CHAPTER II

LITERATURE REVIEW

2.1 Honey

Honey is made by bees and other insects. Bees are the only insects that produce a delicious food consumed by people. Honeys are primarily made of water and carbohydrates (Sampath *et al.,* 2010) and it is produced worldwide where the bees are able to find nectar source. The use of natural honeys by mankind has been in existence from the time of immemorial. Honeys are the oldest sweetener consumed by humans (Abdulwahid *et al*., 2012). Copper, niacin, calcium, magnesium, potassium, riboflavin, iron, and zinc can be found in honey. Also, it contains a blend of flavonoids and phenolic acid which are considered as strong antioxidants due to ability of eliminate potentially destructive free radicals in the human body (Sampath *et al.,* 2010). It was noted that feeding on honey (as food or medicine) can bring high nutritional benefits and therapeutic promises (Nicola, 2009).

Honey bees are social insects (Nicola, 2009). They are classified in the family Apidae, subfamily Apinae, tribe Apini. Close relatives of the honeybees are the orchid bees (*Euglossini*); they belong to an order of Hymenoptera. They live in colonies with a queen, some males and higher number of females workers (Mary, 2007) with a marked division of labor among the various bees in the hive. The bees developed specialized mouthparts and adaptations for collection of pollen. All bees have at least a few plumose hairs and broadened hind legs; they are necessary to gather the pollen and to transport it back to the colony. A colony contains one queen, 500 to 1,000 drones and about 30,000 to 60,000 workers; these numbers are varied according to the bee species. There are two types of bees that produce honey for human consumption (Nicola, 2009).

2.2.1 Sting Bee Species That Produce Honey

There are 4,000 species of native sting bees worldwide. The members of the four most common families are Apidae, Halictidae, Andrenidae and Megachilidae, from remote wildernesses to gardens and backyards. Bees are absent at high mountains (Moisset, 2011). The scientific classification of sting bee species is shown in Table 1.

Table1. Scientific Classification of Sting Bee Species

Kingdom	Anima
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Suborder	Apocrita
Subfamily	Apinae
Tribe	Apini
Genus	Apis
Species: *Apis mellifera*	(ex: *Apis mellifera*)

2.2.2 Stingless Bee Species That Produce Honey

Stingless bee is called stingless honey bees or simply meliponines. They are a large group of honey bee (approximately 500 species) but, they are small in size compared to the sting bee and many of which are poorly known (Rasmussen & Cameron, 2010). Stingless bees (Apidae and Meliponinae) are found in all tropical area of the world. Comparatively, little attention has been given to these bees in beekeeping development programs and limited studies were done on their honey.

However, it is now realized that stingless bees are not less important than the common bee; they are good resources for the production of a special type of honey and other products with high nutrition value (Marinus *et al.*, 1999). The nests of stingless bees are more elaborate and complicated than those of common honey bees. The nests of most species are built within protective cavities like a hollow tree and sometimes in the ground. Few species built their colonies in exposed position because they cannot protect the nest as well as the sting bees. Most of stingless bee built the nest shaped as a flower (Figure 1). Cerumen, a mixture of beeswax and plant resins is the main material they used to build the nest (Marinus, 1999; Vera, 2009). Stingless bee is from a diverse group of highly eusocial bees (meliponines) comprising the tribe Meliponini in the family Apidae. Stingless bees have played an important role in supplying excellent honey (David, 2006). These bees are found in tropical and subtropical regions, of which they are more diverse and numerous in tropical south and Central America (Alain *et al.,* 2013).

Figure 1: Stingless bee nest (Pyper, 2008)

Honey is sour, bitter flowery liquid and have long consumption tradition to which several medical uses are attributed (Kwapong *et al*., 2013). Table 2 describes the scientific classification of one of the stingless bee, *Meliponula ferruginea*.

Table2. Scientific Classification of Stingless Bee Species

Kingdom	Anima
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Family	Apidae
Subfamily	Melipininae
Tribe	Meliponini
Genus: *Meliponula ferruginea*	(eg: *Meliponula ferruginea*).

