Compliance of Plants Phenolic and Microbial Contaminant Limits of Commercial Stingless Bee Honey to Malaysian Standard (MS) 2683:2017

Norsyahira binti Roslan
F15A0123

A thesis submitted in fulfilment of the requirement for the degree of Bachelor of Applied Science (Product Development Technology) with Honours

Faculty of Agro Based Industry

UNIVERSITY MALAYSIA KELANTAN

2019

DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Student

Name: Norsyahira binti Roslan

Date:

I certify that the report of this final year project entitled "**Compliance of Plants Phenolic and Microbial Contaminant Limits of Commercial Stingless Bee Honey to Malaysian Standard (MS) 2683:2017**" by **Norsyahira binti Roslan**, matric number **F15A0123** have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

_____

Supervisor

Name: Dr. Noor Hafizoh binti Saidan

Date:

ACKNOWLEDGEMENT

All praises to Allah, the Almighty, the only one who gives me opportunity and strengths to complete this thesis. First and foremost, my deepest gratitude to my supervisor, Dr. Noor Hafizoh bt Saidan and my co-supervisor, Dr. Kumara Thevan for their willingness to accept me as their student and gave endless support, guideline, knowledge, supervision and suggestion in completing my final year project research. Without their guidance and persistent help, this thesis cannot be completed.

I would also like to express my appreciation to beekeepers whose supplied the sample of honey. Also, to Faculty of Agro-Based Industry, Universiti Malaysia Kelantan for providing the facilities. Not forgetting to laboratory assistants especially Mr. Suhaimi bin Omar, Mr. Qamal bin Othman, Madam Nor Hidayah binti Hamzah, Mr. Nik Fakruddin bin Nik Dzulkefli and Madam Nur Aiashah binti Ibrahim for their cooperation and technical support while conducting my experiment in the laboratory.

My deepest appreciation to my family, especially my father, Roslan bin Husain and my mother, Norini binti Ramli, who constantly offered their moral support, encouragement, and prayers throughout my study. A special thanks also to my beloved friends especially Nur Rahiiqin, Fatin Naimah, Alia Athirah and Siti Rahimah whose have always give me a generous support during my thesis writing. Without them, I am nothing. I will not forget this valuable journey in my life. Lastly, I would like to thank all the parties involved directly or indirectly in this work.

Pematuhan Fenolik Tumbuhan dan Had Pencemaran Mikrobial dalam Komersial Madu Kelulut Komersial dengan Standard Malaysia (MS) 2683: 2017

ABSTRAK

Baru-baru ini, madu kelulut komersial berkembang dengan pesat di pasaran Malaysia kerana sifat nutrisi dan penyembuhan terutama dalam kosmetik, makanan dan minuman dan industri farmaseutikal. Namun, walaupun madu itu asli, kadang-kadang mereka tidak mematuhi spesifikasi Malaysian Standard (MS) 2683: 2017. Oleh itu, matlamat kajian ini bertujuan untuk menentukan mutu 13 sampel madu kelulut dari lokasi terpilih di Kelantan mengenai kandungan fenolic tanaman dan analisis mikrobiologi mengenai had pencemaran mikrob mengikuti spesifikasi MS 2683: 2017. Kandungan fenolik tanaman telah dinilai dengan menggunakan Kromatografi Cecair Kinerja Tinggi (HPLC) manakala had pencemaran mikrob ditentukan dari segi jumlah kiraan plat (TPC), yis dan kulat (YM), dan jumlah kolifom (TC). Hasil kajian menunjukkan kehadiran fenolic tumbuhan asid gallic dalam semua sampel madu kelulut. Tidak terdapat perbezaan yang ketara dalam semua sampel madu kelulut dari segi jumlah kiraan plat (TPC) dan yis dan kulat (YM). Tidak ada kehadiran jumlah koliform dalam semua sampel. Secara ringkasnya, kajian ini menunjukkan kesemua 13 madu kelulut komersil mematuhi spesifikasi MS 2683: 2017 dari segi fenolik tumbuhan dan had pencemaran microbial bagi jumlah kiraan plat (TPC) dan jumlah kolifom (TC). Namun begitu, satu sampel tidak mematuhi spesifikasi MS dari segi yis dan kulat (YM). Untuk masa depan, perbandingan perlu dilakukan untuk jenis madu yang lain separti madu Tualang, madu Acacia dan madu Manuka.

Kata kunci: Fenolik tanaman, had pencemaran mikrobial, madu kelulut, Standard Malaysia 2683: 2017

Compliance of Plants Phenolic and Microbial Contaminant Limits of Commercial Stingless Bee Honey to Malaysian Standard (MS) 2683:2017

ABSTRACT

Recently, the commercial stingless bee honey is growing rapidly in market of Malaysia due to their nutritional and healing properties especially in cosmetic, food and beverage and pharmaceutical industry. However, even the honey is pure, sometimes they did not comply the specification of Malaysian Standard (MS) 2683:2017. Thus, the objective of this study aims to determine the quality of 13 samples of stingless bee honey from selected location in Kelantan in term of plants phenolic content and microbiological analysis of microbial contaminant limits by following the MS 2683:2017 specification. The plants phenolic contents were evaluated by using High Performance Liquid Chromatography (HPLC) while microbial contaminant limits were determined the total plate count (TPC), yeast and molds (YM), and total coliforms (TC). Result showed the presence of plants phenolic of gallic acid in all of the stingless bee honey samples. No significant differences were found in all sample of stingless bee honey in term of total plate count (TPC) and yeast and mold (YM). There were absent of total coliform in all of the samples. In summary, this study shows all of 13 commercial stingless bee honey do comply to MS 2683:2017 specification in term of plants phenolic and microbial contaminant limits of total plate count (TPC) and total coliform (TC). Meanwhile there was one sample that did not comply with MS specification in term of contamination of yeast and mold (YM). For the future, a comparison needs to be done for other types of honey such as *Tualang* honey, Acacia honey, and *Manuka* honey.

Keywords: Plants phenolic, microbial contaminant limits, stingless bee honey, Malaysian Standard 2683:2017

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CDC	Centres for Disease Control and Prevention
CFU	Colony Forming Units
DAD	Diode Array Detector
dH_2O	Distilled water
FDA	Food and Drug Administration
HCL	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
KH_2PO_4	Potassium dihydrogen phosphate anhydrous
MEA	Malt Extract Agar
MOA	Ministry of Agriculture and Agro-based Industry
MOSTI	Ministry of Science, Technology and Innovation
MS	Malaysian Standard
SD	Standard Deviation
SPE	Solid Phase Extraction
UV	Ultra Violet
TC	Total Coliforms
TPC	Total Plate Count
VRBA	Violet Red Bile Agar
YM	Yeast and Mold

LIST OF UNITS AND SYMBOLS

CFU/mL	Colony Forming Units per millilitre
g	Gram
GAE/kg	Gallic acid equivalent per kilograms
h	Hours
L	Litre
mg/kg	Milligram per kilogram
min	Minutes
mm	Millimetre
mS/cm	Milli Siemens per centimetre
mL	Millilitre
mL/min	Millilitre per minutes
nm	Nanometre
s	Second
µL	Microlitre
µm	Micrometre
µg/mL	Microgram per millilitre
µL/mL	Microlitre per millilitre
%	Percent
℃	Celcius

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Honey is defined as natural sweet substance produced by honeybees from flower nectars (Izwan and Zainol, 2016). Its look like a supersaturated sugar solution with colour ranges from nearly colourless to dark brown. It is appeared in term of sticky and viscous supersaturated solution which is mainly composed of a complex mixture of 80–85% carbohydrate (mainly glucose and fructose), 15–17% water, 0.1–0.4% protein, 0.2% ash and minor substances of minerals, amino acids, enzymes, vitamins, organic acids, lipids as well as other substances like phenolic antioxidants (Buba, Gidado, & Shugaba, 2013; Ummulkhair, 2014). However, by depending on the geographical and botanical origin, the composition of chemical is much more complex and extremely variable (Ciulu, Spano, Pilo, & Sanna, 2016). In addition, differences in type of flora, climatic conditions and geographical region also influence the physical and chemical properties of honey (Rao, Krishnan, Salleh, & Gan, 2016).

Nowadays, as stated by Ngoi (2016) the researchers is interested to stingless bee honey due to its higher nutritional values as compared to ordinary honey. Other than that, the growing interest to stingless bee honey due to their composition that have

been proved contain “antiseptic, antimicrobial, anticancer, anti-inflammatory, and wound-healing properties and promote cell functions in (Silva et al., 2013). Hence, researchers began to explore and study the chemical and biological composition of stingless bee honey.

Phenolic compound is secondary metabolites divided into two categories, which are non-flavonoids and flavonoids. The non-flavonoids of phenolic compound are including phenolic acid whereas flavonoids are including flavones, flavanols, flavanones, flavanols, anthocyanidin, isoflavones and chalcones. Studied by Izwan and Zainol (2016) reported that plants phenolic such as phenolic acid and flavonoid is a chemical component that acts as bioactivity in honey in term of antioxidant capacity. The component is depends on their flower sources that the bees collect the nectar. Meanwhile, phenolic compound also acts as antioxidant agents that supply to antioxidant activity of honey (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016).

There are some cases regarding to the infant botulism due to the presence of *Clostridium botulinum* in honey. Because of the issue, a recommendation to not feed honey to infants under one year old is created by Food and Drug Administration (FDA), the Centres for Disease Control and Prevention (CDC), and the American Academy of Paediatrics (Lani, Zainudin, Abdul, Mansor, & Hassan, 2017). Honey can be contaminated through primary sources; pollen, floral nectar, dust, soil and the bodies and digestive tracts of bees or through secondary sources; either during their extraction or processing (Ananias, de Melo, & de Moura, 2013; Izwan & Zainol, 2016; Lani et al., 2017). Certain microorganisms cause honey to ferment, thus could acidified and modified their taste (Ananias et al., 2013). Thus, microbiological analysis were performed in term of determination of total plate count, yeasts and molds and counts of

total coliforms that are indicates of the sanitary quality that can cause foodborne illnesses (Fernandes et al., 2018).

Furthermore, Bradbear (2009) states the fermentation process of honey is occasionally giving a problem to the industry of honey production in market. The several factors contributing to the fermentation in honey is due to high temperature, high content of moisture which is above 20 percent, while high yeast counts which is above 10 of colonies forming units per gram. Basically, honey has properties of longer shelf life with a stable commodity. However, if the honey is harvested and handled hygienically and have suitable containers of storage with tight-fitting lids, it will maintain their shelf life and well- conditioned for several years.

Recently, the issue on quality and artificial honey is widespread in market. Therefore, specification of honey was established by Ministry of Science, Technology and Innovation (MOSTI) and Ministry of Agriculture and Agro-based Industry (MOA) through the Department of Standards Malaysia by developed the Malaysian Standard (MS 2683) Honey Bee-Specification. The honey products in market should follow the guideline and specification under Malaysian Standard 2683:2017 for quality and safety to customers (*Kelulut (Stingless bee) honey - Specification Malaysian Standard MS 2683 : 2017*, 2017).

In conclusion, the stingless bee honey must presence the plants phenolic compound and the microbial contamination must not exceeding the limits specified by the government for the quality and safety to the consumer. Consequently, the aims of this study were to investigate the quality of commercial stingless bee honey products produced in certain region in Kelantan in term of plants phenolic content and microbial contaminant limit by comparing to Malaysia Standard (MS) 2683:2107 specifications.

1.2 Problem Statement

Stingless bee honey is cottage industry where there is no proper quality control is conducted. Furthermore, even the honey is pure, they might not completely safe to be consumed. Therefore, it difficult to consumer to choose the qualified stingless bee honey since not conducting the quality control. To ensure the quality of stingless bee honey, this assessment is done. A specification was set up by regulation of Standards of Malaysia 2683:2017 that acts as the guideline for the quality and safety assessment to stingless bee honey in market.

1.3 Hypothesis

Hypothesis 1

H_0: All of commercial stingless bee honey samples are presence the plant phenolic to be complied with Malaysian Standard 2683:2017

H_1: Some of commercial stingless bee honey samples are presence plant phenolic to be complied with Malaysian Standard 2683:2017

Hypothesis 2

H_0: All of commercial stingless bee honey samples contains microbial contaminant limits complies to Malaysian Standard 2683:2017

H_1: Some of commercial stingless bee honey samples contains microbial contaminant limits complies to Malaysian Standard 2683:2017

1.4 Objective

This study specifically aims:

1. To determine the plants phenolic and microbial contaminant limits in commercial stingless bee honey based on Malaysian Standards (MS) 2683:2017.

1.5 Scope of Study

This study was focused on the plants phenolic contents and microbiological analysis of microbial contaminant limits (MCL) on stingless bee honey products. High Performance Liquid Chromatography (HPLC) technique was used to determine the presence of plants phenolic contents whereas the microbial contaminant limit was accomplished by focusing on determination of total plate counts, yeast and molds and total coliforms. The Malaysian Standard 2683:2017 was referred as the specification and guideline to assess and analyse the quality of the stingless bee honey.

1.6 Significance of Study

This study seeks to analyse the quality of commercial stingless bee honey in term of plants phenolic and microbial contaminant limit specified by Malaysian Standard 2683:2017. This study give advantage to community especially for consumer to choose and purchase the qualified commercial stingless bee honey product followed the Malaysian Standard 2683:2017. In addition, stingless bee honey industry especially for entrepreneurs and manufacturers also should emphasize about the standardisation of stingless bee honey by following the Malaysian Standard before produce and sell to

consumer. In addition, this study also give knowledge especially about plants phenolic and microbiological analysis that can be used for the future studies as the reference to the researchers since the scientific research and literature about this study is still limited.

1.7 Limitation of Study

Some limitations regarding the study are sample collection and condition of the stingless bee products. The availability of stingless bee honey product is limited compare to honeybee because of their production. Besides, there are raw and processed stingless bee honeys products and many species of stingless bee cause blended honey which might also affect the parameter of study. In addition, the preparation of agar media needs a proper handling to avoid the contamination from other organism. Furthermore, this research will not be identifying what are the specific microbes grown on the agar plate but just count their colonies forming units (CFUs). Besides, the limited frame timing also considered as one of the limitations to finish this study.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Honey

In the early 1900's, a group of archaeologists have discovered about the honey on a cave painting at Arana Caves in Valencia, Spain. This discovering is acts as the evident that humans began using the honey at least 10,000 years ago. Long time ago, the Egyptians use honey as the sweeteners for cakes and biscuits, and many other dishes. The ancient of Egyptians and Middle-Eastern also use honey for the mummification of corpse. Besides, according to Aristotle honey can prolongs the life meanwhile Hippocrates stated that honey can be consumed and be used for the treatment of many diseases because it supply good food and health.

Furthermore, a Roman surgeon in army also has noted in his book that honey could acts as treatment for stomach disease, wound healing, haemorrhoids and cure the coughing. Several Roman authors also has wrote the uses of honey as the sweetening agent in Roman recipes and were available in the book Roman cookery (Nayik, Shah, Muzaffar & Wani, 2014). Besides, stingless bee honey is highly valued as a food source by indigenous people of northern Australia and acts as cultural significance, besides plays a role in the social traditions and rituals of the people.

2.2 Background of Stingless Bee Honey

Bees are flying insects which produces wax and honey that lives in large communities. In fact, there are more than 20,000 kinds of bees found over the world including honeybees, bumblebees, stingless bees, carpenter bees and others (Hrncir, Jarau, & Barth, 2016). For general, there are two major groups of honey producing bees, which are have sting (*Apis* spp.) and stingless (*Meliponini*) (Zamora & Arias, 2011). For stingless bees, there are approximately 500 species within the stingless bee genus in the world, with the majority of these species being located in Latin America, the mainland of Australia, Africa, and Eastern and Southern Asia (Abd Jalil, Kasmuri, & Hadi, 2017; Boorn et al., 2009; Hrncir et al., 2016; Kelly, Farisha, Kumara, & Marcela, 2014) whereas 43 species were recognised in Asia (Kumara et al., 2016).

The stingless bees which are from *Meliponini* tribe consist of more than one type of genera including *Melipona spp.*, *Scaptotrigona spp.*, and *Trigona spp* (Kek, Chin, Yusof, Tan, & Chua, 2014). In Malaysia, the genus *Trigona* were explored varies between 17 to 32 species including *Heterotrigona itama, Geniotrigona thoracica, Lepidotrigona terminata*, *Tetragonula laevicep*, and others (Kelly et al., 2014; Kumara et al., 2016). Recently, the subgenera have been upgraded to genera (Rasmussen, 2008).

The colony of stingless bee honey consists of honey, pollen, bee bread and propolis. They store pollen and honey in egg-shaped pots made of beeswax (Figure 2.1(a)). Most the beekeepers kept their stingless bees in log hive and kept them in a wooden box, as for their safety and they can control the hive easier (Figure 2.1(b)) (Villanueva-G, Roubik, & Colli-Ucán, 2005). The production of the honey from stingless bee is least compared to honeybee. However, stingless bees have good quality and properties, and reported to have antitumoral, antimicrobial and antioxidant

activities. Due to the reported health properties and benefits, the demands of honey from stingless bee in Malaysia are rising (Lani et al., 2017). Nowadays, the modern collection and harvesting of stingless bee honey is done by using disposable syringes. Whereas by using a suction pump for more efficiency (Souza et al., 2006).

Figure 2.1(a) The egg-shaped pots that stored the stingless bee honey.

Figure 2.1(b) The log hive of stingless bee in wooden box.

2.3 Composition and Characteristic of Stingless Bee Honey

Stingless bee honey has their own unique characteristic in term of colour, taste, viscosity, water and sugar content that give specialty compared to other types of honey. One of the characteristics of the stingless bees is they do not sting thus it is easier to extract and collect the honey, pollen, and propolis frequently. Furthermore, stingless bees are easier to handle compared to honey bees that are often lost, always abandon their hive, and are vulnerable to disease (Abd Jalil et al., 2017). Studied by Lani et al. (2017) stated the taste of stingless bee honey is sour and not over sweet compare to the honeybees. Moreover, according to Ngoi (2016) states stingless bees honey is storing in resin pots instead of honey combs. Other than that, production of stingless bee honey is also least compared to other honey. Hence, their availability is limited and more expensive compared to honeybees in the market due to their limitation of production.

