CHARACTERIZATION AND ULTRASONIC ASSISTED HYDROLYTIC EXTRACTION OF SELECTED MALAYSIAN PROPOLIS

by

YEO KAI LIANG

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LIST OF ABBREVIATIOINS

AEP	Aqueous extracted propolis
CAPE	Caffeic acid phenethyl ester
DOA	Department of Agriculture
EEP	Ethanolic extracted propolis
GCMS	Gas chromatography mass spectometry
HPLC	High performance liquid chromatography
MAE	Microwave assisted extraction
RISDA	Rubber Industry Small Holders Development Authority
RRIM	Rubber Research Institute Malaysia
SFE	Supercritical fluid extraction
TP	Total phenolic
UAE	Ultrasonic assisted extraction

LIST OF SYMBOLS

EtOH	Ethanol
H_2O	Water

PENCIRIAN DAN PENGEKSTRAKAN PROPOLIS MALAYSIA TERPILIH MELALUI HIDROLISIS TERBANTU ULTRASONIK

ABSTRAK

Propolis telah dibuktikan mempunyai pasaran yang unik dalam nutraseutikal disebabkan oleh keserbabolehannya dalam aktiviti-aktiviti farmakologi: antimikrob, antioksidan, antitumor dan lain-lain. Aktiviti-aktiviti farmakologi propolis ini adalah dipengaruhi oleh faktor utama iaitu asal-usul geografi, sejajar dengan flora tempatan, spesis lebah dan musim. Propolis dari pelbagai asal usul geografi telah dikaji secara meluas, tetapi sedikit yang diketahui tentang propolis Malaysia. Dengan, empat sumber propolis Malaysia iaitu Parit Nibong, Sibu, Serian dan 13th Miles telah disiasat untuk mendapatkan ciri-ciri proksimat, kandungan bioaktif, aktiviti gautan radikal bebas DPPH dan aktiviti anti-bakteria mereka. Ciri-ciri ini kemudiannya dibandingkan dengan propolis Brazil yang telah dikaji secara komprehensif. Propolis Malaysia, terutamanya yang berasal dari Sibu dan Parit Nibong mempunyai kualiti yang setanding dengan propolis Brazil. Propolis Sibu mengandungi kandungan bioaktif tertinggi dengan jumlah fenolik sebanyak 155.41 ± 2.70 mg GAE / g EEP dan jumlah flavonoid sebanyak 212.27 ± 35.20 mg / g EEP. Kandungan bioaktif untuk semua propolis- yang dikaji mencerminkan ciri-ciri antioksidan dan anti-bakteria mereka. Analisis gas kromatografi–spektrometri jisim (GC-MS) menunjukkan kehadiran komposisi utama seperti asid fenolik dan derivatifnya, asid alifatik dan karbohidrat. Cabaran dalam pengekstrakan propolis yang disebabkan oleh masa yang panjang dan penggunaan pelarut yang tinggi (24 jam dan 70% (i/i) ethanol) telah membawa kepada keadah alternatif pengekstrakan propolis melalui ultrasonik bersama dengan manipulasi pH. Gabungan teknik-teknik ini tidak pernah dilaporkan, justeru menunjukkan kepentingan penyelidikan ini. Parameter-parameter yang dikaji adalah kepekatan pelarut, pH dan kitaran ultrabunyi. Tempoh masa pengektrakan optimum ialah 1 jam dan 40 minit, pada pH 10 dan kepekatan pelarut sebanyak 30%(i/i) etanol. Ekstrak yang dihasilkan daripada keadaan optimum ini mempunyai kandungan bioaktif sebanyak 421.43 ± 20.92mg GAE / L bagi jumlah kandungan fenolik dan 233.34 ± 4.22mg Quercitin / L bagi jumlah kandungan flavonoid, kandungan tersebut adalah 80% dan 62% jumlah fenolik dan flavonoid masing-masing dibandingkan dengan ekstrak yang dihasilkan dengan kaedah lazim. Oleh itu, kaedah pengekstrakan yang dicadangkan boleh menjadi kaedah pengekstrakan propolis alternatif yang baik dengan pengurangan masa pengekstrakan dan pengunaan pelarut..