Stingless bee live in the tropical area of the world, and in Malaysia they can be found in and around lowland, hill and upper hill dipterocarp forest as shown in Figure 2. Salim *et al.* (2012) reported that there are 17 species of stingless bees in Peninsular Malaysia as shown in Table 3.

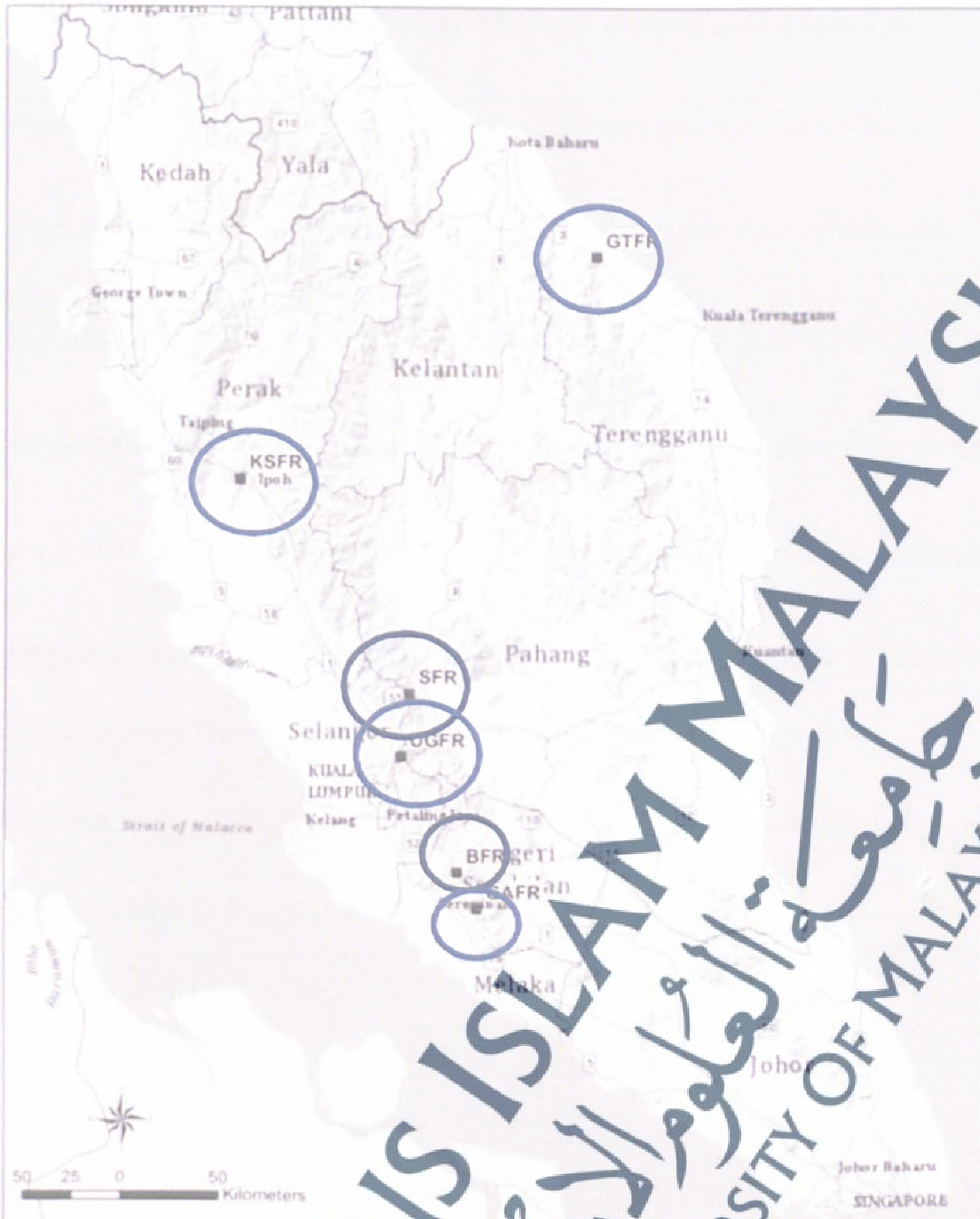

Figure 2: Locations of where stingless bees are found in Malaysia. BFR = Berembun Forest Reserve; GAFR = Gunung Angsi Forest Reserve; GTFR = Gunung Tebu Forest Reserve; KSFR = Kledang Saiong Forest Reserve; SFR = Semangkok Forest Reserve; UGFR = Ulu Gombak Forest Reserve (Salim *et al.*, 2012)

