To characterize the structure of sugar molecules isolated from honey extract.
- To determine the physicochemical properties of honey.
- To evaluate the antioxidant capacities of honey.
- To assess the antibacterial properties of honey against selected human pathogenic bacteria.
- To assess the physicochemical and antioxidant differences between honeydew and blossom honey via chemometric methods.

CHAPTER 2

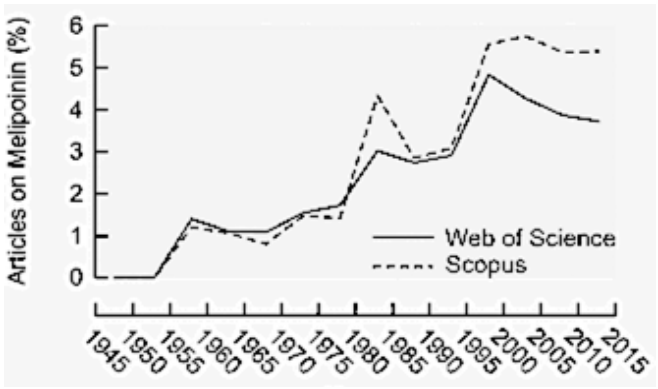
LITERATURE REVIEW

2.1 Scientific Research on Honey

Notwithstanding their ecological importance, as the primary pollinators in most pantropical ecosystems, scientific information about the stingless bee and related products is still rather moderate as compared to that about honey bee. Scientific investigations on stingless bees started only during the 19th century, at a time when scientific research on the honey bee had already been conducted for almost 200 years (Quintal and Roubik, 2013). The delayed and low interest in stingless bees could be due to the fact that these bee colonies making far less honey and therefore have less economic appeal and lower economic value, compared to honey bee (Roubik, 2006). Besides, it was more difficult to access to their natural habitats and it was not possible to maintain their colonies outside of the tropics, and most of the scientific studies were carried out by the Europeans (Hrncir *et al*., 2016). Most importantly, the vast and broad knowledge of the Apini bees, particularly the Western or European honey bee, *Apis mellifera* has limited or halted the investigation of another types of eusocial bee (Figure 2.1).



a



b

a Cumulative number of publications on honey bee and stingless bee.
b Proportion of articles on stingless bee relative to the total of publications on highly eusocial bee.

Figure 2.1: Article publications on honey bees and stingless bees registered in the ISI Web of Science and Scopus Citation Databases (Hrncir *et al*., 2016).

Hence, due to the high market demand and commercial value of stingless bee honey, adequate legislation is definitely needed to ensure its authenticity based on measurements of the physicochemical parameters, as has been achieved for honey produced by *A. mellifera* (Ruoff and Bogdanov, 2004). Furthermore, significant differences have been found in the physicochemical characteristics of honey produced by different stingless bee species, which is one of the obstacles to establish quality standards consistent with the entomological origin of honey in different regions (Bogdanov *et al*., 1996; Vit *et al*., 2009). Although there is a specification standard for Malaysian stingless bee honey (Department of Standards Malaysia, 2017), the standard is not specific enough to differentiate between blossom honey and honeydew honey that are produced

by stingless bees. At present, unlike the Codex Alimentarius Commission (2001), there are no studies that provide physicochemical descriptions of honeydew and blossom honeys produced by stingless bees.

2.2 Physicochemical Properties of Honey

Other than seasonal and climatic factors, the quality of honey is suggested to be greatly influenced by bee species and botanical sources (El Sohaimy *et al*., 2015; Azonwade *et al*., 2018). Moreover, certain beekeepers would treat immature or non-ripened honey with various processing methods in order to fulfill market demand. Extensive processing is also known to alter the quality of honey (Subramanian *et al*., 2007). Generally, the quality of honey cannot be verified by its appearance. Hence, in order to differentiate a raw and genuine honey from any fake, adulterated or processed honey, the quality of honey must be determined with its physicochemical properties (Azonwade *et al*., 2018).

Depending on bee species, the physicochemical properties of honey can be evaluated by referring to the standards established by the Codex Alimentarius Commission (2001) and the Department of Standards Malaysia (2017). The Codex Alimentarius Commission (2001) published a standard guideline for physicochemical properties of honey bee honey, specifically those produced by the western honey bee *A. mellifera*. However, the physicochemical properties of honey produced by honey bee are greatly different to those of honey originating from stingless bees. Due to high market demand and commercial value of

stingless bee honey, and the blooming of the stingless bee industry or meliponiculture in Malaysia, a standard guideline for the physicochemical parameters of stingless bee honey was released by the Department of Standards Malaysia (2017). This guideline is believed to ensure the quality of local stingless bee honey in market.

According to the standards stated earlier, physicochemical parameters including moisture content, reducing sugar content, electrical conductivity, ash content, diastase, pH, free acidity and hydroxymethylfurfural (HMF) are proposed as markers for the determination of honey quality. Apart from these parameters, color intensity, water activity, total sugar content, proline content, hydrogen peroxide, organic acid and mineral content are also known to be associated with the quality of honey (Azonwade *et al*., 2018).

2.2.1 Color

Color is the first attractive attribute of honey, and it is very important for commercialization. It is an important parameter in the judgement of quality, acceptance and preference of consumers (da Silva *et al*., 2016). The range of color intensity in honey can be very different from colorless, pale yellow, golden, amber, dark-brown and even close to black (Codex Alimentarius Commission, 2001). Light colored honeys generally have higher market value, although dark honeys are also appreciated in certain regions (Subramanian *et al*., 2007).

Although the color of honey is influenced by the pollens, moisture

content, amount of sugars and minerals present in honey, the major components that are involved in the color of honey are phenolic compounds (Terrab *et al*., 2003a; Terrab *et al*., 2003b; Baltrusaityte *et al*., 2007; do Nascimento *et al*., 2018). The presence of phenolic compounds in honey is highly associated with the botanical origin of honey (Terrab *et al*., 2003a; Terrab *et al*., 2003b; Baltrusaityte *et al*., 2007). Hence, color intensity of honey can be used as a reliable indicator for the presence of phenolic pigments that also possess antioxidant properties (Moniruzzaman *et al*., 2013b). Several studies have shown that honeys with higher color intensity have higher phenolic contents, thus higher antioxidant capacity is estimated in a darker colored honey (Ferreira *et al*., 2009; Saxena *et al*., 2010; Perna *et al*., 2012; Khalil *et al*. 2012). Additionally, the color intensity of honey is usually used to estimate the overall content of phytochemicals, which also contribute to the antioxidant capacity of honey. Still, color intensity of honey also could be influenced by different processing methods and storage conditions (Ahmed *et al*., 2016).

The color intensity of honey can be indicated by ABS450 which is a parameter to approximate the contribution of phytochemical pigments that are normally linked to antioxidant properties. Hence, a higher ABS450 value is found to be positively correlated with greater antioxidant capacity present in darker colored honey (Moniruzzaman *et al*., 2013a; Moniruzzaman *et al*. 2013b).

2.2.2 Acidity and Organic Acids

Ordinary honey that is produced by honey bees is acidic in nature, with a pH value falling between 3.0 to 4.5 (Geiling, 2013). However, depending on its botanical origin, the pH value of some honey can go up to 6.5 (Machado De-Melo *et al*., 2018). An acidic environment is not an ideal growth condition for microorganisms, as the optimum pH for most microorganisms is between 7.2 and 7.4, thus the acidity of honey is crucial to inhibit the growth of pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp. and *Streptococcus pyogenes* (Mandal and Mandal, 2011). Stingless bee honey generally has higher acidity than that of honey bee honey due to higher organic acid content (Nordin *et al*., 2018). According to the Department of Standards Malaysia (2017), the pH of stingless bee honey should be between 2.5 and 3.8.

The acidity of honey is mainly due to the presence of gluconic acid. This organic acid is a product generated in the enzymatic action of glucose oxidase on glucose. Other than gluconic acid, there are other non-aromatic organic acids present in honey, which include maleic, fumaric, citric, formic, acetic and lactic acids (Mato *et al*., 2006). According to Shamsudin *et al*. (2019), only four organic acids include gluconic, lactic, acetic and citric acids have been detected in all stingless bee honey samples investigated. Gluconic acid was detected as the main organic acid in all stingless bee honey samples; however, honey produced by *Heterotrigona itama* showed higher levels of gluconic acid than those from *Geniotrigona thoracica*. Besides, stingless bee honeys originating from different floral sources also showed significantly different levels of

gluconic acid. Differences in the gluconic acid levels were suggested to be due to different amount of glucose and glucose oxidase enzymatic activities in the honey samples. Glucose oxidase is believed to be produced by *Gluconobacter* spp., one of the major gut microflora of the bees (Mato *et al*., 2006). Apart from being the intermediates of biological oxidation in bees, some organic acids also originate from plants (Machado De-Melo *et al*., 2018). Thus, organic acid levels can be a useful parameter to identify both the bee and botanical origins of honey.

Although different origins and climates influence the acidity in different honey types, honey adulterated with sugar syrup was found to have higher pH values than authentic honey. Furthermore, higher acidity can be related to the deterioration of honey due to the formation of more organic acids such as lactic acid by microbial fermentation (Bogdanov *et al*., 1999). Microbial fermentation normally happens in immature or non-ripened honey because of the higher moisture content. Hence, acidity is an important indicator for the authenticity and quality of a honey (de Rodríguez *et al*., 2004, da Silva *et al*., 2016).

In terms of measurement, pH expresses the amount of free hydrogen ion concentration (H^+) in a solution. Hence, if the concentration of free hydrogen ions in a solution is high, it will be reflected in a lower pH value (Gump, 2014). On the other hand, titratable acidity measures the amounts of organic acids in equilibrium with lactones, esters and inorganic ions such as chloride, phosphate and sulfate in honey (Coulter *et al*., 2004). Hence, it can be said that titratable acidity is a measurement of the total amount of hydrogen ions, including both free hydrogen ions and hydrogen ions that are bound to weak acids. Although

solutions with lower pH are seen with higher titratable acidity, pH is not directly correlated with the concentration of acids present. There is no direct relationship between pH and titratable acidity, but pH is influenced by the ability of acids to dissociate (Coulter *et al*., 2004). Titratable acidity has been proposed as a more accurate representation of perceived acidity because both free and bound hydrogen ions can be tasted palatably whereas the pH relates more to microbial stability and susceptibility to microbial spoilage (Taylor, 2015). According to the Codex Alimentarius Commission (2001), the acidity of honey bee honey should not be more than 50 mEq/kg. However, the quality requirements set by the Department of Standards Malaysia (2017) use pH measurement, whereby the pH value of raw stingless bee honey should be between 2.5 to 3.8.

2.2.3 Sugar Content

Sugar makes up to 95 % of the total dry weight of honey (Bogdanov *et al*., 2002). Other than sweetness, the viscosity and hygroscopicity of honey are also highly associated with its sugar content (Kamal and Klein, 2011). Stingless bee honey generally has a lower sugar content than honey bee honey (Moniruzzaman *et al*., 2013a; Moniruzzaman *et al*. 2013b; Nweze *et al*., 2017; Shamsudin *et al*., 2019) and such variation was suggested to be caused by the higher moisture content in stingless bee honey (Kek *et al*., 2017b; Nweze *et al*.; 2017). High sugar level in honey correlates to high osmotic pressure and less free water molecules, limiting microbial growth (Machado De-Melo *et al*., 2018).

The amount of sugar in honey is highly related to the nectar source and degree of maturity or ripeness (Belay *et al*., 2013). Out of over 25 types of sugar, the monosaccharides, fructose and glucose are the main sugars found in honey. These reducing sugars constitute 85 to 95 % of the total sugar content (Ajibola, 2015). Ideally, the reducing sugar content in honey bee honey must be more than 60% (Codex Alimentarius Commission, 2001); whereas the reducing sugar content in stingless bee honey must not exceed 85 % (Department of Standards Malaysia, 2017). Crystallization in honey is determined by the fructose to glucose ratio. Due to its high solubility, fructose is able to slow down the rate of crystallization; whereas glucose speeds up the rate of crystallization due to its lower solubility (Gleiter *et al*., 2006).

There are also disaccharides in honey, mainly sucrose. However, the sucrose level remains low in honey due to the enzyme invertase secreted by bees, which converts sucrose to glucose and fructose (Bogdanov *et al*., 2002). High levels of sucrose in honey indicate early harvest as the conversion of sucrose into reducing sugars by the enzymatic action of invertase is incomplete (Belay *et al*., 2013). Hence, a matured raw honey should consist of less than 5% sucrose (Bogdanov *et al*., 1996). Other than honey immaturity, honey that has been adulterated with sugar syrup and honey produced by bees that have been fed with sugar solution have been found to have high sucrose levels as well (Adebiyi *et al*., 2004; Tornuk *et al*., 2013; Machado De-Melo *et al*., 2018). More recently, adulteration of honey has been monitored by $^{13}C/^{12}C$ analysis using an isotope ratio mass spectrometer coupled with an elemental analyser (EA-IRMS). Although this technique was useful to detect honey adulteration, especially due

to the addition of corn-sugar cane syrups, it failed to detect the addition of beet sugar syrups (Tosun, 2013).

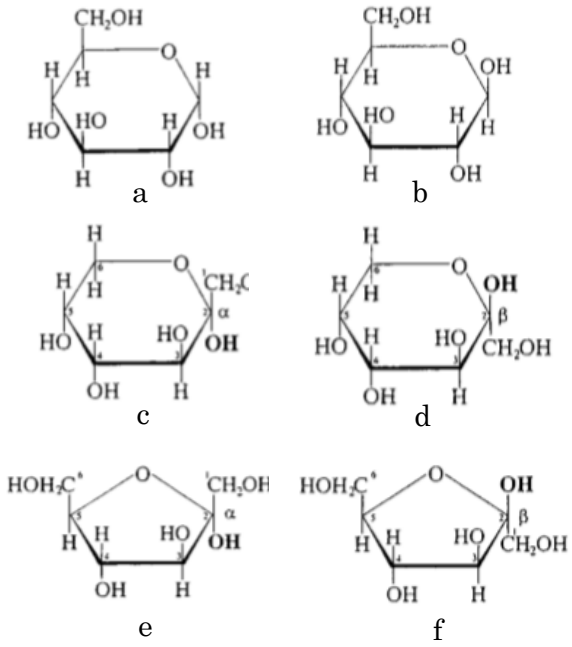
The food industry uses the measurement of °Brix as a reference value to measure any sweet solids in a product including honey (Toledo, 2014). The sugar concentration can be measured with either a densometer or refractometer, since both instruments are capable of converting the measured experimental data automatically into °Brix. Both methods are operator independent and are able to generate rapid and highly reproducible results (Toledo, 2014). However, the °Brix measurement is influenced by temperature; therefore, it is recommended to take measurements at a specified temperature. Newer versions of digital density meters and refractometers have built-in temperature compensations for °Brix measurements (Toledo, 2014). Since the majority of sugars in honey are fructose and glucose instead of sucrose, the reading is referred to as apparent °Brix (Toledo, 2014). Several studies including by Khalil *et al*. (2012), Moniruzzaman *et al*. (2013b) and Nweze *et al*. (2017) adopted the 3,5-dinitrosalicyclic acid (DNSA) method to measure the reducing sugar content in honey. DNSA detects the presence of the free carbonyl group (C=O) of reducing sugars. This involves the oxidation of aldehyde and ketone functional groups which are present in glucose and fructose, respectively. Althoug the DNSA method was found to be sensitive to pH variation, this method was validated by Garriga *et al.* (2017) to be selective, linear, accurate and robust.

According to Angyal (1984), reducing sugars have one property which is different from other organic compounds. When a pure organic compound is

dissolved in a solvent, the solution will usually contain only one compound. However, when a reducing sugar is dissolved in water, it undergoes mutarotation. Mutarotation occurs when the hemiketal ring opens and closes, sometimes resulting in different α-/β-configuration ratios to that of the original molecule (Angyal, 1984; Kazalaki *et al*., 2015). As a result, the obtained reducing sugar solution will always have several components which include pyranoses, furanoses and the acyclic (open-chain) carbonyl form and its hydrate (Angyal, 1984; Kazalaki *et al*., 2015). These various components are often referred to as tautomers or tautomeric forms of the sugar. Each of these tautomers is a distinct compound, with different chemical, physical and biological properties (Angyal, 1984). However, the relative composition of tautomers varies widely amongst different reducing sugars and for the same sugar dissolved in different solvents (Angyal, 1984; Angyal, 1991).

One of the most common reducing sugars, D-glucose exists as a mixture of six tautomers in solution naturally, i.e., the two α- and β-anomers in the pyranose form (α-D-glucopyranose and β-D-glucopyranose), very small quantities of α- and β-anomers in the furanose form (α-D-glucofuranose and β-D-glucofuranose), the corresponding aldehyde and the hydrated aldehyde (Mazzoni *et al*., 1997). On the other hand, D-fructose exists in five tautomeric forms in solution, i.e., the major two α- and β-anomers in the pyranose form (α-D-fructopyranose and β-D-fructopyranose), the α- and β-anomers in the furanose form (α-D-fructofuranose and β-D-fructofuranose) and lastly the open-chain form or acyclic form (keto-D-fructose) (Mazzoni *et al*., 1997). Although very few of these tautomeric forms of sugars have been isolated, the presence of

tautomers in a solution can still be detected by nuclear magnetic resonance (NMR) spectroscopy, and their percentage in an equilibrium mixture can be estimated (Angyal, 1984; Angyal, 1991; Angyal, 1994). In aqueous solutions, D-glucose and D-fructose exist in different tautomeric forms as shown in Figure 2.2. Table 2.1 contains the equilibrium composition of the tautomeric forms of D-glucose and D-fructose in aqueous solutions as determined by ^{13}C NMR spectroscopy (Fuchs and Kaatze, 2001; Kazalaki *et al*., 2015).



a. α-D-glucopyranose b. β-D-glucopyranose
c. α-D-fructopyranose d. β-D-fructopyranose
e. α-D-fructofuranose f. β-D-fructofuranose

Figure 2.2: Different tautomers of D-glucose and D-fructose in aqueous solution.

Table 2.1: Equilibrium composition of the tautomeric forms of D-glucose and D-fructose in aqueous solution (Fuchs and Kaatze, 2001; Kazalaki *et al*., 2015).

Tautomer	Weight %
α-D-glucopyranose	37.5-38
β-D-glucopyranose	62-62.5
α-D-fructopyranose	2.0
β-D-fructopyranose	67.0-70.0
α-D-fructofuranose	5.0-6.0
β-D-fructofuranose	23.0-25.0

For the studies of carbohydrates, several techniques have commonly been employed, especially gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) (Mazzoni *et al*., 1997). However, these analytical procedures have disadvantages and can be time consuming. For example, the poor volatility of carbohydrates requires chemical pre-treatment of the samples (e.g., acetylation, methylation, oximation and trimethylsilylation) before injection for GC analysis (Tisza *et al*., 1996; Veness and Evans, 1996); while HPLC, which allows the analysis of underivatized or thermolabile carbohydrates, also requires sample preparation such as centrifugation, filtration, precipitation and extraction (Herbreteau *et al*., 1992; Coquet *et al*., 1994). On the other hand, NMR spectroscopy has been widely used for molecular dynamics and structural studies. Although this technique is commonly used for the identification and quantitation of compounds, there are not many scientific papers that describe the use of NMR for the analysis of natural mixtures of carbohydrates, especially in honey (Mazzoni *et al*., 1997; Kazalaki *et al*., 2015; Gerginova *et al*., 2020).

Only a few reports have been published on the application of NMR spectroscopy to the analysis of carbohydrates in honey. A computer-aided analysis of ^{13}C NMR spectra was described by Mazzoni *et al*. (1997) which allowed the identification of individual carbohydrates in authentic honeys of different botanical origins harvested in France. Other than the identification and quantification of fructose, glucose, turanose, maltulose, maltose, isomaltose, nigerose, isomaltotriose, melezitose and erlose, the tautomeric forms of some sugars of honey were also identified in the ^{13}C NMR spectrum. Another study demonstrated the potential of ^{13}C NMR spectroscopy to detect and quantify a large number of simple and complex carbohydrate molecules in Greek honey samples (Kazalaki *et al*., 2015). In the study, a total of 28 sugar molecules including glucose, fructose, maltose, isomaltose, nigerose, sucrose, turanose, maltulose, erlose, maltotriose, isomaltotriose, panose, melizitose, kestose and their tautomeric forms were quantified. Differences in sugar composition and concentration in honey samples were also suggested as useful indices of different botanical origins. The outcomes of a study conducted by Gerginova *et al*. (2020) from chemometric analyses and semiquantitative ^{13}C-NMR data on individual sugars including three monosaccharides (glucose, fructose, quinovose), 13 disaccharides (sucrose, kojibiose, α,α- and α,β-trehalose, trehalulose, maltose, isomaltose, maltulose, isomaltulose, nigerose, leucrose, turanose, gentiobiose), five trisaccharides (raffinose, melezitose, 1-kestose, panose, erlose) and some other constituents, led to classification of honey samples by botanical and geographical origin.

In 2020, an unusual disaccharide trehalulose (Figure 2.3) was identified by NMR spectroscopy and ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis as a major component in stingless bee honey from five different stingless bee species, *Tetragonula carbonaria* and *Tetragonula hockingsi* in Australia, from *Geniotrigona thoracica* and *Heterotrigona itama* in Malaysia and from *Tetragonisca angustula* in Brazil (Fletcher *et al*., 2020). This distinctive sugar ~~was~~ is reported to have several health benefits including antidiabetic and acariogenic activities and low glycemic index (Ooshima *et al*., 1991). Therefore, the presence of trehalulose was suggested as a marker of authenticity for stingless bee honey (Fletcher *et al*., 2020).

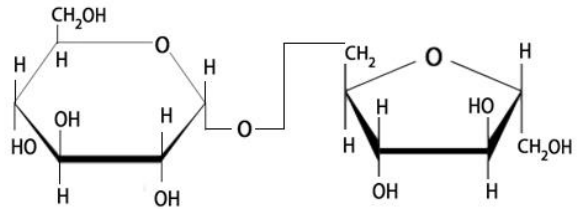


Figure 2.3: Chemical structure of trehalulose.

2.2.4 Moisture Content

Water is the second largest constituent of honey, influencing physical properties such as color, taste, viscosity and crystallization (Escuredo *et al.*, 2013). Other than the botanical origin of honey, the maturity level achieved in the bee nest, processing methods and storage conditions can affect the moisture content in honey significantly (da Silva *et al*., 2016).

Due to hygroscopicity, the moisture content in honey can be influenced

by the surrounding atmosphere, thus good storage conditions are required to keep honey in low moisture condition and to prevent it absorbing water from the atmosphere (Camara and Laux, 2010). Furthermore, the moisture content in honey can also vary in regions with different relative humidities or according to season, as honey is more likely to have a fermentation process in the rainy season rather than the in dry season (Karabagias *et al*., 2014).

According to the international standard established by the Codex Alimentarius Commission (2001), the moisture content present in honey bee honey should less than 20%; whereas according to the standard established by the Department of Standards Malaysia (2017), the moisture content in raw stingless bee honey should be less than 35 %, while processed honey should be less than 22 %. Determination of moisture content in honey can be achieved using refractometry. The moisture content in honey must be low so that microbial fermentation can be prevented (El Sohaimy *et al*., 2015). The fermentation of fructose and glucose in honey caused by osmotolerant yeasts leads to the production of ethanol and carbon dioxide. The sour taste of honey is due to the further oxidation of the ethanol to acetic acid. Hence, low moisture content is crucial to prolong the shelf life (Khalil *et al*., 2012).

In honey, the strong interaction between sugar molecules limits the amount of water molecules accessible for microbes. Hence, water activity (*aw*) is used to measure the availability of free water molecules in honey, and it is a major factor in inhibiting the growth of microbes (Olaitan *et al*., 2007). In the food industry, water activity is used to measure the availability of free water

molecules for microbial growth. Hence, water activity is a principal parameter responsible for the stability of food, modulating microbial reaction and estimating the type of microbes that can be found in food (Chirife *et al*., 2006). Water activity levels that permit growth of bacteria, yeasts and molds are 0.90, 0.80 and 0.70 respectively; hence honey with water activity lower than 0.60 can inhibit the growth of osmophilic yeasts that cause fermentation (Machado De-Melo *et al*., 2018). Compared with honey bee honey, stingless bee honey is reported to have higher water activity, which means it is more susceptive to microbial fermentation (Vit *et al*., 2013).

2.2.5 Proline

Proteins in honeys are attributed to both bee and botanical sources, with secretions of the salivary gland of bees, and pollen being the main source of proteins in honey (Sak-Bosnar and Sakac, 2012; Escuredo *et al*., 2013). Amino acids are responsible for 1 % of the constituents in honey and the relative proportions depend on the botanical origin of the honey (Hermosín *et al*., 2003). The most abundant amino acid in honey and pollen is proline, constituting 49 % in blossom honey and 59 % in honeydew honey of the total amino acid content. Other important amino acids present in honey include phenylalanine and glutamic acid (Iglesias *et al*., 2006; Puscion-Jakubik *et al*., 2020). Other than proline, Popova *et al.* (2021) also detected pyroglutamic acid as one of the major amino acids in stingless bee honey. Additionally, there are other amino acids present in honey including arginine, alanine, asparagine, aspartic acid, cysteine, gamma-aminobutyric acid, glutamine, glycine, histidine, isoleucine, leucine,

lysine, threonine, methionine, ornithine, serine, tryptophan, tyrosine and valine (Hermosín *et al*., 2003; Rebane and Herodes, 2010; Keckes *et al*., 2013; Popova *et al*., 2021).

Proline originates mainly from the salivary secretions of bees during the conversion of nectar into honey. In honey, proline represents up to 85 % of the total amino acids (Machado De-Melo *et al*., 2018). Proline has been used as a criterion for the evaluation of the maturation of honey, and adulteration with sugar. According to the International Honey Commission, the proline level in honey should be more than 180 mg/kg (Bogdanov *et al*., 2002). Hence, a lower proline level could suggest that the honey was either harvested in immature state or has been adulterated with sugar syrup (Machado De-Melo *et al*., 2018). Still, the proline level can be different in various types of honey as it is also influenced by the floral nectar that the bees have collected (Moniruzzaman *et al*., 2013a). A spectrophotometric method that is usually used to measure proline content in honey can be achieved by its reaction with ninhydrin. This method makes use of the colored complex developed between proline and ninhydrin, permitting the amino acid to be quantified (Czipa *et al*., 2012).

2.2.6 Hydrogen Peroxide

In honey, hydrogen peroxide is formed together with gluconic acid during glucose oxidation, a reaction catalysed by glucose oxidase. Glucose oxidase is an enzyme synthesized in the salivary gland of bees that is added into nectar (Ohashi *et al.*, 1999). Glucose oxidase is usually detected in all raw honey

types but its concentration in each honey is different based on the age and health condition of the foraging bees, along with the diversity and richness of foraged diet (Brudzynski *et al*., 2011). The final level of hydrogen peroxide in honey is determined by the difference between its production rate and its destruction by catalase. Catalase is an enzyme that is found in pollens that hydrolyses hydrogen peroxide to oxygen and water (Weston *et al.*, 2000).

It was proposed that the existence of hydrogen peroxide prevents the spoilage of unripe honey during periods when the sugar level is not high enough to inhibit microbial growth (Kwakman and Zaat, 2012). It has been found that the hydrogen peroxide level is positively correlated with the microbial inhibitory potency of honey (Brudzynski *et al*., 2011) and the presence of hydrogen peroxide in honey is believed to contribute to its antibacterial properties (Bang *et al*., 2003; Brudzynski, 2006 and Strelec *et al*., 2018). Hydrogen peroxide kills bacteria by producing free hydroxyl radicals that lead to oxidative damage to bacterial proteins and the lipid membrane. The oxidizing radicals were found to induce bacterial DNA oxidation as well (Linley *et al*., 2012). However, several studies showed the synergistic interaction between hydrogen peroxide and phenolic compounds is actually the key factor that contributes to the antibacterial activity of honey (Brudzynski *et al*., 2011; Chen *et al*., 2012; Bucekova *et al*., 2017). As suggested by several studies, the presence of hydrogen peroxide can be detected via the oxidation of ferrous ions to ferric ions. Such oxidation is mediated by peroxy radicals that are generated after hydrogen peroxide is reacted with sorbitol (Kang *et al*., 2006; Yagi *et al*., 2013; Guzman-Soto *et al*., 2020).

2.2.7 Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is a cyclic aldehydic compound that is formed as an intermediate compound when monosaccharides, mainly fructose is degraded or dehydrated in the Maillard reaction. This non-enzymatic browning reaction usually happens in honey during improper and prolonged storage or on excessive heating (Shapla *et al*., 2018). Hence, HMF content is widely used to determine the freshness of honey, as fresh raw honey has only small amount of HMF, but the content rises slowly during storage. On the other hand, if immature honey has been heated to reduce the moisture content, higher amounts of HMF can be detected in the honey (Khalil *et al*., 2010; Machado De-Melo *et al*., 2018). Thus, HMF content is also considered as an indicator for honey deterioration.

The Codex Alimentarius Commission (2001) has set a maximum value of 40.00 mg/kg for processed honey and a maximum value of 80.00 mg/kg if the honey or blends of these honeys originated from the tropical regions. This is because a higher HMF content is commonly found in honeys harvested from warm climate regions (Sodre *et al*., 2011). In a study conducted by Biluca *et al*. (2014), the HMF content of one honeybee and 13 stingless bee honey samples were measured using capillary electrophoresis and none of the raw honey samples presented HMF. However, when the honey samples were treated at 75 °C for 15 minutes, honeybee honey formed 8.05 mg/kg of HMF, while the stingless bee honey did not exceed the limit of quantitation (LOQ). Furthermore, when the honey samples were subjected to 75 °C for 24 hours, the HMF content of honeybee honey was 695 mg/kg while that of the stingless bee honey was

only 238 mg/kg. Such observations were ascribed to higher water activity and acidity in stingless bee honey. This is because the formation of HMF by Maillard reaction is slower in high water activity and acidic conditions (Guerrini *et al*., 2009; Silvano *et al*., 2014). Furthermore, lower HMF formation in stingless bee honey was suggested to be due to the higher fructose content. This is because the rate of Maillard reaction is faster in the presence of higher glucose content; thus higher HMF formation was observed in honey bee honey (Biluca *et al*., 2014).

Hence, the maximum value for HMF content in raw stingless bee honey set by the Department of Standards Malaysia (2017) is 30 mg/kg, which is lower than the values set by the Codex Alimentarius Commission (2001) for honey bee honey. However, HMF cannot be used as the only parameter to determine the severity of heat processing, as the level of HMF can be influenced by other factors, such as sugar profile, acidity and moisture content. Therefore, HMF content can only serves as an indicator of overheating or improper storage conditions (da Silva *et al*., 2016). Although the International Honey Commission recommended three methods for the determination of HMF in honey, Zappala *et al*. (2005) stated high performance liquid chromatography (HPLC) and the White method usually give similar values; whereas the Winkler method gave higher values in all tested honey samples than the other two methods.

2.2.8 Diastase

Natural honey contains enzymes originating from the hypopharyngeal

glands of bees, usually integrated into honey during regurgitation of the nectar (Pasias *et al*., 2017). Enzymes such as diastase, invertase and glucose oxidase are commonly found in honey. These enzymes are thermolabile and able to indicate overheating and the degree of preservation of honey (da Silva *et al*., 2016).

Diastase, also known as α-amylase, breaks down starch molecules into a mixture of disaccharides (maltose) and trisaccharides (maltotriose). Diastase is one of the enzymes in honey with best heat resistance, thus diastase enzyme level is used widely as an indicator for honey freshness and adulteration (Machado De-Melo *et al*., 2018). If the diastase enzyme level is too low, it could indicate that the diastase enzymes have been degraded due to heat treatment, or the honey has been diluted by sugar syrup (Subramanian *et al*., 2007).

Although the diastase activity in honey was found to be reduced almost by half after a long heating period (Subramanian *et al*., 2007), diastase level alone does not represent the general quality of honey (Oddo *et al*., 1990). Stated in the Codex Alimentarius Commission (2001), the diastase enzyme level in honey bee honey should be more or equal to 8 Schade units in general; whereas for honeys that have naturally low enzyme content should have a value of not lower than 3 Schade units. For honey with low diastase activity, only a maximum of 15 mg/kg of HMF is permitted, as this indicates that the honey bee honey has not undergone heat treatment or prolonged storage (da Silva *et al*., 2016). On the contrary, no reference value is available for diastase level in stingless bee honey (Department of Standards Malaysia, 2017).

Other than botanical origins, the differences in enzyme content present in honey may depend on the age and health status of the bees, the nectar collection period, the quantity of nectar flow and its sugar content, because concentrated nectar could lead to lower enzyme content and pollen consumption (Oddo *et al*., 1990). Low diastase activity in honey could be due to the honey bees being fed with sugar syrup artificially (Guler *et al*., 2014). These researchers stated that bees should not be fed glucose in excessive amounts because this would promote enzyme deficiencies including diastase and enzymes are important in sugar conversion during honey ripening.

2.2.9 Electrical Conductivity and Total Dissolved Solids

Other than sugars, honey contains other minor components such as organic acids and minerals which are able to dissociate into ions (Zivkov-Balos *et al*., 2018). In addition, according to Kropf *et al*. (2008), the color of honey can possibly indicate its electrical conductivity as darker-colored honey has a higher conductivity than that of lighter-colored honey. However, higher conductivity of electricity does not necessarily stipulate high ash content in honey (Escuredo *et al*., 2013).

Electrical conductivity (EC) is a good criterion for probing the botanical origin of honey bee honey especially for discrimination between blossom and honeydew honeys (Pita-Calvo and Vazquez, 2017). The Codex Alimentarius Commission (2001) states that blossom honey must have EC values not more than 0.8 mS/cm while honeydew honey must have values not less than 0.8

mS/cm. Studies also found out the EC values of honeydew honey were significantly higher than for blossom honey (Manzanares *et al*., 2011; Olga *et al*., 2012; Bergamo *et al*., 2019).

However, high electrical conductivity could indicate the presence of contaminants or heavy metals in honey due to improper processing or honey originating from polluted nectar due to fertilizers or pesticides (Aghamirlou *et al*., 2015). Therefore, electrical conductivity is the easiest way to give a general estimation of metal pollution in honey. On the other hand, total content of dissolved solids is a parameter to measure the presence of inorganic and organic substances in honey. These substances can be present in three forms: ionized, molecular, or micro-granular suspended forms (Khalil *et al*., 2012). The total dissolved solids of honey are always positively correlated with the electrical conductivity of honey (Khalil *et al*., 2012; Moniruzzaman *et al*., 2014).

2.2.10 Mineral Content

Minerals are also important for the characterization and classification of honey, since they are stable and dependent on plant absorption from the soil and environment. Although aluminum (Al) and heavy metals such as cadmium (Cd), lead (Pb) and nickel (Ni) are naturally present in the environment, anthropogenic sources are the major source and these are toxic if maximum residue levels are exceeded (Biluca *et al*., 2017). Hence, honey can be a good environmental indicator because the mineral content can reflect the presence of toxic elements of the surrounding water and soil (Czipa *et al*., 2015).

Based on the amounts, mineral elements detected in honey can be classified into macrominerals and microminerals. According to Atanassova *et al*. (2016), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), sulfur (S), sodium (Na) and iron (Fe) were the macrominerals detected in honeydew honey while Al, copper (Cu), strontium (Sr), zinc (Zn) and manganese (Mn) were the trace minerals or microminerals. The heavy metals Cd and cobalt (Co) and toxic elements arsenic (As) and chromium (Cr) were under the detection limits in the honey samples investigated. Similarly, in another analysis conducted by Olga *et al*. (2012), the most abundant mineral in honey was K, followed by P, Mg, and Ca, while Fe, Zn and Cu were present in low quantities.

The mineral content of honey is commonly analysed by inductively-coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS) after pre-treatment with nitric acid. Olga *et al*. (2012) determined the levels of K, Ca, Fe, Mg, Na, P, Zn and Cu of honey with AAS. However, due to spectral interference, Biluca *et al*. (2017) used AAS to measure the levels of Ca and K in stingless bee honey; while Na, Mg, Mn, Al, Fe, Co, Cu and Zn were analysed by ICP-MS. While for Atanassova *et al*. (2016), macrominerals K, Ca, Mg and P and microelements As, Cd, Co, Cr, Cu, Fe, Mn, Na, Ni, Pb and Zn were determined by atomic emission spectrometry with an inductively-coupled plasma system (ICP-AES).

Ash content of honey is indicative of its mineral content. This physicochemical parameter is claimed to be a criterion of honey quality that enables differentiation between blossom and honeydew honey. Terrab *et al*.

(2003a), Terrab *et al*. (2003b) and Nalda *et al*. (2005) also reported that mineral content could be used to differentiate honey based on botanical origins. Generally, the ash content in blossom honey is not more than 0.6 % (w/w) while honeydew honey is less than 1.2 % (w/w) (Terrab *et al*., 2003a; Terrab *et al*., 2003b; Ouchemoukh *et al*., 2007; Habib *et al*., 2014; El Sohaimy *et al*., 2015; Majewska *et al*., 2019). Honeydew honey has higher ash content than blossom honey as the former contains more minerals (Pita-Calvo and Vazquez, 2017). Olga *et al*. (2012) also detected the mineral content in blossom honey is lower compared to honeydew honey, particularly for K and P. According to Majewska *et al*. (2019), together with electrical conductivity, ash content which is linked with mineral content, appeared to be the most reliable markers in determining the botanical origin of honey.

2.3 Chemical Profiling

The biological activities of honey including stingless bee honey are usually associated with its minor components, including phenolic compounds, especially flavonoids and phenolic acids (da Silva *et al*., 2013, Chuttong *et al*., 2016). Hence, the phenolic compounds in honey are not only responsible for its color, flavor and aroma but are also highly involved in its antioxidant and antibacterial properties.

These phytochemicals are plant-derived metabolites or constituents of nectar, plant exudate, pollen, propolis and resin (Tomas-Barberan *et al*., 1993). In addition, these phytochemicals are also believed to be transformed by the

salivary enzymes of the bee during the honey maturation process in the hive (Tomas-Barberan *et al*., 1993). Therefore, these minor components can serve as chemical markers for the botanical origin of different types of honey and the bee species involved in its production (Estevinho *et al*., 2008; da Silva *et al*., 2013; Sergiel *et al*., 2014; da Costa *et al*., 2018).

In this context, chemical profiling appears to be valuable in enhancing the characterization of stingless bee honey. However, the analysis of these minor components is rather challenging, because honey is a complex food matrix that is rich in polar sugar molecules and with large variation of metabolites that are present in small quantities (Truchado *et al*., 2011). Tabulated in Table 2.2 are the phenolic compounds that have been profiled in different studies (Vit *et al*., 1997; Guerrini *et al*., 2009; Truchado *et al*., 2011; da Silva *et al*., 2013; Ranneh *et al*., 2018). The examples of nectar and pollen derived flavonoids in stingless bee honey are also displayed in Figure 2.4.

Table 2.2: Phenolic compounds identified in different stingless bee honey samples.

Compounds	Study
Coumarins	
Bergamottin, fraxin, umbelliferone	Guerrini *et al*. (2009); Biluca *et al*. (2017)
Flavonoids	
Apigenin, aromadendrin, catechin, chalcone, chrysin, luteolin, isorhamnetin, eriodictyol, hesperidin, hispidulin, isoquercitrin, kaempferol, methoxykaempferol, mirecetrin, myricetin, naringenin, pinobanksin, quercetin, quercitrin, taxifolin, tectochrysin, tricetin	Vit *et al*. (1997); Guerrini *et al*. (2009); Truchado *et al*. (2011); da Silva *et al*. (2013); Biluca *et al*. (2017); Ranneh *et al*. (2018); Avila *et al*. (2019)
Phenolic acids	
abscisic acid, caffeic acid, caffeic acid phenethyl ester, chlorogenic acid, cinnamic acid, coumaric acid, dihydroxybenzoic acid, ellagic acid, ferulic	Vit *et al*. (1997); da Silva *et al*. (2013); Biluca *et al*. (2017); Ranneh *et al*. (2018); Avila *et al*. (2019)

acid, gallic acid, hydroxybenzoic acid, hydroxycinnamic acid, mandelic acid, protocatechuic acid, rosmarinic acid salicylic acid, sinapic acid, syringic acid, vanillic acid	
Phenolic aldehydes	
Sinapaldehyde, syringaldehyde, vanillin	Biluca e*t al*. (2017)
Diterpene	
Carnosol	Biluca *et al*. (2017)

In 1997, Vit and her team used high-performance liquid chromatography (HPLC) equipped with a photodiode array detector (PDA) to profile the phenolic compounds of honey bee honey and stingless bee honey. However, the study was unable to identify entomological markers that can differentiate between these two honey types. Although flavonoids such as myricetin, an unidentified chalcone and a flavonol glycoside were only detected in stingless bee honey, not all of the 11 tested honey samples produced by *Melipona compressipes* and *Melipona favosa* have these constituents (Vit *et al*., 1997). According to Guerrini *et al*. (2009), coumarins and flavonoids are the compounds typically characterizing plant-derived products, including honey. In their study, analyses of coumarins and flavonoids were performed by densitometric high performance thin layer chromatography (HPTLC). The coumarins, fraxin and bergamotin were identified only in Ecuadorian stingless bee (Meliponinae) honey; while luteolin, quercitrin and isorhamnetin were the flavonoids detected in other honeys, with luteolin as the most abundant detected phytochemical compound.

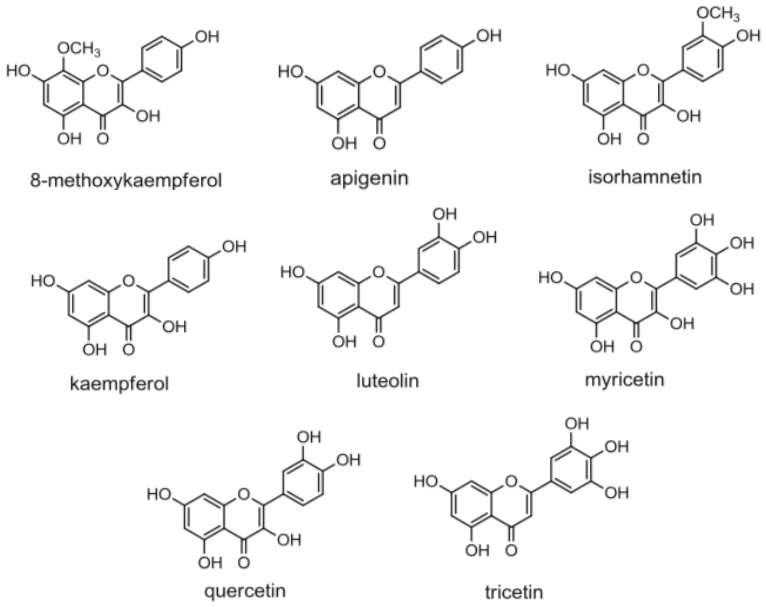


Figure 2.4: Examples of nectar- and pollen-derived flavonoids in stingless bee honey.

In Brazil, da Silva *et al*. (2013) profiled phenolic compounds present in seven honey samples produced by the stingless bee *Melipona seminigra merrillae*. A total of 14 phenolic compounds was identified using high-performance liquid chromatography (HPLC). By correlating the presence of certain phenolic compounds with the predominant pollen type in each honey sample, da Silva *et al*. (2013) proposed that the floral source may determine the phenolic profile in stingless bee honey. By using liquid chromatography-mass spectrometry (LCMS), Ranneh *et al*. (2018) successfully identified a total of 18 flavonoids and phenolic acids in two Malaysian honey samples produced by the Trigona stingless bee. The majority of identified polyphenols are classified as phenolic acids, while some of the flavonoids that are bound to sugar moieties are more hydrophilic.

On the other hand, Truchado *et al*. (2011) were the first researchers to report the identification of flavonoid-C-glycosides in honey. Twelve stingless

bee honey samples produced in Venezuela from *Melipona favosa* were analysed using high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-MSn/ESI), more than 18 phenolic compounds were detected in the honey samples tested, with different relative amounts. The analyses revealed that flavonoid glycosides were the main constituents in stingless bee honey. The honey samples analysed contained a consistent flavonoid pattern composed of flavone-C-glycosides, flavonol-O-glycosides and flavonoid aglycones (Figure 2.5).

However, not all the phenolic compounds in honey result from botanical origins. According to Ferreres *et al*. (1991), more lipophilic flavonoids such as pinocembrin, chrysin, genkwanin and tectochrysin are produced from propolis and beeswax, therefore these phytochemicals are suggested not to be related to the botanical origin of the honey. On the other hand, hydrophilic flavonoids are directly related to the botanical origin of the honey because these phytochemicals originate from the nectar or the pollen foraged by bees. The variations in total phenolic compounds were also verified in honeys originating from different bee species, which likely result from the floral preference of each bee species (Guerrini *et al*., 2009; Avila *et al*., 2019).

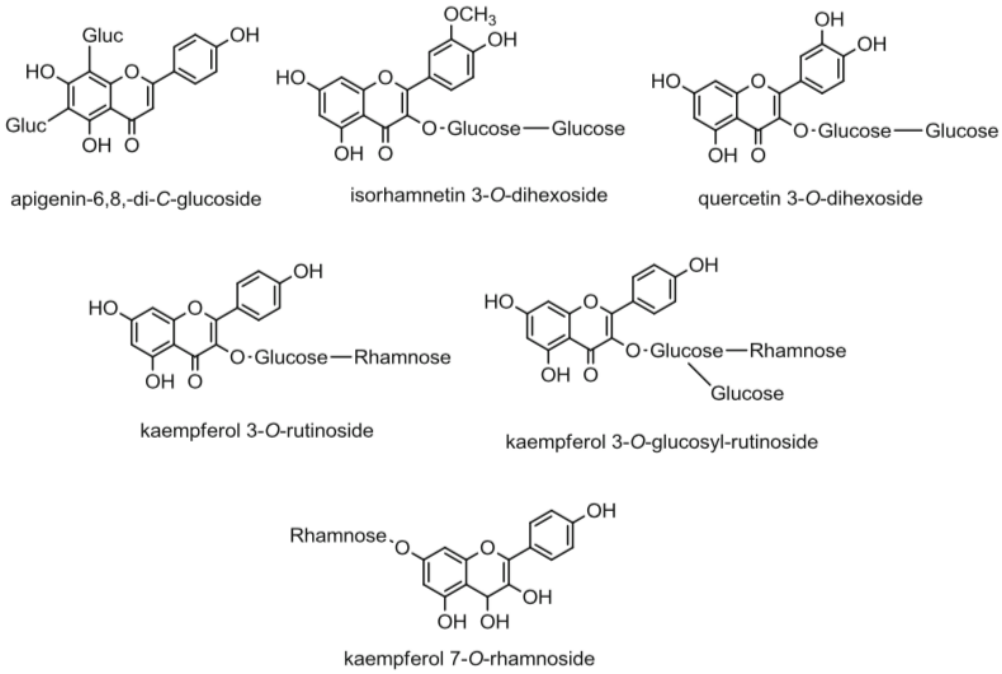


Figure 2.5: Examples of flavonoid glycosides in stingless bee honey.

Generally, stingless bee honey was proposed to have a greater variety of floral flavonoids than honey bee honey because of a wider range of floral resources or a richer flavonoid composition of the foraged nectar (Ferreres *et al*., 1991; Vit *et al*., 1997). In addition, a higher proportion of lipophilic flavonoids is found in stingless bee honey, suggesting infiltration of these phytochemicals from resins into the honey. This is because stingless bee honey is stored in honey pots made from cerumen, which is a mixture of resin and wax (Ferreres *et al*., 1991; Vit *et al*.; 1997; Abd Jalil *et al*., 2017).

