STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF BEES PROPOLIS

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations	Details of the abbreviations
%	Percentage
Å	Angstrom
p	Probability
°C	Degree Celsius
cm	Centimeter
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDX	Energy Dispersive X-ray
FESEM	Field emission scanning electron microscopy
g	Gram
GC-MS	Gas chromatography–mass spectrometry
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
kg	Kilogram
m	Meter
M	Molar
mg	Milligram
µg	Microgram
ml	Milliliter
µl	Microlitre
min	Minute
mm	Millimeter
nm	Nanometer
SEM	Scanning electron microscope
SPSS	Statistical Package for Social Sciences
VPSEM	Variable Pressure Scanning Electron Microscope
WD	Working distance

PENCIRIAN STRUKTUR DAN BIOKIMIA DARIPADA PROPOLIS LEBAH

ABSTRAK

Kajian kimia farmakologi dan klinikal dalam mengenalpasti keberkesanan perubatan tradisional dan masyarakat setempat kebanyakannya bermula dari penentuan ‘status quo’ kepelbagaian bio flora dan tumbuhan botani. Kepelbagaian bio tumbuh-tumbuhan ini telah menjadi asas kepada perubatan tradisional sedia ada. Perkatan propolis merupakan nama generik kepada campuran resin semulajadi yang dihasilkan lebah yang diperoleh daripada pelbagai bahagian tumbuhan, pucuk dan eksudat di kawasan geografi berhampiran. Produk lebah propolis telah digunakan secara global dalam perubatan tradisional disebabkan oleh ciri-ciri antioksida, stimulasi imun, anti-keradangan dan anti-kanser. Pada era industri, penggunaan propolis dalam amalan penjagaan kesihatan telah mula popular, namun keberkesanannya dalam kerangka bukti saintifik masih belum dirungkaikan. Kini, paradigma berkaitan sifat kimia propolis secara umumnya telah berubah. Jumlah kajian tentang sifat propolis terutamanya propolis *Trigona spp* dari aspek ciri semulajadi, bahan yang terkandung, dan keselamatan penggunaan. Di dalam kajian ini, kaedah semi-kuantitatif, kualitatif berserta kajian secara pengimejan telah digunakan untuk mencirikan propolis *Trigona spp*. Propolis *Trigona spp* telah diambil di Kubang Kerian, Kelantan pada Jun 2011 dan dicirikan menggunakan kaedah HPTLC, GC-MS, analisis permukaan SEM dan mikroanalisis EDX dalam menentukan ciri-ciri kimia dan bahan yang terdapat pada sampel. Kajian HPTLC menunjukkan 7 jenis bahan fenolik dengan 3 bahan antioksida dikesan didalam propolis *Trigona spp* manakala 3 bahan antioksidan dikesan dalam propolis *Apis mellifera*. Bahan fenolik tersebut tidak dapat dipastikan apabila jalur sebatian negatif pada perbandingan menggunakan 5 jenis bahan piawaian (Caffeic acid, Naringenin, Naringin, Kaempferol and Luteolin). Data GC-MS menunjukkan terdapat 2 jenis bahan fenolik mudah meruap (iaitu Cardol bagi kedua-dua propolis manaka Cardanol bagi propolis *Trigona spp* dan 1,3-Benzenediol,5-pentadecyl bagi propolis *Apis mellifera*). Kajian lanjutan menggunakan kaedah in vitro bagi menentukan kadungan bahan fenolik dan antioksida menunjukkan propolis *Trigona spp* mengandungi bahan fenolik yang lebih tinggi dan antioksidan yang setara berbanding propolis *Apis mellifera*. Data ultra-mikroskopi menunjukkan keadaan propolis yang terdiri daripada lapisan homogen dan rata dimana propolis *Trigona spp* menujukkan kehadiran bahan-bahan yang lebih banyak berbanding *Apis mellifera*. Kehadiran debunga, struktur berbentuk benih dan urat kayu mikro di dalam sampel propolis *Trigona spp* menyokong perbezaan tingkah laku lebah *Trigona spp* berbanding *Apis mellifera*. Propolis *Trigona spp* dan *Apis mellifera* mengandungi bahan fenolik yang menyerupai propolis dari Thailand dan Indonesia. Kedua-dua propolis tidak menunjukkan persamaan bahan fenolik seperti propolis Eropah. Propolis juga mengandungi bahan surih serta selamat dari bahan toksik.

STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF BEES PROPOLIS

ABSTRACT

Pharmacological and clinical evidence based related chemical studies to harness local folk or traditional medicines are predominantly initiated and were derived from exploitation of geo-located local ‘status quo’ flora and botanical plants biodiversity. As such this was the basis platform of most early medicines. Propolis is is the generic name of a natural resinous mixture produced by honey bees from substances collected from parts of plants, buds, and exudatesin different geographic areas. Propolis honeybee product have been used in global traditional folk medicine signatures for antioxidant, immune-stimulating, anti-inflammatory and anti-cancer effects. While in the industrial era, propolis application in health care has become more trending but much evidence base extrapolation still need to be explore. For acknowledgement, the paradigm concerning propolis chemistry radically changed. There are very few studies that characterize the content of *Trigona spp* and comparison with *Apis mellifera* propolis in terms of the nature of the propolis, the compound present, the safety issue and also the stability of the substances in formulation product. In this research, semi-quantitative and qualitative approaches had been used to characterize *Trigona spp* and *Apis mellifera* propolis. *Trigona spp* propolis was collected from Kubang Kerian, Kelantan in June 2011 and was characterized using HPTLC, GC-MS, SEM surface analysis and EDX microanalysis to determine the chemical properties and compounds present in the sample. HPTLC result showed 7 types of phenolic compounds with 3 antixodiant compounds present in *Trigona spp* propolis while 3 antioxidant compounds in *Apis mellifera* propolis. The compounds

