# STUDY ON ANTIOXIDANT CAPACITY, ANTIBACTERIAL ACTIVITY, PHENOLIC PROFILE AND MICROBIAL SCREENING OF SELECTED MALAYSIAN AND TURKISH HONEY

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FACULTY OF MEDICINE UNIVERSITY OF MALAYA KUALA LUMPUR

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# DESSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

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# UNIVERSITY OF MALAYA ORIGINAL LITERARY WORK DECLARATION

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Study on Antioxidant Capacity, Antibacterial Activity, Phenolic Profile and

Microbial Screening of Selected Malaysian and Turkish Honey.

Field of Study:

Natural Product Biochemistry and Bacteriology

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### ABSTRACT

Honey has been implicated in promoting healing process of superficial wounds like burn injury and decubitus ulcer. It is attributed to high antioxidant and antibacterial activities through prevention of bacterial infection. Phytochemical contents, hydrogen peroxide, low pH and high osmolarity are the key factors that affect honey bioactivity. These properties are unique to different honey type depending mainly on their geographical origin, bee species, and floral source. Influence of these variations to honey production has drawn questions to the level of bioactivity between honey, especially from the different geographical location. To date, there are very limited data on Malaysian and Turkish honey bioactivities to answer such questions. Therefore, the present study aims to investigate the antioxidant and antibacterial activities of five Malaysian honey and five Turkish honey. The antioxidant activity of honey was evaluated using spectrophotometric measurements including 2,2-diphenyl-1picrylhydrazyl 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphuric (DPPH), acid) (ABTS) and Ferric Reducing Antioxidant Power (FRAP) assays. Total phenolic content (TPC) and Liquid Chromatography-Mass spectrophotometry (LC-MS) were employed to characterize the phenolic profile of honey samples. Antibacterial properties of honey were evaluated via Minimum Inhibitory Concentration (MIC)/Minimum Bactericidal Concentration (MBC) assays and agar well diffusion assay against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus cereus. 16S rDNA gene sequencing analysis was conducted for pathogenic bacterial screening. Kelulut honey possessed the highest antioxidant activity with the scavenging ability of 0.00421±0.03µeq AAE/mg and FRAP value of 13.37±0.49µMx100/mg. Gelam honey, however, detected highest in ABTS assay with 93.74% inhibition while 2.216±0.02µg GAE/mg in TPC. LC-MS detected six phenolic acids in honey tested which present in various combination of; 2,3-dihydroxybenzoic acid, caffeic acid, gallic acid, p-salicylic

acid, syringic acid and vanillic acid. Gelam honey exerted highest antibacterial activity against S. aureus with 5% MIC and 6.35% MBC, E. coli with 12.5% MIC and 15% MBC and against P. aeruginosa with 10% MIC and 12.5% MBC. B cereus however only inhibited by gelam, tualang, carob blossom and spring honey at 15% while killed at 20% by those honey. *Kelulut* honey gave equal effects on all bacteria tested for both MIC and MBC assays (20%). In diffusion assay, the highest antibacterial activity was demonstrated by spring honey against B. cereus with 28.54 EPC of total activity while 27.72 EPC of non-peroxide activity. Two honey failed to inhibit bacterial growth neither for total activity nor non-peroxide activity, namely lavender honey against E. coli and P. aeruginosa and pine honey against E. coli. Significant differences were found between total and non-peroxide activities of acacia honey against B. cereus, lavender and wildflowers honey against S. aureus as well as wildflowers and carob blossom honey against E. coli and B. cereus. Sixty-one Gram-positive bacilli were isolated from tested honey with the dominance of Bacillus pumilus (40.90%). In conclusion, Gelam and kelulut honey from Malaysia and spring honey from Turkey possessed excellent antioxidant capacities and antibacterial properties against bacterial species, especially Gram-positive ones. It was likely due to variation in their physicochemical properties that influence by their floral source, place of origin as well as their bee species.

#### ABSTRAK

Madu dikatakan membantu menggalakkan proses penyembuhan luka-luka yang terdedah seperti kecederaan akibat terbakar dan kudis akibat tekanan (decubitus ulcer). Penyumbang kepada keadaan itu adalah aktiviti antioksida dan antibakteria yang tinggi dimana IA mampu mencegah jangkitan. Kandungan fitokimia, hidrogen peroksida, pH rendah, dan kadar osmolariti yang tinggi adalah faktor utama yang memberi kesan kepada kadar bioaktiviti madu. Ini adalah ciri-ciri unik madu bergantung kepada tempat asal madu tersebut, spesis lebah dan sumber tumbuhan madu tersebut dihasilkan. Pengaruh keunikan ini terhadap penghasilan madu telah mengundang persoalan berkaitan tahap bioaktiviti madu terutamanya yang dihasilkan dari lokasi yang berbeza. Dewasa ini, tidak banyak data tentang tahap bioaktiviti madu tempatan (Malaysia) dan madu Turki bagi menjawab persoalan tersebut. Untuk itu, kajian ini dijalankan bertujuan untuk menyiasat tahap aktiviti antioksida dan antibakteria lima madu tempatan (Malaysia) dan lima madu Turki. Aktiviti antioksida dinilai menggunakan kaedah spektrofotometrik termasuklah ujian 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphuric acid) (ABTS) dan Ferric Reducing Antioxidant Power (FRAP). Ujian kandungan phenol keseluruhan (TPC) dan Liquid Chromatography-Mass Spectrophotometry (LC-MS) digunakan untuk mengesan kandungan phenol dalam sampel madu. Aktiviti antibakteria madu dinilai melalui ujian Kepekatan Minimum Perencatan (MIC)/Kepekatan Minimum Bactericidal (MBC) dan ujian serapan agar ke atas Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa dan Bacillus cereus. Analisis 16S rDNA jujukan gen digunapakai untuk menyaring bacteria berbahaya dalam madu. Madu kelulut mengandungi activity antioksida yang paling tinggi dengan kadar penyahoksidaan sebanyak 0.00421±0.03µeq AAE/mg dan nilai FRAP sebanyak 13.37±0.49µMx100/mg. Madu gelam pula dikesan paling tinggi dalam ujian ABTS dengan nilai 93.74% perencatan manakala

2.216±0.02µg GAE/mg dalam ujian TPC. LC-MS mengesan enam asid phenol madu dalam pelbagai kombinasi yang terdiri daripada; asid 2,3-dihydroxybenzoic, asid caffeic, asid gallic, asid *p*-salicylic, asid syringic dan asid vanillic. Madu gelam juga dikesan mengandungi kandungan antibakteria paling tinggi ke atas S. aureus dengan 5% MIC dan 6.35% MBC, ke atas E. coli dengan 12.5% MIC dan 15% MBC juga ke atas P. aeruginosa dengan 10% MIC dan 12.5% MBC. Walaubagaimanapun, pertumbuhan *B cereus* hanya direncat oleh madu gelam, tualang, *carob blossom* dan *spring* pada 15% manakala membunuhnya pada kepekatan 20%. Madu kelulut memberikan kesan yang sama ke atas kesemua bakteria yang diuji untuk kedua-dua ujian, MIC dan MBC (20%). Dalam ujian serapan agar, aktiviti antibakteria yang paling tinggi ditunjukkan oleh madu spring ke atas B. cereus dengan 28.54 EPC untuk aktiviti keseluruhan manakala 27.72 EPC untuk aktiviti tanpa peroksida. Dua madu gagal merencat pertumbuhan bakteria sama ada untuk aktiviti menyeluruh mahupun aktiviti tanpa peroksida, iaitu madu lavender yang diuji ke atas E. coli and P. aeruginosa dan madu pine diuji ke atas E. coli. Perbezaan ketara dikesan diantara aktiviti keseluruhan dengan aktiviti tanpa peroksida madu akasia ke atas B. cereus, madu lavender dan madu wildflowers ke atas S. aureus juga madu wildflowers dan carob blossom ke atas E. coli dan B. cereus. Enam puluh satu bakteria Gram positif telah berjaya disaring daripada madu-madu tersebut dengan didominasi oleh Bacillus pumilus (40.90%). Kesimpulannya, Madu gelam dan kelulut dari Malaysia dan madu spring dari Turki mengandungi kapasiti antioksida berserta aktiviti antibakteria yang tinggi terutamanya terhadap bakteria Gram positif. Ianya berkemungkinan disebabkan kerana variasi dalam ciri-ciri fisiokimia madu dimana dipengaruhi oleh sumber tumbuham, tempat penghasilannya dan spesis lebah.

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# LIST OF SYMBOLS AND ABBREVIATIONS

<	:	Less than
>	:	More than
±	:	Plus/minus
-	:	Minus/negative
~	:	Approximately
ø	:	Diameter
α	:	Alpha
β	:	Beta
γ	:	Gamma
%	:	Percent
°C	:	Degree Celcius (centigrade)
Δ	•	Changes/difference
		Microequivalents
μος	:	Microgram
μ6 μ1	:	Microlitre
μΩ	:	Micromolar
μα/ul	:	Microgram per microlitre
μg/μι μg/l	:	Microgram per litre
$\mu g/1$	•	Microgram per mililitat
µg/mi	÷	Microgram per miniture
$\mu L/mL$	:	Microlitre per millitre
(AnR)-CYP	:	(Aryl hydrocarbon Receptor)-Cytochrome P-450
A. baumannu	:	Acinetobacter baumannii
A <sub>593</sub>	:	Absorbance at 593 nm
ABCA I	:	ATP-binding Cassette, sub-family A, member 1 gene
ABTS	:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphuric acid)
AAE	:	Ascorbic acid equivalent
AFB	÷	American Foulbrood (disease)
AGE	:	Agarose gel electrophoresis
AUC	•	Area Under Curve
ATCC	:	American Type Culture Collection
ATP	:	Adenosine Triphosphate
B. cereus	:	Bacillus cereus
B. stearothermophilus	:	Bacillus stearothermophilus/Geobacillus stearothermophilus
B. subtilis	:	Bacillus subtilis
BBCA	:	Brilliance Blue Coliform Agar
BHI	:	Brain Heart Infusion
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
Ċ	:	Carbon atom
CAPE	:	Caffeic Acid Phenetyl Ester
Cd	:	Cadmium
cells/ml	:	Cells per mililitre
cfu/ml	:	Colony forming unit per mililitre
CERP	•	Cholesterol Efflux Regulatory Protein
CFU	•	Colony Forming Units
CIA	:	Clostridium isolation agar
cm	•	Centimeter
CoNS	:	Coagulase Negative Stanbulococci
CUID	•	Coagarase regarive stapitytoeoeer

CRPA	:	Ciprofloxacin-Resistant Pseudomonas aeruginosa
CYP1A1	:	Cytochrome P-450; family 1, subfamily A, polypeptide 1
		protein gene
DNA	:	Deoxyribonucleic Acid
dNTPs	:	Deoxyribonucleotide triphosphates
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
E. aerogenes	:	Enterobacter aerogenes
E. cloacae	:	Enterobacter cloacae
E. coli	:	Escherichia coli
E. faecalis	:	Enterococcus faecalis
ĔČ	:	Enzyme Commission (number)
EDTA	:	Ethylenediamine tetraacetic acid
EPC	:	Equivalent Phenol Concentration
ESBL	:	Extended Spectrum β-lactamase
et al.	:	et alia (Latin), and other
FeCl <sub>3</sub> .6H <sub>2</sub> O	:	Ferric Chloride Hexahydrate
FeSO <sub>4</sub> .7H <sub>2</sub> O	:	Ferric sulfate heptahydrate
FRAP	:	Ferric Reducing Antioxidant Power
g	:	Gram
g	:	Gravity
GAE	:	Gallic acid equivalent
h	:	Hour
H <sub>2</sub> O <sub>2</sub>	:	Hydrogen Peroxide
HepG2	:	Human Liver Hepatocellular Carcinoma Cell Line
HMF	:	Hydroxymethylfurfural
HPLC	:	High Performance Liquid Chromatography
i.e.	:	id est (Latin), that is
in vitro	:	Latin. in glass
in vivo		Latin, in a living thing
kDa	:	Kilodalton
K. oxvtoca		Klebsiella oxytoca
K. pneumonia	÷	Klebsiella pneumonia
kg	•	Kilogram
KH <sub>2</sub> PO <sub>4</sub>		Potassium Dihydrogen Phosphate
L	:	Litre
L. acidophilus	:	Lactobacillus acidophilus
L. monocytogenes	:	Listeria monocytogenes
LC-MS	:	Liquid Chromatography Mass Spectrometry
LLE	:	Liquid Phase Extraction
Ltd.	:	Limited
Μ	:	Molarity
M. luteus	:	Micrococcus luteus
MBC	:	Minimum Bactericidal Concentration
MCA	:	Mac Conkey agar
MCF-7	:	Michigan Cancer Foundation-7/Breast Cancer Cell Line
mg	:	Milligram
MgCl <sub>2</sub>	:	Magnesium Chloride
mg/kg	:	Milligram per kilogram
mg/ml	:	Milligram per microlitre
MĬC	:	Minimum inhibitory Concentration
min	:	Minute
ml	:	Mililitre

mM	:	MiliMole
MH	:	Müller-Hinton
MRSA	:	Methicillin-Resistant Staphylococcus aureus
MSA	:	Mannitol Salt Agar
MSSA	:	Methicillin-Sensitive <i>Staphylococcus aureus</i>
m/7		Mass-to-Charge ratio
n	:	Number
N	:	Normality
NaCl	:	Sodium Chloride
NacO.	:	Sodium Carbonate
	:	Sodium Hudrovido
NaOП	•	Nonometer
	•	National Density
OD OU	•	Updical Density
OH	:	Hydroxyl group
P	:	Probability
p	:	para-
P. aeruginosa	:	Pseudomonas aeruginosa
P. fluorescens	:	Pseudomonas fluorescens
Pb	:	Lead (plumbum)
PBS	:	Phosphate buffer saline
PCA	:	Plate Count Agar
PCR	:	Polymerase Chain Reaction
pH	:	Power of the concentration of Hydrogen ion
pKa	:	Power of acid-dissociation equilibrium constant
pmol	:	Picomole
PST-P	:	Phase II P-form of Phenol Sulfotransferase
PTFE	:	Polytetrafluorethylene
R	:	Registered trademark
rDNA	: '	Ribosomal Deoxyribonucleic Acid
ROS	:	Reactive Oxygen Species
rpm	:	Revolutions per minute
S	:	Second
S. aureus	÷	Staphylococcus aureus
S. epidermidis	:	Staphylococcus epidermidis
S. marcescens	:	Serratia marcescens
S. typhimurium	:	Salmonella typhimurium
s.d	:	Standard Deviation
SPE	:	Solid Phase Extraction
spp.	:	Species
$T_{\rm m}$	:	Melting Temperature
TEA	:	Tris-Acetate EDTA
THP-1	:	Human Acute Monocytic Leukemia Cell Line
TS	:	Trypticase Soy
TPTZ	:	2,4,6-tripyridyl-triazine
UMF	:	Unique Manuka Factor
UK	:	United Kingdom
UMMC	:	University Malaya Medical Centre
USA	:	United State of America
UPLC	:	Ultra-high Performance Liquid Chromatography
UV	:	Ultraviolet
V	:	Volt/Voltage
Vice versa	:	Latin, conversely/with the order reversed

VRE :	:	Vancomycin-Resistant Enterococci
VSE :	•	Vancomycin-Sensitive Enterococci
v/v :	•	Volume per volume
w/v :	•	Weight per volume
w/w :	•	Weight per weight
X :		Times

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#### **CHAPTER 1: INTRODUCTION**

Honey is a natural supersaturated substance made by bees from flower nectars and has been widely recognized as a wonderful gift from Allah to humankind. In ancient times it was referred to as "nectar of the Gods". Honey has been reported to possess many health benefits including agents of antioxidant, antimicrobial, anti-inflammatory, wound healing as well as an excellent dietary supplement to boost up the energy and optimizing immunity of a healthy person. Its therapeutic implications extended from superior nutritive values to preventing health disorder such as cancer, cardiovascular diseases, neurological degeneration as well as diabetic ulcers (M. I. Khalil, 2010).

In addition to its health-related advantages, honey is also useful as a preservative due to its high osmolarity that can prevent microorganism growth. Its favourable smell and taste makes it suitable to be used as a food's taste enhancer. In addition, it is also used as a table sweetener, an alternative to sugar. To date, medical grade honey is easily available over the counter where its production and processing were highly monitored to meet the 'medical grade' standard.

In preventing microbial growth, honey was reported to have a varying degree of antimicrobial activity. The level of its antimicrobial potency is directly related to its physicochemical characteristics and the microbes of interest. The physicochemical properties of honey are highly dependent on its origin (geographical area, climate, season, and hive), foraging bees and floral sources. It however can be influenced by human activities during processing such as harvesting methods, storage, packaging, transportation and physical contact. Microbes like enterobacteriaceae, healthcare acquired pathogens and food spoilage organisms were reported to be susceptible to some types of honey in different degrees and effects. Generally, bacteria like *Staphylococcus aureus*, *Bacillus subtilis*, *Eschericia coli*, *Pseudomonas aeruginosa*,

Klebsiella pneumonia, Salmonella typhimurium, Enterococcus faecalis, Listeria monocytogenes, and fungi like Penicillium expansum, Aspergillus niger and Alcaligenes faecalis were killed by honey at certain concentration (Kwakman et al., 2010, Mundo et al., 2004a). Nevertheless, microbes like spore-forming organisms and yeasts were frequently reported to be major honey contaminants due to their ability to survive high osmolarity and low pH of honey. *Clostridium botulinum* is one of the most significant contaminants which raises concern among medical practitioners and food providers as it could cause infant botulism due to its spores in honey given to children below 2 years old (Midura, 1996).

In the present millennium, antibiotic resistant bacteria have been reported as a progressive critical challenge that needs an immediate robust solution. Rapid emergence of these 'super bugs' together with a slow new antibiotic discovery has initiated insecurity in the medical and health industry (Arias and Murray, 2009). Limited resources, high cost and time are the reasons why this industry seems to fail to produce excellent antibiotics in time. Antibiotic multi-resistant bugs just add the fuel to the flame. Startled by this phenomenon, scientists have started to look for potential alternatives to combat this seemingly endless issue. Honey has been listed as one of the potential agents together with other herbs and natural products. This is due to the numerous literature reported the antimicrobial activity of various types of honey across the globe which may contribute to development of antimicrobial agent to cope with the drug resistance problem. Ironically, most of them claim to possess effective antibacterial potency against well-known clinically isolated multidrug-resistant bacteria strains.

Apart from antibacterial activity, honey is also popular for its antioxidant capacity. This is said to be reflected from its chemical components, which contains high amounts of phenolic compounds such as phenolic acids and flavonoids. These organic components are derived from the floral source where the bees collect nectar, which primarily comes from minerals of that particular geographical area. To date, many researchers are interested in the phenolic compounds due to their potential as a dietary supplement to overcome oxidative stress and many clinical ailments like cardiovascular diseases (Vermerris and Nicholson, 2009), neurodegenerative diseases (Uttara et al., 2009) and cancers (Sosa et al., 2013). Phenolic acids are ubiquitous in honey. These heat and light-stable compounds are suitable for honey floral authenticity. Recently, the honey phenolic component has been used as a reference to the standardization purpose by several researchers especially in antimicrobial quality of honey (Allen et al., 1991, Irish et al., 2011). Therefore, more extensive study of this particular subject should be conducted to enhance understanding of the phenolic acids contribution to honey bioactivities.

In Malaysia, honey production is not a major contribution to its economic, healthrelated as well as food industries. However, due to its high availability, bioactivity potential, low cost, and numerous selections, honey production in Malaysia have a bright potential to be commercialized to meet the market demand of the world, or at least to meet the local demand. Well known to the locals, there are various types of honey waiting to be investigated. Lack of scientific research which is confirmed by limited scientific literature (of our knowledge) makes them invisible in the market. They include: *tualang*, *gelam*, *kelulut*, coconut, pineapple, acacia, *hutan* (forest), *pucuk daun*, rambutan, durian, *getah* (rubber) and a few others. Only a few reports were found investigating different aspect of several batches of *tualang* and some *gelam* honey (Aljadi and Kamaruddin, 2004, Almahdi Melad Aljadi, 2003b, Ghashm et al., 2010, Moniruzzaman et al., 2013, Nasir et al., 2010, Nurul Syazana et al., 2011, Tan et al., 2009, Zaid et al., 2010, Tumin et al., 2005). The importance of research on Malaysian honey should not be neglected. With a huge tropical forest, located in close proximity to the equator with no seasonal variation the honey produced may show different bioactivities and physicochemical properties as compared to honey from Europe, New Zealand, Australia, America and other regions.