Furthermore, a study showed that the level of flavonoid glycosides in stingless bee honey was considerably higher than the level of flavonoid aglycones (Truchado *et al*., 2011). However, the opposite outcome was observed in honey bee honey that consistently showed higher levels of flavonoid aglycones and lower flavonoid glycoside levels. The same study proposed that

this was mainly due to the action of saliva enzymest produced by the honey bee. These enzymes can hydrolyse flavonoid glycosides into flavonoid aglycones such as quercetin, kaempferol and isorhamnetin. That could be the reason why stingless bee honey was found to be rich in flavonoid glycosides due to the absence of hydrolase enzymes (Truchado *et al*., 2011). Both kaempferol-3-O-(2,6-dirhamnosyl) hexoside and kaempferol-3-O-(2-(hexosyl)rhamnosyl,6-rhamnosyl) hexoside were found to be the major flavonoid glycosides in stingless bee honey samples.

2.4 Antioxidant Capacities of Honey

Reactive oxygen species (ROS) are oxidants that are usually generated endogenously during cellular metabolism or due to exposure to exogenous stressors such as toxicants. ROS including superoxide, hydroxyl and peroxyl radicals are harmful because these free radicals can initiate the breakdown of cellular membranes, proteins and nucleic acids. The exact antioxidant mechanism of honey is unknown, but the proposed mechanisms include free radical sequestration, proton donation and metallic ion chelation (Al-Mamary *et al*., 2002; Tuksitha *et al*., 2018). Hence, antioxidants are crucial to protect cells from being damaged by these harmful radicals (Ahmad *et al*., 2012).

Honey has long been known to be a natural source of antioxidants. Such an antioxidant feature is mainly attributed to the enzymatic and nonenzymatic constituents present in honey. The enzymatic substances are, for example catalase, peroxidase and glucose oxidase; whereas the nonenzymatic substances

are amino acids, proteins and most importantly phenolic compounds (Gheldof and Engeseth, 2002; Ferreira *et al*., 2009). Honey also contains minute quantities of vitamins that also contributs to its antioxidant properties. Other than vitamin C, the most abundant class of vitamin derived from pollen grains is vitamin B complex including vitamin B_1 (thiamine), B_2 (riboflavin), B_3 (nicotinic acid), B_5 (pantothenic acid), B_6 (pyridoxine), B_8 (biotin) and B_9 (folic acid). The acidic environment of honey helps in the preservation of these vitamins (Leon-Ruiz *et al*., 2013).

Phenolic compounds, which are highly dependent on the botanical source of honey are important in determining the antioxidant properties of a honey (Chua *et al*., 2013). Some of the phenolic compounds and flavonoids that were identified in honey include ferullic acid, chlorogenic acid, ellagic acid, chrysin, caffeic acid, gallic acid, herperetin, luteolin, quercetin, myricetin, kaempferol, *p*-coumaric, pinobanksin, apigenin, pinocembrin, genistein, naringenin, syringic acid and vanillic acid (Hussein *et al*., 2011). Phenolic constituents have been reported to be solely responsible for the antioxidant and other medicinal effects of honey (Erejuwa *et al*., 2012). Hence, total phenolic content can be a reliable parameter to indicate the antioxidant capacity of a honey (Kek *et al*., 2014). Generally, dark colored honey contains higher amounts of phenolic compounds, which is always associated with greater antioxidant properties (Al-Farsi *et al*., 2018).

Liu and colleagues demonstrated that the total phenolic and flavonoid contents of honeybee honeys in Taiwan were variable and depended greatly on

the floral source. Except for anti-inflammatory properties, the outcomes suggested that antioxidant levels and antibacterial activities of the honeys are attributable to the total phenolic and flavonoid contents (Liu *et al*., 2013). Valdes-Silverio *et al*. (2018) showed that a saturated sugar solution displayed significantly lower antioxidant properties including reducing power, metal chelating capacity and radical scavenging activity than all raw honey samples tested. Hence, it was concluded that sugars are not involved in the antioxidant properties of honey.

Other than antibacterial properties, manuka honey from the Manuka tree (*Leptospermum scoparium*) originated from New Zealand, is also well known for its excellent antioxidant activities (Stephens *et al*., 2010; Boateng and Diunase, 2015). However, phenolic compound content and antioxidant activities of sourwood honey (*Oxydendrum arboretum*) from Malaysia and buckwheat honey (*Fagopyrum esculentum* Moehch) from China were significantly higher than Manuka honey (Moniruzzaman *et al*., 2013a; Deng *et al*., 2018). It may be explained by the amounts of several phenolic compounds including protocatechuic acid, chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, benzoic acid, quercetin, apigenin, kaempferol, chrysin and galangin in buckwheat honey being significantly higher than Manuka honey (Deng *et al*., 2018).

According to a study conducted by Dzugan *et al*. (2018), which used 90 honey bee honey samples from Poland, generally unifloral honeys had greater antioxidant capacities than multifloral honeys. Among unifloral honeys,

buckwheat honey was found to have greater antioxidant capacities than honeydew honey. However, studies conducted in Czechoslovakia and Croatia discovered that honeydew honey had exceptionally high values of phenolic content and better antioxidant activities than other blossom honeys (Lachman *et al*., 2010; Flanjak *et al*., 2016). Despite the contrasting outcomes, the studies indicate that antioxidant activity can be a useful parameter for determining the botanical origin of monofloral honey (Flanjak *et al*., 2016; Dzugan *et al*., 2018). Interestingly, the phenolic content and antioxidant activity of honey bee honey were found to correlate with the mineral content, suggesting the influence of minerals on the polyphenol synthesis in plants and consequently in honey (Perna *et al*., 2012). Furthermore, the interaction between polyphenols and metal ions was hypothesized to induce the antioxidant properties and biological effects of the polyphenols (Perna *et al*., 2012).

In another study, honey from a *Hypotrigona* stingless bee was found to possess higher phenolic content and antioxidant activities when compared with honey from a honey bee, but another stingless bee honey from *Melipona* sp. had lower antioxidant capacity than the honey bee honey (Nweze *et al*., 2017). Tuksitha *et al*. (2018) also showed honey samples from three different stingless bee species displayed significant variation of phenolic content and antioxidant properties, including radical scavenging activities and reducing power. Malaysian stingless bee honey was also found to exhibit greater antioxidant properties with significant higher values of ascorbic acid equivalent antioxidant content (AEAC) and FRAP than honeys produced by the honey bee (Kek *et al*., 2017b).

Variation of antioxidant properties is commonly found in different honey samples. The antioxidant properties of Malaysian honeys produced by different honey bee species namely *Apis cerana*, *Apis dorsata* and *Apis mellifera* were found to be significantly different (Moniruzzaman *et al*., 2013b). Furthermore, variations of phenolic compounds and antioxidant activities were also observed in honey samples of the same species of stingless bee, but from distinct geographical origins (da Silva *et al*., 2013; Biluca *et al*., 2017). Such variations could be associated with the nectar source and climate. Biluca *et al*. (2017) detected wide variations of phenolic compounds in honeys originating from the same apiary and from the same harvest period, but from different bee species, probably resulting from the floral preference of each bee species.

Honey might be an antioxidant food that can prevent many oxidative stress diseases directly or indirectly. A study conducted by Yao *et al*. (2011) showed use of honey as a dietary supplement was able to reduce DNA damage and plasma malondialdehyde (MDA) level in young and middle-aged rats. Honey was also found to reduce oxidative damage by modulating antioxidant enzyme activities including glutathione peroxidase (GPx) and catalase (CAT). The antioxidant properties of honey were found to prevent ethanol-induced gastric ulcer formation in rats (Almasaudi *et al*., 2016). Honey significantly increased gastric mucosal enzymatic (GPx and superoxide dismutase (SOD)) and nonenzymatic (glutathione (GSH) and nitric oxide (NO)) antioxidants that lowered the concentrations of gastric mucosal MDA and plasma inflammatory cytokines (TNF-α, IL-1β, and IL-6). Hence, honey was proposed to protect gastric mucosa from lesions and to preserve gastric mucosal glycoproteins. Zhao

et al. (2017) also disclosed that their studies indicated that administration of honey could protect livers in mice from acute alcohol-induced toxicity due to its antioxidant properties and prevention of oxidative stress.

The antioxidant protective effect of honey was also found to be effective against damage and oxidative stress induced by cigarette smoke in rat testis (Mohamed *et al*., 2011). The study showed that honey dietary supplementation significantly reduced histological damage and lipid peroxidation level, increased total antioxidant status, as well as significantly restored activities of GPx, SOD and CAT in rat testis after cigarette smoke exposure. Erejuwa *et al*. (2012) found honey-treated diabetic rats not only had significantly reduced blood glucose levels compared with the diabetic control group, but MDA levels were significantly reduced as well. In addition, honey treatment also restored SOD and CAT antioxidant activities, which suggested that hypoglycemic effect of honey might be attributed to its antioxidative effect on the pancreas. Erejuwa *et al*. (2011a; 2011b) also showed that honey potentiated the effects of antidiabetic drugs glibenclamide and metformin to protect the pancreas and kidneys of diabetic rats against oxidative stress and damage induced by streptozotocin. Another study also mentioned that the antioxidant property of honey provided protective effects against carbon tetrachloride (CCl_4)-induced liver and kidney toxicity by maintaining the balance of the antioxidant defense system (El-haskoury *et al*., 2018). The studies suggested that honey administered alone or as adjuvant therapy might be a potential natural antioxidant medicinal agent warranting further experimental and clinical research (Ahmed *et al*., 2018).

2.4.1 Impacts of Storage and Processing on Antioxidant Capacities

Other than physicochemical properties, both storage and processing have been found to influence the antioxidant properties of honey. Honey is mostly not consumed immediately after production, especially if it is bought in a supermarket and not directly from a bee farm. According to the obtained data, Saric *et al.* (2012) reported that phenolic and flavonoid content of honey decreased significantly after one year of storage. Moreover, the antioxidant properties including radical scavenging and reducing activities decreased in a much bigger extent in unifloral honey than in multifloral honey.

The main reason of performing filtration is to remove impurities in honey; however, consumers claim that the quality of filtered honey could be lower than that of unfiltered one because filtration would eliminate essential and beneficial constituents of honey (Wilczynska, 2014). Although about 90 % of pollens in honey were removed after filtration, the color and phenolic compound content were not significantly different between filtered and unfiltered honey (Wilczynska, 2014). Furthermore, the radical scavenging activities of filtered honey were not significantly lower than the unfiltered one. Hence, it can be said that filtration should not deteriorate the quality of honey, including its antioxidant capacity. Although it was found that the level of HMF was higher and the enzyme activities reduced after filtration, Wilczynska (2014) explained that it was because the honey had been heated prior to the filtration process.

On the other hand, most commercialized honey is subjected to heat

treatment for two main reasons, to prevent crystallization and to inactivate microorganisms, especially yeasts, which would lead to fermentation. Several studies have found that heating (45 – 70 °C) was able to increase the antioxidant activity in honey (Turkmen *et al*., 2006; Molaveisi *et al*., 2019). Turkmen *et al*. (2006), Brudzynski and Miotto (2011) and Molaveisi *et al*. (2019) explained that the higher antioxidant activities in heated honey were mostly due to the formation of Maillard reaction products (MRP), but this browning reaction is not desirable from the viewpoint of the consumer. HMF is formed in the Maillard reaction, hence higher HMF content in honey due to heat treatment is not permitted and accepted by the Codex Alimentarius Commission (2001) and the Department of Standards Malaysia (2017). It was believed that these MRP are acting as antioxidants. Thus, the losses of natural antioxidants during heating could be minimized or compensated for by the formation of non-nutrient antioxidants such as MRP (Turkmen *et al*., 2006; Elamine *et al*., 2020)

However, Zarei *et al*. (2019) reported that honey showed significant increase in HMF content but lower antioxidant capacity when the honey was heated at 63 °C for up to 30 minutes. Lower DPPH radical scavenging activity and FRAP value were found to be associated with a decrease in total phenolic content in heated honey. Elamine *et al*. (2020) also observed that, owing to higher HMF formation, DPPH and ABTS radical scavenging activities in honey were increased by the heat processing, but these radicals are not present in the biological systems. Due to lower quantities of flavonoids, chelating ability was totally removed, and NO radical activity was greatly reduced after the honey was heated; this indicates that the capacity of heated honey to scavenge this important

mediator of inflammation is reduced. The same authors concluded that heating would not improve the antioxidant capacity of honey and the evaluation of such property in heated honey is dependent on the methods used.

2.4.2 Evaluation of Antioxidant Capacities

Due to the complex nature of phytochemicals, there is no ideal method to evaluate the antioxidant activity of honey, as each method allows the measurement of a different group of antioxidants. Hence, a single method is not sufficient to conclude on the antioxidant potential of honey. In this context, different standard methods were proposed and used to validate the antioxidant bioactivities of honey (Moniruzzaman *et al*., 2012). The first approach is to evaluate the total content of phenolic compounds, since the antioxidant properties of honey have been reported to be solely contributed by phenolic constituents (Erejuwa *et al*., 2012). The Folin-Ciocalteu reagent is commonly used to quantify the total phenolic content of a honey. This method is performed using a mixture of sodium molybdate and sodium tungstate salts acidified with hydrochloric acid and phosphoric acid. The yellowish color of the mixture turns bluish due to the formation of molybdenum-tungsten complexes in the presence of phenolic compounds. Hence, the blue intensity which can indicate the number of hydroxyl groups of phenols can be measured spectrophotometrically (Sereia *et al*., 2017).

The scavenging activities of honey against several types of free radical are commonly evaluated to determine the antioxidant capacity of a honey. DPPH

(2,2-diphenyl-1-picrylhydrazyl) radicals are employed due to their more selective reaction with hydrogen-donors, while ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radicals are freely soluble in both organic and aqueous solvents so ABTS can be used to screen both hydrophilic and lipophilic antioxidant compounds (Alzahrani *et al*., 2012). The antioxidants present in honey donate hydrogen atoms to the unpaired electrons of DPPH radicals, that eventually change the purple color of the DPPH solution to a yellow color (Chua *et al*., 2013); whereas the greenish blue color of the ABTS solution would decolorize due to the action of hydrogen donor antioxidants (Shalaby and Shanab, 2013).

In living organisms, superoxide radicals ($O_2^{\bullet-}$) are being continuously formed in the reduction of oxygen to water while peroxy-radicals ($ROO^{\bullet}$) are formed due to the attack by oxygen on fatty acids. Both superoxide and peroxy-radicals are involved in lipid peroxidation that leads to enormous damage to cellular components (Lipinski, 2011; Gangwar *et al*., 2014). In analytical assays, superoxide radicals are generated within a system consisting of phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide (NADH) by the oxidation of NADH and are assessed by the reduction of nitroblue tetrazolium (NBT). Decreased intensity of the bluish reaction mixture indicates the presence of superoxide anion scavenging activity (Chai *et al*., 2014). On the other hand, peroxy-radicals produced by a free radical initiator quench the fluorescent probe over time. The presence of antioxidants can inhibit the peroxy-radical oxidation of the fluorescent probe hence the antioxidant capacity is determined based on the fluorescence decay curve (Yang *et al*., 2020).

Many radical reactions are formed from ferrous iron (Fe^{2+}) by its ability to transfer single electrons. Therefore, reduction of the formation of reactive oxygen species can be achieved by the chelation of ferrous ions by both phenolic and non-phenolic constituents (Chai *et al*., 2014; Sudan *et al*., 2014). This reaction results in a decrease of the red color of the ferrozine-Fe^{2+} complex. Halvorsen *et al*. (2002) stated the ferric reducing power represents the corresponding amounts of electron-donating antioxidants with the reduction in the ferric iron (Fe^{3+}) to the ferrous ion (Fe^{2+}). Hence it can be said to be the only assay that directly measures the total antioxidant (or reductant) content in a honey sample compared to other assays that measure antioxidant activity. The total antioxidant content in honey can be estimated by measuring the reduction reaction of antioxidants with colorless ferric tripyridyltriazine (Fe^{3+}-TPTZ) complexes to produce a blue colored form of ferrous tripyridyltriazine (Fe^{2+}-TPTZ) (Rajurkar and Hande, 2011).

2.5 Chemometric Analysis

Due to the complex composition of honey and presence of different antioxidants, a single assay method is definitely not sufficient to represent the overall physicochemical properties and antioxidant capacities of honey. Hence, given the high variability found in different honey types, honey is always evaluated with a combination of methods based on different principles and reaction mechanisms (Perna *et al*., 2012).

Several chemometric tools, in particular principal component analysis (PCA), discriminant analysis (DA) and cluster analysis (CA), are used to obtain a comprehensive overview of significant variables and similarities or differences in physicochemical properties and antioxidant capacities of different honey types based on botanical and geographical origins (Flanjak *et al*., 2016; Dzugan *et al*., 2018).

Using PCA, a study conducted by Flanjak *et al*. (2015) was able to characterize and differentiate five different types of Croatian honey (black locust, lime, sage, chestnut and honeydew) based on their physicochemical properties (moisture, electrical conductivity, HMF content and color) and antioxidant capacities (total phenolic content, DPPH scavenging activity and FRAP). However, the study concluded that the antioxidant capacity played a minor role in honey type identification with respect to the botanical origin.

On the contrary, Dzugan *et al*. (2018) indicated that antioxidant activity can be a useful parameter for determining the botanical origin of honey. Together with several antioxidant assays including photochemiluminescence (PCL), FRAP, DPPH and Folin-Ciocalteu methods, the study was able to differentiate nine different varieties of Polish honey (buckwheat, dandelion, goldenrod, rape, tilia, coniferous honeydew, leafy honeydew, nectar-honeydew and multifloral) using multivariate statistical analyses, PCA and clustering methodologies.

2.5.1 Differentiation of Stingless Bee Honey and Honey Bee Honey

Although the classification of honey based on entomological origin is rather limited, there are few studies that were able to identify and characterize honey using physicochemical properties and antioxidant capacities. Duarte *et al*. (2012) classified Brazilian honeys into honey bee origin, *A. mellifera*, and stingless bee origins *Melipona* spp., and *Plebeia* spp. using both physiochemical and antioxidant parameters, including humidity, pH, electrical conductivity, total sugar, sucrose and flavonoid content and DPPH scavenging activity. Da Silva *et al*. (2013) also managed to differentiate Brazilian honey into *A. mellifera* and *Melipona* spp. using three physicochemical parameters (free acidity, soluble solids and moisture) and four mineral contents (Na, Ca, Mn and Sr). Discriminant models obtained for Romanian honey types confirmed that the differentiation of honey samples according to botanical origins was mainly based on mineral elements composition, K, Mg and Ca being the dominant elements strongly associated with the principal component (Oroian *et al*., 2015).

In Malaysia, Kek *et al*. (2017a) classified honey into groups of honey bee (*Apis dorsata*, *A. mellifera* and *Apis cerana*) and stingless bee (*H. itama*) by chemical profiles (ash, protein, carbohydrate, fructose, glucose and sucrose contents) and minerals (K and Na) with hierarchical cluster analysis (HCA) and PCA. Another study conducted by the same research team was able to classify the same honey samples based on the entomological origins using different variables, including color intensity, moisture content, free acidity, electrical conductivity, AEAC and FRAP (Kek *et al*., 2017b). Kek *et al*. (2017b) also

emphasized the importance of differentiating stingless bee honey from honey bee honey to ensure the quality and authenticity of stingless bee honey to consumers.

Although blossom honey, also known as floral honey is the most common type of honey worldwide, honeydew honey is highly valued by consumers, especially Europeans, due to its higher nutritional value and bioactivity (Gonzalez-Paramas *et al*., 2007; Seraglio *et al*., 2016; Seraglio *et al*., 2019). Therefore, due to increasing demand, honeydew honey fetches higher market prices than blossom honey (Pita-Calvo and Vazquez, 2017; Pita-Calvo and Vazquez, 2018).

2.5.2 Differentiation of Honeydew Honey and Blossom Honey

With higher market demand and consumption of honeydew honey, the differentiation between honeydew honey and blossom honey is needed to avoid adulteration and fraud (Pita-Calvo and Vaazquez, 2017). In order to ensure the authenticity and quality of both honeydew and blossom honey, relevant quality parameters for each type of honey are defined by regulatory organizations such as the Codex Alimentarius Commission (2001) and the Council Directive of the European Union (2002).

Melissopalynological analysis is a conventional method to determine the nectar source of honey based on the identification and quantification of pollens by microscopic examination (Aronne and De Micco, 2010). Although honeydew

honeys mainly contain gathered pollen of non-nectareous plants, there are small amounts of inevitable pollens from other nectareous flowers and air-borne pollens from other plants that accumulate on the surfaces. Hence, due to the limitations of melissopalynological analysis for the identification of honey sources, other methods have been suggested for a more accurate identification (Vasic *et al*., 2019).

A review has reported several comparative studies that successfully differentiate honeydew and blossom honeys using physicochemical parameters (Pita-Calvo and Vazquez, 2017). Olga *et al*. (2012) found that honeydew honeys from Northwest Spain are commonly dark or dark amber, with high electrical conductivity, pH, enzymatic activity, mineral content (especially potassium, calcium, magnesium and phosphorus) and low moisture content. Similarly, according to a study conducted by Bergamo *et al*. (2019), bracatinga (*Mimosa scabrella* Bentham) honeydew honey from Brazil was found to exhibit high free acidity, electrical conductivity and antioxidant activities, and low glucose content when compared to various blossom honeys. As summarized by Pita-Calvo and Vazquez (2017) and Seraglio *et al.* (2019), honeydew honey is usually darker in color, with lesser amounts of monosaccharides and higher values of acidity, electric conductivity, proteins and minerals. Greater biological activities of honeydew honey are explained due to the presence of higher levels of phenolic compounds especially flavonoids and phenolic acids (Vasic *et al*., 2019). Other than the phenolic profile, antioxidant capacity is also one of the possible authenticity assessments of honeydew honey. In addition, honeydew honey is said to possess higher antibacterial properties when compared to

blossom honey (Pita-Calvo and Vazquez, 2018).

Despite being present in low amounts, fungal spores can be found in honey (Olga *et al*., 2012). These spores are produced by plant pathogens particularly *Leptosphaeria* and *Stemphylium*, that grow over the leaves and green parts of plants. Such fungal elements can be introduced into honey when the bee collects honeydew from the infected plants. Hence, the presence of these biotic elements could be indicative of the identity of honeydew honey (Olga *et al.,* 2012).

The number of consumers who appreciate honeydew honey is definitely increasing. Growing demand for honeydew honey significantly contributes to its higher commercial value in the market. However, this specific type of honey, especially from stingless bees, is less studied and less understood than the blossom type. Furthermore, international quality parameters for honeydew honey are defined for honey produced by the Western honey bee (*A. mellifera*) (Codex Alimentarius, 2001; European Commission, 2002). Therefore, scientific research to obtain detailed knowledge of the composition and properties of stingless bee honeydew honey is very much needed. Furthermore, standardized methods for the authenticity and quality of honeydew honey produced by stingless bees are required to avoid adulteration and fraud (Pita-Calvo and Vazquez, 2018). The application of multivariate analysis to physicochemical parameters has been used in several studies to differentiate types of monofloral honey, honeydew and blossom honeys (Duarte *et al*., 2012; Flanjak *et al*., 2016; Kek *et al*., 2017a; Kek *et al*., 2017b).

Hence, this study was conducted not only to obtain physicochemical and antioxidant descriptions of the stingless bee honey, but also to determine the critical parameters that differentiate between honeydew and blossom type honeys based on multivariate analysis. Such analysis will be helpful to contribute in the setting of quality standards in the future.

2.7 Antibacterial Effects of Honey

The emergence of multi-drug resistant bacteria is reducing the effectiveness of antibiotic therapy for a range of infections, including respiratory tract and urinary tract infections (WHO, 2017). Many scientists consider plant products including honey to be important alternative sources of new and innovative antibacterial drugs against multi-drug resistant bacteria. Furthermore, plant natural product-antibiotic combinations are increasingly recognized as a promising strategy in tackling the issue of antibiotic resistance (Simoes *et al*., 2009; Ayaz *et al*., 2019).

As cited by Kwakman and Zaat (2012), the antibacterial activity of honey has been known since the 19th century. Recently, the potent inhibitory activity of honey has further increased the interest in application of honey to eradicate antibiotic-resistant bacterial infections. Using agar diffusion, agar dilution, broth microdilution and time-kill methodology, Australian *Trigona carbonaria* stingless bee honey was found to display greater and broader spectrum inhibitory activities than honey produced by *Apis mellifera* honey bee, against Gram-positive *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus*

pyogenes, *Streptococcus pneumoniae*, *Enterococcus faecalis*, and Gram-negative *Escherichia coli*, *Salmonella* serotype Typhimurium, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, but limited antifungal activity (Boorn *et al.*, 2010).

Nishio *et al*. (2016) demonstrated that two different honeys produced by *Scaptotrigona bipunctata* and *Scaptotrigona postica* stingless bees in Brazil possessed inhibitory activities against Gram-positive and Gram-negative bacteria, including multidrug-resistant strains. Although Gram-positive bacteria were more susceptible to the action of honey, no significant difference was observed with Gram-negative bacteria. The same study also showed a combination of two different stingless bee honeys exhibited greater antibacterial effects.

Chan-Rodriguez *et al*. (2012) also demonstrated that stingless bee honey from *Melipona beecheii* was found to be more effective as an antibacterial agent than honey bee honey produced by *Apis mellifera* honey because stingless bee honey was able to inhibit more bacteria species including *S. aureus* and *E. coli* at lower concentrations. Additionally, no particular bacterial susceptibility to honey between Gram- positive and Gram-negative bacteria was observed (Chan-Rodriguez *et al*., 2012). Ewnetu *et al*. (2013) also stated that both antibiotic sensitive and resistant strains of *S. aureus*, *E. coli* and *K. pneumoniae* were sensitive to the inhibitory effects of honey from Ethiopia. Of the three types of honey analysed, stingless bee honey was found to have the highest antibacterial activities compared to two other types of *Apis mellifera* honey. Due to different

floral sources, different antibacterial potency was observed in the two honey bee honeys (Ewnetu *et al*., 2013). Agar diffusion assays showed stingless bee honey possessed higher antibacterial potency of total and non-peroxide activities than other honey bee honeys, plus, the correlations between antibacterial effects of honey were proven to be dependent on bacterium species and honey origin (Zainol *et al*., 2013).

De Queiroz Pimentel *et al.* (2013) showed that, although honey produced by honey bees showed greater antibacterial activity than honey produced by stingless bee *Melipona compressipes manaosensis*, stingless bee honey showed broader spectrum antibacterial activities against Gram-positive and Gram-negative bacteria including *S. aureus*, *E. coli* (0157: H7), *Proteus vulgaris*, *Shigella sonnei* and *Klebsiella* sp. However, the antibacterial ability of honey was determined to be different among eight different stingless bee species (Rosli *et al*., 2020). At 50 % concentration, *Heterotrigona erythrogastra* honey showed no inhibitory effect at all; *Tetrigona bingami* and *G. thoracica* honeys only inhibited *S. aureus*; *Lepidotrigona terminata*, *Tetrigona apicalis* and *H. itama* honeys inhibited *S. aureus* and *Bacillus subtilis* while honeys produced by *Tetrigona melanoleuca* and *Homotrigona fimbriata* inhibited two Gram-positive bacteria species and three Gram-negative *E. coli*, *Serratia marcescens* and *Alcaligenes faecalis* (Rosli *et al*., 2020). Furthermore, Tuksitha *et al*. (2018) stated that stingless bee honey samples produced by *G. thoracica*, *H. itama* and *H. erythrogasta* that had been diluted fourfold were able to inhibit *S. aureus*, *Staphylococcus intermedius*, *Staphylococcus xylosus*, *Streptococcus alactolyticus*, *E. coli*, *Citrobacter koseri* and *P. aeruginosa*.

Manuka honey, originating from New Zealand, is well known for its excellent antibacterial activities (Stephens *et al*., 2010). Nevertheless, several studies on honeydew honey have indicated it to have greater inhibitory effects than manuka honey. Majtan *et al*. (2011) showed that the ability of honeydew honey to eradicate multidrug resistant *Stenotrophomonas maltophilia* clinical isolates was more efficient than manuka honey. In addition, honeydew honeys produced by honey bees demonstrated equivalent or, in some cases, higher inhibitory activity against *S. aureus* and *P. aeruginosa* than medical-grade manuka and kanuka honey (Bucekova *et al*., 2018). Generally, the antibacterial activity of honey is highly complex due to the presence of different compounds and due to the large variation in the levels of these compounds among honeys that are produced by different bee species and originate from different botanical sources (Kwakman and Zaat, 2012).

Honey also inhibits biofilm-embedded bacteria, which are 10–1000-fold more resistant to antibiotics than the more vulnerable form of planktonic bacteria (Ng *et al*. 2017; She *et al*. 2018). The antibiofilm activity of honey was reported with the ability of honey to repress curli, quorum sensing and virulence genes, thus preventing biofilm colonization and virulence of pathogenic *E. coli* O157:H7 (Lee *et al*. 2014).

2.7.1 Antibacterial Factors

The antibacterial activity in most honeys is ascribed to the glucose oxidase enzymatic production of hydrogen peroxide. However, another type of

honey, which is also known as non-peroxide honey, displays significant antibacterial effects even when the hydrogen peroxide is blocked or neutralized by catalase. Hence, non-peroxide antibacterial mechanism could be related to the hyperosmolarity and acidity of honey that hinder the growth of bacteria. Furthermore, phenolic compounds originating from plant nectar have also been proposed as important factors for the non-peroxide antibacterial activity of honey (Kwakman *et al.*, 2011; Mandal and Mandal, 2011). Several unique antibacterial compounds can be detected in certain honey types; for example methylglyoxal (MGO) and the antibacterial peptide bee defensin-1 were identified as important antibacterial factors in medical-grade manuka honey and Revamil honey, respectively (Kwakman and Zaat, 2012).

Ripened honey bee honey contains less than 18 % of moisture content, consisting of 80 % sugars, mainly the reducing sugars glucose and fructose and some sucrose. Hence, the high concentration of sugars combined with a low moisture content and water activity lead to osmotic stress and inhibit the growth of bacteria (Kwakman *et al.*, 2011). However, a study conducted by Nishio *et al*. (2016) showed that sugar syrup did not exhibit any inhibitory action on any bacterial species, indicating that hyperosmolarity does not play a major role in the antibacterial properties of honey. In any event, stingless bee honey generally has more than 20% moisture content, hence, the involvement of hyperosmolarity in the antibacterial effects of stingless bee honey should be less in this case.

Honey is acidic with pH between 3.2 and 4.5, mainly because of the conversion of glucose into gluconic acid by glucose oxidase. Such a pH is

believed to be low enough to be inhibitory to pathogenic bacteria (Koochak *et al*., 2010; Kwakman *et al*., 2011). The minimum pH values for the growth of *E. coli*, *P. aeruginosa*, *Salmonella* spp., *Shigella* spp., *S. aureus* and *S. pyogenes* are between 4.0 to 4.9; thus the acidity of undiluted honey can be a significant inhibitory factor. Kwakman *et al*. (2011) has conclusively shown the role of pH in the antibacterial activity of honey. In that study, together with neutralization of hydrogen peroxide, MGO and bee defensin-1, the pH level in the honey was titrated from 3.4 to 7.0, resulting in a diminished level of the bactericidal activity of honey to a level equivalent to that of sugar syrup.

As cited by Molan (1992), hydrogen peroxide that is also one of the products of glucose oxidase action, has been identified as a major antibacterial factor in honey since the 1960s. The role of hydrogen peroxide in honey is believed to prevent spoilage of unripe honey when the sugar concentration or osmolarity is not high enough to prevent microbial growth (Kwakman *et al.*, 2011). However, several studies have shown the antibacterial activities of some honey types are highly associated with non-peroxide factors. The involvement of hydrogen peroxide in the antibacterial activity of honey was assessed with the neutralization of this compound by catalase. The minimal inhibitory concentrations (MIC) of catalase-treated stingless bee honey were found to be higher than those of the non-treated honey samples, implicating the involvement of hydrogen peroxide in the antibacterial activity of stingless bee honey (Nishio *et al*., 2016).

However, a different study showed hydrogen peroxide played no essential role in the antibacterial activity of honey bee honey (Garedew *et al*., 2004). In addition, due to weaker acidity, the study demonstrated that the antibacterial activity of the honey bee honey was mainly due to the presence of phytochemicals (Garedew *et al*., 2004). Bucekova *et al*. (2018) noted that the antibacterial activity of honeydew honey is not highly dependent on hydrogen peroxide or the presence of bee-derived antibacterial peptide defensin-1. In fact the interaction of hydrogen peroxide with polyphenolic compounds was the main factor responsible for the antibacterial activity of honeydew honey.

Similarly, Boorn *et al*. (2010) explained the variation in antibacterial activity observed amongst the stingless bee honey samples tested, could be due to the differences of phytochemicals and bee-derived components, because there was no obvious correlation between antibacterial activity and any of the physicochemical properties examined; including pH, reducing sugar content and water content. Chan-Rodriguez *et al*. (2012) and de Queiroz Pimentel *et al*. (2013) also stated that antibacterial activities of both stingless bee honey and honeybee honey are not only related to osmolarity and acidity, but other chemical components that confer antibacterial properties to the honey. De Queiroz Pimentel *et al*. (2013) managed to identify the flavonoid rutin and other phenolic compounds that appear to be involved in the antibacterial effect of stingless bee honey. Tuksitha *et al*. (2018) also concluded that the significant antibacterial effect of stingless bee honey was strongly associated with the amount of phenolic and flavonoid compounds.

Methylglyoxal (MGO) and dihydroxyacetone (DHA) are the chemical markers of *Leptospermum* nectar. MGO, which is produced non-enzymatically from DHA is known to be associated with the non-peroxide antibacterial properties in manuka honey (Irish *et al*., 2011; Cokcetin *et al*., 2016). However, no MGO is detected in Australian stingless bee honey (Massaro *et al*., 2014). Although the hydrogen peroxide level of stingless bee honey was not high enough to achieve a bactericidal effect, the phenolic extracts of honey proved to be bactericidal against both *S. aureus* and *K. pneumoniae*. Therefore, the antibacterial effects of stingless bee honey could be partly ascribed to the hydrogen peroxide content, and the presence of other phenolic compounds including 3-phenyl lactic acid, lumichrome, diglycosylflavonoids and norisoprenoids (Zainol *et al*., 2013; Massaro *et al*., 2014). According to a study conducted by Deng *et al*. (2018), despite possessing a lower content of MGO than manuka honey, buckwheat honey which contained more phenolic compounds, was found to exhibit comparable antibacterial activity against *S. aureus* and *P. aeruginosa*. Among the phenolic compounds, *p*-hydroxybenzoic acid, chlorogenic acid and *p*-coumaric acid were present in higher levels in the buckwheat honey extracts than those in manuka honey.

Although phenolic compounds originating from plant nectar have been shown to be important non-peroxide factors for the antibacterial activity of honey, the amount of each individual compound identified in honey is too low to contribute substantially to antibacterial activity (Weston *et al*., 2000). Hence, a combination of different factors might contribute synergistically and substantially to the antibacterial activity of honey (Kwakman *et al*., 2011;

Kwakman and Zaat, 2012).

The coupled chemistry between hydrogen peroxide and phenolic compounds in honey, may exert oxidative effects that inhibit bacterial growth (Brudzynski *et al*., 2011; Brudzynski and Lannigan, 2012). Although phenolic compounds in honey are commonly known to exert antioxidant activities, the same oxidized phenolic compounds could become powerful pro-oxidants. Oxidized phenolic compounds further generate hydrogen peroxide which, in the presence of transition metals such as Fe^{2+}or Cu^{2+}, produces hydroxyl radicals through the Fenton reaction (Brudzynski and Lannigan, 2012). Together with hydrogen peroxide, hydroxyl radicals can oxidize cellular biomolecules. A study has demonstrated that the inhibitory effect of honey on methicillin resistant *S. aureus* (MRSA) and vancomycin resistant Enterococci (VRE) was dose-dependently associated with the generation of hydroxyl radicals from hydrogen peroxide (Brudzynski and Lannigan 2012). The study suggested that oxidative stress in bacteria could be attributed to the action of hydroxyl radicals, rather than from the action of hydrogen peroxide. Therefore, an association between the generation of hydroxyl radicals from hydrogen peroxide and bacterial growth inhibition has been tentatively established. Furthermore, the mechanism of action of hydroxyl radicals did not discriminate between antibiotic-sensitive and antibiotic-resistant bacteria (Brudzynski and Lannigan, 2012).

2.7.2 Interactive Effects Between Honey and Antibiotics

Due to the emergence of antibiotic resistant bacteria, the sustainability of

antibiotics in modern medical applications is in question. Multi-drug resistant bacteria or "superbugs" are generally resistant to three or more antibiotics (Styers *et al*., 2006), which limits the effectiveness of standard antibiotic therapy. Hence, combination antibiotic treatments are now widely practiced in the clinic to address antibiotic resistant bacteria. Such an approach results in enhancement and synergism with increased antibacterial efficacy and, at the same time reduces the amount of each antibiotic used, which can lower the risk of possible side effects including toxicities and treatment costs. In this context, synergism refers to interactions of two or more drugs that are significantly more effective than the sum of the individual parts (van Vuuren and Viljoen, 2011). Furthermore, the combination of antibiotics with different modes of action can minimize the development of antibiotic resistance (Wagner and Ulrich-Merzenich *et al*., 2009; Leibovici, 2009). This is particularly crucial for chronic wound infections because the antibiotic therapy often runs on long-term. Other than Gram-positive *S. aureus* and *Enterococcus* spp., *E. coli* is another most prevalent Gram-negative bacterium isolated from complicated skin and soft tissue infections (Kaye *et al.*, 2019).

As mentioned previously, there have been several reports about the antibacterial effects of various honey types that managed to inhibit various pathogenic bacteria, including antibiotic resistant strains. Moreover, no development of antibiotic resistance was observed after exposure of pathogenic bacteria including *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* to sub-inhibitory concentrations of honey (Cooper *et al*., 2010). A combination of antibiotics and honey has been suggested as an alternative antibacterial therapy

that might provide broader spectrum coverage and greater inhibitory effects, with the potential to prevent the emergence of resistant bacterial strains (Muller *et al*., 2013; Roberts *et al*., 2019).

For example, medical grade manuka honey has been shown to have a synergism with tetracycline, imipenem, mupirocin, oxacillin, gentamicin, clindamycin and rifampicin against MRSA and *S. aureus* biofilms (Jenkins and Cooper, 2012b; Muller *et al*., 2013; Liu *et al*., 2015; Liu *et al*., 2018). The combination of manuka honey with each rifampicin, tetracycline, colistin, ciprofloxacin, ceftazidime and tobramycin was also found to exhibit higher antibacterial activity against *P. aeruginosa* planktonic cells and biofilms (Jenkins and Cooper, 2012a; Roberts *et al*., 2019). The combination of manuka honey and oxacillin was shown to down-regulate the antibiotic resistant gene of MRSA and restored susceptibility towards beta-lactam antibiotics (Jenkins and Cooper, 2012b). However, the major antibacterial compound in manuka honey, methylglyoxal (MGO) did not act synergistically with rifampicin and is therefore not the contributing factor in the synergism (Muller *et al*., 2013).

Other than medical grade honey, a synergistic effect of a honey bee honey was also observed when it was combined with antibiotics including imipenem, ciprofloxacin, amoxicillin/ clavulinic acid, ceftriaxone, amikacin and aztreonam against *P. aeruginosa*, *Enterobacter* spp. and *Klebsiella* spp. The combination of the same honey with imipenem, ciprofloxacin, amoxicillin/ clavulinic acid and vancomycin was found to exert greater inhibitory effect against MRSA (Abd-El Aal *et al.*, 2007). A combination of stingless honey and ampicillin also exhibited

a higher degree of antibacterial activity against *S. aureus*, including antibiotic resistant strains. Significant morphological alterations were observed on *S. aureus* due to the action of both stingless bee honey and ampicillin (Ng *et al*., 2017). As supported by previous studies mentioned above, the combined use of honey and antibiotics presents a potential alternative for the treatment of chronic wounds and serious skin infections, to improve efficacy and minimize the development of antibiotic resistance (Muller *et al*., 2013).

Combination antibiotic therapies are widely practiced in the clinic to treat antibiotic resistant bacterial infections. Such an approach results in increased antibacterial efficacy, at the same time reducing the amount of each antibiotic used, which can lower the risk of possible side effects and treatment costs. Furthermore, combination use of antibiotics with different modes of action can minimize the development of antibiotic resistance (Wagner and Ulrich-Merzenich, 2009; Leibovici, 2009). Other than Gram-positive *S. aureus* and *Enterococcus* spp., *E. coli* is another prevalent Gram-negative bacteria isolated from complicated skin and soft tissue infections that often require long-term treatment (Kaye *et al.*, 2019).

2.7.3 Assessment of Antibacterial Effect

The determination of antibacterial activity in honey is always a hurdle when assessing and interpreting results because there is no standardized method of assessment. Different protocols have been used to examine the ability of antimicrobial agents to inhibit bacteria. Each method has its own advantages and

hence it depends on the nature of the antimicrobial agent as well as the kinetic characteristic of the molecules present. Generally, these protocols fall under the classification of agar diffusion and broth dilution methods (Balouiri *et al*., 2016).

The agar well diffusion method is widely used to evaluate the inhibitory activity of antibacterial agents. In this method, a bacterial inoculum is introduced by spreading it evenly over the entire agar surface. Then, a well with a diameter of 6 mm is punched aseptically with a sterile cork borer, followed by the introduction of the antibacterial agent into the well and whole medium incubated (Balouiri *et al*., 2016). Agar well diffusion has a relatively low sensitivity, because tested samples are further diluted as soon as they diffuse into the agar with the expanding area of the circle. However, due to the complexity of honey with various sizes of compounds and substances, this method is one of the most employed to determine antibacterial activity of honey. In addition, this method allows the rapid study of a large number of samples (Balouiri *et al*., 2016).

Agar well diffusion enables direct contact between honey constituents and the bacteria and hence this method imitates *in vivo* conditions similar to the way honey is applied onto wounds, providing information regarding the kinetic properties of honey application (Zainol *et al*., 2013). The agar well diffusion method determines the inhibitory effect of a honey with a zone of inhibition surrounding the well in which the honey sample was loaded (Szweda, 2017). Agar well diffusion is also the most commonly used method to estimate the antibacterial activity of medical-grade honey (Allen *et al*., 1991). The inhibitory activity of manuka honey is often assessed by this method with *S. aureus* as the

target microorganism. The antibacterial effect of manuka honey, which is expressed as unique manuka factor (UMF), represents the concentration of a phenol solution yielding a similar zone of growth inhibition (Allen *et al*., 1991). The agar well diffusion method is recognized to be a rapid and low-cost screening method to distinguish honey samples with and without antibacterial activity (Oses *et al*., 2016).

Broth dilution is also one of the most straightforward antibacterial susceptibility testing methods to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of a sample. The procedure involves preparing two-fold dilutions of the antibacterial agent in a liquid growth medium dispensed in tubes or a 96-well microtitration plate. Then, each tube or well is inoculated with a standardized microbial inoculum and incubated. The microbial growth can be determined using counting devices or colorimetric methods based on the use of dye reagents (Balouiri *et al*., 2016). However, the main disadvantages of the broth dilution method are the tedious analysis and risk of errors in the preparation of antibacterial solutions for each test (Reller *et al.*, 2009), especially honey samples with high viscosity. Hence, reproducibility can be a concern when the antibacterial effects of honey are assessed using the broth dilution method.

On the other hand, the destruction of bacteria, especially Gram-negative bacteria leads to the release of endotoxin from cell membrane, as demonstrated in *in vitro*, *in vivo* and clinical studies involving antibacterial agents (Prins *et al*., 1994; Trautmann *et al*., 1998; Braunwarth and Brill, 2014). Endotoxins can be

measured quantitively by a chromogenic limulus amoebocyte lysate (LAL) assay, which is a sensitive method that can reflect the biological activity of endotoxins (Prins *et al*., 1994). Since endotoxins are liberated from the compromised outer membrane of the cell wall in Gram-negative bacteria, the morphological changes in that bacterium can be further examined and verified by scanning electron microscopy (Crosby *et al*., 1994; Trautmann *et al*., 1998). Hence, the bactericidal effects of antibacterial agents including honey can be assessed by the measurement of endotoxin release.

Notably, the inhibitory effects of honey against different human pathogenic bacteria suggest that they are promising alternatives for future development of antibacterial agents against infections. As substantiated by promising findings from previous studies, honey-antibiotic combination therapy can be a promising strategy to combat antibiotic resistance issue.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Design

The general view of the study is summarized in Figure 3.1.

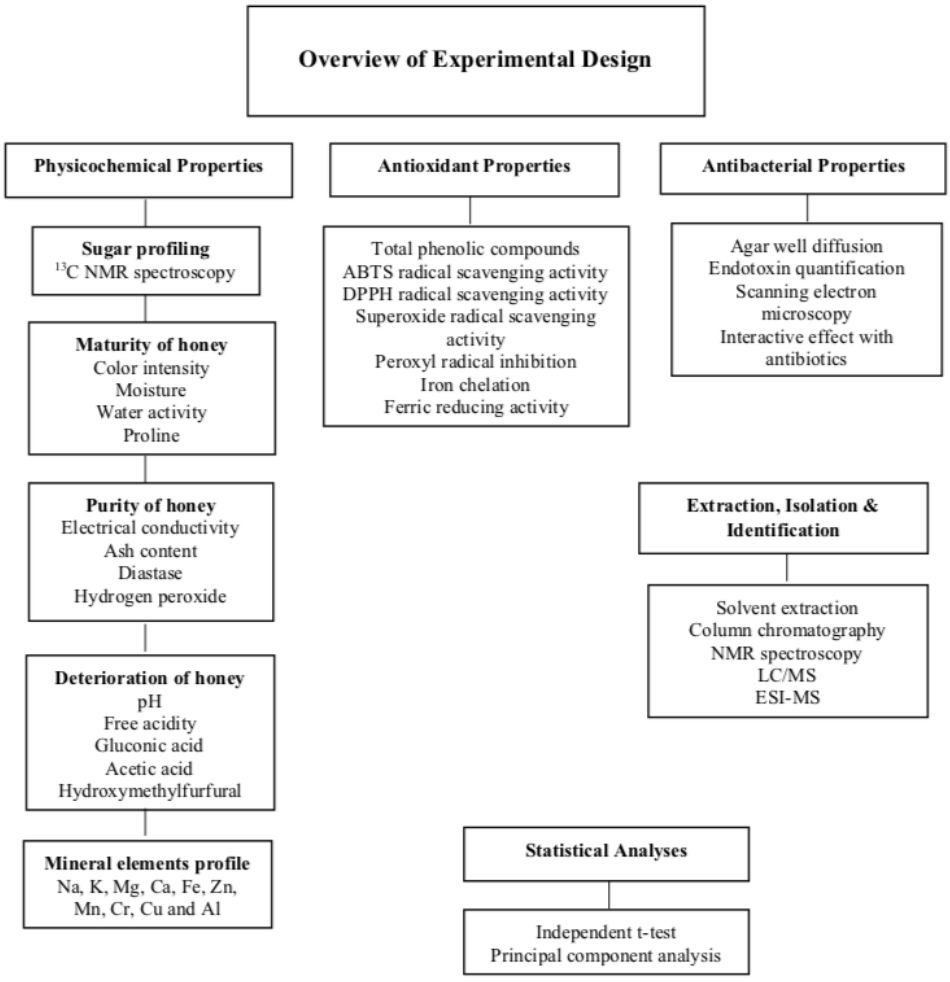


Figure 3.1: Overview of this study.