were currently unidentified as the banding showed no similarities with standards used in this study (Caffeic acid, Naringenin, Naringin, Kaempferol and Luteolin). The GC-MS data detected 2 types of volatile phenolic compound (Cardol in both propolis, Cardanol in *Trigona spp* and1,3-Benzenediol,5-pentadecyl in *Apis mellifera* propolis). The in vitro evaluation showed *Trigona spp* contained higher phenolics compound with almost similar level of antioxidant capactiy compared to *Apis mellifera* propolis. Ultra-microscopy data showed presence of majority flat layer homogenized layer in both propolis where *Trigona spp* propolis showed more presence of compounds. Presence of more pollen, seed-like structure and microfibre wooden chip in *Trigona spp* propolis support chemical finding of different foraging behavior compared to *Apis mellifera*. *Trigona spp* and *Apis mellifera* propolis obtained in Malaysia contain phenolic compounds similar to propolis reported in Thailand and Indonesia. The propolis did not contain well known phenolic compounds commonly seen in the European country. It also contained several trace elements, and free from volatile toxic compound

CHAPTER 1

INTRODUCTION

1.1 Overview of the research

Propolis, a bee natural product is a global etnobotanical signature mixtures optimised from variety of compound gathered from various plants anatomical compartment (ie: stem, leaves, sap and exudates) orginated from different geographic habitat, composed mainly of bee wax, volatile compounds (ie: terpenes, phenolics, hydrocarbons and alcohols) and resins (Nikolaev, 1978; Giuseppina Negri, *et al.*, 2000; Bankova, *et al*., 2014). As such propolis have a local plant ecosystem origin (as such a geographical specified natural product) and contain biological inert substances with active biological mechanism activity (thus with potential medicinal value). Globally these natural product features as mass with different presentation and texture. Some of the propolis are brittle and solid and others are solf, flexible and viscous in nature (Ghisalberti 1978) according to the bee species. Due to their wide range of botanical origin, the propolis can be found with variety of colour from lighter yellow, green to more darker black and brown. They generally taste bitter, sticky and resinous with aromatical odor (Antonio Salatino, *et al*., 2011). The current data on benefits of propolis such as antibacterial properties triggered attention and interest among global community to further characterized and exploring propolis chemical compounds and its therapeutic effect (Bankova, de Castro and Marcucci, 2000, Bankova, 2005).

Propolis is formed by mixing of plant resin and beeswax β-glycosidase enzyme of the bee saliva glands. These partially digested beeswax have been associated with the formation of collective immune system among the community of bees which act as defence mechanism of the hive from diseases and parasite transmission through the colony as such important role in protecting, reinforcing and repairing the hives

(Simone-Finstrom and Spivak, 2010). However there is chemodiversity in nature. Determination of bee wax resin botanical origin is an important intial step in defining propolis especially in the characterization phase. Despite the abundant published data on propolis biochecmical and therapeutic effects, concise and detail data on botanical origin still a lacunae. In tandem to these, the determination of propolis plant sources (of each local ecosystem signature) is an important for characterization and standardization of local etnobotanical propolis for commercial purposes. The inert composition of every geographical located vouchered propolis varied. It is worth mentioning that this composition differs significantly with botanical and geographical origin and depends on climatic conditions, terrain, water availability and other environmental factors on its geographical location and the season for harvesting. In a typical preparation, propolis can contains about 50 variable constituents, including resins, vegetable balsams, waxes, essential oils, and pollen. As such, the paradigm concerning propolis chemistry radically changed and botanical origin becoming the important factor in explaining the variety of chemical composition of each propolis.

Various geographical plants, such as *Clusia rosea*, conifer and poplar trees (black poplar *Populus nigra*) are associated to different types of propolis present. The propolis produced from these plants can are often associated with high level of polyphenolic compounds. Country such as Greece, Italy and Poland are known to produce such propolis. Previous study on poplar origin propolis, the most abundant type of polyphenols are flavonoid (chrysin, galangin), phenolic acids (benzoic acid, caffeic acid), and terpenes (Gardini, *et al.*, 2018; Bankova and Marekov, 1984; Okinczyc, *et al.*, 2018; Popova, *et al.*, 2017). From all the research, possible correlation can be further explore based on tendencies of special plant metabolites to be found in each different botanical origin propolis. This will allow a better and organized characterization and classification of propolis. Generally, the various inert compounds identified in various etnobotanical origin propolis as stated by Marcucci in 1995 are caused by three main factors. The factors are botanical plant which the exudate orginate from, byproduct secretion produced by metobolism of the bee and other compounds utilized by the bees in forming biocomposite to strengthen the propolis.

The stickiness and glue like properties of resinous propolis compound enable this substance to be utilize as building block for honey bees in constructing and maintaining the hive. The nature of propolis allow it to be used for hive contruction and maintainence. The viscosity of propolis is used to fill any hive fracture and also function as building block for regular internal walls. The propolis aromatic odor is also usefull to repel invaders while the stickiness entrap any unwelcome intruders. The propolis will be wrapped around carcass to maintain the hygenic condition of the hive. The outer surface of hive made out of propolis helps to maintain hive’s temperature and protect bees from dangerous weather (Viuda-Marto, *et al.*, 2008).

As a natural byproduct, propolis are reported to exhibit antioxidant (Bolfa, *et al.,* 2013), antibacterial (Borba and Spivak 2017), antiinflammatory (Barroso *et al.*, 2012) and antiproliferative (Sforcin 2007) properties which spurs interest among global and local researchers to harness its therapeutic values for human wellness. As such research have extrapolate propolis availability to stimulated mammalian tissue regeneration, as it caused strong activation of mitosis of cells cultured ‘*in vitro’* and it potential to able to enhanced biosynthesis (Elkhenany, et al., 2019; Gabrys, *et al.*, 1986; Popeskovic, *et al.*, 1977; Scheller, *et al.,* 1977, Zarei, et al., 2016).