Table 3: Stingless bee species in Peninsular Malaysia

1. *Geniotrigona thoracica* Smith	10. *Tetragonilla collina* Smith
2. *Heterotrigona itama* Cockerell	11. *Tetragonula fuscobalteata* Cameron
3. *Homotrigona fimbriata* Smith	12. *Tetragonula geissleri* Cockerell
4. *Lepidotrigona nitidiventris* Smith	13. *Tetragonula iridipennis* Smith
5. *Lepidotrigona terminata* Smith	14. *Tetragonula laeviceps* Smith
6. *Lepidotrigona ventralis* Smith	15. *Tetragonula melina* Gribodo
7. *Lophotrigona canifrons* Smith	16. *Tetragonula reepeni*
8. *Sundatrigona moorei* Schwarz	17. *Tetrigona apicalis* Smith
9. *Tetragonilla atripes* Smith	

Source: (Salim *et al.*, 2012)

An example of widely distributed stingless bee is *Trigona* that is the largest group of stingless bees; including many more subgenera than the present assemblage (Kwapong *et al.*, 2013). There about 150 species presently included in the genus in 11 subgenera worldwide. These bees differ from each other in only minor structural details, primarily of the hind leg.

Trigona bees are black and like all stingless bees species are divided into two castes: queen and worker. The queens can be easily identified by their long, pale abdomen and by the short wings. Stingless bees are too small. *T. hockingsi* is the largest bee, that measuring approximately 4.5 mm in length, while the smallest one is *T. clypearis*, 3.5mm (Figure 3) (Halcroft *et al.*, 2013). There are over 30 species of *Trigona* bees in Malaysia (Rasmussen and Cameron, 2010).