CHARACTERIZATION AND ULTRASONIC ASSISTED HYDROLYTIC EXTRACTION OF SELECTED MALAYSIAN PROPOLIS

ABSTRACT

Propolis was proven to possess a niche market in the nutraceuticals due to its proven versatilities in pharmacological activities: antimicrobial, antioxidant, antitumor etc. The extents of pharmacological activities are affected by their geographical origins with the local floras, bees species and season as the key influencing factors. Propolis of various geographical origins was studied extensively, but little is known about Malaysian propolis. Therefore, four sources of Malaysian propolis, i.e. Parit Nibong, Sibu, Serian and 13th Miles, were investigated for their proximate characteristics, bioactive contents, DPPH free radical scavenging and antibacterial activities. These characteristics were then benchmarked with the comprehensively studied Brazilian propolis. Malaysian propolis, especially those from Sibu and Parit Nibong possessed quality comparable to Brazilian propolis. Sibu propolis contain the highest bioactive content with 155.41 ± 2.70 mg GAE equiv. / g EEP of total phenolic content and 212.27 ± 35.20 mg equiv. / g EEP of total flavonoids content respectively. The bioactive content of all studied propolis reflected well on their antioxidant and antibacterial properties. The gas chromatography- mass spectrometry (GC-MS) analysis indicated the presence of chief compounds like polyphenols and their derivatives, flavonoids and carbohydrates. Due to challenges faced by maceration extraction of propolis, which are time consuming and high solvent consumption (24 hours and 70% (v/v) ethanol, an alternative propolis extraction at varied pH is furthered with ultrasonication. Such combination of techniques have never been reported and thus the importance of this study. The parameters studied were solvent concentration, pH and the ultrasonication period. The optimum time of extraction was 1 hour and 40 minutes, at pH 10 with 30% (v/v) ethanol. Extract produced from the optimum condition yielded bioactive content of 421.43 ± 20.92mg GAE equiv./L and 233.34 ± 4.22mg Quercitin equiv./L for total phenolic and flavonoids content respectively, which are 80% and 62% of the respective bioactive content found in maceration extracted propolis. Thus, the proposed extraction technique can be a good propolis extraction alternative with significant reduction in extraction time and solvent used.

Chapter 1

Introduction

1.1 Research Background

Propolis is a resinous material collected by bees from buds and exudates of the plants and transform using bee enzymes. Depending on the origins of the propolis, the colour may vary from green, red to dark brown. In general, propolis *in natura* is composed of wax, resin and balsam, essential and aromatic oils, pollen and other substances (Burdock, 1998). Propolis in its natural form is as shown in Fig. 1.1.

Fig. 1.1 Propolis before being harvested from the super.

Propolis has long been used in the European folk medicine (Toreti et al., 2013) and the traditional Chinese medicine. Propolis was recognized and widely used by the Greek and the Roman physicians, like Aristotle, Dioscorides, Pliny and Galen. Propolis was used as antiseptic and cicatrizant in wound treatment and also mouth disinfectant, these uses were perpetuated until the Middle Ages and among Arab physicians. The London pharmacopoeias of the seventeenth century listed propolis as an official drug. It has become a very popular drug between the seventeenth and twentieth century in Europe due to its anti-bacterial activity (Toreti et al., 2013). During the World War II, doctors have also applied propolis on wounds of soldier. Asides, the Orthodox medicine in USSR had also accepted the use of propolis in 30% (v/v) alcoholic solution as a treatment. To make the subject more relevant, propolis has been recognized as a diet supplement in 1995 by the National Food Institute (INAL) in Argentina (Lotfy, 2006).