Other than that, Izwan and Zainol (2016) state the properties in term of phytochemical contents, hydrogen peroxide, low pH and high osmolarity is differ between type of honey by depending on their origin of geographical, species of bee, and flora's source. However, the physical properties such as high acidity, low pH value, low water content or moisture and electrical conductivity in all type of honey is commonly same. Besides that, reducing sugar, phenolic compounds, flavonoids, hydrogen peroxide, proteins, enzymes, minerals and vitamins are another molecule that present in all types of honey. In addition, the stingless bee honey has major of composition of sugars which is mainly fructose, glucose and water, and also contains small amounts of other compounds such as organic acids, proteins, amino acids, enzymes, vitamin and phenolic compounds such as phenolic acids and flavonoids. The comparison of physicochemical properties of *Tualang* honey (Erejuwa et al., 2010),

Manuka honey (Stephens et al., 2010) and stingless bee honey (Souza et al., 2006) were shown in Table 2.1.

Table 2.1 Comparison of physicochemical properties between stingless bee honey, *Tualang* honey, and *Manuka* honey

Physicochemical properties	Types of honey		
	Stingless bee honey (Souza et al., 2006)	*Tualang* honey (Erejuwa et al., 2010)	*Manuka* honey (Stephens et al., 2010)
Colour appearance	Amber brown	Dark brown	Light-dark brown
Moisture content (%)	19.9-41.9	23.30	18.70
pH	3.15-4.66	3.55-4.00	3.20-4.20
Fructose (%)	31.11-40.20	29.60	40.00
Glucose (%)	8.20-30.98	30.00	36.20
Sucrose (%)	1.1-4.8	0.60	2.80
Total reducing sugars	58.00-75.70	67.50	76.00
Electrical conductivity (mS/cm)	0.49-8.77	0.75-1.37	0.53
HMF (mg/kg)	8.80-69.00	46.17	40.00
Ash content (%)	0.01-0.12	0.19	0.03

Other than that, honey is undergoes fermentation process. The fermentation of honey is happened due to “action of sugar-tolerant yeasts upon the sugars dextrose and levulose, resulting in the formation of ethyl alcohol and carbon dioxide” (White and Doner, 1980). The presence of oxygen in alcohol is then break down to water and acetic acid. Consequently, the honey that was undergoes the fermentation process cause taste of sour. Besides, White and Doner (1980) states there are other substances which are acts as minor constituents of honey such as amino acids, enzymes, proteins, flavouring materials, minerals, and pigments that were different among types of honey.

For the acidity of honey, it is categorized as acidic, which is usually the pH within the range of 3.7 - 4.5. The sweetness of honey is commonly depending on content of high fructose and acidity. Furthermore, some plants even produce a bitter taste of honey. Honey also has properties of hygroscopic that acts as moisture absorber and regulate the humidity. Honey which is rich in fructose is very hygroscopic. The properties of hygroscopic of honey cause the storage of container to be closed tightly or other else it will absorb the moisture from the air. Hence, this condition might increase the water content and lead to possible fermentation in honey. Thus, it is important to always store the honey in tight fitting lids of container (Bradbear, 2009).

Besides, another importance component in honey is pollen which can acts as major of protein sources (Michener, 2013). A previous study states "pollen is carried to the bees' nest (hive) and stored inside it quite separately from nectar"(Bradbear, 2009). Ironically, microscope can be used to determine the pollen in honey and can identified their plants sources that have been collected by the bees. Other than that, the honeys that contain a very high content of pollen make them look cloudy. In addition, the variations colour of honey is due to plants sources and climate that modified their colour by darkening action of heat. On the other hand, darker honey has a more strong in flavour compared to light-coloured honey (White & Doner, 1980).

2.4 Applications and Benefits of Honey

In general, honey has wide and huge values especially in food and beverage, cosmetic, and medicinal industry. In industry of food and beverage, honey is widely used as a source of sugars especially for making honey's wines and beers. Recently, the uses of honey is also importance in manufacture of secondary products such as

breakfast cereals, bakery goods, and other value-added products (Bradbear, 2009). Next for cosmetic industry, Ediriweera and Premarathna (2012) claimed that honey and beeswax are used as a moisturizer, softener and to heal the skin tissue. Next, other application of honey in cosmetic industry is including face wash, facial cleansing scrub, to heal the pimples, hair lustre, and conditioners.

Traditionally, honey has been used for many purposes such as a laxative, as a natural cure for diarrhea and stomach illness, for coughs and sore throats. Besides, honey also has been known for their healing properties and used for its antibacterial properties in treating wounds. Other than that, honey can be used for the therapeutic purposes such as arthritis, bladder infections, heart disease, toothache, immune system, pimples, skin infections, cancer, digestive system, respiratory system, and eyes disorder (Kowsalya, Anitha, & Gandhi, 2012).

Apart from that, Izwan and Zainol (2016) mentioned honey contain biological activity that give many benefits which is can enhance the health. The main properties are including "antioxidant, antimicrobial (antibacterial, antifungal and antiviral), anti-inflammatory, wound healing promoter, energy booster and anti-aging activities". Meanwhile, stingless bee honey contain high level of antimicrobial that acts as a medicinal properties and reported to have antitumor, and antioxidant activities (Lani et al., 2017). For the antibacterial properties of honey, a study investigated the bacteria cannot survive within a few hours or days after put common pathogenic bacteria to honey (White & Doner, 1980). The result from the studies shows that the bacteria are not suitable to survive in honey. This situation is because of the properties of acidity and high in sugar content in honey. Consequently, the bacteria were killed by high sugar content is because of the osmotic effect.

Indeed, a study from Adenekan, Amusa, Lawal and Okpeze (2010) states the honey have functions of ability to ruin and kill the pathogen organism that give infection of surgical and have function to control the infections of post-operative wound resulting from the action of infective agents including *Salmonella choleraesuis, Samonella thyphi, Serratia marcescens, Escherichia coli, Bacillus anthracis* and *Staphylococcus aureus.*

Furthermore, the content of the phenolic compound of honey acts as antibacterial and antioxidant properties. Besides, the honey also contains anti-carcinogenic properties that act as agents to kill the cancer cells by immune cells. Besides, the study also claimed that honey is beneficial and not harmful to the diabetic patients. This is because the fructose contents in honey could slow down the intestinal absorption of glucose. Thus, this situation can prolong the intestinal absorption besides can limit the absorption of carbohydrates because of the consumption of fructose (Ying, 2015).

2.5 Plants Phenolic in Stingless Bee Honey

Plants phenolic are phytochemical compound found in plant and known as secondary metabolites that can be split into difference main groups which are simple phenols, flavonoids and tannins. Firstly, simple phenols such as phenolic acid, like caffeic acid, gallic acid, rosmarinic acid. Flavonoids which have more complex structure can be categorized into flavanols, flavanones, flavonols, flavones while for tannins that contains relatively high molecular weight can be divided to hydrolysable tannins and condensed tannins. The compounds mostly appear in free form or bound with sugar (Sroka, 2005).

In general, phenolic compounds acts as antioxidants agents that provides antioxidants activity to honey. The degree of antioxidants activity in honey is depends on level of phenolic compound. The difference type of honey contain difference level of antioxidant activity (Izwan & Zainol, 2016). Besides, the darker colour of honey contain higher contents of phenolic compound with higher of antioxidants activity compare to lighter colour of honey (Ying, 2015).

Ironically, all honey over the world contains similar types of phenolic acids, flavonoids and antioxidants contents. The content of phenolic acid such as caffeic, ellagic, ferulic and p-coumaric acids, whereas for flavonoids content are including apigenin, chrysin, galangin, hesperetin, kaempferol, pinocembrin and quercetin while the contents of antioxidants are like tocopherols, ascorbic acid, superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) (Rao et al., 2016).

The previous studies from Almeida, Da Silva, Camara, Queiroz, Magnani, De Novais, Soledade, Lima, de Souza, and de Souza (2013) states the phenolic standards solution selected for phenolic content of stingless honey honey were “vanillic acid, catechol, gallic acid, toxifolin, apigenin, isorhamnetin, kaempferol, luteolin, myricetin, quercetin, tricetin, naringenin, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, caffeic acid, p-coumaric acid, cinnamic acid, sinapic acid, 4-methoxycinnamic acid, chlorogenic acid, 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, syringic acid, (trans–trans)-abscisic acid, (cis–trans)-abscisic acid, and salicylic acid”.

Furthermore, Almeida et al. (2013) investigated the presence of gallic acid also have discovered in stingless bee honey from another several countries including Portugal, New Zealand, Australia and Brazil. According to study by Oliveira, Jain, Luna, and Freitas (2017), honey samples from six species of stingless bees which are

species of *Melipona quadrifasciata, Melipona asilvai, Melipona subnitida, Melipona scutellaris, Melipona compressipes* and *Melipona mandacaia* is analysed the presence of phenolic compounds. From the result, phenolic compound of gallic acid is detected in all of the six species of the stingless bee. General structure of gallic acid is shown in Figure 2.2.

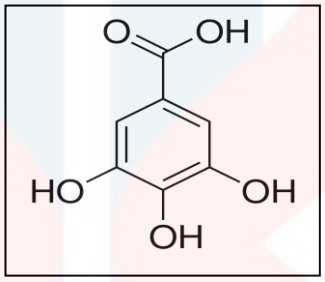

Figure 2.2 General chemical structure of gallic acid.

Besides, a studies by Kek et al. (2014) is to determine the total phenolic contents and colour intensity of Malaysian honeys from the *Apis* spp. and stingless bees. From the finding, species of stingless bee honey gave higher total phenolic content with average of 784.3 mg GAE/kg than the *Apis* spp. honey at average 590.5 mg GAE/kg. The studies also showed the total phenolic content is related with its colour intensity. The colour intensity of honey is a parameter that indicates the presence of pigments which has antioxidant activities such as carotenoids and flavonoids.

A study by Chong, Chin, and Yusof (2017) represented the content of total phenolic in processing stingless bee honey is higher compare to its raw form. Recently, many researchers are interested in the phenolic compounds due to their potential as a dietary supplement to overcome oxidative stress. The oxidative stress can lead to nervous system affection of Alzheimer's disease and Parkinson's disease, and other disease like atherosclerosis, joint disorders, cardiovascular diseases, lung and

kidney disorders, eye disorders, cancer, aging and other degenerative diseases" (Yaacob, Rajab, Shahar, & Sharif, 2018).

In general, total phenolic content differs among the honey regarding their type of bee species, region, season and type of floral sources (Almeida et al., 2013; Yaacob et al., 2018). According to Khoddami, Wilkes, and Roberts (2013), High Performance Liquid Chromatography (HPLC) is a common method to determine and quantified the plants phenolic. On the other hand, MS 2683:2017 also recommend that HPLC is a method to determine the plants phenolic in stingless bee honey.

2.5.1 High Performance Liquid Chromatography (HPLC) Analysis

HPLC is a short term for method of High Performance Liquid Chromatography. As stated by Khoddami et al. (2013) and Moniruzzaman et al. (2014), HPLC analysis is a new method was developed for the detection and quantification of plants phenolic including phenolic acid and flavonoid by employing an HPLC equipped with a photodiode array detector. Based on Malaysian Standard (MS) 2683:2017 mentioned "plants phenolic contents in stingless bee honey are determined by HPLC with photo diode array detector (PDA) and with mass spectrometry detector". In general, the principle of HPLC are analysed according to the external standard method of peak areas by identified on the retention times, UV spectra and reference standards. Besides, the eluted compounds were detected at 280 nm because most of the phenolic compounds show consequent of high absorbance at this value (Aljadi & Yusoff, 2003).

A studied by Moniruzzaman et al. (2014) have used HPLC equipped with a UV detector method for the identification of the phenolic acids and flavonoids in monofloral honey from Bangladesh. The previous studies from Bárbara, Machado, Sodré, Dias,

Estevinho and de Carvalho (2015) also mentioned have used HPLC method to detect the compound of phenolic and flavonoid contents by using gallic acid and quercetin as the reference standard solutions.

2.6 Microorganisms in Stingless Bee Honey

Honey has several possible sources of microorganisms and has their effect. Hence, the microorganism contain in stingless honey product must not exceeding the level limits specified by the Malaysia Standard 2683:2017. The microbiological analysis were done by checking the microorganisms that are indicates of the sanitary quality of food or that can cause foodborne illnesses (Fernandes et al., 2018). The availability of microorganisms in stingless bee honey is presence because of the microbial contamination. According to Izwan and Zainol (2016) and Lani et al. (2017) states there are two possible sources of the microbial contamination in honey. It can be divided into two sources which are primary and secondary sources. The primary sources are including the raw material of the honey which is nectar and pollen. The environment surrounds the honey hive like air and dust is also categorized as primary sources.

Besides, the possible secondary sources of microbial contamination is from the processing procedure of honey such as from human, equipment, containers and their packaging materials. The contamination in honey from post-harvest can also occur due to lack intention regarding to hygienic especially in processing, handling and storage of honey. Furthermore, the bacteria of *Bacillus sp., Clostridium sp. and Micrococcus sp.* are resulting from sources of air and dust. For the solution, the method of sterile and heating the honey can kill the microorganisms which might spoilage the honey's

condition (Fernández, Ghilardi, Hoffmann, & Gallez, 2015; Izwan & Zainol, 2016; Lani et al., 2017).

Furthermore, as mentioned by Kowsalya et al., (2012), contamination of bacteria of *Clostridium botulinum* in honey produce a dangerous problem health to infants as the endospores might change into toxin producing bacteria in their immature intestinal tract, causing to illness and even death. Thus, infants and weakened immune system's people recommend to not consuming the honey to avoid any risk of bacterial or fungal infection. Other than that, Fernández, Ghilardi, Hoffmann and Gallez (2015) revealed 456 cases of infant botulism in Argentina were reported between 1982 and 2007 and some of those cases were also caused by *C. botulinum* spores in honey.

On the other hand, Izwan and Zainol (2016) states that "over-colonized of microbes may lead to infections and worsen the pathological conditions of a patient which may delay wound healing, caused bacteraemia which is presence of bacteria in blood and sometimes may cause death". It is evidence that it will give a bad effect when consume honey that was contaminate with the microbes since the honey is commercially used as the health beneficial. In addition, based on the Malaysian Standard (MS) 2683:2017, the microbial contaminant limit in stingless honey is determined by using method of Total Plate Counts (TPC), yeast and molds, and coliforms. In summary, stingless bee honey shall not contain microorganism exceeding the limits specified by the standardisation.

Besides, Lani et al. (2017) states yeast and spore-forming bacteria are microbes that concerned in honey industry. In other words, yeast is metabolically active and able to grow even in sugary condition of stingless bee honey. Basically, US agriculture handbook (1980) mentioned honey that was not undergoes pasteurized process will

occur fermentation of yeast. The risk of fermentation is depend on the moisture content, yeast spore concentration and temperature storage of honey (Lani et al., 2017).

However, the presence of microorganism is also giving useful role to the stingless bees. The major microorganisms in stingless bee are bacteria, yeast and molds. However, the information is still limited because most of studies just mention about their occurrence but not their function. Recently, there are two genera of bacteria have been discovered, which are Bacillus and Streptomyces. The microorganisms in the stingless bee honey plays an importance role including could secrete the enzymes that can aids in fermentation process of honey and conversion of pollen constituents. The enzyme is acts as food digestion to stingless bee (Menezes, Vollet-Neto, Contrera, Venturieri, & Imperatriz-Fonseca, 2013). Furthermore, the studies from Menezes et al. (2013) also claimed Bacillus and Streptomyces species can secrete antibiotics compound. The antibiotic compounds can defend the larvae from the fungi and other pathogens.

2.6.1 Determination of Total Plate Count

Based on Malaysian Standard (MS) 2683:2017, Total Plate Counts (TPC) is one of method to determine the total number of aerobic bacterial population on stingless bee honey. Basically, TPC method is used to determine the Total Bacterial Count (TBC) besides to indicate the level of microorganism in stingless bee honey. TPC is used to count the bacteria population as the bacteria is dilute with a diluent solution until the bacteria are diluted enough to be counted accurately when spread on the medium. Other than that, the expectation of plate each viable bacterial cell will produce into a single colony.

In general, the bacterial cell numbers must to be reduced by dilution because the colony will be produced too close to each other and difficult to be differentiating as distinct colony-forming units (CFUs). CFU is a microbiology term used to quantify the number of bacteria exist in a solution by depending on their concentration (*Kelulut (Stingless bee) honey - Specification Malaysian Standard MS 2683 : 2017*, 2017).

According to Adenekan et al. (2010) claimed the raw honey normally contains low number of aerobic bacteria. However, due to their improper handling and packaging of honey, the bacteria might be increased (Silva, Rabadzhiev, Eller & Iliev, 2016). Besides, according to studies by Lani et al., (2017) claimed the population aerobic plate count which was contaminated in the stingless bee honey was related to the duration of storage and their species which were the species of *Heterotrigona itama* had slightly higher count compared to species of *Geniotrigona thoracica*.

2.6.2 Enumeration of Yeast and Molds

In general, yeasts are classified as non-filamentous fungi that usually disseminate by wind and air currents and insect vectors. Furthermore, moulds are filamentous and multicellular fungi that available in water, soil, air, and raw organic decomposition. Due to range of optimum temperature which is at around 25 and 30 ℃, the yeast growth require more moisture than needed to moulds and least compared to by bacteria, (Sereia, Sereia, & Ferreira, 2017).