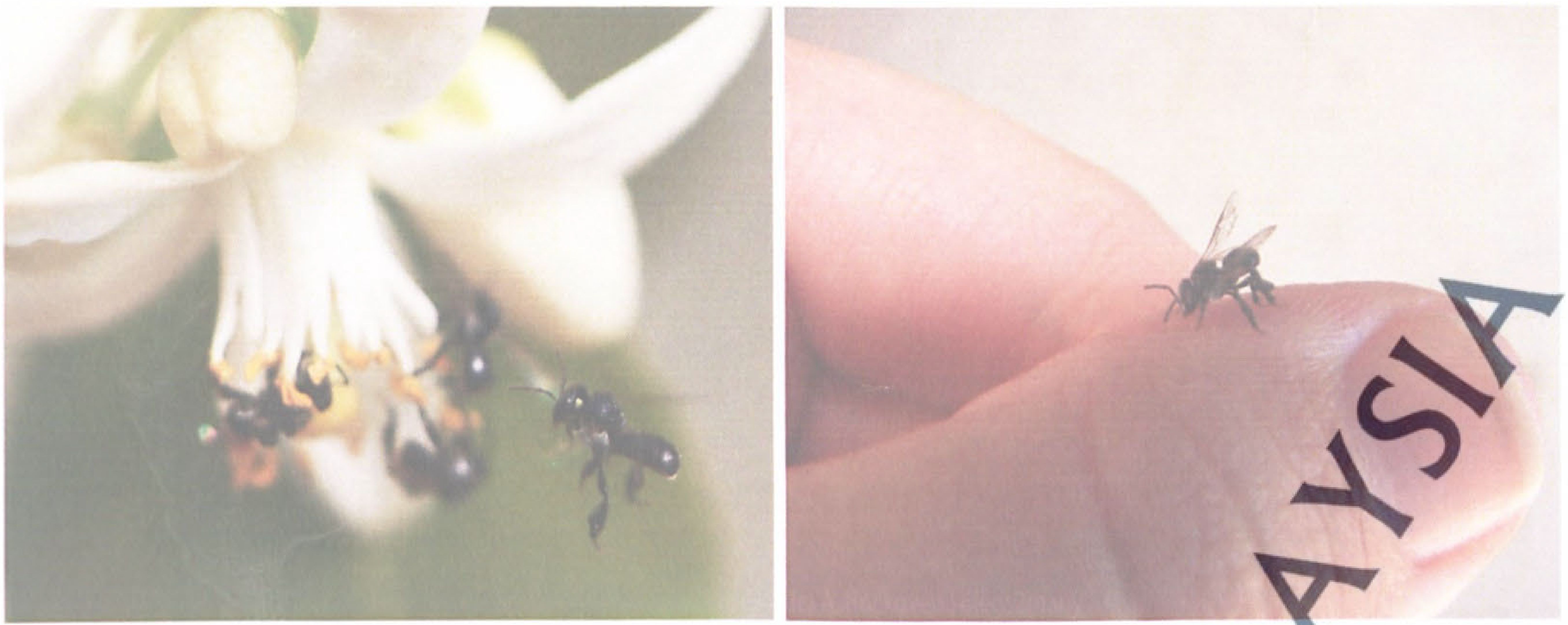

Figure 3: *T. carbonaria* (Halcroft *et al.*, 2013)

In Malaysia, stingless bees are generally known as "Kelulut". The honey from Kelulut bees is rich in antioxidants. Honey from the Kelulut bees is light in color, and the taste is sourish not like the other types of honey. Kelulut honey is still in the research and potential commercialization. A study found that *Trigona* bees in Malaysia have many species of Kelulut bees. In Malaysia, there are 30 over species *Trigona* bees (Rasmussen and Cameron, 2010). Figure 3, 4, 5 and 6 are images of stingless bees commercially reared in Malaysia.