3.2 Materials

3.2.1 Honey Samples

Raw honey samples (n = 23) were harvested from jungles and secondary forests of Southern Negeri Sembilan, Northern Johor and South-Western Pahang in the Malaysian Peninsular (Figure 3.2). Stingless bee honey samples produced by *Heterotrigona itama* and *Geniotrigona thoracica* were collected from August 2016 to September 2018 (Table 3.1). Honey samples were manually filtered and bottled without heat treatment. All samples were kept at room temperature (23 - 26°C) prior to analysis.

Figure 3.2: Locations of honey sample collections in the Malaysian Peninsular.

Table 3.1: Bee type and origin information of honey samples.

Sample	Bee species	Nectar source	Origin	Collection
S1	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	August 2016
S2	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	November 2016
S3	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	April 2017
S4	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	July 2017
S5	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	September 2017

S6	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	April 2018
S7	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	July 2018
S8	*H. itama*	Acacia tree (*Acacia mangium*)	Honeydew	September 2018
S9	*H. itama*	Multifloral	Blossom	August 2016
S10	*H. itama*	Multifloral	Blossom	November 2016
S11	*H. itama*	Multifloral	Blossom	May 2017
S12	*H. itama*	Multifloral	Blossom	July 2017
S13	*H. itama*	Multifloral	Blossom	September 2017
S14	*H. itama*	Multifloral	Blossom	April 2018
S15	*H. itama*	Multifloral	Blossom	May 2018
S16	*H. itama*	Multifloral	Blossom	July 2018
S17	*G. thoracica*	Multifloral	Blossom	October 2016
S18	*G. thoracica*	Multifloral	Blossom	December 2016
S19	*G. thoracica*	Multifloral	Blossom	April 2017
S20	*G. thoracica*	Multifloral	Blossom	July 2017
S21	*G. thoracica*	Multifloral	Blossom	March 2018
S22	*G. thoracica*	Multifloral	Blossom	June 2018
S23	*G. thoracica*	Multifloral	Blossom	October 2018

3.2.2 Bacterial Samples

As listed in Table 3.2, reference strains of Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923 and ATCC 33591) and *Escherichia coli* (ATCC 25922 and ATCC 35218) provided by the Faculty of Science, UTAR were used for antibacterial evaluation of honey samples. Furthermore, in the investigation of interactive effect between honey and antibiotics, four identified clinical isolates of *E. coli* were used. These isolates were obtained from a private hospital located in Penang, Malaysia. *S. aureus* and *E. coli* were cultured and maintained on mannitol salt agar and MacConkey agar, respectively.

Table 3.2: Bacterium samples used.

Bacteria sample	Origin of isolate
S. aureus	Reference strain, ATCC 25923
S. aureus	Reference strain, ATCC 33591
E. coli	Reference strain, ATCC 25922
E. coli	Reference strain, ATCC 35218
E. coli 1	Clinical strain isolated from urine sample
E. coli 2	Clinical strain isolated from urine sample
E. coli 3	Clinical strain isolated from urine sample
E. coli 4	Clinical strain isolated from ascitic fluid

3.2.3 Chemicals

All the chemicals and media used in this study are listed in Table 3.3.

Table 3.3: List of chemicals used and respective manufacturers.

Chemicals	Manufacturers
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)	Roche Diagnostic, Germany
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich, Germany
2,4,6-tri(2-pyridyl)-s-triazine (TPTZ)	Nacalai Tesque, Japan
2-propanol	QReC, Singapore
3,5-dinitrosalicylic acid (DNSA)	Merck KGaA, Germany
Absolute ethanol	Systerm ChemAR, Poland
Acetic acid	QReC, Singapore
Acetic acid assay kit K-ACETRM 06/18	Megazyme, Ireland
Ampicillin sodium salt	VWR Life Science, USA
Amylazyme tablet	Megazyme, Ireland
Calcium chloride dihydrate	Merck KGaA, Germany
D-gluconic acid/ D-glucono -δ-lactone assay kit K_GATE 08/18	Megazyme, Ireland
D-glucose	SYSTERM, Malaysia
Ethylene glycol monoethyl ether	Nacalai Tesque, Japan
Ferric chloride	R&M, UK
Ferrous sulphate heptahydrate	R&M, UK
Ferrozine	Sigma-Aldrich, USA
Folin and Ciocalteu's phenol reagent	Merck KGaA, Germany
Formic acid	Fisher Co., UK
Gallic acid	Bio Basic, Canada
Gentamicin sulphate	Gold Technology, USA
Glacial acetic acid	QReC, Singapore
Glutaraldehyde	Sigma-Aldrich, USA
Hydrochloric acid, 37%	QReC, Singapore
Hydrogen peroxide, 30%	HmbG Chemicals, Germany
L-proline	Merck KgaA, Germany
MacConkey agar	Merck, Germany
Mannitol salt agar	Merck, Germany
Maleic acid	ACROS Organics, UK
Methanol	SYSTERM, Malaysia
Mueller-Hinton agar	CONDA, Spain
Mueller-Hinton broth	Scharlab, S.L., Spain

n-butanol	Systerm ChemAR, Poland
Nicotinamide adenine dinucleotide, disodium salt, trihydrate, reduced	Bio Basic, Canada
Ninhydrin	Nacalai Tesque, Japan
Nitroblue tetrazolium chloride	Bio Basic, Canada
Nutrient agar	Merck KgaA, Germany
OxiSelect™ ORAC activity assay kit	Cell Biolabs, USA
Phenazine methosulfate	Sigma-Aldrich, USA
Phosphate-buffered saline tablet	Takara Bio, Japan
Pierce™ quantitative peroxide assay kit	Thermo Scientific, USA
Potassium ferrocyanide	Merck KgaA, Germany
Potassium persulphate	SYSTERM, Malaysia
Potassium sodium tartrate tetrahydrate	Merck KgaA, Germany
QCL-1000™ Limulus Amebocyte Lysate	Lonza, USA
Sodium sulphite	SYSTERM, Malaysia
Sodium acetate trihydrate	Merck KgaA, Germany
Sodium bisulphite	Bio Basic Canada Inc, Canada
Sodium carbonate	R&M, UK
Sodium chloride	Merck KgaA, Germany
Sodium hydroxide	Merck KgaA, Germany
Trizma base	Sigma-Aldrich, USA
Zinc acetate	Merck KgaA, Germany

3.2.4 Equipment and Labware

All the equipment and labware used in this study are listed in Table 3.4.

Table 3.4: List of equipment and labware used and respective manufacturers.

Equipment/ Labware	Manufacturers
96-well plate, flat-bottomed	Becton Dickson, USA
Analytical balance	Copens Scientific, Malaysia
Beaker	GQ, Malaysia
Bunsen burner	CAMPINGANZ®, Netherlands
Centrifuge machine	Eppendorf, Malaysia
Centrifuge tube	Nest Lab, USA
Colorimeter	Technology & Services Sdn Bhd, Malaysia
Cotton swab	Premier Diagnostics, Malaysia
Cuvette	Greiner Bio-one, Malaysia
Electrical furnace	Nabertherm, Germany
Filter paper	Whatman TM, China
Flame atomic absorption spectroscopy	Agilent Technologies, USA
High resolution liquid chromatograph mass spectrometer (6520 Accurate-Mass Q-TOF LC/MS)	Agilent Technologies, USA
Incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Inoculating loop	HiMedia, India
Laminar flow cabinet	Streamline® Laboratories Products, Singapore
Melting point apparatus (Stuart SMP10)	Cole-Parmer Ltd., United Kingdom
Measuring cylinder	GQ, Malaysia
Microcentrifuge tubes	AXYGEN® Scientific, USA

Micropipette set	Hercuvan Lab Systems Inc., USA
Micropipette tips	Nest Lab, USA
Microplate reader (FLUOstar® Omega)	BMG Labtech, Germany
Multi parameter (PCSTestr™ 35)	Oakton, USA
NMR spectrometer (JNM-ECX400)	JEOL Ltd, Japan
Petri dish	Nest Lab, USA
pH meter	Sartorius, Germany
Refractometer	ATAGO, Japan
Refractometer for sugar	Hanna Instruments, USA
Scanning electron microscope (JSM-6701F)	JEOL, Japan
Spectrophotometer (CM-600d)	Konica Minolta, USA
Spectrophotometer (WPA Lightwave II UV)	Biochrom, UK
Schott bottle	DURAN, Germany
Vortex mixture	Gemmy Industrial Corp., Taiwan
Water activity meter	Novasina, Switzerland
Water bath	Memmert GmbH + Co.KG, Germany

3.3 Methods

3.3.1 Preparation of Solution and Reagent

2-propanol, 50 % (v/v)

2-propanol solution was prepared by mixing approximately 10 mL of 2-propanol (99.9 %) with 10 mL of distilled water.

2,4,6-tripyridyl-s-triazine (TPTZ), 10 mM

TPTZ solution was prepared by dissolving approximately 0.031 g of 2,4,6-tripyridyl-s-triazine (TPTZ) powder in 10 mL of 40 mM hydrochloric acid (HCl) in a water bath at 50 °C.

3,5-dinitrosalicyclic acid (DNSA)

DNSA solution was prepared by mixing approximately 1 g of 3,5-dinitrosalicyclic acid with 1 g of sodium hydroxide (NaOH) and 0.125 g of sodium sulfite with distilled water to reach a final volume of 100 mL.

ABTS stock solution, 7 mM

ABTS stock solution was prepared by dissolving approximately 0.384 g of ABTS in 100 mL of distilled water.

Acetate buffer, 30 mM

Acetate buffer was prepared by adding approximately 0.31 g of sodium acetate trihydrate into 1.6 mL of glacial acetic acid and topping up with distilled water to 100 mL. The pH value was adjusted to pH 3.6 with either sodium acetate trihydrate (99.5 %) or glacial acetic acid (99.8 %).

Acetic acid, 25 % (v/v)

Acetic acid solution was prepared by adding 25 mL of glacial acetic acid to 75 mL of distilled water.

Ampicillin solution

A stock solution with concentration of 10 mg/mL was prepared by dissolving 0.01 g of ampicillin sodium salt powder in 1 mL of distilled water. After that, 6.4 µL of ampicillin stock solution was added with distilled water to reach a final volume of 1 mL to achieve a concentration of 64 µg/mL.

Carrez solution I, 150 mg/mL

The reagent was prepared by dissolving approximately 3 g of potassium ferrocyanide in distilled water to make a final volume of 20 mL.

Carrez solution II, 300 mg/mL

The reagent was prepared by dissolving approximately 6 g of zinc acetate in distilled water to make a final volume of 20 mL solution.

Ethanol solution, 25 %, 50 %, 75 %, 95 % (v/v)

A 25 % ethanol solution was prepared by adding 25 mL of absolute ethanol to 75 mL of distilled water; 50 % ethanol solution was prepared by adding 50 mL of absolute ethanol to 50 mL of distilled water; 75% ethanol solution was prepared by adding 75 mL of absolute ethanol to 25 mL distilled water; 95 % (v/v) ethanol solution was prepared by adding 95 mL of absolute ethanol to 5 mL of distilled water.

Ferric chloride solution, 20 mM

Ferric chloride solution was prepared by dissolving approximately 0.054 g of ferric chloride in 10 mL of distilled water.

Ferric reducing antioxidant power (FRAP) reagent

FRAP reagent was prepared by mixing approximately a 10 mL aliquot of 30 mM acetate buffer (pH 3.6) with 1 mL of 10 mM TPTZ solution and 1 mL of 20 mM ferric chloride solution.

Ferrozine, 0.25 mM.

Ferrozine solution was prepared by dissolving 0.13 g of ferrozine in distilled water to make a final volume of 1 L solution.

Ferrous sulfate standard solution, 1 mM

Ferrous sulfate standard solution was prepared by dissolving approximately 0.028 g of ferrous sulfate heptahydrate in 100 mL of distilled water.

Formic acid, 50 % (v/v)

Formic acid solution was prepared by dissolving 25 mL of formic acid in distilled water and topping up to a final volume of 50 mL.

Gallic acid standard solution, 100 µg/mL

Gallic acid standard solution was prepared by dissolving 0.01 g of gallic acid in 100 mL of distilled water.

Gentamicin solution

A stock solution with concentration of 10 mg/mL was prepared by dissolving 0.01 g of gentamicin sulfate powder in 1 mL of distilled water. Then, a final concentration of 16 µg/mL was achieved by diluting 1.6 µL of gentamicin stock solution with distilled water to a final volume of 1 mL.

Glucose standard solution, 1000 µg/mL

Glucose standard solution was prepared by adding 0.05 g of glucose into 50 mL of distilled water.

Glutaraldehyde solution, 2.5 % (v/v)

Glutaraldehyde in 0.01 M phosphate buffer solution (PBS) was prepared by mixing 2.5 mL of glutaraldehyde with 97.5 mL of PBS.

Hydrochloric acid (HCl), 0.1 N

HCl solution was prepared by adding 8.3 mL of 37% aqueous HCl to distilled water to a final volume of 1 L.

Hydrogen peroxide standard solution, 1000 μM

Hydrogen peroxide standard solution was prepared by mixing distilled water with 11 μL of 30 % hydrogen peroxide to a final volume of 100 mL.

L-proline standard solution, 500 μg/mL

Proline standard solution was prepared by dissolving 0.25 g of L-proline in distilled water and then diluting to reach a final volume of 500 mL.

MacConkey agar

MacConkey agar was prepared by suspending 50 g of agar powder in 1 L of distilled water. The mixture was then autoclaved at 121 ˚C and 100 kPa for 2 hours. Approximately 25 mL of molten agar was poured into each sterile petri dish.

Mannitol salt agar (MSA)

MSA was prepared by suspending 111 g of agar powder in 1 L of distilled water. The medium was then autoclaved at 121 ˚C and 100 kPa for 2 hours. Approximately 25 mL of molten agar was poured into each sterile petri dish.

Methanolic DPPH solution, 0.024 mg/mL

Methanolic DPPH solution was prepared by adding approximately 0.1 mg of DPPH powder into 5 mL of methanol.

Mueller-Hinton agar (MHA)

MHA was prepared by suspending 38 g of agar powder in 1 L of distilled water. The mixture was then autoclaved at 121 ˚C and 100 kPa for 2 hours. Approximately 25 mL of molten agar was poured into each sterile petri dish.

Mueller-Hinton broth (MHB)

MHB was prepared by adding 21 g of MH broth powder into 1 L of distilled water. The medium was then sterilized by autoclaving at 121 ˚C and 100 kPa for 2 hours.

Nicotinamide adenine dinucleotide solution, 468 μM

NADH solution was prepared by dissolving 0.357 g of NADH in distilled water to reach final volume of 1 L.

Ninhydrin solution, 3 % (w/v)

Ninhydrin solution was prepared by mixing 3 g of ninhydrin with ethylene glycol monoethyl ether to reach a final volume of 100 mL.

Nitroblue tetrazolium (NBT) solution, 150 µM

NBT solution was prepared by dissolving 0.123 g of NBT in distilled water to reach final volume of 1 L.

Phenazine methosulfate (PMS) solution, 60 µM

PMS solution was prepared by dissolving 0.018 g of PMS in distilled water to reach a final volume of 1 L.

Phosphate-buffered saline (PBS)

PBS was prepared by dissolving one PBS tablet in 100 mL of distilled water.

Potassium persulfate, 140 mM

Potassium persulfate was prepared by dissolving 0.378 g of potassium persulfate in distilled water to reach a final volume of 10 mL.

Rochelle salt solution, 40 % (w/v)

Rochelle salt solution was prepared by dissolving approximately 2 g of potassium sodium tartrate tetrahydrate powder in distilled water to reach a final volume of 5 mL.

Reagents for acetic acid assay

As provided in acetic acid assay kit K-ACETRM 06/18 (Megazyme, Ireland), NAD+ plus ATP, polyvinylpyrrolidone (PVP) and coenzyme A were dissolved in 5.5 mL of distilled water. NADH, ATP, phosphoenolpyruvate (PEP) and PVP lyophilized powder were dissolved in 15 mL of distilled water. Coenzyme A lyophilized powder was dissolved in 0.8 mL of distilled water. Buffer (pH 7.4) and sodium azide (0.02 % w/v), D-lactate dehydrogenase, phosphotransacetylase and pyruvate kinase suspension, acetate kinase suspension and acetic acid standard solution (0.10 mg/mL) were used as supplied.

Reagents for D-gluconic acid assay

As provided in the D-gluconic acid/ D-glucono -δ-lactone assay kit K_GATE 08/18 (Megazyme, Ireland), NADP+ plus ATP was dissolved in 12.5 mL of distilled water. D-gluconic acid solution (0.25 g/L) was prepared by dissolving 0.25 g of sodium D-gluconate in 1 L of distilled water. Buffer (pH 7.6) plus sodium azide (0.02 % w/v), 6-phosphogluconate dehydrogenase suspension and gluconate kinase suspension were used as supplied.

Reagents for endotoxin assay

As provided in QCL-1000™ *Limulus Amebocyte* Lysate kit (Lonza, USA), *Limulus Amebocyte* Lysate (LAL) solution was prepared by reconstituting the lyophilized lysate with 1.4 ml of LAL reagent water. Endotoxin stock solution (24 EU/mL) was prepared by reconstituting the lyophilized endotoxin with 1.0 ml of LAL Reagent Water. The mixture was mixed for 15 minutes at high speed

on a vortex mixer. The chromogenic solution (2 mM) was prepared by reconstituting the lyophilized substrate with 6.5 ml of LAL Reagent Water.

Reagents for hydrogen peroxide assay

As provided in the Pierce™ quantitative peroxide assay kit (Thermo Fisher Scientific Inc., USA), a working reagent was prepared by mixing 50 µL of Reagent A (25 mM solution of ammonium ferrous (II) sulfate in 2.5 M H_2SO_4) with 5000 µL of Reagent B (100 mM sorbitol, 125 µM xylenol orange in water).

Reagents for oxygen radical antioxidant capacity assay

As provided in the OxiSelect™ ORAC activity assay kit (Cell Biolabs, USA), assay diluent was diluted to 1:4 with deionized water to prepare 1X assay diluent. The fluorescein probe was diluted to 1:100 with 1X assay diluent. The free radical initiator solution (80 mg/mL) was prepared by reconstituting 160 mg of free radical initiator powder with 2 mL of 1X PBS and mixing well.

Saline solution, 0.85 % (w/v)

Normal saline solution (100 mL) was prepared by dissolving 0.85 g of sodium chloride in distilled water. Then, the saline solution was autoclaved at 121 °C and 100 kPa for 2 hours.

Sodium carbonate, 10 % (w/v)

Sodium carbonate solution was prepared by dissolving 5 g of anhydrous sodium carbonate in distilled water to reach final volume of 50 mL.

Sodium bisulfite, 0.20 % (w/v)

Sodium bisulfite solution was prepared by dissolving 0.4 g of sodium bisulfite int distilled water to reach a final volume of 200 mL.

Sodium hydroxide (NaOH), 0.1 N

NaOH solution was prepared by dissolving 4 g of NaOH in distilled water to obtain a final volume of 1 L.

Sodium maleate buffer, 100 mM

Sodium maleate buffer was prepared by dissolving approximately 11.6 g of maleic acid and 0.735 g of calcium chloride dihydrate in distilled water and making up to a total volume of 800 mL.

Trizma base solution

Trizma base solution (900 mL) was prepared by dissolving approximately 20 g of Trizma base in distilled water.

3.3.2 Physicochemical Properties

Major sugar composition

For ^{13}C NMR analysis, 0.2 moles of each model compound solution (glucose, fructose and sucrose) were prepared in deuterated water (D_2O). Isoglucose (45 % glucose and 55 % fructose) and artificial honey (41.27 % glucose, 50.79 % fructose and 7.94 % sucrose) solutions were prepared with D_2O. Each honey sample was prepared by dissolving 200 µL (~260 mg) of honey

in 300 μL of D_2O. An internal reference, 0.2 moles of 1,4-dioxane (δ_C 67.19) was used for quantification. All samples were left overnight to equilibrate fully prior to analysis. ^{13}C NMR analysis was conducted on a JEOL JNM-ECX400 NMR spectrometer operating at 100 MHz for carbon-13 nuclei. ^{13}C NMR spectra of model compounds were obtained with a 90° pulse width 7.25 μs; relaxation delay 2 s, 1000-2000 scans and four pre-scans. For the isoglucose, artificial honey and honey samples, the number of scans was increased to 10,000, to achieve a better resolution and sensitivity.

The assignment of ^{13}C NMR chemical shifts of all sugar model compounds including glucose, fructose and sucrose and their respective tautomers was completed following the chemical shifts reported in the literature (Mazzoni *et al*., 1997; Kazalaki *et al*., 2015). Integration of ^{13}C signals allows the direct quantitative determination of tautomers of glucose, fructose and sucrose. The concentration of each sugar tautomer was calculated based on the signals that were unique for this particular tautomer and overlapped signals were not used for quantification (Mazzoni *et al*., 1997; Kazalaki *et al*., 2015). The quantification of the sugar molecules was achieved by integration of non-overlapping signals with the known concentration of 1,4-dioxane, the internal standard. The applicability of ^{13}C NMR method to quantify sugar molecules was validated by correlating the amount of glucose and fructose in isoglucose, and the amount of glucose, fructose and sucrose in the artificial honey determined by ^{13}C NMR with the actual weighed amount. The masses of all sugar compounds were calculated in g/100g, taking into account the moisture content in stingless bee honey.

Isolation and characterization of sugars

Raw stingless bee honeydew honey produced by *Heterotrigona itama* was first extracted with *n*-butanol in a ratio honey (1): butanol (2). The obtained crude extract was further extracted by liquid-liquid extraction, using chloroform and water, with ratio 1:1. The obtained water extract was freeze dried and subjected to Sephadex-LH 20 column chromatography with methanol as the mobile phase.

NMR spectra were obtained using a JNM-ECX400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. ^{1}H NMR spectra were obtained with a 45° pulse width 11.25 μs; relaxation delay 5 s, 1 prescan, 8 total scans. ^{13}C NMR spectra were obtained with a 30° pulse width 7.25 μs; relaxation delay 2 s, 4 prescans, 10,000 total scans. All chemical shifts are quoted in parts per million (ppm). The chemical shift scales are internally referred to the 1,4-dioxane singlets at 3.75 ppm and 67.19 ppm in D_2O for ^{1}H and ^{13}C, respectively. Structural elucidation of obtained pure compounds was assigned using 1D ^{1}H-NMR, 1D ^{13}C-NMR, 1D ^{13}C-DEPT, 2D Cosy, 2D HMQC and 2D HMBC techniques.

Molecular masses of compounds dissolved in H_2O were confirmed using an Agilent 6520 Accurate-Mass Q-TOF LC/MS, with methanol (7): water (3) (injection volume: 0.4 μL, 19 scans, 180.0 V). Dual electrospray ionization (ESI) technique (injection volume: 0.4 μL, 16 scans, 180.0 V) was used to fragment the sample. The sugar samples were dissolved in acetone until saturated and

followed by a drop of water. The solution was stored at 4 °C. Crystallization occurred after several days. The capillary melting temperature of the isolated sugar crystals was measured with a Stuart SMP10 melting point apparatus.

Color intensity

The color intensity of honey samples was determined according to the procedure of Beretta *et al*. (2005). Each honey sample was diluted to 50 % (w/v) with distilled water followed by filtration through a 0.45 µm filter. The absorbances were measured at 450 nm and 720 nm and the difference in the absorbance readings is expressed as mAU.

Moisture content

The moisture content of honey samples was measured by using a refractometer (Atago, Japan) and calculated using the following equation (AOAC, 1990):

Moisture (g/ 100 g, %) = 100 % - Total °Brix

Water activity

The water activity (a_w) of honey samples was measured using a water activity meter (Novasina, Switzerland).

Total soluble solids

The total soluble solid content of honey samples was measured using a digital refractometer for sugar (Hanna Instruments, USA). The honey sample was placed onto the measuring platform and the refractive index was measured against distilled water. The reading was recorded in % °Brix (g/100g).

Proline

The proline content in the honey samples was determined using an established method by Bogdanov *et al*., (2002). Firstly, 0.5 mL of honey solution (0.05 g/mL) was mixed with 1 mL of 50 % (v/v) formic acid and 1 mL of 3 % (w/v) ninhydrin solution. The mixture was then incubated in a boiling water bath for 15 minutes followed by incubation at 70 ºC for another 10 minutes. Then, 5 mL of 50 % (v/v) 2-propanol solution was added into the mixture following by immediate capping. The mixture was left to cool at room temperature (25 ºC) before the absorbance was measured at 510 nm. A standard curve was constructed using proline solution (100 - 500 µg/mL), and the proline level was calculated based on the equation obtained from the curve. The final value of proline content is expressed in milligram per a kilogram of honey (mg/kg).

Electrical conductivity

According to harmonized methods of the International Honey Commission (Bogdanov *et al.*, 2002), the electrical conductivity of the honey

solution (0.2 g/mL) was measured using a multiparameter tester (Oakton Instruments, USA) and the result is expressed as millisiemens per centimeter (mS/cm).

Ash content

Two grams of each honey sample were put in a porcelain crucible and dried in an oven at 110 °C for four hours. The crucible was then cooled in a desiccator for about four hours and weighed with the evaporated sample. The materials were then ashed in an electrical furnace (Nabertherm, Germany) at 600 °C for six hours, followed by cooling in a desiccator and then weighed. The ash content was calculated according to the following equation (Bogdanov *et al*., 2002):

Ash content % (g/100g) = (C-A)/(B-A) × 100

Where:

A = weight of the crucible

B = weight of crucible and sample after evaporation

C = weight of crucible and sample after ashing

Diastase

One milliliter of the honey solution (0.05 g/mL in 100 mM sodium maleate buffer) was incubated at 40 ºC in a water bath for 5 minutes. An amylazyme tablet (Megazyme, Ireland) was added to the honey solution and the mixture incubated for another 10 minutes followed by addition of 10 mL of

Trizma base solution. The mixture was then left at room temperature for 5 minutes, filtered and the absorbance of the sample solution was measured at 590 nm. The diastase activity of honey is expressed as the diastase number (DN), which indicates the amount of diastase that hydrolyses 1 mL of 1 % (w/v) starch solution using a gram of honey per hour at 40 ºC:

Diastase activity (Schade/gram of honey) = $(26.4 \times A_{590}) + 0.06$

Where:

A_{590} = absorbance value at 590 nm

Hydrogen peroxide assay

The assay was carried out by using an oxidation reaction assay (Thermo Fisher Scientific Inc., USA). To each 20 µL of 10 times diluted honey sample and hydrogen peroxide standard (7.81 – 1000 µmol/L) was added 200 µL of working reagent (ammonium ferrous (II) sulfate, sulfuric acid, sorbitol and xylenol orange) respectively. The mixture was incubated at room temperature for 20 minutes and the absorbance was determined at 595 nm. The average concentration of hydrogen peroxide in each honey sample was calculated in µmol/L with reference to the equation obtained from the standard curve.

pH

A honey solution containing 10 g of honey dissolved in 75 mL of distilled water was prepared, homogenized and the pH value was determined using a calibrated pH meter (Sartorius, Germany).

Free acidity

Ten grams of honey was dissolved in 75 mL of distilled water. Next, the pH was raised to 8.30 using 0.1 M of NaOH. Each titration was completed within 2 minutes. Free acidity was calculated with the following formula (Bogdanov *et al*., 2002):

Free acidity (mEq/kg) = *x* mL of 0.1 M NaOH × 10

Where:

x = volume of NaOH used

D-gluconic acid assay

Each honey sample and standard solution (10 µL) was diluted with 200 µL of distilled water, followed with addition of 20 µL buffer (pH 7.6) plus sodium azide (0.02 % w/v) and NADP+/ATP solution, respectively. Next, 2 µL of 6-phosphogluconate dehydrogenase suspension was added. After 4 minutes, the absorbance (A1) was measured at 340 nm. Lastly, 2 µL of gluconate kinase suspension was added. The absorbance (A2) was measured again at 340 nm after 6 minutes. The D-gluconic acid level was calculated with the following formula (Megazyme, Ireland):

D-gluconic acid level (g/L) = $A_{sample}/A_{standard}$ × standard × F

Where:

A_{sample} or $A_{standard}$ = A2-A1; F = dilution factor

Acetic acid assay

The assay was carried out using an enzymatic method (Megazyme, Ireland). Each honey sample and standard solution (10 µL) was diluted with 200 µL of distilled water, followed by addition of 20 µL buffer (pH 7.6) plus sodium azide (0.02 % w/v) and NADP/ATP/PEP/PVP solution, respectively. Next, 2 µL of each coenzyme A solution and a mixture of D-lactate dehydrogenase, phosphotransacetylase and pyruvate kinase was added. After 2 minutes, the absorbance (A1) was measured at 340 nm. Lastly, 2 µL of acetate kinase suspension was added. The absorbance (A2) was measured again at 340 nm after 4 minutes. The acetic acid level was calculated with the following formula:

Acetic acid level (g/L) = $A_{sample}/A_{standard}$ × standard × F

Where:

A_{sample} or $A_{standard}$ = A2-A1, F = dilution factor

Hydroxymethylfurfural assay

The assay was carried out by adding 0.2 g/mL of honey solution to Carrez solution I (150 mg/mL potassium ferrocyanide) and Carrez solution II (300 mg/ml zinc acetate). 5 mL of the mixture was diluted with the same volume of distilled water and 0.20 % (w/v) sodium bisulfite as the blank. The absorbance was measured at 284 nm and 336 nm. Hydroxymethylfurfural (HMF) content of honey samples was calculated using the following equation (Khalil *et al*., 2010):

HMF (mg/kg) = (A284 – A336) × 149.7 × D/W

Where:

A284 and A336 = the absorbance value at 284 nm and 336 nm, respectively

D = dilution factor

W = weight of honey sample (kg)

Mineral elements assay

Together with standards, the quantitative determination of minerals, including sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), zinc (Zn), manganese (Mn), chromium (Cr), copper (Cu) and aluminum (Al) in each honey sample was analysed by a flame atomic absorption spectroscopy (AAS) (Agilent Technologies, USA). Honey samples were digested with 70 % nitric acid and 30 % hydrogen peroxide before analysis. The final reading of each mineral is expressed in milligram per 1 kg of honey (mg/kg) (Vanhanen *et al.*, 2011).

3.3.3 Antioxidant Properties

Total phenolic compounds

The total phenolic composition in each honey sample was determined by using a modified Folin-Ciocalteu method (Khalil *et al*., 2012). To 0.5 mL of honey solution (0.2 g/mL) and 0.5 mL of gallic acid standard solution (200 – 1000 µg/mL) was added 0.5 mL of Folin and Ciocalteu phenol reagent. After 3 minutes, 0.5 mL of 10 % (w/v) sodium carbonate solution was added and the solution was topped up with distilled water to a final volume of 5 mL. The reaction mixture was incubated in the dark at room temperature for 90 minutes.

The absorbance of each reaction mixture was evaluated at 725 nm. The final value was calculated using the formula below:

Total phenolic compounds (mg GAE/kg) = C × V/M

Where:

C = concentration of gallic acid obtained from standard curve (mg/mL)

V = volume of honey sample used (mL)

M = mass of honey sample used (kg)

ABTS radical scavenging activity

The radical scavenging activity of each honey sample was measured by using a modified method proposed by Moniruzzaman *et al*. (2013a). The prepared ABTS radical cation ($ABTS^{+}$) solution was incubated in the dark at room temperature for 12 to 16 hours before use. Prior to the assay, the $ABTS^{+}$ solution was diluted with phosphate-buffered saline (PBS) to achieve an absorbance of 0.700 ± 0.020 at 734 nm. Then, approximately 200 µL aliquots of each honey solution (0.2 g/mL) were added to 4 mL of $ABTS^{+}$ solution. After 6 minutes, the reduction of absorbance was determined. A negative control was prepared by adding 200 µL of PBS instead of honey solution. The free radical scavenging activity was calculated by using the formula below:

Radical scavenging activity (% RSA) = $(A_b - A_a)/A_b \times 100$

Where:

A_a = absorbance of tested sample at sixth minutes;

A_b = absorbance of negative control at zero minutes.

DPPH radical scavenging activity

The radical scavenging activity of each honey sample was evaluated using the method of Ferreira *et al*. (2009). Approximately 0.5 mL of honey solution (0.2 g/mL) was added to 2.7 mL of a methanolic solution containing DPPH radical (0.024 mg/mL). A negative control was prepared with 2.7 mL of methanolic DPPH radical solution only. After 15 minutes in the dark at room temperature, the absorbance of the mixture was read at 517 nm. The free radical scavenging activity was calculated by using the formula below:

Radical scavenging activity (% RSA) = $[1 – (A_s/A_c)] \times 100$

Where:

A_s = absorbance of sample

A_c = absorbance of negative control

Superoxide anion radical scavenging activity

The radical scavenging activity of each honey sample was evaluated using the method proposed by Chai *et al*. (2014). To 1 mL of phenazine methosulfate solution (60 μM) was added to 2 mL of superoxide radical solution (1 mL of nitroblue tetrazolium, 150 μM and 1 mL of NADH, 468 μM) and 1 mL of honey solution (0.2 g/mL). After incubation at room temperature for 5 minutes, the absorbance was measured at 560 nm. A control was prepared by replacing the honey solution with distilled water. The free radical scavenging activity was calculated by using the formula below:

Radical scavenging activity (% RSA) = $(A_o – A_1)/A_o \times 100$

Where:

A_o = absorbance of the control

A_1 = absorbance of sample

Peroxy-radical inhibitory activity

The assay was carried out according to the method provided by Cell Biolabs, USA. In each microplate well, 25 µL of honey solution (0.2 g/mL) and Trolox standard solution was added together with 150 µL of fluorescein solution, followed by incubation for 30 minutes at 37°C. Then, 25 µL of peroxy-radical solution was added to each well. The absorbance of samples and standards was immediately read at and excitation wavelength of 480 nm and an emission wavelength of 520 nm, every 5 minutes for a total of 60 minutes. The antioxidant activity of each honey sample is expressed as µmol Trolox Equivalents (TE) per volume of sample (µmol TE/L) using the calculated area under the curve (AUC).

Iron chelating activity

The ferrous ion chelation potency of honey was studied using the ferrous ion-ferrozine complex method (Chai *et al*., 2014). The reaction mixture was prepared by addition of 0.2 mL honey solution (0.2 g/mL) to 0.2 mL of 0.10 mM ferrous sulfate and 0.4 mL of 0.25 mM ferrozine solutions. A control was prepared by replacing the honey solution with distilled water. After 10 minutes at room temperature, the absorbance was read at 562 nm. The chelating activity was calculated using the formula below:

Chelating activity (%) = $1 – (A_s/A_c) \times 100$

Where:

A_s = absorbance of sample

A_c = absorbance of the control

Ferric reducing activity

The ferric reducing antioxidant power **(**FRAP) assay was conducted according to a modified method proposed by Benzie and Strain (1999). A 1.5 mL aliquot of FRAP reagent was added to 200 µL of honey solution (0.2 g/mL) and ferrous sulfate standard solution (0.2 - 1.0 mmol/L), respectively. After, the reaction mixture was incubated at 37 °C for 4 minutes and the absorbance was read at 593 nm. The FRAP value was calculated using the formula below:

FRAP value (mmol Fe [II]/kg) = $C \times V/M$

Where:

C = concentration of ferrous sulfate obtained from standard curve (mmol/L);

V = volume of honey sample used (ml)

M = mass of honey sample used (kg)

3.3.4 Chemometric Analysis

Analyses were carried out in triplicates for each honey sample and conducted at room temperature (23 – 26 °C) unless stated otherwise. The data was expressed as a mean ± standard deviation. An independent t-test was performed to determine the significance of mean value differences at a level of

significance of 0.05 between honey samples based on botanical origins. Principal component analysis (PCA) was employed to interpret interdependence and visualize relatedness between data. Microsoft Excel Analyse-it Standard Edition v5.50 software was used to perform the statistical analyses.

3.3.5 Antibacterial Properties

Agar well diffusion

The inhibitory effect of each honey sample was evaluated in triplicate based on a modified agar well diffusion method of Boorn *et al*. (2010) and Mohapatra *et al*. (2011). Fresh overnight bacterial cultures of *S. aureus* (ATCC 25923 and ATCC 33591) and *E. coli* (ATCC 25922 and ATCC 35218) were inoculated with 8 mL of sterile 0.85 % normal saline. The turbidity of each bacterial suspension was adjusted to 0.5 McFarland (optical density reading 0.08-0.13 at the wavelength of 625 nm, which is equivalent to 1×10^8 CFU/mL (Andrews, 2009). The tip of a cotton swab was soaked in the bacterial suspension and pressed firmly to remove excess fluid. Then, the bacterial suspension was streaked evenly over the surface of agar. A sterile 6-mm diameter of cork borer was used to make a well in the center of the agar. Approximately 90 µL of honey sample was added into each well and the same volume of distilled water, which served as a negative control, was added into a third well (ampicillin solution, 10 µg/mL), serving as the positive control was added into different well. After the inoculation of samples, the agar plates were incubated at 37 °C overnight (16 – 20 hours). The diameter of zone of inhibition (if any) was measured to the nearest centimeter (cm).

Endotoxin quantification

The bactericidal effect of honey samples against *E. coli* was determined by measuring the level of endotoxin utilizing Limulus Amebocyte Lysate (LAL) (Lonza, USA). Firstly, a 0-hour sample was prepared by mixing 1800 µL of honey with 200 µL of 0.5 McFarland bacterial suspension. Then, a 24-hour sample was prepared by incubating 1 mL of the prepared mixture at 37˚C for 24 hours. Each 0-hour and 24-hour sample was adjusted to a pH range of 6.0 - 8.0 using sodium hydroxide (0.1 N) and hydrochloric acid (0.1 N) prior to assay. Next, a 50 µL of the sample or standard (0.0.125 – 1.0 EU/mL) were dispensed into an endotoxin-free reaction tube. A blank was prepared with the same volume of LAL reagent water. At time T = 0, 50 µL of LAL was added to each reaction tube, after 10 minutes, 100 µL of substrate solution which had prewarmed to 37 ˚C was added. At T = 16 minutes, acetic acid was added to stop the reaction. Absorbances were read at 410 nm. Each sample was assessed in triplicate and the average value was calculated. The endotoxin level is expressed as endotoxin units per milliliter (EU/mL). One EU equals approximately to 0.1 to 0.2 ng endotoxin/mL of solution.

Scanning electron microscopy

Prior to processing, 0.50 mL of 0.5 McFarland *E. coli* suspension was incubated with 4.50 mL of honey at 37 ˚C for 24 hours. The sample was then centrifuged at 3500 rpm for 5 minutes, the pellet was fixed with 2.5 % (v/v) glutaraldehyde in 0.01 M phosphate buffer solution (PBS) for overnight. The

sample was washed thrice for 10 minutes with 0.01 M PBS and subsequently by distilled water for another 10 minutes. The sample was dehydrated with ascending concentrations of ethanol solution, starting with 25 % (v/v) ethanol solution for 5 minutes followed by 50 % (v/v) ethanol solution for 10 minutes, 75 % (v/v) ethanol solution for 10 minutes, 95 % (v/v) ethanol solution for 10 minutes and lastly with absolute ethanol for 10 minutes. After dehydration, the sample was subjected to freeze drying for 24 hours. Thereafter, the sample was transferred to a carbon tape on copper stage, coated with platinum and viewed under a JEOL JSM-6701F scanning electron microscope. The steps were repeated for a negative control, by replacing honey with normal saline added to the bacterial suspension.

Determination of antibacterial factors

In order to determine the physicochemical properties that are mainly involved in antibacterial effects of honey, four solution samples including sugar solution (43 % fructose, 28 % glucose and 2.0 % sucrose, g/100g) (Cheng *et al*., 2019), hydrogen peroxide solution (184 µmol/L), hydrochloric acid solution (pH 3.3) and gallic acid solution (104 mg GAE/kg) were formulated based on the physicochemical properties of honeydew honey produced by *H. itama*. The inhibitory and bactericidal effects of these samples were also assessed with agar well diffusion method and endotoxin assays.

Interactive effect with antibiotics

A modified agar well diffusion method of Mohapatra *et al*. (2011) was performed to assess the interactive effect between honey and antibiotics. Each *E. coli* suspension with 0.5 McFarland was prepared as stated earlier. A cotton swab was used to streak the prepared bacterial suspension evenly over the surface of agar. Wells with a diameter of 0.6 cm were cut on the surface of the agar using a sterile cork borer. Each well was inoculated with 90 µL of honey (50 %, v/v), ampicillin (32 µg/mL), mixture of honey (50 %, v/v) and ampicillin (32 µg/mL), gentamicin (8 µg/mL), and mixture of honey (50 %, v/v) and gentamicin (8 µg/mL). Distilled water, as the negative control, was also inoculated in a separate well. The agar plates were incubated at 37 °C overnight (16 – 20 hours). After incubation, the diameter of zone of inhibition (if any) was measured to the nearest centimeter (cm).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physicochemical Properties

4.1.1 Major Sugar Composition

^{1}H NMR spectroscopy is commonly used in food analysis, however highly complex ^{1}H NMR spectra can complicate the interpretation and profiling of food metabolites including honey (Kazalaki *et al*., 2015). Such issues can be overcome by ^{13}C NMR spectroscopy that has been largely employed for structural studies. The ^{13}C NMR spectra obtained under proton decoupling have resonances spread over a larger chemical shift range and this makes it easier to identify the sugar resonances in honey (Kazalaki *et al*., 2015). To our best knowledge, the number of relevant scientific reports about using ^{13}C NMR for the analysis of sugars in honey is very limited.

The ^{13}C NMR chemical shifts of seven sugar tautomers (α-D-glucopyranose, β-D-glucopyranose, α-D-fructopyranose, β-D-fructopyranose, α-D-fructofuranose, β-D-fructofuranose and sucrose) relative to 1,4-dioxane are reported in Table 4.1. Both glucose and fructose are the major monosaccharides found in honey formed by the hydrolysis of sucrose by the enzyme invertase (da Silva *et al*., 2016). These reducing sugars undergo mutarotation and form two or more species known as tautomers. Mutarotation happens when the hemiketal

ring opens and closes, and with α or β configuration (Angyal, 1984; Angyal, 1991; Angyal, 1994). For example, D-glucose in aqueous solution appears in two forms, which are α-D-glucopyranose and β-D-glucopyranose; fructose appears in four forms in aqueous solution; namely α-D-fructopyranose, β-D-fructopyranose, α-D-fructofuranose and β-D-fructofuranose. Still, the composition of tautomers varies for each respective reducing sugar in different solvents (Angyal, 1984; Angyal, 1991; Angyal, 1994).

Table 4.1: ^{13}C chemical shifts of sugars in each model compound.

Sugar	Tautomer %	C1	C2	C3	C4	C5	C6	C1´	C2´	C3´	C4´	C5´	C6´
						Glucose							
α-D-glucopyranose (α-GP)	37.23	92.70	72.10	73.38	70.20	72.03	61.20	-	-	-	-	-	-
β-D-glucopyranose (β-GP)	62.77	96.52	74.74	76.37	70.26	76.55	61.36	-	-	-	-	-	-
						Fructose							
α-D-fructopyranose (α-FP)	3.64	65.75	-	-	70.64	71.07	61.70	-	-	-	-	-	-
β-D-fructopyranose (β-FP)	69.18	64.48	98.67	68.14	70.29	69.81	63.96	-	-	-	-	-	-
α-D-fructofuranose (α-FF)	5.97	63.52	105.03	82.58	76.62	81.88	61.68	-	-	-	-	-	-
β-D-fructofuranose (β-FF)	21.21	63.23	102.11	75.95	75.05	81.29	62.99	-	-	-	-	-	-
Sucrose	-	92.75	71.66	73.16	69.80	72.98	60.71	61.93	104.27	76.98	74.56	81.97	62.95

The ^{13}C NMR chemical shifts of isoglucose (45 % glucose and 55 % fructose) and artificial honey (41.27 % glucose, 50.79 % fructose and 7.94 % sucrose) are listed in Table 4.2. The comparison of actual concentration and ^{13}C NMR determined concentration of sugars and tautomers is also reported in Table 4.3. No significant changes in chemical shifts for each tautomer in isoglucose and artificial honey relative to the individual model compounds can be seen indicating that the ^{13}C chemical shifts of a sugar tautomer are not influenced by the presence of other sugars in a solution including honey (Mazzoni *et al*., 1997; Kazalaki *et al*., 2015). The ^{13}C NMR spectra of model compounds, isoglucose and artificial honey are provided in Figure 4.1.

Table 4.2(A): Assignment of the carbon resonances in the ^{13}C NMR spectra of isoglucose.

Sugar	Tautomer %	Chemical shift δ_C	Average integration value
Glucose			
α-D-glucopyranose (α-GP)	39.17	72.01, 72.07, 73.38, 92.68	0.2350
β-D-glucopyranose (β-GP)	60.83	74.72, 96.49	0.3650
Fructose			
α-D-fructopyranose (α-FP)	3.82	65.80, 70.71, 71.05	0.0268
β-D-fructopyranose (β-FP)	69.13	64.50, 68.16, 69.80, 98.65	0.4850
α-D-fructofuranose (α-FF)	5.20	63.54, 81.87, 82.56, 105.03	0.0345
β-D-fructofuranose (β-FF)	21.85	63.00, 63.29, 75.05, 75.98, 81.26, 102.10	0.1533

Table 4.2(B): Assignment of the carbon resonances in the ^{13}C NMR spectra of artificial honey mixture.

Sugar	Tautomer %	Chemical shift δ_C	Average integration value
Glucose			
α-D-glucopyranose (α-GP)	38.46	72.01, 72.08, 73.37	0.15
β-D-glucopyranose (β-GP)	61.64	96.50	0.24
Fructose			
α-D-fructopyranose (α-FP)	2.51	65.77	0.0106
β-D-fructopyranose (β-FP)	70.87	64.49, 68.15, 98.65	0.3000
α-D-fructofuranose (α-FF)	5.32	63.53, 82.60, 105.02	0.0225
β-D-fructofuranose (β-FF)	21.31	63.27, 75.05, 75.98, 81.27, 102.10	0.0902
Sucrose	-	60.73, 71.64, 72.97, 73.16, 76.97, 104.26	0.0357

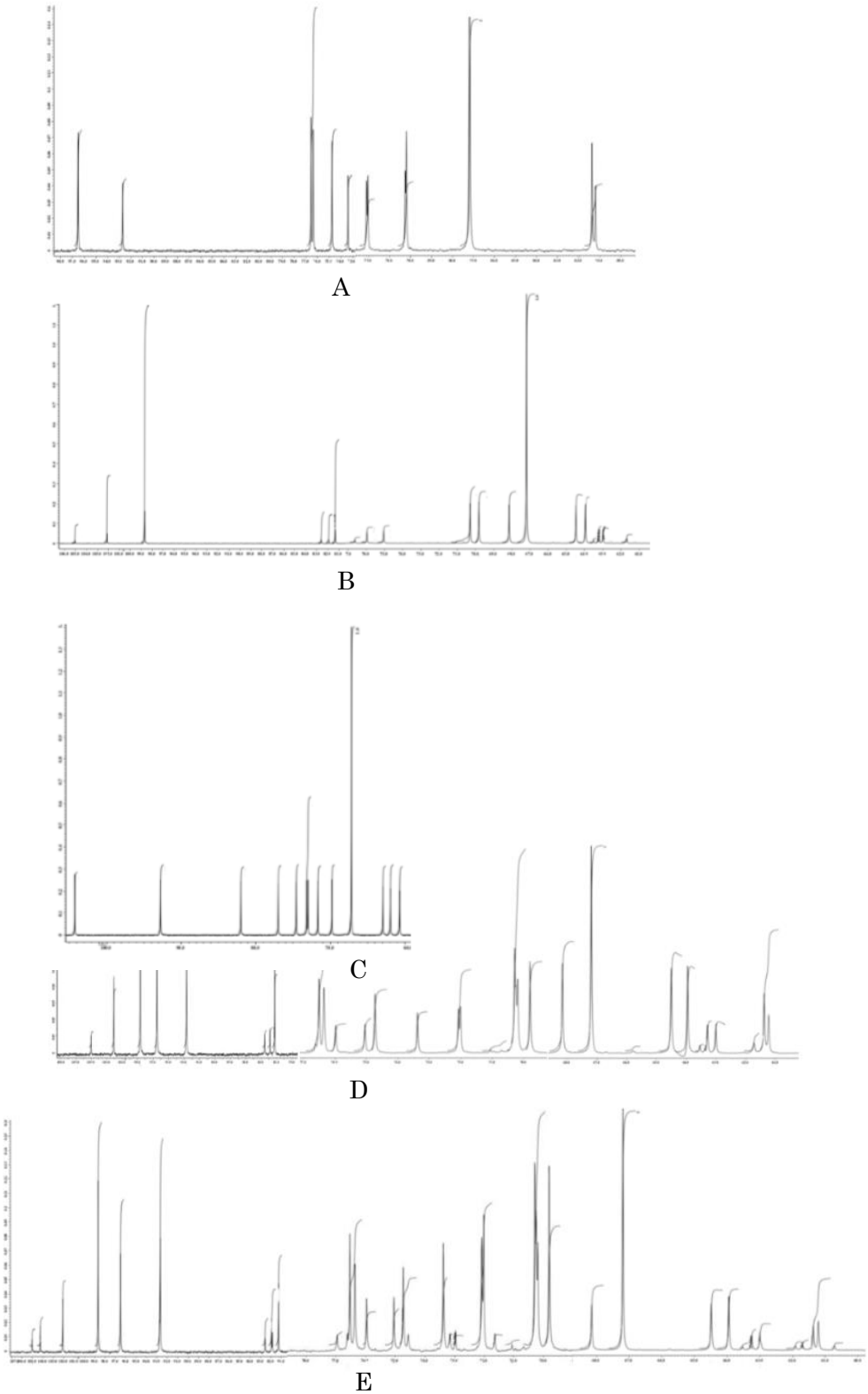


Figure 4.1: ^{13}C chemical shifts of glucose (A), fructose (B) and sucrose (C) model compound, isoglucose (D) and artificial honey (E).