Basically, there are ten yeast genera that had been identified in stingless bee colonies. The genera that frequently appear in pollen and honey are including *Candida* and *Starmerella.* Next, other genera are assumed to be occurred from contamination of external environment besides from the plant's sources. In general, the potential role of

yeast is corresponding to the bacterial roles which is plays a function in secreting the enzyme of honey and also plays function to dehydrate the pollen stored in the stingless bee honey. The process of dehydration acts to avoid decomposition that can cause serious damage to colony (Menezes, Vollet-Neto, Contrera, Venturieri, & Imperatriz-Fonseca, 2013).

A research from Lani et al. (2017) reported the number of yeast count in honey is depends to factor of storage. This is proved that the number of yeast count is increases rapidly after the storage. However, yeasts have ability to withstand to high concentrations of acids and sugar. Thus, this situation gives a problem for the honey production in industry. Besides that, since yeast is required moisture condition, hence, the amount of yeast is related to the humidity of honey. The amount of yeast is increase as the humidity level is higher.

The fermentation that occurred naturally in honey is resulting from the present yeast. However, the higher concentration of sugar can retard the growth of yeast. Besides, another factor that was emphasizing in spoilage of fermentation is water content of the honey. The treatment of heating method is used to destroy the yeast in order to avoid the occurrence of fermentation in honey (White & Doner, 1980). In addition, moulds are classified as an aerobic and less required in term of humidity, pH, temperature and nutrients than yeasts for the growth. Furthermore, any carbon sources derived from food can be absorbed by moulds. The organism of moulds can use nitrate, ammonia and organic nitrogen for growth as honey is acts as a nitrogen source. Besides, when honey is exposed to contact with air, the moisture is increase thus lead a high possibility for molds to grow on the surface of honey, with aids of properties of acids and carbohydrates in honey (Sereia et al., 2017).

A previous studies from Adenekan et al. (2010) reported the present of yeast and molds in 10 sample of honey obtained from difference area of Ibadan, Oyo State in Nigeria is at low counts of CFU. Other than that, Morais, Calaça, and Rosa (2013) states two new species of yeasts were discovered which are *Candida riodocensis* and *Candida cellae* in two species of solitary bees, *Megachile sp*. and *Centris tarsata* in Atlantic, Brazil. They also mentioned new species of yeast, *Zygosaccharomyces siamensis* were detected in raw honey of *Apis mellifera, Apis dorsata,* and *Tetragonula pagdeni* in Thailand and these yeast acts as agent of pot-honey spoilage for the bees. In addition, other yeast that isolated from stingless bee honey is *Hyphopichia burtonii* and *Priceomyces mellissophilus*.

Besides, Camargo et al. (1992) states the yeast has been detected in honey bees, stingless bee and solitary bees. The Amazon species of honey bee, *Ptilotrigona lurida* claimed to require the mutualistic interaction with yeast for the dehydrating and retarding the deterioration of pollen in bee nest. Other than that, Calaca (2011) also reported the number of yeast cells was higher in unripe pot-honey than in ripe honey of *Melipona quinquefasciata* that was collected in Brazil. Thus, indicates the diversity of yeast decreases during honey ripeness (Morais et al., 2013).

2.6.3 Determination of Coliforms

For the principle of total coliforms, the coliform group are including aerobic and facultative anaerobic, gram negative, non-sporing rods that ferment lactose, with acid and gas production within 48 hours at temperatures between 30 ℃ and 37 ℃. The coliforms test detects the bacteria from member of several genera within the family *Enterobacteriacae* including *Escherichia, Enterobacter, Klebsiella* and *Citrobacter*.

Basically, the sample like stingless bee honey is inoculated into the medium containing lactose, which is normally made selective by addition of bile salts, and other surface active agents of dyes (*Kelulut (Stingless bee) honey - Specification Malaysian Standard MS 2683 : 2017*, 2017).

Viable Red Bile Agar (VRBA) is a lactose containing selective medium for the detection and enumeration of coliforms or lactose fermenting gram negative bacteria. The medium contains bile salt and crystal violet as selective agents and the fermentation of lactose is referred by the pH indicator dye, and neutral red. The medium is pasteurised by boiling. Organisms which are rapidly attack lactose produce purple (dark) colonies surrounded by purple haloes (reddish zone of precipitate bile). The coliforms on the medium produce dark red colonies usually greater than diameter of 0.5 mm and often surrounded by a reddish zone. All the colonies are in the depth of the agar due to the overlay (*Kelulut (Stingless bee) honey - Specification Malaysian Standard MS 2683 : 2017*, 2017).

Previous studies from Bárbara, Machado, Sodré, Dias, Estevinho and de Carvalho (2015) claimed there are absent of total coliform in 21 samples of stingless bee pollen (*Melipona mandacaia*) from two regions of Bahia, Brazil which is João Dourado and Uibaí. In addition, the studies of Adenekan et al. (2010) also reported the total coliform were not detected in any of 10 sample honey obtained from difference area of Ibadan, Western Nigeria.

2.7 Development of Malaysian Standards (MS)

A Malaysian Standard is a document developed by consensus and approved by a recognized body that contains rules, guidelines or characteristics for products or

methods of processing and related expenditure, including the terms of the administration in which compliance is not mandatory**.** The main function of Malaysia Standard is to foster and promote standards, standardisation and accreditation as a means of advancing the national economy, promoting industrial efficiency and development, benefiting the health and safety of the public, protecting the consumers, facilitating domestic and international trade and furthering international cooperation in relation to standards and standardisation.

Malaysian Standards (MS) are developed through consensus by committees which comprise balanced representation of producers, users, consumers and others with relevant interests, as may be appropriate to the subject at hand. To the greatest extent possible, Malaysian Standards are aligned to or are adoption of international standards. Approval of a standard as a Malaysian Standard is governed by the Standards of Malaysia Act 1996 [Act 549]. Malaysian Standards are reviewed periodically. The use of Malaysian Standards is voluntary except in so far as they are made mandatory by regulatory authorities by means of regulations, local by-laws or any other similar ways (*Kelulut (Stingless bee) honey - Specification Malaysian Standard MS 2683 : 2017*, 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials, Apparatus and Equipment

The material, apparatus and equipment were used in this study are airtight glass containers, membrane filter (pore size 0.45 µm), HPLC (consisting of HPLC pump, liquid chromatograph (LC-20AT) , degasser (DGU-20A5), auto sampler (SIL-20AC HT), diode array detector (DAD) (SPD-M20A), column oven (CTA-10AS VP, Shimadzu Prominence), Luna C18 LC column (5µm, 4.6 mm x 250 mm), analytical balance, solid phase extraction (SPE) C18 column cartridge, Bunsen burner, glass rod, sterile test tubes and caps, test tube rack, laminar flow, autoclave machine, fume hood, sterile petri dishes (plastic), pipets and sterile pipet tips, dilution bottles, autoclave bottle, circulating water bath, incubators (25 and 37 ℃), sterile measuring cylinder (100 mL), spatula, parafilm M PM999 All-Purpose Laboratory Film, gloves, mask, tissue, chiller, and aluminium foil.

3.2 Chemicals and Reagents

The chemicals and reagents used in this study were methanol (HPLC grade), eluent solution for HPLC (methanol : 0.05 % phosphoric acid in dH_2O), double distilled deionised water, gallic acid (Merck KGaA), hydrochloric acid (HCl) (Bendosen), sterile distilled water, sterile potassium dihydrogen phosphate anhydrous (KH_2PO_4) (Bendosen), sterile plate count agar (PCA) (Himedia), sterile malt extract agar (MEA) (Himedia), , sterile violet red bile agar (VRBA)(Himedia), and 70 % ethanol.

3.3 Experimental Design

The quality of assessment of stingless bee honey samples were carried out in term of plants phenolic and microbial contaminant limits by following the Malaysian Standard 2683:2017 specification. The independent variable is 13 samples of stingless bee honey while the dependent variable is plants phenolic and microbial contaminant limits without related each analysis. The stingless bee honey samples were collected from selected local bee keeping. The samples were identified their type of species either contain single species or blended species, and either in raw or processed type of honey. The plant phenolics were detected by using High Performance Liquid Chromatography (HPLC). Gallic acid and was used as the reference standard for detection of plants phenolic whereas microbial contaminant limits were determined by total plate count, yeast and molds, and total coliforms. The microbial counts were expressed as colony forming units per millilitre of honey (CFU/mL). The statistical analysis of one way of analysis of variance (ANOVA) was performed to determine their significance differences among the samples.

3.4 Methods

3.4.1 Collection and Preparation of Stingless Bee Honey Samples

A total of 13 stingless bee honey samples ($n = 13$) were collected and purchased from difference location in Kelantan. Each of the stingless bee honey products was identified either it is raw or processed product and blended or single species. Their flora sources and date of harvest also were determined. The stingless bee honey samples were placed in clean, dry and airtight glass containers. All of the collected sample were refrigerated in a chiller at the temperature between 4-5 ℃ in airtight glass containers until further analysis. Both raw and processed stingless bee honey were harvested and processed according to MS 2679. Unwanted and foreign material such as wax stick, dead bees and particles of combs were removed by using a sieve before further analysis. The details of the stingless bee honey, including the honey's location, type of honey either raw or processed and either single or blended species, plant or pollen sources, date of harvesting, and their age, were collected from interviewing the beekeeper and was described in Table 3.1. The analysis was performed according to procedure in Malaysian Standard 2683 : 2017 specification.

Table 3.1 The detail information of collected stingless bee honey samples*

Sample no.	Honey location	Types of Honey (Raw or Process)	Honey species (Blended or Single species)	Pollen / Plant sources	Date of harvesting	Age of honey
1	Kubang Kerian	Raw	Blended *(Geniotrigona thoracica, Heterotrigona itama, Lepidotrigona terminata, Tetragonula laevicep)*	Multifloral	4 July 2018	1 week
2	Kg. Sering	Process	Blended (*Geniotrigona thoracica, Heterotrigona itama, Tetragonula laevicep*	Multifloral	June 2018	1 month
3	Pengkalan Chepa	Raw	Single *(Heterotrigona itama)*	Multifloral	March 2018	4 months
4	Kok Lanas	Raw	Single *(Heterotrigona itama*)	Multifloral	April	3 months
5	Ketereh	Raw	Single *(Heterotrigona itama*)	Multifloral	April	3 months
6	Wakaf Bharu	Raw	Single *(Heterotrigona itama)*	Multifloral	February 2018	5 months
7	Wakaf Bharu	Raw	Single *(Heterotrigona itama)*	Multifloral	15 July 2018	0 day
8	Wakaf Bharu	Raw	Single *(Heterotrigona itama)*	Multifloral	April 2018	3 months
9	Wakaf Bharu	Raw	Single *(Heterotrigona itama)*	Multifloral	June 2018	2 weeks
10	Wakaf Bharu	Raw	Single *(Heterotrigona itama)*	Multifloral	14 July 2018	1 day
11	Jedok	Raw	Single *(Heterotrigona itama*)	Multifloral	June 2018	1 month
12	Gemang	Raw	Single *(Gneotrigona thoracica)*	Multifloral	June 2018	1 month
13	Jeli	Raw	Single *(Gneotrigona thoracica)*	Multifloral	21 July 2018	1 day

* Detail information of samples were supplied by beekeepers

3.4.2 High Performance Liquid Chromatography (HPLC) Analysis of Plant Phenolic

3.4.2(a) Validation of HPLC Method

The method to analyse the sample was validated according to the MS 2683: 2017. The validation characteristic of linearity was evaluated.

3.4.2(b) Instrumentation and HPLC Condition

As recommended by MS 2683: 2017, the chromatographic separation of stingless bee sample by HPLC-DAD was conducted using chromatograph Prominence Shimadzu equipped with HPLC pump, liquid chromatograph (LC-20AT), degasser (DGU-20A5), auto sampler (SIL-20AC HT), with diode array detector (DAD) (SPD-M20A) thermostated at 30 ℃, temperature regulated column oven (CTA-10AS VP) at 30 ℃. The analysis was performed using a reverse phase C18 (Phenomenex Luna C18 LC column) (5 µm particle size×4.6 mm×250 mm internal column dimension). The isocratic elution system used in HPLC are mobile phase composed of 0.05% phosphoric acid in deionized water (solvent A), and methanol HPLC grade (solvent B), at a flow 0.8 mL/min. (Table 3.2) and injection volume of 10 µL with 25 min run time at wavelength 254 nm. Peaks are identified on the basis of their retention times, DAD spectra against the reference standards. Quantifications are performed according to the external standard method on peak areas. Some modification was done by referring several previous studies by others.

Table 3.2 The isocratic elution system used in HPLC system

Solvent	Solvent ratio (%)
A (Methanol, HPLC grade)	80
B (0.05% phosphoric acid in deionized water)	20

3.4.2(c) Preparation of Standards Gallic Acid

The stock solution (1000 µg/mL) for standard (gallic acid) was prepared by weighing 1 mg of standard, and dissolved in methanol and then up the volume completed to 1 mL in volumetric flask with methanol. The working standard solutions were prepared by diluting the stock solution (1000 µL/mL) to concentrations of 80 µL/mL, 160 µL/mL, 240 µL/mL, 320 µL/mL, and 400 µL/mL for obtaining the calibration curve.

3.4.2(d) Extraction of Phenolic Compound in Stingless Bee Honey

A modified solid-phase extraction (SPE) procedure was developed to extract the phenolic compounds present in honey. Initially, 4.0 g sample of stingless bee honey product was acidified to pH 2.0 with 10 ml of hydrochloric acid (HCl). For the preparation of HCl (pH 2), 200µL of pure HCl is mixed with 500 mL of deionized water to get pH of 2. The solid phase extraction (SPE) C18 column cartridge (Discovery® DSC-18 SPE, 52603-U, Sigma-Aldrich) was used for the sample purification. The cartridge was pre-conditioned with methanol (3 mL) and HCl (pH 2) and then loading with the acidified sample (5 mL). After the acidified sample was added, the cartridge was washed with 2 mL methanol. The extracted honey was collected, and then was

reduced to dryness *in vacuo* using nitrogen gas. The extract of sample was analysed by using HPLC technique (*Kelulut (Stingless bee) honey - Specification Malaysian Standard MS 2683 : 2017*, 2017).

3.4.2(e) Linearity

Linearity was determined by injecting 10 µL of the standard in 5 concentrations ranges from 80 to 400 µg/mL. The calibration curve was obtained for the compound by obtained the value of the peak area (Y) against the concentration (µg/mL). Linearity of the standard was presented in term of correlation coefficient (R^2). In order to determine the linearity of the calibration graph, regression (R^2) analysis was performed.

3.4.3 Determination of Microbial Contaminant Limits Analysis

The microbial contaminant limits analysis was conducted in stingless bee honey products to determine the total plate count (TPC), yeast and molds (YM) and total coliforms (TC). Total Plate Count (TPC) method is used to determine the microorganisms in food. The procedures were followed the Specification Malaysian Standard MS 2683 : 2017.

3.4.3(a) Preparation of Agar Medium

Different type of agar medium was used to conduct the microbial contaminant limits which are Plate Count Agar (PCA), Malt Extract Agar (MEA) and Violet Red Bile Agar (VRBA). PCA was used for determination of total plate count (TPC), MEA for enumeration of yeast and molds and VRBA for determination of total coliforms.

In preparation of a litre of plate count agar (PCA), 23.5 g of prepared plate count agar powder was added to distilled water until the volume up to 1.0 L while for the preparation of a litre of MEA, 61 g of prepared MEA powder was mixed to distilled water until volume up of 1 L. For the preparation of a litre of VRBA, 41.45 g of prepared VRBA powder was mixed with distilled water until volume up of 1 L. The mixture was stirred and distributed into autoclave bottles and were autoclaved for 20 min at 15 psi pressure 121 ℃. The 12 mL - 15 mL of agar were pour to each petri dish after added the diluted of stingless bee honey sample. Make sure the agar medium was cooled to 55 ℃ before added into the petri dish.

3.4.3(b) Determination of Total Plate Counts (TPC)

For determination of total plate counts, 10 g sample of stingless bee honey product was added into 90 mL of sterile potassium dihydrogen phosphate anhydrous (KH_2PO_4) solution and was shake for 25 times within 7 s. Decimal dilutions of 10^{-1}, 10^{-2},10^{-3}, and 10^{-4} were prepared by transferring 1.0 mL of previous dilution to 9.0 mL of diluents. Separate sterile pipet tips were used for difference dilution. Then, 1 mL of each dilution was pipetted into duplicate separate petri dishes. Before that, the petri dishes were marked to avoid the experimental error. The previous preparation of plate

count agar was cooled to 45 ℃ ± 1 ℃. Then, 12 mL to 15 mL of plate count agar was added to each plate within 15 min of original dilution. The sample dilution and agar medium were mixed thoroughly and uniformly by using alternate rotation and back-and-forth motion of plates on flat level surface. Agar was solidified. The solidified petri dishes were inverted, and were incubated promptly for 48 h ± 2 h at 37 ℃. The colonies were counted and recorded.

3.4.3(c) Enumeration of Yeast and Molds

For the determination of yeast and molds, 10 g sample of stingless bee honey product was added into 90 mL of sterile potassium dihydrogen phosphate anhydrous (KH2PO4) solution and was shake for 25 times within 7 s. Decimal dilutions of 10^{-1}, 10^{-2},10^{-3}, and 10^{-4} were prepared by transferring 1.0 mL of previous dilution to 9.0 mL of diluents by using separate sterile pipets tip. 1 mL of each dilution was pipetted into duplicate separate petri dishes. The petri dishes were marked before pour the sample. The previous preparation of Malt Extract Agar was added with 1.2 mL 10 % tartaric acid of every 100 mL of agar and was cooled at 45 ℃ ± 1 ℃. Next, 12 mL to 15 mL of Malt Extract Agar was added to each plate within 15 min of original dilution. The sample dilution and agar medium were mixed thoroughly and uniformly by using alternate rotation and back-and-forth motion of plates on flat level surface. Agar was solidified. The solidified petri dishes were inverted, and were incubated promptly for 72 h ± 2 h at 25 ℃. The colonies were counted and recorded.

