PHYTOCHEMICAL PROFILING, ANTIOXIDANT ACTIVITIES AND EFFECTS OF PROPOLIS DERIVED FROM LOCAL STINGLESS BEE (TRIGONA APICALIS) ON THE EXPRESSION OF ADHESION MOLECULES IN ENDOTHELIAL CELLS

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by

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LIST OF ABBREVIATION

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
$AlCl_3$	Aluminium chloride
CO_2	Carbon dioxide
^0C	Celsius
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DPPH	1-diphenyl-2-picrylhydrazyl
EEP	Ethanolic extract of propolis
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
g	Gram
GE	Gallic acid equivalent
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
hr	Hour
H_2O	water
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
IC_{50}	A concentration required for 50% inhibition
ICAM-1	Intracellular adhesion molecule-1
ICH	International Conference on Harmonisation

IFN-γ	Interferon gamma
Ig	Immunoglobulin
kg	Kilogram
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molar
MeOH	Methanol
mg	Miligram
mL	Mililiter
mM	MiliMolar
MeCN	Acetonitrile
min	Minute
NaCl	Sodium chloride
Na2CO3	Sodium carbonate
NaNO2	Sodium nitrate
NaOH	Sodium hydroxyl
nm	Nanometer
PBS	Phosphate buffered saline
PDA	Photodiode-array
QTOF LC-MS	Quantum of Flight Liquid Chromatography coupled Mass Spectrometry
QE	Quercetin equivalent
Rf	Retention factor
ROS	Reactive oxygen species
S.D.	Standard deviation

SEM	Standard error of mean
T. apicalis	*Trigona apicalis*
TEAC	Trolox equivalent antioxidant capacity
TFC	Total Flavonoid Content
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
TPC	Total Phenolic Content
µg	Microgram
µL	Microliter
µm	Micrometer or micron
µM	Micromolar
uv	Ultraviolet
VCAM-1	vascular cells adhesion molecules-1

MEMPROFILE FITOKIMIA, AKTIVITI ANTI-OKSIDAN SERTA KESAN PROPOLIS LEBAH KELULUT TEMPATAN (TRIGONA APICALIS) TERHADAP EKSPRESI MOLEKUL LEKANTAN SEL ENDOTHELIAL

ABSTRAK

Propolis ditakrifkan sebagai bahan yang melekit berwarna gelap dihasilkan oleh lebah dari gabungan air liurnya dan bahagian tumbuhan. Kinibanyak bukti yang menunjukkan bahawa propolis mempunyai pelbagai sifat biologi yang bermanfaat seperti anti bakteria, anti-kulat, anti-virus, anti-keradangan, anti-ulser, aktiviti anti-oksidan dan immunomodulasi. Kajian ini bertujuan untuk menganalisa komposisi fitokimia propolis *Trigona apicalis* (*T. apicalis*) dari lokasi yang berbeza (B, M, S, K dan T) serta menilai aktiviti anti-oksidan dan anti-keradangan propolis.Sampel-sampel propolis diekstrak dengan kaedah pengekstrakan yang telah dioptimumkan iaitu melalui pensteran berterusan untuk 72 jam pada suhu 37^0C dengan nisbah sampel kepada pelarut 1:10. Kemudian komposisi fitokimia propolis dianalisa melalui beberapa kaedah kromatografi. Aktiviti anti-oksidan seterusnya dinilai dengan menggunakan kaedah yang diterima pakai umum seperti pengurangan radikal bebas iaitu 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), 2, 2'-azinobis- (3-etilbenzotiazoline-6-sulfonat asid (ABTS) dan kuasa penurunan Ferric. Propolis yang menunjukan kesan anti-oksidan paling tinggi akan dipilih untuk menilai potensi anti-keradangannya melalui ujian perekatan molekul intraselular-1(ICAM-1) dan molekul sel-sel vaskular-1 (VCAM-1). Walaupun kaedah pengekstrakan propolis dan spesies lebah yang digunakan adalah sama, tetapi kandungan fitokimia yang perolehi dari analisa HPLC (High Performance Liquid Chromatography) atau TPC (Total Phenolic Content) dan TFC (Total Flavanoid Content) adalah didapati berbeza untuk sampel berlainan.

Penemuan ini mencerminkan bahawa komposisi fitokimia bergantung pada faktor-faktor seperti geografi, musim dan flora di kawasan sekitar lokasi persampelan. Komposisi kimia didapati berlainan bagi setiap sampel dan seterusnya memberikan potensi anti-oksidan juga berlainan. Kajian ini menunjukan kaedah pengekstratan yang telah dioptimumkan melalui pengacauan berterusan 72jam pada suhu 37^0C dengan nisbah sampel kepada pelarut 1:10 menghasilkan hasil ekstrak yang tinggi. Manakala aktiviti anti-oksidan dinilai berdasarkan TEAC (mM/g bagi setiap sampel kering) menunjukan terdapat perbezaan bagi setiap lokasi iaitu K, B, T, S and Mdengan nilai masing-masing adalah 320.66, 279.75, 225.00, 136.95 dan 152.09 bagi DPPH. Manakala aktiviti ABTS masing-masing adalah 13285.35, 12023.91, 9522.83, 7122.38 dan 7018.13. Bagi FRAP nilai aktiviti mereka adalah 11.79, 10.11, 2.92, 2.74 dan 0.58. Nilai TFC dan TPC juga didapati bekolerasi positif terhadap aktiviti anti-oksidan dengan nilai R^2 melebihi 0.5. Sampel dari k juga menunjukan kandungan TPC dan TFCnya adalah berkolerasi secara positif terhadap ICAM-1 dan VCAM-1. Nilai r^2 pearson bagi korelasi TPC untuk ICAM-1 and VCAM-1 adalah 0.94961 dan 0.98968 dan nilai r^2 pearson bagi TFC untuk ICAM-1 and VCAM-1 0.86301 dan 0.96171. Propolis sampel K mempunyai kandungan fitokimia lebih tinggi berbanding sampel lain berdasarkan profile HPLC. Berdasarkan analisa GCMS, ia juga mempunyai tiga (3) bahan fitokimia yang tidak dimiliki oleh sample lain. Keunikan ini mungkin menyebabkan sampel K lebih poten berbanding yang lain.