Malaysia has a great biodiversity as our country is densely covered by rainforest dating back to 70 million years ago (Park, 2002). But the apiculture industry in Malaysia still remains in its infancy with little development and integration of advance technology in producing apiculture products. The government attempted to promote this industry since the 1980s. Efforts of promoting this industry were being carried out by agencies like the Department of Agriculture (DOA), Rubber Research Institute Malaysia (RRIM), Rubber Industry Small Holders Development Authority (RISDA) and others. Locally, apiculture is practiced by companies to commercialize locally produced honey and bee pollen running in small scale, scattered in suburbs and rural area in the country, while propolis was usually treated as side product (Ismail, 2009).

The very first work indexed by *Chemical Abstracts* on propolis was in 1903 while the first patent was patented in 1904 (Toreti et al., 2013), the number of publication in journals and patents on propolis escalated drastically since then. Most of the works that carried out to date were on the chemical composition and biological activities of propolis of different geographical origins. Propolis produced in the South America like Brazil, Argentina and Portuguese are well studied. In terms of Asian propolis, those that are produced in China, Taiwan Korea and Japan are better understood as their botanical origins were traced and identified. Furthermore, the unique compounds in the propolis, e.g. propolis C and D, were being isolated and their biological attributions were being verified (Popova et al., 2010). At present, there are only limited studies being carried out on South East Asia propolis, e.g. Thai and Indonesian propolis. The studies on the propolis produced in these areas were focused in finding its uniqueness aside from the evaluation of its quality and biological attributions (Trusheva et al., 2011, Siripatrawan et al., 2013).

To date, propolis is understood to possess bio-attributions like antibacterial (Grange and Davey, 1990), anti-fungal, anti-viral (Gekker et al., 2005), hepatoprotective, anti-tumoural (Moreira et al., 2008), antineurodegenerative (Farooqui and Farooqui, 2012) and anti-inflammatory (Banskota et al., 2001, Bueno-Silva et al., 2013, Toreti et al., 2013). For this reason, the demand of the application of propolis in the nutraceuticals has escalated. Furthermore causing it to be the subject of intense pharmacological and chemical research for the past 30 years. These researches had widened the understanding of researchers towards propolis. In the 1960s, propolis was thought to be very complex, but with more or less constant chemistry, like bee wax and bee venom. Studies had been carried out for propolis of different geographical region, results have shown that the composition of propolis was highly variable and dependent upon the local flora at the site of collection, species of bees collecting, region of origin of the propolis and the local weather. Thus the term propolis cannot be characterized with respect to the chemical composition and made general upon study on one sample, unlike the bee venom for example.

Food products like fruits and vegetables, coffee, cocoa and tea are complex mixture of vitamins, sugars, proteins and lipids, fibres, aromas, pigments, antioxidants and other organic and mineral compounds. These products have to be processed and extracted for their food ingredients before they were commercialized. The same goes to crude propolis which is a mixture of ash, wax, bioactive compounds and pollen, therefore cannot be directly consumed. Thus extraction process is required to extract the bioactive compounds prior to its consumption. Up to now, the most efficient extraction method is still the conventional maceration extraction method which requires high amount of solvent and time consuming. Thus many different modern extraction methods of propolis extraction were developed and studied to overcome these shortcomings. There has been trend in propolis extraction research in finding the alternative solvent, e.g. water and oil, for propolis extraction. The integration of modern technology like ultrasonication and microwave helps to reduce the extraction time required.

1.2. Problem Statement

The global consumption of propolis was estimated to be around 700 – 800 tons/year (da Silva et al., 2006). Up till now, Brazilian propolis is the most commercialized propolis globally. Brazil has been dominating the propolis market internationally. It was reported by the Ministry of Development Industry and Foreign Trade of Brazil (Bureau of Foreign Trade, 2012), that Brazil has exported 41,721 kg of propolis in 2012 with the value of 129.47 USD/kg, a total export value of 5,401,643 USD (Toreti et al., 2013). From the statistic shown, the price of propolis increases every year showing that the market of propolis is good. According to Aga et al. (1994), Japan is the main importer of Brazilian propolis compared to other countries. Malaysia should have similar geographical advantage with Brazil judging from the richness in the country's flora and fauna and the whole year of sunshine and rain.