Table 4.3(A): Comparison of the measured amount (%, g/100 g) of each tautomer of D-fructose and D-glucose in the isoglucose mixture from the integration of the ^{13}C NMR signals to that of the actual weight amount.

Sugar	Actual concentration	Concentration determined by ^{13}C NMR	Similarity (%)
α-D-glucopyranose (α-GP)	17.63	18.08	97.45
β-D-glucopyranose (β-GP)	27.37	28.09	97.37
Total glucose	45.00	46.17	97.40
α-D-fructopyranose (α-FP)	2.10	2.06	98.10
β-D-fructopyranose (β-FP)	38.02	37.21	97.87
α-D-fructofuranose (α-FF)	2.86	2.80	97.90
β-D-fructofuranose (β-FF)	12.02	11.76	97.84
Total fructose	55.00	53.83	97.87

Table 4.3(B): Comparison of the measured amount (%, g/100 g) of each tautomer of D-fructose, D-glucose and sucrose in the artificial honey mixture from the integration of the ^{13}C NMR signals to that of the actual weight amount.

Sugar	Actual concentration	Concentration determined by ^{13}C NMR	Similarity (%)
α-D-glucopyranose (α-GP)	15.87	17.02	92.75
β-D-glucopyranose (β-GP)	25.44	27.28	92.77
Total glucose	41.27	44.26	92.76
α-D-fructopyranose (α-FP)	1.27	1.21	95.28
β-D-fructopyranose (β-FP)	35.99	34.05	94.61
α-D-fructofuranose (α-FF)	2.70	2.56	94.81
β-D-fructofuranose (β-FF)	10.82	10.24	94.64
Total fructose	50.79	48.04	94.59
Total sucrose	7.94	7.69	96.85

As shown in Table 4.4, the assignment of all carbon signals for honeydew and blossom honey is based on the assigned ^{13}C NMR spectra of the model compounds, isoglucose and artificial honey. The ^{13}C NMR spectra of the honey samples are provided in Figure 4.2.

Table 4.4(A): Assignment of the carbon resonances in the ^{13}C NMR spectra of stingless bee honeydew honey.

Sugar	Tautomer %	Chemical shift δ_C	Average integration value
Glucose			
α-D-glucopyranose (α-GP)	41.70	72.00, 72.09, 73.41, 92.68	0.2825
β-D-glucopyranose (β-GP)	58.30	74.74, 96.50	0.3950
Fructose			
α-D-fructopyranose (α-FP)	2.59	65.82	0.0188
β-D-fructopyranose (β-FP)	69.59	63.95, 64.53, 68.18, 98.66	0.5050
α-D-fructofuranose (α-FF)	6.01	63.59, 81.86, 82.58, 105.03	0.0436
β-D-fructofuranose (β-FF)	21.81	63.03, 63.33, 75.07, 76.01, 81.25, 102.11	0.1583
Sucrose	-	-	-

Table 4.4(B): Assignment of the carbon resonances in the ^{13}C NMR spectra of stingless bee blossom honey produced by *H. itama*.

Sugar	Tautomer %	Chemical shift δ_C	Average integration value
Glucose			
α-D-glucopyranose (α-GP)	40.87	72.00, 72.09, 92.68	0.2500
β-D-glucopyranose (β-GP)	59.13	74.74, 76.36, 76.48, 96.50	0.3725
Fructose			
α-D-fructopyranose (α-FP)	4.17	65.81, 70.73, 71.07	0.0286
β-D-fructopyranose (β-FP)	68.47	63.95, 64.54, 68.19, 98.65	0.47
α-D-fructofuranose (α-FF)	6.24	63.59, 76.64, 82.58, 105.03	0.0428
β-D-fructofuranose (β-FF)	21.12	63.02, 63.35, 75.08, 76.03, 81.25, 102.11	0.145
Sucrose	-	104.24	0.0075

Table 4.4(C): Assignment of the carbon resonances in the ^{13}C NMR spectra of stingless bee blossom honey produced by *G. thoracica*.

Sugar	Tautomer %	Chemical shift δ_C	Average integration value
Glucose			
α-D-glucopyranose (α-GP)	40.16	72.01, 72.10, 73.41, 92.69	0.2450
β-D-glucopyranose (β-GP)	59.84	74.75, 76.37, 76.49, 96.51	0.3650
Fructose			
α-D-fructopyranose (α-FP)	4.17	70.73, 71.07	0.0270
β-D-fructopyranose (β-FP)	69.50	63.96, 64.54, 68.19, 98.66	0.4500
α-D-fructofuranose (α-FF)	6.16	63.60, 76.64, 81.87, 82.59, 105.04	0.0399
β-D-fructofuranose (β-FF)	20.17	63.03, 63.34, 75.08, 76.02, 81.26, 102.11	0.1306
Sucrose	-	-	-

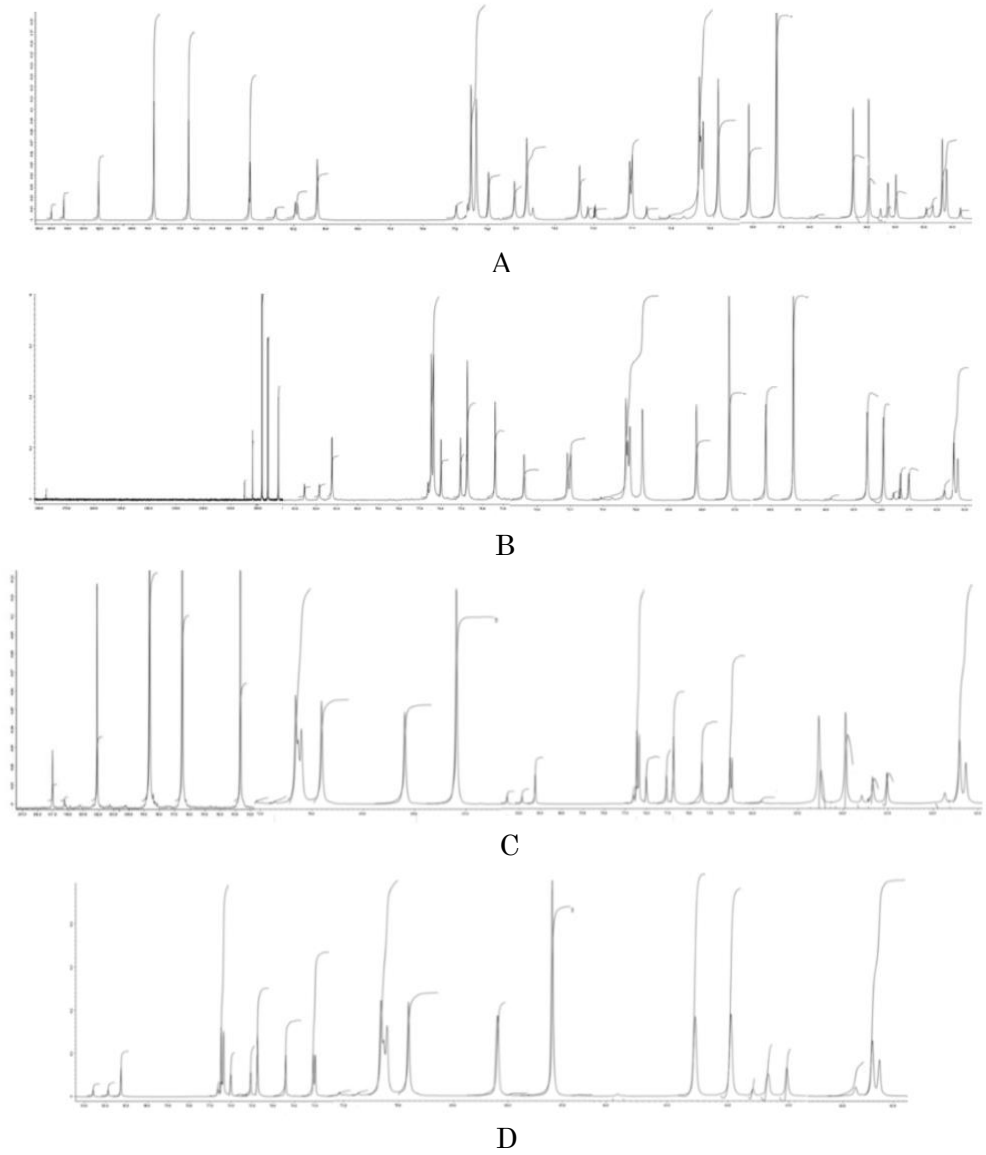


Figure 4.2: ^{13}C NMR spectra of stingless bee honey samples originating from (A-B) honeydew and (C-D) blossom.

Table 4.5 summarizes the average concentration of each tautomer in honeydew and blossom honey. The content of sucrose in honeydew honey was too low to be integrated by ^{13}C NMR spectroscopy. Although the average amount of major sugars in honeydew honey (73 %) was lower than blossom honey (74 %), there was no significant difference in the total content of glucose and fructose between these two types of stingless bee honey. The composition of tautomers in stingless bee honey obtained in this study is in agreement with the literature values. For instance, the composition of the two glucose tautomers α-

D-glucopyranose and β-D-glucopyranose ranged from 9.9 to 16.1 g/100g and from 14.8 to 26.5 g/100g, respectively; while the composition of the four fructose tautomers α-D-fructopyranose, β-D-fructopyranose, α-D-fructofuranose and β-D-fructofuranose from non-detected to 1.1 g/100g, from 19.4 to 25.8 g/100g, from 1.5 to 4.8 g/100g, from 7.0 to 13.3 g/100g in Greek honey samples (Kazalaki *et al*., 2015).

Table 4.5: Quantification of sugar tautomers present in stingless bee honey samples using ^{13}C NMR spectroscopy.

Sugar	**Honeydew honey**			**Blossom honey**		
	Tautomer %	Average integration value	g/100g	Tautomer %	Average integration value	g/100g
α-D-glucopyranose (α-GP)	41.70	0.2825	14.41 ± 0.42	40.50	0.2475	14.45 ± 0.31
β-D-glucopyranose (β-GP)	58.30	0.3950	20.99 ± 0.62	59.50	0.3688	20.96 ± 0.52
Total glucose			35.39 ± 0.20			35.36 ± 0.76
α-D-fructopyranose (α-FP)	2.59	0.0188	1.02 ± 0.05	4.17	0.0278	1.60 ± 0.01
β-D-fructopyranose (β-FP)	69.59	0.5050	25.99 ± 0.40	68.99	0.4600	26.19 ± 0.14
α-D-fructofuranose (α-FF)	6.01	0.0436	2.29 ± 0.02	6.20	0.0414	2.39 ± 0.01
β-D-fructofuranose (β-FF)	21.81	0.1583	8.32 ± 0.12	20.65	0.1378	8.08 ± 0.04
Total fructose			37.61 ± 0.20			38.25 ± 0.21
Total sucrose	-	-	-	-	0.0038	0.40 ± 0.57
Fructose to glucose ratio (F/G)			1.06 ± 0.01			1.08 ± 0.03
Glucose to moisture ratio (G/M)			1.31 ± 0.01			1.38 ± 0.03

Each total content of glucose and fructose between honeydew honey and blossom honey was not significantly different at $p < 0.05$.

The reducing sugar content in stingless bee honey samples ranging from 73 to 74 % also met the requirement set by the Department of Standards Malaysia (2017), which is not more than 85.0 %. Honeydew honey was found to possess very minimal sucrose by ^{13}C NMR spectroscopy and sucrose content obtained from blossom honey in this study (0.4 %) met the requirement established, which is not more than 9.5 %. The values obtained by other authors in stingless bee honeys also met this requirement (Nweze *et al*., 2017; Shamsudin *et al*., 2019).

Disaccharide trehalulose (Figure 4.3) was previously identified as maltose in several studies (Vit *et al*., 1998; Oddo *et al*., 2008; Tuksitha *et al*., 2015; Shamsudin *et al*., 2019). Fletcher *et al*. (2020) was the first who reported that this trehalulose is a major sugar component (from 13 to 44 g/100g) of stingless bee honey. In this previous study, NMR and UPLC-MS/MS analyses uniformly confirmed the identity of trehalulose isolated from stingless bee honey samples, from *Tetragonula carbonaria* and *Tetragonula hockingsi* in Australia, from *Tetragonisca angustula* in Brazil and from *H. itama* and *G. thoracica* in Malaysia. Thus, trehalulose was suggested as an indicator of authenticity to be incorporated in the development of stingless bee honey standards. Hungerford *et al*. (2021) concluded that nectar high in sucrose will result in stingless bee honey high in trehalulose.

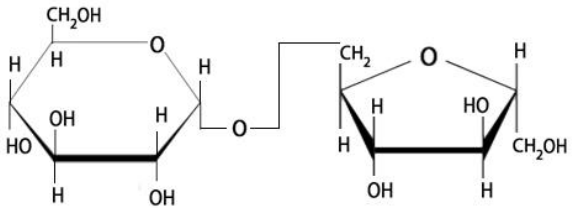


Figure 4.3: Chemical structure of trehalulose.

However, the presence of trehalulose in tested stingless bee honey samples was not detected by ^{13}C NMR spectroscopy. The chemical shift for C-1 of the α-D-glucopyranose unit in trehalulose (99.3 - 101.3 ppm) (Bates *et al*., 1990; Thompson *et al*., 2001; Fletcher *et al*., 2020) was not observed in stingless bee honey samples in this study. Besides, the obtained chemical shift for C-1 of α-D-glucopyranose tautomer was consistent with the value from a standard glucose compound as that reported in another study (92.7 ppm) (Mazzoni *et al*., 1997). Contrary to an earlier study (Fletcher *et al*., 2020), such a disaccharide

could be absent or present only in a very low quantity in the tested stingless bee honey samples produced by *H. itama* and *G. thoracica*. This could be due to a low sucrose content in the nectar foraged by the stingless bees (Hungerford *et al.,* 2021). Sucrose transformation to trehalulose is more prevalent than is observed for stingless bees than honey bees. The same study also stated stingless bees were unable to form threhalulose directly from glucose and fructose (Hungerford *et al*., 2021).

It has been reported that honeydew honey presents a lower value of the sum of glucose plus fructose than blossom honey because blossom honey is usually measured with a higher glucose content (Manzanares *et al*., 2011; Pita-Calvo *et al*., 2017). However, this study and those of other authors showed different results in which there were no significant differences for both types of honey (Vela *et al*., 2007). Glucose tends to precipitate and crystalize because it is less water soluble than fructose. The tendency of crystallization in honey can be evaluated based on the fructose to glucose ratio (F/G) and the glucose to moisture ratio (G/M). According to a previous study, F/G not more than 1.14 is associated with honey crystallization; whereas a value of 1.33 and above indicates a slower crystallization (Smanalieva and Senge, 2009). Other authors have suggested that the G/M ratio could be a better indicator for honey crystallization with values not more than 1.7 indicating slow or no crystallizations, while higher values up to 2 suggest rapid crystallization (Manikis and Thrasivoulou, 2001; Manzanares *et al*., 2011; Pita-Calvo *et al*., 2017). In this study, the F/G values of honeydew and blossom honeys were 1.06 and 1.08, respectively, whereas the G/M values of honeydew and blossom

honeys were 1.31 and 1.38, respectively. Hence, these values suggest a slower crystallization of both honeydew and blossom honeys. Although the average value of total sugars in honeydew honey (73 %) was lower than blossom honey (74 %), there was no significant difference in the total content of glucose and fructose between honeydew honey and blossom honey. Hence, the major sugar composition is suggested to be consistent among stingless bee honey.

4.1.2 Identification and Characterization of *n*-butyl β-D-glucopyranoside

In order to study the major sugar compounds in stingless bee honeydew honey, raw honey sample was first extracted with *n*-butanol, then the obtained crude extract was further subjected to a liquid-liquid partition using chloroform and water. As the sugar compounds under study are relatively polar, Sephadex-LH 20 was utilized to separate the sugars with methanol as the eluant. One pure compound was obtained as white crystals, with a capillary melting point of 52 - 54 °C. The ^{1}H and ^{13}C NMR spectra of this compound resembled those of glucose but exhibited extra signals shown in Figures 4.4 and 4.5.

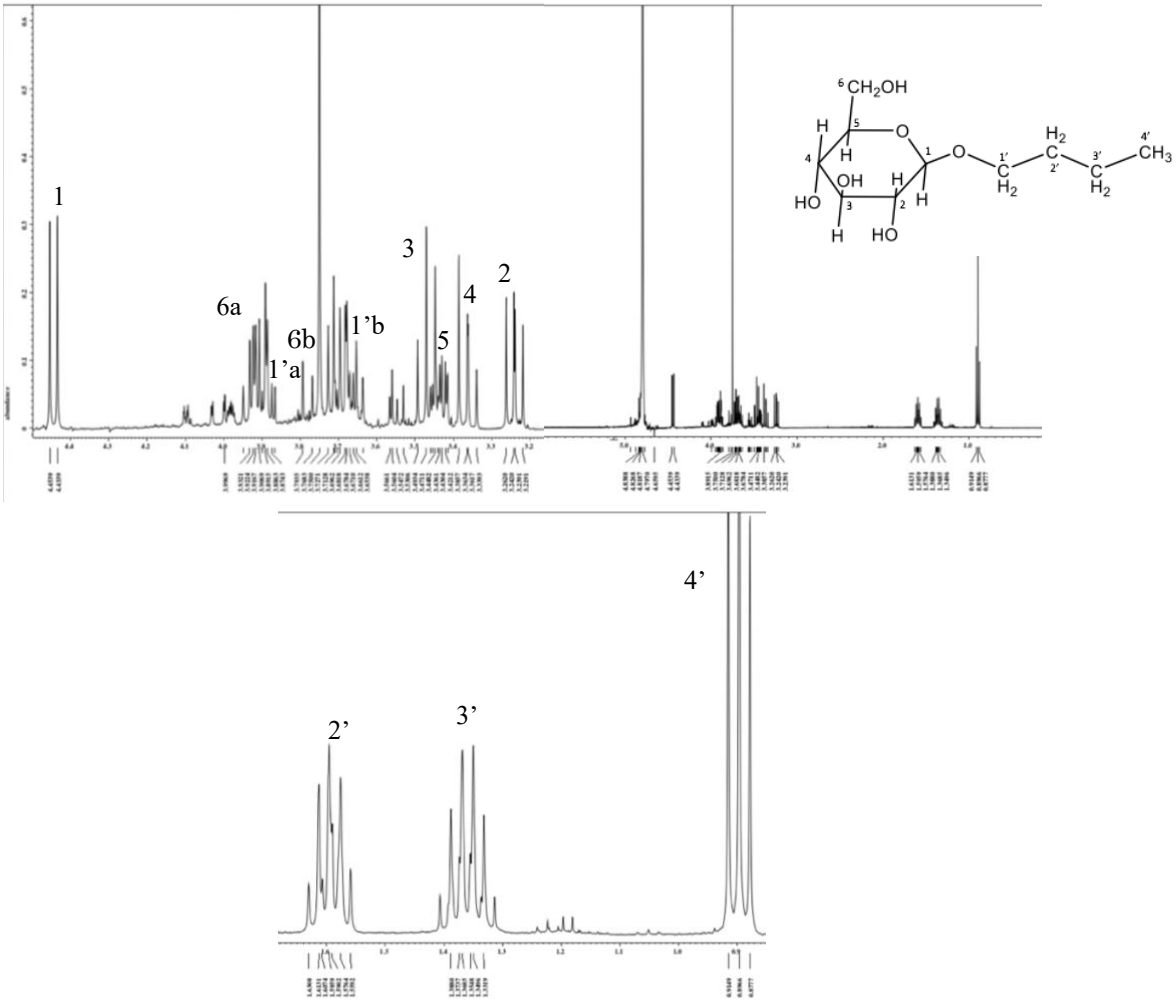


Figure 4.4: ^{1}H NMR spectrum of butylated glucoside isolated from stingless bee honey.

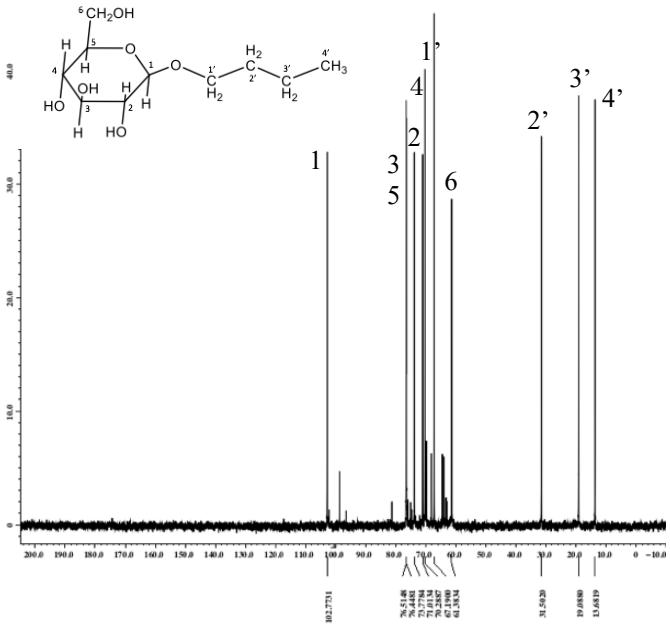


Figure 4.5: ^{13}C NMR spectrum of butylated glucoside isolated from stingless bee honey.

Based on the ^{1}H and ^{13}C NMR spectra shown above, it can be seen that extra resonances appear in the upfield region compared to those of glucose indicating possible substitution of a proton with an alkyl group; whereas the other signals of glucose seem to be intact. Peaks at δ_H (multiplicity); 0.90 (triplet), 1.36 (sextet), 1.60 (quintet), and a pair of double triplets at a lower field of 3.67 and 3.88 respectively suggested the presence of an *n*-butyl group in the molecule. This proposition was further supported by the corresponding resonances in the ^{13}C NMR spectrum at δ_C 13.7, 19.1, 31.5 and 71.0 respectively. The resonances of the above highlighted signals at the lower field indicate the presence of an electronegative atom nearby and, based on the structure of glucose and the C-13 chemical shifts this could only point to an oxygen atom. Since the data taken together suggest the compound as a butyl glucoside, the next question was to determine the configuration of this glucoside. This could be achieved unequivocally because both signals H-1 and H-2 do not overlap with the other signals. H-1 appears as a doublet with a coupling constant of 8.0 Hz

which is possible only if the butoxy group is in a β configuration. The full structure was solved by analysis of data from 2D Cosy, 2D HMQC and 2D HMBC techniques (Appendix 1) and the assignments are summarized in Tables 4.6 and 4.7. The chemical shift values obtained are similar to those reported by Matin *et al* (2013) and Kazalaki *et al* (2015). Figure 4.6 illustrates the structure of *n*-butyl β-D-glucopyranoside.

Table 4.6: ^{1}H NMR spectrum of isolated butylated glucoside.

Proton	Chemical shift δ_H (ppm)	Literature value Matin et al (2013)
H-1	4.44 (*J* = 8.0 Hz, *d*)	4.43 (*J* = 8.0 Hz, *d*)
H-2	3.24 (*J* = 9.2, 8.0 Hz, *dd*)	3.24 (*J* = 9.6, 7.9 Hz, *dd*)
H-3	3.47 (*J* = 9.2 Hz, *t*)	3.47 (*J* = 9.6 Hz, *t*)
H-4	3.36 (*J* = 9.6, 9.2 Hz, *dd*)	3.36 (*J* = 9.8, 9.6 Hz, *dd*)
H-5	3.44 (*J* = 9.6, 6.2, 2.3 Hz, *ddd*)	3.41 (*J* = 9.8, 4.8, 2.0 Hz, *ddd*)
H-6a	3.92 (*J* = 12.5, 6.2 Hz, *dd*)	3.90 (*J* = 12.8, 6.9 Hz, *dd*)
H-6b	3.70 (*J* = 12.5, 6.2 Hz, *dd*)	3.70 (*J* = 12.4, 5.5 Hz, *dd*)
H-1'a	3.88 (*J* = 6.9, 3.2 Hz, *dt*)	3.89 (*J* = 9.5, 7.3 Hz, *dt*)
H-1'b	3.67 (*J* = 6.9, 3.2 Hz, *dt*)	3.62 (*J* = 9.5, 7.4 Hz, *dt*)
H-2'	1.60 (*J* = 7.4 Hz, *qu*)	1.57 (*m*)
H-3'	1.36 (*J* = 7.4 Hz, *sext*)	1.34 (*m*)
H-4'	0.90 (*J* = 7.4 Hz, *t*)	0.89 (*m*)

Table 4.7: ^{13}C NMR spectra of spectrum of isolated butylated glucoside.

Carbon	Chemical shift δ_C (ppm)	Literature value Matin et al (2013)
C-1	102.8	98.5
C-2	73.8	71.9
C-3	76.5	73.3
C-4	70.3	70.4
C-5	76.4	72.8
C-6	61.4	61.0
C-1'	71.0	66.5
C-2'	31.5	31.3
C-3'	19.1	19.0
C-4'	13.7	13.9

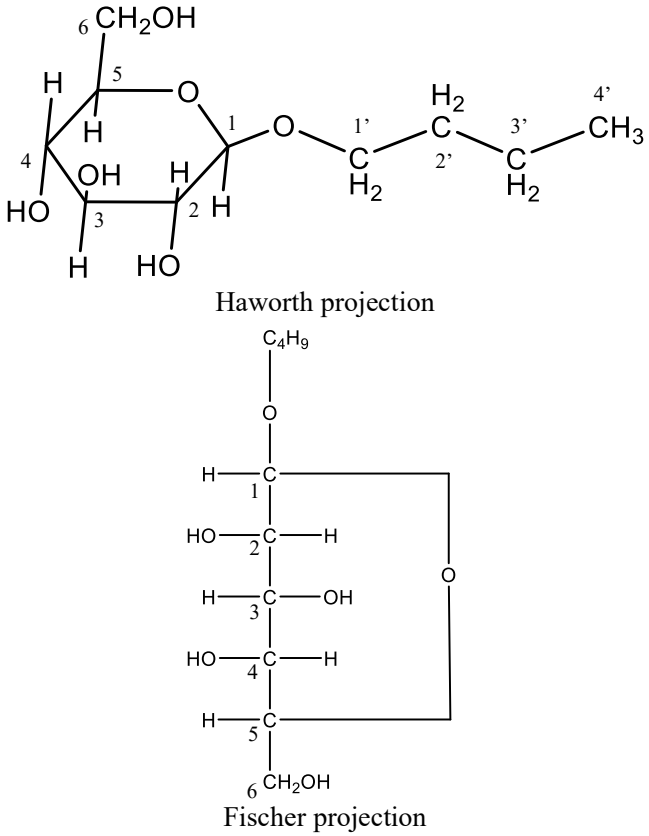


Figure 4.6: Structure of *n*-butyl β-D-glucopyranoside isolated from stingless bee honey.

The molecular formula of the isolated butylated glucoside ($C_{10}H_{20}O_6$) was further confirmed by HR-LCMS. A major polyatomic ion was observed at $^{m}/_{z}$ 237 and is shown in Appendix 2. The mass spectrometric details of the polyatomic ion are tabulated in Table 4.8.

Table 4.8: Mass spectrum of butylated glucoside.

Ion formula	Calculated mass	Observed *m/z*	Error (mDa)	Error (ppm)	Score (MFG, MS)	Score (MGF, mass)
$C_{10}H_{21}O_6^+$	237.2701	237.1333	0.71	3.00	92.05	95.57

There is very limited literature about the NMR spectra of *n*-butyl β-D-glucopyranoside except Matin *et al.* (2013), which is consistent with the chemical shifts obtained in this study. The capillary melting point of the

colo~~u~~rless butyl-glycoside solid was 52 - 54 °C, which is in agreement with Antkowiak *et al*. (2003) and Matin *et al*., (2013), 52 – 54 °C and 53 – 54 °C, respectively.

Next, a distinctive fragmentation pattern of the butylated glucoside was observed. The product ion mass spectrum is shown in Figure 4.7; a total of seven fragment ions was formed from the protonated molecule. To date, this is the first report for the fragmentation pathways of *n*-butyl β-D-glucopyranoside. Proposed fragmentation pathways and structures of the product ions are given in Figure 4.8, in which the step-by-step fragmentation of the precursor ion is depicted. There are two possibilities for the loss of the first molecule, which loses a $C_4H_{10}O$ molecule or a CH_4 molecule. After the loss of a $C_4H_{10}O$ molecule at $^m/_z$ 163, the protonated molecules undergo loss of two H_2O molecules to form ions at $^m/_z$ 145 and 127, respectively. The species at $^m/_z$ 127 undergoes further dehydration, resulting in ring cleavage to form an ion at $^m/_z$ 109. On the other hand, after the loss of CH_4 at $^m/_z$ 221, the protonated molecule undergoes reductive loss of an oxygen atom and dehydration to form ions at $^m/_z$ 205 and 187, respectively. According to Taylor *et al*. (2005), the ESI-MS/MS product ion mass spectrum of a glucose solution also showed fragmentation pathways involving neutral losses of a water molecule.

Alkyl glycosides including butyl-glycosides are a new generation of surfactants prepared using renewable agricultural resources, namely starch and fats or their derivatives. These surfactants are utilized in the cosmetic industry due to their high biodegradability and low toxicity (Ismail *et al*., 1998). This

compound was shown to exhibit moderate inhibitory effects against bacteria (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pastunella maltosida*, *Salmonella gallinarium*, *Salmonella typhi*, *Shigella dysenteriae* and *Vibro cholera*). Moderate antifungal activities of this compound were also seen on *Aspergillus acheraccus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nodusus*, *Fuserium equiseti* and *Candida albicans* (Matin *et al*., 2013). No significant higher antimicrobial activities were observed with the incorporation of *n*-butyl group at the glycosidic position (Matin *et al*., 2013).

This is the first report of the isolation and identification of *n*-butyl β-D-glucopyranoside from a honey sample. However, due to the absence of any evidence of butyl glucoside in any raw honey sample, the isolation of this compound is clearly an artifact from the extraction with *n*-butanol. Although butyl-glucosides are commonly synthesized in laboratory when ß-glucosidase and glucose were added with butanol (Ismail *et al*., 1998), such butylated glucosides can be synthesized when a catalytic amount of concentrated sulfuric acid is added to a mixture of glucose and butanol, followed by heating to reflux at 100 °C for five hours (Matin *et al*., 2013). Similar conditions could be linked with this study, whereby glucose in highly acidic stingless bee honey (pH 3.3) reacts with an excess of *n*-butanol during extraction and the mixture was subjected to relatively high temperature (45 – 55 °C) during drying and rotary evaporation. The proposed chemical reaction is shown in Figure 4.9 (Matin *et al*., 2013). Therefore, *n*-butyl β-D-glucopyranoside is considered to be a by-product derived from the extraction process.

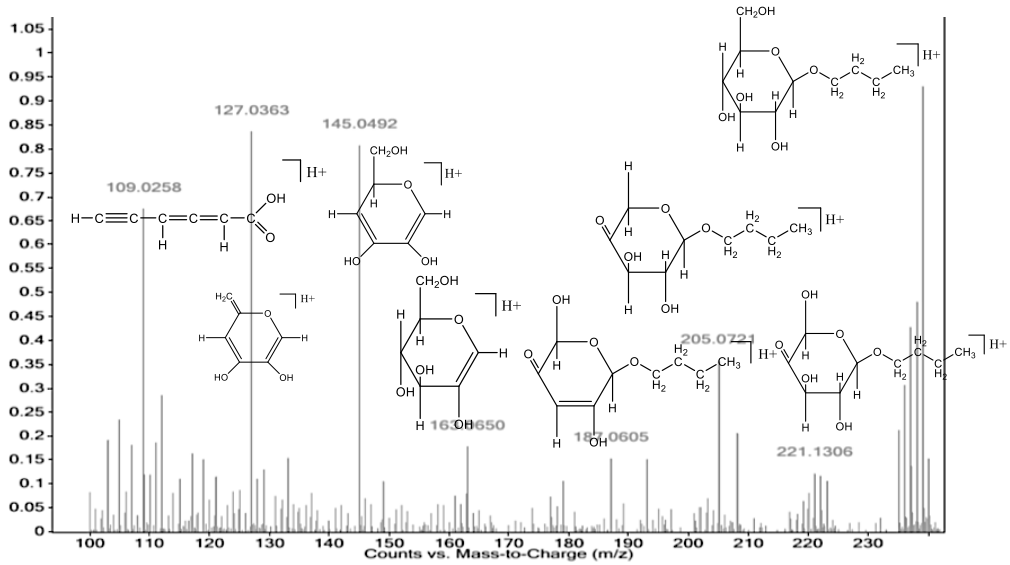


Figure 4.7: Fragmentation mass spectrum of protonated butyl glucoside.

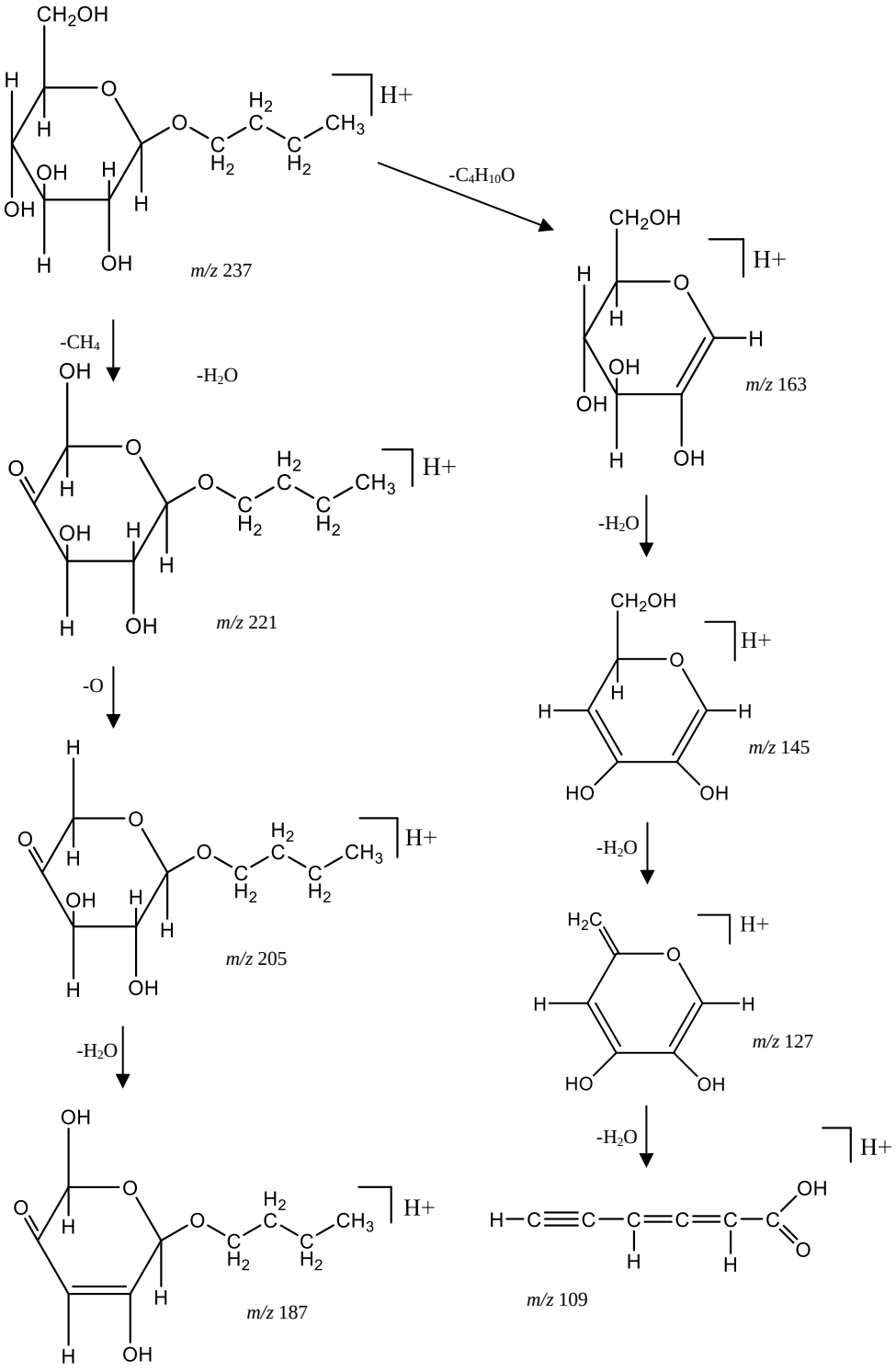


Figure 4.8: Proposed formation, structure and fragmentation of protonated butyl glucoside molecules.

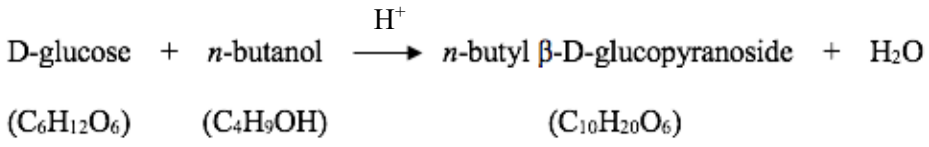


Figure 4.9: Proposed chemical equation for the formation of butyl glucoside.

4.1.3 Maturity of Honey

The maturity of honey was assessed based on several physicochemical parameters including the moisture content and total soluble solids (Bergamo *et al*., 2019). In addition, the color intensity and water activity of honey were also evaluated. According to Bogdanov *et al*. (2004), proline content also can be used as a criterion of honey ripeness and adulteration. The investigation into maturity of stingless bee honey samples is shown in Table 4.9.

Table 4.9: Maturity parameters of stingless bee honey samples.

Sample	Color intensity (mAU)	Moisture (%, g/100g)	Water activity	Total soluble solid (◦Brix)	Proline (mg/kg)
S1	180 ± 0	27.30 ± 0	0.62 ± 0	72.70 ± 0	537.13 ± 6.86
S2	190 ± 0	27.13 ± 0.06	0.60 ± 0	72.87 ± 0.06	513.13 ± 5.00
S3	200 ± 0	26.40 ± 0	0.62 ± 0	73.60 ± 0	522.93 ± 20.01
S4	190 ± 0	26.37 ± 0.06	0.63 ± 0	73.63 ± 0.06	542.37 ± 37.50
S5	190 ± 0	26.33 ± 0.06	0.63 ± 0	73.67 ± 0.06	547.43 ± 16.01
S6	150 ± 0	27.87 ± 0.06	0.62 ± 0	72.13 ± 0.06	514.50 ± 3.90
S7	140 ± 0	27.40 ± 0	0.65 ± 0	72.60 ± 0	454.80 ± 11.39
S8	150 ± 0	27.43 ± 0.06	0.65 ± 0	72.57 ± 0.06	546.73 ± 10.83
S9	60 ± 0	23.50 ± 0.10	0.54 ± 0	76.50 ± 0.10	577.00 ± 2.00
S10	80 ± 0	22.57 ± 0.06	0.54 ± 0	77.43 ± 0.06	546.00 ± 2.69
S11	60 ± 0	22.50 ± 0	0.55 ± 0	77.50 ± 0	578.37 ± 4.47
S12	70 ± 0	26.60 ± 0	0.54 ± 0	73.40 ± 0	588.80 ± 35.76
S13	60 ± 0	26.73 ± 0.06	0.57 ± 0	73.27 ± 0.06	514.23 ± 14.70
S14	50 ± 0	24.57 ± 0.06	0.64 ± 0	75.43 ± 0.06	525.27 ± 7.28
S15	50 ± 0	24.30 ± 0	0.63 ± 0	75.70 ± 0	525.70 ± 4.62
S16	70 ± 0	24.20 ± 0	0.63 ± 0	75.80 ± 0	525.10 ± 7.82
S17	250 ± 0	26.20 ± 0	0.64 ± 0	73.80 ± 0	670.27 ± 6.78
S18	250 ± 0	27.20 ± 0	0.67 ± 0	72.80 ± 0	598.50 ± 3.50
S19	240 ± 0	27.10 ± 0	0.67 ± 0	72.87 ± 0.06	612.40 ± 4.25
S20	280 ± 0	27.30 ± 0	0.67 ± 0	72.70 ± 0	627.57 ± 7.10
S21	220 ± 0	27.37 ± 0.06	0.67 ± 0	72.63 ± 0.06	510.67 ± 5.86
S22	250 ± 0	28.00 ± 0	0.69 ± 0	72.00 ± 0	510.47 ± 1.50
S23	210 ± 0	26.97 ± 0.06	0.67 ± 0	73.03 ± 0.06	535.70 ± 4.96
Average	156.09 ± 76.59	26.14 ± 1.64	0.62 ± 0.05	73.85 ± 1.64	548.94 ± 47.74
Honeydew (S1 – S8)	173.75 ± 22.23	27.03 ± 0.56*	0.63 ± 0.02	72.97 ± 0.56*	522.38 ± 32.61*
Blossom (S9 – S23)	146.67 ± 92.44	25.67 ± 1.83	0.62 ± 0.06	74.32 ± 1.83	563.11 ± 48.74
H. itama (S1 – S16)	62.50 ± 9.89^	24.37 ± 1.54^	0.58 ± 0.04^	75.63 ± 1.54^	547.63 ± 30.68^
G. thoracica (S17 – S23)	242.86 ± 21.71	27.16 ± 0.51	0.67 ± 0.01	72.83 ± 0.51	580.80 ± 59.41

* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.

Color intensity of honey is a reliable parameter that indicates the presence of pigments with antioxidant activities such as carotenoids and flavonoids (Moniruzzaman *et al*., 2013b). Kek *et al*. (2014), Selvaraju *et al*. (2019) and Shamsudin *et al*. (2019) reported that the range of color intensity of stingless bee honey can be very wide from as low as 32.25 to 1480 mAU. Higher color intensity of honey is generally observed from matured honey, which is also commonly correlated with the phenolic and flavonoid content of the honey (Kek *et al*. 2014). A previous study also stated the color intensity of raw honey is mainly related to the botanical origin and composition including mineral, pollen, phenolic compounds (Ramalhosa *et al*., 2011). In this study, higher color intensity was observed in honeydew honey (174 mAU) but the value was not significantly different from blossom honey (147 mAU). Considering honey samples produced by honey bee, although the color intensity of honeydew honey (625 mAU) was found to be significantly higher than multifloral honey (275 mAU) and Acacia honey (51 mAU), there was no significant difference with chestnut honey (754 mAU) (Choi and Nam, 2020). Other than that, a significant difference in color intensity was seen between the honey samples produced by *H. itama* (63 mAU) and *G. thoracica* (243 mAU). In another study, the color intensity of stingless bee honey was found to be significantly different between *H. itama* (0.23 mAU) and *G. thoracica* (0.09 mAU) (Maringgal *et al*., 2019). The differences in color intensity can be attributed to the different botanical sources used by different bees to produce honey (Shamsudin *et al*., 2019).

Moisture is an important quality parameter for honey shelf life because it determines its stability to resist spoilage by yeast fermentation. Lower moisture content is generally observed in matured honey samples (Omar *et al*., 2019). However, the moisture content in stingless bee honey samples was in the range from 22.5 % to 28.0 %, exceeding the limits set by the regulatory organizations Codex Alimentarius Commission (2001) and the Council Directive of the European Union (2002). Still, the value was in compliance with the Malaysian standard for stingless bee honey established by the Department of Standards Malaysia (2017), in which the moisture content of stingless bee honey should not exceed 35.0 %. Stingless bee honey is naturally high in moisture content. The moisture content values of all raw honey samples ranges from 14.67 to 47.00 % (Biluca *et al*., 2016; Chuttong *et al*., 2016; Shamsudin *et al*., 2019; Umana *et al*., 2021). The higher moisture content characteristic of stingless bee honey is suggested to be due to the influence of the humid tropical environment and the honey storage sites; specifically stingless bee honey pots that are made of cerumen and honey bee honeycombs that made of pure propolis (do Nascimento *et al*., 2015; Omar *et al*., 2019). Other than that, nectar collections from undergrowth flowers and ripe fruits rich in water content have also been suggested to contribute to the higher moisture content in stingless bee honey (Biluca *et al*., 2016). Hence, the moisture content in stingless bee honey is one of the unique parameters demonstrating the importance of an international legislation geared towards stingless bee honey. In this study, although stingless bee honeydew honey was found to have significant higher moisture content (27.0 %) than blossom honey (25.7 %), opposite outcome was observed in another study of honeybee honey, whereby the blossom honey (17.5 %) was found to

have significantly higher moisture content than honeydew honey (16.7 %) (Olga *et al*., 2012). However, other studies have found no significant differences for moisture content between honeydew and blossom honey (Choi and Nam, 2020; Manzanares *et al*., 2011). In addition, honey samples produced by the stingless bee *G. thoracica* (27.2 %) were found to have significant higher moisture content than those of *H. itama* (24.4 %), agreeing with a study conducted by Omar *et al*. (2019), where the moisture content of *G. thoracica* honey (27.6 %) was found to be higher than that of *H. itama* honey (26.6 %). The difference in the moisture content of stingless bee honey may be due to preferred nectar sources used by different stingless bees to produce honey (Shamsudin *et al*., 2019).

Although moisture content is positively correlated with water activity, water activity can determine the amount of free water available for microorganisms. In nature, bees reduce the water activity of honey by evaporation and hydrolysis of sucrose to fructose and glucose (Subbiah *et al*., 2020). Thus, this parameter is a better indicator of microbial growth. The water activity in stingless bee honey (0.54 – 0.69) was not significantly different between honeydew and blossom origins. Still, the values obtained in this study were lower than a previously reported values of 0.76 to 0.87 from stingless bee honey (Shamsudin *et al*., 2019). Another study also found no significant difference for water activity between honeydew and blossom honeys (Manzanares *et al*., 2011). Water activity lower than 0.60 is able to prevent microbial growth including osmophilic yeasts, hence stingless bee honey is generally more susceptible to microbial fermentation. This parameter should be considered to be included in legislation to ensure the stability of honey towards

microbial fermentation (Chen, 2019).