PHYTOCHEMICAL PROFILING, ANTIOXIDANT ACTIVITIES AND EFFECTS OF PROPOLIS DERIVED FROM LOCAL STINGLESS BEE (TRIGONA APICALIS) ON THE EXPRESSION OF ADHESION MOLECULES IN ENDOTHELIAL CELLS

ABSTRACT

Propolis is defined as a sticky, dark–coloured, resinous substance which produced by bees through combination of their saliva and plants exudates. Currently, there is substantial evidence indicating that propolis acquires many beneficial biological properties such as anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, anti-ulcer, antioxidative and immunomodulating activities. This study aimed to analyze the phytochemical compositions of *Trigona apicalis* (*T. apicalis*) propolis from different location (B, M, S, K dan T) and evaluate their antioxidant and anti-inflammatory activities. Samples were extracted with an optimized extraction method, which was continuous stirring for 72 hour at 37^0C with sample to solvent ratio of 1:10. Its phytochemical compositions were gathered using various methods of chromatography. The antioxidant activities were evaluated using the established1,1-diphenyl-2-picryl-hydrazyl(DPPH),2,2'-azinobis-(3-ethylbenzothiazoline -6-sulfonic acid) (ABTS) radical scavenging and Ferric Reducing Ability of Power (FRAP) assays. While the anti-inflammatory activity of the selected most potent propolis was evaluated by intracellular adhesion molecule-1 (ICAM-1) and vascular cells adhesion molecules-1 (VCAM-1) assays. Despite the extraction method and bee, species were similar, the phytochemical contents exhibited either by HPLC (High Performance Liquid Chromatography) or TPC (Total PhenolicContent) and TFC (Total Flavanoid Content) analysis were found to be different between samples. Due to the

dissimilarities in their chemical compositions, the antioxidant activities of propolis from different areas were therefore found to be diverse. This study highlighted the optimized extraction method (continuous stirring for 72 hour at 37^0C with sample to solvent ratio 1:10) produced the highest yield, while the antioxidant activity, represented as TEAC (mM/g of dry sample). The antioxidant activities of samples were found differ from each location namely, K, B, T, S and M as 320.66, 279.75, 225.00, 136.95 and 152.09 respectively for DPPH assay and for ABTS assay13285.35, 12023.91, 9522.83, 7122.38 and 7018.13 respectively. FRAP values for propolis gathered from K, B, T, S and M were11.79, 10.11, 2.92, 2.74 and 0.58. The antioxidant capacity of propolis samples were found to be positively correlated with TFC and TPC in all sample with their R^2 above 0.5. Equivalent to antioxidant activity, the TPC and TFC of sample from K was found positively correlated to ICAM-1 and VCAM-1 assay. Their pearson (r) correlation values were 0.94961 and 0.98968, respectively for TPC and pearson (r) values of 0.86301 and 0.96171, respectively for TFC. According to HPLC profile, K sample contain the highest phytochemical content. From GCMS analyse K sample was known to have three (3) unique compounds which did not exist in other samples. Probably, these unique compounds may contribute to the potency of sample K.

CHAPTER 1

INTRODUCTION

Malaysia is one of the tropical countries which are blessed with great diversity of fauna and one of them is stingless bee. Stingless bee is known as “kelulut” by local people of Malaysia and it is becoming popular lately. It is most likely due to the emergence of various modern techniques of stingless bee cultivation. Even though the honey production of stingless bee is less compared to the honey bee species, the stingless bees are cultivated due to the beneficial medicinal properties found in their honey, propolis, royal jelly and bee pollen. This study focused on one of the important products of stingless bee which is propolis.

Propolis is made of beeswax, bee saliva and resins collected by the bees from various parts of plants. It is believed that bees produce propolis to protect their hive from external invaders such as bacteria, virus, parasites and small insects. Apart from its role in protecting the bees, it also being used for sealing holes, covering dead animals or insects which are too large to be carried out, smoothing out the internal walls and act as an antiseptic to prevent microbial infection of larvae and honey stores in the combs. Propolis compositions mainly depend on the type of plants near the hives and seasonal factors. Therefore, the physical appearance of propolis varies in odour, colour, and texture including its medicinal characteristics.

Since stingless bee cultivation in Malaysia is still new, there are many areas in relation to stingless bee’s product research and development need to be explored. In fact, research on stingless bee propolis in Malaysia is lacking. In addition, globally

there is limited data being published regarding stingless bee propolis and unfortunately most of them is preliminary data. Therefore, this study attempted to investigate the antioxidant and anti-inflammatory activities, in association with the phytochemical contents of propolis from *Trigona apicalis (T. apicalis)* species, collected from different locations.