Turkey however has a temperate Mediterranean climate with dry summers and wet winters. This geographical difference may affect honey constituents and contribute to honey variations. As the world's second largest honey producer (as in 2012), the production of Turkish honey should move together with its research progression (Eliot Masters, 2014). However, to date, very limited data have been published by researchers investigating the analytical and bioactivity in respect to Turkish honey. The difference between Malaysian honey (tropical honey) and Turkish honey (Mediterranean honey) should provide a more comprehensive understanding about their bioactivities and physicochemical components, therefore justifying the present study.

Lack of scientific information limits the utilization of Malaysian and Turkish honey. To date, numerous studies have been done investigating the biological profile of honey around the world. This study should provide the same basic information in reference to ten tested honey from Malaysia and Turkey. The level of bioactivities analysed like antioxidant and antibacterial activities should assist in the application of these honey. In addition to their bioactivity profile, the contaminant screening of unpasteurized honey will provide useful information regarding to honey safety especially to those harvested from different origin such as Malaysia and Turkey.

#### **CHAPTER 2: LITERATURE REVIEW**

### 2.1 History of Honey in Medicine

Dating back to the earliest history, various civilizations had claimed to glorify honey as one of their superior remedy to treat illnesses as well as a food supplement. The oldest record known emphasizing honey as aforementioned was a prescript on a clay tablet from Nippur in Euphrates valley, *c*. 2000 BC. The Sumerians believed that honey was the best medicine to treat skin infections and ulcers (Jones, 2009). Smith papyrus (*c*. 2600-2200 BC) followed by Ebers papyrus (*c*. 1550 BC) listed various potions and grease for wound application including honey (Jones, 2009, Zumla and Lulat, 1989). Xia dynasty of Chinese use honey as food and medicine for centuries (*c*. 2000 BC) (Altman, 2010). Ancient Hindu text, the *Rig-Veda* (*c*. 1500-1000 BC) mentioned the bees and honey as a special emanation from gods (Jones and Sweeney-Lynch, 2011). Honey was also religiously endorsed by both Islam (Quran, 16:68-69) and Christianity (Bible, Genesis: 43.11) where they valued the therapeutic importance of honey (Zumla and Lulat, 1989, Jones, 2009).

Dated back to ancient Greece (*c.* 460-377 BC), the father of modern Western Medicine, Hippocrates introduced "oxymel" which contains honey and vinegar to release pain while "hydromel" was said to contain honey and water to ease thirst, probably refers to fever (Zumla and Lulat, 1989, Jones, 2009). According to Crane, (1999), Marcellus Empiricus is a medical writer who recorded that honey with butter and oil of roses will aid in ear pain, sight dullness and white in the eyes. In 1623, the father of English Beekeeping, Charles Butler wrote a practical manual of beekeeping "The Feminine Monarchie" which includes the basic skills of honey applications for various physical wounds as well as health disturbances (Jones, 2009).

Today, although honey is widely endorsed by various traditional cultures across the globe, it is said to be under-utilized in conventional medicine. Modern therapeutic methods such as conventional antibiotics and generic drugs are still on top of the list to treat typical yet recurring ailments. Only a minority of people especially those who live in remote areas constantly use honey as their main curative agents and health-promoting supplement.

## 2.2 Physicochemical Composition of Honey

Every natural occurring substance has its own unique characteristics that confer its specialty in the ecosystem. The same goes for honey as it contains several unique factors contributing to its various bioactivities. The diversity of honey physicochemical components have been reported widely by several researchers emphasizing that different honey exhibits different patterns of physicochemical properties (Khalil et al., 2012, Vit et al., 1998, Bogdanov, 1997, Mendes et al., 1998, Gomes et al., 2010). Generally, all honey share similar physical properties such as high acidity, low pH value, low water content or moisture and electrical conductivity. Molecules like reducing sugar, phenolic compounds, flavonoids, hydrogen peroxide, proteins, enzymes, minerals and vitamins are ubiquitously present in all types of honey. The concentrations of these components differentiate the level of bioactivity of one honey to another. In addition to that, some honey also contains rare chemical elements like methyl syringate, methyglyoxals and others (Adams et al., 2009, Tuberoso et al., 2009).

### 2.2.1 pH and Acidity

Honey is an acidic substance. pH of blossom honey varies between 3.5 and 4.5. Whilst honeydew honey have a slightly higher pH value which is between 4.5 and 6.5 due to their higher mineral contents (Bogdanov, 2009). Some organic acids could be found in honey in low amount. These are: formic acid, acetic acid, citric acid, lactic acid, maleic acid, malic acid, oxalic acid, pyroglutamic acid and succinic acid. Even though gluconic acid does not contribute to honey's active acidity, it is found to be the major acid composition (lactone). Its presence is due to glucose breakdown by glucose oxidase. The presence of phosphates, carbonate and some other mineral salts awarded the buffering capacity of honey.

### 2.2.2 Water Content and Water Activity

Honey humidity is one of the key factors that determine its quality and shelf life. Higher humidity will lead to increase fermentation activity. On average, water content of honey lies between 15-20% base on honey origin (Bogdanov, 2009). In some cases however, the water content can extend up to 22% like honey from a tropical country, Malaysia (Almahdi Melad Aljadi, 2003a). It is due to the climate of the country, which is humid throughout the year with high precipitation recorded annually. High water content also could be found in honey harvested by stingless bees which can reach up to 29.5% water compositions (Bogdanov, 2009). Honey is a very hygroscopic substance. High humidity in ambient air will reflect honey water content as it can absorb moisture from the air.

Water activity is a measurement to estimate free water content in food. It is important in determining microbiological risk of honey spoilage. High amount of free water molecules will cause high microbiological activity, which increases the fermentation process. In honey, majority of water molecules are bound to carbohydrate, thus are unavailable for microorganism. The water activity of honey varies between 0.55 to 0.75 units with the value less than 0.60 are considered microbiologically stable (Bogdanov, 2009). Water content also influences honey crystallization. Medium water content (15-18%) causes honey to crystallise optimally while low or high water content slows the process.

# 2.2.3 Electrical Conductivity, Density and Specific Gravity

As honey contains acids and minerals, it can conduct electrical current. As an electrolyte, electrical conductivity is a suitable parameter to evaluate honey quality. This parameter is used to replace the ash content of honey because of their linear relationship. Moreover electrical conductivity requires less time to evaluate with cost effective procedure and necessary for unifloral honey characterization (Bogdanov et al., 1999). In general, blossom honey should have less than 0.8mS/cm while honeydew honey and a few exceptions of blossom honey should have more than that value.

Honey density is regularly expressed as specific density. It is greater than water density despite the fact that it's dependent on the water content. High water content usually settles above the denser dried honey. Specific gravity of honey at 20°C is recorded to be between 1.3 and 1.5 with typical 15-18% water content.

#### 2.2.4 Colour

Colour of honey depends on its pigmented chemicals that include carotenoid-like substances, Millard reaction products, phytochemicals and possibly its water content. It is noteworthy to say that darker and opaque honey possess the highest level of total phenolics as reported by Blasa *et al.* (2006). Total phenolic contents were recorded higher in darker honey while lower in lighter honey (Kaškonienė et al., 2009). Significant correlations were reported between honey colour and pigmented contents with antioxidant activities (Bertoncelj et al., 2007). Regularly, honey colour was expressed in millimeter Pfund scale, which has a linear relationship with its optical density. Alternatively, some researchers used differences in light absorption spectrums at two wavelengths (450 and 720 nm) as the colour measurement (Irina Dobre, 2010, Khalil et al., 2012, Bertoncelj et al., 2007). The range of honey's colour reported lies between light yellow and amber. Depending on the botanical origin, some honey may have white colour or dark amber to black appearance. Study on Zambian honey reported darker honey to possess stronger ability in reducing enzymatic browning in white cabbage suggesting color as an important indicator in determining honey bioactivities (Nyawali et al., 2015).

### 2.2.5 Sugar

Carbohydrate is the most extensive component of honey investigated and documented so far. It is a major component of honey, which contributes more than 90% of honey dry matter. Studies recorded that monosaccharides, disaccharides, trisaccharides and oligosaccharides are present in honey in different proportions. Monosaccharides, mainly fructose and glucose are the most abundant reducing sugars detected in honey followed by the other three carbohydrate classes. Other carbohydrates may include: sucrose, maltose, turanose, trehalose, palatinose, cellobiose, melibiose, laminaribiose, isomaltose, melezitose, raffinose and panose. All of these minor sugars are present in trace amounts and may be absent in some types of honey.

One of the major contributions of honey carbohydrate content is being used as a measure to distinguish between nectar honey from honeydew honey (de la Fuente et al., 2011). Sugars like sucrose and maltose are usually used to evaluate the possible adulteration of honey (Cotte et al., 2003, Kaškonienė et al., 2010). Detection of high levels of these sugars demonstrates the impurity of honey. This is because their presence is normally very low in natural honey. The reason is bee-origin enzymes in pure honey will catalyse the transformation most of those disaccharides to fructose and glucose. The proportion of major monosaccharides, fructose to glucose is frequently used as an indicator of honey stability. Ratio between the two particular sugars can also be used as a tool for honey authentication.

The level of carbohydrate is a key player in determining various physicochemical properties of honey. Reason being, carbohydrate especially monosaccharides will be used as substrate for various molecular sequential mechanisms to produce vital end products such as gluconic acid and hydroxymethylfurfural (HMF). For that reason, its analysis is considered essential when assessing honey quality. This is because by knowing the level of carbohydrate present, determination of honey shelf life and its "active" period can be expected for optimal consumption by the consumers.

### 2.2.6 Phytochemicals

Another important component of honey that draws interest to recent researchers is its phytochemicals. These elements are reported to be abundant in honey since the original source of honey is the nectar from the plant flowers. Therefore, their presence in honey is directly related to the plant's phytochemical contents. Several phytochemicals especially simple phenolic compounds in class of benzoic, cinnamic and coumaric acids have been reported in honey (Weston et al., 1999, Gómez-Caravaca et al., 2006). These

compounds existed either in free form or bounded to sugars, esterified or nonesterified. Their stability to heat and sunlight make them very useful in honey analysis and bioactivity test. Some researchers use the phenolic bioactivity as a reference for honey quality. Allen et al. (1991) for example use phenol as standard to assess the antibacterial potency of New Zealand honey. It is followed by others as phenolic compounds are ubiquitous in honey (Irish et al., 2011). Researchers suggested that individual phenolic acid could be the floral marker for some honey (Anklam, 1998, Yaoa et al., 2005, Zhou et al., 2014). This is based on the presence of specific phenolic acids frequently detected in a particular honey in high proportions. It is also considered as another criterion for honey authentication after the sugar profile. In addition to that, polyphenols are also in the best interest to researchers nowadays. They mainly focus on flavonoids rather than lignans, lignins or tannins. Flavonoids like pinocembrin pinobanksin and chrysin are components of propolis which also can be found in honey (Yao et al., 2003b). Flavonoid contents are strongly associated with botanical species of honey origin, which are contributed by dominant pollen presented (Iurlina et al., 2009). Phytochemicals also influence the colour, smell and taste of honey. High phytochemical contents also contribute to the darker colour of honey and vice versa.

## 2.2.7 Phenolic acid

Phenolic compounds are referred to as substances or compounds that are composed of phenol(s) attached to one of their skeleton carbons. There are several alternative classifications available to categorized phenolic compounds. Most common one is the classification by their carbon number. This was first done by Harborne and Simmonds in 1964. According to them,  $C_6$  phenolic structure is referred as simple phenolics.  $C_6$ - $C_1$ structures are the phenolics acids and its related compounds;  $C_6$ - $C_2$  are phenones; and  $C_6$ - $C_3$  should be coumarins, cinnamic and coumaric acids and their derivatives. Polyphenols however are compounds comprised more than one phenolic hydroxyl group attached to their benzene ring(s). Their classification have includes;  $C_{15}$  as flavonoids (flavans, flavones, flavanones, flavanonols), anthocyanidins and anthocyanins;  $C_{18}$  refers as betacyanins; and  $C_{30}$  is biflavonyls.

Class	Skeletor	n structure	Examples	Derivatives
Simple phenolics	$C_6$ $C_6$ $c_6$ $c_6$ $c_6$	ho-, (1,2-) ta-, (1,3-) yra-, (1,4) u-tri, (1,3,5- ) tri, (1,2,6-)	Resorcinol (1,3- dihydroxybenzene) phloroglucinol ((1,3,5- trihydroxybenzene)	
Phenolic acids and aldehydes	$C_6$ Sub	ooxyl group stituted on phenol	Benzoic acid p-hydroxybenzoic acid gallic acid gentisic acid protochathechuic acid salicylic acid syringic acid vanillic acid veratric acid	Syringealdehyde vanillin
Acetophenones and phenylacetic acids	C	<sub>6</sub> -C <sub>2</sub>	2- hydroxyacetophenone 2-hydroxyphenyl acetic acid	
Cinnamic acids	С	<sub>6</sub> -C <sub>3</sub>	Cinnamic acid <i>p</i> -coumaric acid Caffeic acid Ferulic acid 5-hydroxyferulic acid Sinapinic acid	Chlorogenic acid (caffeic acid + quinic acid) Sinopyl malate Sinopyl choline
Flavonoids	C <sub>6</sub> -	C <sub>3</sub> -C <sub>6</sub>	Kaemferol (5,7,4'- Hydroxyflavone) Quercetin (5,7,3',4'- hydroxyflavone) Myricetin (5,7,3',4',5'- hydroxyflavone)	

Table 2.1: Phenolic compounds classification, adapted from Robbins (2003) &Vermerris et al. (2009).

Polyphenols refer to compounds comprising of more than one phenol group attached to their basic molecular structure. This term is easily misleading to be a multiple repetition of the same phenolic compounds in one single large molecular structure, even though it is not exclusively wrong. Some compounds may contain repetitive dominant phenolic structures, but their 'polyphenols' identity will be determined by the present of other phenolics structures in the same molecule. Tannic acid and flavonoids are the most common polyphenols widely studied.

Phenolic acids are described as compounds composed of phenol(s) and carboxylic acid in their molecular structure. Predominant phenolic acids in most plants are hydroxybenzoic and hydroxycinnamic acids. Basic structures of both are  $C_6$ - $C_1$ , which is distinguished by their pattern of hydroxylation and methoxylations. Hydroxybenzoic acid is usually found attached to carbohydrate to form sugar derivatives. Gallic acid and ellagic acid are common examples of hydroxybenzoic acid found in plant. Caffeic, ferulic, and sinapic acids are predominant Hydroxycinnamic acid regularly found in fruits. They also exist mainly in bound form in various conjugated identity. The direct association between plant and honey production has contributed to the detection of this compounds in honey, which vary greatly depending to its origin (Andrade et al., 1997, Khalil et al., 2011, Estevinho et al., 2008, Yao et al., 2003a).



## Figure 2.1: Structures of phenolic acid derivatives.



Figure 2.2: continued.

### 2.2.8 Hydrogen Peroxide

"Inhibine" was used to grade honey bioactivity in early days. Dold *et al.* (1937), as quoted by White *et al.* (1963) postulated inhibine as the major antibacterial factor that ran parallel with invertase activity. The term "inhibine" basically refers to hydrogen peroxide  $(H_2O_2)$  content, a product from honey-glucose oxidase system (White Jr et al., 1963). The authors in their report successfully proved that antibacterial activity has a direct relationship with honey's H<sub>2</sub>O<sub>2</sub> content. The presence of this heat-labile and lightsensitive compound depends entirely on its destruction-production system. Its accumulation is under the influence of the nectar origin. Its production in honey is mainly due to glucose oxidase action on glucose producing gluconic acid, which is also the contributing factor for honey low pH and acidity. Its destruction however may be due to catalase enzyme, which transferred to honey either from plants or honey bees. The balance between these two enzymatic activities determines the net level of H2O2. In undiluted honey, the glucose oxidase is said to be almost completely inactive which cause very little or no  $H_2O_2$  production. In diluted honey particularly less than 50%, these authors found that  $H_2O_2$  was actively produced up to 50000 times higher (White et al., 1962). In 2011, Brudzynski et al. when re-examining the role of H<sub>2</sub>O<sub>2</sub> concluded its involvement in oxidative damage, which is directly related to antibacterial efficacy by causing degradation of the bacterial DNA. They successfully demonstrated that H<sub>2</sub>O<sub>2</sub> destructive principle is highly influenced by four factors, which were: 1) bacterial sensitivity to oxidative stress, 2) bacterial growth phase 3) bacterial survival strategy (sporulation) and 4) modulation of other honey compounds.

### 2.2.9 **Protein and Enzymes**

A few proteins variety reported in honey are present as amino acids and antimicrobial polypeptides such as proline and bee defensin-1 respectively (Kwakman et al., 2010). The physiological roles of proline which act as osmoregulator, protein and membrane stabilizer, reactive oxygen species (ROS) scavenger, inhibitor of ice crystal formation and lowering agent for melting temperature ( $T_m$ ) of DNA may contribute to biological activities of honey. In addition, apidaecin originally isolated from honey bees is known to kill various pathogenic strains such as *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella enteritidis*, *Klebsiella pneumonia* and many more (El-Gendy, 2010). Although recent finding reported that apolipophorin III from bees (*A. cerana*) exhibited antibacterial activity, there are no evidence reporting this lipid transporter protein are present in honey (Kim and Jin, 2015). In most studies, the detected proteins remained unknown as no further investigations were conducted (Won et al., 2008, Azeredo et al., 2003).

Enzymes however have been detected in honey from the very early scientific studies on honey particularly the one that relates to carbohydrate hydrolysis. Different honey exhibits different enzyme activities due to physiological stage of the plant's organelles and bee's glands during the productive seasons. Bee-origin enzyme, glucose oxidase and plant-origin enzyme, catalase are the two most important enzymes. They are directly related to honey-glucose oxidase system, which is involved in numerous bioactive potency of honey.

Glucose oxidase (GOD) also known as  $\beta$ -D-glucose:oxygen 1-oxidoreductase (EC 1.1.2.3.4) is responsible in producing gluconic acid in honey via oxidation of  $\beta$ -D-glucose. It reduces atmospheric oxygen molecules into H<sub>2</sub>O<sub>2</sub>, which is known as major antibacterial agents of honey. GOD in honey originated from hypopharyngeal glands of bee. It is transported into honey and remains in inactive state until honey is diluted to certain level where it can freely catalyse the reaction of glucose hydrolysis into gluconic acids. The optimum pH range of GOD varies from 5 to 7, depending on the species origin (Bankar et al., 2009). Therefore it is only being activated when honey reaches this level of pH value while remains inactivated at normal honey pH value (3.5-4.5).
Catalase (CAT, EC 1.11.1.6) is an important enzyme that removes the potential harm generated from oxidative stress particularly  $H_2O_2$ . It is classified into three major classes: mono-functional, bi-functional catalase-peroxidase (KatG) and non-heme (manganese) with some minor catalases. Mono-functional catalase is the most abundant subfamily that can be found in animal, plants, fungi, and bacteria. Its mechanism of action involves two simple steps. The heme-containing CAT will be oxidized by  $H_2O_2$ to generate oxyferryl species that then reduced by the second  $H_2O_2$  molecule. These reactions take place in sequential manner and exclusive only to mono-functional CAT. Its final products are water, oxygen and resting state of CAT enzyme (Eason and Fan, 2014). Most prokaryotes and eukaryotes secrete CAT as self-defense mechanism to protect against oxidative damages. It can be found mainly in peroxisomes, mitochondria, cytosol and chloroplast. CAT in honey is suggested to be mainly originating from plants. It is transferred via nectar from nectar synthesis process that shares similar organelles.

Invertase is generally related to the level of sucrose in honey. This enzyme is responsible in transforming sucrose to fructose and glucose. Commonly found in plants and some microorganisms, it is also known as sucrose or  $\beta$ -fructofuranosidase (EC 3.2.1.26). As its official name implies, it catalyses the hydrolysis of  $\alpha$ -1,4-glycosidic bond of  $\beta$ -fructofuranoside producing  $\alpha$ -D-glucose and  $\beta$ -D-fructose. The resulted invert sugars are important to determine honey purity and shelf life. Due to its stability in a wide range of pH, invertase is generally active in undiluted honey and reflects the ability of honey in crystalisation process. Major contribution of invertase in plants includes the formation of hexose-rich nectars which occur in nectariferous tissue and/or in the secreted nectar itself (Heil, 2011). Therefore, it is common to find invertase in nectar of plants, which later on transported into the hive by bees to be stored as honey.