3.4.3(d) Determination of Total Coliforms

For determination of total coliforms, 10 g sample of stingless bee honey product was added into 90 mL of sterile potassium dihydrogen phosphate anhydrous (KH_2PO_4) solution for the dilution and then was shake for 25 times within 7 s. Decimal dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were prepared by transferring 1.0 mL of previous dilution to 9.0 mL of diluents. Separate sterile pipets tip was used for the transferring. The petri dishes were marked to avoid experimental error. 1 mL of each dilution was pipetted into duplicate separate petri dishes. The previous preparation of Violet Red Bile Agar (VRBA) was cooled to 45 ℃ $\pm$ 1 ℃. Then, 10 mL of Viable Red Bile Agar (VRBA) was added to each plate within 15 min of original dilution. The sample dilution and agar medium were mixed thoroughly and uniformly by using alternate rotation and back-and-forth motion of plates on flat level surface. Agar was solidified. Another layer of agar was added onto previous solidified agar and was solidified. The solidified petri dishes were inverted and incubated promptly for 48 h $\pm$ 2 h at 37 ℃. The colonies were counted and recorded.

3.4.3(e) Counting the Colonies

After incubation period, the growth colonies were counted. The population of the microbial counts were expressed in colony forming units per millilitre (CFU/mL) by using formula:

$$CFU/ml = \frac{(no.of\ colonies \times dilution\ factor)}{volume\ of\ culture\ plate} \quad (3.1)$$

All colony forming units (CFUs) that grow in the petri dishes were counted. The dilution used and total number of colonies counted was recorded correctly. For normal plate, the growth of colony is between 25 colonies to 250 colonies. When number of CFU per plate exceeds 250 colonies, for all dilutions, the counts is recorded as too numerous to count (TNTC). When plates from all dilutions have no colonies, APC as less than 1×10^1 the corresponding lowest dilution used is reported.

3.4.4 Statistical Analysis of Data

For microbial contaminants limit, the data was analysed in average of reading and was expressed in means value ± standard deviations (SD) by calculated using one way of analysis of variance (ANOVA) to determine their significance differences among variables ($p < 0.05$) by using IBM SPSS version 21.0. Plants phenolic analysis is determined by using Excel. For plant phenolic content analysis, a calibration curve of standard (gallic acid) was obtained for concentration versus peak area.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determination of Plants Phenolic Content by HPLC Method

According to Kostić et al. (2014) the major problem to analyse the plants phenolic in honey since contain very high level of sugar content, make the plants phenolic extraction and sample preparation using High Performance Liquid Chromatography (HPLC) analysis is difficult. However, this problem has been solved by using modified solid-phase extraction (SPE) for the sample preparation to extract the plants phenolic compounds as recommended by method from Malaysian Standard MS 2683: 2017. A studied by Moniruzzaman et al. (2014) also have mentioned to use the SPE for the sample purification of honey from Bangladesh.

A phenolic compound namely gallic acid for the phenolic acid were investigated by using HPLC technique to determine the plants phenolic in 13 samples of stingless bee honey. Chromatographic analyses were performed using a Shimadzu Prominence LG2OAT HPLC equipped with a photodiode array detector (SPDM2O) and a reversed-phase column (Luna C18 LC column, 5µm, 4.6 mm x 250 mm). The isocratic elution system used in HPLC are mobile phase composed of 0.05% phosphoric acid in deionized water (solvent A), and methanol HPLC grade (solvent B), at a flow 0.8 mL/min.

Previous study has revealed the presence of gallic acid compound in honey (Almeida et al., 2013; Silva et al., 2013; Oliveira et al., 2017). Hence, the current study was to detect the gallic acid in sample stingless bee honey. A reverse phase HPLC-DAD method for determining the standard gallic acid compound was performed. Gallic acid was used as the standard or marker. The standard compound was detected with a better peak shape and good resolution within 25 min. A 5 series of concentration of standard gallic acid range from 80 to 400 µg/mL to establish a calibration curve. Figure 4.1 shows representative of HPLC chromatogram for gallic acid standard at concentration of 240 µg/mL. The figure showed the peak area and retention time of gallic acid at concentration of 240 µg/mL which were 5,880,958 and 3.332 min respectively.

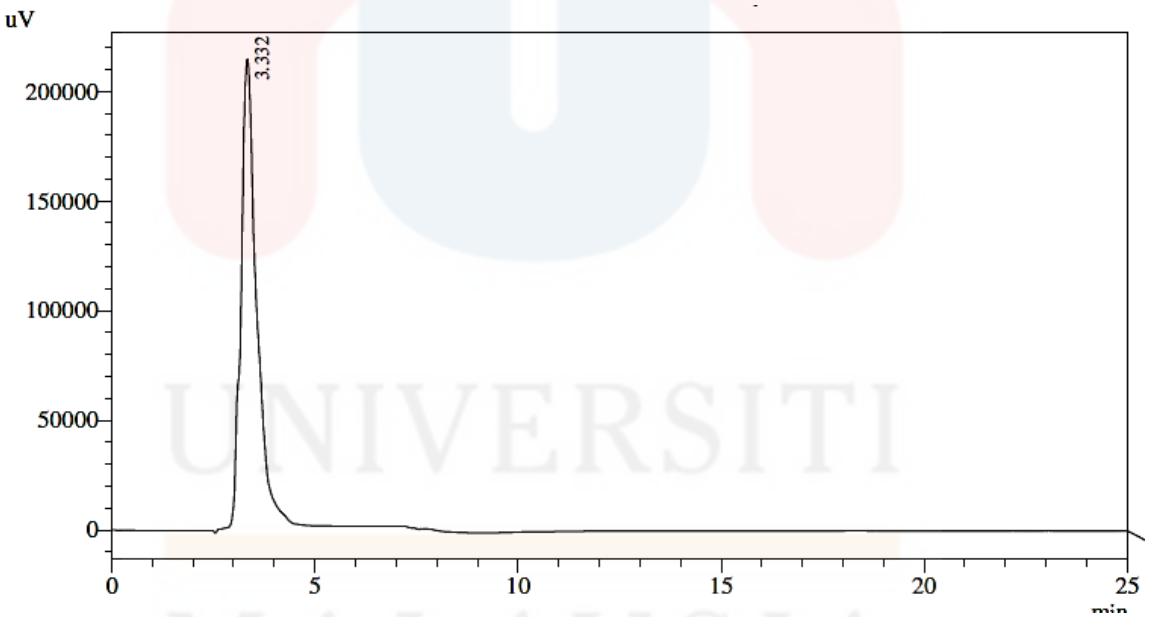

Figure 4.1 HPLC chromatogram of gallic acid standard at concentration of 240 µg/mL.

Besides, Table 4.1 shows the retention time and peak area of different concentration of standard gallic acid. From the concentration and peak area data, a calibration curve is plotted. Figure 4.2 represented the calibration curve of the standard compound of gallic acid was plotted after obtaining the value of the peak area (Y)

against the concentration (µg/mL). Linearity of the standard was presented in term of correlation coefficient (R^2). The regression equation of the standard also was shown in Figure 4.2. The graph showed the good linearity with correlation coefficient (R^2) by obtaining range of 0.997. A perfect line should be R^2 value of 1. However, the value of R^2 obtained for standard gallic acid is not value of 1.00 since the standard gallic acid used is not a HPLC grade.

Table 4.1 The retention time and peak area of different concentration of standard gallic acid

Standard concentration (µg/mL)	Retention time	Peak area
80	3.345	2,571,859
160	3.381	4,070,894
240	3.332	5,880,958
320	3.337	7,978,267
400	3.323	9,509,906

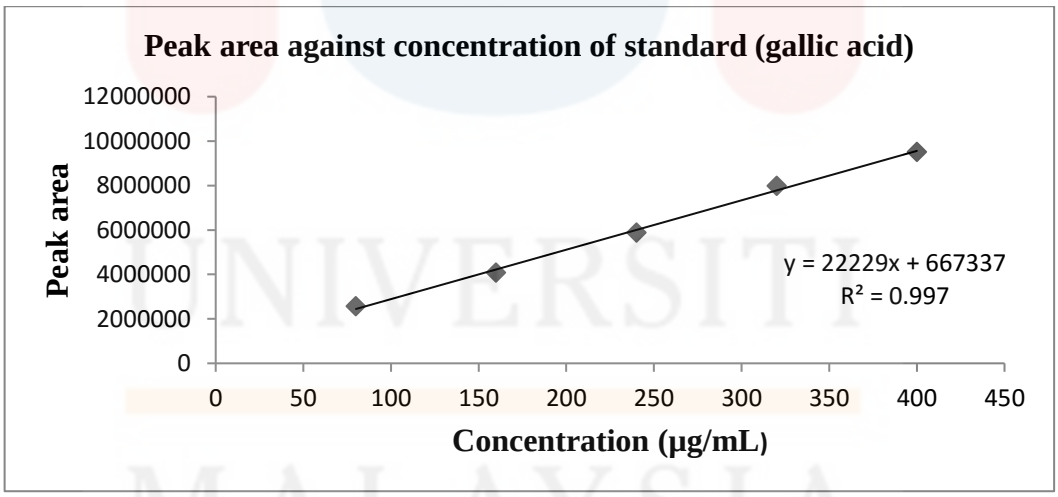

Figure 4.2 The calibration curve of peak area and concentration of standard gallic acid

4.1.1 Screening of Gallic Acid in Stingless Bee Honey

Firstly, A modified solid-phase extraction (SPE) for extraction of phenolic compounds in sample is performed. The SPE method is done for the sample purification by removing big molecule such as glucose and fructose compound in stingless bee honey sample. If not, the HPLC machine will be clogged and thus cannot detect the compound. Firstly, the concentration of the extracted honey is cannot be calculated after undergoes SPE process for sample purification. Then, after obtaining the HPLC data, majority value of peak area in samples were shown out of values since the value was out of range in calibration curve of standard gallic acid. Figure 4.3(a) until Figure(m) represented HPLC chromatogram of gallic acid in all 13 sample of stingless bee honey, respectively.