T. apicalis was chosen because the honey production by this species is relatively high amongst other stingless bees' species. This study aimed to determine the variability on the quality of propolis produced by the same stingless bee species, gathered from different location. The bee species was identified and confirmed as *T .apicalis* by an entomologist in the Centre for Insect Systematic (CIS) located in School of Environmental and Natural Resource Sciences, Faculty of Science and Technology of Universiti Kebangsaan Malaysia (UKM).

It is believed that the quality of propolis extract is directly influenced by the extraction method. Therefore, the optimization of various extraction methods was done by considering several factors such as concentration of solvent used (1:10 versus 1:20), ultrasonication versus stirring techniques, the duration of extraction (30min versus 60min) and different temperature conditions (25^0C versus 37^0C). The best method was determined by the highest amount of yield obtained at a minimum cost of extraction. The optimized method was later utilized to extract the propolis samples used in current study.

The antioxidant capacity of different propolis samples were analysed using three different methods which were DPPH (1-diphenyl-2-picrylhydrazyl), ABTS

(2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and FRAP (Ferric reducing antioxidant power). While the phytochemical content of propolis samples were analysed using the Total Phenolic (TPC) and Total Flavonoid Content (TFC) techniques. The phytochemical profile of propolis content was performed using High Performance Liquid Chromatography (HPLC), Gas Chromatography coupled Mass Spectrometry (GC-MS) and Quantum of Flight Liquid Chromatography coupled Mass Spectrometry (QTOF LC-MS).

The most potent propolis sample according to the antioxidant capacity was further analysed for its anti-inflammatory activity by observing the percentage of reduction in intracellular adhesion molecule-1 (ICAM-1) and vascular cells adhesion molecules-1 (VCAM-1) production in human umbilical vein endothelial cells (HUVEC EA.hy926). The antioxidant and anti-inflammatory activities were eventually analysed in order to evaluate their correlations with TPC and TFC of the propolis extracts.

1.1 Problem statement and rationale of the study

Beekeeping in Malaysia, especially stingless bee industry is still at its infancy, therefore several areas in regards to the stingless bee potential development should be improved. For example, development of quality control measures and standardized product guidelines need to be given a serious attention. In addition, research and development for clinical or therapeutic applications of stingless bee products should be focused, and encouragement towards sustainable colonies must be applied to ensure sustainability issues in the long run of the industry. Therefore it is very crucial to make

the first move in order to explore the phytochemicals profile, antioxidant activity and their effects as an anti-inflammatory agent.

1.2 Significance of the study

According to anecdotal data propolis has been used by old folks since ancient time for maintaining their health. Although there were many studies done on the chemical contents and biological activities of propolis but most of them possess limited clinical relevance. It was mainly due to the lack of quality standardization of propolis. One of the major obstacles regarding this issue is the variability of phytochemical compositions between propolis samples which may be influenced by different geographical, local flora or even seasonal factors.

Therefore the aim of this study was to look into the different phytochemical contents of propolis gathered from different locations, and relate them with its effects on the antioxidant and anti-inflammatory activity. These data will help us to analyse the relationship between phytochemicals content and biological activity of the propolis extract. More over these data will help our local farmers to decide the best surrounding they should have around their bee colonies which will help them to produce the best product. Therefore this research could contribute to the development and expansion of stingless bee-keeping industries in Malaysia.

1.3 Objective of the study

In view of the above mentioned issues, the objectives of this study were:

1. To profile the phytochemical contents of propolis extract, using chromatographic fingerprint method.
2. To assess the antioxidant activity of each propolis samples using antioxidant assays, which include ABTS, DPPH and FRAP.
3. To analyse the correlation between antioxidant activity of each propolis samples with the TPC and TFC.
4. To evaluate the effects most potent sample on the anti-adhesion molecules assay using flow cytometry

CHAPTER 2

LITERATURE REVIEW

2.1 Propolis as natural healer

Natural products are gaining popularity nowadays, as an alternative medicine because of its positive reliability, safety and efficacy. The ancient civilization such as the Egyptians, Romans and Greeks had used natural products, including propolis, as their medicine due to the effectiveness and relatively less side effect properties of these products. Unfortunately, there is very little scientific research that has been carried out with standardised approachon their biological activity, particularly in regards to the stingless bee propolis (Aminimoghadamfarouj & Nematollahi, 2017; Campos et al., 2015; Santos et al., 2017).

Stingless bees produce several beneficial products such as honey, pollen or bee bread, propolis and royal jelly. These products have been used by old folks as medicinal remedies. In the last decade, there were remarkable numbers of compounds with excellent pharmacological effects that have been identified from propolis. It was found that propolis possessed antibacterial, antifungal, anticancer, antiviral, antiparasitic, and anti-inflammatory properties, which are beneficial to human health (Campos et al., 2015; Carneiro et al., 2016; Miguel & Antunes, 2011). Therefore propolis has beenone of the most important bee products which are currently being used in traditional and complementary medicine (dos Santos et al., 2017; Maruyama, Sumitou, Sakamoto, Araki, & Hara, 2009; Massaro et al., 2013).

2.1.1 Benefits of propolis

Propolis has been used by mankind for ages. There are records suggesting the use of propolis by ancient Romans, Persians and Egyptians. The ancient Egyptians used vase made from propolis for drink to improve their health. They also had learnt from bees which use propolis as "embalming" substance. The bees covered their dead invader with propolis in order to restrain the spread of infection caused by decomposing carcass (Kuropatnicki, Szliszka, & Krol, 2013).