1.2 Justification of the study

Codex Standard For Honey had been developed in 1981 aiming to standardized and maintain quality of each batch of honey. The codex are consist of monitoring of several parameter such as sugar percentage, moisture content and heavy metal contamination. Botanical origin of honey in general are more developed such as in manuka honey. Little is known of its macroscopic and macroscopic characteristics and in relation to this the propolis. The Codex defined honey botanical origin can only be properly classified when it is confirmed that majority of the nectar come from stated botanical source proven through pollen count microscopic analysis, mathcing physcio-chemical data and accurate organoleptic characteristic of the honey (Codex Alimentarius Commission, 2001). Thus, it is academically well received that honey from any geochemical location will never be completely pure mono floral of origin since it will be impossible to prohibit and manage different floral collection by honey bees to mix in any batch of honey collected (Winston, 1987). The bees work within 1 km radius from the bee hive, although there is data mentioned a maximum of 14 km distance ever been recorded. The bees will forage any available floral nectar within the vicinity. Researcher stated that bees behaviour in terms of foraging activity as supergeneralist (Hawkins, et al., 2015; Seeley, 1995; Stephens, 2006; Stephens, *et al.*, 2015).

Although there is a revival of interest in etnobotanical propolis and its bees, poor profusion of taxonomic and biological literatures are made available especially here in Malaysia. The research effort hopes to create interest among local scientists thus stimulate tangible biological and behavioral discoveries by this thesis characterization studies. Hopefully the undertakings of this research attempts will determine, enhance and value-added the available biological information and concern in optimisation strategies towards propolis management and upscale its potentials as therapeutics natural products. Little is known of the properties of the local etnobotanicals Trigona's propolis especially characterised via macroscopic and high-resolution microscopical inspection analysis. Therefore, evaluation of local vouchered etnobotanicals propolis morphological features and properties reflecting the bees and its ecosystem, as well as its related biological therapeutic compounds mask in the propolis wax are needed.

1.2 a. Relevant of the study:

Propolis is a geographical specified natural product. Thus propolis can be acknowledged to comprise combination of various biocompound that sourced from specific geographical botanical radius and others varied enviromental contributing factors. Therefore propolis might have display high chemical diversity or biochemical specificity. The ancient application of honey, one of the more highly reported bee product as wound treatment has been reviewed by some, mainly due their antimicrobial properties (Zumla, *et al.*, 1989; Drouet, 1983; Harris, 1994). In current medical advances, antimicrobial resistance has become a growing threat. This factor contributes to growing interest among researchers on alternative treatment. With previous ancient application of bee product in general lead to more modern scientific approach on understanding and standardizing the usage of bee products. The increased in reported exploitation should not only be look as stratergies to sustain a comercial exploitation but also to allocate proper taxonomy properties and databases that will enriched local vouchered propolis.

Globally the most widely used and reliable standard of honey botanical classification is using microscopical melissopalynlogy; a basic pollen count that can be found in honey sample. The number of pollen will determined the botanical origin of a vouchered honey through light microscopical examination (Anklam, 1998). The downside of this methodology is that it required a lot of time to be conducted and the confirmation of the pollen rely solely on taxonomic resolution of the pollen characteristic. More importantly palynology method is only more established and reliable in determining honey botanical source compared to propolis due to the nature of the collection of both bee products. Little is known on local Trigona's propolis harvested from local Malaysia hive of such macroscopic and microscopic properties and characteristics to enhance its taxonomy and and related this charcteristic to its much purported and reported potential in health and human wellness.

This fundamental chemical knowledge is not only to identify novel lead structures favourable to drug discovery but will also serve to properly characrterized and compare them from list of biotechnology manufactured and composite compounds found commercially in the global market. The efficacies of propolis application in health condition is not properly documented. Without proper data based on modern scientific findings, it is hard for this traditional product to even be considered as alternatives to the modern approach. The mechanism of action of local geographical vouchered propolis, and its related properties still remains obscure and needs further scientific evidence to reinforce a standard database profile base on microscopical and biochemical fingerprinting for authentication of local propolis and honeys.

1.3 Objectives

1.3.1 General Objective

To determine the physical, morphological characteristics and biochemical properties of bees propolis.

1.3.2 Specific Objectives

1. To evaluate biochemical composition and toxic profiles of *Trigona spp* and *Apis mellifera* propolis via GC-MS.
2. To evaluate antioxidant compounds of vouchered etnobotanical *Trigona's spp* and *Apis mellifera* propolis antioxidants (phenolic) compounds via HPTLC analysis and DPPH assay.
3. To evaluate biological trace minerals of *Trigona's spp* and *Apis mellifera* propolis via EDX microdiffraction study.
4. To characterize the gross and ultra topographical morphological characteristics of crude *Trigona's spp* and *Apis mellifera* propolis.

1.4. Hypothesis

This study hypothesized that the *Trigona spp* and *Apis mellifera* bee propolis contain high biochemical diversity associated with the topographical features of the propolis.

CHAPTER 2

LITERATURE REVIEW

2.1 Honey bees and propolis

Integration of natural traditional medicine require scientific approach as a bridge in connecting it to modern medicine. The traditional medicine needs more data and research in order for it to be considered as an alternative to the current therapeutical drug. One of the important aspect to be properly explore is the geobotanical profile of the product (Molan and Rhodes, 2015). Among these natural product of tangible signature is propolis. Propolis is define as resinous compound produced by honey bee by collecting resin exudates from multiple different parts of plant to be mixed with beeswax and pollen. The propolis is used by honey bees as building material of the hive and as a general hygenic maintainence substances (Sun, *et al*., 2015).