Diastase is a group of enzymes, consists of  $\alpha$ - and  $\beta$ -amylase that hydrolyze starch into disaccharides particularly maltose. Alpha-amylase (EC 3.2.1.1) is an endoamylase which catalyse the breakdown of  $\alpha$ -1,4-glycosidic bond present in inner part of amylose or amylopectin chain. It produces  $\alpha$  configuration of oligosaccharides with varying lengths including branched oligosaccharides and  $\alpha$ -limit dextrin. Beta-amylase (EC 3.2.1.2) however belongs to exoamylase group which cleaves  $\alpha$ -1,4- and  $\alpha$ -1,6glycosidic bonds. The substrate involved the external glucose residue of amylose and amylopectin therefore produce glucose, maltose and  $\beta$ -limit dextrin (van der Maarel et al., 2002). Diastase activity has been used as the earliest measure to evaluate the quality of honey which expressed as diastase index (White et al., 1962, Azeredo et al., 2003). Diastase number (DN) is a measurement used by International Honey Commission to determine the diastase activity which stated that it must not be less than or equal to 8. It is based on the Gothe scale number which defined as each gram of starch hydrolysed in 1 hour duration at 40°C per 100g of honey (Tosi et al., 2008).

Acid phosphatase is another enzyme that plays crucial role in honey fermentation. It originates from pollen and nectar of plants. Easily ferment honey contains high level of acid phosphatase activity as compared to unfermented honey. This enzyme is dependent on the pH of honey. The higher the pH value, the greater the acid phosphatase activity hence increase fermentation rate of that particular honey (Alonso-Torre et al., 2006).

#### 2.2.10 5-Hydroxymethyl-2-furaldehyde (HMF)

With the dominant contents of monosaccharides (fructose and glucose) in honey, formation of 5-Hydroxymethyl-2-furaldehyde or hydroxymethylfurfural (HMF) is unavoidable. It is specifically produced by acidic-catalysed dehydration of hexoses. Alternative mechanism of its formation is Maillard reactions. Literally, it appears naturally in any natural product that contains monosaccharides and water at acidic condition. High fructose level in honey makes this compound of important consideration in honey analysis. It is used as an indicator to evaluate honey quality and adulterations. Codex Alimentarius has limited the presence of HMF in European honey after processing and blending to be not more than 80 mg/kg depending on honey type (White, 1992, Zappalà et al., 2005). It is closely related to the toxicity issues in the food industry. The high amount of this compound is said to affect human health. Temperature and pH are two factors that strongly influence the formation of this compound. Increase temperature lead to increase formation of HMF and low pH value (acidic) will enhance its production (Fallico et al., 2004).

#### 2.2.11 Minerals, Vitamins and Trace Elements

Besides the major compositions of honey, minerals, vitamins and trace element also play their own role in determining the organoleptic characteristic of honey. They can be used as an indicator for honey geographical origin, climatic and seasonal changes of foraging activity. Potassium was detected to be the most abundant mineral in honey (Anklam, 1998). Honeydew honey was said to be richer in mineral content as compared to floral honey. Minerals like chlorine, sulphur, calcium, sodium, phosphorus, magnesium, and silica are present in honey in range of 22-113 p.p.m (White and Doner, 1980). Their existences were higher in darker honey than lighter honey. As for trace elements, the distributions are shown in table 2.1. Toxic elements content of honey are also important and can be used as environmental indicator for a particular geographical area. High amount of lead (Pb) and cadmium (Cd) may indicate environmental pollution. Concentration of toxic elements in Hungarian honey reported to be very low as compared to honey from several other countries such as New Zealand, Croatia, Turkey and Brazil indicating variation of contents across the globe (Czipa et al., 2015).

Honey possesses various important vitamins especially from group B and C. To date, very limited studies were conducted documenting the content of vitamins in specific type of honey around the world. As cited by Haydak *et al.* (1942), there were various studies conducted by different researchers demonstrating the presence of ascorbic acid (vitamin C) in Estonian and diverse nectar honey. Riboflavin, (vitamin B<sub>2</sub>), nicotinic acid (vitamin B<sub>3</sub>), pantothenic acid (vitamin B<sub>5</sub>) and folic acid (vitamin B<sub>9</sub>) were also detected in honey (Ciulu et al., 2011).

The physicochemical properties of honey strongly affected its organoleptic characteristics like colour, texture, viscosity, taste as well as odour. In general, it is dependent upon the biological background of one particular honey ranging from bee species to the hive conditions provided that honey production happens naturally.

Elements	Concentration (µg/l)	95% CI (μg/l)	Certified (µg/l)
Cadmium	23.7	$\pm 0.87$	22.8
Lead	28.5	±0.92	27.9
Chromium	36.2	±2.69	38.6
Manganese	123.0	±12.0	121.0
Iron	35.6	±5.16	34.3
Nickel	27.3	±7.28	27.4
Copper	88.8	±14.2	85.2
Zinc	54.5	±29.2	53.2

 Table 2.2: Level of trace elements in honey from Swiss production area

 (Bogdanov, Haldimann, Luginbuhl, & Gallmann, 2007).

# 2.3 Biological Activity of Honey

Most of natural occurring products have specific biological activity. Similar to honey, it possesses numerous beneficial bioactivities that may enhance health. It includes antioxidant, antimicrobial (antibacterial, antifungal and antiviral), antiinflammatory, wound healing promoter, energy booster and anti-aging activities. Until now, the mechanisms of most of these activities remain unknown. Despite of lacking in scientific evidence regarding to mechanism of actions, studies on honey bioactivities are gaining more attention from researcher around the globe. This may be due to several economic and environmental factors which persistently rose such as rapidly emerging multi-resistance bacteria strains, high cost and time consuming antibiotics research as well as ethical issues regarding to certain pharmaceutical research. Honey seems to be a perfect candidate to overcome these restraints since it has been reported to have the opposite effect against all those obstacles.

# 2.3.1 Antimicrobial Activity

Microbes such as bacteria, fungi and virus are among the major player in biosystem. They can be beneficial to human kind as well as potential cause of harm. Overcolonized of microbes may lead to infections and worsen the pathological conditions of a patient which may delay wound healing, caused bacteremia and sometimes may cause death. Recent studies found that honey can be an excellent antimicrobial agent to overcome this infection and promote wound healing (Kwakman et al., 2010, Aljady et al., 2000, Subrahmanyam et al., 2001).

## 2.3.1.1 Antibacterial Potency

Antibacterial potency is a major biological activity of honey which indisputably becoming the limelight in today's research field. Several studies have successfully proven the prominent antibacterial activity of honey against different opportunistic pathogens. These studies involved clinical isolated-, laboratory maintained- as well as standard commercially available strains, which confer the diverse perspective of bactericidal effect of different honey. Bacteria reported to be susceptible to honey was listed in table 2.3. Some of them were opportunistic human pathogen, multi-drugs resistant strains or normal flora to human being. Most of the studies showed good susceptibility profiles of bacteria species with a few exceptions. Among those researchers, some of them used antibacterial property of honey as an indicator for evaluation of honey quality by referring to phenolic activity equivalent (Allen et al., 1991, Irish et al., 2011).

Table 2.3: Antibacterial activity of honey against various pathogenic bacteriaspecies.

No.	Bacteria species	Honey	Reference
1	E. coli, B. Subtilis, B. cereus, S. aureus, S. epidermidis, P. aeruginosa, K. pneumonia, S. typhimurium, M. luteus, E. aerogenes, & S. marcescens,	Talah, Dhahian, Sumra & Sidr	Noori Al-Waili <i>et al.</i> 2013.
2	Streptococcus mutans	Saudi Arabian natural honey	H. M. Nassar <i>et al</i> . 2012.
3	<i>E. coli, S. typhimurium, Y.</i> <i>enterocolitica, E. aerogenes, E.</i> <i>cloacae, S. flexneri, S. sonnei</i> & Extended spectrum β-lactamase (ESBL)- producing organisms	Manuka honey (UMF +16)	Lin S. M., Molan P. C., & Cursons R. T. 2011 .
4	Methicillin-resistant <i>Staphylococcus</i> <i>aureus</i> (MRSA) & vancomycin- sensitive enterococci (VSE)	Manuka honey	Jenkins <i>et al.</i> 2011; Cooper <i>et al.</i> 2002.

# Table 2.3: continued.

	No.	Bacteria species	Honey	Reference
	5	E. coli, S. aureus & P. aeruginosa	Ulmo & manuka honey	Sherlock et al. 2010.
(	6	E. coli, S. aureus, B. Subtilis, P. aeruginosa, MRSA, K. oxytoca, vancomycin-resistant Entero- coccus faecium (VREF), extended- spectrum •-lactamase-pro- ducing E. coli (E. coli ESBL) & ciprofloxacin-resistant P. aeruginosa (CRPA)	Unprocessed Revamil honey	Kwakman et al. 2010; Kwakman et al. 2008.
	7	Campylobacter spp.	Manuka honey	Lin S. M., Molan P. C. & Cursons R. T. 2009.
	8	Chromobacterium violaceum	Italian & Slovakian honey mainly; chesnut, linden and orange honey	P. Truchado <i>et al.</i> 2009.
	9	A. baumannii, E. cloacae, E.faecalis, E. coli, P. aeruginosa, K. pneumonia, S. aureus & VRE	Medihoney	George et al. 2007.
	10	E. coli, B. cereus, S. aureus, S. enterica Ser. Typhimurium, L. monocytogenes, P. fluorescens, L. acidophilus, B. stearothermophilus	American honey	Mundo <i>et al.</i> 2004.
	11	E. coli, E. cloacae, P. aeruginos, S. dysenteriae, Klebsiella spp., H. influenza, Proteus spp., S. aureus & S. hemolyticus group B.	New, heated, UV- exposed and heated- stored honey	Noori Al-Waili, 2004.
	12	<i>E. coli, S. aureus</i> , methicillin- resistant <i>Staphylococcus aureus</i> (MRSA) & methicillin-sensitive <i>Staphylococcus aureus</i> (MSSA)	<i>Gelam &amp;</i> coconut honey	Almahdi <i>et al</i> . 2003.
	13	E. coli, P. aeruginosa, K. pneumonia, P. mirabilis, C. diversus, C. freundii, P. vulgaris, S. aureus (coagulase positive & negative)	Jamnhul honey (Syzygiu cumini)	Subrahmanyam <i>et al.</i> 2001.
	14	P. aeruginosa	Manuka honey	Cooper et al. 1999.
	15	Helicobacter pylori	Manuka honey	Al Somal, Coley, Molan, & Hancock, 1994.
-	16	S. aureus	Various New Zealand honey	Allen et al. 1991.

Since the discovery of the first antibiotic, penicillin in 1928 by Alexander Fleming, the industry was blooming with commercialization of new antibiotic of different types and classes. Following this successful pharmaceutical era is bacteria evolution and adaptation to survive the eradication attempt. Late 1930s was known as the starting point for the first specific bacterial resistant discovered against sulfonamide (Davies and Davies, 2010). Two modes of bacterial adaptation are by accumulation of various genes on resistance plasmid which each one encoded to a single drug resistance mechanism or increase gene expression for the same purpose (Nikaido, 2009). This is done through five possible mechanisms: (1) the production of antibiotics metabolizing enzymes, (ii) efflux pumps activity to eliminate antibiotics, (iii) modification of cellular target to prevent antibiotics binding, (iv) alternative pathways to bypass the antibiotics transporter (Liu and Pop, 2009).

This phenomenon leads to a serious clinical manifestation called "Superbugs" or literally means high level of multiple drugs resistant bacteria species characterized by enhance morbidity and mortility. There are strains that becoming resistant to almost all antibiotics including disinfectant such as *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA). *P. aeruginosa* together with *Acinetobacter baumanii* are referred as "pan-resistant" Gram negative strains which are said to have no antibiotics can be used effectively against them so far (Nikaido, 2009). It was noted that superresistant bacteria also have capability to enhance virulence and transmissibility which considered as their progressive virulence factor (Davies and Davies, 2010).

Until now, multi-drugs resistance remains a challenging global public health problem which required a continuous and robust solution. Due to this protracted impasse especially the emergence of multidrugs resistant bacteria among the hospital-acquired strains, the physicians were urged to seek alternative remedy since new antibiotics finding is taking too much time and cost as well as too laborious. This is where the utilization of herbs, ginseng, propolis, honey, aloe vera and many more natural products came into the pictures. However, honey seems to be a perfect candidate based on several factors such as easily available, affordable price (cheap in some places), no adverse effect reported so far, and not to mention packed with various health benefits. Studies on the antibacterial property of honey revealed numerous factors that contribute to this biological action. They include;  $H_2O_2$  production, osmolarity (due to high sugar content), low pH, high acidity, low water activity, presence of phenolic compounds, enzymes, proline and antibacterial peptides (Kwakman et al., 2010, Bogdanov, 1997, Bogdanov, 2009, Almahdi Melad Aljadi, 2003b). All these factors depended on honey geographical origin, botanical source of nectar, bee species, season, foraging activity of bees as well as environmental influences. In additions, secondary factors may also affect the quality of honey including processing procedure, transport, harvesting method, packaging and time of processing.



Figure 2.2: Antibacterial factors of honey.

#### 2.3.1.2 Antifungal Activity

Beside bacteria, honey has also been reported to be effective against fungi and yeast. Clinically significant fungi like Tinea capitis and Tinea versicolor which cause superficial skin infection especially in poverty and poor sanitation area were reported to be sensitive to honey (Ngatu et al., 2011). Aspergillus Niger is an opportunistic pathogen, which showed negative effects with the presence of honey; worsen by the addition of starch which increase the diastase number of that honey (Boukraâ et al., 2008, Mundo et al., 2004b). Another aspergillus species named Aspergillus nidulans also showed susceptibility against Saudi Arabian honey (Al-Waili et al., 2013). Phenolic compound particularly flavonoids were reported to have the ability to inhibit the dimorphic conversion of Candida albicans (Candiracci et al., 2012, Al-Waili et al., 2013). Anticandidal activity also demonstrated in Iranian honey against different candida species including C. parapsilosis, C. tropicalis, C.kefyr, C. globrata and C. dubliniensis (Khosravi et al., 2008). Cassia (Cassia javanica), citrus (Citrus reticulate) and ziziphus (Ziziphus spina-Christi) honey were reported to have various degree of antifungal activity against wide range of dermatophytes (trichophyton spp. and Microsporum spp.) being ziziphus as the most effective ones (El-Gendy, 2010). *Rhotorula spp.* is a pigmented yeast which also reported to be susceptible to honey (Moussa et al., 2012).

# 2.3.2 Antioxidant

Free radicals and reactive oxygen species (ROS) are the vital molecules lead to many cellular specific reactions. They are produced via several pathways; (i) cellular respiration, (ii) enzymatically synthesized by phagocytic cell, neutrophils and macrophage, (iii) ionization radiation by ultraviolet (UV) ray and (iv) chemically induced by cigarettes, pesticides, herbicides and fried food. Their general function in normal circumstance includes; defensive mechanism against microbes and pathogens, helped in hormone production (thyroxine), involve in cell function and cell signaling. However, imbalance generation of these unstable molecules may lead to pathological conditions such as oxidative stress, deleterious reaction of some indigenous structures, drug and oxygen toxicities, fibrosis, inflammation, carcinogenic reactions, aging and lipid peroxidation of cellular membrane. Persistence exposure may cause more severe conditions such as atherosclerosis and cardiovascular disease, degenerative neurological ailments, diabetes, reperfusion injury, cancer, cataracts, gastrointestinal inflammatory illness and many more (Almahdi Melad Aljadi, 2003b, Kaur et al., 2012, Singh and Jialal, 2006, Uttara et al., 2009, Sosa et al., 2013, Wright et al., 2006, Baynes and Thorpe, 1999).

Gelam honey was reported to possess protective effect against diabetes- and hyperglycemia-induced oxidative stress (Batumalaie et al., 2013). It has been reported that honey exerted numerous protective effect against oxidative stress of different disease mechanism such as gastroprotective, hepatoprotective, antihypertensive, hypoglycemic and reproductive (Erejuwa et al., 2012). In a review wrote by Khalil *et al.* (2010), they stated that honey components particularly polyphenols reduced the risk of coronary heart disease. Phenolic compound like phenolic acids and flavonoids are among the well-known antioxidant agents, which may contribute to honey antioxidant activity. Different level of phenolic compounds reflected different degree of antioxidative activity of honey. In addition to phytochemicals, antioxidant activity of honey may be attributed to other enzymatic and non-enzymatic factors such as catalase, glucose oxidase, ascorbic acid, organic acids, amino acids, proteins, peptides, Maillard reaction byproducts and carotenoid derivatives (Baltrušaitytė et al., 2007). Antioxidative property of honey has extensively been studied since the finding of phytochemicals content in honey and its role in disease pathogenicity. Associated with its phytochemical content, honey has been reported to have anticancer, anticarcinogenic, antimitogenic and anti-inflammatory activities (Khalil et al., 2010a). Honey contains caffeic acid and other phenolic acids which known to be able to suppress colon tumors in rat. It also contains quercetin, which has antiproliferative effect against glioma and breast cancer cells. Acacetin is one type of flavonoids, which can induce apoptosis and block the cell cycle progression at G1 phase. Antiproliferative effect of honey was also contributed by the presence of proline. Proline-rich compound was reported to have specific inhibitory effects on proliferation of several cancer cell lines; human THP-1, liver cancer HepG2 and breast cancer MCF-7 cells (El-Gendy, 2010).

Many studies reported the scavenging activity of honey against various pro oxidant (Aljadi and Kamaruddin, 2004, Baltrušaitytė et al., 2007, Beretta et al., 2005, Bertoncelj et al., 2007, Blasa et al., 2006, Estevinho et al., 2008, Ferreira et al., 2009, Frankel et al., 1998, Irina Dobre, 2010, Islam et al., 2012, Küçük et al., 2007, Moniruzzaman et al., 2013). It happens either by electron transfer or hydrogen atom transfer to stabilize the reactive molecules, eventually breaking the free radical chain reaction to produce inert low energy products. It is postulated that antioxidant capacity of honey depends on its botanical origin, season, environmental and processing (secondary) factors (Baltrušaitytė et al., 2007). Therefore, different type of honey contains different level of antioxidant capacity. It is directly reflected by the amount of phytochemical as well as other plant-origin antioxidant component in a particular honey.

#### 2.3.3 Wound Healing

Increasing number of available literature showed attention of research in honey wound healing property. Scientific community has begun to accept the fact that honey has the ability to promote wound healing based on the reliable scientific evidence provided by laboratory-based research and clinical trials (Molan, 2004). Al-Waili *et al.* (2011) summarized some of the scientific reports related to wound healing potential of honey around the world. The synergistic effect of various honey components include osmotic effect, acidity,  $H_2O_2$ , hygroscopicity, antioxidant content, and some possible unidentified compounds were said to be associated with honey ability to promote wound healing. It was propagated through stimulation of tissue growth, enhanced epithelialization and minimal scar formation.

Study conducted on Malaysian *gelam* honey demonstrated the stimulation of fibroblast function, enhancement of the synthesis of glycosaminoglycan and deposition of collagen. It was shown to increase the rate of wound contraction and epithelialization as well as improved the nutritional state of experimental animal when administered orally (Aljady et al., 2000). Acceleration of wound healing by honey treatment was also reported in urethral injury (Ayyıldız et al., 2007).the efficacy of manuka honey was suggested to be related to stimulation of cytokines from monocytes, which is widely known to have a significant role in wound healing (Visavadia et al., 2008). In a study involving 32 patients with neuropathic diabetic foot ulcer, manuka honey-impregnated dressing was proven to accelerate healing and wound cleanliness (Kamaratos et al., 2012). In burn wound management, specifically on patients with partial thickness burn (<30%), *tualang* honey was reported to be effective especially in preventing bacterial colonizations (Nasir et al., 2010).

In the application of honey wound dressing, antibacterial potency plays a vital function. It is because wound healing is closely associated with infection eradication and wound hygiene. However, exact mechanism of honey wound healing activity was not fully understood and more studies have to be conducted to elucidate this. Some studies were in disagreement with the aforementioned results where they found that honey did not exert a significant wound healing ability to certain course of wound injuries. For example, manuka honey was reported to give an insignificant effect on the severity of radiation-induced oral mucositis (Hawley et al., 2013). Despite the reported superior outcomes of honey usage, there are certain unconfident vibes on the actual usefulness of honey in superficial and burn treatments (Moore et al., 2001). It is noteworthy to say that wound healing potential of honey is dependable to various factors including the conditions of the wound (exudate), wound location (which may reflect the blood supply), severity of the wound, infections, type of honey used, mode of application, and honey preparation.

#### 2.4 Microorganisms in Honey

Antibacterial activity of honey is attributed to several internal and external factors. Internal factors consist of physicochemical properties of honey and its freshness while external factors may include the medium, bacteria species and growth conditions. Despite of strong inhibitory effect of physicochemical characteristics, there were microorganisms reported to be able to survive in honey. The concern of honey microorganism contamination raised after the report on infant botulism (Arnon, 1998). It is obvious that some microorganisms are able to survive extreme osmotic pressure and low pH level of honey. The possible explanation was the microorganism has adaptation mechanism to overcome the unfavourable conditions, in most cases, spore production ability.