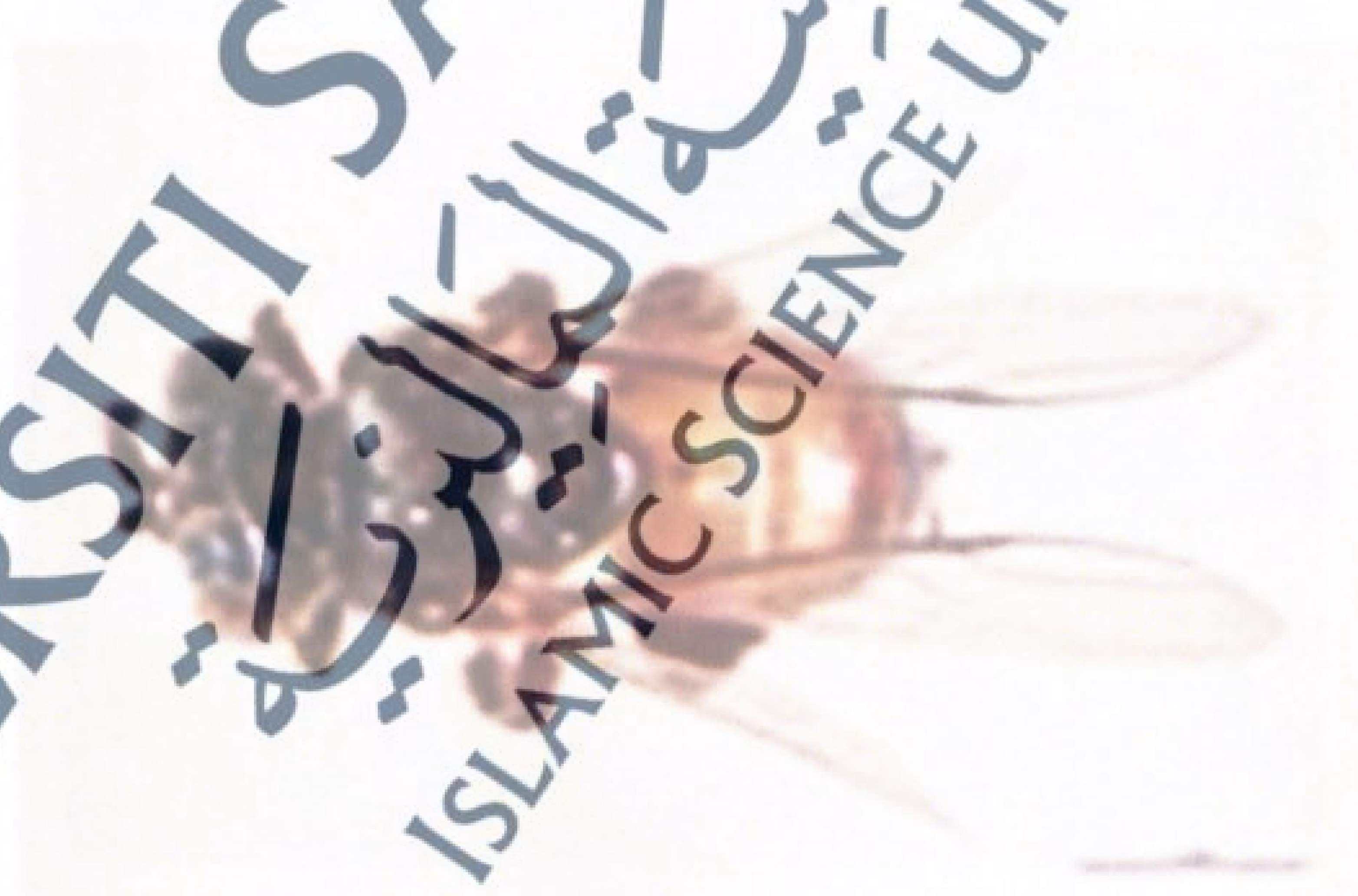

Figure 4: *Hetrotringona laeviceap* (Dollin *et al.*, 2011)

Figure 5: *Heterotringona thoracica* (Anem, 2014)

Figure 6: *Heterotringona Itama* (Anem, 2014)

Figure 7: *Hetrotringona terminata* (Bahari *et al*., 2014)

2.3. Physico-chemical characteristics of honey

Honey is classified into four different categories which are (1) blossom honey, this honey is obtained predominantly from flowers that are the main source of nectar; (2) honeydew honey, which is produced by workers bees after they collect the "honeydew" (secretions of insects belonging to the genus, *Rhynchota*), which pierce plant cells, ingest plant sap and then secrete it again; (3) monofloral honey, in which the bees forage predominantly on a type of plant and which is named according to this plant, and (4) multifloral honey (also known as polyfloral) which has several botanical sources, none of which is predominant, e.g., meadow blossom honey and forest honey (Alvarez *et al.,* 2014).

In Malaysia, Tualang honey is related to the Tualang tree (*Koompassia excels*) in which the bees (*Apis dorsata*) make their nests (Zuraidah, 2010). The Tualang tree can harbour more than 80 combs; each one has between 30.000 and 70.000 bees. This honey is considered as the prized possessions of the rainforest; particularly, for the medicinal benefits it has. According to Zuraidah (2010) Tualang honey collected in Ulu Muda is among the best in the world as the bees gathered nectar from 180 species of flowers.

The physical properties of honey vary depending on the source (type of flora used to produce it), temperature, and the proportion of specific sugars that the honey contains. Fresh honey is a supersaturated liquid, containing more sugar than the water that can typically dissolve at ambient temperatures. However, at room temperature, honey is a super cooled liquid, it is supersaturated solution of four main sugar including glucose, fructose, sucrose and maltose (Weston & Brocklebank, 1999), in which the glucose will precipitate into solid granules. This forms a semisolid solution of precipitated glucose crystals in a solution of fructose and other ingredients (Andrés *et al.*, 2011).

2.3.1 Viscosity

The viscosity of honey is normally affected greatly by the temperature and the water content which probably related to changes in weather (dry and wet seasons). Generally, normal ripened honey has a moisture content of 17.5 to18%, with a water activity of 0.58 (Matther, 1997).

The higher the humidity, the easier honey will flow. However, above the melting point of honey, the water has little effect on the viscosity. Aside from water content of honey, the compositions also have little effect on the viscosity, with the exception of a few types. The viscosity of honey can be increased or decreased depending on the temperature that occurs very slowly at first. A type of honey containing about 16% humidity and at 70°C will have a viscosity of around 2 poises, while at 30°C the viscosity of this honey will be 70 poises. As cooling progresses, honey will attend to become more viscous at an increasingly rapid rate reaching 600 poises around 14°C (Mohammad *et al*., 2012).