Entering the millennium, propolis is now considered a “natural antibiotic”, nanostructure natural product adapted to human wellness as therapeutic systems because of its pharmaceutical properties, which include antioxidant, anti-inflammatory, and antimicrobial activities. However globally there are different types of etnobotanical propolis vouchered present. These propolis inert chemical complexity and geographical flora signatures variation are preselected by the bees. As such these contributed to categorical differentiation derived from physico-chemical based and their colour presentation for each propolis (Sena-Lopes, *et al.*, 2018, Trusheva, *et al.*, 2006).

Colour is one of the traits that can be used in early classification of the propolis. Previous researches had managed to associate colour trait of propolis to the botanical source. For example, *Baccharis dracunculifolia* plant is a type of signature plant among Brazillian propolis. Propolis derived from this plant share common colour trait that is green in colour. The green coloured propolis has been found to be associated with therapeutic potential compound such as hydroxycinnamic acids, terpenes, and phenolic acids (Machado, *et al.*, 2016; Zaccaria *et al*., 2018). The red coloured propolis is often associated to *Dalbergia ecastophyllum (L.) Taub*. *(Fabaceae)* mangrove plant. The red propolis has be shown to contain flavonoids, flavones, catechins and terpenes. While the brown coloured propolis is associated with *Copaifera* plant. Red propolis has been shown to contain galangin, chrysin, flavonoids and terpenes (Curti, *el al*., 2019; de Mendonça, *et al*., 2015; Franchi, *et al.*, 2012).

Propolis varies from one to the other. The variation are caused by several factors. The propolis is can differ simply due to different foraging territory or seasonal differentiation. Foraging territory and seasonal differentiation are linked to different botanical sources available in radius of the foraging bee. Different botanical sources of propolis will contain different biological compound. In general there are 5 major botanical sources that have been properly linked based on phytochemical matching pattern to propolis production. The botanical sources are Poplar, Pacific, Tropical, Birch, and Mediterranean (Bankova, 2005).

Propolis has been used as traditional approaches in health and disease management for a very long time. Propolis was utilized by the Greeks in treating abscesses. While among Assyrians, propolis had been applied in treating wounds. Others have different ways of utilizing this bee product. The Egyptians was using the propolis as a component in preserving the corpse through mummification. As such propolis have been historically reported to be able to provide various beneficial and supportive wellness effect on daily mangement human health and even for the life after (Frade, *et al.*, 2012, Kuropatnicki, *et al*., 2013).

Developments trends in propolis research involve biological studies performed with chemically characterized samples. However, different geographic regions propolis samples potentiate a completely different chemistry, efficacies and biological activity (See table 2.1 below for an Indian representaives of the chemistry activities). Previous researches have concluded that raw propolis compounds are divided to 58 -62% of terpenes, 25-30% of beeswax and 5-10% of phenolic compounds raw propolis (Huang, *et al.*, 2014). Propolis samples taken from European and Asian region shown to contain phenolic acids (Bankova, *et al.*, 2002). In tropical climate the propolis are shown to contain a high level of lignans (Petrova, *et al.*, 2010). Among all of the compounds found so far, one of the compound has been associated to be potentially therapeutic related compound that is caffeic acid. Caffeic acid can be found in propolis collected from Asia, America and European region (Omene, *et al.*, 2013). In South American, especially in Brazil, green propolis is one of the most popular propolis due to its potential for therapeutic value. The compounds with promising therapeutic potentials are hydroxycinnamic acid derivatives artepillin C, prenylated cinnamic acids and caffeic acid derivatives (Marcucci, *et al.*, 2001). Organic acids, aldehydes, hydrocarbons and mineral can also be found (Wagh, 2013).

	India continent geographical region	Related cientific evidence activities	Solvent used in the extraction	References
1.	The state of Karnataka	Antibacterial	Petroleum ether, chloroform, ethanol, and 40% methanol	Selvan, Singh, and Prabhu, 2011.
2.	The state of West Bengal	Antioxidant	Ethanol, water, petroleum ether, and chloroform	Laskar, Roy, and Begum, 2010.
3.	The state of Gujarat	Antioxidant, antimicrobial	Ethanol, methanol, and 40% methanol	Kumar, Ahmad, Dang, and Husain, 2008.
4.	The state of Madhya Pradesh	Antimicrobial, Hepatoprotective	Ethanol	Deepak M. Kasote, 2017.
	The state of Maharashtra	Antimicrobial, Antibacterial	Ethanol	Shubharani Ramnath, *et al.*, (2015).

Table 2.1: A tabulated representative of vouchered propolis honey: geographical origin, activities, and chemical compounds identified and extrapolated from the Indian continent.

Propolis is usually presented with a pleasant aromatic odour. Botushanov, *et al.*, 2001 have recognized propolis as “veritable cascade of aromatic nutrient” which possess capabilities in combating disease caused by viruses, bacteria, fungi and parasites. The composition of propolis (of different colors) depends on the phytogeographical location, seasonal collection time, and botanical source. For example, yellow resinous coat observed on Thailand Chanthaburi propolis is reported to originate from the fruit surface of plant origin in Thailand: *G. mangostana1* (Kumazawa 2018).