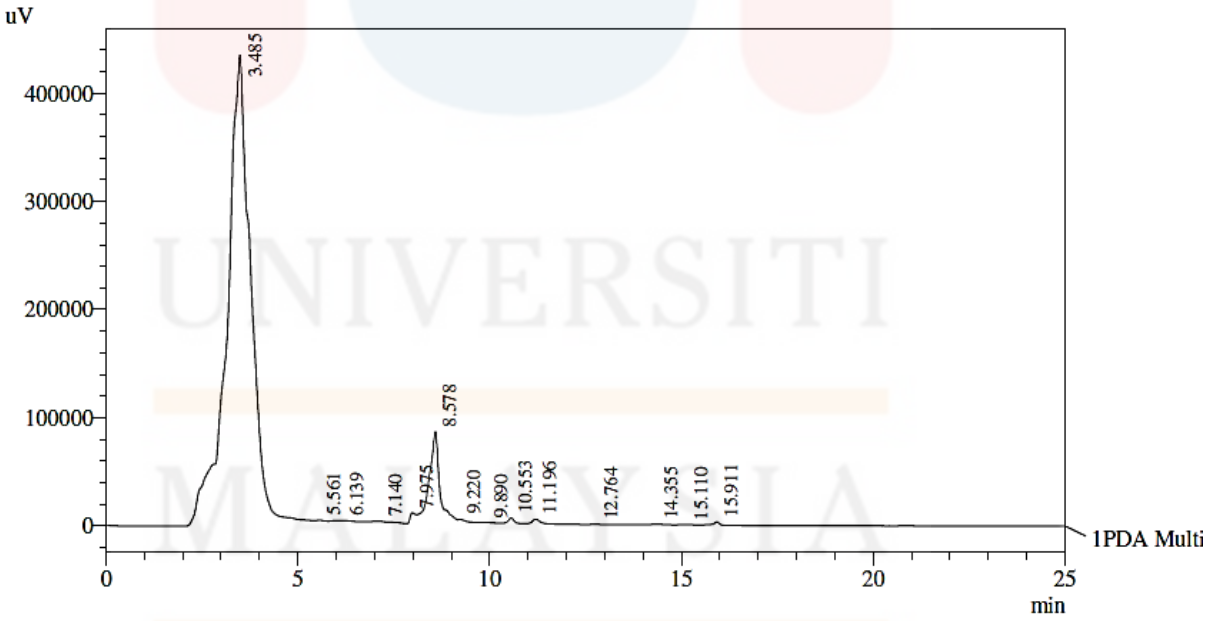

Figure 4.3(a) HPLC chromatogram of gallic acid in Sample 1

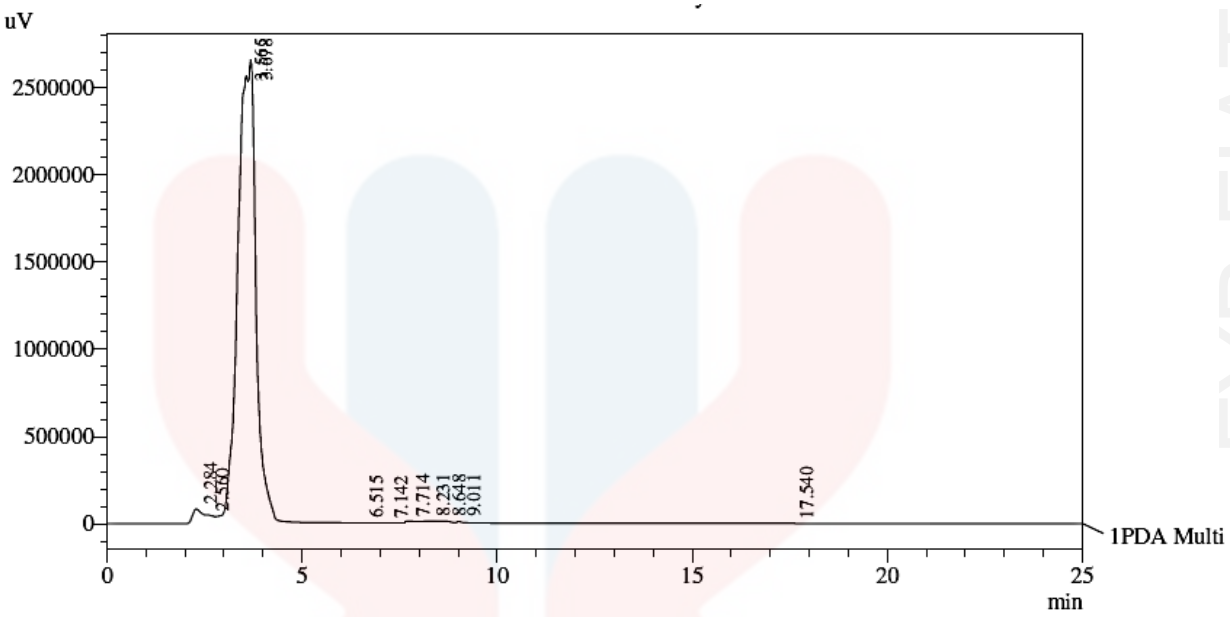

Figure 4.3(b) HPLC chromatogram of gallic acid in Sample 2

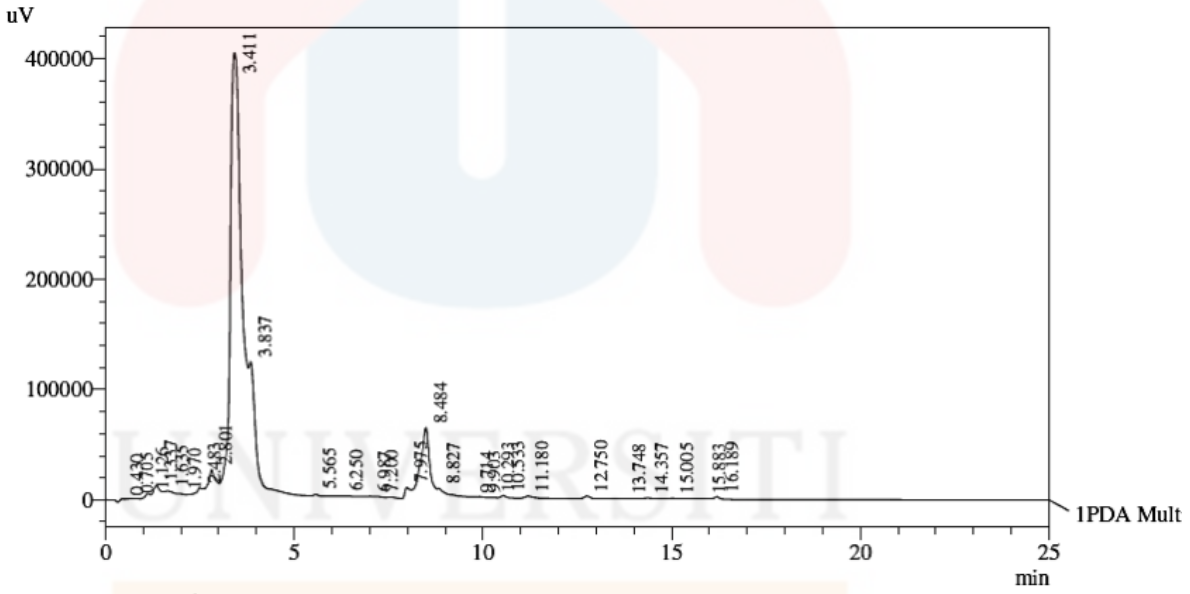

Figure 4.3(c) HPLC chromatogram of gallic acid in Sample 3

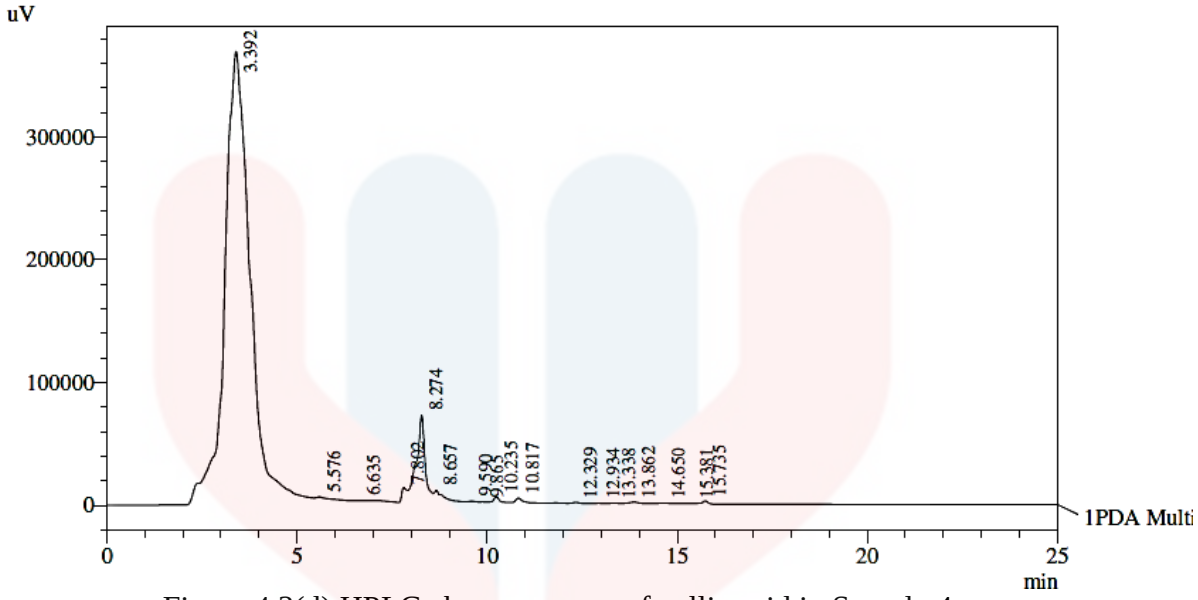

Figure 4.3(d) HPLC chromatogram of gallic acid in Sample 4

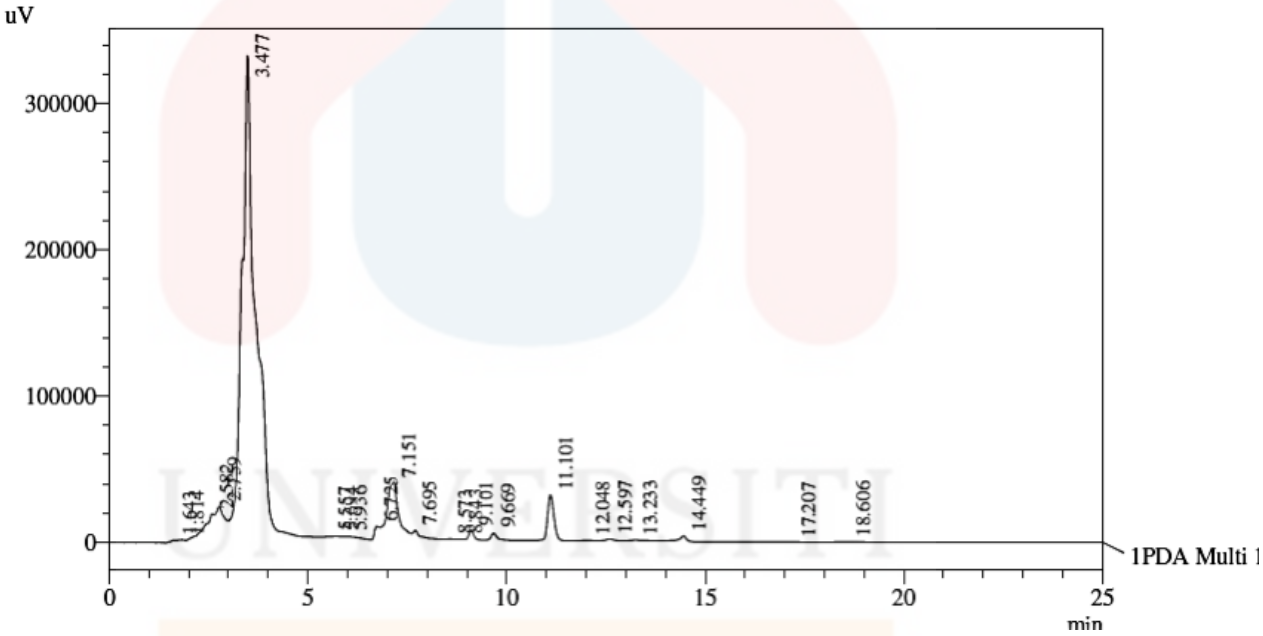

Figure 4.3(e) HPLC chromatogram of gallic acid in Sample 5

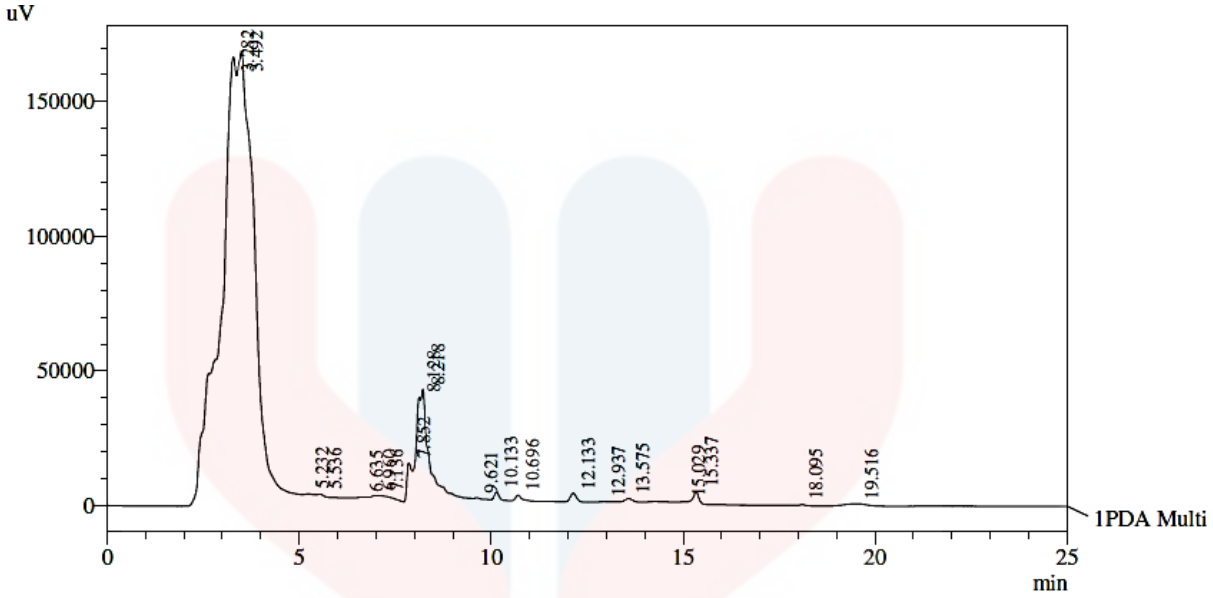

Figure 4.3(f) HPLC chromatogram of gallic acid in Sample 6

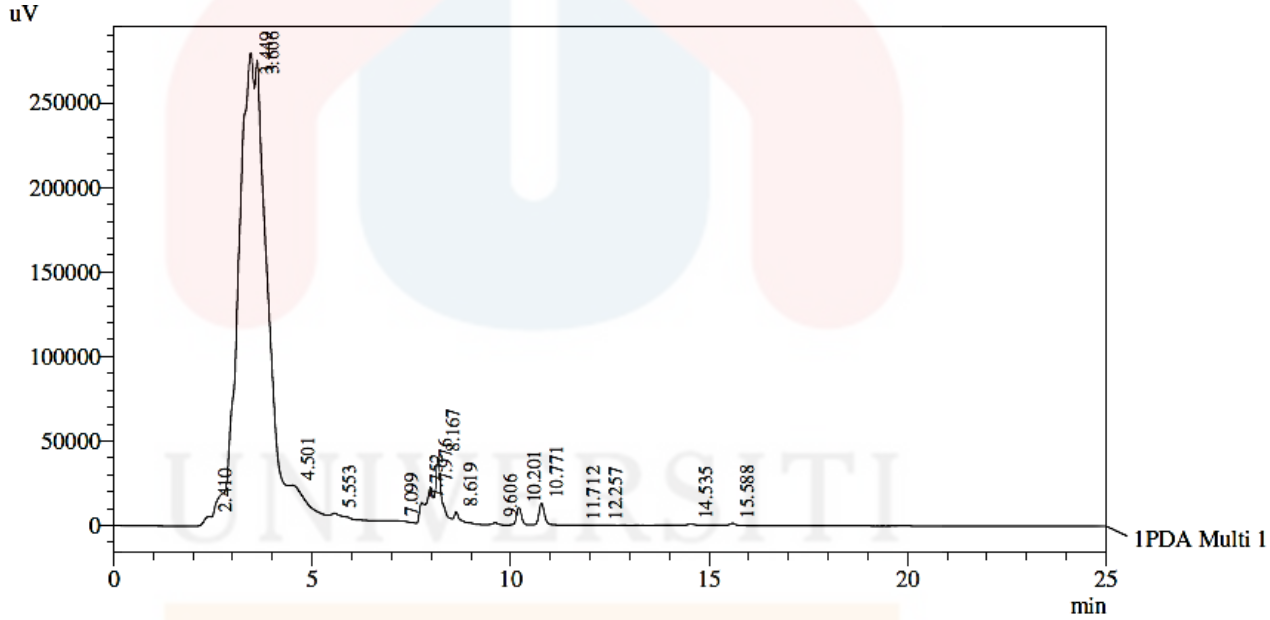

Figure 4.3(g) HPLC chromatogram of gallic acid in Sample 7

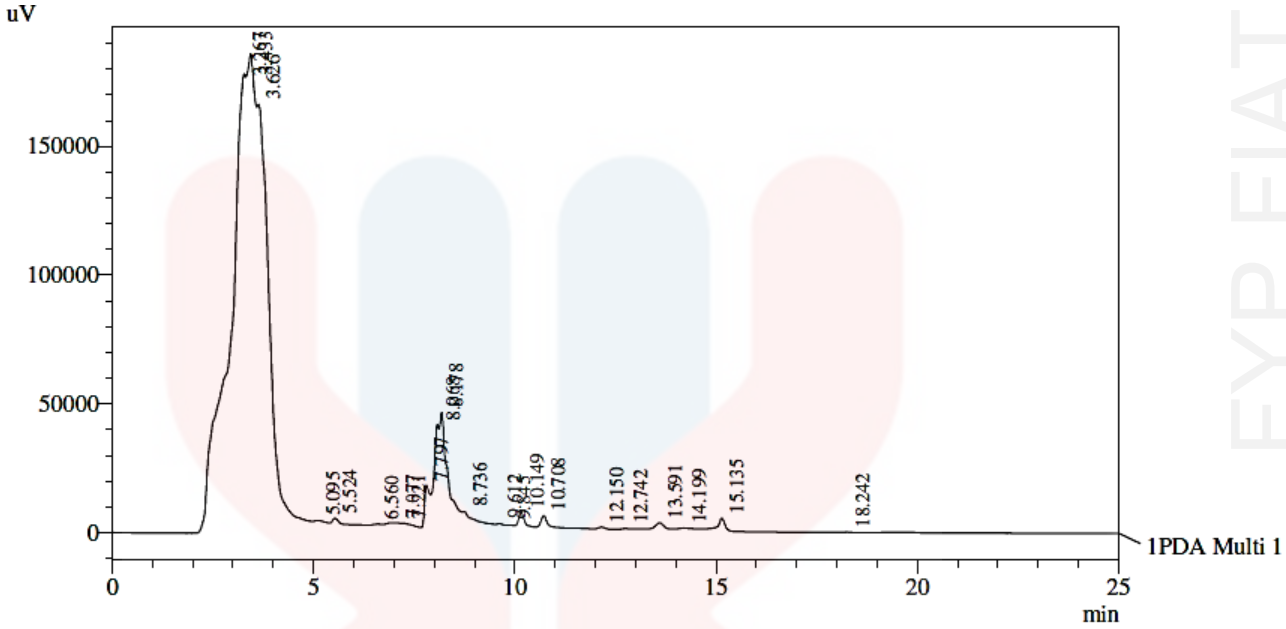

Figure 4.3(h) HPLC chromatogram of gallic acid in Sample 8

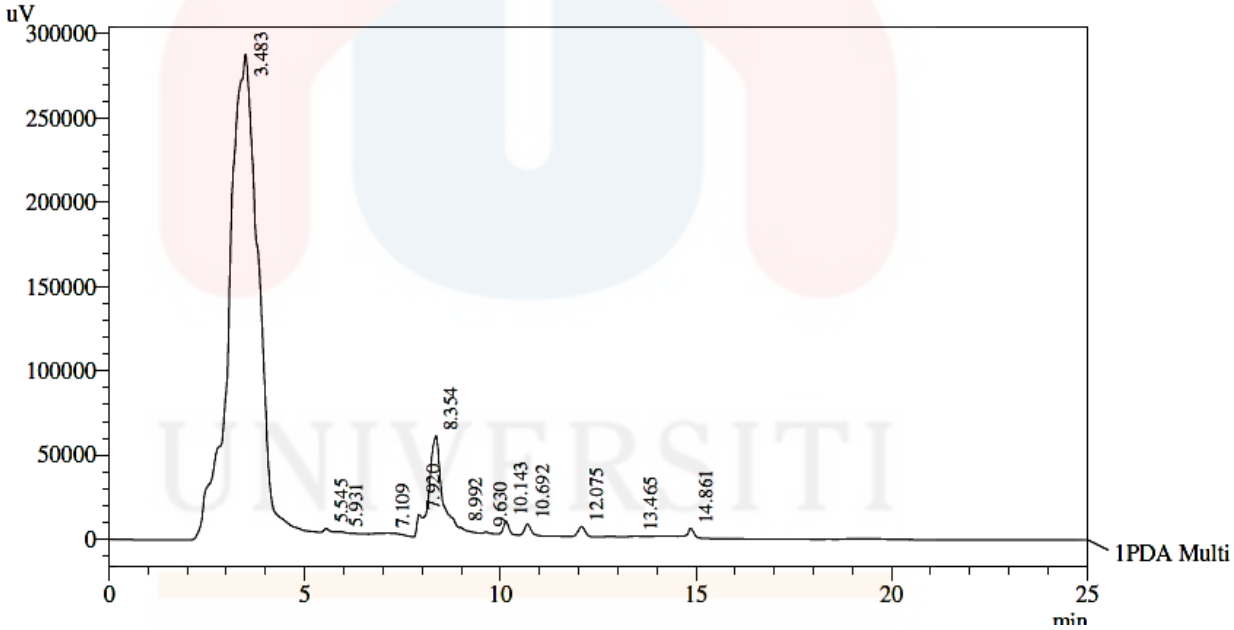

Figure 4.3(i) HPLC chromatogram of gallic acid in Sample 9

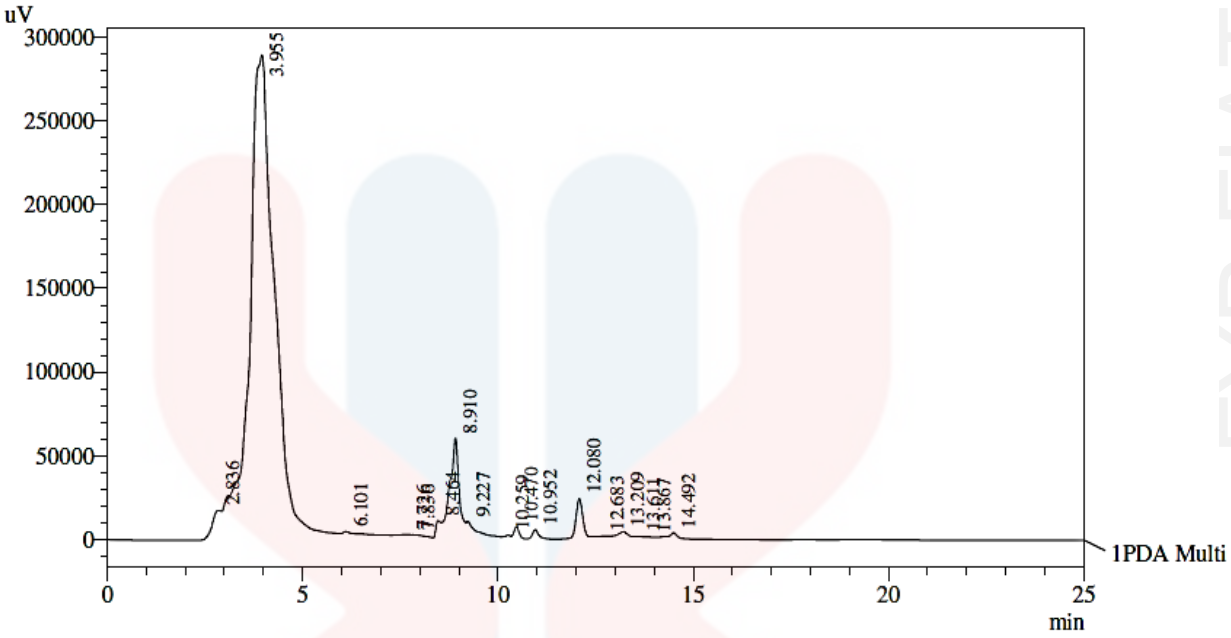

Figure 4.3(j) HPLC chromatogram of gallic acid in Sample 10

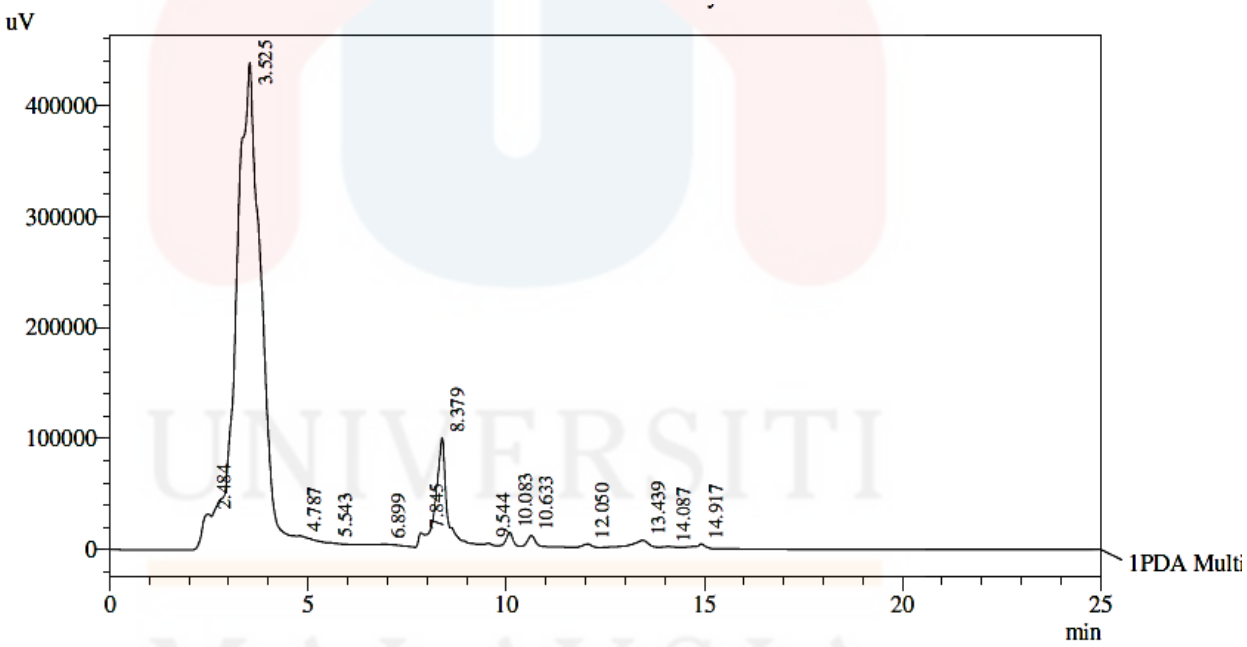

Figure 4.3(k) HPLC chromatogram of gallic acid in Sample 11

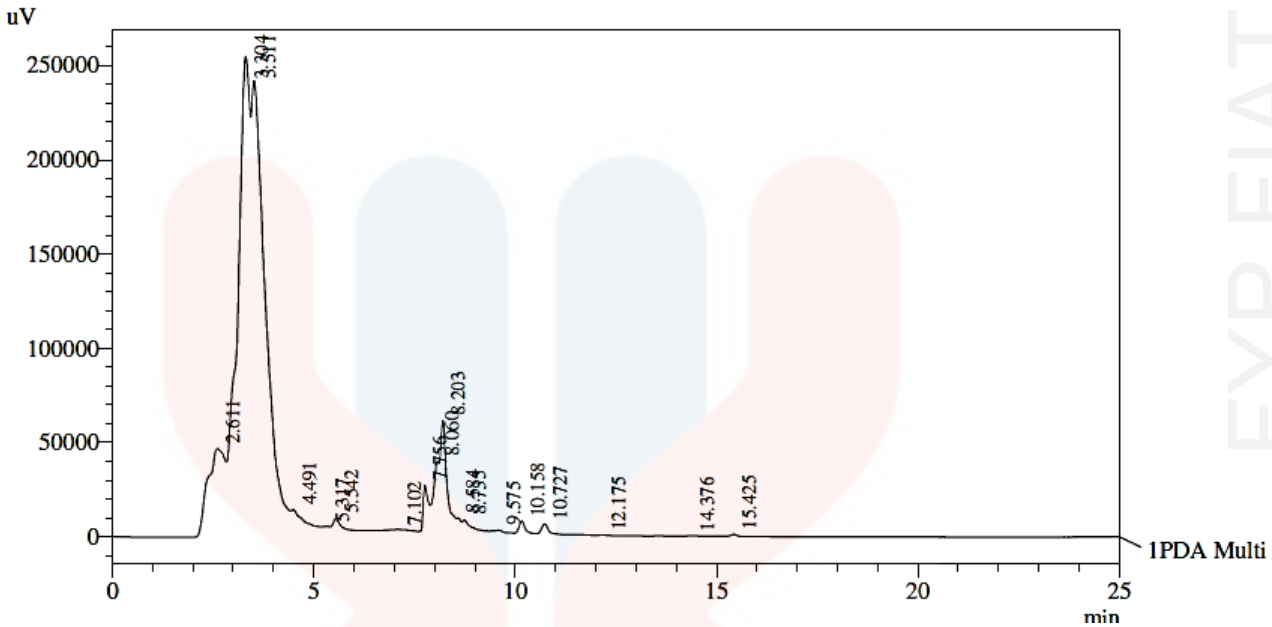

Figure 4.3(l) HPLC chromatogram of gallic acid in Sample 12

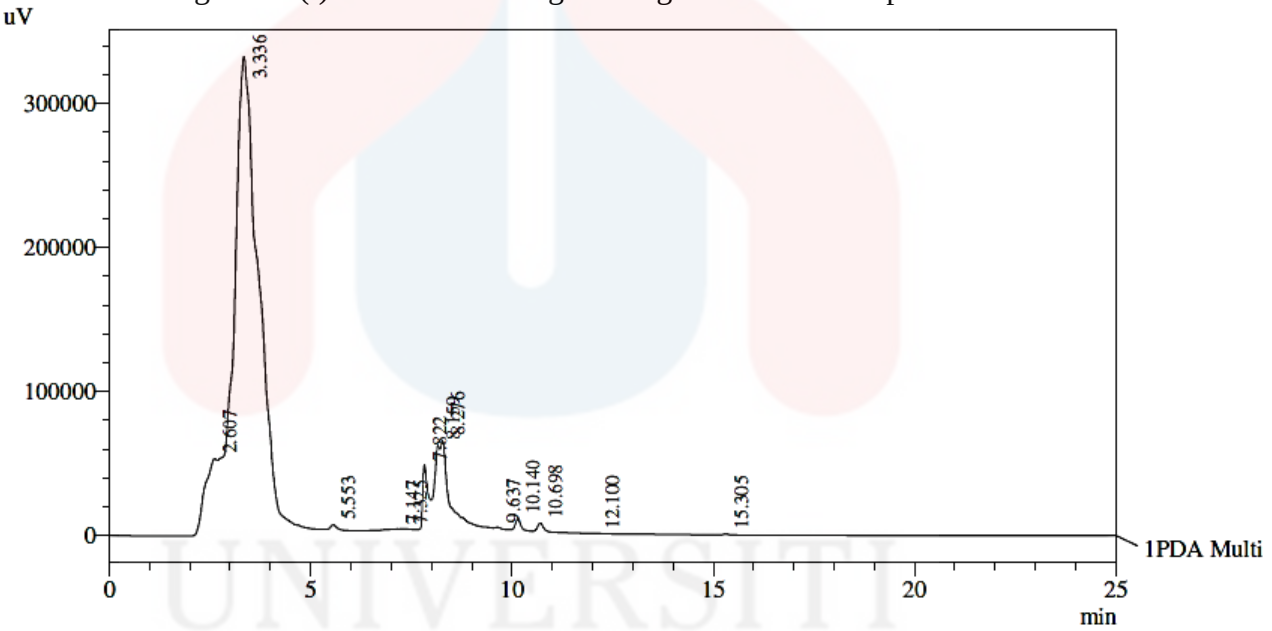

Figure 4.3(m) HPLC chromatogram of gallic acid in Sample 13

Figure 4.3(a) to Figure 4.3(m) illustrates HPLC chromatogram of gallic acid in all sample stingless bee honey which were showed their peak area and retention time within 25 min. From peak area of sample obtained shows all of the 13 sample are presence with the gallic acid. The sample of honey shows contained high concentration compared to concentration of standard. From data of peak area, concentration of gallic acid in the honey sample is calculated by using equation of $y = 22229x + 667337$ from the calibration curve. Table 4.2 represents the total peak area of 13 samples of stingless

bee honey. From the data from Table 4.2, a comparison of the peak area in 13 samples of stingless bee honey was shown in bar chart in Figure 4. 4. From the data, sample 2 was obtained the highest of peak area while sample 8 was recognised as the lowest of peak area.

Table 4.2 The peak area of honey samples.

Honey samples	Peak area
1	19,494,511
2	49,205,402
3	8,527,091
4	17,211,083
5	8,693,251
6	5,201,979
7	7,589,614
8	4,940,540
9	15,592,765
10	13,477,617
11	19,119,412
12	6,286,795
13	14,320,609

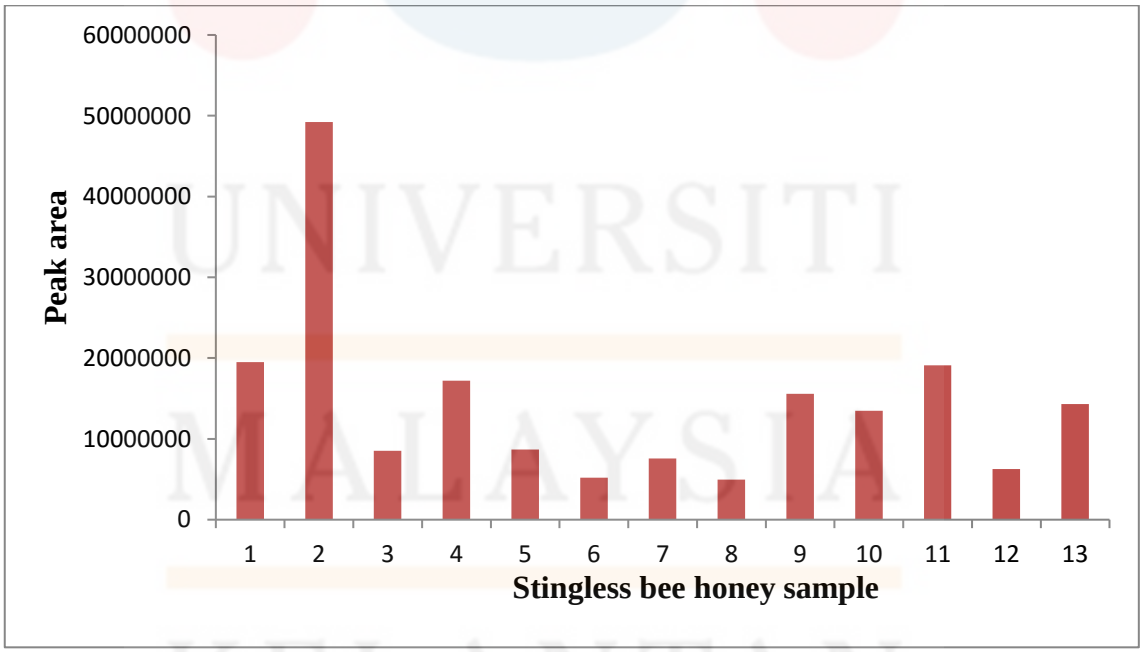

Figure 4.4 The peak area of gallic acid detected in 13 sample of stingless bee honey

The absorption of the wavelength is acts as the indication of the detection or absence of plant phenolic compound in samples. In this study the chromatograms were monitored at 254 nm, since majority of the honey plant phenolic have their UV absorption maxima around these wavelengths (Kostić et al., 2014). Basically, the area of peaks was related to the concentration of the gallic acid compound in the sample. Table 4.3 tabulated the concentration of gallic acid in honey sample after calculated using the equation of calibration curve of standard gallic acid. Hence, the concentration of gallic acid is the samples were determined. The table shows all of the 13 samples of stingless bee honey was presence with gallic acid compound in difference concentration. The different amount of plant phenolic in honey was influenced by floral sources that primarily depend on their geographical, seasonal and environmental factors (Silva et al., 2013).

Table 4.3 The amount of concentration (µL/mL) of gallic acid compound in stingless bee honey sample.

Sample	**Concentration of gallic acid in sample (µL/mL)**
1	845.12
2	2177.66
3	353.23
4	742.71
5	360.69
6	204.10
7	311.19
8	192.38
9	670.13
10	575.27
11	828.30
12	252.76
13	613.07

From the data, concentration of gallic acid compound in sample 2 was the highest (2177.66 µg/mL) compared to other. This is because sample 2 was applied with thermal process at below 40 ℃. Studies by Ngoi (2016) and Šarić et al. (2013) mentioned the levels of plants phenolics were significantly increased when undergoes processing with heat treatment, hence increases the antioxidant activity. Thus, this statement was proved successfully from finding of this study. The higher level of plant phenolic, the higher of degree of antioxidants activity in honey (Izwan & Zainol, 2016). This is meant sample 2 contained highest antioxidant activity.

The antioxidant activities in this sample is related to Maillard reaction that can be explained in terms of the excellent radical scavenging properties of melanoidins and their reactions with oxygen (O_2) and hydroxide (OH) (Hendek Ertop & Öztürk Sarikaya, 2017). This Millard reaction in honey is related to a toxic compound namely 5-hydroxymethylfurfural (HMF) that might be formed due reducing sugar and heating process in acidic environment. Several studies have reported that HMF possesses antioxidative effect. Thus, the reaction is occurred in sample 2 since it undergoes heating treatment. This compound might be mutagenic, carcinogenic and cytotoxic to the human body (Shapla, Solayman, Alam, Khalil, & Gan, 2018). Besides, sample 2 also having darker in colour effect from the Maillard reaction that can increase the plants phenolic content. In short, sample 2 was not good to be consumed since have toxic compound.