In terms of total soluble solids, stingless bee honey generally had a significantly lower Brix value (73.9 – 74.9 °Brix) than honey bee honey (76.4 °Brix). Similar results were reported by de Sousa *et al*. (2016) in Brazilian stingless bee honey, with values between 71.1 and 74.7 °Brix. The Brix value of stingless bee honey was also found to be different among bee species from as low as 55.2 °Brix produced by *Melipona quadriasciata* to 76.1 °Brix produced by *Scaptotrigona bicunctata*. Such outcomes were also shown in this study whereby a honey sample produced by *H. itama* (75.6 °Brix) was significantly higher than that from *G. thoracica* (72.8 °Brix). The total soluble solids are generally associated with sugar and moisture in honey (Shamsudin *et al*., 2019). Therefore, stingless bee honey with lower total soluble solids possesses a lower sugar content and consequently higher moisture content. This parameter is not regarded in any of the current quality standards for honey. Although there are no reports in the literature about the total sugar content of honeydew and blossom honey produced by stingless bees, several authors have reported the average value of total sugar content honeydew honey produced by honey bees to be generally lower than honey of blossom origin (Conti *et al*., 2007, Terrab *et al*., 2003a; Manzanares *et al*., 2011). This is in agreement with the Brix value of stingless honey honeydew honey (73.0 °Brix) which was significantly lower than blossom origin (74.3 °Brix).

Proline is the major component (50 – 85 %) of the total amino acids in honey (Iglesias *et al.*, 2004; Czipa *et al.*, 2012). The proline content is used as a criterion of honey ripeness and to indicate sugar adulteration (Can *et al*., 2015). Although there is no standard for the proline content in stingless bee honey, proline content in honey bee honey has to be at least 180 mg/kg to be accepted internationally (Bogdanov *et al*., 2002). Honey with less than 180 mg/kg proline can be considered as either unripe or adulterated honey (Bogdanov *et al*., 1999). All stingless bee honey samples in this study met this requirement with the range of 455 to 670 mg/kg. The values obtained were higher the reports in previous studies by Nweze *et al*. (2017) and Gela *et al*. (2021) ranging from 124 to 499 mg/kg. Gela *et al*. (2021) also stated that a higher proline content was detected in stingless bee honey than in honey bee honey. In this study, the proline contents in honey samples produced by *H. itama* (548 mg/kg) and *G. thoracica* (581 mg/kg) were found to be different significantly. Differences in proline content may be attributed to the different species of bees involved in honey production. It has been reported that proline originates from the bees' secretion and nectar used to make honey (Anklam, 1998). Several studies have reported that high values of proline are typical for honeydew honey (Ouchemoukh *et al*., 2007; Manzanares *et al*., 2011). However, another study stated that honeydew honey had higher proline content (932 – 1192 mg/kg) than certain blossom honey (186 – 1155 mg/kg), although it was found that another blossom honey had the highest proline content (2148 – 2404 mg/kg) (Czipa *et al*., 2012). The high variability of the proline content makes it impossible to characterize the honeydew or blossom origin of the honey by this criterion (Sanchez *et al*., 2001; Manzanares *et al*., 2011).

4.1.4 Purity of Honey

The purity of honey was assessed using electrical conductivity, ash content and diastase level (Codex Alimentarius Commission, 2001; Council Directive of the European Union, 2002; Bergamo *et al*., 2019). Antibacterial effects of honey are often associated with the presence of hydrogen peroxide, which is produced by glucose oxidase, an enzyme introduced into nectar by the bees. Hence, the hydrogen peroxide level could serve as a honey-specific or activity-associated biomarker that could allow predicting the antibacterial effects of pure honey (Brudzynski, 2006; Brudzynski *et al*., 2011). The evaluations of purity of honey in honeybee and stingless bee honeys are shown in Table 4.10.

Table 4.10: Purity parameters of stingless bee honey samples.

Sample	Electrical conductivity (mS/cm)	Ash content (%, g/100g)	Diastase (Schade unit/g)	Hydrogen peroxide (μmol/L)
S1	0.36 ± 0	0.18 ± 0.01	3.04 ± 0.08	177.58 ± 19.80
S2	0.39 ± 0	0.16 ± 0.01	2.98 ± 0.05	185.43 ± 7.29
S3	0.42 ± 0	0.15 ± 0	2.45 ± 0.03	187.05 ± 20.39
S4	0.45 ± 0	0.13 ± 0.01	2.51 ± 0.09	177.19 ± 18.63
S5	0.41 ± 0	0.13 ± 0.01	2.71 ± 0.04	189.77 ± 18.09
S6	0.52 ± 0	0.13 ± 0	2.22 ± 0.08	184.07 ± 13.14
S7	0.51 ± 0	0.13 ± 0.01	2.05 ± 0.02	192.82 ± 20.58
S8	0.52 ± 0	0.14 ± 0.01	2.34 ± 0.07	174.18 ± 6.22
S9	0.38 ± 0	0.04 ± 0	2.63 ± 0.59	111.40 ± 1.77
S10	0.35 ± 0	0.04 ± 0	2.09 ± 0.02	111.97 ± 1.82
S11	0.29 ± 0	0.04 ± 0.01	2.25 ± 0.03	118.20 ± 10.78
S12	0.29 ± 0	0.04 ± 0.01	2.15 ± 0.04	112.40 ± 2.50
S13	0.41 ± 0	0.04 ± 0.01	2.30 ± 0.04	112.95 ± 3.49
S14	0.42 ± 0	0.04 ± 0.01	2.17 ± 0.18	119.53 ± 8.88
S15	0.45 ± 0	0.04 ± 0.01	2.39 ± 0.02	114.15 ± 9.31
S16	0.45 ± 0	0.04 ± 0	2.38 ± 0.03	111.07 ± 11.28
S17	0.38 ± 0	0.05 ± 0.01	1.83 ± 0.15	168.37 ± 21.51
S18	0.35 ± 0	0.05 ± 0.01	2.23 ± 0.35	146.17 ± 30.41
S19	0.30 ± 0	0.05 ± 0.01	2.18 ± 0.14	166.73 ± 12.74
S20	0.28 ± 0	0.05 ± 0.01	2.16 ± 0.14	160.17 ± 17.36
S21	0.36 ± 0	0.05 ± 0.01	2.43 ± 0.02	169.63 ± 4.57
S22	0.38 ± 0	0.05 ± 0.01	2.36 ± 0.03	178.61 ± 2.01
S23	0.38 ± 0	0.05 ± 0	2.29 ± 0.08	162.77 ± 21.68
Average	0.39 ± 0.07	0.08 ± 0.05	2.35 ± 0.31	153.57 ± 33.12
Honeydew (S1 – S8)	0.45 ± 0.06*	0.14 ± 0.02*	2.54 ± 0.34*	183.51 ± 15.11*
Blossom (S9 – S23)	0.36 ± 0.05	0.04 ± 0.01	2.26 ± 0.24	137.61 ± 28.74
H. itama (S1 – S16)	0.38 ± 0.06^	0.04 ± 0^	2.30 ± 0.25	113.96 ± 6.86^
G. thoracica (S17 – S23)	0.35 ± 0.04	0.05 ± 0.01	2.21 ± 0.23	164.63 ± 18.03

* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.

The electrical conductivity has been found to be positively correlated with ash content. Unlike honey bee honey, the reference range for the electrical conductivity and ash content are stated in the international standards (Codex Alimentarius Commission, 2001; Council Directive of the European Union, 2002), although there are no such guidelines for stingless bee honey. The obtained electrical conductivity (0.28 to 0.52 mS/cm) for stingless bee honey in this study was found to be similar to the previously reported values by Suntiparapop *et al*. (2012), ranging from 0.32 to 1.10 mS/cm, Moniruzzaman *et al*. (2013a), ranging from 0.35 to 0.76 mS/cm and de Sousa *et al*. (2016), ranging from 0.30 to 0.67 mS/cm. The ash content of stingless bee honey samples fulfilled the requirement set by the Department of Standards Malaysia (2017), of being not more than 1.00%.

In this study, the values of both electrical conductivity and ash content showed great variability among botanical origins whereby values found in honeydew honey (0.45 mS/cm; 0.14 %) were significantly higher than blossom honey (0.36 mS/cm; 0.04 %), which indicate that honeydew honey is richer in both organic and inorganic substances including organic acids (Table 4.11) and minerals (Table 4.12). Furthermore, honeydew honey produced by honeybees had significant higher electrical conductivity (0.830 mS/cm) than blossom honey (0.540 mS/cm) (Olga *et al*., 2012). Nesovic *et al*. (2020) also found that obtained electrical conductivity values for honeydew honey (0.94–1.13 mS/cm) were higher than polyfloral honey (0.27 to 0.78 mS/cm).

A study conducted by Shamsudin *et al*. (2019) revealed that the ash content of Acacia honey and starfruit honey produced by different stingless bee species namely, *H. itama* and *G. thoracica* showed no significant difference. However, Gelam honey produced by *H. itama* was found to have significantly higher ash content than that of *G. thoracica*. In the same study, honey samples from different floral sources produced by the same stingless bee species were found to have significantly different ash content (Shamsudin *et al*., 2019). Therefore, it can be suggested that, instead of bee species, the foraged nectar source is the main factor for the variations in electrical conductivity and ash content (Suntiparapop *et al*., 2012). Likewise, it can also be due to the plants absorbing minerals from different soils, which eventually end up in the nectar (Nordin *et al*., 2018). Although based on honey bee honey samples, both Pita-Calvo *et al*. (2017) and Recklies *et al*. (2021) suggested higher electrical conductivity and ash content are the suitable parameters for the differentiation of honeydew honey from blossom honey. Based on the outcomes in this study, these parameters could be suitable to differentiate between honeydew honey and blossom honey produced by stingless bees as well.

Diastase is the major enzyme in honey, followed by invertase and glucose oxidase. Diastase catalyses the breakdown of starch into maltose which originates from nectar, exudate and the bees (Ahmed *et al*., 2012) and is commonly used as an indicator of honey purity (Machado De-Melo *et al*., 2018). This enzyme is sensitive to heat and prolonged poor storage; hence it also can be used to monitor the freshness of honey (Bergamo *et al*., 2019). Diastase activity is commonly used in Europe as an indicator of freshness of honey.

According to the international standards established by Codex Alimentarius Commission (2001) and Council Directive of the European Union (2002), the diastase enzyme level in honey bee honey should be more than 8 Schade units, or 3 Schade units for honey bee honey with low natural enzyme content. To date, there is no standard for the diastase level of stingless bee honey samples. The diastase level in stingless bee honey samples reported in this study was rather low, ranging from 1.83 to 3.04 Schade units as compared to the diastase level reported by Souza *et al*. (2006), ranging from 0.9 to 23.0 Schade units. Other studies discovered a wide variation in diastase activity of different stingless bee honey samples. Among 28 stingless bee honey samples from Thailand, there were six samples without any enzyme detected, with the value range for the remaining 22 honey samples being 0.05 to 4.9 Schade units (Chuttong *et al.*, 2016). The diastase activity in South American stingless bee honey samples also showed a wide variation, with a range of 2.4 to 21 Schade units from 14 stingless bee species (Vit *et al*., 2013).

Although previous studies showed that there was no significant difference in the mean values of diastase activity between honeydew and blossom honeys (Manzanares *et al*., 2011; Nesovic *et al*., 2020), honeydew honey (2.54 Schade units) was found to have significantly higher enzymatic activity than blossom honey (2.26 Schade units) in this study. This outcome is in agreement with Olga *et al*. (2012) whereby the diastase content in honeydew honey (18.8 Shade units) was significantly higher than that in blossom honey (15.7 Shade units). Olga *et al*. (2012) postulated that the variations of enzyme level in honeydew and blossom honeys could be due to the duration of honey

production. Blossom honey production is generally completed in a shorter period; hence these honeys have lower enzyme content. Conversely, the production of honeydew honey takes longer, and the enzyme content is higher.

Other than honey adulteration and deterioration, low diastase levels in honey samples could be due to the acidic pH of the honey and storage in a hot climate. According to Babacan and Rand (2007), diastase activity would be inactivated in pH values lower than 4.6 to 5.3. Therefore, low diastase levels could be explained due to higher acidity, as the average pH value of stingless honey samples in this study range from 3.16 to 3.54. Bergamo *et al*. (2019) also stated higher temperature in tropical regions may also diminished diastase number in honey. A revision of this parameter can be considered in legislation considering the different natures of tropical honey and stingless bee honey.

Although hydrogen peroxide is known to be an important antibacterial factor in raw honey, to date, there is no legislation available for this parameter - possibly due to the large variation of hydrogen peroxide levels in different honey samples. Both Chen *et al*. (2012) and Lehmann *et al*. (2019) reported that the hydrogen peroxide level of honey bee honey samples was as low as 0 to 1017 µmol/L. Other than the environmental conditions such as climates and health status of bees, which can affect glucose oxidase activity, the presence of catalase that is introduced into honey with pollen grains can suppress hydrogen peroxide levels (Lehmann *et al*., 2019). The hydrogen peroxide level of stingless bee honey samples in this study ranged from 111.4 µmol/L to 192.8 µmol/L, which was slightly higher than the hydrogen peroxide level of stingless bee honey

reported by Massaro *et al*. (2014), 155.8 ± 10 µmol/L. Significantly higher levels of hydrogen peroxide were detected in honeydew honey (183.5 µmol/L) than in blossom honey (137.6 µmol/L). Such a variation may be associated with the greater antibacterial activity exhibited by honeydew honey as shown in Tables 4.15 and 4.16.

Although hydrogen peroxide is an important factor in the inhibition of bacterial growth, it is not the sole parameter that determines the antibacterial potency of a honey. Chen *et al*. (2012) reported that honey samples with no hydrogen peroxide were found to have little or no antibacterial activity, and some honey samples failed to exhibit any antibacterial activity despite having relatively high levels of hydrogen peroxide. Studies showed phenolic compounds and their interaction with hydrogen peroxide are the key factors responsible for the antibacterial activity of honey (Brudzynski *et al*., 2011; Chen *et al*., 2012; Bucekova *et al*., 2018). Information about hydrogen peroxide level in stingless bee honey is still very limited. Hence, more studies are required to investigate the correlation of hydrogen peroxide with the antibacterial effects of stingless bee honey.

4.1.5 Deterioration of Honey

The deterioration of honey due to microbial fermentation, prolonged storage, poor storage condition and heating was assessed by the measurement of pH, free acidity, organic acids and hydroxymethylfurfural (HMF) (Table 4.11).

Table 4.11: Deterioration parameters of stingless bee honey samples.

Sample	pH	Free acidity (mEq/kg)	Gluconic acid (g/kg)	Acetic acid (g/kg)	HMF (mg/kg)
S1	3.26 ± 0.02	89.33 ± 0.58	0.34 ± 0.01	0.09 ± 0.01	19.69 ± 0.04
S2	3.26 ± 0.01	87.33 ± 0.58	0.31 ± 0.01	0.11 ± 0.02	19.41 ± 0.56
S3	3.18 ± 0.02	85.00 ± 1.00	0.38 ± 0.06	0.13 ± 0.03	19.48 ± 0.36
S4	3.16 ± 0.02	93.00 ± 2.00	0.34 ± 0.01	0.12 ± 0.04	17.31 ± 0.81
S5	3.21 ± 0.02	90.33 ± 0.58	0.37 ± 0.03	0.09 ± 0.03	16.62 ± 0.22
S6	3.54 ± 0.01	94.67 ± 0.58	0.71 ± 0.06	0.09 ± 0.03	11.45 ± 0.37
S7	3.54 ± 0.01	92.67 ± 0.58	0.62 ± 0.03	0.08 ± 0.02	14.85 ± 0.32
S8	3.51 ± 0.01	95.33 ± 0.58	0.62 ± 0.04	0.09 ± 0.03	15.64 ± 0.11
S9	3.46 ± 0.01	72.00 ± 1.00	0.57 ± 0.01	0.09 ± 0.03	19.87 ± 0.46
S10	3.51 ± 0.01	70.67 ± 0.58	0.54 ± 0.05	0.16 ± 0.05	19.17 ± 0.38
S11	3.28 ± 0.01	72.00 ± 0	0.58 ± 0.03	0.12 ± 0.02	17.22 ± 0.72
S12	3.27 ± 0.01	78.00 ± 0	0.45 ± 0	0.09 ± 0.02	27.10 ± 0.92
S13	3.22 ± 0.01	75.00 ± 0	0.35 ± 0.01	0.08 ± 0.01	13.13 ± 1.51
S14	3.25 ± 0.02	62.00 ± 2.00	0.56 ± 0.05	0.10 ± 0.02	13.54 ± 0.44
S15	3.17 ± 0.02	60.67 ± 0.58	0.67 ± 0.02	0.10 ± 0.02	14.22 ± 0.41
S16	3.22 ± 0.03	61.67 ± 0.58	0.72 ± 0.06	0.13 ± 0.02	12.71 ± 0.55
S17	3.21 ± 0	80.67 ± 0.58	0.34 ± 0.01	0.02 ± 0.01	20.24 ± 0.46
S18	3.25 ± 0.01	81.67 ± 0.58	0.35 ± 0.03	0.05 ± 0.02	20.39 ± 0.46
S19	3.21 ± 0.01	81.67 ± 0.58	0.43 ± 0.04	0.03 ± 0.01	19.53 ± 0.27
S20	3.28 ± 0.01	85.00 ± 0	0.25 ± 0.01	0.03 ± 0.02	21.10 ± 1.28
S21	3.51 ± 0	86.67 ± 0.58	0.34 ± 0.02	0.04 ± 0.01	24.54 ± 0.04
S22	3.51 ± 0	91.00 ± 1.00	0.30 ± 0.03	0.03 ± 0.02	19.69 ± 0.19
S23	3.50 ± 0	91.00 ± 0	0.65 ± 0.05	0.04 ± 0.01	18.88 ± 1.04
Average	3.33 ± 0.14	81.62 ± 10.70	0.47 ± 0.15	0.08 ± 0.04	18.08 ± 3.74
Honeydew (S1 – S8)	3.33 ± 0.16	90.96 ± 3.54*	0.46 ± 0.15	0.10 ± 0.03*	16.81 ± 2.74*
Blossom (S9 – S23)	3.32 ± 0.13	76.64 ± 9.88	0.47 ± 0.15	0.07 ± 0.05	18.76 ± 4.05
H. itama (S1 – S16)	3.30 ± 0.12	69.00 ± 6.40^	0.56 ± 0.12^	0.11 ± 0.03^	17.12 ± 4.72^
G. thoracica (S17 – S23)	3.35 ± 0.14	85.38 ± 4.18	0.38 ± 0.13	0.03 ± 0.02	20.63 ± 1.86

* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.

Although no limit for pH values is set by the Codex Alimentarius Commission (2001) and the Council Directive of the European Union (2002), some studies consider it is an indicator for honey deterioration as a result of microbial contamination and fermentation (Khalil *et al*., 2012, da Silva *et al*., 2016). da Silva *et al*. (2016) stated that honey with a more highly acidic pH is able to inhibit the growth of microorganisms, as most spoilage bacteria grow best around neutral pH values. Similar to many studies, pH values of stingless bee honey are one of the most consistent parameters with the least variability. The pH values of stingless bee honey in this study were in the range from 3.16 to 3.54, in compliance with the reference range suggested by the Malaysian Standard, between 2.5 and 3.8 (Department of Standards Malaysia, 2017). Both

Suntiparapop *et al*. (2012) and Chuttong *et al*. (2016) also reported pH values with a range of 3.48 to 3.76 and 3.1 to 3.9, respectively.

The maximum level of free acidity permitted in honey bee honey is 50 mEq/kg (Codex Alimentarius Commission, 2001; Council Directive of the European Union, 2002). Considering the limit, none of the stingless bee honey samples with free acidity values of 60.67 to 95.33 mEq/kg were in accordance with the regulations. Although results in this study were higher than the acidity of stingless bee honey evaluated by Almeida-Muradian *et al*. (2013), with a range of 31.79 to 33.19 mEq/kg, similar acidity of stingless honey samples (72.84 to 89.90 mEq/kg) was obtained by another study (Suntiparapop *et al*., 2012). However, a large variation of total acidity was detected from 28 stingless honey samples with the range from 25 to 592 mEq/kg. Four honey samples from *Homotrigona fimbriata*, *Tetrigona apicalis* and *Tetrigona melanoleuca* exhibited very high total acidity with a range from 440 to 592 meq/kg (Chuttong *et al*., 2016).

In this study, the free acidity of honeydew honey (90.96 mEq/kg) produced by stingless bee was also significantly higher than blossom honey (76.64 mEq/kg), but the pH value was not significantly higher. Similar outcomes were also observed in other studies, Manzanares *et al.* (2011) recorded that the acidity level of honeydew honey (34.65 mEq/kg) was significantly higher than that of blossom honey (24.44 mEq/kg). Although the acidity of honeydew honey (29.84 mEq/kg) was higher than blossom honey (27.18 mEq/kg), the difference was not significantly higher than blossom honey (Nesovic *et al*., 2020).

Furthermore, the pH of honeydew honey was not significantly different to that of blossom honey (Olga *et al*., 2012).

Stingless bee honey is known to possess a higher moisture content. Studies show that honey with a higher water content can permit the enzymatic activity of glucose oxidase to produce gluconic acid, while the presence of more unbound water molecules encourages microbial fermentation (Olaitan *et al*., 2007; Kretavicius *et al*., 2010). Both glucose oxidase activity and microbial fermentation lead to higher organic acid content in honey, particularly gluconic acid and acetic acid (Pita-Calvo and Vazquez, 2017).

Organic acids are minor constituents in honey, accounting for less than 0.5 % of the fresh weight of honey. Still, the acidity of honey is strongly related with the organic acids in honey, such as gluconic acid derived from the glucose oxidase enzymatic pathway, or acetic acid from microbial fermentation (Dardon *et al*., 2013; Silvano *et al*., 2014). Shamsudin *et al*. (2019) stated the major organic acid, which is gluconic acid in stingless bee honey, was detected in the range from 0.07 to 1.48 g/kg. Their result is similar to the current study whereby the measured gluconic acid level was in the range of 0.25 to 0.72 g/kg. These findings indicate the contribution of gluconic acid to the acidity of stingless bee honey. However, wide variation of gluconic acid content can be due to different amount of glucose and glucose oxidase enzymatic activity in honey (Shamsudin *et al*., 2019).

Although the acetic acid level of stingless bee honey in this study (0.02

– 0.16 g/kg) was lower than in other studies, the values are still within the range of values reported by Shamsudin *et al*. (2019), ranging from 0.01 to 0.39 g/kg. Mato *et al*. (2003) stated that excessive acetic acid concentration in honey may indicate fermentation, even though acetic acid is generally found in most honeys. Therefore, a lower acetic acid level is a good indicator to show honey samples have not deteriorated due to microbial fermentation. However, to date, there is no study of normal and fermentation levels of acetic acid in honey. The possibility of other nonaromatic acids as indicators of honey fermentation should be further investigated.

With higher free acidity values, honeydew honey was found to have significantly higher acetic acids content (0.10 g/kg) than blossom honey (0.07 g/kg), although the gluconic acid levels were not significantly different between these two types of stingless bee honey. Hence, the acidity of honey may also be result of contributions by other organic acids such as succinic, acetic, formic, and malic acids (Tezcan *et al.*, 2011; Shamsudin *et al*., 2019). Despite of the crucial contributions of organic acids to organoleptic properties, especially flavor, and to physicochemical properties such as pH and free acidity, information about organic acids in honey especially stingless bee honey is still very limited.

Due to the fact that formation of hydroxymethylfurfural (HMF) results from the degradation of sugars, HMF content is one of the parameters used to assess the deterioration state of honey (Bergamo *et al*., 2019). HMF is also formed slowly during storage or at a faster rate if the honey is heated (da Silva

et al., 2016). The HMF content measured of all stingless bee honey samples was in the range of 11.45 - 27.10 mg/kg, which was higher than the HMF values reported by Shamsudin *et al*. (2019). No HMF was detected in all stingless bee honey samples produced by *G. thoracica*, while the HMF level of *H. itama* honey ranged from undetectable to 0.14 mg/kg (Shamsudin *et al*., 2019). Still, the obtained HMF values in this study were in accordance with the reference value established by the Department of Standards Malaysia (2017) for stingless bee honey at maximum level of 30 mg/kg. Such expected values indicate that the honey samples had been harvested and stored properly, without undergoing any heating process or exposure to high ambient temperature during storage (Biluca *et al*., 2016). According to Chuttong *et al*. (2016), the HMF level in stingless bee honey samples showed wide variability both between and among bee species, with a range undetectable to 46 mg/kg. Likewise, the stingless bee honey analysed by Vit *et al*. (2013) also gave highly variable HMF levels, from undetectable to 25 mg/kg. Other authors also recorded that no HMF was detected in stingless bee honey (Biluca *et al*., 2016; de Sousa *et al*., 2016).

Stingless bee honeys are often accussed of adulteration due to their high HMF content (Nordin *et al*., 2018). However, stingless bee honey has been reported to be more resistant to HMF formation than honey bee honey when subjected to thermal treatment at 75 to 95 °C, possibly due to higher moisture content and lower pH (Biluca *et al*., 2014). Although no significant difference in HMF levels between honeydew honey and blossom honey were recorded by Olga *et al*. (2012), honeydew honey analysed by Manzanares *et al*. (2011) had significantly higher HMF levels (10.8 mg/kg) than blossom honey (7.7 mg/kg).

However, in this study, honeydew honey was found to have significant lower HMF levels (16.8 mg/kg) than blossom honey (18.8 mg/kg). Therefore, due to the wide variation of HMF levels in honey, a revision of the HMF standard set for stingless bee honey is necessary.

4.1.6 Mineral Profile

Mineral profile is also particularly important in terms of the characterization and classification of honey, since minerals in nectar are stable and dependent on plant absorption from the soil and environment (de Alda-Garcilope *et al*., 2012). Although minerals such as Na, K, Mg, Ca are crucial for physiological and biochemical reactions in the body; heavy metals such as Cr and Cu, can be toxic if maximum residue levels are exceeded (Biluca *et al*., 2017).

As listed in Table 4.12, the four major mineral elements detected in stingless bee honey are Na, K, Ca and Mg, which is in agreement with a review article authored by Nordin *et al*. (2018). In this study, Na was the most abundant element in all stingless bee honey samples, ranging from 224 to 327 mg/kg, followed by K ranging from 165 to 302 mg/kg. To our knowledge, this is the first report that Na but not K is the most abundant element detected in honey. Other studies have stated K was the most abundant amount of mineral element, ranging from less than 106 to 4980 mg/kg (Biluca *et al*., 2016; Biluca *et al*., 2017; Cheng *et al.*, 2019). One study even recorded that Mg was the major mineral element in their stingless bee honey samples with the highest value of

83 mg/kg; while Na was the second largest mineral with the range from 23 to 33 mg/kg (Maringgal *et al*., 2019). Nevertheless, Na content recorded in the study was found to be within the range of values from other studies, ranging from 73 to 589 mg/kg in stingless bee honey (Biluca *et al*., 2016; Biluca *et al*., 2017; Cheng *et al*., 2019).

Table 4.12(A): Mineral element profiles of stingless bee honey samples.

Sample	Na (mg/kg)	K (mg/kg)	Mg (mg/kg)	Ca (mg/kg)	Fe (mg/kg)
S1	283.40 ± 6.01	298.27 ± 11.06	50.51 ± 0.64	67.27 ± 2.85	12.17 ± 1.13
S2	295.42 ± 1.95	285.92 ± 7.17	55.39 ± 1.29	70.24 ± 2.89	11.82 ± 1.51
S3	326.75 ± 22.74	269.35 ± 40.91	58.19 ± 0.42	69.76 ± 2.96	12.86 ± 0.35
S4	305.63 ± 14.03	296.93 ± 6.39	59.95 ± 0.38	65.54 ± 3.05	14.29 ± 0.89
S5	312.14 ± 25.09	274.47 ± 28.46	54.54 ± 0.49	70.73 ± 0.96	12.77 ± 1.22
S6	300.13 ± 9.81	302.40 ± 4.27	52.64 ± 0.86	66.43 ± 3.83	13.77 ± 1.97
S7	308.57 ± 10.66	285.60 ± 14.20	52.99 ± 0.44	69.03 ± 2.43	12.26 ± 0.86
S8	316.87 ± 32.21	273.73 ± 59.26	54.29 ± 1.46	69.77 ± 2.36	12.50 ± 0.56
S9	256.60 ± 27.52	190.69 ± 11.00	42.02 ± 1.89	47.23 ± 5.72	12.34 ± 0.80
S10	242.68 ± 12.61	178.61 ± 14.91	35.95 ± 3.03	37.72 ± 0.79	10.41 ± 0.74
S11	223.87 ± 16.54	167.20 ± 11.02	32.77 ± 1.04	38.24 ± 2.70	10.73 ± 1.50
S12	263.65 ± 11.12	166.67 ± 9.62	34.57 ± 3.59	47.45 ± 2.12	10.68 ± 1.21
S13	243.53 ± 48.50	165.27 ± 14.55	42.09 ± 5.21	45.45 ± 4.39	10.90 ± 1.57
S14	286.20 ± 6.91	191.37 ± 6.25	42.53 ± 5.12	53.90 ± 7.37	12.73 ± 0.81
S15	277.03 ± 23.49	204.00 ± 16.52	47.41 ± 4.04	58.13 ± 2.75	12.50 ± 0.66
S16	283.70 ± 5.79	179.83 ± 17.82	44.78 ± 2.69	63.27 ± 5.16	11.87 ± 0.61
S17	278.83 ± 12.71	223.65 ± 14.98	44.58 ± 1.51	61.75 ± 2.08	11.26 ± 0.92
S18	278.59 ± 28.74	232.70 ± 16.22	46.36 ± 1.35	63.73 ± 0.50	11.38 ± 0.78
S19	260.30 ± 43.61	209.05 ± 18.20	44.82 ± 2.06	63.39 ± 1.17	10.71 ± 0.93
S20	263.88 ± 28.77	224.23 ± 13.89	40.25 ± 1.85	63.99 ± 2.93	12.09 ± 0.70
S21	275.60 ± 25.84	189.27 ± 12.66	46.13 ± 5.16	70.30 ± 2.09	12.33 ± 1.46
S22	274.60 ± 7.45	184.07 ± 11.60	50.83 ± 4.49	69.33 ± 0.90	10.57 ± 1.10
S23	292.27 ± 6.55	179.93 ± 1.15	47.02 ± 2.34	63.77 ± 6.77	11.57 ± 0.80
Average	280.45 ± 31.09	224.92 ± 51.17	46.98 ± 7.56	60.71 ± 10.70	11.94 ± 1.34
Honeydew (S1 – S8)	306.11 ± 19.79*	285.83 ± 26.51*	54.81 ± 3.00*	68.60 ± 2.96*	12.80 ± 1.26*
Blossom (S9 – S23)	266.76 ± 27.16	192.44 ± 24.00	42.81 ± 5.68	56.51 ± 10.98	11.47 ± 1.14
H. itama (S1 – S16)	259.66 ± 28.68	180.46 ± 17.36^	40.27 ± 5.82^	48.92 ± 9.38^	11.52 ± 1.25
G. thoracica (S17 – S23)	274.87 ± 23.41	206.13 ± 23.49	45.71 ± 3.93	65.18 ± 4.02	11.42 ± 1.03

* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.

Table 4.12(b): Mineral elements profiles of stingless bee honey samples.

Sample	Zn (mg/kg)	Mn (mg/kg)	Cr (mg/kg)	Cu + Al (mg/kg)	Total mineral elements (mg/kg)
S1	3.40 ± 0.26	0.44 ± 0.14	0.42 ± 0.08	< LOQ	715.88 ± 7.39
S2	3.74 ± 0.32	0.29 ± 0.21	0.66 ± 0.38	< LOQ	723.47 ± 5.73
S3	3.78 ± 0.38	0.59 ± 0.25	0.27 ± 0.20	< LOQ	741.56 ± 38.62
S4	3.63 ± 1.10	0.75 ± 0.16	0.44 ± 0.10	< LOQ	747.15 ± 16.63
S5	2.66 ± 0.58	0.63 ± 0.04	0.27 ± 0.20	< LOQ	728.21 ± 54.06
S6	3.88 ± 0.30	0.63 ± 0.06	0.63 ± 0.32	< LOQ	740.52 ± 3.59
S7	4.25 ± 0.28	0.72 ± 0.28	0.53 ± 0.40	< LOQ	733.95 ± 2.96
S8	4.14 ± 0.46	0.67 ± 0.15	0.60 ± 0.26	< LOQ	732.56 ± 26.36
S9	3.47 ± 0.65	0.62 ± 0.13	0.61 ± 0.11	< LOQ	553.58 ± 13.19
S10	3.41 ± 0.19	0.54 ± 0.07	0.56 ± 0.12	< LOQ	509.88 ± 9.36
S11	2.27 ± 0.85	0.42 ± 0.14	0.45 ± 0.06	< LOQ	475.94 ± 24.77
S12	2.98 ± 0.11	0.40 ± 0.13	0.51 ± 0.07	< LOQ	526.91 ± 15.85
S13	2.60 ± 0.38	0.52 ± 0.06	0.67 ± 0.11	< LOQ	511.02 ± 44.73
S14	3.03 ± 0.39	0.68 ± 0.10	0.82 ± 0.07	< LOQ	591.27 ± 13.90
S15	2.71 ± 0.33	0.69 ± 0.12	0.42 ± 0.12	< LOQ	602.90 ± 42.12
S16	3.31 ± 0.44	0.67 ± 0.12	0.61 ± 0.36	< LOQ	588.03 ± 20.94
S17	2.39 ± 0.78	0.45 ± 0.19	0.53 ± 0.25	< LOQ	623.44 ± 17.96
S18	2.00 ± 0.92	0.37 ± 0.05	0.63 ± 0.12	< LOQ	635.77 ± 31.56
S19	1.81 ± 0.19	0.39 ± 0.18	0.48 ± 0.09	< LOQ	590.96 ± 54.17
S20	1.76 ± 0.24	0.42 ± 0.27	0.54 ± 0.04	< LOQ	607.17 ± 19.59
S21	2.94 ± 0.24	0.60 ± 0.17	0.73 ± 0.16	< LOQ	597.89 ± 46.93
S22	3.32 ± 0.49	0.65 ± 0.05	0.59 ± 0.08	< LOQ	593.96 ± 9.66
S23	3.09 ± 0.17	0.80 ± 0	0.79 ± 0.17	< LOQ	599.24 ± 8.09
Average	3.07 ± 0.81	0.56 ± 0.19	0.55 ± 0.21	-	629.19 ± 88.03
Honeydew (S1 – S8)	3.68 ± 0.65*	0.59 ± 0.21	0.48 ± 0.27	-	732.91 ± 23.96*
Blossom (S9 – S23)	2.74 ± 0.70	0.55 ± 0.17	0.60 ± 0.17	-	573.86 ± 51.74
H. itama (S1 – S16)	2.97 ± 0.57^	0.57 ± 0.14	0.58 ± 0.18	-	544.94 ± 49.24^
G. thoracica (S17 – S23)	2.47 ± 0.75	0.53 ± 0.20	0.61 ± 0.16	-	606.92 ± 30.82

LOQ – Limits of quantitation.
* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.

Other major mineral elements in stingless bee honey are Ca and Mg, ranging from 38 to 71 mg/kg and 33 to 60 mg/kg, respectively. Other studies also reported similar values for Ca and Mg, ranging from 11 to 352 mg/kg and 4 to 231 mg/kg, respectively (Biluca *et al*., 2016; Biluca *et al*., 2017; Cheng *et al*., 2019). Other trace elements including Fe, Zn, Mn, and Cr were found to be present in low quantities in stingless bee honey (Suntiparapop *et al*., 2012; Biluca *et al*., 2016; Biluca *et al*., 2017; Cheng *et al*., 2019). Cu and Al were not detected in any of the stingless bee honey samples in the study by Biluca *et al*., (2017). Although elements including Al are naturally present in the environment, nowadays the presence of such elements is more related with anthropogenic sources especially from industrial discharges and agricultural outputs. Therefore,

honey can serve as a good indicator to reflect the accumulation of toxic elements in the environment (Czipa *et al*., 2015).

Significant differences of total mineral content in honey were found between *H. itama* (545 mg/kg) and *G. thoracica* (607 mg/kg). This result was similar to results reported by Biluca *et al*. (2017) and Maringgal *et al*. (2019), wherein their studies showed that honey samples produced by different species of stingless bee in the same location possessed different mineral compositions. This could be due to the characteristics and preference of each bee species during the construction of hive and nectar collection. Mineral elements and other substances can be transferred to the honey through the hive, due to contact during honey maturation and storage (Biluca *et al*., 2017).

Different mineral contents were observed in different honey samples due to the nature of the nectar foraged by the bees (Escuredo *et al*., 2015) and it has been concluded that, instead of bee species, botanical and geographical origins are the primary factors of mineral content (Kek *et al.*, 2017a). Cheng *et al*. (2019) also demonstrated that stingless bee honey collected from forests was richer in mineral content compared to that with suburban origin. A previous study also found that the total mineral content (235 mg/100g) of honeydew honey including K, Mg, Ca and phosphorus was significantly higher than that in blossom honey (149 mg/100g) (Olga *et al*., 2012). Significantly higher total mineral content in honeydew honey was also reflected in electrical conductivity and ash content analyses (Table 4.10).

4.2 Antioxidant Properties

Natural compounds such as phenolic compounds, carotenoid substances and others significantly contribute to the antioxidant capacity of honey (Pita-Calvo and Vazquez, 2017). The presence of these phenolic compounds in honey is also important in the assessment of authenticity (Pita-Calvo and Vazquez, 2017). The major phenolic compounds in stingless bee honey comprise salicylic acid, *p*-coumaric acid, naringin and taxifolin. Moreover, the presence of mandelic acid, caffeic acid, chlorogenic acid, rosmarinic acid, aromadendrin, isoquercetrin, eriodictyol, vanillin, umbelliferone, syringaldehyde, sinapaldehyde and carnosol in stingless bee honeys has also been reported (Biluca *et al*., 2017). Gheldof and Engeseth (2002) indicated that the antioxidant capacity is due to a combination of a wide range of honey active compounds beyond phenolics. Hence, due to the presence of various phytochemicals, Munteanu and Apetrei (2021) recommended that different methods are necessary for assessing the antioxidant capacity of a functional food to obtain a reliable outcome. Nevertheless, assessment of the total phenolic compounds are a marker for the antioxidant capacity of honey, and it is generally used as an antioxidant test (Can *et al*., 2015). ABTS radicals are freely soluble in both organic and aqueous solvents so both hydrophilic and lipophilic antioxidant compounds can react with ABTS radicals; whereas DPPH radicals are more selective in the reaction with hydrogen-donating antioxidants (Alzahrani *et al*., 2012). Both superoxide and peroxy-radicals, which are the endogenous radicals that associated with lipid peroxidation were found to be sensitive towards the antioxidant actions. The ability of honey to reduce reactive oxygen species

formation was also proven with the ferrous ion chelation (Chai *et al*., 2014; Sudan *et al*., 2014). Lastly, the ability of honey to serve as a reducing agent was also proven with the reduction of ferric irons to ferrous ions (Halvorsen *et al*., 2002).

As recorded in Table 4.13, this study revealed that stingless bee honey from honeydew origin possessed greater antioxidant capacities than blossom honey. Other than significantly higher total phenolic content (104 mg GAE/kg), scavenging activities against ABTS, DPPH and superoxide radicals (63 %, 36 % and 76 %), peroxy-radical inhibition (5.71 µmol TE/g), iron chelation (19 %) and ferric reducing power (3.18 mmol Fe(II)/kg) were all significantly higher than with blossom honey. Most of the honeydew honey samples demonstrated higher total phenolic compound content (77 to 131 mg GAE/100 g) as compared to blossom honey (29 to 90 mg GAE/100 g). These results are in agreement with those reported by Jaafar *et al*. (2017), for Lebanese honeydew honey and blossom honey. Several authors have also stated that honeydew honey, which is usually darker in color, possesses higher antioxidant activities (Tezcan *et al*., 2011; Pita-Calvo and Vazquez, 2017). Another study also found that the mean values of total phenolic content (105 mg GAE/100 g), DPPH radical scavenging activity (42 mg AAE/100 g) and ferric reducing power (861 µmol Fe(II)/100 g) of honeydew honey produced by Brazilian honey bees were higher than blossom honey at 61 mg GAE/100 g, 19 mg AAE/100 g and 354 µmol Fe(II)/100 g, respectively (Bergamo *et al*., 2019). In addition, honeydew honey was found to exhibit greater reducing capacity in the FRAP assay compared to blossom honey, as reported by Can *et al*. (2015). Nesovic *et al*. (2020) also recorded that

honeydew honey with greater total phenolic compound content (72 mg GAE/100 g) had higher DPPH radical scavenging activity (10 %) than blossom honey (64 mg GAE/100 g; 8 %). Nesovic *et al*. (2020) also notd that individual phenolic compounds in honey samples were insufficient to exhibit any significant antioxidant effect. Therefore, it can be suggested that the antioxidant capacity of honey is due to synergism among phenolic compounds, other than the simple ratio of individual phenolic compounds. Furthermore, the presence of other compounds in honey such as peptides, organic acids, enzymes and Maillard substrates, are also involved in interactions that lead to antioxidant activity.

Table 4.13: Antioxidant properties of stingless bee honey samples.

Sample	Total phenolic compounds (mg GAE/kg)	ABTS radical scavenging activity (%)	DPPH radical scavenging activity (%)	Superoxide radical scavenging activity (%)	Peroxyl radical inhibition (µmol TE/g)	Iron chelation (%)	Ferric reducing power (mmol Fe(II)/kg)
S1	105.60 ± 1.63	59.13 ± 1.87	34.83 ± 0.56	76.60 ± 0.36	5.47 ± 0.04	17.38 ± 1.53	3.20 ± 0.02
S2	105.60 ± 4.37	63.28 ± 0.41	36.03 ± 0.33	77.51 ± 0.34	5.61 ± 0.30	14.29 ± 0.44	3.18 ± 0.03
S3	120.06 ± 1.29	70.90 ± 0.36	36.26 ± 0.25	76.00 ± 0.10	5.81 ± 0.16	16.02 ± 0.81	3.31 ± 0.02
S4	109.74 ± 1.03	68.17 ± 0.82	34.52 ± 1.27	75.30 ± 0.26	5.61 ± 0.12	16.08 ± 0.47	3.29 ± 0.02
S5	113.59 ± 1.69	68.69 ± 0.66	37.37 ± 1.50	76.50 ± 0.40	6.12 ± 0.33	16.18 ± 0.70	3.58 ± 0.31
S6	95.39 ± 2.63	55.68 ± 0.70	35.44 ± 0.40	73.98 ± 0.03	5.88 ± 0.32	22.51 ± 0.93	3.06 ± 0.04
S7	83.28 ± 16.36	58.00 ± 0.70	35.25 ± 0.69	76.02 ± 0.79	5.48 ± 0.24	21.74 ± 1.87	2.96 ± 0.05
S8	99.51 ± 1.62	61.33 ± 0.58	35.49 ± 0.93	76.92 ± 0.24	5.70 ± 0.33	27.29 ± 1.52	2.87 ± 0.05
S9	65.58 ± 2.03	53.89 ± 0.45	36.14 ± 0.42	70.80 ± 0.80	4.58 ± 0.18	16.43 ± 1.56	1.33 ± 0.01
S10	73.54 ± 2.11	62.07 ± 0.53	32.62 ± 2.17	71.07 ± 0.67	4.90 ± 0.17	13.08 ± 0.91	1.71 ± 0.01
S11	62.25 ± 0.94	53.44 ± 0.68	35.48 ± 2.20	69.73 ± 0.70	5.19 ± 0.27	10.83 ± 0.26	1.64 ± 0.03
S12	75.51 ± 2.25	54.33 ± 0.31	34.33 ± 0.31	74.13 ± 2.80	5.18 ± 0.20	8.26 ± 1.23	1.63 ± 0.04
S13	61.01 ± 2.22	44.77 ± 0.66	34.67 ± 0.51	73.87 ± 2.34	5.97 ± 0.20	10.54 ± 0.56	1.56 ± 0.04
S14	57.35 ± 0.98	51.28 ± 0.24	33.38 ± 0.72	74.06 ± 1.43	5.60 ± 0.17	12.91 ± 1.67	1.57 ± 0.05
S15	56.78 ± 2.24	56.38 ± 0.70	32.84 ± 1.74	75.03 ± 0.45	4.85 ± 0.27	10.18 ± 1.02	1.83 ± 0.01
S16	59.32 ± 0.85	51.82 ± 0.35	33.09 ± 0.51	72.45 ± 1.33	4.44 ± 0.10	11.62 ± 0.69	1.56 ± 0.04
S17	97.04 ± 1.45	58.37 ± 0.47	31.59 ± 0.80	71.13 ± 0.81	5.61 ± 0.24	12.37 ± 0.68	1.62 ± 0.08
S18	99.94 ± 2.69	60.44 ± 0.87	33.44 ± 1.55	75.07 ± 0.50	5.60 ± 0.18	14.79 ± 0.26	2.25 ± 0.05
S19	94.67 ± 2.05	56.01 ± 0.22	31.56 ± 0.69	79.36 ± 0.55	5.65 ± 0.11	19.15 ± 0.48	1.90 ± 0.07
S20	101.13 ± 1.21	62.00 ± 0.26	32.83 ± 1.80	73.73 ± 1.42	5.66 ± 0.06	11.78 ± 0.38	2.20 ± 0.02
S21	78.22 ± 0.59	66.28 ± 0.48	33.16 ± 0.69	77.22 ± 0.70	5.64 ± 0.20	12.89 ± 0.25	2.00 ± 0.01
S22	88.14 ± 0.85	66.74 ± 0.82	32.17 ± 0.69	78.89 ± 1.40	5.51 ± 0.11	15.07 ± 0.61	2.29 ± 0.08
S23	77.11 ± 2.68	56.92 ± 0.58	31.90 ± 1.63	78.90 ± 1.50	5.47 ± 0.22	14.85 ± 1.57	2.25 ± 0.34
Average	86.10 ± 19.69	59.13 ± 6.33	34.10 ± 1.89	74.97 ± 2.80	5.46 ± 0.45	15.05 ± 4.43	2.29 ± 0.71
Honeydew (S1 – S8)	104.09 ± 12.06*	63.15 ± 5.38*	35.65 ± 1.12*	76.10 ± 1.08*	5.71 ± 0.30*	18.94 ± 4.36*	3.18 ± 0.23*
Blossom (S9 – S23)	76.51 ± 15.89	56.98 ± 5.77	33.28 ± 1.69	74.36 ± 3.23	5.32 ± 0.47	12.98 ± 2.79	1.82 ± 0.30
H. itama (S1 – S16)	63.92 ± 6.98^	53.50 ± 4.68^	34.07 ± 1.66^	72.64 ± 2.24^	5.09 ± 0.52^	11.73 ± 2.51^	1.60 ± 0.14^
G. thoracica (S17 – S23)	90.89 ± 9.58	60.97 ± 4.10	32.38 ± 1.25	76.33 ± 3.09	5.59 ± 0.16	14.41 ± 2.42	2.07 ± 0.24

* – Significant differencce between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.