Pharmacological and clinical evidence based related chemical studies to harness local folk or traditional medicines are predominantly initiated and were derived from exploitation of geo-located local 'status quo' flora and botanical plants biodiversity. As such this was the basis platform of most early medicines. In tandem to this, the current progress on propolis experimentation involve application of biological analysis on chemical characterization to provide a better understanding of the samples. The dilemma and problem related to these biological studies are mainly caused by the wide range of chemical compounds in propolis. In this millennium various effort and industrialized optimization based on scientific evidences in the development and research of commercially viable propolis (either as extracts or formulations) remain as significant challenges per se to establish policies at national and international level that provide better compatibility and purported tangibles in the propolis market and industry. The knowledge lacunae among researcher, industrial player, policy maker and consumer in general is to be the main obstacle in moving forward despite increased emphasis upon evidence-based policy. Effort need to be channelled somewhere to kickstart the integration of this natural treasure into the modern medicine. Hence a gold standard guidelines or recommendations in respect to propolis (extract and formulation) standardization and practical applications in therapy application and research development is found wanting.

2.2 The taxonomy, biology and anatomy of *Trigona spp* and Honey Bees.

In pertinent to their taxonomy, stingless bees are a monophyletic group of bees hibernating and pollinating in the tropical and subtropical geographical areas of America, Asia, Africa and the Australia continent (Roubik, 1989; Michener, 2000). The oldest known fossil: *Trigona prisca* (was identified about 96 to 74 M years ago). As such stingless bees are a large and diverse taxon with more than 500 species recorded worldwide, thus are important pollinators of cultivated and wild plants, contributing significantly to biodiversity and food security. The most common genera of stingless bees were: *Trigona, Meliponula, Dectylurina* and *Lestrimelitta.*

Most of the Asian and African stingless bees' species belong to the genera Trigona (Michener, 2000). In the state of Kelantan, eastcoast of Malaysia, the taxonomy study conducted on 161 stingless bee hive at local apiary (06°07'N, 102°19'E) revealed the present of five species of stingless bees that were: *Tetragonula laeviceps*, *Geniotrigona thoracica*, *Lepidotrigona terminata*, *Lisotrigona scintillans* and *Heterotrigona itama*, that accounted for 83.2%, 11.2%, 2.5%, 1.9%, 0.6% and 0.6%, respectively. In this publication it was acknowledged that of 17-32 known species of stingless bees in Malaysia, only *T. itama* and *T. thoracica* were mostly used in meliponiculture. As such the scientific evidences studies on *Trigona spp* species diversity are still found lacking and in tandem to this literature on characteristics features to propolis associated to this species globally are also in lacuna. Till date the present taxonomy understanding to these stingless bees is as highlighted in the table below:

Scientific classification /	**Taxonomy**
Kingdom:	Animalia
Phylum:	Euarthropoda
Class:	Insecta
Order:	Hymenoptera
Family:	Apiidae
Tribe:	Meliponini
Genus:	***Trigona*** Jurine, 1807

Table 2.2: Scientific taxonomy representation of *Trigona* stingless honey bees. (Cited and enhanced from Kelly *et al.*, 2014 and Mark L. Winston. 1987. *The Biology of the Honey Bee*. Harvard University Press. Cambridge, MA).

Scientific classification of Subspecies: *Apis mellifera*

Geographic distribution	Subgenus
Central Mediterranean Sea and South West Europe:	Ligustica, Carnica, Macedonia, Sicula, Cecropia
Western Mediterranean sea and West Europe:	Mellifera, Iberica, Sahariensis, Intermissa
Middle East:	Meda, Adami, Cypria, Caucasica, Armeniaca, Anatolica
Africa:	Intermissa, Major, Sahariensis, Adansonii, Unicolor, Capensis, Monticola, Scutellata, Lamarkii, Yementica, Litorea

Table 2.3: Scientific classification of Subspecies: *Apis mellifera*
Cited from: https://en.wikipedia.org/wiki/Subspecies

A honey bee is a eusocial, flying insect within the genus *Apis* of the bee clade. They are known for construction of perennial, colonial nests from wax, for the large size of their colonies, and for their surplus production and storage of honey, distinguishing their hives as a prized foraging target of many animals, including honey badgers, bears and human hunter-gatherers. The honey bee (*A. mellifera* L.) is the highly widespread economic bees. It accounted for over 90 % of the world honey production and majority of insect's pollination in plants. Since they exist in abundance and also due to their behaviour, *Apis mellifera* prone to work around a lot of different type of plants. This factor contribute to major difference in characterization among propolis samples. There were 7 species and 44 subspecies that have been properly documented until early 2000 (Engel, 1999; Michener, 2000). Honey bees are hymenopterans, a group that generally feed on pollen and nectar and constitute about

20,000 species throughout the world, known taxonomically as the superfamily Apoidea (Michener, 2000).

A. mellifera originated from Europe, Asia and Africa and have about 25 races that includes important ones such as European bee (*Apis mellifera mellifera*), Asian bee (*Apis cerena*, West African bee (*Apis mellifera adansonii*), China bee (*Apis cerena cerena*), Central and East Africa bee (*Apis mellifera scutellata*), North Africa bee (*Apis mellifera intermissa*), Italian bee (*Apis mellifera ligustica*) (Seegeren, *et al.*, 1996).

Similar to most insect, honey bees' outer anatomy is covered by exoskeleton. This exoskeleton is made up of chitin. The main function of exoskeleton is to provide protection for the internal component and organ of the honey bee. Exoskeleton also function as a form of barrier to keep moisture from leaving the inner part of the bodies. (Gary, 1992; Snodgrass, 1925). As other insect, honey bee growth are completed through shedding process of the exoskeleton. Honey bees anatomy consist of three main sections. The first section, the head consist of antennae, compound eyes and mouthparts. The second section is the thorax. The thorax consists of wings, legs and appendages. The legs are further subdivided into prothoracic, mesothoracic and metathoracic which are determined by the position of the legs. Compared to the others leg, metathoracic leg possesses corbicula. The corbicula served as a basket for pollen storage. The corbicula was also used by the honey bee to collect resin for propolis production. The third section is abdomen. The abdomen is where multiple organ of honey bees are located. These organs function as digestive system, reproduction system and also defence system. (Snodgrass and Erickson, 1992; Snodgrass, 1925; Snodgrass, 1956; Gary, 1992).