Basically, besides factor of thermal process could increase the plant phenolic content, the colour of the honey also indicates the total amount of phenolic content. Based on colour of sample, sample 1,2, 4, 11, 13 have darker colour compared to others. The darker colour of honey contains higher total phenolic content (Ngoi, 2016; Ying, 2015). This statement is strongly proved when the sample 1, 2, 4, 11, 13 have higher

concentration of 845.12 µg/mL, 2177.66 µg/mL, 742.71 µg/mL, 828.30 µg/mL and 613.07 µg/mL respectively.

According to study by Almeida et al. (2013) discovered the detection of gallic acid in stingless bee honey from several countries which were Brazil, Australia, Portugal, and New Zealand. Besides, Oliveira, Jain, Luna, & Freitas (2017) also detects the phenolic compound of gallic acid all of the six species of the stingless bee. Other than that, a study by Kek et al. (2014) was determined the comparison of plant phenolic content between *Apis spp*. and stingless bee honey. The result shows stingless bee honey contains high level of total phenolic content with average of 784.3 mg GAE/kg than the *Apis spp.* honey at average 590.5 mg GAE/kg.

Regarding Malaysian Standard (MS) 2683:2017, raw and processed stingless bee honey shall contain naturally occurring of plants phenolic for the quality requirements. Overall, the plant phenolic compounds were detected in all the 13 samples of stingless bee honey from selected location in Kelantan. Presence of plants phenolic in stingless bee honey sample was indicated it do comply with the MS 2683:2017 specifications.

4.2 Microbial Contaminant Limit Analysis

Microbial contamination such as aerobic bacteria, yeast, mold and coliforms could give a bad effect to the quality of the honey samples. The contamination might obtain from various sources such as pollen, the digestive tracts of honey bees, dust, nectar, and others. Poor harvesting, handling and storage may also one of the factors that cause the contamination of the honey sample. The microbial contaminant limits of the stingless bee honey products must not beyond the acceptable limits to ensure the

quality and safety for the consumers. For this study, three analysis of microbial contaminant limits is determined which were total plate count (TPC), yeast and molds (YM) and total coliforms (TC) by following the specification of Malaysian Standard 2683:2017.

The analysis is used difference medium agar for the analysis. For the determination of total plate count (TPA), plate count agar (PCA) is used. While for determination of the yeast and molds (YM) and total coliform (TC) were used malt extract agar (MEA) and violet red bile agar (VRBA) respectively. For the colony count, this study was interpreted into colony forming unit per millilitre (CFU/mL). The CFU per milliliter was obtained by dividing the number of colonies by the dilution factor by using the formula (3.4).

4.2.1 Determination of Total Plate Count Analysis

The inhibitory properties such as high content of sugar, low level of water activity (a_w), low pH value, presence of hydrogen peroxide and other compounds are contributed to the antimicrobial properties against different types of microorganisms. However, honey was not considered as 100 percent safe even it contains the inhibitory properties that can acts as the antimicrobial agents to honey. Thus, certain microorganisms such as spore-producing bacteria (*Clostridium spp*.), xerophilic moulds and osmophilic yeasts can tolerate with the extreme conditions owned by the honey (Gradvol, Atlaban, Lenart, & Pavlović, 2015). Hence, the microbiological analysis is determined in this study. The microbial contaminant limits in term of total plate count was presented in Table 4.4. The data was analysed in form of colony forming unit per millilitre (CFU/mL).

Table 4.4 Microbial contaminant limit of total plate count (TPC) in stingless bee honey samples in CFU/mL

Honey Samples $(\boldsymbol{n = 2})$	**Microorganism count (CFU/mL) ***
1	$<1.0\times10^{1}$**
2	$<1.0\times10^{1}$**
3	$5\pm 7.07\times10^{2}$
4	$<1.0\times10^{1}$**
5	$5\pm 7.07\times10^{2}$
6	$<1.0\times10^{1}$**
7	$<1.0\times10^{1}$**
8	$<1.0\times10^{1}$**
9	$<1.0\times10^{1}$**
10	$5\pm 7.07\times10^{2}$
11	$5\pm 7.07\times10^{2}$
12	$<1.0\times10^{1}$**
13	$<1.0\times10^{1}$**

*Values represented as mean ± std. deviation (*n*=2)
**Plates with no CFU

From the result, 30.77% from all the samples were considered as contaminated with the microorganism of total plate count which were samples 3, 5, 10, and 11. The detection of the contamination in the samples were similar with an average of $5 \pm 7.07\times10^{2}$ CFU/mL. On the other hand, majority of other samples were absent with the contamination of total plate count. Most bacteria cannot grow or reproduce in honey due to presence of inhibitory properties in the stingless bee honey like antibacterial or antimicrobial properties that was stated previously (Olaitan, Adeleke, & Ola, 2007; Pucciarelli, Schapovaloff, Kummritz, & Señuk, 2014).