As mentioned, the apiculture industry in Malaysia still remains in its infancy with little development and integration of advance technology in producing apiculture products. Malaysia apiaries products are not diverse as the industry is generally oriented towards honey and bee pollen production (Lim and Baharun, 2009). Propolis also possesses the potential to strive in the market as a profitable product with a distinctive selling point than honey and bee pollen.

Prior to commercializing locally produced propolis as a therapeutic agent or value-added products, it is crucial to establish the fundamental understanding of Malaysian propolis by evaluating the quality of propolis through the physical characteristics, possessed bio-attributions and identify the general constituents. These characteristics were then compared with other established propolis, e.g. Brazilian and American propolis. Lim and Baharun (2009) have also mentioned that these products are usually being marketed with misleading statement to convince customers who have little knowledge on bee products, not to mention when propolis was being marketed. Furthermore, these products were not being labeled correctly with correct information, e.g. the content of flavonoids which is one of the main constituent contributing to propolis wide spectrum of biological attributions. Such problems arise due to the lack of understanding of locally produced apiaries products, including propolis.

The understanding of propolis will also benefit the local apiculture especially the local farmers who sell honey by raising bees. By understanding the characteristic of locally produced propolis, the uniqueness of the propolis can be identified to set it apart from propolis produced elsewhere. According to market research carried out by Lim and Baharun (2009) on locally produced honey, it was stated that 77% of the studied consumers prefers locally produced honey over those imported. By using honey as a proxy, we will be able to say that locally produced propolis product will be well received.

The complexity of the propolis chemistry is the major challenge in propolis research, thus the profiling and understanding of propolis produced in different region is important. By doing so, it will be able to provide the chemical signature of different region easing the categorization and understanding of its properties. The most common categories of propolis are Temperate, Birch, Tropical, Mediterranean and Pacific (Bankova, 2005). Whereas, the type of propolis found in the Asia Pacific region are categorized under Pacific propolis and is believed to be produced from *Macaranga tanarius L.* (Kumazawa et al., 2008). There was a study being carried out by Wiryowidagdo et al. (2009), who have proposed that the botanical origins of Javanese propolis are from *Ceiba petandra*, *Euphoria longan* and *Hevea brasiliensis*. Such findings have shown that Javanese propolis is distinctively different from that produced from the Eastern Asia.

To address the issue of propolis extraction, the shortcomings of current most favorable extraction technique, the conventional maceration or the soxhlet extraction method should be identified. The drawbacks of these conventional methods are high solvent consumption, high energy costs, high operating temperature, injurious for thermolabile substances and solvent residue in the solute causing lower quality extracts (Paviania et al., 2011). The most commonly used solvent in propolis extraction is ethanol. However, the disadvantages of using ethanol are the strong residual flavor, adverse effects and intolerance to alcohol by some consumers (Mello and Hubinger, 2012). Methanol has also been proven for its high extraction efficiency of flavanones and flavonols than ethanol, but its toxic effect impaired its application in nutraceuticals (Jug et al., 2014). Thus water is chosen as an alternative for propolis extraction. However water only extracted only a small portion of the bioactive compounds but research have shown that altering the extraction condition like the addition of tensoactive compounds (Konishi et al., 2004) or introduction of acid or basic compounds (Mello and Hubinger, 2012) would help to increase the extraction efficiency. The addition of base in facilitating extraction efficiency, is believe to be done through the triggering of hydrolysis of ester, thus increasing the solubility of the compound.