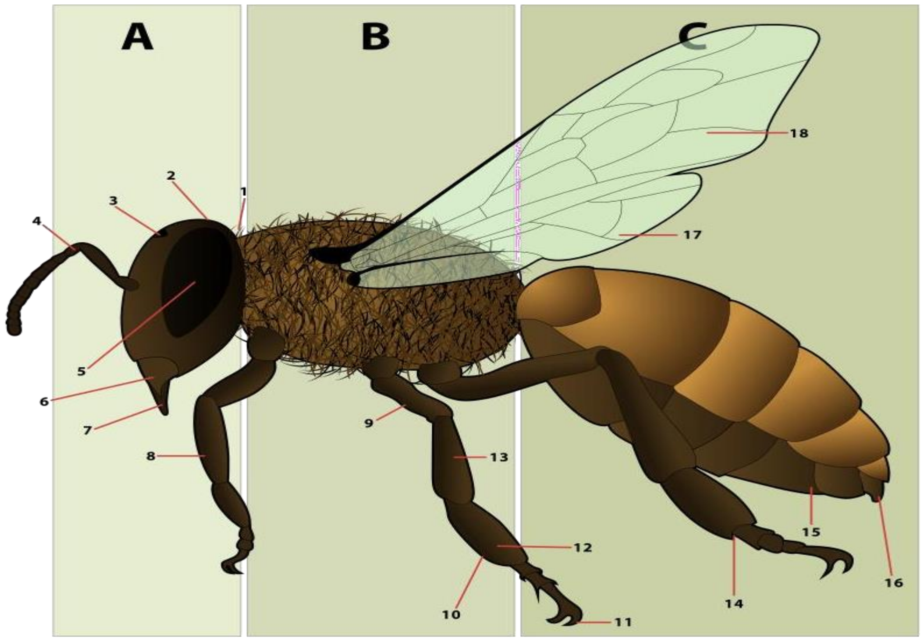

Figure 2.1: Morphology of a female honey bee

The above female honey bee morphology can be identified as a female by both the number of divisions on its antenna and by its sting. **A**: Head, **B**: Thorax, **C**: Abdomen **1**: Gena, **2**: Vertex, **3**: Ocelli, **4**: Antenna, **5**: Compound Eye, **6**: Feelers, **7**: Proboscis, **8**: Foreleg, **9**: Femur, **10**: Middle Leg, **11**: Tarsal Claw, **12**: Tarsus, **13**: Tibia, **14**: Hind Leg, **15**: Sternum, **16**: Sting, **17**: Hind Wing, **18**: Forewing.
[Adapted and cited from: HoneyBeeAnatomy.svg].

Figure 2.2: A digital representative of *Trigona (Tetragonula) iridipennis* stingless bee. Most *Trigona* are oval-shaped creatures with golden-yellow and brown bands. The total body length ranges from 2.5–3.5 mm. This photomicrograph of the stingless bee was cited *in the Journal of Threatened Taxa* 2014. Note: For acknowledgement the above said bees till date has not been properly scientifically evaluated.

2.3 Global etnobotanical plant origin (Taxonomy and Source)

In tandem to commercial exploitation and with the rise of evidence-based complementary medicine globally, as signified by various clinical trial studies to show the efficacy of propolis for various ailments, it will be of tangible benefits' if a consensus of these efforts will demonstrate the similarity of propolis functions and therapeutic actions although of different generic geo-location. Different location produce designated propolis. For example, one of the most widely characterized propolis come from poplar plant. Poplar plant are often located in temperate zone (Roumen, *et al*., 2006). Another type of propolis found in a lot of publication is propolis associated with Baccharis spp plant (Park, *et al*., 2004). This type of propolis can be found in Brazil. Geographical neighboorhood country like Indonesia's propolis has been reported to be related to *Macaranga tanarius L.* and *Dalbergia ecastaphyllum* (Trusheva, *et al*., 2011).

Poplar plant bud excretion is the major source for propolis in North Africa, South America, Europe and West Asia (König, 1985). While Canadian poplar plants originated from Section Aigeiros: *Populus deltoides* Marsh, *Populus fremontii Wats* or *Populus maximoviczi* Henry was described by Garcia-Viguera, Ferreres, & Tomas-Barberan, 1993). Besides poplar botanical sources of propolis also include European region plant alder, birch, hazel, oak and willow; South African region plant *Acacia Karro*; Australian region plant *Xanthorrhoea pressii* and *X australis;* Hawaii region plant *Plumeria acutifolia*, *P. accuminata*, *Psidium guajava* and *Schinus terebinthifolius* and Cuba, Venezuela South American region *Clusia spp*. (Warakomska and Maciejewicz, 1992).

The important compounds which were stated in multiple publication in the past can be classified as phenolic acid and derivatives, flavonoid and derivatives, volatile terpenes, aldehyde, fatty acids, aromatic hydrocarbon, steriod and stilbene (Hrobonova *et al*., 2008; Gardana, *et al*., 2007; Awale, *et al*., 2008; Li *et al*., 2008; Sahinler and Kaftanoglu, 2005; Abu-Mellal, *et al*., 2012). Every geographical location provide propolis with different major compounds and therapeutic related compounds. In temperate region, where poplar plant derived propolis can be found, the propolis were found to contain higher amount of flavonoids and phenolic acids. Poplar plant is also available in Mediterranean region. The propolis orignated from this region contain high amount of diterpenes. In Eastern region such as Japan and Taiwan, the propolis contain high amount of flavanones. While in Australian the propolis contain high amount of stilbenes. South American region provide propolis with high level onf benzophenones, terpenes and flavonoid. Tropical region originated propolis contain various kind of compound such as cinnamic acid, flavonoid, lignan and benzophenons (Bankova, *et al*., 2000; Popova, *et al*., 2010; Chen, *et al*., 2003; Kumazawa, *et al.*, 2004; Abu-Mellal, *et al*., 2012; Russo, *et al*., 2004). These subtances not only marked important botanical origin, but also related to geographical and most importantly will influence the output of therapeutic effects.