The first infant botulism was reported in California, the United States of America which was firstly misdiagnosed as encephalitis in 1931 (Nevas, 2006, Midura et al., 1979). The possible food items associated with infant botulism are honey and milk. The main reason of this clinical manifestation is the production of toxin by Clostridium *botulinum* whenever in unfavourable conditions especially in poorly developed immune system of infants. This is the reason why detection of honey contaminants is becoming more important. Since then, a few research studies have been conducted to elucidate the possible honey contaminants. Until now, only simple cultivable microorganisms have been screened and isolated from unpasteurized honey. Sinacory et al. (2014) has successfully isolated 464 pure bacteria culture and 117 filamentous fungi strains from 31 nectar and 7 honeydew honey. The molecular-based detection showed the dominancy of spore-forming Bacillus spp. including pH tolerant lactic acid bacilli (LAB). Carvalho et al. (2010) have evaluated three different approaches of yeast identification from honey and found that partial sequence of the 26S rDNA method produced the best results. They were able to isolate Rhodotorula mucilaginosa, Candida magnoliae and Zygosaccharomyces mellis as the predominant species. Microbiological screening of honey also being performed to detect the presence of *Paenibaccilus larvae*, an atiological agent of America Foulbrood (AFB) disease in honeybees (Gilmore et al., 2010).

In spite of potential risk factors, screening and detection of honey microbiological contaminant also may be conducted to evaluate the possible bioactivities. Author Lee *et al.* (2008) concluded that antimicrobial activity of honey may in part be attributed to the production of antimicrobial compounds by bacteria present, notably bacteriocin-like

compounds and peptide antibiotics. A study devoted to isolation of *Lactobacillus acidophilus* from Malaysian honey revealed that their presence may contribute to antibacterial activity of those honey (Mustafa Aween et al., 2012). It is worth noting that microbial contaminant in honey may directly relate to bee species, floral sources, and geographical location and environmental factors which play significant role in microbial survival and spreading.

In general, there are two possible sources of honey microbial contaminations. The primary source includes the raw material such as nectar and pollen. Microbial contaminants may be originating from normal flora of honeybees. Environment surrounds the hive including air and dust also may be classified as primary source of microbial contamination. Secondary source contamination involves the processing procedures of honey. It includes human, equipment and containers as well as packaging materials (Snowdon and Cliver, 1996). However, this mode of contamination usually involves soil- and environment-dwelling bacteria that seldom cause clinical manifestation on human being. On the whole, there are many reasons for us to evaluate the possible contaminants in honey. It is becoming significantly important since honey has been proposed as an alternative remedy in various medical- and health-related disciplines.

# 2.5 Objectives

The general objective of this study is to investigate the antioxidant capacity, phenolic profile and antibacterial activity of Malaysian and Turkish honey.

This study specifically aims:

- I. To determine the antioxidant capacity of phenolic extract of selected Malaysian and Turkish honey
- II. To isolate and identify phenolic acids of selected Malaysian and Turkish honey
- III. To evaluate the antibacterial potency of selected Malaysian and Turkish honey
- IV. To isolate and detect the bacterial contaminant of selected Malaysian and Turkish honey

#### **CHAPTER 3: MATERIALS AND METHODS**

#### **3.1** Honey sample collection

Ten types of honey were used throughout this study. Five of them were collected locally (Malaysia) and remaining five were acquired from Turkey. The identifications of honey's floral origin were performed by the bee hunter based on their geographical hunting area and floral availability at the location of bee hives. It was supported by organoleptic confirmation of every honey.

Malaysian honey included acacia, gelam, kelulut, pineapple and tualang while Turkish honey consisted of lavender, wildflowers, pine, carob blossom and spring honey. Among these honey (table 3.1), six of them were monofloral namely acacia, gelam, pineapple, lavender, pine and carob blossom while four others were polyfloral honey. Acacia honey is derived from tropical acacia species, *Acacia mangium*, plant known to be used widely in forest plantation industry in Sarawak state of Malaysia. *Gelam* is derived from mangrove swamp known as *Malaleuca cajupati powell* while pineapple honey from pineapple flower, *Ananas comosus*, both from Johore state. *Kelulut* is harvested by stingless bees (*Trigona spp.*) and derived from multifloral foraging activity of bees. *Tualang* is wild polyfloral honey produced by giant bee, *Apis dorsata* that built their hives on one of the tallest tropical rainforest tree from species *Koompassia excelsa*. Unifloral honey of Turkish origin are derived from their respective flower named *Lavandula spp*. (lavender), *Pinus spp*. (pine) and *Ceratonia spp*. (carob blossom). Wildflowers honey is derived from multifloral foraging action of bees as well as spring honey, which was produced during the spring season.

All honey samples were kept in dark plastic containers away from direct sunlight at room temperature. To ascertain the reproducibility and reliability of the results, standard commercially available medical grade honey derived from manuka bush (*Leptospermum scoparium*) was included (Comvita Wound Care UMF 18+, New Zealand).

No	Honey	Floral source	Classification	Origin
1	Acacia	Acacia mangium	Unifloral	
2	Gelam	Malaleuca cajupati powell	Unifloral	
3	Pineapple	Ananas comosus	Unifloral	Malaysia
4	Kelulut	Various tropical flower species	Polyfloral	1
5	Tualang	Various tropical flower species	Polyfloral	
6	Lavender	Lavandula spp.	Unifloral	
7	Carob blossom	Ceratonia spp.	Unifloral	
8	Pine	Pinus spp.	Unifloral	Turkey
9	Wildflowers	Various flower species	Polyfloral	
10	Spring	Various flower species	Polyfloral	

#### Table 3.1: Honey samples.

# **3.2** Honey samples preparation

#### 3.2.1 Liquid-liquid extraction (LLE)

To investigate the phenolic profile of honey, phenolic components were extracted using saponification or base hydrolysis liquid-liquid extraction procedure followed by solid phase extraction to recover the phenolics. The procedure was adapted from Wahdan (1998), which was modified by Aljadi *et al.* (2004). Ten grams of wellhomogenized honey were diluted to 50 ml with deionized distilled water (Milli-Q Merck Millipore, Darmstadt, Germany). After thorough mixing, 25 ml of 3 N sodium hydroxide (Merck, Darmstadt, Germany) were added and mixed again. It was then incubated under nitrogen (Linde, Selangor, Malaysia) at room temperature for four hours.

At the end of the incubation period, pH of the hydrolysate was adjusted to 3.5 with 4 N hydrochloric acid (VWR, Singapore). One gram of sodium bisulfite (Sigma, Steinheim, Germany) and 50 ml of ethyl acetate (Merck, Darmstadt, Germany) were added to the hydrolysate in a separating funnel and mixed by shaking the funnel vigorously for five minutes. Organic layer of the mixture was collected and the aqueous part was repeatedly extracted through the same steps up to six times. All organic parts of 10 g honey sample (approximately 600 ml) were combined and concentrated using rotary evaporator (Bunchi, Flawil, Switzerland) under vacuum at 35°C and 40 rpm until the volume was reduced to 10 ml before drying under nitrogen. The dry honey extract was kept at -20°C until further analysis.

#### 3.2.2 Phenolic acid recovery

The recovery of phenolic compounds from ethyl acetate extract was performed according to Seo and Morr (1984) via solid phase extraction at dropwise flow rate. This method utilized  $C_{18}$  column (Waters Corporation, Massachusetts, USA) to absorbed phenolics and eluted using methanol (Merck, Darmstadt, Germany). The  $C_{18}$  cartridge was first preconditioned by passing 5 ml of each methanol and pH 3.5 acidified deionized water sequentially. Dried ethyl acetate extract from section 3.2.1 was dissolved in 5 ml of pH 3.5 acidified deionised water, and passed through that preconditioned cartridge. The elution of adsorbed phenolics was done by passing through 5 ml of 50% (v/v) methanol in water. The recovered fraction was then dried under nitrogen, weighed and stored at -20°C.

#### **3.3** Antioxidant assays

The present study utilised three different antioxidant assays, water soluble as well as total antioxidant activities were evaluated to give a better perspective of antioxidant capacity of honey extracts. They were: (i) 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, to determine the scavenging activity of honey extract by absorbance reduction measurement caused by hydrogen or electron transfer, (ii) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay to measure absorbance inhibition of ABTS<sup>\*+</sup> radical cation and (iii) ferric reducing antioxidant power (FRAP) to measure colour reduction in acidic condition. In addition to that, total phenolic content (TPC) was also performed to evaluate the antioxidant capacity by reduction of antioxidant through electron transfer mechanism. All tests were conducted in two independent experiments.

# 3.3.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Antiradical activity of honey phenolic extract was assessed according to Chen *et al.* (2000). Fresh phenolic extract of honey 1.0 mg/ml was prepared using warm deionized water. An amount of 750 µl was then added to 1.5 ml of 0.09 mg/ml 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma, Steinheim, Germany) solution in methanol. The mixture was left for five minutes at room temperature before mixing with 2 ml xylene (Sigma-Aldrich, USA). Mixture was shaken vigorously, allowed to separate and centrifuged at 300 rpm for 3 minutes after that, the aqueous layer was removed. The absorbance was measure at 517 nm against blank of xylene (X<sub>1</sub>). The absorbance of the aqueous layer, which contains the phenolic extract, was measured at the same wavelength (A<sub>1</sub>). The readings were subtracted to eliminate the additional absorbance of sample,  $A_s = A_1$ -X<sub>1</sub>]. The resulting absorbance  $A_s$  then was compared against a

calibration curve constructed from ascorbic acid (Merck, Darmstadt, Germany) in water (mg/ml). It was expressed as antioxidant microequivalents ( $\mu$ eq) as one antioxidant  $\mu$ eq was referred to the ability to reduce one  $\mu$ mole of pro-oxidant. One  $\mu$ mole of ascorbic acid standard has two antioxidant  $\mu$ eq due to its ability of reducing two molecules of pro-oxidant.

#### 3.3.2 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphuric acid) (ABTS) assay

The ABTS assay based on the determination of the delay in oxidation was adapted from Re at al. (1999). The 2 mM ABTS stock solution was first prepared by dissolving 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphuric acid) diamonium salt (ABTS) (Sigma, Steinheim, Germany) in 50 ml phosphate buffer saline (PBS) (Sigma, Steinheim, Germany) containing the mixture of 8.18 g NaCl (Merck, Darmstadt, Germany), 0.27 g KH<sub>2</sub>PO<sub>4</sub> (Merck, Darmstadt, Germany), 3.58 g NaHPO<sub>4</sub>.11H<sub>2</sub>O (Merck, Darmstadt, Germany) and 0.16 g KCl (Merck, Darmstadt, Germany) in 1 L of deionized water. This solution was adjusted to pH 7.4 with 0.1 M NaOH. An amount of 50 ml of this stock solution was than mixed with 200 µl of 70 mM potassium peroxydisulfate and left in the dark for 16 hours prior to use. This procedure was done to generate ABTS radical cation (ABTS<sup>+</sup>). Reaction mixture then was prepared by dilution of ABTS<sup>++</sup> solution with PBS to reach absorbance of 0.800 ( $\pm 0.030$ ) at 734 nm. Ten  $\mu$ l of honey phenolic extract (10 mg/ml) were added to 3 ml of ABTS<sup>++</sup> solution in 1 cm cuvette giving the final phenolic extract concentration of 0.033 mg/ml. Readings of absorbance were taken in triplicate after 10 minutes at 734 nm against PBS as blank. The percentage of absorbance decrease was calculated using formula of:  $I=[(A_b-A_a)/A_b]$ X 100; where: I = percentage of ABTS<sup>++</sup> inhibition,  $A_a =$  absorbance of honey extract (t=10 min),  $A_b$  = absorbance of the blank (t=0 min).

#### **3.3.3** Ferric Reducing Antioxidant Power (FRAP)

Electron transfer-based assay called Ferric Reducing Antioxidant Power (FRAP) was adapted in this study (Benzie and Strain, 1996). Working FRAP reagent was prepared fresh prior to use. It contained 1% (w/v) ferric tripyridyltriazine (Fe<sup>III</sup> – TPTZ) (Merck, Darmstadt, Germany) solution, 1% (w/v) ferric chloride hexahydrate (FeCl<sub>3</sub>.6H<sub>2</sub>O) (Merck, Darmstadt, Germany) solution and 10 % (w/v) acetate buffer (Merck, Darmstadt, Germany). To start the assay, 300 µl working FRAP reagent was pipetted into 96 wells microtitre plate (NUNC TC Microwell, Denmark). Ten µl of 1.0 mg/ml phenolic extract, deionized water (reagent blank) and standard of 1000  $\mu M$  ferric sulfate heptahydrate (FeSO<sub>4</sub>.7H<sub>2</sub>O) were added into the corresponding wells accordingly. The plate was shaken to aid mixing and the absorbance (A<sub>0</sub>) was measured at 593 nm (Bio-Rad, USA). It was allowed to stand 37°C for 30 minutes before being read again to obtain final absorbance (A<sub>30</sub>). To eliminate the possible interference, sample blank was used containing 300 µl deionized water and 10 µl phenolic extract. FRAP values were expressed in  $\mu M$ , representing the absorbance differences by calculation using the following formula:  $\Delta A_{593}$  sample/ $\Delta A_{593}$  standard X FRAP value of standard (1000  $\mu M$ ) (Almahdi Melad Aljadi, 2003a, Benzie and Szeto, 1999). ΔA<sub>593</sub> was defined as absorbance difference between initial time  $(A_0)$  and 30 minutes incubation time  $(A_{30})$ ,  $(A_0 - A_{30}).$ 

## **3.3.4** Total phenolic content (TPC)

Total phenolic content of honey extract was measured by spectrophotometric procedure adapted from Almahdi *et al.* (2003) which was first described by An *et al.* (2001) utilizing Folin-Ciocalteau reagent. It is based on the reduction of

phosphomolybdic-phosphotungstic acid (folin) reagent to a blue-coloured complex in an alkaline solution in the presence of phenolic compounds. Honey extract from section 3.2.1 was dissolved in deionized distilled water to meet 1.0 mg/ml concentration. The solution was diluted by mixing 0.5 ml into 9.5 ml of deionized distilled water. It was then further mixed with 3 ml 20% (w/v) sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) (Sigma, Steinheim, Germany) saturate solution followed by 1 ml Folin-Ciocalteau reagent (Sigma, Steinheim, Germany), mixed well and kept at room temperature. After 1 hour, the absorbance reading was measured at 750 nm against deionized distilled water blank. Estimation of phenolic content was determined against calibration curve constructed from gallic acid (Merck, Darmstadt, Germany) standard, ranged from 1.25 to 10  $\mu$ g/ml.

# 3.4 Phenolic acids profiling via Liquid chromatography-mass spectrometry (LC-MS)

The honey crude extract (20 mg/ml) from section 3.2.2 was diluted with methanol to meet the concentration of 1 mg/ml and analysed according to Kassim *et al.* (2010). Following the dilution, 1 mL of the honey extract was filtered through a 0.22  $\mu$ m hydrophobic polytetrafluorethylene (PTFE) filter (Millipore, Darmstadt, Germany) into an autosampler vial for LC-MS analysis. An Agilent 1290 Liquid Chromatography system (Agilent Technologies, Santa Clara, CA, USA) coupled to a 6520 Q-TOF tandem mass spectrometer was used to separate compounds from the honey sample. The mass detector was a Q-TOF accurate mass spectrometer equipped with electrospray ionization (ESI) interface and controlled by MassHunter software. Four  $\mu$ l of the crude sample comprising mixture of phenolic compounds were loaded on a 2.1 mm (*i.d*) Narrow-Bore SB-C18 (length 150 mm) analytical column (particle size 3 mM) used with a flow rate of 0.21 mL/min in solution A (0.1% formic acid in water) and solution

B (100% Methanol with 0.1% formic acid) (Merck, Darmstadt, Germany). The gradient run was as follows: 10% B for 7.5 minutes, 10–40% B for 2.5 minutes, 40-45 for 20 minutes, 45-60 for 10 minutes, 60-80 for 2 minutes and 80-100 for 10 minutes. The total gradient time for the LC-MS run was 52 minutes. The ionization conditions were adjusted at 300 °C and 4000 V for capillary temperature and voltage, respectively. The nebulizer pressure was 45 psi and the nitrogen flow rate was 10 L/min. All mass spectrometry data were recorded in both positive and negative ion modes. The acquisition rate was at 1.03 spectra across the ranges 100-2500 m/z for positive mode and 115-3200 m/z for negative mode. Finally, The MS data were analysed using Agilent MassHunter Workstation Qualitative Analysis Software and the compounds were identified using MassHunter Workstation METLIN Metabolite PCD/PCDL Software (Agilent technologies Inc., California, USA).

## 3.5 Antibacterial activity of honey

#### 3.5.1 Inoculum preparation

Four bacteria species were used in this study which were; *Staphylococcus aureus* (ATCC 25923) *Eschericia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus cereus* (ATCC 11778). All bacteria were supplied by Department of Medical Microbiology, University of Malaya except for *B. cereus* that was obtained from Molecular Bacteriology laboratory, Department of Biomedical Science, University of Malaya. All the bacteria supplied were reconstituted into Trypticase Soy broth, (TS) (Difco, USA) and incubated at 37°C. After 24 hours, they were sub-cultured on Muèller Hinton Agar (MH) (Lab M, UK) and incubated again at 37°C overnight before they were transferred into cryogenic vials containing brain heart infusion broth (BHI) (Difco, USA) and 15% glycerol (R & M Chemicals, UK) for long term storage at -70°.

Working bacteria culture was prepared prior to test by inoculating a loopful of primary culture from -20°C storage into universal bottle containing 10 ml of TS broth. The inoculum was incubated at 37°C for 24 hours before proceeding to subsequent assay.

#### **3.5.2** Minimum inhibitory Concentration (MIC)

Minimum inhibitory Concentration (MIC) assay was adapted from Patton *et al.* (2006) and Tan *et al.* (2009) with slight modifications. Working bacteria culture was prepared as described in section 3.7.2 and adjusted to reach 0.5 Mc Farland standards (1 X  $10^8$  cfu/ml). It was then further diluted to meet 5 X  $10^5$  cfu/ml by mixing 1 part of the adjusted culture with 199 parts of TS broth. Volumes of 10 ml TS broth were transferred into 5 properly labeled screw-capped test tubes (Lab Chem, Malaysia). Another empty tube served as the first tube of honey stock solution where it was used to prepare 50% (w/v) honey solution. In that particular tube, 5 g of honey sample was diluted with sterile deionized water up to 10 ml. It was mixed well and filtered through 0.22 µm sterile filters (Merck Millipore, Darmstadt, Germany). A Two-fold serial dilution was prepared accordingly using all five pre-filled tubes. In addition to that, 4 extra tubes containing honey dilution of 5, 10, 15 and 20 % (w/v) were included. All tubes were vortex to aid mixing.

Volumes of 190  $\mu$ l of each honey dilution were aseptically transferred into 96 well flat-bottom microtitre plate (figure 3.5.3) in eight replicates per dilution. The first two wells of every honey dilution served as dilution sterility control (added with 10  $\mu$ l TS broth only). The remaining six wells were mixed with 10  $\mu$ l bacteria culture. Column number 11 and 12 were reserved for batch sterility control and growth control respectively. Volumes of 200  $\mu$ l TS broth were used as assay sterility control in all wells of column 11 while 10  $\mu$ l bacteria culture in 190  $\mu$ l TS broth served as assay growth control in all wells of column 12.

Plate was kept in a shaker incubator at 120 rpm, 37°C for 24 hours. The absorbance of each well was read at 590 nm using microtitre plate reader after incubation. The percentage of inhibition of bacteria growth was calculated by using formula: 1– (Absorbance of test well – Absorbance of corresponding control well) / (Absorbance of assay growth control – Absorbance of sterility control) x 100. Standards were prepared according to well-established two-fold dilution method comprising phenol (1-10%, w/v) (Merck, Darmstadt, Germany), ampicillin (10 mg/L) (Oxoid, UK), ciprofloxacin hydrochloride (5 mg/L) (Oxoid, UK) and tetracycline (30 mg/L;) (Oxoid, UK) (Aljadi and Kamaruddin, 2004, NCCLS, 2001).

# **3.5.3 Minimum Bactericidal Concentration (MBC)**

Streak plate method for MBC test was started by selecting each honey dilution with no bacterial growth from MIC test in section 3.5.3. For each of them, two wells of the corresponding honey dilution were randomly selected and one loopful (10 µl) bacteria suspension was transferred from each well onto MH agar in duplicate. It was spread evenly onto 90 mm in diameter, circular agar media using sterile hockey stick and incubated at 37°C for 24 hours. For verification purposes, the incubation period of any inoculated plate with no bacterial growth was prolonged up to 72 hours. MBC were determined by the minimum concentration that allowed less than 1% of bacterial growth. The MIC-MBC assays were conducted in duplicate for every honey tested.