The antioxidant capacity was found to be significantly different between stingless bee species. The honey produced by *G. thoracica* had higher total phenolic compounds (91 mg GAE/kg), ABTS (61 %), superoxide radical scavenging activities (76 %), peroxy-radical inhibition (5.59 µmol TE/g), iron chelation (14 %) and ferric reducing power (2.07 mmol Fe(II)/kg) than *H. itama* with 64 mg GAE/kg, 54 %, 73 %, 5.09 µmol TE/g, 12 % and 1.60 mmol Fe(II)/kg, respectively. However, other studies showed that the antioxidant capacity of *H. itama* honey was higher than *G. thoracica*. Stingless bee honey evaluated by Tuksitha *et al*. (2018) revealed that the honey produced by *H. itama* showed higher DPPH radical scavenging activity at 47 %; superoxide radical scavenging activity at 61 % and a FRAP value of 50.66 mM Fe(II)/100 g than *G. thoracica* with 17 %, 54 % and 38.0 mM Fe(II)/100 g, respectively. Shamsudin *et al*. (2019) recorded that total phenolic content and FRAP values were higher in honey produced by *H. itama* (33 – 52 mg GAE/100 g; 0.62 – 1.64 µmol/kg) than *G. thoracica* (27 – 56 mg GAE/100 g; 0.53 – 0.93 µmol/kg). In addition, Maringgal *et al*. (2019) also reached similar conclusions in which *H. itama* honey had higher total phenolic compound content (4.47 mg GAE/100 g) and DPPH radical scavenging activity (30 %) than *G. thoracica* (3.39 mg GAE/100 g; 12 %). In their study, other than *H. itama* and *G. thoracica*, the antioxidant capacities were found to be different among other stingless bee species, including *T. laeviceps* and *L. terminate* (Maringgal *et al*., 2019). Other bee species also showed similar outcomes, whereby the total phenolic content and FRAP values in stingless bee honey produced by *Hypotrigona* sp. (527 mg GAE/kg; 667 µM Fe(II)/100 g) were significant higher than *Melipona* sp. (372 mg GAE/kg; 427 µM Fe(II)/100 g) (Nweze *et al*., 2017). Hence, it can be said

that there is no definite comparative outcome regarding the antioxidant properties in honey produced by different bee species. Since phenolic compounds in honey originate from the nectar plants, the phenolic content is greatly affected by the nectar source harvested by the bee types (Truchado *et al*., 2015). Hence, there are significant differences in the phenolic compound levels of honey produced by different stingless bees, followed with the variations in antioxidant activities.

4.3 Chemometric Analysis

In this study, although all stingless bee honey samples were sourced from the same geographical area, stingless bee honey from honeydew and blossom origins showed variations in both physicochemical and antioxidant properties. Therefore, principal component analysis (PCA) a multivariate statistical technique that can uncover the relationships between and among the variables was applied for the differentiation of stingless bee honey samples based on botanical types.

As shown in Table 4.14, the first principal component (PC1) and the second principal component (PC2) represented 56.4 % of the variance. According to correlation coefficients, the parameters that are most associated with PC1 were ash content (-0.822), hydrogen peroxide (-0.886), free acidity (-0.842), total mineral elements (-0.916), K (-0.817), Mg (-0.876), Ca (-0.863), total phenolic content (-0.817) and ferric reducing power (-0.907). Considering only these parameters, another PCA was generated with 84.6 % of total data

variance. The correlation coefficients of these parameters were ash content (-0.891), hydrogen peroxide (-0.878), free acidity (-0.811), total mineral elements (-0.945), K (-0.892), Mg (-0.876), Ca (-0.810), total phenolic content (-0.851) and ferric reducing power (-0.945). The most suitable parameters were highlighted in this analysis in the differentiation of honey samples based on botanical origin.

Furthermore, as displayed in Figures 4.10 and 4.11, this statistical analysis was able to differentiate stingless bee honey samples into two distinctive clusters which relate to honeydew and blossom origins. Therefore, based on the correlation coefficient, it is suggested that parameters including ash content, hydrogen peroxide, free acidity, total mineral elements, K, Mg, Ca, total phenolic compounds and ferric reducing power are able to differentiate stingless bee honey between honeydew and blossom origins.

Table 4.14: Factor loadings for parameters of stingless bee honey samples.

Principal component (PC) number	**Data related to Figure 4.9**		**Data related to Figure 4.10**	
	1	**2**	**1**	**2**
Eigenvalue	11.693	5.220	6.952	0.666
% variance	39.00%	17.40%	77.20%	7.40%
Component score correlation				
Colour intensity	-0.634	-0.698		
Moisture content	-0.764	-0.407		
Water activity	-0.560	-0.390		
Total soluble solid	0.763	0.408		
Proline	0.285	-0.608		
Electrical conductivity	-0.465	0.720		
Ash content	-0.822	0.334	-0.891	0.310
Diastase	-0.330	0.206		
Hydrogen peroxide	-0.886	-0.147	-0.878	-0.324
pH	-0.107	0.179		
Free acidity	-0.842	-0.201	-0.811	-0.438
Gluconic acid	0.288	0.686		
Acetic acid	0.164	0.715		
HMF	-0.040	-0.666		
Na	-0.710	0.268		
K	-0.817	0.235	-0.892	0.307
Mg	-0.876	0.245	-0.876	0.158
Ca	-0.863	-0.127	-0.810	-0.229
Fe	-0.452	0.413		
Zn	-0.375	0.654		

Mn	-0.137	0.450		
Cr	0.159	0.005		
Total mineral elements	-0.916	0.250	-0.945	0.219
Total phenolic compounds	-0.817	-0.285	-0.851	-0.204
ABTS radical scavenging activity	-0.653	-0.196		
DPPH radical scavenging activity	-0.300	0.478		
Superoxide radical scavenging activity	-0.632	-0.259		
Peroxyl radical inhibition	-0.598	-0.302		
Iron chelation	-0.658	0.290		
Ferric reducing power	-0.907	0.178	-0.945	0.110

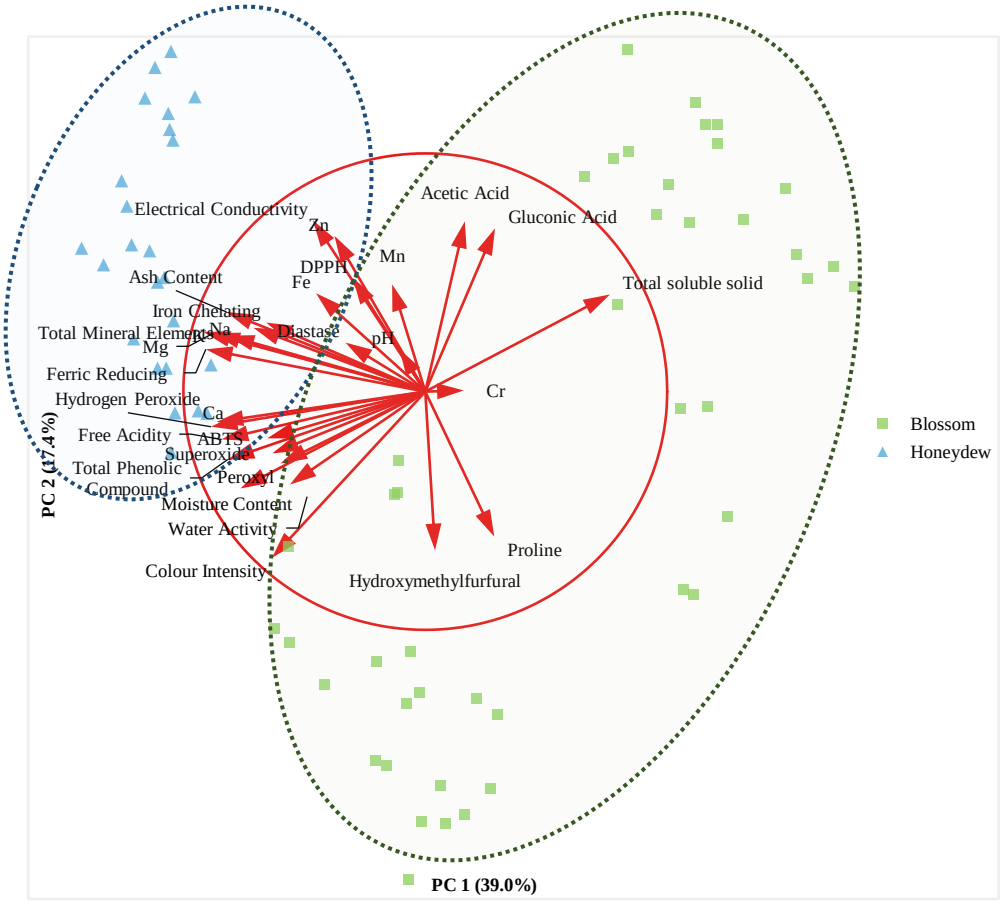


Figure 4.10: Plot of principal component loading of stingless bee blossom and honeydew honey samples and the descriptors including physicochemical and antioxidant properties.

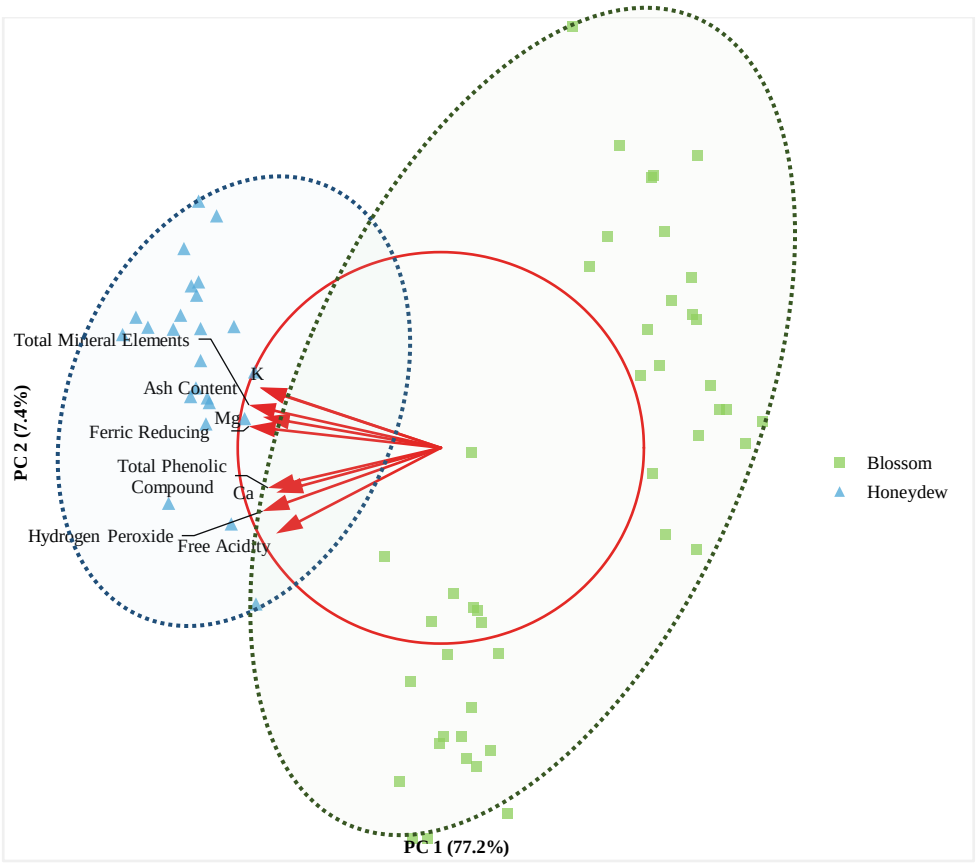


Figure 4.11: Plot of principal component loading of stingless bee blossom and honeydew honey samples and the descriptors including physicochemical and antioxidant properties.

To date, this is the first differentiation of stingless bee honey using physicochemical and antioxidant properties. PCA has also been previously employed in a study to differentiate honey bee honey into honeydew and blossom types (Bergamo *et al*., 2019). In their study, free acidity, total phenolic content, ferric reducing power, electrical conductivity, glucose level and DPPH scavenging activity were suggested to be suitable parameters to differentiate between honeydew honey and blossom honey. Therefore, these PCA outcomes suggest that raw stingless bee honey samples from Malaysia are classifiable into honeydew and blossom origins by physiochemical and antioxidant properties.

4.4 Antibacterial Properties

4.4.1 Inhibitory Effects

The antibacterial effects of stingless bee honey samples were tested against two types of bacteria, Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* which are the two most common bacterial nosocomial infections (Bereket *et al*., 2012). The assessment of inhibitory potency of various honeys was carried out by the agar well diffusion method. As tabulated in Table 4.15, all stingless bee honey samples were able to inhibit the growth of *S. aureus* (ATCC 25923 and ATCC 33591) and *E. coli* (ATCC 25922 and ATCC 35218). The diameter of the inhibition zone exerted on *S. aureus* ranged from 0.7 to 1.4 cm ,while the range for *E. coli* was 0.7 to 1.7 cm. Stingless bee honeys from Malaysia were also found to exhibit broad a antibacterial spectrum with the formation of inhibition zones on Gram-positives *S. aureus* (0.8 – 1.7 cm), *Staphylococcus intermedius* B (0.3 – 1.7 cm), *Staphylococcus xylosus* (1.6 – 2.1 cm), *Streptococcus alactolyticus* (1.5 – 2.5 cm) and Gram-negative *Citrobacter koseri* (3.0 – 3.4 cm), *E. coli* (0.5 – 1.8 cm*), Klebsiella pneumoniae (*0 – 1.3 cm), *Pseudomonas aeruginosa* (1.1 – 1.6 cm), *Salmonella cholerasuis* (0 – 1.8 cm) and *Vibrio parahaemolyticus* (0 – 2.0 cm) (Tuksitha *et al*., 2018). In another study conducted by Omar *et al*. (2019), with the exception of one stingless bee, the honey of which was found to demonstrate very weak antibacterial effects, two other stingless bee honey samples were able to inhibit wound pathogens including *Streptococcus pyogenes*, methicillin resistant *S. aureus* (MRSA), *S. aureus*, *P. aeruginosa*, *E. coli* and *K. pneumoniae* with diameters of inhibition zone 2.1 to 2.4 cm, 1.8 to 2.1 cm, 1.5 to 1.6 cm, 1.7 to 1.8 cm, 1.1 to 1.2 cm and

1.0 to 1.4 cm, respectively.

Table 4.15: Zones of inhibition (cm) of stingless bee honey samples against pathogenic bacteria.

Sample	*S. aureus* (ATCC 25923)	*S. aureus* (ATCC 33591)	*E. coli* (ATCC 25922)	*E. coli* (ATCC 35218)
S1	1.2 ± 0.1	1.0 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
S2	1.1 ± 0	0.9 ± 0.2	1.4 ± 0.1	1.3 ± 0.1
S3	1.1 ± 0	0.9 ± 0	1.6 ± 0	1.3 ± 0.1
S4	1.4 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	1.5 ± 0
S5	1.0 ± 0.1	0.9 ± 0	1.1 ± 0.1	1.2 ± 0
S6	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0
S7	0.9 ± 0.1	0.8 ± 0	1.2 ± 0.1	1.2 ± 0
S8	0.9 ± 0.1	0.8 ± 0	1.0 ± 0	1.1 ± 0
S9	0.7 ± 0.1	0.8 ± 0.1	1.1 ± 0	0.9 ± 0
S10	0.8 ± 0.1	0.8 ± 0	1.0 ± 0.1	0.9 ± 0.1
S11	0.9 ± 0.1	0.8 ± 0	1.1 ± 0.1	0.9 ± 0
S12	0.9 ± 0.1	0.7 ± 0	0.9 ± 0.1	0.7 ± 0
S13	0.8 ± 0.1	0.7 ± 0	1.0 ± 0.1	0.7 ± 0.1
S14	0.8 ± 0.1	0.8 ± 0	1.0 ± 0.1	0.7 ± 0.2
S15	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
S16	0.8 ± 0.1	0.8 ± 0	0.9 ± 0.1	0.7 ± 0
S17	1.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.1	1.0 ± 0
S18	1.0 ± 0.1	0.8 ± 0	1.3 ± 0.1	1.0 ± 0.1
S19	1.2 ± 0.1	0.9 ± 0	1.3 ± 0.1	1.0 ± 0
S20	0.9 ± 0	0.9 ± 0	1.1 ± 0.1	0.8 ± 0.1
S21	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0	0.8 ± 0.1
S22	1.0 ± 0.1	0.8 ± 0	1.3 ± 0.1	1.0 ± 0.1
S23	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.1 ± 0
Average	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.2[a]	1.0 ± 0.2[a]
Honeydew (S1–S8)	1.1 ± 0.2*	0.9 ± 0.1*	1.3 ± 0.3	1.3 ± 0.2*
Blossom (S9 – S23)	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
H. itama (S1–S16)	0.8 ± 0.1^	0.8 ± 0^	1.0 ± 0.1^	0.8 ± 0.1^
G. thoracica (S17–S23)	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1

* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.
a – The zone of inhibition exhibited on *E. coli* was significantly larger than *S. aureus* at $p < 0.05$.

Although comparative investigations of antibacterial effects between stingless bee honey and honey bee honey are rather limited, stingless bee honey has been known to exhibit greater antibacterial activities. Ewnetu *et al.* (2013) stated that honey of the stingless bee exhibited the highest mean inhibition (2.2 cm) compared to other honey bee honeys (2.1 cm and 1.8 cm) on all tested bacterial strains including *S. aureus*, *E. coli* and resistant clinical isolates *S. aureus*, *E. coli* and *K. pneumoniae*. Compared with honey bee honey (phenol equivalence = 0 – 3.4 % w/v; MIC = 16 – 32 % w/v; MBC = 32 % w/v), stingless

bee honey with higher phenol equivalence (0 – 28.6 % w/v) and lower minimum inhibitory concentration (MIC) (1 – 16 % w/v) and minimum bactericidal concentration (MBC) (2 – 16 % w/v) values was found to exhibit a broader spectrum of inhibitory and bactericidal activities against Gram-positive and Gram-negative bacteria including reference and clinical strains of *S. aureus*, *S. pyogenes*, *E. coli* and *Haemophilus influenzae* (Brown *et al*., 2020).

Although honeydew honey is increasingly valued due to its pronounced antibacterial potential (Bucekova *et al*., 2018); to date, there are no scientific data regarding the antibacterial effects of stingless bee honeydew honey. The antibacterial effects of stingless bee honey reported in scientific articles only focusing on blossom honey. As summarized in Table 4.15, among the stingless bee honey samples, honeydew honey had the greatest antibacterial effect with the largest zone of inhibition exerted on *S. aureus* ATCC 25923 (1.1 cm), ATCC 33591 (1.1 cm), *E. coli* ATCC 25922 (1.3 cm) and ATCC 35218 (0.9 cm), compared with blossom honey with 0.9 cm, 0.8 cm, 1.1 cm and 0.9 cm, respectively.

Honeydew honey produced by honey bees was found to exhibit greater antibacterial effects than blossom honey. In a study conducted by Majtan *et al*., (2011), Slovakian honeydew honey was found to have exceptional antibacterial activity against multi-drug resistant *Stenotrophomonas maltophilia* isolated from cancer patients and it was more efficient than Manuka honey. The MIC for honeydew honey ranged from 6.25 to 17.5 %, while Manuka honey ranged from 7.5 to 22.5 %. Furthermore, Italian honeydew honey was found to have higher

bacteriostatic and bactericidal activities on *S. aureus*, *Enterococcus faecalis*, *E. coli*, *Proteus mirabilis* and *P. aeruginosa* than other blossom honeys (Grego *et al*., 2016). In particular, honeydew honey produced a larger inhibition zone (0.8 – 3.1 cm) and lower MIC and MBC values (3.1 - 12.5 % and 3.1 – 50 %, respectively), compared to Manuka honey. Tramuta *et al*. (2017) also demonstrated that honeydew honey-based membranes had strong antibacterial activities against multidrug-resistant strains of *E. coli*, *P. aeruginosa*, *P. mirabilis* and *Staphylococcus pseudointermedius* were isolated from canine wound infections. Honeydew honey-based membranes were able to achieve 100 % inhibition of bacterial growth within 24 hours, with the shortest duration seen with *S. pseudointermedius* (1 hour), followed by *E. coli* and *P. aeruginosa* (6 hours) and lastly *P. mirabilis* (24 hours).

Regarding the susceptibility of bacteria towards the antibacterial action of stingless bee honey, Ewnetu *et al*. (2013), Chanchao (2019) and Omar *et al*. (2019), it was found that *S. aureus* was found to be more susceptible to the inhibitory action of stingless bee honey than *E. coli*. In another study, stingless bee honey was found to exert similar antibacterial effects on *S. aureus*, *E. coli*, *P. aeruginosa* and *B. cereus* with the same MIC and MBC values (Zainol *et al*., 2013). In the same study, stingless honey appeared to be the most consistent in inhibiting bacterial growth regardless of bacterial species, with almost similar growth inhibition curves or similar increments in inhibition percentages of bacterial growth. However, in this study *E. coli* was found to be more sensitive to the antibacterial action of stingless bee honey. Significantly larger zones of inhibition were observed on *E. coli* (1.0 – 1.2 cm) than *S. aureus* (0.8 – 1.0 cm).

These results are in agreement with Al-Naama (2009) who showed that honey had a greater inhibitory effect on Gram-negative *E. coli* and *Pseudomonas* spp. with larger inhibition zones (2.2 cm and 2.3 cm respectively) and lower MIC (6.25 mg/mL and 1.5 mg/mL respectively) than *S. aureus* with an inhibition zone of 2.0 cm and MIC 12.5 mg/mL. Methanol, ethanol and ethyl acetate extracts of raw and processed honey were found to be more effective on Gram-negatives *E. coli*, *P. aeruginosa* and *Salmonella typhi* than Gram-positives *S. aureus*, *B. cereus*, *Bacillus subtilis* and *Micrococcus luteus* (Mohapatra *et al.*, 2011). The inhibition zones exerted on Gram-negative bacteria (1.3 – 3.8 cm) were significantly larger than Gram-positive bacteria (0.7 – 2.4 cm). According to Brudzynski and Sjaarda (2014), honey was found to target the cell wall and lipopolysaccharide outer membrane of *E. coli*, causing cell wall destruction and increased permeability of the outer membrane, eventually leading to cell lysis.

However, this agar well-diffusion method may not reflect the actual total antibacterial activity of stingless bee honey because this method does not differentiate between bacteriostatic and bactericidal activity, nor allow the quantification of bactericidal activity (Boorn~~,~~ *et al.*, 2010; Kwakman and Zaat, 2012). Therefore, endotoxin assay and electron microscopic examination were carried out to confirm the bactericidal effect of stingless bee honey against *E. coli*.

4.4.2 Bactericidal Effects

The bactericidal effect of stingless bee honey on *E. coli*, assessed with endotoxin assay is tabulated in Table 4.16, with the measurement of endotoxin levels in this assay used to to signify death of *E. coli*. According to Brudzynski and Sjaarda (2014), honey treatment was able to cause destruction of the cell wall and disintegration of the lipopolysaccharide outer membrane of *E. coli* with endotoxin release at bactericidal concentrations. In the same table, it can be observed that the treatment of *E. coli* with stingless bee honey led to the release of endotoxins.

Table 4.16: Endotoxin level (EU mL^{-1}) released by *E. coli* treated with stingless bee honey samples after 0-hour and 24-hour incubation.

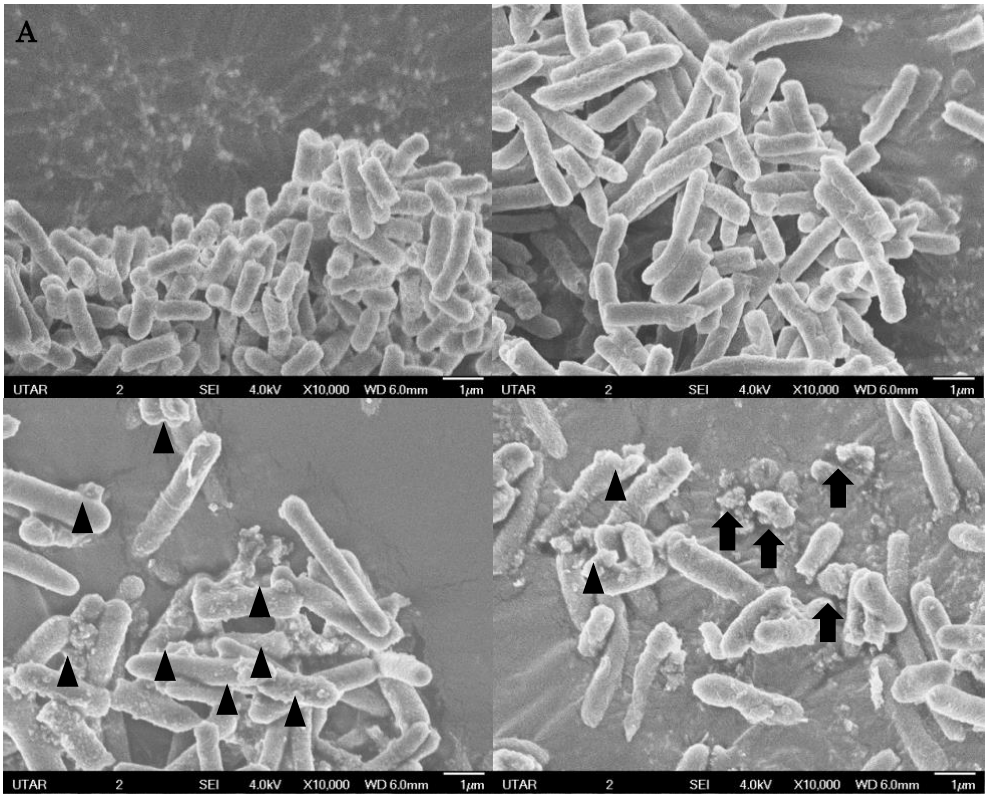
Sample	*E. coli* (ATCC 25922) 0-hour	*E. coli* (ATCC 25922) 24-hour	*E. coli* (ATCC 35218) 0-hour	*E. coli* (ATCC 35218) 24-hour
S1	1.77 ± 0	2.32 ± 0	1.68 ± 0	2.32 ± 0
S2	1.58 ± 0	2.22 ± 0	1.51 ± 0	2.21 ± 0
S3	1.88 ± 0	2.30 ± 0	1.58 ± 0	2.20 ± 0
S4	1.56 ± 0	2.21 ± 0	1.56 ± 0	2.21 ± 0
S5	1.72 ± 0	2.29 ± 0	1.62 ± 0	2.25 ± 0
S6	1.89 ± 0	2.35 ± 0	1.69 ± 0	2.30 ± 0
S7	1.97 ± 0	2.38 ± 0	1.67 ± 0	2.30 ± 0
S8	1.88 ± 0	2.32 ± 0	1.88 ± 0	2.32 ± 0
S9	1.33 ± 0	2.12 ± 0	1.31 ± 0	2.10 ± 0
S10	1.38 ± 0	2.12 ± 0	1.35 ± 0	2.11 ± 0
S11	1.27 ± 0	2.02 ± 0	1.37 ± 0	2.09 ± 0
S12	1.38 ± 0	2.05 ± 0	1.38 ± 0	2.05 ± 0
S13	1.27 ± 0	2.00 ± 0	1.25 ± 0	2.00 ± 0
S14	1.38 ± 0	2.15 ± 0	1.30 ± 0	2.05 ± 0
S15	1.37 ± 0	2.13 ± 0	1.35 ± 0	2.10 ± 0
S16	1.38 ± 0	2.12 ± 0	1.33 ± 0	2.12 ± 0
S17	1.67 ± 0	2.22 ± 0	1.56 ± 0	2.20 ± 0
S18	1.68 ± 0	2.31 ± 0	1.58 ± 0	2.22 ± 0
S19	1.57 ± 0	2.22 ± 0	1.50 ± 0	2.23 ± 0
S20	1.57 ± 0	2.19 ± 0	1.57 ± 0	2.18 ± 0
S21	1.57 ± 0	2.17 ± 0	1.57 ± 0	2.16 ± 0
S22	1.50 ± 0	2.15 ± 0	1.48 ± 0	2.14 ± 0
S23	1.57 ± 0	2.19 ± 0	1.58 ± 0	2.29 ± 0
Average	1.57 ± 0.21	2.20 ± 0.10[a]	1.51 ± 0.16	2.18 ± 0.09[a]
Honeydew (S1–S8)	1.78 ± 0.15*	2.30 ± 0.06*	1.65 ± 0.11*	2.26 ± 0.05*
Blossom (S9 – S23)	1.46 ± 0.14	2.14 ± 0.08	1.43 ± 0.12	2.14 ± 0.08
H. itama (S1–S16)	1.35 ± 0.05^	2.09 ± 0.06^	1.33 ± 0.04^	2.08 ± 0.04^
G. thoracica (S17–S23)	1.59 ± 0.06	2.21 ± 0.05	1.55 ± 0.04	2.20 ± 0.05

* – Significant difference between honeydew honey and blossom honey at $p < 0.05$.
^ – Significant difference between blossom honey produced by *H. itama* and *G. thoracica* at $p < 0.05$.
a – Significant difference between 0-hour and 24-hour at $p < 0.05$.

The release of endotoxins due to bacterial destruction was shown to be increased significantly from 0-h to 24-h of incubation, indicating that more bacteria were killed over a longer duration of incubation. This outcome was consistent with the study reported by Boorn and colleagues (2010) whereby incubation with stingless bee honey for 60 minutes resulted in a significant decrease in the viability of *S. aureus* and *P. aeruginosa* as compared to 0 minutes of incubation. Based on the outcomes of the endotoxin assay, it can be confirmed that stingless bee honeydew honey had a greater antibacterial effect, specifically bactericidal action, than blossom type due to detection of significantly higher levels of endotoxin released in assays with both *E. coli* ATCC 25922 and ATCC 35218 at 0-hour and 24-hours of treatment. The highest levels of endotoxin were detected in *E. coli* ATCC 25922 with 1.56 to 1.97 EU/mL at 0-h then 2.21 to 2.38 EU/mL at 24-h, after treated with honeydew honey. For *E. coli* ATCC 35218, the highest endotoxin levels, 1.51 to 1.88 EU/mL were detected at 0-h then 2.20 to 2.32 EU/mL at 24-h after treatment with the same honey type.

The impacts of stingless bee honeydew honey on the morphology of *E. coli* were further verified by scanning electron microscopy. The normal morphology and intact structures of *E. coli* ATCC 25922 and ATCC 35218 are shown in Figures 4.12(A) and (B), respectively. In Figure 4.12(C), *E. coli* was observed to suffer loss of structural integrity with the formation of bulges on the rough surfaces after treatment with honeydew honey. *E. coli* treated with honey was reported to possess longer rod and filamentous shapes, indicative of the inhibition of septation and cell division. Ruptured and lysed *E. coli* due to the action of honeydew honey can be seen in Figure 4.12(D). Furthermore,

spheroplasts, smaller cells and cell debris were previously observed in honey-treated *E. coli* samples (Brudzynski and Sjaarda, 2014). Together with the results of the endotoxin assay, it is clear that structural changes in *E. coli* and the damage to the cell wall and outer membrane constitute the mechanism underlying the antibacterial effects of stingless bee honeydew honey.



(A) Negative control *E. coli* ATCC 25922 (without honey); (B) Negative control *E. coli* ATCC 35218 (without honey); (C) *E. coli* ATCC 25922 treated with honeydew honey, showing that the cells form bulges with rough surface (arrowhead); (D) *E. coli* ATCC 35218 treated with honeydew honey, showing ruptured and lysed cells (arrow).

Figure 4.12: SEM images of the antibacterial effect of honeydew honey against *E. coli*.

4.4.3 Antibacterial Factors

In order to investigate the physicochemical properties that are mainly responsible for the antibacterial action of stingless bee honeydew honey, sugar solution (43 % fructose, 28 % glucose, and 2.0 % sucrose, g/100 g), hydrogen

peroxide solution (184 μmol/L or μM), acid solution (pH 3.3) and gallic acid solution (104 mgGAE/kg) samples were formulated based on the physicochemical properties of honeydew honey. However, as recorded in Table 4.17, none of the prepared solutions displayed any inhibition and only minimal bactericidal effects on *E. coli*.

Table 4.17: Zone of inhibition (cm) and endotoxin level (EU mL^{-1}) released by *E. coli* treated with different solutions.

Sample	Zone of inhibition		Endotoxin level			
	E. coli (ATCC 25922)	*E. coli* (ATCC 35218)	*E. coli* (ATCC 25922)		*E. coli* (ATCC 35218)	
			0-h	24-h	0-h	24-h
Sugar solution	Nil	Nil	1.20 ± 0	1.25 ± 0	1.07 ± 0	1.10 ± 0
Hydrogen peroxide solution	Nil	Nil	1.41 ± 0	1.52 ± 0	1.22 ± 0	1.35 ± 0
Acid solution	Nil	Nil	1.54 ± 0	1.58 ± 0	1.29 ± 0	1.34 ± 0
Gallic acid solution	Nil	Nil	1.22 ± 0	1.23 ± 0	1.11 ± 0	1.10 ± 0

Nil—No zone of inhibition.

Hyperosmolality is claimed to be one of the antibacterial factors in honey due to its high sugar content, which limits the uptake of water molecules by bacteria for growth. However, in this study, the prepared sugar solution was unable to inhibit both *E. coli* strains. A review by Albaridi (2019) mentioned several studies that also used 'artificial honey' that was prepared by mixing monosaccharides and disaccharides with the same total sugar content as in honey but these were also unable to inhibit the growth of bacteria. Molan (1992) proposed that hyperosmolality was not the predominant antibacterial factor in honey. In an experiment, honey was shown to have 18% of minimum inhibitory concentration in agar well diffusion method but the same concentration of 'artificial honey' was unable to exhibit the same antibacterial effect. Additionally, a study conducted by Al-Waili *et al.* (2005) demonstrated that honey collected from the United Arab Emirates was able to exert potent inhibitory effects against Gram-positive and Gram-negative bacteria. However,

the modelled glucose solution that was tested against the same pathogens showed no antibacterial activity. Thus, the author claimed that osmotic stress in honey was not sufficient to exhibit antibacterial effects (Al-Waili, 2004). Both Nishio *et al*. (2016) and Brown *et al*. (2020) have noted that 'artificial honey' which was a mixture of fructose, glucose, maltose and sucrose, failed to inhibit any bacteria. All these outcomes specifically show that high osmolarity is not the major antibacterial factor in honey. Furthermore, due to the lower sugar content in stingless bee honey, Brown *et al*. (2020) remarked that the contribution of hyperosmolarity in antibacterial activity of stingless bee is minimal compared with acidity.

The acidic environment in honey has been noted to alter the metabolism of bacteria by interfering with several enzymes' activity and by disruption of plasma membrane integrity (Jin and Kirk, 2018). In this study, the acidity of honey was mimicked by using hydrochloric acid to test against *E. coli* strains but failed to display any zone of inhibition. Although the acidic environment in honey is due to the presence of gluconic acid, claimed to be one of the antibacterial factors, Al-Waili *et al*. (2011) observed no significant decrease in antibacterial effect after the acidity of honey was neutralized. Thus, it is suggested that acidity could be an assisting role in the antibacterial effect of honey. In this study, stingless bee honey with the lowest pH was found to exhibit a more potent antibacterial effect, and hence it was suggested that the antibacterial effect of stingless bee honey could be influenced by acidity in combination with other factors.

Hydrogen peroxide possesses the ability to cause extensive protein degradation and cellular damage in bacteria. However, the prepared hydrogen peroxide solution in this study was unable to inhibit the growth of *E. coli*. A similar outcome was observed in a study conducted by Brudzynski *et al*. (2011) whereby a hydrogen peroxide solution with concentration of 256.3 μM was unable to inhibit *E. coli* growth. A possible reason of such an outcome could be due to insufficient levels of hydrogen peroxide in the prepared solution. According to Mohapatra *et al*. (2010), a concentration of 3% hydrogen peroxide in which is commonly used as an antiseptic is approximately 880 mM. Thus, the difference between the concentration of prepared hydrogen peroxide solutions and 3% hydrogen peroxide was too large, therefore, the strength of hydrogen peroxide in the prepared solution was not sufficient to exhibit any inhibitory effect against bacteria. Furthermore, Albaridi (2019) claimed that hydrogen peroxide may have a synergistic interaction with other honey components to exhibit antibacterial effects. This was because the antibacterial effect of honey was not completely removed despite the honey having been treated with catalase to neutralize the presence of hydrogen peroxide. The possible synergistic effect of hydrogen peroxide with other compounds in honey was discussed by Al-Waili *et al*. (2011), whereby the ascorbic acid in honey was suggested to potentiate the action of hydrogen peroxide to exhibit an antibacterial effect. Additionally, honey also contains nitric oxide, which works synergistically with hydrogen peroxide and is claimed to induce DNA double-strand damage in the bacterial genome. Nitric oxide has also been claimed to alter cellular respiration and deplete glutathione level in bacteria, resulting in lipid, DNA, RNA and protein damage by reactive oxygen species (Thannickal and Fanburg, 2000).

Nishio *et al*. (2016) also noted that, after stingless bee honey was treated with catalase, although the honey was still able to inhibit bacterial growth, the antibacterial potency was reduced five-fold. Such data show the central importance of hydrogen peroxide for the antibacterial effect of stingless bee honey, but it is undeniable that there are also other components present in the honey that may inhibit bacterial growth. Massaro *et al*. (2014) postulated that the non-peroxide antibacterial activity of stingless bee honey must be contributed to by another factor, for example phytochemicals.

In this study, a gallic acid solution was used to represent phenolic compounds in the honey but it did not inhibit *E. coli*. This could be an indication that total food extracts may be more beneficial and efficient than isolated constituents, since a bioactive individual compound can change its properties in the presence of other compounds, corresponding to a synergistic antibacterial effect (Estevinho *et al*., 2008). In the same study, a honey replacement consisting of five flavonoids (naringenin, kaempferol, apigenin, pinocembrin and chrysin) and nine phenolic acids (protocatequic acid, *p*-hydroxibenzoic acid, caffeic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, benzoic acid, ellagic acid, and cinnamic acid) was able to exhibi inhibitory effects against *S. aureus*, *B. subtilis*, *Staphylococcus lentus*, *K. pneumoniae* and *E. coli*. The study also concluded that the phenolic compounds in honey are partially responsible for the antibacterial activity of honey (Estevinho *et al*., 2008). The antibacterial mechanisms of phenolic compounds were claimed to destroy the bacterial membrane, prevent biofilm formation and inhibit virulence factors including toxins and enzymes. Furthermore, phenolic compounds were found to diminish antibiotic resistance

of pathogenic bacteria (Miklasińska-Majdanik *et al*., 2018).

According to Bucekova *et al*. (2018), just hydrogen peroxide or phenolic compounds were insufficient to exhibit antibacterial effects in honey. However, hydrogen peroxide was said to be able to accelerate the auto-oxidation process of phenolic compounds to generate more reactive oxygen species. Thus, the synergism between hydrogen peroxide and phenolic compounds can lead to greater DNA damage and inhibit the multiplication of bacterial cells. Still, the exact phenolic compounds to have synergism with hydrogen peroxide has yet to be identified. Therefore, it could be another reason to explain the inability of prepared gallic acid solution to exhibit any inhibitory effect against *E. coli* in this study. Other authors have mentioned the antibacterial factors in honey are not limited to hyperosmolarity, acidic stress, hydrogen peroxide and phenolic compounds. It is also possible that other components such as, methylglyoxal, antimicrobial peptides, methyl syringate and 5-hydroxymethylfurfural may contribute to the antibacterial effect of honey (Nishio *et al*, 2016). The outcomes of this study suggest that the antibacterial effect of stingless bee honey is due to an interaction among different components instead of depending solely on one of these physicochemical properties.

4.4.4 Interactive Effects with Antibiotics

Combination antibiotic therapies are widely practiced clinically to treat chronic wound infections especially when involving antibiotic resistant bacteria. In this study, the combined inhibitory effects of stingless bee honeydew honey

and antibiotics were tested on four clinical isolates of *E. coli*, together with *E. coli* reference strains ATCC 25922 and 35218. The antibiotic susceptibility profile of each clinical strain is presented in Table 4.18. Other than *E. coli* 1, which was resistant to ampicillin, other isolates were resistant to at least two antibiotics, particularly *E. coli* 3, which was resistant to three out of four tested antibiotics.

Table 4.18: Antibiotic susceptibility profile of each *E. coli* clinical isolate.

Antibiotic	*E. coli* 1	*E. coli* 2	*E. coli* 3	*E. coli* 4
Ampicillin (10 µg)	R	R	R	R
Chloramphenicol (30 µg)	S	S	R	S
Gentamicin (10 µg)	S	S	S	S
Tetracycline (30 µg)	S	R	R	R

R—Resistant; S—Susceptible.

As displayed in Table 4.19, a combination of honey and antibiotic was considered synergistic when the scored zone of inhibition for the combination was bigger than the zone of inhibition of honey and antibiotic separately (Hegazi *et al*., 2014). The results revealed that the addition of honeydew honey showed synergistic antibacterial effects with ampicillin, with larger diameters of inhibition zones against *E. coli* 1, from 0.7 cm for honeydew honey alone and no zone of inhibition for ampicillin alone to 0.9 cm for the combination; *E. coli* 2, from 0.7 cm for honeydew honey alone and 0.7 cm for ampicillin alone to 1.3 cm for the combination; *E. coli* 3, from 1.0 cm for honeydew honey alone and no zone of inhibition for ampicillin alone to 1.4 cm for the combination; *E. coli* ATCC 25922, from 1.2 cm for honeydew honey alone and 1.0 cm for ampicillin alone to 1.7 cm for the combination.

Table 4.19: Antibacterial activity of honeydew honey, ampicillin, gentamicin separately and combined against *E. coli* isolates.

Sample	*E. coli* 1	*E. coli* 2	*E. coli* 3	*E. coli* 4	*E. coli* (ATCC 25922)	*E. coli* (ATCC 35218)
Honey	0.7 ± 0.1	0.7 ± 0	1.0 ± 0.1	0.7 ± 0.1	1.2 ± 0	1.0 ± 0
Ampicillin	Nil	0.7 ± 0.1	Nil	Nil	1.0 ± 0.1	Nil
Honey + Ampicillin	0.9 ± 0 (S)	1.3 ± 0 (S)	1.4 ± 0.1 (S)	0.7 ± 0.1	1.7 ± 0 (S)	1.0 ± 0.1
Gentamicin	1.3 ± 0	1.0 ± 0	Nil	1.3 ± 0	2.0 ± 0	1.6 ± 0
Honey + Gentamicin	1.3 ± 0.1	1.3 ± 0.1 (S)	1.4 ± 0 (S)	0.9 ± 0.1	2.2 ± 0 (S)	1.3 ± 0.1

Nil – No zone of inhibition.
(S) – Synergistic effect achieved.

E. coli isolates, including antibiotic resistant clinical strains, were found to have higher susceptibility to the mixture of stingless bee honeydew honey and antibiotics. Larger inhibition zones exhibited by the combination of honey and ampicillin were considered to indicate synergy when the scored zone of inhibition for the combination was bigger than the zone of inhibition of honey or antibiotic separately (Hegazi *et al*., 2014). Although not all of the *E. coli* strains tested responded in the same way to these combination treatments, the honey–ampicillin combination was considered as the most promising, with larger inhibition zones and higher endotoxin levels. Honey has previously been found to work better with beta-lactam antibiotics to inhibit bacteria (Liu *et al*., 2015). The synergistic antibacterial effects of stingless bee honey with antibiotics against multidrug-resistant bacteria isolated from infected wounds was also reported by Ng *et al*. (2017). In that study, greater inhibitory activity against *S. aureus* was observed in the combination of honey and ampicillin with the largest inhibition zone and the highest bactericidal rate. The synergistic effect was believed to cause significant morphological alteration and subsequently bacterial cell lysis.

Greater antibacterial effects are believed to derive from the involvement of honey antibacterial factors and ampicillin together. One of the ways in which a combination of antibacterial compounds works is when both compounds act sequentially, achieving a 'like plus like' effect (Kalan and Wright, 2011). Although sugars are the important components of honey, none of the tested antibiotics in combination with sugar at an equivalent concentration to honey showed larger zones of inhibition in any of the tested bacteria (Liu *et al*., 2015). Such an outcome suggests that the sugar content of honey is unlikely to contribute to the synergistic effects observed. On the other hand, hydrogen peroxide, which is naturally present in honey, can diffuse through the bacterial cell membrane easily and lead to the generation of hydroxyl free radicals. Oxidative stress caused by the free radicals encourages lipid peroxidation, which would disrupt the integrity of cell membrane (Brudzynski *et al*., 2011). Furthermore, the formation of hydroxyl free radicals could destroy bacterial DNA (Jantakee and Tragoolpua, 2015). It is proposed that DNA damage could result in inhibition of the formation of the enzyme β-lactamase, which would greatly enhance the susceptibility of bacteria towards the action of ampicillin (Ng *et al*., 2017). Jenkins and Cooper (2012b) also reported that honey was found to interact synergistically with oxacillin, a beta-lactam antibiotic in the inhibition on MRSA. Antibiotic resistance in MRSA is associated with the *mec* gene complex whereby *mecA* encodes a penicillin-binding protein with low binding affinity for beta-lactam antibiotics that allow peptidoglycan biosynthesis. On the other hand, *mecA* is regulated via *mecR1*, which encodes for a two-component sensor/signal transducer protein, and *mecI*, which encodes for a repressor protein (Meng *et al*., 2006). In that study, microarray analysis

showed that exposure of MRSA to honey resulted in down-regulation of *mecR1*. Hence, the synergistic antibacterial effect could be attributed to restored oxacillin resistance in MRSA treated with honey.

On the other hand, gentamicin, which is an aminoglycoside, inhibits bacteria by targeting the 30S subunits of the ribosome (Schlunzen *et al*., 2001). Since honey alters the production of proteins, including ribosomal proteins in bacteria (Blair *et al*., 2009; Packer *et al*., 2012), the synergistic effect of honey in combination with gentamicin may be due to these impacts on protein synthesis pathways, thus inhibiting the growth of bacteria more effectively. However, the combination of honey and gentamicin produced little to no synergistic effects in this study. Although slight larger zones of inhibition were observed with the combination of honey and gentamicin against *E. coli* 2, *E. coli* 3 and *E. coli* ATCC 25922, similar or smaller zones of inhibition were observed in the combination of honeydew honey with gentamicin for *E. coli* 1, *E. coli* 4 and *E. coli* ATCC 35218. Equally, Jenkins and Cooper (2012a) also reported honey and gentamicin combinations had no effect against methicillin-resistant *S. aureus* (MRSA).

Interestingly, not all *E. coli* strains were found to have higher susceptibility towards the honey-antibiotic combination. This may be due to different responses in these strains toward the stresses induced by the honey and/or antibiotics such as efflux systems or barriers that prevent the entry, accumulation or action of these antibacterial agents (Liu *et al*., 2015). Although the combination of honey and antibiotic may not always work synergistically,

honey still can be recommended a good antibiotic adjuvant. Liu *et al*. (2015) also stated the antibiotic can act systemically, entering from the bottom of the wound bed, while honey acts topically from the top of the wound. The overall effectiveness of stingless bee honeydew honey and antibiotic combinations shown in this study can be suggested as an alternative for wound infection treatment, since *E. coli* is one of the most common bacterial species associated with acute and chronic wound infections (Bowler *et al*., 2001; Bessa *et al*., 2015).

4.5 Future Studies

This study has shown that the physicochemical and bioactivity of stingless bee honey are significantly affected by honeydew and blossom origins. Hence in future, the impact of different monofloral origins on the properties of stingless bee honey should be further investigated. Furthermore, as shown in this study, the comparison of properties of honey originating from the same monofloral origin but produced by different species of stingless bee needs to be investigated.

The presence of toxic substances in stingless bee honey including heavy metals mercury (Hg), cadmium (Cd), lead (Pb), nickel (Ni) and arsenic (As), and pyrethroids, organochlorine and organophosphate pesticide residues can be detected and quantified. Such information could be an important indicator for environmental pollution.

High bioactivities of the stingless bee honey are associated with the infiltration of phytochemicals from honey pots that made of cerumen (Abd Jalil *et al*., 2017). However, the bioactivities and active compounds of cerumen have not been widely studied. Thus, such properties of cerumen from different nectar origins and stingless bee species should be further investigated.