In the Quran there is a chapter about bees which mentionsthe use of products of bees as healing substances (An-Nahl 16:69). Propolis is also mentioned in the Old Testament as "tzori". The ancient Jews consider "tzori" (the Hebrew word for propolis) as a medicine. In the Bible there is a word "Balm of Gilead" which refers to propolis that was given by Queen of Sheba to King Solomon.

Propolis had been used as the main ingredient in polyanthus perfume and aromatic herbs by the Greek. Hippocrates used propolis to treat wounds and ulcers (Kuropatnicki et al., 2013). Meanwhile the Persian manuscripts mentioned propolis as a drug against eczemas, myalgia, and rheumatism (Kuropatnicki et al., 2013; Miguel & Antunes, 2011). Avicenna was known to have documented the characteristic of different types of propolis (Kuropatnicki et al., 2013).

In the medieval period, propolis was not a very popular topic. There were only a few manuscripts dealing with propolis. Nevertheless, propolis was extensively used as one of the remedies in herbal medicine. During that time propolis was often called "Russian penicillin". In early modern time, John Gerard in his famous herbal book,

The History of Plants (1597), made reference to the use of "the resin substance of the black poplar tree buds" for healing ointments. In the seventeenth century, propolis was found written in England pharmacopoeias as a major ingredient of healing (Kuropatnicki et al., 2013).

In 1967 until 1973 a series of studies were performed in Denmark that proved the effectiveness and safety of propolis to be usedas medicine. Dr Karl Lund Aagaard, a Danish biologist observed the effect of propolis on more than 50,000 patients in Scandinavia and concluded that propolis can be used as treatment for various medical conditions. They include cancer, infection of the urinary tract, swelling of the throat, gout, open wounds, sinus congestion, colds, influenza, bronchitis, gastritis, diseases of the ears, periodontal disease, intestinal infections, ulcers, eczema eruptions, pneumonia, arthritis, lung disease, stomach virus, headaches, Parkinson's disease, bile infections, sclerosis, circulation deficiencies, warts, conjunctivitis and hoarseness (Kuropatnicki et al., 2013; Miguel & Antunes, 2011).

In 1976, Aagaard patented extraction method for purifying propolis. He also discovered that it is not necessary to extract individual compounds from propolis, instead the substance in its natural form has a powerful curative effect on diseases for instance pharyngitis, rheumatism, chronic colitis and conjunctivitis (Kuropatnicki et al., 2013).

2.2 Stingless bees

Stingless bees are amongst the longest evolving group of bees. Stingless bees have populated tropical forest for over 65 million years longer than honey bees. They

developed before the continents drifted apart from each other. Stingless bees have been found preserved inside pieces of amber. Approximately 400 to 500 different species in 32 genera of stingless bees have been recorded (Chuttong, Chanbang, & Burgett, 2014). However, new species are being identified yearly.

Stingless bees live in colonies of a few hundred up to several thousands of individuals. Stingless bees are also usually known as Trigona or Meliponine species,“sugar-bag bee”, “sweat bee”,“native honey bee” and “drunken baymen”. They are a subfamily of the Apidae family, of the Hymenoptera order of the insects. Stingless bees are insects with four wings. Generally, their length is less than 15mm long and relatively absence of hair. It also has buccal parts that form a structure of the tongue. This structure helps them to collect liquid food such as nectar and plants wax.

The characteristics which make stingless bee differs from Apidae members are the reduced veins in the previous wings, atrophied sting and their composite eyes which are devoid of pilosity (Pino, Marbot, Delgado, Zumárraga, & Sauri, 2006). On the other hand, unlike the Apis or honeybees, stingless bees build their nest with the wax from plants or mud and mixed with their enzymes (Sara Diana Leonhardt & Blüthgen, 2009; Alexandra Christine Helena Frankland Sawaya, Barbosa da Silva Cunha, & Marcucci, 2011). This resinous nest is also known as propolis. Stingless bees stored their honey in propolis pots which are known as cerumen, whereas honeybees keep their honey in waxy hexagonal combs. Beside storing honey in the pots, they also used it to keep pollens and eggs (Chuttong et al., 2014).

Figure 2.1: Artificial stingless bee hive. (A) modified log hive of stingless bee coloni. (B) The upper part of the modified log was removed, exposing the stingless bee nest matrix, adapted from Chuttong et al (Chuttong et al., 2014).

Stingless bees are known for their well-organized colonies. This can be seen in how they arrange their nest. The pots (combs) are arranged horizontally. They also segregate the food storage pots with the brood combs. Stingless bees are also known in maintaining single egg-laying queen in their colony. The egg-laying queen may live more than three (3) years. Although new queens develop repeatedly, most of them will be killed (Chuttong et al., 2014; Sara D. Leonhardt, Wallace, & Schmitt, 2011; Sanches, Pereira, & Serrão, 2017).