#### 3.5.4 Agar well diffusion assay

Agar well diffusion assay was adapted from Allen *et al.* (1991) with slight modifications. A volume of 150 ml nutrient agar (NA) (Difco, USA) was prepared according to manufacturer's instructions. It was allowed to cool after autoclaving (at high pressure,  $121^{\circ}$ C for 10 minutes) and standing at 50°C before being seeded with 100 µl of pre-prepared 24 hours bacteria culture (from section 3.5.1). The culture was first prepared by measuring it absorbance to meet 0.5 at 540 nm using TS broth as the diluent and blank. After uniform swirling, the agar was poured into large square bioassay dishes  $245 \times 245 \times 25$  mm dimension (Nunc, Denmark) (figure C5, appendix C). Solidified plate was stored overnight at 4°C upside down to be used on the following day.

Prior to assay, the agar plate was allowed to stand at room temperature for 20 minutes in biosafety cabinet to aid temperature stabilization and to dry the agar surface. Wells were cut into the agar using a sterile cork borer with 8 mm diameter. Honey sample was freshly prepared for each assay and filter-sterilized with 0.22 µm sterile filters. Twenty-five percent (w/v) honey in deionized distilled water was prepared for the total activity test and 25% (w/v) honey in catalase solution (5 mg/mL) was also prepared for the non-peroxide activity test. Aliquots of 100 µl well-mixed honey samples were transferred randomly into each corresponding well in quadruplicate. Sterile deionized water and catalase solution were used as blank. Phenol standards 1% (w/v) to 10% (w/v) were prepared and transferred in the same manner as the samples application. Phenol standards can be used up to one month when stored at 4°C. The plate was then incubated at 37°C for 24 hours. Ampicillin (10 µg), ciprofloxacin (5 µg) and tetracycline (30 µg) were included to ascertain the reproducibility and reliability of the assay and the bacterial resistant profiles. The random location of samples, blanks, controls and standards were recorded properly for references.

The diameter of zones of inhibition of the wells were measured using digital vernier calipers (Mitotoyo, Japan) by measuring them in at least 2 directions perpendicular  $(90^{\circ})$  to each other (figure 3.3). The measurements were performed before all the samples and standards were re-identified to avoid bias. The mean of diameters of inhibition zone for each well and honey sample was calculated and squared. A standard curve was plotted to show phenol concentration (%, v/v) against the mean of square diameter of inhibition zone. The best-fit linear line was drawn and the equation generated was used to calculate honey antibacterial activity from the readings obtained. They were expressed as Equivalent Phenol Concentration, EPC (%, w/v).



Figure 3.1: Measurement of inhibition zones using digital vernier caliper after overnight incubation of 25% Tualang honey. Red arrows showed the perpendicular measurement between one reading to another.

#### **3.6** Isolation and identification of cultivable bacteria in honey

#### **3.6.1** Isolation and collection bacteria strains

Prior to assay procedures, all honey samples were warmed in 37°C water bath for 30 minutes. Five grams of each honey sample was diluted to reach 50% (w/v) with sterile deionized water. It was mixed properly. Inoculating loop (10 µl) was used to inoculate the honey solution onto different 90 mm in diameters, circular agar media and sterile hockey stick was used to spread the solution evenly. Agar media used include; nutrient agar, 5% Columbia blood agar (Oxoid, UK), Mac Conkey agar (MCA) (Oxoid, UK), mannitol salt agar (MSA) (Oxoid, UK), Brilliance blue coliform agar (BBCA) (Oxoid, UK), *Clostridium* isolation agar (CIA) (Sigma, Missouri, USA) and plate count agar (PCA) (Oxoid, UK). Inoculation on nutrient and 5% Columbia blood agar were prepared in duplicate in which one was subjected to anaerobic incubation together with *Clostridium* isolation agar. PCA inoculations were done in quadruplicates. All inoculated media were incubated at 37°C for 48 hours.

Centrifugation method was adapted from Midura *et al.* (1979) with some modifications. Ten grams of each honey samples was weight in 15 ml falcon tube (Becton Dickinson Labware, New Jersey, USA). Twenty ml of sterile deionized water were mixed and the tube was vortexed for homogenization. The tube then was centrifuged at 3300 X g for 30 minutes in 4°C (Eppendorf, Germany). After decanting the supernatant, the pallet was resuspended into 2 ml of sterile deionized water. The suspension was inoculated onto the same microbiological media as mention above. The remaining suspension was subjected to DNA extraction and amplification via polymerase chain reaction (PCR).

Every single bacterial colony observed after 48 hours incubation on any agar plate was properly identified and labeled. Colony morphology was performed and the observations were recorded. For every colony, it was subjected to Gram stain and subculture procedures. The isolates were subcultured on the same agar plate they were previously grown at the same incubation conditions. After that, bacteria grown on the subcultured plates were reexamined again for their colony morphology for confirmation and to avoid contaminations. From the subculture plates, 5-8 colonies were transfer into 2 tubes of brain heart infusion broth with 15% glycerol for long term storage at -70°C. The remaining colonies were subjected to DNA extraction and PCR amplification.

# 3.6.2 Morphology characterization

Colony morphology of bacteria isolates was done twice, immediately after the isolating inoculation step and after subculture step. This is to ensure we were subculturing the same bacteria species and to avoid cross contamination. Colony morphology were done in accordance to Sneath *et al.* (1986) and Goldman & Green (2008) with the determination of colony's size (mm), elevation, colour, margin, pigmentation, texture, appearance and optical property. The colour changes on indicator-based agar media like MSA, MCA and BBCA also observed. Colony growth on 5% Columbia blood agar was observed for their hemolytic property.

## 3.6.3 Gram staining

Gram staining was conducted to all bacteria isolates and observed under oil immersion (Goldman and Green, 2008). In brief, the isolate was aseptically transferred into normal saline on a glass slide, spread uniformly and heat-fixed. The fixed slide then was flooded sequentially with crystal violet, Lugol's iodine, acetone, and safranin for 1 minute each before washed with tap water and blotted gently (Bacton Dickinson & Company, USA). Under 100 times magnification of light microscope, Gram stain characteristics were observed and recorded. It included the Gram staining status (positive or negative), cellular shape of bacteria, arrangement and spore presence. Any unusual and unique characteristics were also recorded for future reference. The slide then was immersed into xylene for at least 10 minutes, blotted gently and mounted using Distyrene-plasticizer-xylene (DPX) mounting medium (Merck, Darmstadt, Germany) for long term storage.

# **3.6.4** Molecular detection and identification of bacteria isolates

#### 3.6.4.1 Bacterial DNA extraction

Bacteria isolate was subcultured accordingly as mention in section 3.6.1. After appropriate incubation period, 6-8 colonies were resuspended into 1 ml sterile saline in 1.5 ml microcentrifuge tube (Eppendorf, Germany). The tube was incubated in the 95°C water bath for 10 minutes before centrifuged at 20000 X g for 1 minute. One  $\mu$ l of the supernatant was subjected to amplification by PCR in next section.

# 3.6.4.2 Polymerase chain reaction (PCR)

The amplification of bacterial DNA was conducted via PCR method adapted from Harris *et al.* (2002) which later was followed by Harris & Hartley (2003). The reaction mixture for PCR was: 1 X PCR buffer (1st Base Laboratory, Selangor, Malaysia), 1.5 mM MgCl<sub>2</sub> (1st Base Laboratory, Selangor, Malaysia), 0.03U/µl of taq DNA polymerase (1st Base Laboratory, Selangor, Malaysia), 400 µM of each dNTPs (1st Base Laboratory, Selangor, Malaysia), 1 µl of DNA template from section 3.6.3.1, 0.4 µM of each primers as in table 3.2 and sterile deionised water to make up 50 µl final volume. The reaction profile was: 94°C initial temperature for 3 minutes, another 26 cycles of 94°C denaturation for 30 seconds, 63°C annealing for 1 minute, 72°C extension for 1 minute and followed by final extension of 72°C for 5 minutes.

Table 3.2: Primers us	ed for PCR reaction	n targeting amp	olification of 2	16S rDNA
	gene seque	ence.		

No	Primer name	Sequence (5'-3')	Tm (°C)
1	16S forward a	GCT CAG ATT GAA CGC TGG	63.1
2	16S forward b	GCT CAG GAY GAA CGC TGG	66.9
3	16S reverse	TAC TGC TGC CTC CCG TA	65.8

#### 3.6.4.3 Agarose gel electrophoresis

Analysis of PCR product was conducted through gel electrophoresis to confirm the presence of amplified target DNA. Two  $\mu$ l of each PCR product were subjected to gel electrophoresis on 1% (w/v) agarose gel (Sigma, Steinheim, Germany) stained with florosafe DNA stain (1st Base Laboratory, Singapore) and ran in 1 X Tris-Acetate EDTA (TAE) buffer (1st Base Laboratory, Selangor, Malaysia) at 100 V for 1 hour together with DNA ladder 100 bp (1st Base Laboratory, Selangor, Malaysia). The visualization of the electrophoresed gel was done under ultraviolet (UV) transilluminator system (Major Science, California USA). The presence of amplified target DNA was indicated by the appearance of 320 bp stained band under the UV.

# 3.6.4.4 PCR product purification

Prior to sequencing, the positive PCR product was purified using Gel/PCR DNA fragments extraction kit<sup>TM</sup> (Geneaid, Taipei, Taiwan) according to manufacturer's protocol. Briefly, the remaining PCR product from section 3.6.3.2 was transfer into 1.5 microcentrifuge tube and five volumes of extraction buffer were mixed. The mixture was centrifuged at 15 000 X g for 30 seconds in spin column. After discarding the flow-through, 600  $\mu$ l wash buffer with ethanol were added and allowed to stand for 1 minute. The spin column then was centrifuged at the same speed for 30 seconds. The flow-

through discarded and the spin column was centrifuged again for 3 minutes at the same speed to dry the column matrix. The purified PCR product was eluted from the column matrix by 50  $\mu$ l elution buffer into a new sterile 1.5 microcentrifuge tube for 2 minutes. The final yield was stored at -20°C until subsequent analysis.

#### 3.6.4.5 Sequencing, database analysis (BLAST)

Purified PCR products were sent to be sequenced by 1st base Laboratories Snd. Bhd. (Selangor, Malaysia). Primers used were the same as mentioned in table 3.2 (1st Base Laboratory, Selangor, Malaysia). The results obtained were aligned and analysed using Chromas Lite version 2.1.1 (Technelysium Pty. Ltd., Queensland, Australia) and BioEdit Sequence Alignment Editor Software version 7.0.5.3 (An Abbott Company, California, USA). Sequences then were checked for any nucleotide discrepancy using Sequence Scanner Software version 1.0 (Life technologies, New York, USA) and adjusted to the most possible alignments. The resulted sequences were compared for their similarity with sequences available in the Genebank Database using Basic Local Alignment Search Tool (BLAST) program (National Centre for Biotechnology Information, National Institute of health; http://blast.ncbi.nlm.nih.gov).

# **3.7** Statistical analysis

All results were analysed using Statistical Package for the Social Sciences (SPSS) Ver. 21.0 (IBM Corp., US 2012). Some method comparisons were analysed using student *t*-test via p value determinations (p<0.05).
#### **CHAPTER 4: RESULTS**

#### 4.1 Sample preparation - phenolic extract

Honey samples underwent preparative phenolic extract procedures before subjected to antioxidant assays (section 3.3) and phenolic profile analysis (section 3.4). The highest weight was obtained from *gelam* honey with 104.9 mg followed by *Kelulut*, *Tualang*, Spring, pineapple, carob blossom, acacia, wildflowers and lavender honey with weights of 104.2 mg, 104.1 mg, 103.8 mg, 103.6 mg, 103.5 mg, 102.8 mg, 102.7 mg, 102.6 mg respectively. The lowest weight was obtained in pine honey with 102.3 mg. The average dry weight of ethyl acetate extraction of honey phenolics was  $103.5\pm0.0083$  mg (table 4.1).

No	Honey sample <sup>n</sup>	Dry weight (mg)
1	Gelam <sup>m</sup>	104.9
2	Kelulut <sup>m</sup>	104.2
3	Tualang <sup>m</sup>	104.1
4	Spring <sup>t</sup>	103.8
5	Pineapple <sup>m</sup>	103.6
6	Carob blossom <sup>t</sup>	103.5
7	Acacia <sup>m</sup>	102.8
8	Wildflowers <sup>t</sup>	102.7
9	Lavender <sup>t</sup>	102.6
10	Pine <sup>t</sup>	102.3
	Mean (±s.d)	103.5±0.008

Table 4.1: Weight of dry ethyl acetate extraction of phenolic acids forMalaysian and Turkish honey.

<sup>n</sup>; 2, <sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

# 4.2 Antioxidant capacity

#### 4.2.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

*Kelulut* honey possessed highest ability to reduce DPPH radicals  $(0.00421\pm0.027\mu eq AAE)$ , followed by *gelam* honey with  $0.00335\pm0.033\mu eq AAE$ . Only these two honey demonstrated reduction ability of more than  $0.003 \mu eq AAE$ . For Turkish honey,  $0.00286\pm0.113 \mu eq AAE/mg$  of spring honey is the highest scavenging activity recorded followed by carob blossom honey with  $0.00272\pm0.117 \mu eq AAE/mg$  activity. Overall, honey showed DPPH reduction values between  $0.00132\pm0.067$  and  $0.00421\pm0.027 \mu eq AAE/mg$  as presented in table 4.2.

No	Honey extract <sup>n</sup>	µeq AAE/mg extract
1	Kelulut <sup>m</sup>	0.00421±0.027
2	Gelam <sup>m</sup>	0.00335±0.033
3	Tualang <sup>m</sup>	0.00298±0.357
4	Spring <sup>t</sup>	0.00286±0.113
5	Carob blossom <sup>t</sup>	0.00272±0.117
6	Lavender <sup>t</sup>	0.00185±0.261
7	Wildflowers <sup>t</sup>	0.00144±0.145
8	Pineapple <sup>m</sup>	0.00163±0.090
9	Pine <sup>t</sup>	0.00136±0.099
10	Acacia <sup>m</sup>	0.00132±0.067

Table 4.2: Scavenging ability of honey on DPPH radicals.

AAE; Ascorbic acid equivalent, <sup>n</sup>; 2, <sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

#### 4.2.2 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphuric acid) (ABTS) assay

Similar to DPPH assay, ABTS readings for Malaysian honey dominated by *gelam* (93.7%) and *kelulut* (92.77%) honey while spring (83.38%) and carob blossom (80.99%) for Turkish honey. Lowest percentages were showed by two Turkish honey; pine and wildflowers honey with 66.29% and 64.18% colour inhibitions respectively. Range of inhibition readings for this assay fell between ranges of 93.74% and 64.18% as presented in table 4.3.

No	Honey extract <sup>n</sup>	I (%)
1	<i>Gelam</i> <sup>m</sup>	93.74
2	Kelulut <sup>m</sup>	92.77
3	<i>Tualang</i> <sup>m</sup>	88.38
4	Spring <sup>t</sup>	83.18
5	Carob blossom <sup>t</sup>	80.99
6	Lavender <sup>t</sup>	77.90
7	Pineapple <sup>m</sup>	72.46
8	Acacia <sup>m</sup>	71.49
9	Pine <sup>t</sup>	66.29
10	Wildflowers <sup>t</sup>	64.18

Table 4.3: Percentage of absorbance inhibitions (I) by 10 mg of honey extract.

<sup>n</sup>; 2, <sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

# 4.2.3 Ferric Reducing Antioxidant Power (FRAP)

FRAP value for *kelulut* and *gelam* honey recorded the highest antioxidant activity with 13.37  $\mu$ Mx100/mg and 11.84  $\mu$ Mx100/mg respectively. Spring and carob blossom honey consistently dominated Turkish honey antioxidant activity by FRAP value of

11.04  $\mu$ Mx100/mg and 10.12  $\mu$ Mx100/mg respectively. Lowest value was recorded by pine honey with 4.23  $\mu$ Mx100/mg. The range of FRAP value of present study fell between 4.23  $\mu$ Mx100/mg and 13.37  $\mu$ Mx100/mg of honey extract (table 4.4.).

No	Honey extract <sup>n</sup>	$\mu M \ge 100/mg = tract$
1	Kelulut <sup>m</sup>	13.37
2	$Gelam^m$	11.84
3	Spring <sup>t</sup>	11.04
4	Tualang <sup>m</sup>	10.47
5	Carob blossom <sup>t</sup>	10.12
6	Wildflowers <sup>t</sup>	6.66
7	Pineapple <sup>m</sup>	6.46
8	Lavender <sup>t</sup>	6.12
9	Acacia <sup>m</sup>	5.18
10	Pine <sup>t</sup>	4.23

Table 4.4: FRAP values of honey extracts.

<sup>n</sup>; 2, <sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

# 4.2.4 Total phenolic content (TPC)

*Gelam* honey recorded highest TPC value with 2.216±0.019 µg GAE/mg followed by *tualang* honey (1.961±0.014 µg GAE/mg). Spring honey (1.765±0.006 µg GAE/mg) from Turkey recorded slightly higher TPC than *kelulut* honey (1.667±0.019 µg GAE/mg). It is followed by carob blossom honey (1.373±0.006 µg GAE/mg), wildflowers honey (1.196±0.018 µg GAE/mg), pineapple honey (1.039±0.013 µg GAE/mg), pine honey (0.960±0.011 µg GAE/mg) and acacia honey (0.922±0.010 µg GAE/mg). Lowest reading was obtained by lavender honey with 0.765±0.015 µg GAE/mg as presented in table 4.5. Highest phenolic content in honey tested (*gelam* honey) was almost 3 fold higher than the lowest reading obtained (lavender honey).

No	Honey extract <sup>n</sup>	µg GAE/mg extract
1	Gelam <sup>m</sup>	2.216±0.019
2	Tualang <sup>m</sup>	1.961±0.014
3	Spring <sup>t</sup>	1.765±0.006
4	Kelulut <sup>m</sup>	1.667±0.019
5	Carob blossom <sup>t</sup>	1.373±0.006
6	Wildflowers <sup>t</sup>	1.196±0.018
7	Pineapple <sup>m</sup>	1.039±0.013
8	Pine <sup>t</sup>	0.960±0.011
9	Acacia <sup>m</sup>	0.922±0.010
10	Lavender <sup>t</sup>	$0.765 \pm 0.015$

Table 4.5: Total phenolic content (TPC).

GAE; Gallic acid equivalent, <sup>n</sup>; 2, <sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

# 4.2.5 Association of antioxidant activity and phenolic content of honey

Correlations between antioxidant activity and phenolic content of honey were evaluated via Pearson's coefficient (r). All honey showed high positive association between antioxidant activity (DPPH) and their phenolic content (TPC), which indicated by r-values that were close to 1 (table 4.6). Highest correlation showed by spring honey (r=0.999) followed by carob blossom (r=0.994), kelulut (r=0.992), wildflowers (r=0.974), lavender (r=0.963), pineapple(r=0.957), pine (r=0.929), gelam (r=0.929), tualang (r=0.927) and acacia (r=0.901).

No	Honey	Pearson's correlation (r)
1	Spring <sup>t</sup>	0.999
2	Carob blossom <sup>t</sup>	0.994
3	Kelulut <sup>m</sup>	0.992
4	Wildflowers <sup>t</sup>	0.974
5	Lavender <sup>t</sup>	0.963
6	Pineapple <sup>m</sup>	0.957
7	Pine <sup>t</sup>	0.929
8	Gelam <sup>m</sup>	0.929
9	<i>Tualang</i> <sup>m</sup>	0.927
10	Acacia <sup>m</sup>	0.901

 Table 4.6: Pearson's correlation (r) between antioxidant activity (DPPH) and phenolic content (TPC).

<sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

# 4.3 Phenolic profile

The present study focused on the characterization of simple benzoic and cinnamic acids in Malaysian and Turkish honey. Ten honey tested contain a combination between six detected phenolic acids; 2,3-dihydroxybenzoic acid, gallic acid, caffeic acid, *p*-salicylic acid, syringic acid and vanillic acid. Honey with the highest number of different combinations were shown by *kelulut* (absent of caffeic acid) and wildflowers (absent of gallic acid) honey with five phenolic acids detected as compared to others while pineapple honey only contains one phenolic acid. All other honey showed the presence of four aforementioned phenolic acids in different combinations; acacia honey

contains 2,3-dihydroxybenzoic acid, *p*-salicylic acid, syringic acid and vanillic acid; *gelam* honey contains 2,3-dihydroxybenzoic acid, gallic acid, *p*-salicylic acid and syringic acid; *tualang* honey contains 2,3-dihydroxybenzoic acid, *p*-salicylic acid, syringic acid and vanillic acid; lavender honey contains 2,3-dihydroxybenzoic acid, caffeic acid, *p*-salicylic acid and syringic acid; carob blossom honey contains 2,3dihydroxybenzoic acid, *p*-salicylic acid, syringic acid and vanillic acid; pine honey contains 2,3-dihydroxybenzoic acid, caffeic acid, *p*-salicylic acid and syringic acid; spring honey contains 2,3-dihydroxybenzoic acid, *caffeic acid, p*-salicylic acid, syringic acid and vanillic acid. The *p*-salicylic acid was found in all ten honey tested. Gallic acid was detected in two Malaysian honey which are *gelam* and *kelulut* while caffeic acid was found in three Turkish honey namely lavender, pine and wildflowers honey. Only pineapple honey did not contain 2,3-dihydroxybenzoic acid and syringic acid. Summary of results are presented in table 4.7.