Geographic region	**Propolis types**	**Geobotanical plant sources**	**Chemical characteristics**	**References**
Temperate regions	Poplar	Populus section Aigeiros	Flavonoids caffeic acid esters	Bankova, *et al.*, 1994
South America: South Brazil State of Bahia	 Green propolis	 *Araucaria spp.* *Baccharis dracunculifolia*, Artepillin C	 Diterpenoids Prenylated phenylpropanoids,	 Machado, *et al.*, 2016

	(Alecrim propolis) Red propolis. Brown propolis	 *Dalbergia ecastophyllum (L.) Taub.* (Fabaceae) mangrove species Copaifera species	triterpenoids, benzoic acid and chlorogenics Flavonoids and terpenes	 Daugsch, *et al.*, 2007 Piccinelli, *et al.*, 2011. Franchi, *et al.*, 2012.
Northern Argentine and San Juan (Cuyo region, Western Argentine)	Apis mellifera propolis	*Baccharis* sp.,	Galangin (3,5,7-trihydroxyflavone) Antooxidants	Isla, Paredes Guzman and Nieva Moreno, 2005. María, *et al.*, 2009.
Cuba	Red propolis Brown and yellow propolis	Genus *Dalbergia* Floral resin of *Clusia rosea*	Isoflavonoid phytoalexins Polyisoprenylated benzophenones (PPBs)	Ingham, 1979. Marquez Hernandez, *et al.*, 2010.
Asia: Myanmar China Nepal	 Unknown Poplar Nepalese propolis	 *Dalbergia sericea* resin	 Cycloartane triterpenoids, e.g.(24E)-3-oxo-27,28 dihydroxycycloart-24-en-26-oic acid 81,82 (22Z,24E) Oxocycloart-22,24-dien-26-oic acid 6-Cinnamylchrysin 3-O-[(S)-2Methylbutyroyl] pinobanksin Neoflavonoids, chalcones, and pterocarpans	 Zhang, *et al.*, 2013 Awale, *et al.*, 2005
Chitwan Nepal	-	Leguminosae subfamily Faboideae	Dalbergiones, Isoflavones, neoflavonoids, flavanonols, flavonols	Shrestha, *et al.*, 2007. Shrestha, Narukawa and Takeda *et al.*, 2007.

Northern and southern regions of Peninsular Malaysia	*Apis mellifera* Propolis	*Acacia mangium*		Tuan Nadrah Naim, *et al.*, 2018. Nurhamizah, *et al.*, 2016.
Korea	Poplar propolis	Poplars	Flavonoids with an unsubstituted B-ring, caffeic acid esters	Huang, *et al.*, 2014. Choi, Noh and Cho, 2006.
Turkey		*Populus alba*, *P. tremuloides*, *Salix alba*	Flavonoids, B-ring, vanillin, bisabolol	Murat Kartal, *et al.*, 2002.
Laguna and Quezon, Philippines	Tetragonula biroi (Friese) propolis		Total phenolics, total flavonoids, antioxidant activity and antibacterial property	Joy Line T. Cumbao, *et al.*, 2016.

Table 2.4 Selected representation of global propolis geographic region locations and to illustrate honeybee propolis types, geobotanical plant sources, and chemical characteristics.

2.4. Global etnobotanical propolis etnocomposition studies

Literatures have documented that research activities related to extrapolating on chemical composition and profile of geobotanical propolis started at the beginning of the 20th century. In the beginning, the research on propolis characterization mainly involved fractionation processes. Among all these literatures, a research conducted by Dieterich and Helfenberg where they perform among the earliest form of extraction for propolis which utilizes alcohol, chloroform, and ether as the solvent (Dieterich, 1908, Helfenberg, 1908). In 1911, in his later work, Dietrich then identified the presence of vanillin in propolis (Dieterich, 1911), while K¨ustenmacher, identified compound known as cinnamic acid and cinnamyl alcohol in their sample (K¨ustenmacher, 1911). In 1926, Jaubert discovered compound known as chrysin. Chrysin was a flavon based pigment which was isolated from the beeswax. A hypothesis made by Plinius regarding the source of plant compartment in which the resin of the propolis were later confirmed through a resarch in 1927 by German scientist named R¨osch (R¨osch, 1927). Multiple researches performed in United State in between 1940 to 1942 had discover presence of vitamins B1, B2, B6, C, and E along with nicotinic and pantothenic acid in samples of propolis (Haydak and Palmer, 1940, Haydak and Palmer 1941, Haydak and Palmer, 1942). A reseacher and his colleague discover multiple different wax compound in propolis sample, all 4 with different individual colour presentation (Ushkalova and Topolova 1973) . Before 1967 multiple researches on flavonoid compound isolated from propolis sample were conducted to explore its potential therapeutic and physiological functions (Bohm, 1968). Flavonoid showed antibacterial properties through inhibition of 10 different strains of bacteria (Ghisalberti, 1979).