For this study, samples 7 and 13 were acts as control and were free from the contamination of the total plate count bacteria since it was freshly harvested from their hive. Based on study by White and Doner (1980) investigated the effect of antibacterial properties in honey and the result determined the bacteria cannot withstand in honey after few hours or days. The finding from the study reveals the bacteria are not remain alive in honey.

However, even sample 10 is categorized as freshly harvested, it still contaminated with the total plate count bacteria. This is because by according to a studies by Adenekan et al. (2010), even it was a fresh honey, it still have opportunity to be contaminated due to improper handling and packaging of honey that normally presence at low number of aerobic bacteria (Lani et al., 2017) besides from primary sources of contamination such as pollen, nectar, digestive tracts of honey bees, dust, while secondary sources were including the air, handlers, cross-contamination, equipment and buildings (Pucciarelli et al., 2014). Basically, Pucciarelli et al. (2014) stated the contaminations in stingless bee honey are hardly to control which could be originated from primary sources like pollen, the digestive tracts of honey bees, dust, nectar, and others while for the other sources are including air, handlers, cross-contamination, equipment and buildings. The sources of contamination could be managed by applied good manufacturing and hygiene practices

Besides, samples 3, 5 and 11 were also contaminated with the total plate count bacteria. This was due to their storage periods which were 4 months, 3 months and 1 month respectively. These samples were indicated have higher storage time. Basically, the lactic acid bacteria were present at a highest number after harvest and will decrease throughout the period of storage (Lani et al., 2017). A study by Olofsson et al. (2014) mentioned there are large number of lactic acid bacteria (LAB) contain in fresh honey that play a role in inhibiting bacterial and fungal growth and act as antimicrobial activity against various pathogens, bacteria and yeasts. However, when the samples having longer storage time, the lactic acid were decreases.

Furthermore, honey samples that have undergoes sterilize or heat treatment can kill the microorganism (Fernandes et al., 2018; Izwan & Zainol, 2016; Lani et al., 2017). This statement is strongly have been proved in this study as the sample 2 that

undergoes heat treatment is absent with total plate count bacteria. Next, according to the European Commission Health and Consumer Protection (2002), the growth and the survival of bacteria in honey are depends on their honey physicochemical properties theoretically. Thus, sample 4, 6, 8, 9, and 12 that were free from the contamination of total plate count microorganism is may be contained a good physicochemical properties that prevent from the growth of the bacteria even the samples were in longer storage period (Lani et al., 2017).

Studied by White and Doner (1980) and Adenekan, Amusa, Okpeze, and Owosibo (2012) revealed the physicochemical properties such as their pH that categorized as acidity, high in sugar content, and low in moisture content could killed and prevent the growth of microorganism. Coherently, the microorganisms were not alive when contains high content of sugar due to osmotic effect that could drying out the bacteria. Besides, low of moisture content could also protects the honey from been attacked by microorganisms (Adenekan et al., 2012). In addition, proper handling and correct storage condition also could avoid the honey to be contaminated.

In addition, many studies regarding the antimicrobial agents of honey have been evaluated. The honey has been claimed as the bactericidal to many pathogenic microorganisms including *Salmonella spp*, *Shigella spp*; other enteropthogens like *Escherichia coli*, *Vibrio cholerae* and other Gram negative and Gram-positive organisms. The great properties of antimicrobial activity due to factors of osmotic effect, acidity, hydrogen peroxide and phytochemical factors in the honey (Olaitan et al., 2007). In addition, by comparing microbial count in honeybee sample, low level of total plate count (TPC) are found in all of honey samples originated from different locations in Ogun State, Nigeria (Adenekan et al., 2012).

By referring to specification of Malaysian Standard 2683:2017, the microbial contaminants limit for total plate count must not exceeding 1.0×10^3 CFU/mL. Thus, even some of the sample had value of CFU that was indicated as the contamination, it still accepted and complied with the Malaysian Standard 2683:2017 since the value is less than 1.0×10^3 CFU/mL. In the nutshell, the TPC among 13 samples of stingless bee honey was not significantly different ($p > 0.05$). Besides, all of samples stingless bee honey microbial was complied with MS 2683:2017 specification regarding contaminant limit in term of total plate count.

4.2.2 Enumeration of Yeast and Mold Analysis

In honey industry, yeast was microbes that primarily concerned in honey after spore-forming bacteria. Basically, yeast needed good nutrients to grow and to metabolized the sugar into ethanol and then undergoes the process, known as fermentation process. The risk of fermentation was depending on factor of moisture content, yeast spore concentration within the honey, and also the temperature that stored the honey (Lani et al., 2017). Hence, yeast was one of the factors related to fermentation process in honey. The detection of contamination from yeast and mold in the stingless bee honey samples was shown in Figure 4.5 that was presented in colony forming unit per millilitre (CFU/mL).

Table 4.5 Microbial contaminant limit of yeast and mold (YM) in stingless bee honey samples in CFU/mL

Honey Samples $(\boldsymbol{n = 2})$	**Microorganism count (CFU/mL) ***
1	$<1.0\times10^{1}$**
2	$<1.0\times10^{1}$**
3	$<1.0\times10^{1}$**
4	5.00 ± 7.07
5	$<1.0\times10^{1}$**
6	10.00
7	$<1.0\times10^{1}$**
8	$<1.0\times10^{1}$**
9	$<1.0\times10^{1}$**
10	$<1.0\times10^{1}$**
11	$<1.0\times10^{1}$**
12	5.00 ± 7.07
13	$<1.0\times10^{1}$**

*Values represented as mean ± std. deviation (*n*=2)
**Plates with no CFU

In this study, the contamination of yeast and mold were detected in sample 4, 6 and 12. Hence, only 23% of the samples were contaminated with yeast and mold. The CFU per millilitre of sample 4 and 12 was similar with an average of 5.000 ± 7.071 CFU/mL while sample 6 has an average of 10 CFU/mL. By referring to MS 2683:2017 specification, the level microbial contaminant limits of yeast and mold is must less than 1×10^{1} CFU/ml. Thus, the quality of the contaminated sample 4 and 12 were still accepted since the contamination value of CFU is still in value range of MS specification. However, only sample 6 was not qualified due to the CFU per millilitre is out of range of MS 2683:2017 specification. The result also shows that microbial contaminant limits of yeast and mold were not significantly difference ($p > 0.05$) among the 13 samples of the stingless bee honey.

According to study by Pucciarelli et al. (2014), the presence of yeast and molds in honey is due to the ability to grow under high sugar concentrations even the content of water in honey is limited. Thus, sample 4, 6, and 12 contained the yeast and

mold spore that have ability to survive and grow in the sample. There are many sources for the contamination of yeast and mold in honey sample. The main sources were pollen sources and soil. Other sources of contamination of microbial in honey are including handler, equipment, containers, wind, and dust. Yeasts could be from equipment in bee hive that can promote yeast into honey. Besides, the possible routes of transmission into the extracted honey were including air, food handlers such as from skin infections, sneezing or faecal contamination (Saha, Ahammad, & Barmon, 2018).

From the data, the CFU per millilitre for sample 6 is highest which was valued of 10 compared to sample 4 and 12 which was valued of 5.000 ± 7.071. As studied by Lani et al. (2017), as the storage applied, the number of yeast count was increased. This statement is strongly proved since sample 6 that have storage period of 5 months was contained higher value of yeast count than contaminated sample 4 and 12 that have storage period of 3 months and 1 month respectively. The high and low value count of yeast throughout the storage was due to fermentation process in the honey. Hence, a suggestion which is not to store the honey for a longer period is recommended. Other than that, contamination from post-harvest activity also could be occurred due to lack intention regarding to hygienic especially in processing, handling and storage of honey (Izwan & Zainol, 2016; Lani et al., 2017).

However, 77 % of the samples is absent with yeast and mold contamination which were sample 1,2,3,5,7,8,9,10,11, and 13. This is because according to Lani et al. (2017), yeast cannot grow in honey because of their physicochemical properties of moisture content. Based on the studies, yeast cannot survive in moisture content that below than 17 % unless the moisture content is above 17 % and the yeast was grows poorly between 17 and 18.5 %. Other than that, Olaitan et al. (2007) reported that honey also contained an inhibitory effect that cannot survive the growth of around 60 species

of bacteria including aerobes and anaerobes, gram-positives and gram-negatives. The antifungal action also has been investigated for some yeasts and mold species including *Aspergillus* and *Penicillium* that cannot survive. This fact is strongly proved when yeast and molds in majority of sample in this study is absent from the contamination of the bacteria.

By comparing to other studies, 40 samples of stingless bee honey from Piauí, Brazil also contains low levels of contamination molds and yeasts which were 1.88 CFU/g^1 (Fernandes et al., 2018). To be compared with honeybee, a results obtained from Zamora and Arias (2011) also showed that 87 % of 30 samples had bacterial and spore counts of yeast equal or lower than 1.0×10^1 CFU/g. A previous studies by Adenekan et al. (2010) also presented yeast and molds is at low counts of CFU in 10 sample of honey collected from difference area of Ibadan, Oyo State, Nigeria

Furthermore, study by Adenekan et al. (2012) was determined the contamination of yeast and mold in honey collected from different locations in Ogun State, Nigeria. From finding, total yeast count was very low in honey samples collected from Ijebu-Ode and Ogere area. The variation in total yeast may be due to the type of harvesting, processing and freshness of the honey. The absence of this type of microorganism in some of the honey samples due to antimicrobial properties that can delay the growth of many microorganisms. Generally, honey may contain yeast organisms from bees, soil, air and dust that could be contaminated during post-harvest handling.

4.2.3 Determination of Total Coliform Analysis

The degree of microbial contaminant limits of stingless bee honey was determined by total coliform as indicators. The specification of Malaysian Standard (MS) 2683:2017 has established a standard for the acceptable levels of total coliforms in stingless bee honey for the consumer consumption. Thus, commercial stingless bee honey shall not contain the microorganism of total coliform exceeding the limits that was specified. By referred to the MS specification, the limit for the total coliform in stingless bee honey product was must less than 1×10^1 CFU/mL. The microbial contaminant limits in term of total coliforms (in CFU/mL) of 13 samples of stingless bee honey was shown in Table 4.6.

Table 4.6 Microbial contaminant limit of total coliforms (TC) in stingless bee honey samples in CFU/mL

Honey Samples $(\boldsymbol{n = 2})$	**Microorganism count (CFU/mL)**
1	$<1.0\times10^1$**
2	$<1.0\times10^1$**
3	$<1.0\times10^1$**
4	$<1.0\times10^1$**
5	$<1.0\times10^1$**
6	$<1.0\times10^1$**
7	$<1.0\times10^1$**
8	$<1.0\times10^1$**
9	$<1.0\times10^1$**
10	$<1.0\times10^1$**
11	$<1.0\times10^1$**
12	$<1.0\times10^1$**
13	$<1.0\times10^1$**

**Plates with no CFU

In this study, total coliform was not detected in any of 13 samples of stingless bee honey. By comparing to another similar study from Zamora and Arias (2011), all the 30 samples from different geographical areas of Costa Rica had showed the negative result of total coliforms. Besides, similar studied by Fernandes et al. (2018) also proved

all the 40 samples of stingless bee honey species of *Melipona fasciculata* collected from Brazilian State of Piauí also did not show any contamination by total coliforms. Furthermore, by according to their standard that have been set by Legislation Mercado Común del Sur (MERCOSUR) Legislation and Código Alimentario Argentino (CAA), the honey sample must does not presence the total coliforms (Fernández *et al*., 2015). Thus, the studied by Fernández et al. (2015) was complied with the standardisation that have been set since all of the sample is absent of total coliform.

Based on studies by Adenekan et al. (2012) that determined the microbial analysis on honeybee sample from different locations in Ogun State, the total coliform (TC) was very low with a minimum of 0.3 x 10^3 cfu g^{-1} obtained from the honey samples collected from Abeokuta area of Ogun State. Other than that, coliform count in is also not detected in honey samples collected from Ayetoro, Otta, Ibefun, Ifo and Sagamu areas of Ogun State, Nigeria. The studied was concluded by the evidence that honey is well preserved against bacteria so that the organisms of total coliforms cannot survive in the unfavourable conditions.

Basically, sources of coliforms were from the animal faeces contains that pathogenic bacteria. Hygienic environment condition could avoid the total coliform to be alive in the animal stomach. Thus, there are high probability of total coliform contamination towards the country that have bad hygienic control. The selected area in Kelantan that collected the samples can be categorized as free from the total coliform since the sample is absent of total coliform contamination because of the good environment condition of hygienic level.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, objective of this study which was to determine the plants phenolic and microbial contaminant limits in stingless bee honey products based on Malaysian Standards (MS) 2683:2017 is accomplished. For the overall, phenolic compound is detected in all of 13 samples of stingless bee honey from selected location in Kelantan with difference amount concentration of gallic acid. Honey that undergoes thermal process contains higher of plants phenolic while the darker colour of honey also gives a higher plants phenolic content. Besides, the difference concentration of plant phenolic is depends on the floral sources which is primarily depend on their geographical, seasonal and environmental factors.

For the microbiological quality, all sample do comply with MS 3683:2017 in term of total plate count (TPC) and total coliform whereas there are one sample that was not complying to MS in term of yeast and mold (YM) because of the longer period of storage. In short, stingless bee honey should assess the quality for consumer safety.

5.2 Recommendation

For future of study, a full complete analysis of stingless bee honey should be done including all parameters such as sugar content (fructose, glucose, maltose, and sucrose), moisture content, pH, ash content, hydroxymethylfurfural (HMF) content, electrical conductivity, free acidity, pollen analysis, microbial contaminants limits, and heavy metal for honey quality control. All of the analysis can determine all of the parameter of stingless bee honey in various scope of study.

Besides, in term of microbiological analysis, the microorganisms should be included of microscopically and morphologically observation and determine their specific genera and species. The identification of the genus and species of bacteria by using technique of Gram Staining and genetic testing by identified their DNA using Polymerase Chain Reaction (PCR).

The future study also can determine the comparison of antioxidants activity in term of plants phenolics in stingless bee honey by using several methods and techniques such as spectrophotometric Folin-Ciocalteu method, colorimetric assay, Ferric Reducing/Antioxidant Power Assay (FRAP Assay), DPPH Free Radical-Scavenging Activity, Gas Chromatography-Mass Spectrometry (GCMS), and thin-layer chromatography (TLC).

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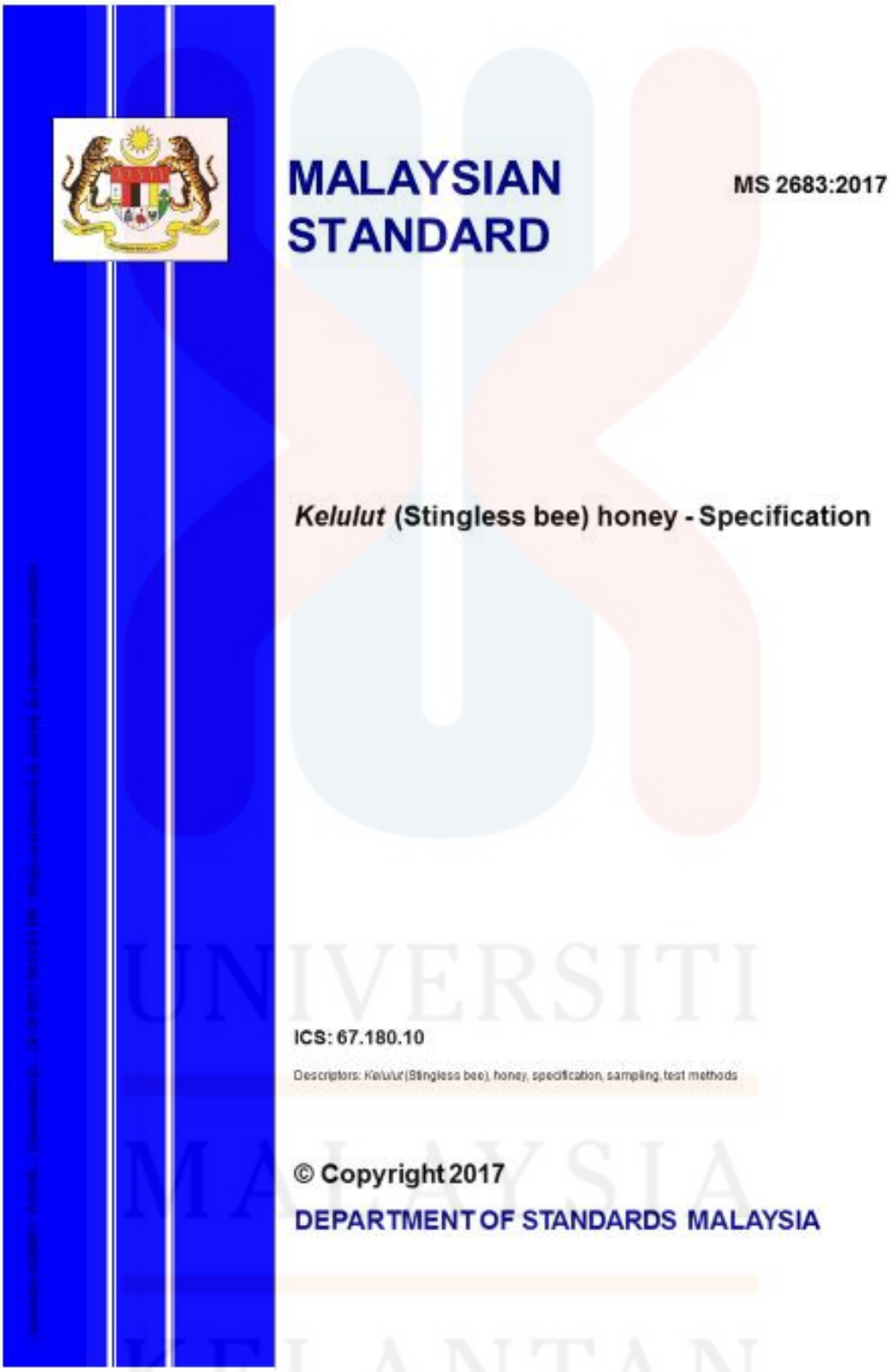
APPENDIX A: MS 2683:2017 Specification



MALAYSIAN STANDARD

MS 2683:2017

Kelulut (Stingless bee) honey - Specification

ICS: 67.180.10

Descriptors: Kelulut (Stingless bee), honey, specification, sampling, test methods

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Kelulut (Stingless bee) honey - Specification

1 Scope

This Malaysian Standard specifies the quality requirements, sampling, preparation of test sample, test methods, hygiene, packaging and labelling for *kelulut* honey produced by stingless bee of Meliponini tribe intended for direct human consumption.