However, while most types of honey is very viscous; they have rather low surface tension (Bogdanov *et al.,* 2008). A few types of honey have unusual viscous properties. Honey from heather or Manuka display thixotropic properties. These types of honey enter a gel-like state when motionless, but then, it liquefies when the honey is stirred (Krell, 1996).

2.3.2 Electrical and optical properties

Electrolytes are found in honey in the form of acids and minerals. It has the ability to exhibit varying degrees of the electrical conductivity. Measurements of the electrical conductivity are used to determine the quality of honey in the term of ash content (Bogdanov *et al*., 2008).

The refractive index for honey usually ranges from 1.504 at 13% to 1.474 at 25%. In addition, honey is affected by polarized light, it will rotate the polarization plane; the fructose content of honey gives a negative rotation, on the other hand, glucose content will give a positive rotation. The overall rotations are used as indicator to evaluate the ratio of the mixture of fructose to glucose in honey (Bogdanov *et al.,* 2008; Jenifer *et al.,* 2012).

2.3.3 Chemical composition of honey

The specific chemical components of honey are varied according to the flora available to the bees, locations, harvest season, management, and especially the bee species that produced the honey (Mohd *et al*., 2003; Carlos *et al*., 2008). Adenekan (2012) reported differences in the nutritional content of honey, for example, the mean nutrient contents of honey samples collected from different sources in Ogun State, Southwestern Nigeria for the year 2008, 2009 and 2010 contained different amount of sugar content. The glucose content was 18.42 and 18.91 g 100 g^{-1} obtained from the samples of honey collected from Abeokuta and Ijebu-Ode, respectively, while the mean value of 25.32 and 30.1 g 100 g^{-1}glucose were obtained from Ogere and Ilisan honey samples, respectively. On the other hand, the fructose content evaluated in honey samples ranged from 25.42 – 38.21 g 100 g^{-1}.

Additionally, honeys contain low amounts of many compounds thought to function as antioxidants, including chrysin, pinobanksin, catalase, and pinocembrin (Martos *et al*., 2000; Gheldof, 2002). Honey also contains a wide array of vitamins, such as vitamin C, vitamin B6, thiamin, niacin, riboflavin and pantothenic acid. Essential minerals are found in honeys including calcium, copper, iron, magnesium, manganese, potassium, phosphorus, sodium and zinc as well as other different amino acids that have been identified in different types of honey.

2.4 Antibacterial Properties of Honey

Honey is well documented to have antimicrobial effect especially against Gram positive bacteria (Molan, 1999; Bogdanov, 2011). Pathogenic bacteria which consist of Gram negative and Gram positive bacteria can be inhibited by honey (Table 4). Honey also has been reported to be effective *in vitro* against range of multiresistant pathogenic bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and other multiresistant Gram negative organisms such as *Pseudomonas aeruginosa* (Cutting and Bogdanov, 2014; Mullai and Menon, 2005).

Table 4: Bacteria that were inhibited by natural honeys

Stenotrophomonas maltophilia	Coagulase negative staphylococci
Acinetobacter baumannii	Methicillin-resistant *Staphylococcus aureus* (MRSA)
Salmonella enterica serovar *typhi*	*Streptococcus agalactiae*
Pseudomonas aeruginosa	*Staphylococcus aureus*
Proteus mirabilis	Coagulase-negative *Staphylococcus aureus* (CONS)
Shigella flexneri	*Hemolytic streptococci*
Escherichia coli	*Enterococcus*
Enterobacter cloacae	*Streptococcus mutans*
Shigella sonnei	*Streptococcus sobrinus*
Salmonella typhi	*Actinomyces viscosus*
Klebsiella pneumonia	*Streptococcus pyogenes*
Stenotrophomonas maltophilia	*Porphyromonas gingivalis*
Burkholderia cepacia	*Campylobacter* spp.
Helicobacter pylori	

Source: (Lim *et al.*, 2014; Abdul-Wahid *et al.*, 2012)

The antimicrobial activity of honey is not only effective against the bacteria species, but some kinds of fungi due to the high sugar content and acidity (Molan, 2012). But unlike an antibiotic which normally targets a specific site within an infective agent, the inhibitory effects of honey varies according to type of honey used and the species treated and, multiple effects are often observed (Cooper, 2014).