In the earlier stages of researches on propolis, it was postulated that propolis as a compound consist of complex composition but was constant in nature much like the beeswax (Lindenfelser, 1967). It was proved later on through numbers of researches using much more latest and sophisticated scientific laboratory approach on multiple different samples that showed significant variation of each samples collected from different geographical origin. Later on, several researcher succesfully isolated and discovered 2 compounds that were flavanones and isovanillin along with six flavonoid pigments in propolis (Popravko, *et al*., 1969; Popravko, *et al.*, 1970). Lavie had performed experiment on antibacterial properties of propolis on several strains of bacteria that were *Bacillus subtilis, Bacillus alvei,* and *Proteus vulgaris* (Lavie, 1957). Several scientists from France later on succesfully extracted flavon compound known as galangin, which they believe was responsible as one of the compound which gave propolis antibacterial properties (Villanueva, *et al*., 1964). The same group of researchers successfully discover and characterized several new compounds extracted from propolis known as pinocembrin, tectochrysin and isalpinin (Villanueva *et al*., 1970). In 1970 Cizmarik and Matel reported separation and identification of 3, 4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid which are present in propolis (Cizmarik and Matel, 1970; Cizmarik and Matel, 1973). Nikiforov with his co-workers detected presence of trace element in propolis samples that were copper and manganese (Nikiforov, *et al.*, 1971), and at the same time Herold conducted experiment on ash residue of propolis and found more trace elements iron, calcium, aluminum, vanadium, strontium, manganese, and silicon (Herold, 1970).

In the 1970s, advances in chromatographic analytical methods allowed for separation and extraction of more components from propolis. In 1975 Schneidweind and his co-workers identified 17 constituents of propolis, including 9 previously identified compounds (Schneidweind, *et al.*, 1975). Simultaneously, Metzner with his co-workers using bioautographic methods proved that only a few compounds detectable in the extracts of propolis have significant antimycotic activity (Metzner, *et al.*, 1975). In 1977, Australian researchers separated and identified four flavones, pinostrobin, sakuranetin, isosakuranetin, pterostilbene, chrysin, 3, 5-dimethoxybenzyl alcohol, and xanthorrhoeol (Ghisalberti, *et al.*, 1977). In 1979 Vanhaelen and Vanhaelen-Fastre used liquid and gas chromatography in propolis analysis. Application of GC-MS led to discovery of sugar profile of propolis. Marcucci and Bankova *et al.* have registered over 300 known substances in propolis (Marcucci, 1995; Bankova, *et al.* 2000). Heinen and Linskens studied fatty acid constituents of propolis where it contain C7– C18 acids (Heinen and Linskens, 1972). Popravko discovered 18 new chemical compounds isolated from propolis sample with 14 of them belong in flavonoid groups (Popravko, 1978).

Propolis compound differ based of several factors that are regional, seasonal, botanical and also individual hive. A range of more than 80 to 100 chemical compounds can be isolated from single sample of propolis at given time (Marcucci, *et al*. 2001). Altogether, at least 180 different compounds have been identified in propolis so far. A broad analysis revealed approximately 50 constituents in “typical” European propolis, which comes usually from trees, such as poplars and conifers. These constituents comprise primarily resins and vegetable balsams, mainly cinnamic acid and derivatives, coumarin acid, prenylated compounds, artepillin C (50%), beeswax (30%), essential oils (10%), bee pollen (5%), and minerals, polysaccharides, proteins, amino acids, amines, amides, and organic debris (5%) (Cirasino, *et al*. 1987). The major constituents of propolis foremost of the sources are flavonoids (Bankova, *et al.*, 2002 and Marcucci, *et al.*, 2001). Some of the principal phenolic esters and flavonoids like caffeic acid phenethyl ester, quercetin, baicalin, pinocembrin, naringin, galangin, and chrysin have been found to be responsible for therapeutic effects of propolis (Savickas, *et al*. 2005).

2.5. The perspectives of a reproducible, standardized biologically active propolis extract.

In order for any natural product based medicine to be accepted as an outsource to modern medicine, the compound must have proper elaborated data of safety, quality control and scientific based proven efficacy. The question involve which biological compounds should become the important point of interest to be screened among the hundreds of available compounds. This will mark the starting point of any natural product based drug research. Each extracts need to be evaluated through the modern scientific method so that one standard, safe and therapeutically effective extract can be move forward to next stage in clinical application.

A scientific elucidation investigation of previously reported synergistic effect of combination of natural and synthetic product inhibitors should be considered for further evaluation. Thus the extracts or assay formulation or preparation parameters needs to be carefully investigated and this include various considerations that include the dose dependent or drug-ability towards the targeted tissues, the screening compatibility of the product, and mechanism of action for the compound in clinical condition.

Propolis was used as one of method of treating illness for centuries, and today many propolis products are available commercially. However, propolis collected from different regions of the world will have different compositions and will vary in activity. Therefore, it is important to establish standards for the composition of propolis, where the presence and amount of important constituents, and absence of contamination can be determined (Bankova & Marcucci, 2000; Bankova, *et al*., 2016). As such, propolis has become the subject of intense global modern scientific based research aiming to transform this natural compound into commercialize ready medicinal product for past few decades. These studies have proven its versatile pharmacological activities: antibacterial, antifungal, antiviral, antiinflammatory, hepatoprotective, antioxidant, antitumor, and others (Banskota, *et al.*, 2001). *Per se*, the chemical variability of propolis is discussed with respect to the problem of standardization. Several chemical types of propolis are formulated, based on their plant source. Reliable criteria for chemical standardization of different propolis types are needed but such generally accepted criteria do not yet exist. In pertinent to propolis, these liquid formulations are often prepared using ethanol containing media to solve the poor water solubility of propolis extracts. Nevertheless, considering the irritating and drying effects of ethanol on the oral mucosal membrane (Muller, *et al.*, 1983) as well as the use of propolis preparations in pediatrics, aqueous formulations of propolis should be preferred to