It is applicable to both raw and processed *kelulut* honey.

2 Normative references

The following normative references are indispensable for the application of this standard. For dated references, only the edition cited applies. For undated references, the latest edition of the normative reference (including any amendments) applies.

MS 1514, *General principles of food hygiene*

MS 2679, *Amalan Pertanian Baik (APB) - Pemeliharaan lebah (tribus Apini) dan kelulut (tribus Meliponini)*

Food Act 1983

Food Regulations 1985

Food Hygiene Regulations 2009

3 Terms and definitions

For the purposes of this standard the following terms and definitions apply.

1. *kelulut* (stingless bee) honey

A natural sweet with certain acidity substance produced by stingless bees of Meliponini tribe from the nectar of plants or from secretions of living parts of plants, which the stingless bees collect, transform by combining with the specific substances of their own, deposit, dehydrate, store and leave in the natural honey pots to ripen and mature.

In this standard it is referred to as *kelulut* honey.

2. processed *kelulut* honey

Raw *kelulut* honey which undergoes drying process at a temperature not more than 40 °C to reduce moisture content.

3. raw *kelulut* honey

Kelulut honey that is collected from natural sealed honey pots.

4 Requirements

1. General

1. *Kelulut* honey shall has its natural characteristic flavour and aroma.

2. *Kelulut* honey shall free from foreign matter.

3. *Kelulut* honey shall not contain any food additives.

4. Both raw and processed *kelulut* honey should be harvested and processed in accordance with MS 2679.

2. Quality requirements

1. *Kelulut* honey shall comply with the requirements given in Table 1.

Table 1. Quality requirements for *kelulut* honey

Characteristics	Requirements		Test method[a]
	Raw honey	Processed honey	
Moisture, %	Not more than 35.0	Not more than 22.0	Annex A
Sucrose, g/100 g	Not more than 7.5	Not more than 8.0	Annex B
Fructose and glucose (sum), g/100 g	Not more than 85.0	Not more than 90.0	
Maltose, g/100 g	Not more than 9.5	Not more than 10.0	
Ash, g/100 g	Not more than 1.0	Not more than 1.0	Annex C
Hydroxymethylfurfural, mg/kg	Not more than 30.0	Not more than 30.0	Annex D
pH	2.5 to 3.8	2.5 to 3.8	Annex E
Plant phenolics[b]	Present	Present	Annex F

[a] Other equivalent and recognised test methods can be used.

[b] *Kelulut* honey shall contain naturally occurring plant phenolic. A typical HPLC pattern of plant phenolics in *kelulut* honey is shown in Figure F.1. of Annex F.

4.3 Microbial contaminant limits

Kelulut honey shall not contain microorganisms exceeding the limits specified in Table 2.

Table 2. Microbial contaminant limits for *kelulut* honey

Characteristics	Limit	Test method[a]
Total plate counts, CFU/ml	1 x 10^2	Annex G
Yeast and mold, CFU/ml	Less than 1 x 10^1	Annex H
Coliforms, CFU/ml	Less than 1 x 10^1	Annex J
[a] Other equivalent and recognised test methods can be used. CFU= colony forming unit		

5 Sampling

1. General

1.Different sampling methods are employed for raw *kelulut* honey (5.2) and processed *kelulut* honey (5.3).

2.The analysis of physicochemical parameters shall be carried out within two to four weeks after collection of samples.

NOTE. Prescribed period indicates the freshness level of the samples for analysis.

5.2 Raw *kelulut* honey

5.2.1 Sampling shall be carried out as the following.

a) Species of the *kelulut* honey shall be identified.

b) The *kelulut* honey shall be harvested from matured honey pots i.e. sealed honey pots by piercing the upper part of the pots using sharp tool followed by suction using clean syringe or specific honey pump.

c) The *kelulut* honey harvested shall be representative of the identified beekeeping area where the sample is taken.

d) The minimum amount of samples to be collected is 350 g.

e) The *kelulut* honey shall be placed in clean, dry and sealed suitable food grade container. It shall be separated according to the specific species.

f) The *kelulut* honey collected shall be kept in a chiller at the temperature between 0 °C to 4 °C.

5.2.2 The samples shall be labelled to include the following information:

a) name of contributors, organisation/company together with contact person and phone number;

b) *kelulut* (stingless bee) species;

c) date of sampling;

d) beekeeping area; and

e) major plant species surrounding the beekeeping area.

5.3 Processed *kelulut* honey

5.3.1 Sampling shall be carried out as the following.

a) Species of the *kelulut* honey and producer of the honey shall be identified.

b) The minimum amount of samples to be collected is 350 g.

c) The *kelulut* honey shall be placed in clean, dry and sealed suitable food grade container. It shall be separated according to the specific species and producer.

5.3.2 The samples shall be labelled to include the following information:

a) name and address of company/manufacturer; and

b) a statement to indicate the samples are processed *kelulut* honey.

6 Preparation of test sample

Crystallised honey should be left at room temperature to allow the crystal to dissolve. Alternatively, the crystallised honey should be heated up to less than 40 °C. Homogenise the sample at room temperature.

7 Test methods

Testing should be carried out as specified in Annexes A to J.

NOTE Alphabet "I" is not used for labelling of annexes.

8 Packaging and labelling

8.1 Packaging

The product shall be packed in suitable, hygienic, food grade packaging materials which are able to withstand the acidity of *kelulut* honey so as to protect the safety and quality of the product in accordance with MS 2679.

2. Labelling

1.The product shall be labelled in accordance with *Food Act 1983* and *Food Regulations 1985*.

2.Each container/bottle of product shall be legibly and indelibly labelled with the following information:

a) name of product;

b) batch or code number;

c) date of packing and expiry date;

d) name, and address of the packer;

e) net weight;

f) country of origin;

g) storage instruction; and

h) packer registered trade mark, if any.

8.2.3 The containers may also be marked with the producer specified branding, place of origin and associated plant source.

9 Hygiene

Kelulut honey shall be processed under the good processing and harvesting of *kelulut* honey in accordance with MS 2679, MS 1514 and *Food Hygiene Regulations 2009*.

10 Legal requirements

The product shall in all other aspects comply with the requirements of the legislation currently in force in the country.

Annex F
(normative)

Determination of plant phenolics

1. Scope

This annex describes the procedure to determine the plant phenolics in *kelulut* honey.

2. Principle

Plant phenolics contents are determined by HPLC (High Performance Liquid Chromatography) with photo diode array detector (PDA) and/or with mass spectrometry detector. Peaks are identified on the basis of their retention times, UV spectra and reference standards. Quantifications are performed according to the external standard method on peak areas.

3. Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

1. **Methanol**, HPLC grade.

2. **Acetonitrile**, HPLC grade.

3. **Eluent solution for the HPLC**, gradient mixing of acetonitrile and water containing 1.0 % formic acid. The gradient elution is carried out over 70 min from 10 % to 30 % acetonitrile.

F.3.4 HPLC water or of equivalent purity, for example double distilled deionised water.

4. Apparatus

Use the usual laboratory apparatus and, in particular, the following.

1.Membrane filter for aqueous solutions, pore size 0.45 µm.

2.HPLC consisting of pump, sample applicator, temperature-regulated PDA-detector thermostated at 30 °C, temperature regulated column oven at 30 °C and integrator.

3.Analytical stainless-steel column, e.g. 5 µm, 4.6 mm x 250 mm.

4.Analytical balance, accurate to 0.001 g.

5. Procedure

1. Sample preparation

See Clause 6.

2. High Performance Liquid Chromatography (HPLC) condition

If a column of the type described above is used, the following conditions are recommended:

Flow rate: 0.2 ml/min.

Mobile phase: Acetonitrile:water containing 1.0 % formic acid. The gradient elution is carried out over 70 min from 10 % to 30 % acetonitrile

Column and detector temperature: 40 C.

Sample volume: 10 µl.

3. Determination

1.Acidify 4.0 g sample to pH 2.0 with 10 ml hydrochloric acid (HCl).

2.Use solid phase extraction (SPE) C18 column cartridge for sample purification.

3.Pre-condition the cartridge with methanol and HCl (pH 2) and load the loading with the acidified sample.

4.After adding the acidified sample, wash the cartridge with methanol. The extract is then reduced to dryness *in vacuo*.

5.Analyse extract of sample in triplicate using HPLC comprising HPLC pump with an auto sampler and a photo diode array detector (PDA) scanning from 200 nm to 550 nm. Plant phenolics can be detected at 280 nm and 365 nm.

F.6 Expression of results

A typical of HPLC pattern of plant phenolics in *kelulut* honey is shown in Figure F.1.

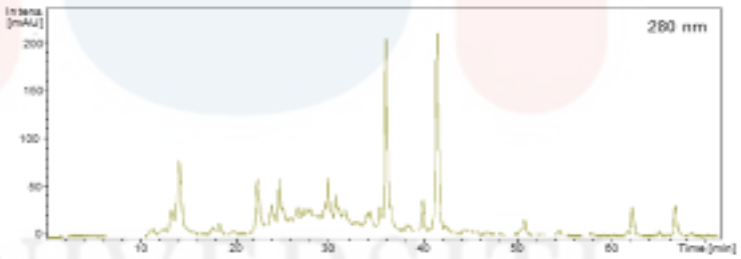

Figure F.1. HPLC chromatogram of plant phenolics in *kelulut* honey

Annex G
(normative)

Determination of total plate count

1. Scope

This annex describes the procedure to determine the total number of aerobic bacterial population on *kelulut* honey.

2. Principle

The total plate count is intended to indicate the level of microorganism in *kelulut* honey. The suitable colony counting range is 25 colonies to 250 colonies. The plate count method means diluting bacteria with a diluent solution (e.g. sterile saline) until the bacteria are diluted enough to count accurately when spread on a plate. The assumption is that each viable bacterial cell will develop into a single colony. Bacterial cell numbers need to be reduced by dilution, because more than 200 colonies on a standard 9 cm plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs).

3. Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

1. **Sterile Ringers solution**, for dilution.
2. **Sterile plate count agar**.

4. Apparatus

Use the usual laboratory apparatus and, in particular, the following.

1. **Sterile petri dishes**, glass or plastic, at least 15 mm × 90 mm.
2. **Pipets and sterile pipet tips**, 1 ml.
3. **Dilution bottles**, 100 ml and 25 ml.
4. **Circulating water bath**, thermostatically controlled to 45°C ± 1°C.
5. **Incubator**, 37°C ± 1°C.
6. **Colony counter**.

G.5 Procedure

G.5.1 Sample preparation

See Clause 6.

2. Determination

1.Weigh 10 g sample into 90 ml Ringers solution and shake for 25 times within 7 s.

2.Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate by transferring 1.0 ml of previous dilution to 9.0 ml of diluents.

3.Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes.

4.Add 12 ml to 15 ml plate count agar (cooled to 45°C ± 1°C) to each plate within 15 min of original dilution.

5.Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify.

6.Invert solidified petri dishes, and incubate promptly for 48 h ± 2 h at 37 °C.

3. Counting of colonies

1.After incubation period, count growth colonies in all petri dish.

2.Normal plates (25 colonies to 250 colonies): Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size, on selected plate(s). Record dilution(s) used and total number of colonies counted.

3.Plates with more than 250 colonies: When number of CFU per plate exceeds 250 colonies, for all dilutions, record the counts as too numerous to count (TNTC).

4.Plates with no CFU: When plates from all dilutions have no colonies, report APC as less than 1 x 10^{1} the corresponding lowest dilution used.

Annex H
(normative)

Enumeration of yeast and mold

1. Scope

This annex prescribes the procedure to enumerate the yeast and mold on *kelulut* honey.

2. Principle

There are many media for enumeration of yeast and mold in food samples. These media are either acidified or incorporated with antibiotic to suppress the growth of bacteria. In general, fungi can grow at an optimum pH much lower than that most of bacteria. Selective medium acidified to around pH 3.5 using sterile lactic or tartaric acid has been traditionally used for enumerating yeast and mold. Acidified media presents two distinct shortcoming- occasional growth of acidic tolerant bacteria and some yeast and mold grow poorly. Although acidified medium is preferred when examining acidic sample, it is not suitable for food media containing high level of acid tolerant bacteria.

3. Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in test.

1. **Sterile Ringers solution**, for dilution.
2. **Sterile Malt Extract Agar (MEA)**.
3. **Tartaric acid**, 10 %.

4. Apparatus

Use the usual laboratory apparatus and, in particular, the following.

1. **Sterile petri dishes**, glass or plastic, at least 15 mm × 90 mm.
2. **Pipets and sterile pipet tips**, 1 ml.
3. **Dilution bottles**, 100 ml and 25 ml.
4. **Circulating water bath**, thermostatically controlled to 45 C ± 1 C.
5. **Incubator**, 37°C ± 1 C.
6. **Colony counter**.

5. Procedure

1. Sample preparation

See Clause 6.

2. Determination

1.Weigh 10 g sample into 90 ml Ringers solution and shake for 25 times within 7 s.

2.Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate by transferring 1.0 ml of previous dilution to 9.0 ml of diluents.

3.Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes.

4.Add 12 ml to 15 ml MEA (added with 1.2 ml tartaric acid 10 % to 100 ml agar) (cooled to 45 °C ± 1 °C) to each plate within 15 min of original dilution.

5.Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify.

6.Invert solidified petri dishes, and incubate promptly for 72 h ± 2 h at 25 °C.

3. Counting of colonies

1.After incubation period, count grown colonies in all petri dish.

2.Normal plates (25 colonies to 250 colonies): Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size on selected plate(s). Record dilution(s) used and total number of colonies counted.

3.Plates with more than 250 colonies: When number of CFU per plate exceeds 250 colonies, for all dilutions, record the counts as too numerous to count (TNTC).

4.Plates with no CFU: When plates from all dilutions have no colonies, report APC as less than 1 x 10^{1} the corresponding lowest dilution used.

Annex J
(normative)

Determination of total coliforms

1. Scope

This annex prescribes the procedure to determine the coliforms count on *kelulut* honey.

2. Principle

The coliform group includes aerobic and facultatively anearobic, gram negative, non-sporing rods that ferment lactose, with acid and gas production within 48 h at temperatures between 30 °C and 37 °C. The bacteria detected by coliform test are members of several genera within the family Enterobacteriacae including *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter*. The sample is inoculated into the medium containing lactose, which is normally made selective by addition of bile salts, other surface active agents of dyes.

Viable Red Bile Agar (VRBA) is a lactose containing selective medium for the detection and enumeration of coliforms or lactose fermenting gram negative bacteria. The medium contains bile salt and crystal violet as selective agents and the lactose fermentation is indicated by the pH indicator dye, neutral red. The medium pasteurised by boiling. Organisms which rapidly attack lactose produce purple (dark) colonies surrounded by purple haloes (reddish zone of precipitated bile). Non-lactose or late-lactose fermenters produce pale or colourless colonies with greenish zones. Coliforms on this medium produce dark red colonies usually greater than 0.5 mm in diameter and often surrounded by a reddish zone. All colonies are in the depth of the agar due to the overlay.

3. Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in test.

1. **Sterile Ringers solution**, for dilution.
2. **Sterile Viable Red Bile Agar (VRBA)**.

4. Apparatus

Use the usual laboratory apparatus and, in particular, the following:

1. **Sterile petri dishes**, glass or plastic, at least 15 mm × 90 mm.
2. **Pipets and sterile pipet tips**, 1 ml.
3. **Dilution bottles**, 100 ml and 25 ml.

J.4.4 **Circulating water bath**, thermostatically controlled to 45°C ± 1 C.

J.4.5 **Incubator**, 37°C ± 1 C.

J.4.6 **Colony counter**.

5. Procedure

1. Sample preparation

See Clause 6.

2. Determination

1.Weigh 10 g sample into 90 ml Ringers solution and shake for 25 times within 7 s.

2.Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate by transferring 1.0 ml of previous dilution to 9.0 ml of diluents.

3.Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes.

4.Add 10 ml VRBA (cooled to 45°C ± 1°C) to each plate within 15 min of original dilution.

5.Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify.

6.Add another layer of agar onto previous solidified agar. Let agar solidify.

7.Invert solidified petri dishes, and incubate promptly for 48 h ± 2 h at 37 °C.

3. Counting of colonies

1.After incubation period, count grown colonies in all petri dish.

2.Normal plates (25 colonies to 250 colonies): Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size on selected plate(s). Record dilution(s) used and total number of colonies counted.

3.Plates with more than 250 colonies: When number of CFU per plate exceeds 250 colonies, for all dilutions, record the counts as too numerous to count (TNTC).

4.Plates with no CFU: When plates from all dilutions have no colonies, report APC as less than 1 x 10^{1} the corresponding lowest dilution used.

APPENDIX B: Survey form for collection of stingless bee honey samples

Samples no.	Honey location	Types of Honey		Honey type		Pollen sources / Plant sources	Date of harvesting	Age of honey	Location (company/ individual/ distributor)
		Raw	Process	Blended species	Single species				
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									