2.2.1 Scientific classification of stingless bee

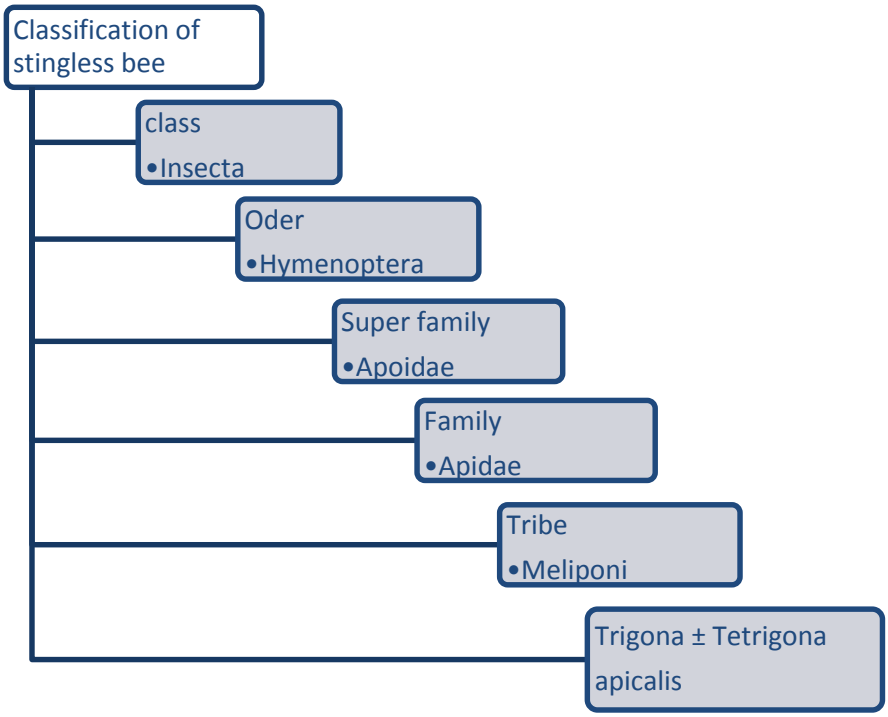


Figure 2.2: Scientific classification of stingless bee.

The *Trigona* is the most prominent stingless bee genus. *Trigona* plays an important role in the pollination of native plants, flowers and crops during the collection of nectar and pollen as sources of food. Stingless bees were observed to pollinate 22.6% plant species in lowland forest in Sarawak (Samejima, Marzuki, Nagamitsu, & Nakasizuka, 2004). Therefore stingless bee colonies were kept in commercial agriculture side such as in Cameron Highlands, Malaysia and Chanthaburi, Thailand in order to pollinate the plants and eventually increase food production (Chuttong et al., 2014). Regrettably, their numbers are declining in most part of the world mainly due to commercial logging and shifting cultivation (Samejima et al., 2004). Several countries have recently taken initiative to conserve it, especially in South America, Australia and Southeast Asia where stingless bees can be found (Chuttong et al., 2014; Samejima et al., 2004).

2.2.2 *Trigona apicalis*

Trigona apicalis can be differentiated from other *Trigona* species by several characteristics. For example the mandibles, except for the extreme base and antennae, are ferruginous in colour. The quadrangle is not so broad in worker bees due to the convergence of their eyes. The eyes are separated from the base of the mandible. The malar space can clearly be seen. Most of thorax is ferruginous in colour but less in tegulae, pronotum and tubercles. Thorax is covered with a low growth of dull greyish hair. Half of the wings especially close to body are dark whereas the apical are lacteous (Schwarz, 1939)

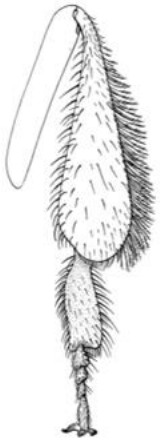


Figure 2.3: Hind leg of male *Trigona apicalis*, adapted from Schwarz (Schwarz, 1939).

Most part of legs is bright and ferruginous in colour. The black hairs appear sparsely on the legs. Mean while the abdomen is mostly dark with reddish colour especially on the ventral side.

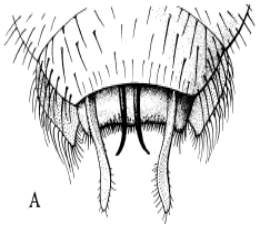


Figure 2.4: Apical end of abdomen viewed dorsally of the male *Trigona apicalis*, adapted from Schwarz (Schwarz, 1939).

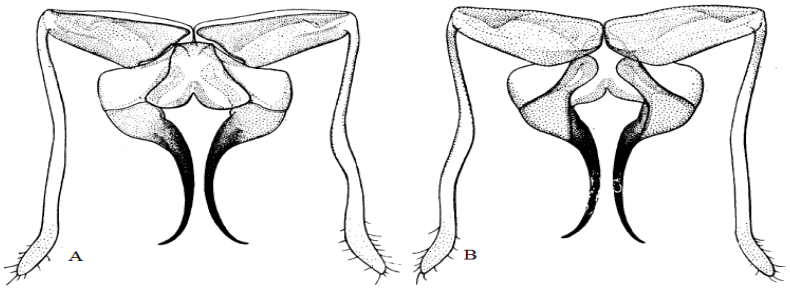


Figure 2.5: A and B Genitalia organ of the male *Trigona apicalis*, respectively dorsal and ventral view, adapted from Schwarz (Schwarz, 1939).

The genitalia organ is long black needle-like sagittae that are of rather uniform narrowness, except at their broadened yellowish base Schwarz (Schwarz, 1939).

2.3 Types and compositions of propolis

Propolis is made up of complex materials which contain many parts of plant and bee's origin. Commonly, propolis consists of three different materials/components. They include plant base substances such as exudates from wounds in plants, lipophilic materials on leaves and buds, resins, mucilage, gums, lattices and others substances collected by bees. Secondly, propolis is also made from bees own substances such as their saliva; and thirdly, materials which are introduced by bees during the making process of propolis for example muds (Chuttong et al., 2014; Alexandra Christine Helena Frankland Sawaya et al., 2011).