		Ph	enolic ac	ids		
Honey	2,3- Dihydroxybenzoic acid	Caffeic acid	Gallic acid	<i>p</i> - Salicylic acid	Syringic acid	Vanillic acid
Acacia <sup>m</sup>				$\checkmark$	$\checkmark$	$\checkmark$
<i>Gelam</i> <sup>m</sup>			$\checkmark$	$\checkmark$	$\checkmark$	
<i>Kelulut</i> <sup>m</sup>			$\checkmark$	$\checkmark$		$\checkmark$
Pineapple <sup>m</sup>				$\checkmark$	<b>NO</b>	
<i>Tualang</i> <sup>m</sup>					$\checkmark$	
Lavender <sup>t</sup>		$\checkmark$		$\checkmark$	$\checkmark$	
Carob blossom <sup>t</sup>	$\checkmark$			Å	$\checkmark$	$\checkmark$
Pine <sup>t</sup>		$\checkmark$	$\square$	$\checkmark$	$\checkmark$	
Wild flowers <sup>t</sup>	$\checkmark$	V		$\checkmark$	$\checkmark$	$\checkmark$
spring <sup>t</sup>	$\checkmark$			$\checkmark$	$\checkmark$	$\checkmark$

# Table 4.7: Phenolic acids detected in Malaysian and Turkish honey.

 $\sqrt{}$ ; Detected, <sup>m</sup>; Malaysian honey, <sup>t</sup>; Turkish honey.

# 4.4 Antibacterial activity of honey

# 4.4.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Growth effects of Gram-positive bacteria species against Malaysian and Turkish honey were tested on *S. aureus* and *B. cereus* in the present study (table 4.8). The lower value of MIC and MBC tests indicated high efficacy of honey in inhibiting and/or eradicating bacterial growth as demonstrated by *gelam* honey. Only 5% (w/v) of *gelam* honey was required to inhibit *S. aureus* growth while 6.25% (w/v) to kill them. It is followed by *tualang* honey that inhibits the growth of *S. aureus* at 10% (w/v) and killed them at 15% (w/v). These values were reported to be equal to the concentration obtained by commercially available manuka honey (Comvita 18+), which served as standard reference honey in this experiment. On the other hand, Turkish honey exhibited higher MICs and MBCs. Most effective Turkish honey were carob blossom and spring honey, which equally inhibited *S. aureus* at 12.5% (w/v) concentration. Lowest MBC however was shown by spring honey by 15% (w/v) concentration. Highest MIC and MBC value against *S. aureus* recorded by lavender and pine honey with value of 25% (w/v) and 50% (w/v) respectively.

*B. cereus* was less affected by honey samples as compared to *S. aureus*. Lowest concentration of Malaysian honey needed to inhibit *B. cereus* was 15% (w/v) as exerted by *gelam* and *tualang* honey. The same concentration also required by *gelam* honey to kill this bacteria species. Turkish honey was represented by carob blossom and spring as the most effective honey to inhibit the growth of *B. cereus* at 15% (w/v) while killed them at 20% (w/v). Identical to *S. aureus*, lavender and pine honey also less effective against *B. cereus* with 25% (w/v) of MIC and 50% (w/v) MBC. Interestingly, *kelulut* honey possessed uniform MIC and MBC concentration. Phenol standards inhibited *S. aureus* at low concentration (0.5%, w/v) while 2% (w/v) were needed to kill them. However, it took 1% (w/v) of phenol concentration to inhibit and eventually kill *B. cereus*. Both strains were generally sensitive to three standard antibiotics used. The MBCs fell between 0.063% (w/v) to 16% (w/v) of antibiotics concentrations.

	Staphylocod	ccus aureus	Bacillus	s cereus
Honey/standards	MIC (%,	MBC (%,	MBC (%,	MBC (%,
	w/v)	w/v)	w/v)	w/v)
Acacia	20	25	20	25
Gelam	5	6.25	15	15
Kelulut	20	20	20	20
Pineapple	15	25	20	25
Tualang	10	15	15	20
Lavender	25	50	25	50
Wildflowers	20	25	20	25
Pine	25	50	25	50
Carob Blossom	12.5	25	15	20
Spring	12.5	15	15	20
Manuka (Comvita 18+)	10	15	10	12.5
Artificial honey	50	>50 <sup>a</sup>	50	>50 <sup>a</sup>
Phenol solution	0.5	2	1	1
Ampicillin	0.25	0.25	16	16
Ciprofloxacin	0.5	1	0.125	0.25
Tetracycline	0.125	0.125	0.063	0.063

 Table 4.8: MIC and MBC of honey against Gram-positive bacteria.

<sup>a</sup>: the highest concentration tested

Higher MICs and MBCs were recorded on antibacterial effect of selected Malaysian and Turkish honey against Gram-negative bacteria as presented in table 4.9. *E. coli* was most susceptible against *gelam* (Malaysian) and spring (Turkish) honey, which recorded MIC of 12.5% (w/v) for both while MBCs of 15% (w/v) for *gelam* and 20% (w/v) for spring honey. The highest MICs against *E. coli* were given by acacia, pineapple, lavender and wildflowers honey as they demonstrated 25% (w/v) concentration. Three of these honey also recorded highest MBCs (50%, w/v) namely acacia, pineapple and lavender while wildflowers honey exerted equal MIC and MBC. Susceptibility analysis of *P. aeruginosa* outlined highest antibacterial activity by *gelam* honey with MIC of 10% (w/v) and MBC of 12.5% (w/v). It was followed by *tualang* honey, which again possessed equal antibacterial level as standard manuka honey (Comvita 18+) with the similar value of MIC (12.5%, w/v) and MBC (20%, w/v). *Kelulut* honey consistently inhibited and killed Gram-negative bacteria at 20% (w/v) concentration as shown in Gram-positive bacterial species. *P. aeruginosa* was more sensitive to phenol solution, which required 0.5% (w/v) of phenol standards for MIC while 1% (w/v) for MBC. *E. coli*, however required 1% (w/v) of phenol standards to be inhibited while 2% (w/v) to be killed. *E. coli* was obviously sensitive to all standard antibiotics tested while *P. aeruginosa* was affected only by ciprofloxacin. It was unaffected against ampicillin while least sensitive against tetracycline with MIC of 64% (w/v) and MBC of 128% (w/v). Artificial honey exerted 50% (w/v) MIC while failed to kill all four bacterial species at all concentrations tested.

	Escher	richia coli	Pseudomona	ıs aeruginosa
Honey/standards	MIC (%,	MPC(0/m/m)	MIC (%,	MBC (%,
	w/v)	$\mathbf{WIDC}(70, \mathbf{W}/\mathbf{V})$	w/v)	w/v)
Acacia	25	50	20	50
Gelam	12.5	15	10	12.5
Kelulut	20	20	20	20
Pineapple	25	50	25	50
Tualang	20	25	12.5	20
Lavender	25	50	25	50
Wildflowers	25	25	20	25
Pine	20	25	25	50
Carob Blossom	20	20	15	25
Spring	12.5	20	15	20
Manuka (Comvita 18+)	20	25	12.5	20
Artificial honey	50	>50 <sup>a</sup>	50	>50 <sup>a</sup>
Phenol solution	1	2	0.5	1
Ampicillin	4	4	>128 <sup>a</sup>	NT
Ciprofloxacin	0.125	0.25	0.25	0.25
Tetracycline	2	4	64	128

Table 4.9: MIC and MBC assay of honey against Gram-negative bacteria.

<sup>a</sup>: the highest concentration tested, NT: Not Tested

Growth kinetic of Malaysian honey against Gram-positive bacteria species were analyzed and presented in figure 4.1. Negative growth inhibition readings were recorded at the lowest two concentrations of acacia honey against B. cereus. The prominent increases in growth inhibition were recorded starting at 12.5% (w/v) acacia honey concentration until it reached maximum inhibition effect. E. coli was the slowest bacteria to reach maximum inhibition effect against acacia honey. It only reached maximum inhibition level when treated with 20% (w/v) concentration. Gelam honey, however, exerted a different degree of growth response curve. At lowest concentration (1.6 %, w/v), gelam honey was able to inhibit more than 50% of S. aureus growth while reached maximum inhibition effect at 5% (w/v) concentration (figure 4.1 b). The least affected strain against gelam honey was recorded by B. cereus that reached the maximum growth inhibition at 15% (w/v). Figure 4.1 (c) exhibits growth inhibition spectrum of *kelulut* honey where all bacteria species were almost equally affected. As honey concentration increase, the growth inhibitions of tested bacteria increased until it reached maximum growth inhibition effects at 20% (w/v) honey concentration. Growth inhibition kinetics against pineapple honey in figure 4.4 (d) also exhibited the same pattern, which S. aureus appeared to be the most susceptible strain. At the lowest pineapple honey concentration, B. cereus exerted negative value. Dose response curve of *tualang* honey in figure 4.1 (e) displays the unique responses on S. aureus and P. aeruginosa. The readings increased sharply to meet maximum growth inhibition (10% w/v and 12.5%, w/v respectively) from considerably lower growth inhibition (less than 50% inhibition) of previous honey concentration (6.3%, w/v and 10% w/v respectively).



Figure 4.1: Dose response curves of Malaysian honey against four different bacteria species. Data are representative of two independent experiments (error bar, s.e.m).



Figure 4.1: continued.

Growth kinetics of Turkish honey against bacteria are presented in figure 4.2. Lavender honey exhibited similar inhibitory effect against all bacterial species as 25% (w/v) honey concentration was required to reach the maximum growth inhibition effect (figure 4.2 a). The similar kinetic pattern of bacterial growth was also exhibited by wildflowers and pine honey as shown in figure 4.2 (b) and (c) respectively. In dose response curve of wildflower honey, maximum growth inhibition effect of S. aureus, B. cereus and P. aeruginosa were achieved at 20% (w/v) concentration while 25% (w/v) for E. coli. Pine honey however exhibited species-dependence response where Grampositive bacteria reached maximum growth inhibition effect at 25% (w/v) concentration meanwhile Gram-negative bacteria were at 20% (w/v) honey concentration. Other negative values of growth inhibition were recorded against B. cereus at the lowest concentration of carob blossom and spring honey (figure 4.2 d and e). The sharp increments of bacterial inhibition were demonstrated by carob blossom honey at 10% w/v concentration against S. aureus to meet maximum inhibition at 12.5% w/v and against B. cereus at 12.5% w/v concentration to achieve maximum inhibition at 15% w/v.



Figure 4.2: Dose response curves of Turkish honey against four different bacteria species. Data are representative of two independent experiments (error bar, s.e.m).



Figure 4.2: continued.

#### 4.4.2 Agar well diffusion assay

The total antibacterial activities of honey inclusive of peroxide components were generally higher than non-peroxide activities. Highest activities recorded by spring honey against *B. cereus* with 28.54 EPC for total activity and 27.72 EPC of non-peroxide activity. Four sets of measurements were recorded above 20 EPC which were: *gelam* (total: 23.04 EPC, non-peroxide: 22.31 EPC), *tualang* (total: 27.61 EPC, non-peroxide: 27.35 EPC) and spring (total: 28.54 EPC, non-peroxide: 27.72 EPC) honey against *B. cereus* and *kelulut* (total: 26.49 EPC, non-peroxide: 25.74 EPC) honey against *S. aureus*. Lavender honey failed to inhibit the growth of Gram-negative bacteria, *E. coli* and *P. aeruginosa* regardless to the presence of peroxide components. Similarly, pine honey showed no inhibitory effect on *E. coli* at the concentrations tested. Notably, antibacterial activities of *tualang* honey closely resembled standard manuka (Comvita +18) honey, especially on Gram-negative bacteria. *Overall*, honey activity against *B. cereus* appeared to be approximately one fold higher than those of Gram-negative bacteria, *E. coli* and *P. aeruginosa*. Table 4.11 summaries the antibacterial activity of Malaysian and Turkish honey.

Despite the difference recorded between total antibacterial and non-peroxide activities, only seven reading were statistically significant (figure 4.3 and 4.4). Among Malaysian honey, only acacia demonstrated significant different between total and non-peroxide antibacterial activity against *B. cereus* with p value of 0.0136. Six Turkish honey which recorded the significant different were; lavender honey when treated against *S. aureus* (p=0.0017), carob blossom honey against *E. coli* (p=0.0234) and *B. cereus* (p=0.0240) and *B. cereus* (p=0.0006).

	S. aı	ireus	Е. с	coli	P. aerı	iginosa	В. се	ereus
Honey samples	Total	Non- peroxide	Total	Non- peroxide	Total	Non- peroxide	Total	Non- peroxide
Acacia	14.56	13.99	7.85	7.59	8.00	7.85	16.12	11.52
Gelam	18.35	18.25	16.28	16.17	14.51	14.20	23.04	22.31
Kelulut	26.49	25.74	10.56	9.67	13.16	12.48	21.01	19.55
Pineapple	19.76	19.71	9.57	9.20	12.48	12.01	13.21	13.94
Tualang	16.94	16.08	14.13	13.12	16.80	16.22	27.61	27.35
Lavender	8.53	6.50	-	-	-	-	14.09	12.06
Wildflowers	9.10	7.33	9.33	6.48	9.57	7.85	21.11	13.10
Pine	6.97	5.67		-	6.71	8.22	14.66	12.69
Carob Blossom	10.19	9.46	13.06	9.89	8.94	8.84	24.65	18.51
Spring	15.86	14.87	11.50	11.58	10.92	10.4	28.54	27.72
Manuka (Comvita +18)	20.38	19.81	14.25	13.52	16.80	16.17	25.84	26.88
Mean (EPC)	15.19	14.31	11.84	10.80	11.79	11.42	20.90	18.69

# Table 4.10: Antibacterial activity of Malaysian and Turkish honey, Equivalent Phenol Concentration (EPC).

Equivalent phenol concentration (EPC) was calculated in undiluted honey. Square of mean diameter of inhibition zone were multiplied by dilution factor and density of Malaysian honey, 1.3 g/ml to obtain 100% honey concentration (Almahdi Melad Aljadi, 2003a).



Figure 4.3. Total and non-peroxide antibacterial activity of Malaysian honey against four tested bacteria. Data are representative of quadruplicate experiments (error bar, s.e.m). \*; Significant different (P $\leq$ 0.05, 95% CI), P values as indicated in parentheses.



Figure 4.3 continued.



Figure 4.4. Total and non-peroxide antibacterial activity of Turkish honey against four tested bacteria. Data are representative of quadruplicate experiments (error bar, s.e.m). \*; Significant different ( $P \le 0.05$ , 95% CI), P values as indicated in parentheses.



Figure 4.4. continued.

The correlation between antibacterial performances was measured by Pearson coefficient (r) between EPC and MIC. All bacteria demonstrated intermediate negative association between EPC and MIC (figure 4.5). It was shown that correlation between EPC and MIC of honey against *S. aureus* was r=-0.462. Almost similar correlations were observed when *E. coli* and *B. cereus* were treated with tested honey with r-values

of -0.494 and -0.556 respectively. The highest association was obtained between EPC and MIC of *P. aeruginosa* with *r*-value of -0.692. As a whole, the total bacteria population obtained moderate negative correlation between EPC and MIC with *r*-value of -0.476.



**Figure 4.5: EPC and MIC association of honey against tested strains.** Pearson's correlation coefficients *r*, were calculated to demonstrate intraspecific bacterial association between EPC and MIC. Each symbol represents individual type of tested honey measured as a mean of two independent experiments.

# 4.5 Isolation and identification of cultivable bacteria in honey

#### 4.5.1 Isolation and morphology characterisation

. A number of 39 colonies were successfully grown on enrichment medium like Columbia blood agar, which was used to isolate the fastidious bacteria under both aerobic and anaerobic conditions. Selective agar media such as MSA were used to isolate the selective bacterial strains and were able to isolate 5 strains in this study. Thirteen isolates were obtained from nutrient agar while 4 isolates were anaerobically grown on CIA. In total, 61 bacteria strains were successfully isolated from 10 samples of Malaysian and Turkish honey. There were no bacteria colony isolated from CIA when incubated anaerobically after 48 and 72 hours. For the centrifugation method as elaborated in section 3.6.1, there were no bacteria isolated from all honey tested. Colony count for PCA inoculation was 7.15 ( $\pm$ 1.44) colonies per 10 µl of 50 % (w/v) honey subjected to 40 determinations. This resulted in approximately 1400 CFU/g honey.

Gram staining of isolates found that all 61 strains were Gram-positive bacilli. Some of the staining slides are displayed in figure 4.9. Out of 61 isolates, 8 (13.11%) were nonspore-forming bacilli. The remaining 86.89% were spore-forming bacteria. Some isolates appeared as Gram variable (figure 4.9 a). After repetition of Gram staining, it was confirmed to be Gram positive as presented in figure 4.9 (b). Polymorphic bacilli were shown in figure 4.9 (c) with abundant of curvy and irregular rod shapes, specifically isolated on MSA. Bacteria isolates with endospores are shown by figure 4.9 (d) (central) and figure 4.9 (e) (subterminal). Large bacilli with no spores are shown in figure 4.9 (f).

# 4.5.2 Molecular detection and identification of bacteria isolates

Agarose gel electrophoresis (AGE) of selected PCR products prior to gene sequence are shown in figure 4.7. All bands showed clear 320 bp PCR products indicating the bacterial DNA extraction and PCR procedures were successful. Bacterial isolates included in figure 4.7 were randomly selected from each type of tested honey. They were selected from acacia, *gelam*, *kelulut*, pineapple, *tualang* (2 isolates), lavender, pine, carob blossom, wildflowers and spring honey respectively.



**Figure 4.6: Gram staining of different bacteria isolated from honey under 1000X light microscope magnification.** (a) Gram-variable bacilli from pineapple honey; (b) Gram-positive bacilli from pineapple honey; (c) polymorphic bacilli from *tualang* honey; (d) bacilli with central endospores from *kelulut* honey; (e) bacilli with subterminal endospores from spring honey and; (f) large bacilli from carob blossom honey.



320 bp

Figure 4.7: AGE amplifying 320 bp PCR products of 16S rDNA gene from isolated bacteria. Lane 1: 1000 bp DNA ladder (First Base Laboratory, Selangor, Malaysia), lane 2 & lane 14: negative control (sterile deionized distilled water), lane 3: isolate A-CIA-CO<sub>2</sub>-1 (acacia) lane 4: isolate G-NA-1 (*gelam*), lane 5: isolate K-CBA-3 (*kelulut*), lane 6: isolate N-CBA-CO<sub>2</sub>-1 (pineapple), lane 7: isolate T-NA-2 (*tualang*), lane 8: isolate T-CBA-CO<sub>2</sub>-3 (*tualang*), lane 9: isolate L-CBA-CO<sub>2</sub>-2 (lavender), lane 10: isolate P-NA-1 (pine), lane 11: isolate C-MSA-1 (carob blossom), lane 12: isolate W-CBA- CO<sub>2</sub>-1 (wildflowers), lane 13: isolate S-CBA-CO<sub>2</sub>-1 (spring).

Gene sequencing analysis for 16S rDNA was done by both forward and reverse primers. Sixty-one isolates belongs to 12 different bacteria species were identified up to their genus level (table 4.11). They were 25 strains of *Bacillus pumilus* (40.90 %), 8 strains of *Paenibacillus mucilaginosus* (13.12 %), 6 strains of *Bacillus clausii* (9.84 %), 4 strains of *Microbacterium testaceum* (6.56 %), 4 strains of *Bacillus cereus* (6.56 %), 2

*Bacillus toyonensis* (3.28 %), and 1 strain each of: *Bacillus halodurans* (1.64 %), *Bacillus megaterium* (1.64 %), *Bacillus subtilis* (1.64 %), *Bacillus thuringiensis* (1.64 %), *Brevibacillus brevis* (1.64 %) and *Solibacillus silvestris* (1.64 %). Six isolates only able to be identified for their species were: 2 strains of *Bacillus spp.* (3.28 %) and 4 of *Paenibacillus spp.* (6.56 %).