Honey is recognized to have antibacterial activity since 1980 when honey has been known to heal wounds as well (Deb *et al.,* 2011; Chanpen, 2009). Honey has the ability to regulate oxidative stress in wounds by the inhibition of reactive oxygen species and activating polymorphonuclear neutrophils in wound healing (Cutting &

Bogdanov, 2014). The antimicrobial action of honey depends on the presence of the components that have antimicrobial properties against pathogenic bacteria, among them, the presence of hydrogen peroxide which is produced enzymatically during the production of honey (Dustman, 1979). Aggad and Guemour (2014) reported that remarkable antimicrobial properties of honeys are mainly attributed to the effect of hydrogen peroxide and gluconic acid. In addition, the antimicrobial activity of honey depends on physical factors; a_w, viscosity, water content and the chemical factors (Pimental *et al.*, 2013).

There are two main theories that have been proposed to explain the antimicrobial activity of honey; first theory is related to of the hydrogen peroxide actions (Dustman, 1979), and the other is the non-peroxide activity that inhibits microbial growth. Sugars components are found to be the major content of honeys, in which themselves possess the antimicrobial action because of the osmotic effect they create, although many reports carried out to test this antimicrobial activity using concentrations at which the sugar content are not osmotically active (Viuda *et al.*, 2008).

Sampath *et al.* (2010) also reported that the antimicrobial properties of honey can be resulted due to (a) the low water activity that causing osmosis; the effect of honey is primarily a saturated mixture of low monosaccharides. The mixture has a very low water activity; most of the water molecules are associated with the sugar and only few remain available for micro-organisms, as a result, this environment is poor for the microorganism to growth; (b) hydrogen peroxide effect that is generated by the action of an enzyme added by the bees during the process of nectar to honey and, (c) high acidity where the pH of honey is reported to range between 3.2 and 4.5. This relatively acidic pH level can prevents the growth of many microorganisms. The antibacterial activity is reported to be related to a number of factors other than the acidity of honey. Additionally, honey viscosity is high enough to create a physical barrel which is able to inhibit the contamination of a wound by the infectious agents present in the air and, by the osmosis due to its high sugar concentration (Pimental *et al*, 2013).

The antimicrobial activity of honey varies with the bacteria species and water activity of honey. The a_w of honey is evaluated to be in the range of 0.94 to 0.9960. Many bacteria species are totally inhibited at this water activity. However, few species that have their maximum growth rate at a_w 0.9910 can also be inhibited; in which the inhibition is caused by the osmotic effect (water-withdrawing) of dilute solutions of honey sample (Peter, 1992). Paulus *et al* (2010) have shown that bees make a protein which added to the honey during the production, called defensing-1. This protein gives the honey the ability to treat burns and skin infections.

Aggad and Guemour (2014) reported that remarkable antimicrobial properties of honeys are mainly attributed to the effect hydrogen peroxide. Hydrogen peroxide and osmosis have long been suggested to be the responsible factors for the antibacterial capacity of honey. However, the verification of non-peroxide antibacterial activity of honey diluted to low concentrations has brought the attention to the other antimicrobial agents present in honey (Pimental *et al*, 2013).

The geographical region and flower from which honey is derived influenced the antimicrobial activity of honey (Andualem, 2013). Studies indicated that compounds present in medicinal plants are detected present in honey, and the researchers have suggested that honey can be used as a vehicle to transfer the medicinal components from plants through bees (Alvarez *et al*., 2014). Sidr honey, for example, has strong antioxidant and antibacterial properties. This honey when used to treat bacterial infection shows a great reaction. Alqurashi *et al*. (2013) reported that sidr honey inhibits the growth of Gram negative bacteria (*Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli),* when used at 40%-80% (w/v) in concentration. Increasing the honey concentration, in general, lead to increase growth inhibition of the tested bacteria (Alqurashi *et al*., 2013). Modern science has made it possible to classify the properties of honey as bactericidal, bacteriostatic, antiviral, antifungal, antioxidant and antitumor (Bardy *et al*., 2008; Estevinho *et al*., 2008).