Research regarding the phytochemical compositions of propolis originated from different places and their pharmacological activity has increased in the last few years. Thus, this bee product has gained popularity as an alternative medicine since then. It has been used as health amelioration and disease preventive agent. Most of the propolis extracts contain amino acids, phenolic acids, phenolic acid esters, flavonoids, cinnamic acid, coumaric acid, terpenes, hesperatin, nicotinic acid, and caffeic acid (Pazin et al., 2017; Santos et al., 2017). The flavonoid and phenolic contents in propolis have been shown to be capable of scavenging free radicals which help to protect the cell (Bonamigo et al., 2017; Miguel & Antunes, 2011; Pazin et al., 2017).

In the beginning of the 20th century there were several scientific researches on chemical compositions of propolis. Early attempts to determine the compositions of propolis was through simple fractionation done by Dieterich and Helfenberg (Kuropatnicki et al., 2013). They present their extraction methods and propolis constituents separated in alcohol, chloroform, and ether. In 1911, a German researcher Dietrich identified vanillin in propolis. Meanwhile, Jaubert identified chrysin pigment, which is responsible for the propolis colour in 1926. A German researcher, K¨ustenmacher, identified cinnamic acid and cinnamyl alcohol as components of propolis. Later in 1927, another German scientist, R¨osch, proved that propolis originates from the buds of plants (Kuropatnicki et al., 2013; Santos et al., 2017).

2.4 Definition of phytochemicals

Phytochemicalis a combination of Greek words. Phyto means "plant" in Greek. Therefore phytochemicals literally means chemicals that are produced naturally by plants. The phytochemicals are responsible for colour, taste and smell of the food.

There are estimated more than 8,000 different phytochemicals that have been identified, but many more remain unknown (Dores et al., 2014). These non-nutritive plants chemicals may have health-promoting properties such as antioxidant, anti-allergic, anti-tumour and others. Daily intake of antioxidants can significantly produce effective prevention against oxidative damage (Dores et al., 2014; Pazin et al., 2017).

2.4.1 Classification of phytochemicals

Phytochemicals can be broken down into four major categories based on the number of phenol rings that are identified in its molecular structure. They include flavonoids, stilbenes, lignans and phenolic acids. Phenolic acid and flavonoid are amongst the popularly known phytochemical compounds in propolis. The presence of these compounds in propolis may be responsible for its physical characteristics and biological activities.

Flavonoids have antioxidant and anti-inflammatory properties, and they consist of 6 members which are flavones, flavonols, flavanones, isoflavones, anthocyanidins and flavan-3-ols.While, phenolic acids consist of 2 members which are hydroxybenzoic acids and hydroxycinnamic acids. Most common member in this class are ferulic, p-coumaric, caffeic acids and their derivatives.

2.4.2 Factors affecting the phytochemical and pharmacological variations

Phytochemical contents of propolis are considered very highly variable. It is due to several factors such as plant species, from which the nectarsare collected by bees, geographical locations, and seasonal variations. A study done by Sanches (Sanches et al., 2017) showed that there were vast varieties of compounds in stingless

bee propolis. However, these compounds vary among bee species and interestingly, they also differed even among same species from different locations (Sanches et al., 2017).

A study on propolis gathered from three different locations in Brazil, was conducted among same stingless bee species, namely *M. fasciculata*. In that study, the researchers used the same extraction method to extract the phytochemical compounds in propolis. However, the compositions of each sample varied in either the phytochemical content or pharmacological activity, as expected. It apparently demonstrates the influence of geographical location on the phytochemical contents of stingless bee propolis. The most reasonable explanation for this finding was the different plants species around the bee hive as described by Liberio (Liberio et al., 2011).

A group of researchers from Thailand reported the antioxidant activity of different stingless bee species from the same location was found to be different. The study compared and identified that the phenolic contents between propolis gathered from different species were also different (Vongsak, Kongkiatpaiboon, Jaisamut, Machana, & Pattarapanich, 2015). Nevertheless, the climatic factors seemed do not influence the phytochemical contents of propolis because of the distribution of stingless bee in tropical rainforest which, the plants composition remain the same throughout the year.

2.4.3 Phytochemical and antioxidant properties of stingless bee propolis

Antioxidant activity of propolis is determined by its ability to scavenge free radicals. The antioxidant ability is proportionate to their chemical contents, particularly the phenolic and flavonoid contents. Free radicals are known to cause damage or mutations to normal cells. For instance, atherosclerosis occurs when free radicals attack the cell membrane. Normal physiological response of human body will react to this situation with numerous biological mechanisms in order to protect the cells form destruction, yet it is still insufficient especially with aging or immunodeficiency states. Thus, consumption of a sufficient amount of antioxidant resources, for example propolis, is necessary to help the body combat free radicals (Pazin et al., 2017).

A group of researcher from Thailand analysed the antioxidant activity of different stingless bee species namely, Lepidotrigona ventralis Smith, Lepidotrigona terminata Smith, and Tetragonula pagdeni Schwarz, from the same mangosteen orchard and found to be different in their activity. On the other hand their study also reported the antioxidant activities (ABTS, DPPH and FRAP) were strongly correlated with the total phenolic content of the propolis samples (Vongsak et al., 2015).