Three species were reported to be isolated from acacia honey, which were Paenibacillus mucilaginosus (2) and unidentified Bacillus spp. Similarly, 3 isolates were grown from gelam honey which included Bacillus pumilus, Bacillus thuringiensis and one unidentified Bacillus spp. Eight bacteria strains were isolated from kelulut honey where half of them were Microbacterium testaceum while the other half were belong to *Bacillus pumilus* species. Six strains were able to be isolated from pineapple honey, where 5 of them were shown to be Bacillus pumilus with one Bacillus subtilis isolate. Tualang honey contained the highest number of bacterial isolates. Fourteen of them were Bacillus pumilus, 4 Bacillus cereus and an isolate of unidentified Paenibacillus spp. Only 2 bacterial strains were isolated from lavender honey namely Paenibacillus mucilaginosus and Solibacillus silvestris. Three isolates of Paenibacillus mucilaginosus were detected from wildflowers honey. Pine honey contained 8 bacterial isolates which were: Bacillus megaterium (1), Bacillus pumilus (1), Bacillus toyonensis (2), Brevibacillus brevis (1), Paenibacillus mucilaginosus (1) and unidentified Paenibacillus spp. (2). Six strains of Bacillus clausii were detected from carob blossom honey with other 2 bacteria, Bacillus halodurans and Paenibacillus mucilaginosus. Lastly, spring honey contains only one bacteria species of Paenibacillus spp. The percentages of the isolates are presented in figure 4.8 with the dominance of B. pumilus (41%), followed by *P. mucilaginosus* (13%) and *B. clausii* (10%).

Honey origin	Honey type	Bacteria isolated	Frequency
	Acacia	Paenibacillus mucilaginosus	2
	Acacia	Bacillus sp.	1
		Bacillus pumilus	1
	Gelam	Bacillus thuringiensis	1
		Bacillus sp.	1
Malaysia	Kolulut	Microbacterium testaceum	4
Whataysta	Кстини	Bacillus pumilus	4
	Pineannle	Bacillus pumilus	5
	Тпеарре	Bacillus subtilis	1
		Bacillus pumilus	14
	Tualang	Paenibacillus sp.	1
		Bacillus cereus	4
	Lavender	Paenibacillus mucilaginosus	1
	Lavender	Solibacillus silvestris	1
	Wildflowers	Paenibacillus mucilaginosus	3
		Bacillus megaterium	1
		Bacillus toyonensis	2
	Dine	Brevibacillus brevis	1
Turkey	1 1110	Paenibacillus mucilaginosus	1
		Paenibacillus sp.	2
		Bacillus pumilus	1
	Carob	Bacillus clausii	6
	Blossom	Bacillus halodurans	1
	Diosson	Paenibacillus mucilaginosus	1
	Spring	Paenibacillus sp.	1
		Total	61

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Figure 4.8: Percentages of cultivable bacterial contaminants isolated from selected Malaysian and Turkish honey.

#### **CHAPTER 5: DISCUSSION**

The present study focused on the biochemical profile of Malaysian and Turkish honey particularly their phenolic composition and antioxidant capacity. The antibacterial activities of honey against four bacterial strains were also successfully determined. In addition, screening of bacterial contaminants may provide a new dogma for "antibacterial activity" definition of honey and warrants a further interesting investigation.

To date, there are very limited studies on Malaysian and Turkish honey. Most of the available literature focuses on the clinical application of Malaysian honey in treating various ailments such as burn wound, alkaline injury of the eyes, diabetic foot wound and anticancer activity (Khoo et al., 2010, Bashkaran et al., 2011, Sukur et al., 2011, Fauzi et al., 2011, Yusof et al., 2007, Sadagatullah Abdul Nawfar et al., 2011). A few studies reported the potential medicinal benefits of Malaysian honey: as wound dressing agent (Mohd Zohdi et al., 2011, Aljady et al., 2000), protective effects on testicular functions in rats exposed to cigarette smoke (Mahaneem et al., 2011), increase fertility of male rats (Asiyah et al., 2011) and prevention of uterine atrophy on menopausal rats (Zaid et al., 2010). The analytical aspects of honey were not extensively reported thus need to be conducted to evaluate the actual biological potential of these honey. Several physicochemical and biological studies on Malaysian and Turkish honey have taken place to initiates the effort but, they were far too little as compared to analytical study done on honey from first world countries (Küçük et al., 2007, Khalil et al., 2010b, Silici et al., 2010, Yilmaz and Kufrevioglu, 2009). The methods of choice in this study were selected to provide a comprehensive evaluation on the analytical aspect so that we can compare Malaysian and Turkish honey to other honey on their biological profiles such as antioxidant capacity, phenolic content, antibacterial potency as well as bacterial contaminants.

Geographical location of honey production is important because it may reflect the composition of honey thus its biological activity. This is because different geographical location inhabited by different plant community which provide different nutritional value to the consumer (Rosenzweig, 1995, Gentry, 1988). Therefore, chemical composition of honey is directly related to the plant community available at the foraging radius where the bees collect nectar. In relation to that, geographical difference also determines the bee species availability. In tropical rainforest which inhabited by giant honey bee (Apis Dorsata), they build their hives on top of the giant and tall tree due to their durability to avoid predator (Oldroyd and Wongsiri, 2009, Robinson, 2012). This type of bee species also foraging nectar in the area of tall and larger tree instead of forest ground plant thus the source of nectar composition most likely be originating from the nutrient where the plant grow. Apis dorsata F, otherwise, known as rock honey bees are the only species working during the full moon night which enables them to survive in limited vegetation of Himalayan ecology (Tiwari et al., 2010). In some places where giant and tall trees are not a part of the plant community, the giant honeybees may not be dominant. The variation in floral availability, more likely in favor to different bee species like Apis Mellifera, Apis florea or Apis Cerana as well as stingless be species like Trigona spp. and Meliponini spp. which foraging nectar at different nature of plants. Based on these variations, Turkish honey was selected as comparison to Malaysian tropical honey to provide geographical location variation on honey bioactivity.

#### 5.1 Antioxidant capacity

Antioxidant capacity evaluation has been a powerful procedure in determining the nutritive value of foods, beverages as well as natural products. It is among the first-line investigations to be taken into consideration whenever food- and nutrition-related studies are being done. This is crucial to the current research environment because most of the clinical issues are related directly or indirectly to oxidative stress. Ailments such as cancers, cataracts, diabetes, atherosclerosis and neurodegenerative diseases are proven to be closely linked to this phenomenon (Baynes and Thorpe, 1999, Kaur et al., 2012, Singh and Jialal, 2006, Sosa et al., 2013, Uttara et al., 2009, Wright et al., 2006). Using suitable approaches and appropriate implementations, high antioxidant capacity products will help in reducing the risk factors of abovementioned illnesses. Therefore, the present study outlined a few antioxidant capacity assays to evaluate the antioxidant activity in selected Malaysian and Turkish honey.

Generally, antioxidant components can be divided into two major groups; watersoluble and lipid-soluble antioxidants. DPPH and ABTS assays were conducted to evaluate the water-soluble antioxidant in different approaches to evaluate the variation in most utilised techniques. Whereas FRAP test was conducted to represent the lipidsoluble antioxidant evaluation (Almahdi Melad Aljadi, 2003a). All antioxidant assays conducted in the present study were based on electron transfer reactions with color changes as a principle indicator.

The scavenging activity of honey in DPPH assay was presented as ascorbic acid equivalent while ABTS test was based on percentage of solution decolorisation. In DPPH assay, *kelulut* honey showed the highest antioxidant activity followed by *gelam* honey. However, in ABTS assay, *Gelam* honey exhibited the highest percentage of absorbance inhibition over *kelulut* honey. The respective subsequent activities were the same for both assays, namely *tualang*, spring, carob blossom, lavender and pineapple honey (in order of their antioxidant capacity level). Other two honey showed deviation of readings between these two tests where wildflowers honey exerted higher antioxidant activity in DPPH assay as compare to pine and acacia honey which showed to be lowest in ABTS test. Pine honey remained as the second lowest in its antioxidant capacity in both tests. The differences were likely due to different principles underlined these two employed tests which targeted different antioxidant molecules in honey. As DPPH assay targeted hydrogen donor species while ABTS assay catalysed electron transfer mechanism, they most likely targeting different chemical constituents in honey.as different botanical origin of honey compose different chemical constituents (Mannina et al., 2015). In addition, *kelulut* honey was produced by stingless bee which might be another reason to the chemical differences in honey composition hence affected its antioxidant capacity.

Previous study on Turkish acacia honey recorded higher DPPH value than lavender and pine honey (Can et al., 2015). This data were opposite to our finding where Malaysian acacia honey was found to be lowest in DPPH value as compared to lavender and pine honey from Turkish. Geographical origin may play significant contribution in this variation thus emphasizing the critical role of geographical and possibly climatic changes over floral sources. It is because, the plant surrounding geographical location of honey production reflect bee foraging radius thus affect the nectar compositions. The nectar, which coming from the plant is influenced by soil composition at that particular area where the plants obtain their nutrient to grow.

Lipid-soluble evaluation of antioxidant capacity of honey by FRAP test revealed an entirely different pattern of antioxidant power. It starts with *kelulut* as honey with the highest antioxidant power followed by *gelam*, spring, *tualang*, carob blossom,

wildflowers, pineapple, lavender acacia and pine honey (in order of their antioxidant capacity level). Our results are in agreement with previous study conducted by Kishore *et al.* (2011) where they found that *gelam* honey possessed greater scavenging activity (DPPH assay) as compared to *tualang* honey. However, in FRAP assay both studies showed a contradiction where the present study exhibited *gelam* honey to possess higher antioxidant power than *tualang* honey while Kishore *et al.* (2011) showed *vice versa.* This contradiction may indicate the presence of lipid-soluble antioxidant components, which should be taken into account when evaluating honey antioxidant power. A significant disagreement in both studies is also presented by the deviation of DPPH assay in pineapple honey. The present study showed lower scavenging activity of pineapple honey as compared to *tualang* and *gelam* honey while Kishore *et al.* (2011) reported the higher pineapple scavenging capacity. Outcomes of FRAP test however showed consistency with the present data.

According to study conducted by Moniruzzaman *et al.* (2013), *tualang* honey possessed higher antioxidant power (FRAP assay) followed by acacia and pineapple, slightly different with the present finding where pineapple honey possessed higher antioxidant power than acacia honey. The deviations were however much anticipated since the authors performed their test using crude honey while the present study utilised honey extract. The complex mixture of non-extracted honey matrix may contain numerous interfering biomolecules which may influence the results obtained as compared to extracted honey therefore they are most likely incomparable. Study conducted by Can et al. (2015) reported that pine honey possessed higher FRAP value than lavender and acacia honey. Present study however found a disagreement in this measure where FRAP value of pine honey was found to be lowest among those three honey discussed, possibly due to variation in collection time.

Commercially available Folin-Ciocalteu reagent was used in TPC method. Although the actual chemical identity of active compound in this reagent is still unclear, it is suggested the phosphotungstates-molybdates in the complex will change colour from yellow to blue when reduce by antioxidant (Huang et al., 2005). The present study found that *gelam* honey has highest TPC followed by *tualang*, spring and *kelulut* honey. Our results were comparable to the previous study conducted by Aljadi & Kamaruddin (2004) which found that TPC value of gelam honey was 2.14(±0.129) µg/mg. In general, the results lie in equal range of methanolic Anatolia's Rhododendron honey which have been reported to be in the range of between 0.21 and 1.21  $\mu$ g GAE/mg honey (Silici et al., 2010). A study conducted on three Malaysian honey showed some disagreement with our data where they found acacia honey exerted higher TPC value than *tualang* honey (A-Rahaman et al., 2013). However, the result is not comparable to our study since they analysed the crude honey while our data are based on honey extract evaluation. Present study showed insignificant different of TPC value among acacia, lavender and pine honey while a study conducted on Turkish honey reported significantly low TPC value of acacia honey as compared to lavender and pine honey (Can et al., 2015). Kucuk et al. (2007) reported higher TPC value of chestnut, heterofloral and *Rhododendron* honey of Turkey with the values of 2.39, 1.98, 1.32 µg GAE/mg honey respectively.

The excellent association was found between antioxidant activity of honey and TPC. The DPPH assay was chosen to represent antioxidant activity of honey in this analysis due to its wide recognition as antioxidant capacity test over the other two tests. This correlation is in strong agreement with Chua *et al.* (2013) where they found that antioxidant activity of honey as evaluated via different antioxidant assays have significantly higher correlation to the total flavonoid content and total phenolic content. This is because phenolic acids possess antioxidant capacity (Robbins, 2003, Silva et al.,
2000). High activity of antioxidant may implicate high phenolic acids contents in honey hence justify the correlation. Some studies found the significant correlation between antioxidant capacity, phenolic content and color of honey (Bertoncelj et al., 2007, Beretta et al., 2005). The correlation between antioxidant capacity and honey colour potentially becomes a limelight in current research interest. A number of studies suggested the interrelation between antioxidant capacity and honey colour (Socha et al., 2009, Blasa et al., 2006, Bertoncelj et al., 2007, Beretta et al., 2005, Alvarez-Suarez et al., 2010, Sant'Ana et al., 2014). This may in part be due to phytochemicals particularly phenolic compounds present in honey at high level. Dark honey color indicates high amount of phytochemicals thus possess higher antioxidant activity. The present study, however did not evaluate this correlation hence this interesting aspect remains to be elucidated.

### 5.2 Phenolic acids profiling

Honey is known to contain a wide range of phenolic compounds. From very simple hydroxybenzoic acid to complex flavonoids, abundant of different phenolics were reported to be present in honey. Phenolics is one of the two major medicinal substances present in plants other than terpenoids that fueled honey with various bioactivity through its nectar. Several benefits were reported to be associated to these organic biomolecules. For example, in medical and health related field where their antioxidant activity are high, phenolic acids may help in preventing cancer and cardiovascular diseases (Tripoli et al., 2005, Khalil et al., 2010a). Some researchers suggested these compounds as suitable candidates to determine honey authenticity (Anklam, 1998, Yao et al., 2004).

In addition to authentication, phenolic compounds have also been used as criteria of standardization by some researchers. Allen *at al.* (1991) used equivalent phenol concentration to screen the bioactivity of New Zealand honey against *S. aureus*, which specifically showed the honey antibacterial activity. This method was then followed by Irish *et al.* (2011) indicating the acceptance of the standardization method among the researchers. The present study also adapted the same evaluation protocol for honey antibacterial activity as described in section 3.5.5 with some modifications and adjustments. The method of choice was selected based on the knowledge that phenolic compounds are ubiquitous in honey and can be used as a benchmark for honey testing procedures.

The same basis had led to the evaluation and profiling of honey phenolic compounds in the present study. Although diverse components of phenolic compounds have been reported to be present in honey, the present study concentrated on simple but major phenolic acids such as hydroxybenzoic and hydroxycinnamic acids. Among the simple phenolic acids existed, six of them were found to be present in the Malaysian and Turkish honey tested as presented in tables 4.7 and 4.8. The *p*-salicylic acid has been found in all tested honey indicating that it is an important component of honey. However, author Can et al. (2015) did not report any salicylic acid presented in Turkish honey particularly acacia, lavender and pine. Salicylic acid is known as a plant defense regime and broadly distributed in plants (Chen et al., 2009). Therefore, its presence in honey suggests that it is from botanical origin of nectar. As one of the key components of aspirin, it may give clinical advantages to honey as a supplement. 2,3-Dihydroxybenzoic acid was detected in all honey except pineapple honey. It is suggested to be a good candidate for iron shutter in chelation therapy of  $\beta$ -thalassemia (Giardina and Grady, 2001). Syringic acid was detected in all tested honey except for pineapple honey. Vanillic acid was reported to be absent in gelam, pineapple, lavender and pine honey. Caffeic acid appeared almost exclusively in Turkish honey specifically in Lavender, pine and wildflower honey whereby gallic acid was only detected in two Malaysian honey, *gelam* and *kelulut*. This finding concurred with Can *et al.* (2015) where no gallic acid was detected in acacia, lavender nor pine honey. Caffeic acid however has been detected in Turkish acacia honey reported by that author while present study did not detect them in Malaysian acacia honey. Geographical variation may be a major factor contributed into this difference. Phenolic compounds such as syringic, caffeic and vanillic acids have been reported to demonstrate excellent antibacterial effect against microbes including *Eschericia coli*, *Klebsiella pneumonia*, *Aspergillus flavus* and *Aspergillus parasiticus* (Aziz et al., 1997). Syringic and vanillic acids were also reported to possess hepatoprotective effect in mice which render an interesting nutritive value to honey (Itoh et al., 2009).

There are very limited literatures reporting the analysis of phenolic acids in Malaysian and Turkish honey. The present study supports the finding made by Kassim *et al.* (2010) where they reported that *gelam* honey contains gallic acid. In addition, the same study also detected caffeic, chlorogenic, *p*-coumaric and ferulic acids from *gelam* honey. In another study, *gelam* honey was reported to also contain benzoic and cinnamic acids (Almahdi Melad Aljadi, 2003b). Sarfarz and Nor Hayati (2013) reviewed that *tualang* honey also contains benzoic acid, gallic acid, *p*-coumaric acid, *p*-coumaric acid and caffeic acid while lacking of 2,3 dihyroxybenzoic acid, *p*-salicylic acid and vanillic acid. The present study did not detect the additional phenolic acids mentioned, which may be explained by the different in honey origin, batch and the method of analysis used. Pine honey from Poland was reported to contain caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, gentisic acid, synaptic acid and syringic acid (Socha et al., 2009). Only caffeic and syringic acids detection was concurred with the present study indicating different geographical origin of common botanical source of

honey may also influence its chemical constituents. The present study did not subjected to enzymatic hydrolysis during sample's preparative phase thus excluded any bounded acids which requires enzymes for their release.

## 5.3 Antibacterial activity

Antibacterial activities of honey have been at the center of interest among researchers around the world. It is postulated to be closely dependent to several factors such as  $H_2O_2$ , osmolarity, pH and other minor yet important constituents such as phenolic acids and flavonoids. Most studies either recorded qualitative evaluation of activity or semi-quantitative effect of honey as reported by Allen *et al.* (1991), Mundo *et al.* (2004), Tan *et al.* (2009), Kwakman *et al.* (2010), and Irish *et al.* (2011). The present study combined both methods of evaluation to obtain better understanding in defining antibacterial activity of selected Malaysian and Turkish honey.

MIC data in the present study were collected by means of a spectrophotometric endpoints evaluation. This measurement was chosen based on a number of reasons, including high sensitivity, reproducibility, minimal time consumption, reduced cost, fewer amounts of sample and reagents required, and most importantly, less subjectivity as it does not involve human observations with the naked eye. Authors Patton *et al.* (2006), Sherlock *et al.* (2010) and Brudzynski *et al.* (2011) used  $T_{24}$ - $T_0$  different times comparison to measure the antibacterial effect of honey. In our preliminary test,  $T_{24}$ - $T_0$ different time comparisons showed a critical problem of inconsistency in the results recorded. In honey sterility control test, the final readings ( $T_{24}$ ) deviated from initial readings ( $T_0$ ) detected. Some honey exerted increased spectrophotometric readings while others showed otherwise even though at high honey concentration. This was expected to be constant due to the absence of bacterial growth. We suspected this could be due to volatile compounds present in honey as suggested by a number of studies (Anklam, 1998, Bogdanov, 1997, Bogdanov, 2009, Bogdanov et al., 1999). At initial time (T<sub>0</sub>), these compounds were still in a complex mixture within the honey solution, hence, were measured as part of the sample. After 24 hours, under incubation temperature of  $37^{\circ}$ C, some volatile compounds could have evaporated, thus, affecting the measurements recorded. A significant reduction in the spectrophotometric reading led to false evaluation of bacterial growth. The degree of reduction of spectrophotometric readings was suggested to be dependent on the amount of volatile compounds in honey. As a complex mixture of different molecules and compounds, the other chemical constituents of honey might also affect its absorbance, including minerals, peptides, amino acids and alkaloids which can produce major interference (Bogdanov, 1997). Therefore, this method of measurement was avoided and single endpoints (T<sub>24</sub>) method of measurement was chosen instead.

This study refers MIC as the lowest concentration of honey solution required to at least inhibit 99% of bacterial growth. MBC however is defined as the lowest concentration of honey solution required to kill at least 99% of the tested bacterial strains. Semi-quantitative methods exhibited equal bacteriostatic and bactericidal effects of *kelulut* honey whereas all other honey showed higher MBC value than MIC. The findings suggest that *kelulut* honey possess unique antibacterial response on the tested bacteria regardless to their species and survival abilities. This could be due to the presence of unique organic antibacterial factors obtained by stingless bee (*Trigona spp.*) rather than typical honeybee (*Apis spp.*), as well as nectar's botanical origin. Tan *et al.* (2009) reported that manuka honey (Kordel's, UMF 10+) contains higher antibacterial activity than *tualang* honey against *S. aureus*, *E. coli* and *P. aeruginosa*. Conversely, the present study obtained a lower MIC for *tualang* honey against all three mentioned bacteria, probably due to different batch of honey and technical variations involved.