The enzymes present in honey were reported to exhibit the antimicrobial properties. However, in most honeys, the antimicrobial properties produced can also be from non-peroxide components. Manuka honey, for example have a high level of non-peroxide components and display significant antimicrobial effects even when the activity of hydrogen peroxide is blocked (Deb *et al*., 2011; Molan, 2012). Cooper (2014) reported that the inhibitory effects of manuka honey on *S. aureus* and methicillin-resistant *S. aureus* (MRSA) are caused by the accumulation of cells with fully formed cross walls that failed to complete the cell division cycle.

Honey also reported to show antibiofilm activity. Cooper (2014) demonstrated that manuka honey prevented biofilm formation of *Streptococcus pyogenes* by decreasing the expression of two important surface proteins that act as adhesins in facilitating bacterial binding to fibronectin. Bacteria such as *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *E. coli* that pose a serious threat to the public health and antibiotics have been controlled by honey. The antimicrobial capacity of most types of honey is related to the high acidity, hydrogen peroxide and other factors (Alqurashi *et al*., 2013). Recent studies have demonstrated the existence of other compounds contributing to the antimicrobial activity of honey such as methylglyoxal and gluconic acid.

2.5 Honeys from Stingless Bees

The properties of honey from stingless bees are slightly different from honey produced by sting bees. Stingless bee honey has been found to contain agents that display antioxidant action like the enzymes glucose oxidase, phenolic acids, flavonoids and catalase as well as antimicrobial activity. These biologically active compounds in stingless bee honey have received a special attention from research groups for example their role in the prevention of illness that associated to the oxidative stress (Andréa *et al*., 2013; Paulus *et al.*, 2012).

2.5.1 Antimicrobial Activity of Stingless Bee Honey

Recently, the medicinal value of stingless bee honey has been evaluated. Several species of these bees including *Friesiomelita nigra, Melipona solani, M. quadrifasciata* and *Trigona australis* demonstrated to produce honey with greatly appreciated therapeutic and antimicrobial properties (Ortiz *et al*., 2013). However, study on honey obtained from singles bee is limited.

Andréa *et al.*, (2013) reported that stingless bee honey to contain flavonoids that have antimicrobial activity. They also reported that stingless bee honey contains polyphenols, making it more resistant to fermentation than the honey produced by the common honeybee, *A. mellifera*. These two components of stingless bee make it important medicinal aid to fight against bacterial infection. Although the antimicrobial effects of various type of honey are well established, the implicated mechanisms remain incompletely understood (Aggad & Guemour, 2014) due to the incomplete knowledge of the whole antibacterial components that could be involved. Additionally, the variability of antibacterial capacity is major obstacles of honey applications as a treatment. The mechanism of the antimicrobial activity of honey is still complex because of the multi-reactions of the components and also due to the variation in the concentration of the components (Paulua *et al.*, 2012).

Peter (1992) evaluated the antimicrobial activity of SBH samples against unidentified micro-organisms obtained from soil, water and air. He reported that the honey inhibited the growth of bacterial colonies at 70 to 90% (w/v) of honey, 30 to 60% (w/v) of honey inhibited growth of fungi isolated from soil, sewage, air and 25% (w/v) honey inhibited microorganisms from tap water. However, the growth of colonies that obtained from air borne contaminants has been reported to be inhibited completely when tested by 20% of honey and partially by 2% of SBH samples.

2.6 Evaluation of the Antimicrobial Activity

2.6.1 Agar-Well Diffusion Assay

The agar diffusion assay has been the most widely used method for determination of antimicrobial activity throughout history. The results of the agar diffusion test are generally qualitative. In this assay, the antimicrobial compound is added into a well within an agar plates and then, the compound diffuses through the agar, which resulted in a concentration gradient that is inversely proportional to the distance from the wells. The degree of inhibition, which is measured as the zone (zone of inhibition), where no growth around the well, is depending on the rate of the diffusion of the compound and the cell growth (Davidson *et al.,* 2005).