Lastly, although *n*-butyl β-D-glucopyranoside isolated in this study is an artifact, the literature reports into the bioactivities of this compound are still limited. Hence, the antioxidant and antibacterial properties of this butylated glucoside and its derivatives should be further studied in future.

CHAPTER 5

CONCLUSION

This is the first report about the isolation and identification of *n*-butyl β-D-glucopyranoside from a honey sample. However, due to the absence of butyl-glucoside signals in the ^{13}C and ^{1}H NMR spectra of raw honey, the presence of this compound is most probably an artifact from the extraction process.

This study also demonstrated the potential of ^{13}C NMR spectroscopy to identify and quantify the major sugar molecules in stingless bee honey samples. Consistent major sugar composition observed in honeydew and blossom honey samples could be useful for the identification of unadulterated stingless bee honey. Furthermore, physicochemical parameters including ash content, hydrogen peroxide, free acidity, total mineral elements, K, Mg and Ca levels, together with total phenolic content and ferric reducing power were identified with principal component analysis (PCA) that can differentiate stingless bee honey samples based on botanical origin. Chemometric analysis has demonstrated the potentials of using these parameters to evaluate the authenticity of stingless bee honey in Malaysia. This is the first report characterizing stingless bee honey collected over the past three years for the differentiation of botanical origins using chemometrics. The obtained data can be useful for legislation when reviewing the existing standard parameters for stingless bee honeys from tropical and subtropical regions.

In terms of bioactivities, stingless bee honeydew honey was found to have significantly higher antioxidant capacities than blossom honey. Stingless bee honeydew honey also exhibited greater antibacterial properties with both inhibitory and bactericidal effects. A synergistic effect was observed between this honey with antibiotics in inhibiting and eradicating antibiotic resistant bacteria. Outcomes of this study reveal the potential of honeydew honey produced by the stingless bee which can serve as an antibacterial agent in health care.

REFERENCES

Abd-El Aal, A.M., El-Hadidy, M.R., El-Mashad, N.B. and El-Sebaie, A.H., 2007. Antimicrobial effect of bee honey in comparison to antibiotics on organisms isolated from infected burns. *Annals of Burns and Fire Disasters*, 20(2), pp. 83-88.

Abd Jalil, M.A., Kasmuri, A.R. and Hadi, H., 2017. Stingless bee honey, the natural wound healer: A review. *Skin Pharmacology and Physiology*, 30(2), pp. 66-75.

Abdul Hamid, Z., 2019. Stingless bee the next "superfood" industry. News Straits Times [Internet]. Aug 2019, available from https://www.nst.com.my/opinion/columnists/2019/08/514289/stingless-bees-next-super-food-industry

Adebiyi, F.M., Akpan, I., Obiajunwa, E.I. and Olaniyi, H.B., 2004. Chemical/physical characterization of Nigerian honey. *Pakistan Journal of Nutrition*, 3(5), pp. 278-281.

Aggad, H. and Guemour, D., 2014. Honey antibacterial activity. *Medicinal & Aromatic Plants*, 3(2), pp. 1-2.

Aghamirlou, H.M., Khadem, M., Rahmani, A., Sadeghian, M., Mahvi, A.H., Akbarzadeh, A. and Nazmara, S., 2015. Heavy metals determination in honey samples using inductively coupled plasma-optical emission spectrometry. *Journal of Environmental Health Science and Engineering*, 13(1), pp.1-8.

Ahmed, M., Djebli, N., Aissat, S., Bacha, S., Meslem, A. and Khiati, B., 2012. Synergistic inhibition of natural honey and potato starch and their correlation with diastase number and sugar content against *Klebsiella pneumoniae* ATCC 27736. *Natural Products Chemistry & Research*, 1(1), pp. 1-4.

Ahmad, A., Shameem, M. and Husain, Q., 2012. Relation of oxidant-antioxidant imbalance with disease progression in patients with asthma. *Annals of Thoracic Medicine*, 7(4), pp. 226-232.

Ahmed, S., Sulaiman, S.A., Baig, A.A., Ibrahim, M., Liaqat, S., Fatima, S., Jabeen, S., Shamim, N. and Othman, N.H., 2018. Honey as a potential natural antioxidant medicine: an insight into its molecular mechanisms of action. *Oxidative Medicine and Cellular Longevity*, 2018, pp. 1-10.

Ahmed, M., Imtiaz Shafiq, M., Khaleeq, A., Huma, R., Abdul Qadir, M., Khalid, A., Ali, A. and Samad, A., 2016. Physiochemical, biochemical, minerals content analysis, and antioxidant potential of national and international honeys in Pakistan. *Journal of Chemistry*, 2016, pp. 1-10.

Ajibola, A., 2015. Novel insights into the health importance of natural honey. *The Malaysian Journal of Medical Sciences: MJMS*, 22(5), pp. 7-22.

Albaridi, N.A., 2019. Antibacterial potency of honey. *International Journal of Microbiology*, 2019, pp. 1-10.

Al-Farsi, M., Al-Amri, A., Al-Hadhrami, A. and Al-Belushi, S., 2018. Color, flavonoids, phenolics and antioxidants of Omani honey. *Heliyon*, 4(10), p. e00874.

Al-Mamary, M., Al-Meeri, A. and Al-Habori, M., 2002. Antioxidant activities and total phenolics of different types of honey. *Nutrition Research*, 22(9), pp. 1041-1047.

Al-Naama, R.T., 2009. Evaluation of in-vitro inhibitory effect of honey on some microbial isolate. *African Journal of Bacteriology Research*, 1(6), pp. 64-67.

Al-Waili, N.S., 2004. Investigating the antimicrobial activity of natural honey and its effects on the pathogenic bacterial infections of surgical wounds and conjunctiva. *Journal of Medicinal Food*, 7(2), pp. 210-222.

Al-Waili, N., Akmal, M., Al-Waili, F., Saloom, K. and Ali, A., 2005. The antimicrobial potential of honey from United Arab Emirates on some microbial isolates. *Medical Science Monitor*, 11(12), pp. BR433-BR438.

Al-Waili, N.S., Salom, K., Butler, G. and Al Ghamdi, A.A., 2011. Honey and microbial infections: a review supporting the use of honey for microbial control. *Journal of Medicinal Food*, 14(10), pp. 1079-1096.

Albaridi, N.A., 2019. Antibacterial potency of honey. *International Journal of Microbiology*, 2019, pp. 1-10.

Allen, K.L., Molan, P.C. and Reid, G.M., 1991. A survey of the antibacterial activity of some New Zealand honeys. *Journal of Pharmacy and Pharmacology*, 43(12), pp. 817-822.

Almasaudi, S.B., El-Shitany, N.A., Abbas, A.T., Abdel-dayem, U.A., Ali, S.S., Al Jaouni, S.K. and Harakeh, S., 2016. Antioxidant, anti-inflammatory, and antiulcer potential of manuka honey against gastric ulcer in rats. *Oxidative Medicine and Cellular Longevity*, 2016, pp. 1-10.

Almeida-Muradian, L.B., Stramm, K.M., Horita, A., Barth, O.M., da Silva de Freitas, A. and Estevinho, L.M., 2013. Comparative study of the physicochemical and palynological characteristics of honey from *Melipona subnitida* and *Apis mellifera*. International *Journal of Food Science & Technology*, 48(8), pp. 1698-1706.

Alzahrani, H.A., Boukraâ, L., Yuva Bellik, F.A., Bakhotmah, B.A., Kolayli, S. and Sahin, H., 2012. Evaluation of the antioxidant activity of three varieties of honey from different botanical and geographical origins. *Global Journal of Health Science*, 4(6), pp. 191-196.

Andrews, J.M., 2009. BSAC standardized disc susceptibility testing method (version 8). *Journal of Antimicrobial Chemotherapy*, 64(3), pp. 454-489.

Angyal, S.J., 1984. The composition of reducing sugars in solution. In Advances in Carbohydrate Chemistry and Biochemistry (Vol. 42, pp. 15-68). Academic Press.

Angyal, S.J., 1991. The composition of reducing sugars in solution: Current aspects. In Advances in Carbohydrate Chemistry and Biochemistry (Vol. 49, pp. 19-35). Academic Press.

Angyal, S.J., 1994. The composition of reducing sugars in dimethyl sulfoxide solution. *Carbohydrate Research*, 1(263), pp. 1-11.

Anklam, E., 1998. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry*, 63(4), pp. 549-562.

Antkowiak, R., Antkowiak, W.Z., Banczyk, I. and Mikolajczyk, L., 2003. A new phenolic metabolite, involutone, isolated from the mushroom *Paxillus involutus*. *Canadian Journal of Chemistry*, 81(1), pp. 118-124.

Aronne, G. and De Micco, V., 2010. Traditional melissopalynology integrated by multivariate analysis and sampling methods to improve botanical and geographical characterisation of honeys. *Plant Biosystems*, 144(4), pp. 833-840.

Association of Official Analytical Chemists (AOAC), 1990. Official Methods of Analysis of the Association of Official Analytical Chemists. *Journal of AOAC International*, 934(6), pp. 1-2.

Atanassova, J., Lazarova, M. and Yurukova, L., 2016. Significant parameters of Bulgarian honeydew honey. *Journal of Central European Agriculture*, 17(3), pp. 640-651.

Ávila, S., Hornung, P.S., Teixeira, G.L., Malunga, L.N., Apea-Bah, F.B., Beux, M.R., Beta, T. and Ribani, R.H., 2019. Bioactive compounds and biological properties of Brazilian stingless bee honey have a strong relationship with the pollen floral origin. *Food Research International*, 123, pp. 1-10.

Ayaz, M., Ullah, F., Sadiq, A., Ullah, F., Ovais, M., Ahmed, J. and Devkota, H.P., 2019. Synergistic interactions of phytochemicals with antimicrobial agents: Potential strategy to counteract drug resistance. *Chemico-biological Interactions*, 308, pp. 294-303.

Azonwade, F.E., Paraïso, A., Agbangnan Dossa, C.P., Dougnon, V.T., N'tcha, C., Mousse, W. and Baba-Moussa, L., 2018. Physicochemical characteristics and microbiological quality of honey produced in Benin. *Journal of Food Quality*, 2018, pp. 1-13.

Babacan, S. and Rand, A.G., 2007. Characterization of honey amylase. *Journal of Food Science*, *72*(1), pp. C050-C055.

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

Baltrušaitytė, V., Venskutonis, P.R. and Čeksterytė, V., 2007. Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry*, 101(2), pp. 502-514.

Bang, L.M., Buntting, C. and Molan, P., 2003. The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing. The *Journal of Alternative & Complementary Medicine*, 9(2), pp. 267-273.

Bates, R.B., Byrne, D.N., Kane, V.V., Miller, W.B. and Taylor, S.R., 1990. NMR characterization of trehalulose from the excrement of the sweet potato whitefly, *Bemisia tabaci*. *Carbohydrate Research*, 201(2), pp. 342-345.

Belay, A., Solomon, W.K., Bultossa, G., Adgaba, N. and Melaku, S., 2013. Physicochemical properties of the Harenna forest honey, Bale, Ethiopia. *Food Chemistry*, 141(4), pp. 3386-3392.

Benzie, I.F. and Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, 299, pp. 15-27.

Bereket, W., Hemalatha, K., Getenet, B., Wondwossen, T., Solomon, A., Zeynudin, A. and Kannan, S., 2012. Update on bacterial nosocomial infections. *European Review for Medical and Pharmacological Sciences*, 16(8), pp. 1039-1044.

Beretta, G., Granata, P., Ferrero, M., Orioli, M. and Facino, R.M., 2005. Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. *Analytica Chimica Acta*, 533(2), pp. 185-191.

Bergamo, G., Seraglio, S.K.T., Gonzaga, L.V., Fett, R. and Costa, A.C.O., 2019. Physicochemical characteristics of bracatinga honeydew honey and blossom honey produced in the state of Santa Catarina: An approach to honey differentiation. *Food Research International*, 116, pp. 745-754.

Bessa, L.J., Fazii, P., Di Giulio, M. and Cellini, L., 2015. Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection. *International Wound Journal*, 12(1), pp. 47-52.

Biluca, F.C., Braghini, F., Gonzaga, L.V., Costa, A.C.O. and Fett, R., 2016. Physicochemical profiles, minerals and bioactive compounds of stingless bee honey (Meliponinae). *Journal of Food Composition and Analysis*, 50, pp. 61-69.

Biluca, F.C., de Gois, J.S., Schulz, M., Braghini, F., Gonzaga, L.V., Maltez, H.F., Rodrigues, E., Vitali, L., Micke, G.A., Borges, D.L. and Costa, A.C.O., 2017. Phenolic compounds, antioxidant capacity and bioaccessibility of minerals of stingless bee honey (Meliponinae). *Journal of Food Composition and Analysis*, 63, pp. 89-97.

Biluca, F.C., Della Betta, F., de Oliveira, G.P., Pereira, L.M., Gonzaga, L.V., Costa, A.C.O. and Fett, R., 2014. 5-HMF and carbohydrates content in stingless bee honey by CE before and after thermal treatment. *Food Chemistry*, 159, pp. 244-249.

Blair, S.E., Cokcetin, N.N., Harry, E.J. and Carter, D.A., 2009. The unusual antibacterial activity of medical-grade Leptospermum honey: antibacterial spectrum, resistance and transcriptome analysis. *European Journal of Clinical Microbiology & Infectious Diseases*, 28(10), pp. 1199-1208.

Blasa, M., Candiracci, M., Accorsi, A., Piacentini, M.P., Albertini, M.C. and Piatti, E., 2006. Raw Millefiori honey is packed full of antioxidants. *Food Chemistry*, 97(2), pp. 217-222.

Boateng, J. and Diunase, K.N., 2015. Comparing the antibacterial and functional properties of Cameroonian and Manuka honeys for potential wound healing—have we come full cycle in dealing with antibiotic resistance? *Molecules*, 20(9), pp. 16068-16084.

Bogdanov, S., Lüllmann, C., Martin, P., von der Ohe, W., Russmann, H., Vorwohl, G., Oddo, L.P., Sabatini, A.G., Marcazzan, G.L., Piro, R. and Flamini, C., 1999. Honey quality and international regulatory standards: review by the International Honey Commission. *Bee World*, 80(2), pp. 61-69.

Bogdanov, S., Martin, P. and Lullmann, C., 2002. Harmonised methods of the international honey commission. *Swiss Bee Research Centre*, *FAM*, *Liebefeld*.

Bogdanov, S., Ruoff, K. and Oddo, L.P., 2004. Physico-chemical methods for the characterisation of unifloral honeys: a review. *Apidologie*, 35(Suppl. 1), pp. S4-S17.

Bogdanov, S., Vit, P. and Kilchenmann, V., 1996. Sugar profiles and conductivity of stingless bee honeys from Venezuela. *Apidologie*, 27(6), pp. 445-450.

Boorn, K.L., Khor, Y.Y., Sweetman, E., Tan, F., Heard, T.A. and Hammer, K.A., 2010. Antimicrobial activity of honey from the stingless bee *Trigona carbonaria* determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology. *Journal of Applied Microbiology*, 108(5), pp. 1534-1543.

Bowler, P.G., Duerden, B.I. and Armstrong, D.G., 2001. Wound microbiology and associated approaches to wound management. *Clinical Microbiology Reviews*, 14(2), pp. 244-269.

Braunwarth, H. and Brill, F.H., 2014. Antimicrobial efficacy of modern wound dressings: oligodynamic bactericidal versus hydrophobic adsorption effect. *Wound Medicine*, 5, pp. 16-20.

Brown, E., O'Brien, M., Georges, K. and Suepaul, S., 2020. Physical characteristics and antimicrobial properties of *Apis mellifera*, *Frieseomelitta nigra* and *Melipona favosa* bee honeys from apiaries in Trinidad and Tobago. *BMC Complementary Medicine and Therapies*, 20(1), pp. 1-9.

Brudzynski, K., 2006. Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. *Canadian Journal of Microbiology*, 52(12), pp. 1228-1237.

Brudzynski, K., Abubaker, K. and Castle, A., 2011. Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. *Frontiers in Microbiology*, 2, pp. 1-9.

Brudzynski, K. and Lannigan, R., 2012. Mechanism of honey bacteriostatic action against MRSA and VRE involves hydroxyl radicals generated from honey hydrogen peroxide. *Frontiers in Microbiology*, 3(36), pp. 1-8.

Brudzynski, K. and Miotto, D., 2011. Honey melanoidins: Analysis of the compositions of the high molecular weight melanoidins exhibiting radical-scavenging activity. *Food Chemistry*, 127(3), pp. 1023-1030.

Brudzynski, K. and Sjaarda, C., 2014. Antibacterial compounds of Canadian honeys target bacterial cell wall inducing phenotype changes, growth inhibition and cell lysis that resemble action of β-lactam antibiotics. *PloS one*, 9(9), p. e106967.

Bucekova, M., Buriova, M., Pekarik, L., Majtan, V. and Majtan, J., 2018. Phytochemicals-mediated production of hydrogen peroxide is crucial for high antibacterial activity of honeydew honey. *Scientific Reports*, 8(1), pp. 1-9.

Bucekova, M., Sojka, M., Valachova, I., Martinotti, S., Ranzato, E., Szep, Z., Majtan, V., Klaudiny, J. and Majtan, J., 2017. Bee-derived antibacterial peptide, defensin-1, promotes wound re-epithelialisation *in vitro* and *in vivo*. *Scientific Reports*, *7*(1), pp. 1-13.

Camara, V.C. and Laux, D., 2010. Moisture content in honey determination with a shear ultrasonic reflectometer. *Journal of Food Engineering*, 96(1), pp. 93-96.
Can, Z., Yildiz, O., Sahin, H., Turumtay, E.A., Silici, S. and Kolayli, S., 2015.

An investigation of Turkish honeys: their physico-chemical properties, antioxidant capacities and phenolic profiles. *Food Chemistry*, 180, pp. 133-141.

Chai, T., Mohan, M., Ong, H. and Wong, F., 2014. Antioxidant, iron-chelating and anti-glucosidase activities of *Typha domingensis* Pers (Typhaceae). *Tropical Journal of Pharmaceutical Research*, 13(1), pp. 67-72.

Chan-Rodríguez, D., Ramón-Sierra, J., Lope-Ayora, J., Sauri-Duch, E., Cuevas-Glory, L. and Ortiz-Vázquez, E., 2012. Antibacterial properties of honey produced by *Melipona beecheii* and *Apis mellifera* against food-born microorganisms. *Food Science and Biotechnology*, 21(3), pp. 905-909.

Chanchao, C., 2009. Antimicrobial activity by *Trigona laeviceps* (stingless bee) honey from Thailand. *Pakistan Journal of Medical Sciences*, 25(3), pp. 364-369.

Chen, C., 2019. Relationship between water activity and moisture content in floral honey. *Foods*, 8(1), p. 30.

Chen, C., Campbell, L., Blair, S.E. and Carter, D.A., 2012. The effect of standard heat and filtration processing procedures on antimicrobial activity and hydrogen peroxide levels in honey. *Frontiers in Microbiology*, 3, pp. 1-8.

Cheng, M.Z.S.Z., Ismail, M., Chan, K.W., Ooi, D.J., Ismail, N., Zawawi, N. and Mohd Esa, N., 2019. Comparison of sugar content, mineral elements and antioxidant properties of *Heterotrigona itama* honey from suburban and forest in Malaysia. *Malaysian Journal of Medicine and Health Sciences*, 15, pp. 104-112.

Chirife, J., Zamora, M.C. and Motto, A., 2006. The correlation between water activity and % moisture in honey: Fundamental aspects and application to Argentine honeys. *Journal of Food Engineering*, 72(3), pp. 287-292.

Choi, S.H. and Nam, M.S., 2020. Classification of honeydew and blossom honeys by principal component analysis of physicochemical parameters. *Korean Journal of Agricultural Science*, 47(1), pp. 67-81.

Chua, L.S., Rahaman, N.L.A., Adnan, N.A. and Eddie Tan, T.T., 2013. Antioxidant activity of three honey samples in relation with their biochemical components. *Journal of Analytical Methods in Chemistry*, 2013, pp. 1-8.

Chuttong, B., Chanbang, Y., Sringarm, K. and Burgett, M., 2016. Physicochemical profiles of stingless bee (Apidae: Meliponini) honey from South east Asia (Thailand). *Food Chemistry*, 192, pp. 149-155.

Codex Alimentarius Commission, 2001. Revised codex standard for honey. Codex Stan, pp. 12-1981.

Cokcetin, N.N., Pappalardo, M., Campbell, L.T., Brooks, P., Carter, D.A., Blair, S.E. and Harry, E.J., 2016. The antibacterial activity of Australian Leptospermum honey correlates with methylglyoxal levels. *PloS one*, 11(12), p. e0167780.

Conti, M.E., Stripeikis, J., Campanella, L., Cucina, D. and Tudino, M.B., 2007. Characterization of Italian honeys (Marche Region) on the basis of their mineral content and some typical quality parameters. *Chemistry Central Journal*, 1(1), pp. 1-10.

Cooper, R.A., Jenkins, L., Henriques, A.F.M., Duggan, R.S. and Burton, N.F., 2010. Absence of bacterial resistance to medical-grade manuka honey. *European Journal of Clinical Microbiology & Infectious Diseases*, 29(10), pp. 1237-1241.

Coquet, A., Haerdi, W., Degli Agosti, R. and Veuthey, J.L., 1994. Determination of sugars by liquid chromatography with post-column catalytic derivatization and fluorescence detection. *Chromatographia*, 38(1), pp. 12-16.

Coulter, A.D., Godden, P.W., Pretorius, I.S. 2004. Succinic acid—How it is formed, what is its effect on titratable acidity, and what factors influence its concentration in wine? *Australian and New Zealand Wine Industry Journal*, 19(6), pp. 16–25.

Council Directive of the European Union, 2002. Council Directive 2001/110/EC of 20 December 2001 relating to honey. *Official Journal of the European Communities*, 10, pp. 47-52.

Crosby, H.A., Bion, J.F., Penn, C.W. and Elliott, T.S.J., 1994. Antibiotic-induced release of endotoxin from bacteria in vitro. *Journal of Medical Microbiology*, 40(1), pp. 23-30.

Czipa, N., Andrasi, D. and Kovacs, B., 2015. Determination of essential and toxic elements in Hungarian honeys. *Food Chemistry*, 175, pp. 536-542.

Czipa, N., Borbely, M. and Gyori, Z., 2012. Proline content of different honey types. *Acta Alimentaria*, 41(1), pp. 26-32.

da Costa, A.C.V., Sousa, J.M.B., da Silva, M.A.A.P., dos Santos Garruti, D. and Madruga, M.S., 2018. Sensory and volatile profiles of monofloral honeys produced by native stingless bees of the Brazilian semiarid region. *Food Research International*, 105, pp. 110-120.

da Silva, I.A.A., da Silva, T.M.S., Camara, C.A., Queiroz, N., Magnani, M., de Novais, J.S., Soledade, L.E.B., de Oliveira Lima, E., de Souza, A.L. and de Souza, A.G., 2013. Phenolic profile, antioxidant activity and palynological analysis of stingless bee honey from Amazonas, Northern Brazil. *Food Chemistry*, 141(4), pp. 3552-3558.

da Silva, P.M., Gauche, C., Gonzaga, L.V., Costa, A.C.O. and Fett, R., 2016. Honey: Chemical composition, stability and authenticity. *Food Chemistry*, 196, pp. 309-323.

Dardon, M.J., Maldonado-Aguilera, C. and Enríquez, E., 2013. The pot-honey of Guatemalan bees. In *Pot-Honey* (pp. 395-408). Springer, New York.

de Alda-Garcilope, C., Gallego-Picó, A., Bravo-Yagüe, J.C., Garcinuño-Martínez, R.M. and Fernández-Hernando, P., 2012. Characterization of Spanish honeys with protected designation of origin "Miel de Granada" according to their mineral content. *Food Chemistry*, 135(3), pp. 1785-1788.

de Queiroz Pimentel, R.B., da Costa, C.A., Albuquerque, P.M. and Junior, S.D., 2013. Antimicrobial activity and rutin identification of honey produced by the stingless bee *Melipona compressipes manaosensis* and commercial honey. *BMC Complementary and Alternative Medicine*, 13(151), pp. 1-13.

de Rodríguez, G.O., de Ferrer, B.S., Ferrer, A. and Rodríguez, B., 2004. Characterization of honey produced in Venezuela. *Food Chemistry*, 84(4), pp. 499-502.

de Sousa, J.M.B., de Souza, E.L., Marques, G., de Toledo Benassi, M., Gullón, B., Pintado, M.M. and Magnani, M., 2016. Sugar profile, physicochemical and sensory aspects of monofloral honeys produced by different stingless bee species in Brazilian semi-arid region. *LWT-Food Science and Technology*, 65, pp. 645-651.

Deng, J., Liu, R., Lu, Q., Hao, P., Xu, A., Zhang, J. and Tan, J., 2018. Biochemical properties, antibacterial and cellular antioxidant activities of buckwheat honey in comparison to manuka honey. *Food Chemistry*, 252, pp. 243-249.

Department of Standards Malaysia, 2017. Kelulut (Stingless Bee) Honey-Specification: MS 2683: 2017. Malaysian Standard, pp. 1-33.

do Nascimento, A.S., Chambó, E.D., de Jesus Oliveira, D., Andrade, B.R., Bonsucesso, J.S. and de Carvalho, C.A.L., 2018. Honey from stingless bee as indicator of contamination with metals. *Sociobiology*, 65(4), pp. 727-736.

do Nascimento, A.S., Marchini, L.C., de Carvalho, C.A.L., Araújo, D.F.D., de Olinda, R.A. and da Silveira, T.A., 2015. Physical-chemical parameters of honey of stingless bee (Hymenoptera: Apidae). *American Chemical Science Journal*, 7(3), pp. 139-149.

Duarte, A.W.F., dos Santos Vasconcelos, M.R., de Menezes, A.P.D., da Silva, S.C., Oda-souza, M. and López, A.M.Q., 2012. Composition and antioxidant activity of honey from Africanized and stingless bees in Alagoas (Brazil): a multivariate analysis. *Journal of Apicultural Research*, 51(1), pp. 23-35.

Dżugan, M., Tomczyk, M., Sowa, P. and Grabek-Lejko, D., 2018. Antioxidant activity as biomarker of honey variety. *Molecules*, 23(8), p. 2069.

El-Haskoury, R., Al-Waili, N., Kamoun, Z., Makni, M., Al-Waili, H. and Lyoussi, B., 2018. Antioxidant activity and protective effect of carob honey in CCl_4-induced kidney and liver injury. *Archives of Medical Research*, 49(5), pp. 306-313.

El Sohaimy, S.A., Masry, S.H.D. and Shehata, M.G., 2015. Physicochemical characteristics of honey from different origins. *Annals of Agricultural Sciences*, 60(2), pp. 279-287.

Elamine, Y., Anjos, O., Estevinho, L.M., Lyoussi, B., Aazza, S. and Miguel, M.G., 2020. Effect of extreme heat processing on the Moroccan Zantaz'honey antioxidant activities. *Journal of Food Science and Technology*, 57(9), pp. 3323-3333.

Erejuwa, O.O., Sulaiman, S.A. and Ab Wahab, M.S., 2012. Honey- a novel antidiabetic agent. *International Journal of Biological Sciences*, 8(6), pp. 913-934.

Erejuwa, O.O., Sulaiman, S.A., Ab Wahab, M.S., Sirajudeen, K.N.S., Salleh, M.S.M. and Gurtu, S., 2011a. Glibenclamide or metformin combined with honey improves glycemic control in streptozotocin-induced diabetic rats. *International Journal of Biological Sciences*, 7(2), pp. 244-252.

Erejuwa, O.O., Sulaiman, S.A., Wahab, M.S.A., Salam, S.K.N., Salleh, M.S.M. and Gurtu, S., 2011b. Comparison of antioxidant effects of honey, glibenclamide, metformin, and their combinations in the kidneys of streptozotocin-induced diabetic rats. *International Journal of Molecular Sciences*, 12(1), pp. 829-843.

Escuredo, O., González-Martín, M.I., Rodríguez-Flores, M.S. and Seijo, M.C., 2015. Near infrared spectroscopy applied to the rapid prediction of the floral origin and mineral content of honeys. *Food Chemistry*, 170, pp. 47-54.

Escuredo, O., Míguez, M., Fernández-González, M. and Seijo, M.C., 2013. Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food Chemistry*, 138(2-3), pp. 851-856.

Estevinho, L., Pereira, A.P., Moreira, L., Dias, L.G. and Pereira, E., 2008. Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. *Food and Chemical Toxicology*, 46(12), pp. 3774-3779.

Eteraf-Oskouei, T. and Najafi, M., 2013. Traditional and modern uses of natural honey in human diseases: a review. *Iranian Journal of Basic Medical Sciences*, 16(6), pp. 731-742.

Ewnetu, Y., Lemma, W. and Birhane, N., 2013. Antibacterial effects of *Apis mellifera* and stingless bee honeys on susceptible and resistant strains of *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* in Gondar, Northwest Ethiopia. *BMC Complementary and Alternative Medicine*, 13(269), pp. 1-7.

Eyer, M., Neumann, P. and Dietemann, V., 2016. A look into the cell: honey storage in honey bees, *Apis mellifera*. *PloS one*, 11(8), p. e0161059.

Ferreira, I.C., Aires, E., Barreira, J.C. and Estevinho, L.M., 2009. Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. *Food Chemistry*, 114(4), pp. 1438-1443.

Ferreres, F., Tomáas-Barberáan, F.A., Gil, M.I. and Tomáas-Lorente, F., 1991. An HPLC technique for flavonoid analysis in honey. *Journal of the Science of Food and Agriculture*, 56(1), pp. 49-56.

Flanjak, I., Kenjerić, D., Bubalo, D. and Primorac, L., 2016. Characterisation of selected Croatian honey types based on the combination of antioxidant capacity, quality parameters, and chemometrics. *European Food Research and Technology*, 242(4), pp. 467-475.

Fletcher, M.T., Hungerford, N.L., Webber, D., Carpinelli de Jesus, M., Zhang, J., Stone, I.S., Blanchfield, J.T. and Zawawi, N., 2020. Stingless bee honey, a novel source of trehalulose: a biologically active disaccharide with health benefits. *Scientific Reports*, 10(1), pp. 1-8.

Fuchs, K. and Kaatze, U., 2001. Molecular dynamics of carbohydrate aqueous solutions. Dielectric relaxation as a function of glucose and fructose concentration. *The Journal of Physical Chemistry B*, 105(10), pp. 2036-2042.

Fuenmayor, C.A., Díaz-Moreno, A.C., Zuluaga-Domínguez, C.M. and Quicazán, M.C., 2013. Honey of Colombian stingless bees: Nutritional characteristics and physicochemical quality indicators. In *Pot-Honey* (pp. 383-394). Springer, New York.

Gangwar, M., Gautam, M.K., Sharma, A.K., Tripathi, Y.B., Goel, R.K. and Nath, G., 2014. Antioxidant capacity and radical scavenging effect of polyphenol rich *Mallotus philippenensis* fruit extract on human erythrocytes: an *in vitro* study. *The Scientific World Journal*, 2014, pp. 1-14.

Garedew, A., Schmolz, E. and Lamprecht, I., 2004. Microcalorimetric investigation on the antimicrobial activity of honey of the stingless bee *Trigona* spp. and comparison of some parameters with those obtained with standard methods. *Thermochimica Acta*, 415(1-2), pp. 99-106.

Garriga, M., Almaraz, M. and Marchiaro, A., 2017. Determination of reducing sugars in extracts of *Undaria pinnatifida* (harvey) algae by UV-visible spectrophotometry (DNS method). *Actas de ingenieria*, 3, pp. 173-179.

Geiling, N., 2013. The Science Behind Honey's Eternal Shelf Life. SmithsonianMag. com.

Gela, A., Hora, Z.A., Kebebe, D. and Gebresilassie, A., 2021. Physico-chemical characteristics of honey produced by stingless bees (*Meliponula beccarii*) from West Showa zone of Oromia Region, Ethiopia. *Heliyon*, 7(1), p. e05875.

Gerginova, D., Simova, S., Popova, M., Stefova, M., Stanoeva, J.P. and Bankova, V., 2020. NMR profiling of North Macedonian and Bulgarian honeys for detection of botanical and geographical origin. *Molecules*, 25(20), p. 4687.

Gheldof, N. and Engeseth, N.J., 2002. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *Journal of Agricultural and Food Chemistry*, 50(10), pp. 3050-3055.

Gleiter, R.A., Horn, H. and Isengard, H.D., 2006. Influence of type and state of crystallization on the water activity of honey. *Food Chemistry*, 96(3), pp. 441-445.

González-Paramás, A.M., García-Villanova, R.J., Gómez Bárez, J.A., Sánchez Sánchez, J. and Ardanuy Albajar, R., 2007. Botanical origin of monovarietal dark honeys (from heather, holm oak, pyrenean oak and sweet chestnut) based on their chromatic characters and amino acid profiles. *European Food Research and Technology*, 226(1), pp. 87-92.

Grego, E., Robino, P., Tramuta, C.L.A.R.A., Giusto, G.E.S.S.I.C.A., Boi, M., Colombo, R., Serra, G., Chiadò-Cutin, S., Gandini, M. and Nebbia, P., 2016. Evaluation of antimicrobial activity of Italian honey for wound healing application in veterinary medicine. *Schweiz Arch Tierheilkd*, 158(7), pp. 521-527.

Guerrini, A., Bruni, R., Maietti, S., Poli, F., Rossi, D., Paganetto, G., Muzzoli, M., Scalvenzi, L. and Sacchetti, G., 2009. Ecuadorian stingless bee (Meliponinae) honey: A chemical and functional profile of an ancient health product. *Food Chemistry*, 114(4), pp. 1413-1420.

Guler, A., Kocaokutgen, H., Garipoglu, A.V., Onder, H., Ekinci, D. and Biyik, S., 2014. Detection of adulterated honey produced by honeybee (*Apis mellifera* L.) colonies fed with different levels of commercial industrial sugar (C3 and C4 plants) syrups by the carbon isotope ratio analysis. *Food Chemistry*, 155, pp. 155-160.

Gump, B.H., 2014. pH and Titratable Acidity - Determination of [H+] and various acid compounds in musts and wines. Florida International University Presentation. Retrieved 22/9/2020. https://www.umpqua.edu/images/areas-of-study/career-technical/viticulture-enology/downloads/conferences/technical-symposia/2014-dec-wine-chemistry/2014-ts-3-ph-ta-n2.pdf

Guzmán-Soto, I., Omole, M., Alarcon, E.I. and McTiernan, C.D., 2020. Lipoic acid capped silver nanoparticles: a facile route to covalent protein capping and oxidative stability within biological systems. *RSC Advances*, 10(54), pp. 32953-32958.

Habib, H.M., Al Meqbali, F.T., Kamal, H., Souka, U.D. and Ibrahim, W.H., 2014. Physicochemical and biochemical properties of honeys from arid regions. *Food Chemistry*, 153, pp. 35-43.

Halvorsen, B.L., Holte, K., Myhrstad, M.C., Barikmo, I., Hvattum, E., Remberg, S.F., Wold, A.B., Haffner, K., Baugerød, H., Andersen, L.F. and Moskaug, Ø., 2002. A systematic screening of total antioxidants in dietary plants. *The Journal of Nutrition*, 132(3), pp. 461-471.

Hegazi, A., AbdEl-Moez, S., Abdou, A.M. and Abd Allah, F., 2014. Synergistic antibacterial activity of Egyptian honey and common antibiotics against Clostridium reference strains. *International Journal of Current Microbiology and Applied Sciences*, 3(8), pp. 312-25.

Herbreteau, B., Lafosse, M., Morin-Allory, L. and Dreux, M., 1992. High performance liquid chromatography of raw sugars and polyols using bonded silica gels. *Chromatographia*, 33(7), pp. 325-330.

Hermosín, I., Chicon, R.M. and Cabezudo, M.D., 2003. Free amino acid composition and botanical origin of honey. *Food Chemistry*, 83(2), pp. 263-268.

Hungerford, N.L., Fletcher, M.T., Tsai, H.H., Hnatko, D., Swann, L.J., Kelly, C.L., Anuj, S.R., Tinggi, U., Webber, D.C., Were, S.T. and Tan, B.L., 2021. Occurrence of environmental contaminants (pesticides, herbicides, PAHs) in Australian/Queensland *Apis mellifera* honey. *Food Additives & Contaminants: Part B*, 14(3), pp. 193-205.

Hussein, S.Z., Yusoff, K.M., Makpol, S. and Yusof, Y.A.M., 2011. Antioxidant capacities and total phenolic contents increase with gamma irradiation in two types of Malaysian honey. *Molecules*, 16(8), pp. 6378-6395.

Hrncir, M., Jarau, S. and Barth, F.G., 2016. Stingless bees (Meliponini): senses and behavior. *Journal of Comparative Physiology A*, 202, pp. 597–601.

Iglesias, M.T., Martín-Álvarez, P.J., Polo, M.C., de Lorenzo, C., González, M. and Pueyo, E., 2006. Changes in the free amino acid contents of honeys during storage at ambient temperature. *Journal of Agricultural and Food Chemistry*, 54(24), pp. 9099-9104.

Irish, J., Blair, S. and Carter, D.A., 2011. The antibacterial activity of honey derived from Australian flora. *PloS one*, 6(3), p. e18229.

Ismail, A., Soultani, S. and Ghoul, M., 1998. Optimization of the enzymatic synthesis of butyl glucoside using response surface methodology. *Biotechnology Progress*, 14(6), pp. 874-878.

Ismail, M.M. and Ismail, W.I.W., 2018. Development of stingless beekeeping projects in Malaysia. E3S Web of Conferences, 52 (00028), pp. 1-5.

Ismail, W.W., 2016. A review on beekeeping in Malaysia: History, importance and future directions. *Journal of Sustainability Science and Management*, 11(2), pp. 70-80.

Jaafar, K., Haidar, J., Kuraydiyyah, S., Ghaddar, T., Knio, K., Ismail, B. and Toufeili, I., 2017. Physicochemical, melissopalynological and antioxidant properties of artisanal honeys from Lebanon. *Journal of Food Science and Technology*, 54(8), pp. 2296-2305.

Jantakee, K. and Tragoolpua, Y., 2015. Activities of different types of Thai honey on pathogenic bacteria causing skin diseases, tyrosinase enzyme and generating free radicals. *Biological Research*, 48(4), pp. 1-11.

Jenkins, R. and Cooper, R., 2012a. Improving antibiotic activity against wound pathogens with manuka honey *in vitro*. *PloS one*, 7(9), p. e45600.

Jenkins, R.E. and Cooper, R., 2012b. Synergy between oxacillin and manuka honey sensitizes methicillin-resistant *Staphylococcus aureus* to oxacillin. *Journal of Antimicrobial Chemotherapy*, 67(6), pp. 1405-1407.

Jin, Q. and Kirk, M.F., 2018. pH as a primary control in environmental microbiology: 1. thermodynamic perspective. *Frontiers in Environmental Science*, 6(21), pp. 1-15.

Jmaludin B., 2020. Local Kelulut honey industry can reap RM3.03 bln a year. The Borneo Post [Internet]. Mar 2029, available from https://www.pressreader.com/malaysia/the-borneo-post/20200302/281809990939203

Kalan, L. and Wright, G.D., 2011. Antibiotic adjuvants: multicomponent anti-infective strategies. *Expert Reviews in Molecular Medicine*, 13(e5), pp. 511-517.

Kamal, M.A. and Klein, P., 2011. Determination of sugars in honey by liquid chromatography. *Saudi Journal of Biological Sciences*, 18(1), pp. 17-21.

Kang, K.A., Lee, K.H., Zhang, R., Piao, M., Chae, S., Kim, K.N., Jeon, Y.J., Park, D.B., You, H.J., Kim, J.S. and Hyun, J.W., 2006. Caffeic acid protects hydrogen peroxide induced cell damage in WI-38 human lung fibroblast cells. *Biological and Pharmaceutical Bulletin*, 29(9), pp. 1820-1824.

Karabagias, I.K., Badeka, A., Kontakos, S., Karabournioti, S. and Kontominas, M.G., 2014. Characterization and classification of *Thymus capitatus* (L.) honey according to geographical origin based on volatile compounds, physicochemical parameters and chemometrics. *Food Research International*, 55, pp. 363-372.

Kaye, K.S., Petty, L.A., Shorr, A.F. and Zilberberg, M.D., 2019. Current epidemiology, etiology, and burden of acute skin infections in the United States. *Clinical Infectious Diseases*, 68(Supplement_3), pp. S193-S199.

Kazalaki, A., Misiak, M., Spyros, A. and Dais, P., 2015. Identification and quantitative determination of carbohydrate molecules in Greek honey by employing ^{13}C NMR spectroscopy. *Analytical Methods*, 7(14), pp. 5962-5972.

Kečkeš, S., Gašić, U., Veličković, T.Ć., Milojković-Opsenica, D., Natić, M. and Tešić, Ž., 2013. The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry. *Food Chemistry*, 138(1), pp. 32-40.

Kek, S.P., Chin, N.L., Yusof, Y.A., Tan, S.W. and Chua, L.S., 2014. Total phenolic contents and colour intensity of Malaysian honeys from the *Apis* spp. and *Trigona* spp. bees. *Agriculture and Agricultural Science Procedia*, 2, pp. 150-155.

Kek, S.P., Chin, N.L., Tan, S.W., Yusof, Y.A. and Chua, L.S., 2017a. Classification of honey from its bee origin via chemical profiles and mineral content. *Food Analytical Methods*, *10*(1), pp. 19-30.

Kek, S.P., Chin, N.L., Yusof, Y.A., Tan, S.W. and Chua, L.S., 2017b. Classification of entomological origin of honey based on its physicochemical and antioxidant properties. *International Journal of Food Properties*, 20(sup3), pp. S2723-S2738.

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

Khalil, M., Moniruzzaman, M., Boukraa, L., Benhanifia, M., Islam, M., Sulaiman, S.A. and Gan, S.H., 2012. Physicochemical and antioxidant properties of Algerian honey. *Molecules*, 17(9), pp. 11199-11215.

Khalil, M.I., Sulaiman, S.A. and Gan, S.H., 2010. High 5-hydroxymethylfurfural concentrations are found in Malaysian honey samples stored for more than one year. *Food and Chemical Toxicology*, 48(8-9), pp. 2388-2392.

Koochak, H., Seyyednejad, S.M. and Motamedi, H., 2010. Preliminary study on the antibacterial activity of some medicinal plants of Khuzestan (Iran). *Asian Pacific Journal of Tropical Medicine*, 3(3), pp. 180-184.

Kretavičius, J., Kurtinaitienė, B., Račys, J. and Čeksterytė, V., 2010. Inactivation of glucose oxidase during heat-treatment de-crystallization of honey. *Žemdirbystė (Agriculture)*, 97(4), pp. 115-122.

Kropf, U., Jamnik, M., Bertoncelj, J. and Golob, T., 2008. Linear regression model of the ash mass fraction and electrical conductivity for Slovenian honey. *Food Technology & Biotechnology*, 46(3), pp. 335-340.

Kwakman, P.H., Te Velde, A.A., De Boer, L., Vandenbroucke-Grauls, C.M. and Zaat, S.A., 2011. Two major medicinal honeys have different mechanisms of bactericidal activity. *PloS one*, 6(3), p. e17709.

Kwakman, P.H. and Zaat, S.A., 2012. Antibacterial components of honey. *IUBMB Life*, 64(1), pp. 48-55.

Lachman, J., Orsák, M., Hejtmánková, A. and Kovářová, E., 2010. Evaluation of antioxidant activity and total phenolics of selected Czech honeys. *LWT-Food Science and Technology*, 43(1), pp. 52-58.

Lee, J.H., Kim, Y.G., Cho, H.S., Ryu, S.Y., Cho, M.H. and Lee, J., 2014. Coumarins reduce biofilm formation and the virulence of *Escherichia coli* O157: H7. *Phytomedicine*, 21(8-9), pp. 1037-1042.

Lehmann, D.M., Krishnakumar, K., Batres, M.A., Hakola-Parry, A., Cokcetin, N., Harry, E. and Carter, D.A., 2019. A cost-effective colourimetric assay for quantifying hydrogen peroxide in honey. *Access Microbiology*, 1(10), pp. 1-8.

Leibovici, L., 2009. Non-antibiotic treatment for bacterial infections: how to validate chance findings. *Clinical Microbiology and Infection*, 15(4), pp. 298-301.

León-Ruiz, V., González-Porto, A.V., Al-Habsi, N., Vera, S., San Andrés, M.P. and Jauregi, P., 2013. Antioxidant, antibacterial and ACE-inhibitory activity of four monofloral honeys in relation to their chemical composition. *Food & Function*, 4(11), pp. 1617-1624.

Lim, Y.W. and Baharun, R., 2008. Apiculture strategies in Malaysia: Planning implementation. *Contemporary Issues in Marketing*. Penerbit UTM, pp. 33-53.

Linley, E., Denyer, S.P., McDonnell, G., Simons, C. and Maillard, J.Y., 2012. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *Journal of Antimicrobial Chemotherapy*, 67(7), pp. 1589-1596.

Lipinski, B., 2011. Hydroxyl radical and its scavengers in health and disease. *Oxidative Medicine and Cellular Longevity*, 2011, pp. 1-9.

Liu, J.R., Ye, Y.L., Lin, T.Y., Wang, Y.W. and Peng, C.C., 2013. Effect of floral sources on the antioxidant, antimicrobial, and anti-inflammatory activities of honeys in Taiwan. *Food Chemistry*, 139(1-4), pp. 938-943.

Liu, M.Y., Cokcetin, N.N., Lu, J., Turnbull, L., Carter, D.A., Whitchurch, C.B. and Harry, E.J., 2018. Rifampicin-manuka honey combinations are superior to other antibiotic-manuka honey combinations in eradicating *Staphylococcus aureus* biofilms. *Frontiers in Microbiology*, 8, p. 2653.

Liu, M., Lu, J., Müller, P., Turnbull, L., Burke, C.M., Schlothauer, R.C., Carter, D.A., Whitchurch, C.B. and Harry, E.J., 2015. Antibiotic-specific differences in the response of *Staphylococcus aureus* to treatment with antimicrobials combined with manuka honey. *Frontiers in Microbiology*, 5, p. 779.

Machado De-Melo, A.A., Almeida-Muradian, L.B.D., Sancho, M.T. and Pascual-Maté, A., 2018. Composition and properties of *Apis mellifera* honey: A review. *Journal of Apicultural Research*, 57(1), pp. 5-37.

Majewska, E., Drużyńska, B. and Wołosiak, R., 2019. Determination of the botanical origin of honeybee honeys based on the analysis of their selected physicochemical parameters coupled with chemometric assays. *Food Science and Biotechnology*, 28(5), pp. 1307-1314.

Majtan, J., Majtanova, L., Bohova, J. and Majtan, V., 2011. Honeydew honey as a potent antibacterial agent in eradication of multi-drug resistant *Stenotrophomonas maltophilia* isolates from cancer patients. *Phytotherapy Research*, 25(4), pp. 584-587.

Mandal, M.D. and Mandal, S., 2011. Honey: its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine*, 1(2), pp. 154-160.

Manikis, I. and Thrasivoulou, A., 2001. The relation of physicochemical characteristics of honey and the crystallization sensitive parameters. *Apiacta*, 36(2), pp. 106-112.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M., 2011. Volatile compounds in honey: a review on their involvement in aroma, botanical origin determination and potential biomedical activities. *International Journal of Molecular Sciences*, 12(12), pp. 9514-9532.

Manzanares, A.B., García, Z.H., Galdón, B.R., Rodríguez, E.R. and Romero, C.D., 2011. Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition. *Food Chemistry*, 126(2), pp. 664-672.