Nonetheless, the pattern of antibacterial response of this particular honey was in agreement, i.e., *S. aureus* was found to be the most susceptible bacteria, followed by *P. aeruginosa* and *E. coli*. In facts, many study concurred with the finding that Grampositive bacteria particularly *S. aureus* is more susceptible to different type of honey (Lusby et al., 2005, Cooper et al., 1999, Willix et al., 1992).

Increment of *B. cereus* growth, denoted by negative values (Figures 4.1a, d, 4.2d and e), in low concentrations of acacia, pineapple and spring honey might be due to the concentration of glucose which is sufficient to support *B. cereus* growth but not concentrated enough to inhibit them by osmotic pressure. This parameter however did not further investigated and remain interesting to be clarified in future. Most honey inhibited more than 20% of bacterial growth at the lowest honey concentration tested (1.6%, w/v) with a few exceptions. *B. cereus* was the most unaffected bacteria when treated with low honey concentration, except for *kelulut* and manuka honey. Despite the adaptive ability of *Bacillus* species which are capable of withstanding alteration of their surrounding environment by generating endospores, the growth of *B. cereus* were still inhibited and eventually killed by all types of honey tested (Logan, 1988). However, no further test was done to ascertain whether *B. cereus* were totally killed or were sporulating to withstand the antibacterial effects of honey.

Antibacterial activity tests by agar diffusion assay are usually performed in many different ways - well/cup diffusion, disk diffusion, agar dilution or dual layer/double diffusions. The method of choice usually depends on the nature of antibacterial agents to be tested and the kinetic properties of molecules inside. The present study utilised well diffusion assay because honey is a complex solution consisting of different sizes of chemicals and compounds (Bogdanov, 1997). The exclusion of large molecules that are not properly absorbed by the paper disk may occur when using disk method and may lead to inaccuracy. The chosen method, agar well exercise allows possible contact of honey components to the bacteria mimicking *in-vivo* condition whenever honey is applied on infected wounds, and therefore, may provide useful information about the kinetic system of honey topical dressing. The duration of this physical contact may affect the overall honey antibacterial performance since over time, honey will be diluted by body fluid and/or wound exudates and will literally generate more  $H_2O_2$  (White Jr et al., 1963). This method was performed to evaluate the antibacterial activity of honey at constant concentration (qualitatively) as compared to semi-quantitative evaluation by MIC/MBC tests, which included different concentrations. The present study was implementing the equivalent phenol concentration (EPC) as the main measurement parameter because it is comparable with unique manuka factor (UMF), a commercially know measurement system to quantify non-peroxide antibacterial activity based on honey density (Snow et al., 2005). Hence it is worth saying that EPC has similar level of antibacterial activity as UMF.

Specifically, *S. aureus* was most susceptible to *kelulut* honey, *E. coli* was most affected by *gelam* honey, *P. aeruginosa* was equally susceptible to *tualang* and manuka (+18) honey and *B. cereus* was highly susceptible to *tualang* and spring honey. Conversely, the growth of *S. aureus* was least affected by pine honey, *E. coli* was not affected by 25 (w/v) of lavender and pine honey while *P. aeruginosa* was not affected by lavender honey but poorly affected by acacia, pine and carob blossom honey. Growth of *B. cereus* however recorded medium to high susceptibility to honey with the lowest shown by pineapple honey at more than 13 EPC (table 4.10 & figure 4.4). Our findings also recorded that some Malaysian and Turkish honey have higher antibacterial activity as compared to well-known manuka (+18) honey as proven by *kelulut* honey against *S. aureus, gelam* honey against *E. coli* and spring honey against *B. cereus* (table 4.10).

In the present study,  $H_2O_2$  was removed from the honey solution to measure the antibacterial effect of honey without the presence of peroxide molecules (Allen et al., 1991). Student's t-test was used to compare the total and non-peroxide activities of tested honey. Only seven readings showed significant difference (p < 0.05) between these two activities that implies the influence of H<sub>2</sub>O<sub>2</sub> presence in honey's antibacterial activity. We found that antibacterial activity of wildflowers honey was highly dependent on  $H_2O_2$ . Without the present of  $H_2O_2$ , wildflowers honey antibacterial activity reduced significantly when treated against all bacteria except P. aeruginosa. This result indicated that wildflowers honey composes lower non-peroxide antibacterial component as compared to other honey tested. P. Aeruginosa was shown to be affected by honey antibacterial activity regardless to their H<sub>2</sub>O<sub>2</sub> composition. All honey, with or without the presence of  $H_2O_2$  proved to inhibit *P. aeruginosa* equally, most likely due to synergistic effect of honey's physicochemical components toward molecular structure of P. aeruginosa that renders inhibition. To our knowledge, no previous study conducted to test the effect of non-peroxide honey component against P aeruginosa, therefore no comparison could be made to draw some hypothesis regarding to this data.

Six-tested honey shown to have high non-peroxide antibacterial activity were; *gelam*, *kelulut*, pineapple, *tualang*, pine, and spring honey. The antibacterial activity was not affected significantly by the absence of  $H_2O_2$ . They were just slightly reduced to some extent (Figure 4.3 & 4.4) indicating that  $H_2O_2$  is still one of the components of honey's antibacterial system. Some of the readings from agar diffusion assay generated interesting information when compared to MIC/ MBC values. For example, *kelulut* honey exerted high MIC/MBC values (20%, w/v), which theoretically means poor antibacterial effect, but gave large zones of inhibition on agar diffusion assay, especially against *S. aureus*, indicating high antibacterial activity. This contradicting result between the two assays might be due to the properties of their chemical constituents. At

high honey concentration, particularly concentrations above MIC value, they easily diffuse throughout the agar and inhibit bacterial growth in a large area. The variation in chemical composition might possibly be due to the unique property of *kelulut* honey as mentioned earlier. Further analysis is required on the chemical composition of the antibacterial compounds to elucidate this.

Contradicting results were also detected in the MIC/MBC assays against EPC measurement for B. cereus. High values of MIC/MBC data (Table 4.8) were recorded for this particular bacteria indicating poor antibacterial effect while agar diffusion assay showed high EPC value (Table 4.10), especially for gelam, kelulut, tualang, carob blossom, spring and manuka (+18) honey. A possible explanation might be the adaptive ability of this species, as discussed earlier, which caused the bacteria to be highly affected at a particular level of honey concentration while remaining unaffected at low concentrations. Our study emphasized that even though honey has high antibacterial potency against some bacteria species, it was not conclusive that they were both quantitatively and qualitatively excellent. In theory, low MIC value should give high EPC value since both are expected to have a high antibacterial potency. As such, the association of these two variables should illustrate a negative correlation, which is consistent with our data (Figure 4.8). Kelulut honey is unique and should be tested and analysed separately from the blossom honey. Our findings concurred with the latest antibiotic resistance issue, i.e., the serious therapeutic challenge presented by Gramnegative bacteria, P. aeruginosa and E. coli, due to its bacterial adaptive mechanism against current available antibiotics (Arias and Murray, 2009). This situation suggests that Gram-negative bacteria are less susceptible to available antibiotics compared to Gram-positive bacteria, which is consistent with our data as shown in figures 4.8, 4.9 and 4.10. Data of present study are also in line with most recent antibacterial testing conducted on the formulation of honey nanofibers where the authors found that synergestic antibacterial effects of chitosan nanofibers with honey were more effective against *S. aureus* than *E. coli* (Sarhan and Azzazy, 2015). The same study highlighted the fact that honey can be used together with other substance to maximize its therapeutic and medicinal affects without losing its bioactivity.

Agar well diffusion assay shows that lavender honey failed to inhibit *E. coli* and *P. aeruginosa*, both are Gram-negative bacterial species. In addition, pine honey also failed to inhibit *E. coli*. This situation occurred regardless to the presence of peroxide component at 25% honey concentration. One possible explanation would be the poor activity of both honeys that prevent them from inhibiting a particular bacterial growth. Higher honey concentration may increase the antibacterial activity of those honeys against those particular bacteria.

Our study used standard laboratory strains because there are very limited studies reporting on the ten types of Malaysian and Turkish honey of interest against these bacteria species. *S. aureus* was included as it is widely used as the standard Grampositive strain of preliminary assay with *E.coli* representing the Gram-negative strain (Allen et al., 1991, Irish et al., 2011, Patton et al., 2006, Moore et al., 2001, Bonev et al., 2008). *P. aeruginosa* represented a prominent healthcare-associated pathogen and *B. cereus* was chosen to represent spore-forming species which might also became clinically important particularly in food poisoning (Arias and Murray, 2009, Bottone, 2010). Reproducibility and repeatability of the tests were verified using commercially available Comvita +18 UMF manuka honey against standard strains, *S. aureus* (ATCC 25923). Allen *et al.* (1991) stated that +18 UMF means that the honey contains at least 18% (w/v) phenol equivalents of non-peroxide activity. This study was proven to be reproducible when the assay on Comvita +18 UMF Manuka honey produced the result of 18.38 UMF, SD  $\pm$  0.14%. Artificial honey was used to demonstrate the osmotic

effect of honey against bacteria preferably to exclude the osmotic factors of natural honey. MIC and diameters of inhibition zones for all antibiotics were reproduced for susceptible strains of all bacteria tested as determined by Clinical Laboratory Standard Institute (CLSI) (data not shown NCCLS, 2001: CLSI, 2005; CLSI, 2007 & CLSI, 2012]). The effectiveness of catalase was assayed to affirm that the catalase added was working well in removing all  $H_2O_2$  molecules and its activity was not affected by other components.

# 5.4 Screening of honey bacterial contaminant

Despite having a widely known antibacterial activity, honey was repeatedly reported to contain various bacterial species. This is due to contamination. There are two types of honey contamination that may occur. Primary contamination is attributed to bacterial transfer from primary sources such as gut of honeybee, pollen-colonised species, and hives dwelling bacterial species. Secondary contamination may come from processing procedures; which include dust and contaminated air, container as well as the food handlers themselves. Decontamination of honey may happen naturally by the high osmotic pressure, acidity and other antibacterial components of honey, which may confer the safeness of honey. However, in several occasions where bacterial adaptive ability enables them to withstand the antibacterial activity of honey, some bacterial species may survive the 'extreme' condition of honey. This may jeopardize the safeness of honey. The spore-forming bacteria may survive and later on infect consumers and cause serious clinical manifestations. For example *Clostridium botulinum* in infant botulism case as discuss in section 2.5. Beholding this concern, the present study outlined the bacterial contaminant screening to evaluate the possible bacterial contaminant, which may be present in tested Malaysian and Turkish honey. This is also to provide comprehensive antibacterial profile of tested Malaysian and Turkish honey.

The 16S rDNA gene sequencing was conducted to detect the bacterial contaminants at genus and species level based on bacterial conserved region on 16S ribosomal DNA. According to Janda & Abbott (2007), this method of choice provide identification at genus level in most cases (>90%) and 65 to 83% of species identifications. They cited that 1 to 14% of bacteria isolates typically remain unidentified. Our statistical result showed that 6 isolates (9.84%) were detected at the genus level while remaining 55 isolates (90.16%) were characterized down to their species identity. However, the similarities of the isolates were in the range of 87 to 100% with mean of 95.5%, median and mode of 99%. To conduct such procedure, there are various set of primers used by different studies and references (Inbakandan et al., 2010, De Clerck et al., 2004, Frank et al., 2008, Fierer et al., 2005, Devereux and Wilkinson, 2004). However, we decided to adapt the primers previously used by Harris & Hartley (2002) due to the clinical relevance of broad-range bacterial isolates. The analysis showed that Bacilus pumilus is the major bacterial contaminant in tested honey (figure 4.11). This particular species was known as soil dwelling bacilli. La Duc et.al (2007) reported that B. pumilus is among the bacteria that is highly resistant to extreme environmental conditions such as limited nutrient availability, low pH and acidity, desiccation, irradiation, H<sub>2</sub>O<sub>2</sub> and chemical disinfections. The endospore enables *B. pumilus* to survive the antibacterial activity existed in honey therefore raised a reasonable alert of their presence in Malaysian and Turkish honey tested. Theoretically, this bacterial species are likely to be the prominent honey contaminant base on the fact that it is a soil and environmental dwelling bacteria, which may be transferred into honey by bees on foraging activity. This finding evoked a new perspective of honey's antibacterial profile as B. pumilus was reported to produce bacteriocin, pumilicin and pumilin, which were proven to be effective against MRSA and VRE (Bhate, 1955, Aunpad and Na-Bangchang, 2007). Could this bacterium contribute to honey's antibacterial activity by producing bacteriocin? This question remains to be elucidated in future investigations.

In total, 14 different isolates were successfully detected from Malaysian and Turkish honey (figure 4.11). The present study found that some bacterial species were isolated solely from a specific type of honey. For example, *Microbacterium testaceum* was isolated only from *kelulut* honey, *Bacillus toyonensis* from pine honey and six isolates of *Bacillus clausii* from carob blossom honey. As for now, there is no evidence suggesting the specific bacterial contaminants may come from specific floral source, but, our study showed some pattern which may initiate a further interesting research on this matter. *B. pumilus* otherwise presented in almost all honey. Above all, *tualang* honey that had been proven to have high antibacterial activity against bacteria as presented in section 4.4, contains the most abundant bacterial contaminants. Nineteen isolates were detected from *tualang* honey with dominancy of *B. pumilus* followed by *B cereus* and one isolate of *Paenibacillus spp*. Conversely, only one bacteria species was isolated from spring honey which was *Paenibacillus spp*.

Study conducted by Mustafa Aween *et al.* (2012) successfully isolated *Lactobacillus acidophilus* from various Malaysian honey using enhanced medium of isolation. However, the present study did not encounter any *Lactobacilli* species, presumably due to non-optimal media used in this study. Some of the isolates from the present study are consistent with bacterial species isolated from Italian nectar and honeydew honey by Sinacori *et al.* (2014). These include; *B pumilus*, *B cereus*, *B subtilis*, *B. thuringiensis* and *B. megaterium*.

The second most abundant species isolated from honey was *Paenibacillus mucilaginosus*. This obligate anaerobe species was also isolated and characterized by La

Duc *et al.* (2007) as one of the strains capable of withstanding an extreme conditions. Six percent of the total isolated bacteria were of unidentified *Paenibacillus sp.* According to Williams (2000), *Paenibacillus larvae* can infect honeybees and cause American foulbrood disease (AFB). A further investigation should be done to confirm the 6% of unidentified *Paenibacillus sp.* and elucidate the uncertainty of AFB occurrence in Malaysian and Turkish honey. Figure 4.11 showed that isolated bacterial species were mainly categorised under genera *Bacillus*, presumably due to endospore characteristic and ability of bacilli to withstand extreme conditions (Nicholson et al., 2000). According to Snowdon and Cliver (1996), the presence of *Bacillus* species was expected to be found in honey. Except for *B. cereus* which has been reported to cause food poisoning, the present study did not detect any clinically important bacteria including *C. botulinum* (Bottone, 2010). Argentinian honey as reported by Iurlina and Fritz (2005) also contained *B. cereus* and *B. pumilus*. Since all isolated bacteria were widely distributed in the soil as environmental community, they are expected to be presented in honey via both primary and possibly secondary contamination routes.

# 5.5 Study limitations and future study prospects

Honey sample for our study only involved one batch representing each type of Malaysian and Turkish honey. A larger sample size should be tested and analysed to obtain a better picture about their correlation. According to Molan (1992), a small number of samples does not represent a particular source of honey as a whole. Therefore, this present study was considered more likely to be a preliminary screening of Malaysian and Turkish honey for their antibacterial potency. It is also worth noting that the results could be different between one batch to another due to various factors such as season, botanical sources, and harvesting time. Four reference bacteria in antibacterial study were standard laboratory strains. Therefore, the results were limited to the susceptibility of laboratory strains and not referred to clinical isolated strains. To diversify our findings, clinical strains should be included. In addition, strains with different degree of antibiotics resistance can also be included to assess the potency of honey against multidrug resistance strains.

The present data were limited to planktonic bacteria. There was no assessment conducted on biofilm effects of tested bacteria although most of the bacteria used are capable of forming biofilm. The effect of honey on bacterial biofilm is another important yet interesting investigation to be conducted in future. This may assure the comprehensive understanding about the mechanism of honey antibacterial effects against bacterial species. Despite the inhibitory effects of honey against clinically important bacterial species, study on honey efficacy on probiotics growth is also important which reveals a new dimension of bioactivities of honey (Das et al., 2015).

Another future prospect of study is to look into the effects of cooking to honey bioactivities. Honey is a well-known "accessory" in culinary industry. Analysis on its bioactivities before and after cooking process is a cornerstone for honey commercialization. The concentrations of HMF, H<sub>2</sub>O<sub>2</sub>, phenolic compounds, enzymes and other heat-sensitive elements should be determined to evaluate honey medicinal consistency before and after cooking. Most importantly, the risk of increase in HMF content and other dangerous molecules should be monitored to avoid health complications in consumers.

Bacterial contamination assay conducted was limited to cultivable bacterial species with growing period up to 48 hours only. Any fastidious bacteria species that requires special growth conditions were excluded due to limited media and assay setup. Slowgrowing bacteria, which may take up to three weeks of growing period, may also have been excluded. Colony forming units of bacterial species were excluded in our study as very few growths were observed on agar plate indicating very low count of bacteria in tested honey.

As suggested earlier, some bacteria isolated from honey were reported to produce bacteriocin which beneficial to treat pathogenic bacterial infections. Therefore, further investigation should be carried out to determine their effectiveness against those strains *in vitro* as well as *in vivo*. The rate of bacteriocin production and its kinetic mechanism should be also included to provide more information for future honey utilization. The occurrence of AFB disease also should be monitored regularly via proper detection analysis among Malaysian and Turkish honey.

#### **CHAPTER 6: CONCLUSION**

The present study has achieved all of its objectives which include the determination of antioxidant capacity, antibacterial activity, phenolic and microbial profiling of Malaysian and Turkish honey. Generally, Malaysian and Turkish honey possessed high to medium level of antioxidant capacity. Investigation of honey antioxidant capacity via three different approaches indicates that *gelam*, *kelulut*, *tualang*, carob blossom and spring honey exerted high scavenging power against radical molecules. A strong correlation between antioxidant and phenolic content of honey emphasized the contribution of phenolic in honey bioactivity.

The isolation of phenolic components of Malaysian and Turkish honey resulted in the characterization of six principle phenolic acids present in those honey. These are 2,3-dihydroxybenzoic acid, gallic acid, caffeic acid, *p*-salicylic acid, syringic acid and vanillic acid. The *p*-salicylic acid was shown to be present in all honey. It is the only simple phenolic acid detected in pineapple honey. Three out of five Turkish honey contain caffeic acid and two honey from Malaysia contain gallic acid. The present study found that at least four phenolic acids (2,3-dihydroxybenzoic acid, *p*-salicylic acid, syringic acid and vanillic acid) were predominant in honey based on their distribution in the tested honey.

The antibacterial potencies of Malaysian and Turkish honey were generally comparable to the well-known New Zealand manuka honey (+18 UMF), with the closest resemblance by *tualang* honey. *Kelulut* honey however showed higher antibacterial potency based on its equivalent phenol concentration, EPC value. Agar diffusion assay proved that all Malaysian honey and three Turkish honeys possess high non-peroxide antibacterial activity. The Malaysian honey, namely *gelam*, *kelulut*, *tualang* and the Turkish honey which is spring honey have high antibacterial potency of total and nonperoxide activities implying that peroxide and other constituents are mutually important contributing factors to the antibacterial system of honey. Antibacterial activity of wildflowers honey was found to be dependent to  $H_2O_2$ component. *P. aeruginosa* was affected to honey regardless to their  $H_2O_2$  content. The correlations between MIC and EPC value of honey were proven to be dependent on bacterial species and honey origin. The spore-forming bacteria, *B. cereus*, were found to be affected differently by tested honey as compared to other bacterial species. *Kelulut* honey has quantitatively poor but qualitatively excellent antibacterial potency. The present study suggested that Gram-positive bacteria are more susceptible to honey as compared to Gram-negative bacterial species.

Sixty-one strains were successfully isolated from unpasteurized honey whereby spore-forming *Bacillus* species are predominant with *B. pumilus* being the most abundant strain. Nineteen cultivable bacteria species were isolated from *Tualang* honey indicating high contamination and relates to improper harvesting procedures. The 16S rDNA gene sequencing method employed was able to detect and characterized only one clinically significant bacteria species from tested honey namely *B. cereus*. No *C. botulinum* species was detected in all the ten samples of honey tested, indicating Malaysian and Turkish honey are generally safe for consumption and unlikely to cause infantile botulism.

The present study had proven that Malaysian (*gelam*, *kelulut* and *tualang*) and Turkish honey (carob blossom and spring) possessed excellent antioxidant capacity and antibacterial potency. The phenolic and contaminant profiles of honey conducted in this study have provided important information regarding honey nutritive value and safeness and can be used as references for further investigation.

### **CHAPTER 7: REFERENCES**

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