2.4.4 Phytochemical and anti-inflammatory properties of stingless bee propolis

The anti-inflammatory effects of propolis derived from Melipona scutellaris, which is one of the stingless bee species, have been evaluated by using induced animal model. The changes in Interleukin-1b (IL-1b) and Tumour Necrosis Factor-α (TNF-α) were observed. The ethanolic extract, hexane and aqueous fractions of propolis showed positive anti-nociceptive activity. Interestingly, researcher also found that

there was significant presence of phenolic and flavonoid compounds in the extract (M. Franchin et al., 2012). This may indicate that the anti-nociceptive activity might be due to the effects of these compounds.

Asthma is one of the common diseases in our community. This disease involved inflammatory process which is also referred as chronic inflammatory disease of the airways. In asthmatics, multiple proteins of inflammatory cascades can be observed (Jos Farias et al., 2014). These inflammation proteins were suppressed by propolis of *Scaptotrigona aff. Postica* from Maranhao, Brazil (Jos Farias et al., 2014).

2.5 Effects of free radicals to health and the role of antioxidants

2.5.1 Free radicals or oxidants

Free radicals are molecules with one or more unpaired electron in its outer shell. They are formed from breakage of chemical bonds. The free radicals exist in the body through several processes, for example from cell metabolisms or from external sources (pollution, cigarette smoke, radiation and medication). Some example of free radicals are hydroxyl ($OH^{\bullet -}$), nitric oxide ($NO^{\bullet}$), lipid peroxyl ($LOO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). The over loaded free radicals in the body cannot be excreted, therefore it becomes toxic to the body organs (Pham-Huy, He, & Pham-Huy, 2008). Biological free radicals are unstable as inorganic free radicals. These unstable molecules are able to react with various organic substances such as protein, DNA and lipids, which consequently will lead to physiological disturbances and thus eventually organ failures and diseases (Brewer, 2011).

2.5.2 Definition of antioxidants

Antioxidants can be defined as substances that are able to protect our body from damage by harmful molecules known as free radicals even at low concentration. Antioxidant activities are mostly contributed by phenolic acids (gallic, protocatechuic, caffeic, and rosmarinic acids), phenolic diterpenes (carnosol, carnosic acid, rosmanol, and rosmadial), flavonoids (quercetin, catechin, naringenin, and kaempferol), and volatile oils (eugenol, carvacrol, thymol, and menthol). They are able to chelate the free radicals by donating H^+ to oxygen radicals. Consequently it will slow down the oxidation process, thus the formation of free radicals in the body (Brewer, 2011).

2.5.3 Sources of natural antioxidants

Antioxidant substances may present naturally in fruits, vegetables, cereals, tea and bees products. The antioxidant phytochemical compounds such as flavonoids and phenolic acids are able to scavenge free radicals such as lipid peroxyl and peroxide. Eventually it will stop the oxidative processes in the human body. Eventhough antioxidant compounds could lose their electrons, they do not become free radicals; instead they are stable in either form.

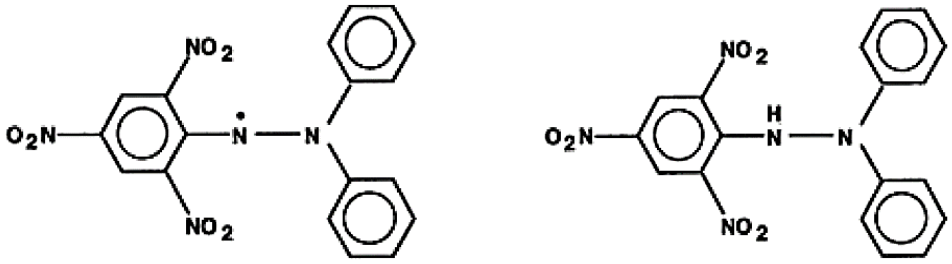
Although human body is capable to produce antioxidants naturally, it is not fully effective in the case of over-production of free radicals. Moreover the efficiency of our body to neutralize this reaction also declines when we become older and during diseased states. Some examples of antioxidant include phenolic compounds (flavonoids, phenolic acids and tocopherol), nitrogen compounds (alkaloids, chlorophyll substances, amino acids peptides and amines), carotinoid derivatives and Ascorbic acid. Scientific evidences suggest that daily intake of high antioxidant

containing food can reduce the risk of chronic diseases. It is related to the anti-inflammatory, anti-allergic, anti-bacterial and anti-cancer effect of the substances (Pazin et al., 2017; Samappito & Butkhup, 2010; Tamuly et al., 2013). Realizing the importance of antioxidants to our body, therefore it is best to know the presence, quality and quantity of these compounds in our food. Moreover the composition of these chemical compounds varies between stingless bee species, location of resources and seasonal factors. Therefore the compositions and quantity of these compounds must be validated and standardized from the beginning of production process.

2.6 Antioxidant assays

2.6.1 DPPH (4-2, 2-Diphenyl-1-picrylhydrazyl)

This method has been developed to determine the antioxidant activity of foods or natural products, which utilizes the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The structure of DPPH and its reduction by an antioxidant are shown in Figure 2.6. The odd electron in the DPPH free radical gives a strong maximum absorption at wavelength of 517nm. The colour turns from purple to yellow as reduction occurs via the absorption of DPPH radicals at 517nm. The odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H resulting in the decolourization of DPPH (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Taira et al., 2010).