Maringgal, B., Hashim, N., Tawakkal, I.S.M.A., Mohamed, M.T.M., Hamzah, M.H. and Shukor, N.I.A., 2019. The causal agent of anthracnose in papaya fruit and control by three different Malaysian stingless bee honeys, and the chemical profile. *Scientia Horticulturae*, 257, p. 108590.

Massaro, C.F., Shelley, D., Heard, T.A. and Brooks, P., 2014. In vitro antibacterial phenolic extracts from “sugarbag” pot-honeys of Australian stingless bees (*Tetragonula carbonaria*). *Journal of Agricultural and Food Chemistry*, 62(50), pp. 12209-12217.

Matin, M.M., Bhuiyan, M.M.H. and Azad, A.K., 2013. Synthesis and antimicrobial evaluation of some *n*-butyl α-and β-D-glucopyranoside derivatives. *RGUHS Journal of Pharmaceutical Sciences,* 3(1), pp. 53-59.

Mato, I., Huidobro, J.F., Simal-Lozano, J. and Sancho, M.T., 2003. Significance of nonaromatic organic acids in honey. *Journal of Food Protection*, 66(12), pp. 2371-2376.

Mato, I., Huidobro, J.F., Simal-Lozano, J. and Sancho, M.T., 2006. Analytical methods for the determination of organic acids in honey. *Critical Reviews in Analytical Chemistry*, 36(1), pp. 3-11.

Mazzoni, V., Bradesi, P., Tomi, F. and Casanova, J., 1997. Direct qualitative and quantitative analysis of carbohydrate mixtures using ^{13}C NMR spectroscopy: application to honey. *Magnetic Resonance in Chemistry*, 35(13), pp. S81-S90.

Menezes, C., Vollet-Neto, A., Contrera, F.A.F.L., Venturieri, G.C. and Imperatriz-Fonseca, V.L., 2013. The role of useful microorganisms to stingless bees and stingless beekeeping. In Pot-Honey (pp. 153-171). Springer, New York, NY.

Meng, J., Hu, B., Liu, J., Hou, Z., Meng, J., Jia, M. and Luo, X., 2006. Restoration of oxacillin susceptibility in methicillin-resistant *Staphylococcus aureus* by blocking the MecR1-mediated signaling pathway. *Journal of Chemotherapy*, 18(4), pp. 360-365.

Michener, C.D., 2000. The bees of the world (Vol. 1). The John Hopkins University Press. United States.

Michener, C.D., 2013. The meliponini. In *Pot-honey* (pp. 3-17). Springer, New York.

Miklasińska-Majdanik, M., Kępa, M., Wojtyczka, R.D., Idzik, D. and Wąsik, T.J., 2018. Phenolic compounds diminish antibiotic resistance of *Staphylococcus aureus* clinical strains. *International Journal of Environmental Research and Public Health*, 15(2321), pp. 1-18.

Mohapatra, D.P., Thakur, V. and Brar, S.K., 2011. Antibacterial efficacy of raw and processed honey. *Biotechnology Research International*, 2011, pp. 1-6.

Mohamed, M., Sulaiman, S.A., Jaafar, H. and Sirajudeen, K.N.S., 2011. Antioxidant protective effect of honey in cigarette smoke-induced testicular damage in rats. *International Journal of Molecular Sciences*, 12(9), pp. 5508-5521.

Mohd Norowi, H., Sajap, A.S., Rosliza, J., Mohd Fahimie, J. and Suri, R., 2010. Conservation and sustainable utilization of stingless bees for pollination services in agricultural ecosystems in Malaysia. Conference: Proceeding of International Seminar on Enhancement of Functional Biodiversity Relevant to Sustainable Food Production in ASPAC: Tsukuba, Japan.

Molan, P.C., 1992. The antibacterial activity of honey: 1. The nature of the antibacterial activity. *Bee World*, 73(1), pp. 5-28.

Molaveisi, M., Beigbabaei, A., Akbari, E., Noghabi, M.S. and Mohamadi, M., 2019. Kinetics of temperature effect on antioxidant activity, phenolic compounds and color of Iranian jujube honey. *Heliyon*, 5(1), p. e01129.

Moniruzzaman, M., Khalil, M.I., Sulaiman, S.A. and Gan, S.H., 2012. Advances in the analytical methods for determining the antioxidant properties of honey: a review. *African Journal of Traditional, Complementary and Alternative Medicines*, 9(1), pp. 36-42.

Moniruzzaman, M., Khalil, M.I., Sulaiman, S.A. and Gan, S.H., 2013a. Physicochemical and antioxidant properties of Malaysian honeys produced by *Apis cerana*, *Apis dorsata* and *Apis mellifera*. *BMC Complementary and Alternative Medicine*, 13(1), pp. 1-12.

Moniruzzaman, M., Sulaiman, S.A., Khalil, M.I. and Gan, S.H., 2013b. Evaluation of physicochemical and antioxidant properties of sourwood and other Malaysian honeys: a comparison with manuka honey. *Chemistry Central Journal*, 7(1), pp. 1-12.

Moniruzzaman, M., Chowdhury, M.A.Z., Rahman, M.A., Sulaiman, S.A. and Gan, S.H., 2014. Determination of mineral, trace element, and pesticide levels in honey samples originating from different regions of Malaysia compared to Manuka honey. *BioMed Research International*, 2014, pp. 1-10.

Moore, J.C., Spink, J. and Lipp, M., 2012. Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *Journal of Food Science*, 77(4), pp. R118-R126.

Müller, P., Alber, D.G., Turnbull, L., Schlothauer, R.C., Carter, D.A., Whitchurch, C.B. and Harry, E.J., 2013. Synergism between Medihoney and rifampicin against methicillin-resistant *Staphylococcus aureus* (MRSA). *PloS one*, 8(2), p. e57679.

Munteanu, I.G. and Apetrei, C., 2021. Analytical methods used in determining antioxidant activity: A review. *International Journal of Molecular Sciences*, 22(7), p. 3380.

Mustafa, M.Z., Yaacob, N.S. and Sulaiman, S.A., 2018. Reinventing the honey industry: opportunities of the stingless bee. *The Malaysian Journal of Medical Sciences*, 25(4), pp. 1-5.

Nalda, M.J.N., Bernal Yagüe, J.L., Diego Calva, J.C. and Martin Gomez, M.T., 2005. Classifying honeys from the Soria Province of Spain via multivariate analysis. *Analytical and Bioanalytical Chemistry*, 382(2), pp. 311-319.

Nešović, M., Gašić, U., Tosti, T., Trifković, J., Baošić, R., Blagojević, S., Ignjatović, L. and Tešić, Ž., 2020. Physicochemical analysis and phenolic profile of polyfloral and honeydew honey from Montenegro. *RSC Advances*, 10(5), pp. 2462-2471.

Ng, W.J., Lye, P.Y., Chan, Y.J., Lau Z.K. and Ee, K.Y., 2017. Synergistic Effect of Trigona Honey and Ampicillin on *Staphylococcus aureus* Isolated from Infected Wound. *International Journal of Pharmacology,* 13(4): pp. 403-407.

Ng, W.J., Sit, N.W., Ooi, P.A.C., Ee, K.Y. and Lim, T.M., 2020. The antibacterial potential of honeydew honey produced by stingless bee (*Heterotrigona itama*) against antibiotic resistant bacteria. *Antibiotics*, 9(12), p. 817.

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

Nishio, E.K., Ribeiro, J.M., Oliveira, A.G., Andrade, C.G.T.J., Proni, E.A., Kobayashi, R.K.T. and Nakazato, G., 2016. Antibacterial synergic effect of honey from two stingless bees: *Scaptotrigona bipunctata* Lepeletier, 1836, and *S. postica* Latreille, 1807. *Scientific Reports*, 6(1), pp. 1-8.

Nordin, A., Sainik, N.Q.A.V., Chowdhury, S.R., Saim, A.B. and Idrus, R.B.H., 2018. Physicochemical properties of stingless bee honey from around the globe: A comprehensive review. *Journal of Food Composition and Analysis*, 73, pp. 91-102.

Nweze, J.A., Okafor, J.I., Nweze, E.I. and Nweze, J.E., 2017. Evaluation of physicochemical and antioxidant properties of two stingless bee honeys: a comparison with *Apis mellifera* honey from Nsukka, Nigeria. *BMC Research Notes*, 10(1), pp. 1-6.

Oddo, L.P., Baldi, E. and Accorti, M., 1990. Diastatic activity in some unifloral honeys. *Apidologie*, 21(1), pp. 17-24.

Oddo, L.P., Heard, T.A., Rodríguez-Malaver, A., Pérez, R.A., Fernández-Muiño, M., Sancho, M.T., Sesta, G., Lusco, L. and Vit, P., 2008. Composition and antioxidant activity of *Trigona carbonaria* honey from Australia. *Journal of Medicinal Food*, 11(4), pp. 789-794.

Ohashi, K., Natori, S. and Kubo, T., 1999. Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.). *European Journal of Biochemistry*, 265(1), pp. 127-133.

Olaitan, P.B., Adeleke, O.E. and Iyabo, O.O., 2007. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *African Health Sciences*, *7*(3), pp. 159-165.

Olga, E., María, F.G. and Carmen, S.M., 2012. Differentiation of blossom honey and honeydew honey from Northwest Spain. *Agriculture*, 2(1), pp. 25-37.

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

Ooshima, T., Izumitani, A., Minami, T., Fujiwara, T., Nakajima, Y. and Hamada, S., 1991. Trehalulose does not induce dental caries in rats infected with Mutans Streptococci. *Caries Research*, 25(4), pp. 277-282.

Oroian, M., Amariei, S., Leahu, A. and Gutt, G., 2015. Multi-element composition of honey as a suitable tool for its authenticity analysis. *Polish Journal of Food and Nutrition Sciences*, 65(2), pp. 93-100.

Osés, S.M., Pascual-Maté, A., Fernández-Muiño, M.A., López-Díaz, T.M. and Sancho, M.T., 2016. Bioactive properties of honey with propolis. *Food Chemistry*, 196, pp. 1215-1223.

Ouchemoukh, S., Louaileche, H. and Schweitzer, P., 2007. Physicochemical characteristics and pollen spectrum of some Algerian honeys. *Food Control*, 18(1), pp. 52-58.

Packer, J.M., Irish, J., Herbert, B.R., Hill, C., Padula, M., Blair, S.E., Carter, D.A. and Harry, E.J., 2012. Specific non-peroxide antibacterial effect of manuka honey on the *Staphylococcus aureus* proteome. *International Journal of Antimicrobial Agents*, 40(1), pp. 43-50.

Pasias, I.N., Kiriakou, I.K. and Proestos, C., 2017. HMF and diastase activity in honeys: A fully validated approach and a chemometric analysis for identification of honey freshness and adulteration. *Food Chemistry*, 229, pp. 425-431.

Perna, A., Simonetti, A., Intaglietta, I., Sofo, A. and Gambacorta, E., 2012. Metal content of southern Italy honey of different botanical origins and its correlation with polyphenol content and antioxidant activity. *International Journal of Food Science & Technology*, 47(9), pp. 1909-1917.

Pita-Calvo, C. and Vázquez, M., 2017. Differences between honeydew and blossom honeys: A review. *Trends in Food Science & Technology*, 59, pp. 79-87.

Pita-Calvo, C. and Vázquez, M., 2018. Honeydew honeys: A review on the characterization and authentication of botanical and geographical origins. *Journal of Agricultural and Food Chemistry*, 66(11), pp. 2523-2537.

Popova, M., Gerginova, D., Trusheva, B., Simova, S., Tamfu, A.N., Ceylan, O., Clark, K. and Bankova, V., 2021. A preliminary study of chemical profiles of honey, cerumen, and propolis of the african stingless bee *Meliponula ferruginea*. *Foods*, 10(5), p. 997.

Prins, J.M., Van Deventer, S.J., Kuijper, E.J. and Speelman, P., 1994. Clinical relevance of antibiotic-induced endotoxin release. *Antimicrobial Agents and Chemotherapy*, 38(6), pp. 1211-1218.

Puścion-Jakubik, A., Borawska, M.H. and Socha, K., 2020. Modern methods for assessing the quality of bee honey and botanical origin identification. *Foods*, 9(8), p. 1028.

Quintal, R.B. and Roubik, D.W., 2013. Melipona bees in the scientific world: Western cultural views. In Pot-Honey (pp. 247-259). Springer, New York, NY.
Roubik, D.W., 2006. Stingless bee nesting biology. *Apidologie*, 37(2), pp. 124-143.

Rajurkar, N.S. and Hande, S.M., 2011. Estimation of phytochemical content and antioxidant activity of some selected traditional Indian medicinal plants. *Indian Journal of Pharmaceutical Sciences*, 73(2), pp. 146-151.

Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T. and Estevinho, L.M., 2011. Mead production: Tradition versus modernity. *Advances in Food and Nutrition Research*, 63, pp. 101-118.

Ranneh, Y., Ali, F., Zarei, M., Akim, A.M., Abd Hamid, H. and Khazaai, H., 2018. Malaysian stingless bee and Tualang honeys: A comparative characterization of total antioxidant capacity and phenolic profile using liquid chromatography-mass spectrometry. *LWT - Food Science and Technology*, 89, pp. 1-9.

Rattanawannee, A. and Duangphakdee, O., 2019. Southeast Asian Meliponiculture for Sustainable Livelihood. In Modern Beekeeping. IntechOpen. DOI: http://dx.doi.org/10.5772/intechopen.90344

Rebane, R. and Herodes, K., 2010. A sensitive method for free amino acids analysis by liquid chromatography with ultraviolet and mass spectrometric detection using precolumn derivatization with diethyl ethoxymethylenemalonate: Application to the honey analysis. *Analytica Chimica Scta*, 672(1-2), pp. 79-84.

Recklies, K., Peukert, C., Kölling-Speer, I. and Speer, K., 2021. Differentiation of honeydew honeys from blossom honeys and according to their botanical origin by electrical conductivity and phenolic and sugar spectra. *Journal of Agricultural and Food Chemistry*, 69(4), pp. 1329-1347.

Reller, L.B., Weinstein, M., Jorgensen, J.H. and Ferraro, M.J., 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases*, 49(11), pp. 1749-1755.

Roberts, C.A. and Buikstra, J.E., 2019. Bacterial infections. In Ortner's Identification of pathological conditions in human skeletal remains (pp. 321-439). Academic Press.

Rosli, F.N., Hazemi, M.H.F., Akbar, M.A., Basir, S., Kassim, H. and Bunawan, H., 2020. Stingless bee honey: Evaluating its antibacterial activity and bacterial diversity. *Insects*, 11(8), p. 500.

Ruoff, K. and Bogdanov, S., 2004. Authenticity of honey and other bee products. *Apiacta*, 38(38), pp. 317-327.

Sak-Bosnar, M. and Sakač, N., 2012. Direct potentiometric determination of diastase activity in honey. *Food Chemistry*, 135(2), pp. 827-831.

Saludin, S.F., Kamarulzaman, N.H. and Ismail, M.M., 2019. Measuring consumers' preferences of stingless bee honey (meliponine honey) based on sensory characteristics. *International Food Research Journal*, 26(1), pp. 225-235.

Sánchez, E., López-Lefebre, L.R., García, P.C., Rivero, R.M., Ruiz, J.M. and Romero, L., 2001. Proline metabolism in response to highest nitrogen dosages in green bean plants (*Phaseolus vulgaris* L. cv. Strike). *Journal of Plant Physiology*, 158(5), pp. 593-598.

Santos, C.G.D., Megiolaro, F.L., Serrão, J.E. and Blochtein, B., 2009. Morphology of the head salivary and intramandibular glands of the stingless bee *Plebeia emerina* (Hymenoptera: Meliponini) workers associated with propolis. *Annals of the Entomological Society of America*, 102(1), pp. 137-143.

Šarić, G., Marković, K., Major, N., Krpan, M., Uršulin-Trstenjak, N., Hruškar, M. and Vahčić, N., 2012. Changes of antioxidant activity and phenolic content in acacia and multifloral honey during storage. *Food Technology and Biotechnology*, 50(4), pp. 434-441.

Saxena, S., Gautam, S. and Sharma, A., 2010. Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry*, 118(2), pp. 391-397.

Schlunzen, F., Zarivach, R., Harms, J., Bashan, A., Tocilj, A., Albrecht, R., etal., 2001. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature*, 413, pp. 814–821.

Selvaraju, K., Vikram, P., Soon, J.M., Krishnan, K.T. and Mohammed, A., 2019. Melissopalynological, physicochemical and antioxidant properties of honey from West Coast of Malaysia. *Journal of Food Science and Technology*, 56(5), pp. 2508-2521.

Seraglio, S.K.T., Silva, B., Bergamo, G., Brugnerotto, P., Gonzaga, L.V., Fett, R. and Costa, A.C.O., 2019. An overview of physicochemical characteristics and health-promoting properties of honeydew honey. *Food Research International*, 119, pp. 44-66.

Seraglio, S.K.T., Valese, A.C., Daguer, H., Bergamo, G., Azevedo, M.S., Gonzaga, L.V., Fett, R. and Costa, A.C.O., 2016. Development and validation of a LC-ESI-MS/MS method for the determination of phenolic compounds in honeydew honeys with the diluted-and-shoot approach. *Food Research International*, 87, pp. 60-67.

Sereia, M.J., Março, P.H., Perdoncini, M.R.G., Parpinelli, R.S., de Lima, E.G. and Anjo, F.A., 2017. Techniques for the evaluation of physicochemical quality and bioactive compounds in honey. *Honey Analysis*, pp. 193-214.

Sergiel, I., Pohl, P. and Biesaga, M., 2014. Characterisation of honeys according to their content of phenolic compounds using high performance liquid chromatography/tandem mass spectrometry. *Food Chemistry*, 145, pp. 404-408.

Shalaby, E.A. and Shanab, S.M., 2013. Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of *Spirulina platensis*. *Indian Journal of Geo-Marine Sciences*, 42(5), pp. 556-564.

Shamsudin, S., Selamat, J., Sanny, M., Abd. Razak, S.B., Jambari, N.N., Mian, Z. and Khatib, A., 2019. Influence of origins and bee species on physicochemical, antioxidant properties and botanical discrimination of stingless bee honey. *International Journal of Food Properties*, 22(1), pp. 239-264.

Shapla, U.M., Solayman, M., Alam, N., Khalil, M. and Gan, S.H., 2018. 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health. *Chemistry Central Journal*, 12(1), pp. 1-18.

She, P., Wang, Y., Luo, Z., Chen, L., Tan, R., Wang, Y. and Wu, Y., 2018. Meloxicam inhibits biofilm formation and enhances antimicrobial agents' efficacy by *Pseudomonas aeruginosa*. *Microbiology Open*, 7(1), p. e00545.

Silvano, M.F., Varela, M.S., Palacio, M.A., Ruffinengo, S. and Yamul, D.K., 2014. Physicochemical parameters and sensory properties of honeys from Buenos Aires region. *Food Chemistry*, 152, pp. 500-507.

Simoes, M., Bennett, R.N. and Rosa, E.A., 2009. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural Product Reports*, 26(6), pp. 746-757.

Simone-Finstrom, M. and Spivak, M., 2010. Propolis and bee health: the natural history and significance of resin use by honey bees. *Apidologie*, 41(3), pp. 295-311.

Smanalieva, J. and Senge, B., 2009. Analytical and rheological investigations into selected unifloral German honey. *European Food Research and Technology*, 229(1), pp. 107-113.

Sodré, G.D.S., Marchini, L.C., Moreti, A.C.D.C.C., Otsuk, I.P. and Carvalho, C.A.L.D., 2011. Physico-chemical characteristics of honey produced by *Apis mellifera* in the Picos region, state of Piauí, Brazil. *Revista Brasileira de Zootecnia*, 40(8), pp. 1837-1843.

Souza, B.A., Roubik, D.W., Barth, O.M., Heard, T.A., Enríquez, E., Carvalho, C., Villas-Bôas, J., Marchini, L., Locatelli, J., Persano-Oddo, L. and Almeida-Muradian, L., 2006. Composition of stingless bee honey: setting quality standards. *Interciencia*, 31(12), pp. 867-875.

Stephens, J.M., Schlothauer, R.C., Morris, B.D., Yang, D., Fearnley, L., Greenwood, D.R. and Loomes, K.M., 2010. Phenolic compounds and methylglyoxal in some New Zealand manuka and kanuka honeys. *Food Chemistry*, 120(1), pp. 78-86.

Streinzer, M., Huber, W. and Spaethe, J., 2016. Body size limits dim-light foraging activity in stingless bees (Apidae: Meliponini). *Journal of Comparative Physiology A*, 202(9-10), pp. 643-655.

Strelec, I., Crevar, B., Kovač, T., Rajs. B.B., Primorac, L. and Flanjak, I., 2018. Glucose oxidase activity and hydrogen peroxide accumulation in Croatian honeys. *Croatian Journal of Food Science and Technology*, 10(1), pp. 33-41.

Styers, D., Sheehan, D.J., Hogan, P. and Sahm, D.F., 2006. Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. *Annals of Clinical Microbiology and Antimicrobials*, 5(1), pp. 1-9.

Subbiah, B., Blank, U.K. and Morison, K.R., 2020. A review, analysis and extension of water activity data of sugars and model honey solutions. *Food Chemistry*, 326, p. 126981.

Subramanian, R., Umesh Hebbar, H. and Rastogi, N.K., 2007. Processing of honey: a review. *International Journal of Food Properties*, 10(1), pp. 127-143.

Sudan, R., Bhagat, M., Gupta, S., Singh, J. and Koul, A., 2014. Iron (FeII) chelation, ferric reducing antioxidant power, and immune modulating potential of *Arisaema jacquemontii* (Himalayan Cobra Lily). *BioMed Research International*, 2014, pp. 1-7.

Suntiparapop, K., Prapaipong, P. and Chantawannakul, P., 2012. Chemical and biological properties of honey from Thai stingless bee (*Tetragonula leaviceps*). *Journal of Apicultural Research*, 51(1), pp. 45-52.

Suntiparapop, K., Prapaipong, P. and Chantawannakul, P., 2012. Chemical and biological properties of honey from Thai stingless bee (*Tetragonula leaviceps*). *Journal of Apicultural Research*, 51(1), pp. 45-52.

Szweda, P., 2017. Antimicrobial activity of honey. *Honey Analysis*, 1, pp. 215-232.

Taylor, K., 2015. Craft Brewers Conference 2015 Presentation. Sour Beers: It's more than just pH. White Labs http://www.craftbrewersconference.com/wp-content/uploads/2015_presentations/W1320_Kara_Taylor.pdf

Taylor, V.F., March, R.E., Longerich, H.P. and Stadey, C.J., 2005. A mass spectrometric study of glucose, sucrose, and fructose using an inductively coupled plasma and electrospray ionization. *International Journal of Mass Spectrometry*, 243(1), pp. 71-84.

Terrab, A., Díez, M.J. and Heredia, F.J., 2003a. Palynological, physico-chemical and colour characterization of Moroccan honeys: I. River red gum (*Eucalyptus camaldulensis* Dehnh) honey. *International Journal of Food Science & Technology*, 38(4), pp. 379-386.

Terrab, A., Díez, M.J. and Heredia, F.J., 2003b. Palynological, physico-chemical and colour characterization of Moroccan honeys. II. Orange (*Citrus* sp.) honey. *International Journal of Food Science & Technology*, 38(4), pp. 387-394.

Tezcan, F., Kolayli, S., Sahin, H., Ulusoy, E. and Erim F.B., 2011. Evaluation of organic acid, saccharide composition and antioxidant properties of some authentic Turkish honeys. *Journal of Food and Nutrition Research*, 50(1), pp. 33-40.

Thannickal, V.J. and Fanburg, B.L., 2000. Reactive oxygen species in cell signaling. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 279(6), pp. L1005-L1028.

Thompson, J., Robrish, S.A., Pikis, A., Brust, A. and Lichtenthaler, F.W., 2001. Phosphorylation and metabolism of sucrose and its five linkage-isomeric α-D-glucosyl-D-fructoses by *Klebsiella pneumoniae*. *Carbohydrate Research*, 331(2), pp. 149-161.

Tisza, S., Molnár-Perl, I., Friedman, M. and Sass, P., 1996. Simultaneous capillary GC of acids and sugars as their silyl (oxime) derivatives: Quantitation of chlorogenic acid, raffinose, and pectin substances. *Journal of High Resolution Chromatography*, 19(1), pp. 54-58.

Toledo, M., 2014. BRIX – Sugar Determination. In Density and Refractometry. pp.1-8. https://beta-static.fishersci.com/content/dam/fishersci/en_US/documents/programs/scientific/technical-documents/technical-bulletins/mettler-toledo-brix-sugar-determination-techinical-bulletin.pdf

Tomás-Barberán, F.A., Ferreres, F., García-Vignera, C. and Tomás-Lorente, F., 1993. Flavonoids in honey of different geographical origin. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 196(1), pp. 38-44.

Tornuk, F., Karaman, S., Ozturk, I., Toker, O.S., Tastemur, B., Sagdic, O., Dogan, M. and Kayacier, A., 2013. Quality characterization of artisanal and retail Turkish blossom honeys: Determination of physicochemical, microbiological, bioactive properties and aroma profile. *Industrial Crops and Products*, 46, pp. 124-131.

Tosun, M., 2013. Detection of adulteration in honey samples added various sugar syrups with $^{13}C/^{12}C$ isotope ratio analysis method. *Food Chemistry*, 138(2-3), pp. 1629-1632.

Tramuta, C., Nebbia, P., Robino, P., Giusto, G., Gandini, M., Chiado-Cutin, S. and Grego, E., 2017. Antibacterial activities of Manuka and honeydew honey-based membranes against bacteria that cause wound infections in animals. *Schweiz Arch Tierheilkd*, 159(2), pp. 117-121.

Trautmann, M., Held, T.K., Susa, M., Karajan, M.A., Wulf, A., Cross, A.S. and Marre, R., 1998. Bacterial lipopolysaccharide (LPS)-specific antibodies in commercial human immunoglobulin preparations: superior antibody content of an IgM-enriched product. *Clinical & Experimental Immunology*, 111(1), pp. 81-90.

Truchado, P., Vit, P., Ferreres, F. and Tomas-Barberan, F., 2011. Liquid chromatography–tandem mass spectrometry analysis allows the simultaneous characterization of C-glycosyl and O-glycosyl flavonoids in stingless bee honeys. *Journal of Chromatography A*, 1218(42), pp. 7601-7607.

Truchado, P., Vit, P., Heard, T.A., Tomás-Barberán, F.A. and Ferreres, F., 2015. Determination of interglycosidic linkages in O-glycosyl flavones by high-performance liquid chromatography/photodiode-array detection coupled to electrospray ionization ion trap mass spectrometry. Its application to *Tetragonula carbonaria* honey from Australia. *Rapid Communications in Mass Spectrometry*, 29(10), pp. 948-954.

Tuksitha, L., Chen, Y.L.S., Chen, Y.L., Wong, K.Y. and Peng, C.C., 2018. Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak). *Journal of Asia-Pacific Entomology*, 21(2), pp. 563-570.

Turkmen, N., Sari, F., Poyrazoglu, E.S. and Velioglu, Y.S., 2006. Effects of prolonged heating on antioxidant activity and colour of honey. *Food Chemistry*, 95(4), pp. 653-657.

Umaña, E., Zamora, G., Aguilar, I., Arias, M.L., Pérez, R., Sánchez, L.A., Solórzano, R. and Herrera, E., 2021. Physicochemical differentiation of stingless bee honeys from Costa Rica. *Journal of Apicultural Research*, 2021, pp. 1-10.

Valdés-Silverio, L.A., Iturralde, G., García-Tenesaca, M., Paredes-Moreta, J., Narváez-Narváez, D.A., Rojas-Carrillo, M., Tejera, E., Beltrán-Ayala, P., Giampieri, F. and Alvarez-Suarez, J.M., 2018. Physicochemical parameters, chemical composition, antioxidant capacity, microbial contamination and antimicrobial activity of Eucalyptus honey from the Andean region of Ecuador. *Journal of Apicultural Research*, 57(3), pp. 382-394.

van Vuuren, S. and Viljoen, A., 2011. Plant-based antimicrobial studies–methods and approaches to study the interaction between natural products. *Planta Medica*, 77(11), pp. 1168-1182.

Vanhanen, L.P., Emmertz, A. and Savage, G.P., 2011. Mineral analysis of mono-floral New Zealand honey. *Food Chemistry*, 128(1), pp. 236-240.

Vasić, V., Gašić, U., Stanković, D., Lušić, D., Vukić-Lušić, D., Milojković-Opsenica, D., Tešić, Ž. and Trifković, J., 2019. Towards better quality criteria of European honeydew honey: Phenolic profile and antioxidant capacity. *Food Chemistry*, 274, pp. 629-641.

Vela, L., de Lorenzo, C. and Perez, R.A., 2007. Antioxidant capacity of Spanish honeys and its correlation with polyphenol content and other physicochemical properties. *Journal of the Science of Food and Agriculture*, 87(6), pp. 1069-1075.

Veness, R.G. and Evans, C.S., 1996. Identification of disaccharides by gas chromatography-fourier transform infrared spectroscopy. *Journal of Chromatography A*, 750(1-2), pp. 311-316.

Vit, P., Pedro, S.R. and Roubik, D. eds., 2013. Pot-honey: a legacy of stingless bees. Springer Science & Business Media.

Vit, P., Rodríguez-Malaver, A., Roubik, D.W., Moreno, E., Souza, B.A., Sancho, M.T., Fernández-Muiño, M., Almeida-Anacleto, D., Marchini, L.C., Gil, F. and González, C., 2009. Expanded parameters to assess the quality of honey from Venezuelan bees (*Apis mellifera*). *Journal of Apiproduct and Apimedical Science*, 1(3), pp. 72-81.

Vit, P., Soler, C. and Tomás-Barberán, F.A., 1997. Profiles of phenolic compounds of *Apis mellifera* and *Melipona* spp. honeys from Venezuela. *Zeitschrift für Lebensmitteluntersuchung und-Forschung A*, 204(1), pp. 43-47.

Wagner, H. and Ulrich-Merzenich, G., 2009. Synergy research: approaching a new generation of phytopharmaceuticals. *Phytomedicine*, 16(2-3), pp. 97-110.

Wang, Z.L., Liu, T.T., Huang, Z.Y., Wu, X.B., Yan, W.Y. and Zeng, Z.J., 2012. Transcriptome analysis of the Asian honey bee *Apis cerana cerana*. *Plos one*, 7(10), p. e47954.

Weston, R.J., Brocklebank, L.K. and Lu, Y., 2000. Identification and quantitative levels of antibacterial components of some New Zealand honeys. *Food Chemistry*, 70(4), pp. 427-435.

Weston, L., 2020. Honeyed words and waxen tablets: Aldhelm's bees and the materiality of Anglo-Saxon literacy. *Mediaevalia*, 41, pp. 43-69.

Wieczorek, J., Pietrzak, M., Pomianowski, J. and Wieczorek, Z., 2014. Honey as a source of bioactive compounds. *Polish Journal of Natural Sciences*, 29(3), pp. 275-285.

Wilczyńska, A., 2014. Effect of filtration on colour, antioxidant activity and total phenolics of honey. *LWT-Food Science and Technology*, 57(2), pp. 767-774.

World Health Organization. WHO Publishes List of Bacteria for Which New Antibiotics are Urgently Needed; 2017. Available from: http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/.

Xu, P., Shi, M. and Chen, X.X., 2009. Antimicrobial peptide evolution in the Asiatic honey bee *Apis cerana*. *Plos one*, 4(1), p. e4239.

Yagi, H., Tan, J. and Tuan, R.S., 2013. Polyphenols suppress hydrogen peroxide-induced oxidative stress in human bone-marrow derived mesenchymal stem cells. *Journal of Cellular Biochemistry*, 114(5), pp. 1163-1173.

Yang, M., Ma, Y., Wang, Z., Khan, A., Zhou, W., Zhao, T., Cao, J., Cheng, G. and Cai, S., 2020. Phenolic constituents, antioxidant and cytoprotective activities of crude extract and fractions from cultivated artichoke inflorescence. *Industrial Crops and Products*, 143, p. 111433.

Yao, L.K., Razak, S.L.A., Ismail, N., Fai, N.C., Asgar, M.H.A.M., Sharif, N.M., Aan, G.J. and Jubri, Z., 2011. Malaysian gelam honey reduces oxidative damage and modulates antioxidant enzyme activities in young and middle aged rats. *Journal of Medicinal Plants Research*, 5(23), pp. 5618-5625.

Zainol, M.I., Yusoff, K.M. and Yusof, M.Y.M., 2013. Antibacterial activity of selected Malaysian honey. *BMC Complementary and Alternative Medicine*, 13(1), pp. 1-10.

Zappala, M., Fallico, B., Arena, E. and Verzera, A., 2005. Methods for the determination of HMF in honey: a comparison. *Food Control*, 16(3), pp. 273-277.

Zarei, M., Fazlara, A. and Alijani, N., 2019. Evaluation of the changes in physicochemical and antioxidant properties of honey during storage. *Functional Foods in Health and Disease*, 9(9), pp. 593-605.

Zhang, Y.Z., Chen, Y.F., Wu, Y.Q., Si, J.J., Zhang, C.P., Zheng, H.Q. and Hu, F.L., 2019. Discrimination of the entomological origin of honey according to the secretions of the bee (*Apis cerana* or *Apis mellifera*). *Food Research International*, 116, pp. 362-369.

Zhao, H., Cheng, N., He, L., Peng, G., Xue, X., Wu, L. and Cao, W., 2017. Antioxidant and hepatoprotective effects of A. *cerana* honey against acute alcohol-induced liver damage in mice. *Food Research International*, 101, pp. 35-44.

Živkov-Baloš, M., Popov, N., Vidaković, S., Ljubojević-Pelić, D., Pelić, M., Mihaljev, Ž. and Jakšić, S., 2018. Electrical conductivity and acidity of honey. *Arhiv Veterinarske Medicine*, 11(1), pp. 91-101.

APPENDIX 1

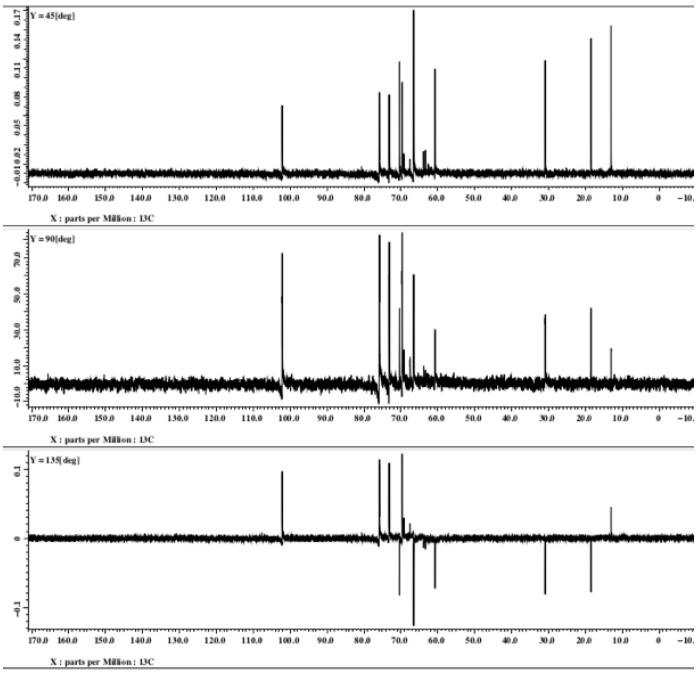


Figure 1: The outcome of DEPT for butylated glucoside isolated from stingless bee honey.

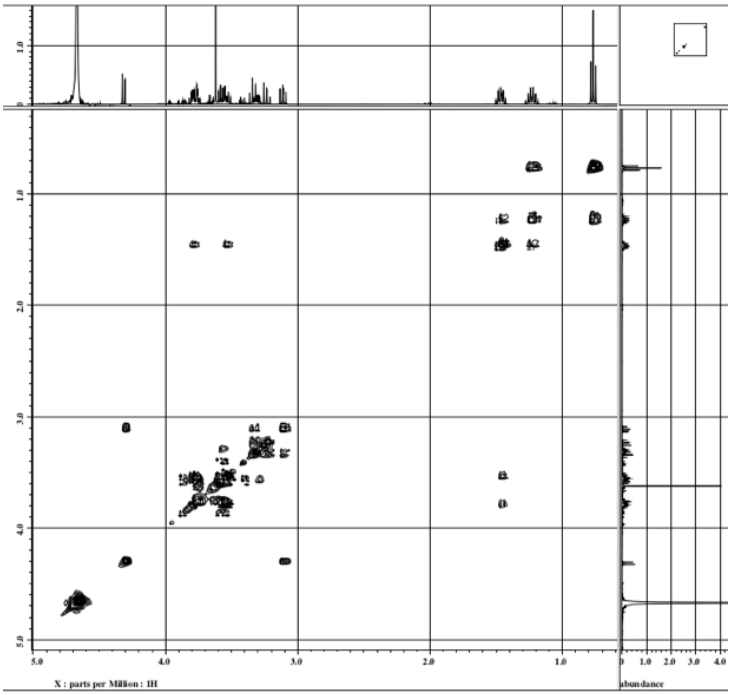


Figure 2: The COSY spectra of butylated glucoside isolated from stingless bee honey.

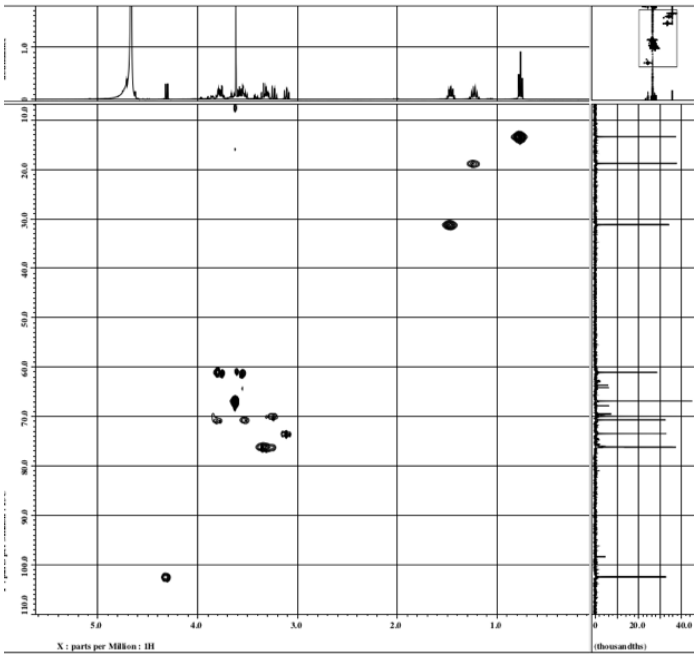


Figure 3: The outcome of HMQC for butylated glucoside isolated from stingless bee honey.

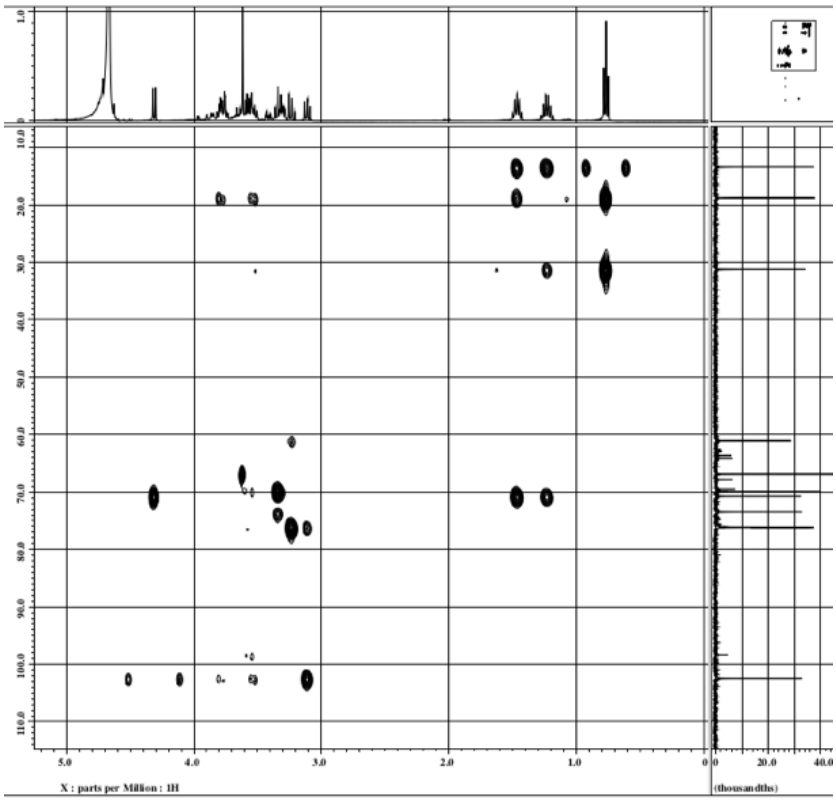


Figure 4: The outcome of HMBC for butylated glucoside isolated from stingless bee honey.

APPENDIX 2

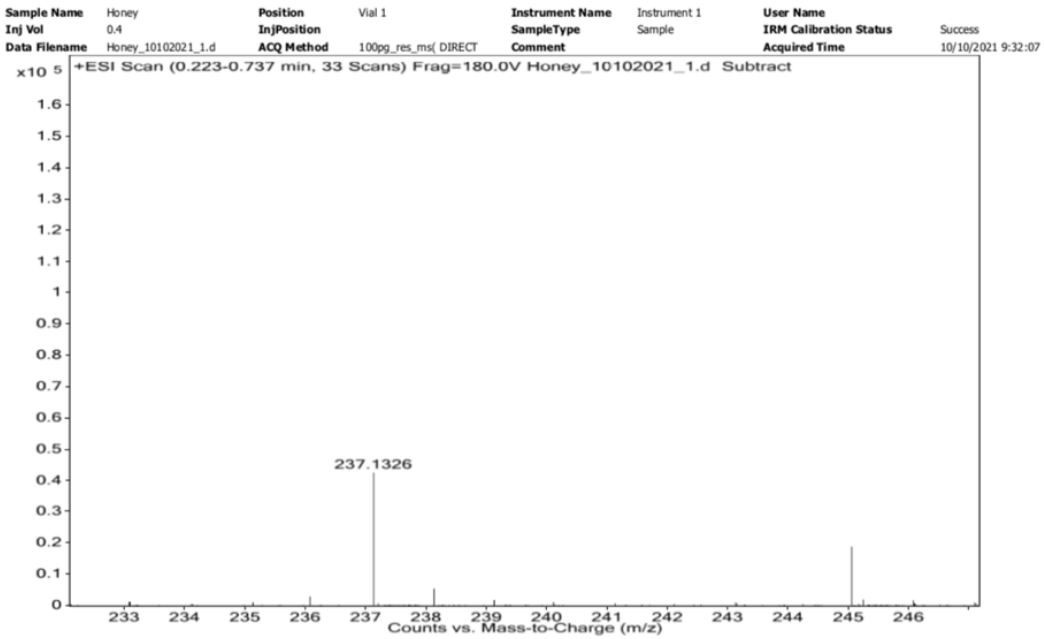


Figure 5: Mass spectrum of protonated butyl glucoside.

Best	Name	Formula	Score	Mass	Mass (Tgt)	Mass (DB)	Mass (MFG)	Diff (ppm)	Diff (abs. ppm)	Diff (mDa)
✓		C10 H20 O6	92.05	236.1253			236.126	3	3	0.71

Species	Ion Formula	m/z	Height	Score (MFG)	Score (MFG, MS)	Score (MFG, MS/MS)	Score (MFG, mass)	Score (MFG, abund)	Score (MFG, iso. spacing)
(M+H)+	C10 H21 O6	237.1333	42438.7	92.05	92.05		95.57	96.77	79.35

m/z	m/z (Calc)	Diff (ppm)	Diff (mDa)	Height	Height (Calc)	Height %	Height % (Calc)	Height Sum %	Height Sum% (Calc)
237.1326	237.1333	3	0.7	42438.7	43262.2	100	100	86.7	88.4
238.1371	238.1367	-1.59	-0.4	5076.2	4882.5	12	11.3	10.4	10
239.1442	239.1384	-24.24	-5.8	1413.4	783.6	3.3	1.8	2.9	1.6

Figure 6: The mass spectrometric details of protonated butylated glucoside.

APPENDIX 3

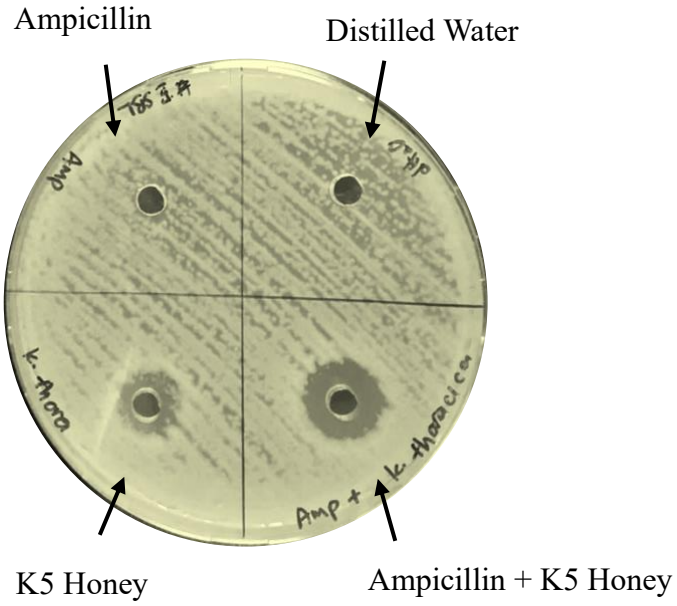


Figure 7: Example of plate of *Escherichia coli* 3 for honey alone, ampicillin alone and mixture of ampicillin and honey.

APPENDIX 4

antibiotics

MDPI

Article

The Antibacterial Potential of Honeydew Honey Produced by Stingless Bee (*Heterotrigona itama*) against Antibiotic Resistant Bacteria

Wen-Jie Ng *, Nam-Weng Sit, Peter Aun-Chuan Ooi, Kah-Yaw Ee and Tuck-Meng Lim

Faculty of Science, Universiti Tunku Abdul Rahman, Kampar 31900, Malaysia; sitnw@utar.edu.my (N.-W.S.); peteracooi@gmail.com (P.A.-C.O.); eeky@utar.edu.my (K.-Y.E.); ltmeng@utar.edu.my (T.-M.L.)
* Correspondence: ngwj@utar.edu.my

Received: 2 November 2020; Accepted: 18 November 2020; Published: 5 December 2020

check for updates

Abstract: Scientific studies about the antibacterial effects of honeydew honey produced by the stingless bee are very limited. In this study, the antibacterial activities of 46 blossom and honeydew honeys produced by both honey bees and stingless bees were evaluated and compared. All bacterial isolates showed varying degrees of susceptibility to blossom and honeydew honeys produced by the

molecules

MDPI

Article

Botanical Origin Differentiation of Malaysian Stingless Bee Honey Produced by *Heterotrigona itama* and *Geniotrigona thoracica* Using Chemometrics

Wen-Jie Ng *, Nam-Weng Sit, Peter Aun-Chuan Ooi, Kah-Yaw Ee and Tuck-Meng Lim

Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Kampar 31900, Malaysia; sitnw@utar.edu.my (N.-W.S.); peteracooi@gmail.com (P.A.-C.O.); eeky@utar.edu.my (K.-Y.E.); ltmeng@utar.edu.my (T.-M.L.)
* Correspondence: ngwj@utar.edu.my

Abstract: Stingless bee honey, specifically honeydew honey, is generally valued for its better health benefits than those of most blossom types. However, scientific studies about the differentiation of stingless bee honey based on honeydew and blossom origins are very limited. In this study, ^{13}C NMR spectroscopy was employed to quantify the seven major sugar tautomers in stingless bee honey samples, and the major sugar compositions of both honeydew and blossom types were found not

Figure 8: Journal article publications in this study.