A. Diphenylpicrylhydrazyl (free radical)

B.Diphenylpicrylhydrazine (nonradical)

Figure 2.6: Comparison between molecular structure of A. free radical and B. stable form of DPPH (Jurzak, Ramos, & Pilawa, 2017; Taira et al., 2010).

The efficiency of scavenging free radicals is an important factor in the quantification of antioxidant activity. Trolox is used as the reference standard and it reacts with DPPH solution in methanol at room temperature. Trolox is vitamin E water soluble analogue. The absorbance changes are measured at 517nm (Campos et al., 2014; Jurzak et al., 2017; Varthamanan & Jayanthi, 2014; Vongsak et al., 2015).

2.6.2 ABTS [2, 2'- azinobis (3-ethylbenzothiazoline-6- sulfonic acid)]

ABTS is frequently used to measure antioxidant activity in various research fields for instance, pharmaceutical and food industries. ABTS turns to radical $ABTS^{+}$ cation after adding sodium persulfate. This blue in colour radical cation absorbs light at 734nm wavelength (Antolovich et al., 2002; Campos et al., 2015). It can be utilized to determine antioxidant activity of phenolics, thiols, vitamin C and a few other substances.

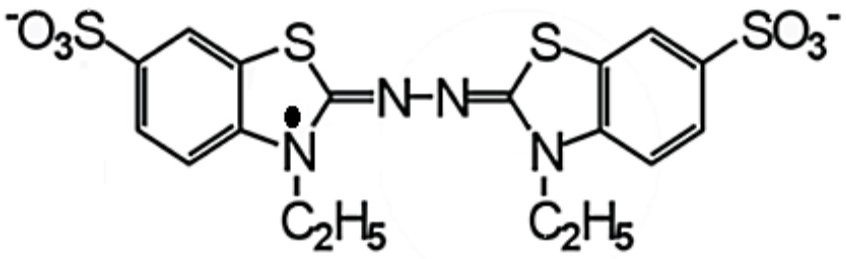


Figure 2.7: Molecular structure of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) kation (Li et al., 2016).

The intensity of the blue radical cation will decrease or eventually turn colourless after cationic $ABTS^{+}$ being converted to its natural form. This changes could be observed by spectrophotometer at 734nm of wavelength (Vongsak et al., 2015). The decreasing in intensity of the blue radical ABTS cation is proportionate to the antioxidant capacity of tested substance (Zheng, Zhao, Xiao, Zhao, & Su, 2016). Therefore, antioxidant capacity of interest substance can be expressed by comparing to Trolox Equivalent Antioxidant Capacity (TEAC), similar to DPPH method.

2.6.3 FRAP(Ferric reducing antioxidant power)

This method is based on reduction of Fe^{3+} to Fe^{2+} by antioxidant substance in acid medium. The stronger antioxidant activity the more intense blue colour will appear. The changes in intensity can be observed by spectrophotometer at wavelength of 593nm. Antioxidant capacity of the interest substance are measured by comparing to Trolox, as the positive reference standard, therefore expressed as Trolox Equivalent Antioxidant Capacity (TEAC) (Antolovich et al., 2002; Vongsak et al., 2015).

2.7 Extraction, separation and identification of compounds in propolis

2.7.1 Types of extraction methods

Chemical compositions of propolis are complex due to the variety of plants visited by bees. Generally, propolis composed of the polar and non-polar compounds. Therefore the choice of solvent with the correct ratio will determine the quality and quantity of the extracted propolis (Alexandra C. H. F. Sawaya, Souza, Marcucci, Cunha, & Shimizu, 2004). Based on the concept of "like dissolves like", a single or combination of different extraction solvents can be deployed to extract as much active compounds as possible from the raw propolis.

The phenolic compounds extracted from raw propolis are proportionate to the polarity index of the solvent used. Whereas the highest flavonoid compounds can be extracted from raw propolis, proportionally with the increase of concentration of the solvent (Alexandra Christine Helena Frankland Sawaya et al., 2011). Therefore, the polarity index and concentrations of a particular solvent have to be considered before it is used as an extraction solvent.

A study performed in Brazil showed that solvents with different polarity dissolved different phytochemicals in stingless bee propolis (Pazin et al., 2017). Thus, the extracts resulted in the differences in their biological activity. Some of the most common extraction methods being used nowadays to extract propolis are maceration process, soxhlet extraction, supercritical fluid extraction or spray dry, microwave-assisted, ultrasonic extraction and surfactant extraction (Cunha et al., 2004).

A study comparing different methods of extraction of propolis concluded that ultrasonic extraction (UE) and microwave-assisted extraction (MAE) are not preferable methods as they involve heat. Furthermore those above mentioned methods produced insignificant yield differences compared to maceration method (B. Trusheva, D. Trunkova, & V. Bankova, 2007).

2.7.2 Analyses of phytochemical compounds in propolis by chromatography techniques

Chromatography is a technique to separate different molecules or components in a mixture form. Separation and identification of compounds in propolis is very important in order to determine the responsible compounds, which may contribute to its biological effects. The chromatographic method enables us to characterize and quantify each identified compound in the sample. There are several chromatographic techniques, based on mode of separation available as adsorption, partition, ion exchange, size exclusion and affinity chromatography.

Above all chromatographic methods mentioned, High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Liquid Chromatography combined with Mass Spectrometry (LC-MS) were chosen to analyse propolis sample in this study. Besides their availability in the laboratory, these methods are inexpensive, less time consuming and reliable. In addition, by combining these methods, it enables us to study both volatile and non-volatile compounds in propolis (Shabir, 2010).