Another problem faced by propolis extraction is the time required, whereby the average time of extraction for maceration is 24 hours. Such a long time of extraction caused propolis researchers to seek alternative advance extraction techniques which allow significant improvement in extraction time like ultrasonic assisted extraction, microwave assisted extraction, supercritical fluid extraction and other. Trusheva et al. (2007) have proven that ultrasound assisted extraction yield excellent results and accelerated the extraction process significantly. However, the combination effect of ultrasound assisted extraction at varied pH has not yet been studied.

1.3 Research Objectives

The objectives of this research are to pioneer the fundamental understanding of Malaysian propolis characteristics and to study the effect of ultrasonication on propolis extraction at varied pH condition.

1. To characterize Malaysian propolis on proximate characteristics, bioactive content, biological attributions and chemical constituents.
2. To study the enhancement effect of ultrasonication on propolis extraction through solvent concentration, pH and sonication cycles.

1.4 Scope of Study

This study was conducted in two parts, mainly the characterization of Malaysian propolis and the study of the enhancement effect of ultrasonic assisted hydrolytic extraction of propolis.

The first part of the study was conducted to establish the fundamental understanding of the characteristics of selected Malaysian propolis of Parit Nibong, Serian, 13th Miles and Sibu respectively. These propolis were then compared to the Brazilian and United States propolis, which was well understood and commercialized as nutraceutical products. The characteristics studied were divided into proximate characteristics, bioactive content, biological attributions and chemical constituents. The study on proximate characteristic of propolis was carried out by investigating the moisture, ash, was and resin and balsam content, such study is essential in understanding the physical characteristic of the studied Malaysian propolis. The study was also furthered with the investigation of bioactive content of Malaysian propolis through their yield, total phenolic, flavone, flavanones and flavonoids content, this part of the study will set the foundation for the bio-attribution study followed, as the bio-attributions possessed by propolis was proven to be contributed by the phenolic and flavonoids compounds (Toreti et al., 2013). Two of the most common bio-attributions possessed by propolis were studied, the anti-bacterial and anti-oxidant properties. This part of the study will enable the understanding of how Malaysian propolis can perform as a potential nutraceutical product. This part of the study was then furthered by carrying out GC-MS study to gain a further insight in the chemical constituent of Malaysian propolis and how different they are compared to Brazilian and United States propolis.

The second part of the study was focusing on developing an alternative extraction technique for propolis extraction by combining ultrasonic technology and basic hydrolytic extraction. Both techniques have never been combined, thus their combined effects have never been explored. In order to achieve the understanding of the combination effect of these two techniques, the parameters of the study were the effects of solvent concentration, pH and ultrasonication cycle. The rational of combining both techniques is to develop an extraction technique that enable shorter extraction time with significant reduction in solvent consumption, which are the benefits of both ultrasonic extraction and basic hydrolytic extraction, respectively. Each optimum condition from respective parameter study was carried forward to obtain the propolis extract with the highest phenolic and flavonoid content. Further investigations on bio-attributions and chemical constituents were carried out to compare the resulting extract to the extract produced through conventional maceration extraction. The bio-attribution studied were antibacterial and antioxidant properties. The chemical constituent of propolis was identified using GC-MS analysis to gain insight on whether different extraction techniques and alteration of solvent content would yield extract with different chemical constituents but still possessed the desired bio-attributions.

Chapter 2

Literature Review

2.1 Approximate Properties of Propolis

Propolis is a generic term used for the resinous substance produced by bees. Propolis, also known as bee glue, comprised of substances actively secreted by plants or exudates from wounds in plants. These resinous products are materials on leaves and leaf buds, resins, mucilages, gums, lattices and other substance from various floras around the beehive (Toreti et al., 2013). The functions of propolis are to prevent the spreading of microbial infections, defending them from invaders, embalming carcass of hive invaders which the bees have killed but cannot transport out from the hive and as the construction material for their hives (Falcão et al., 2010). It is also used as thermal isolation of the beehive, filling eventual cracks or apertures (Moreira et al., 2008). Bees modify propolis by glucodiases, which are the enzymes from the hypophargyngeal glands, during their collection and then processed it. Such enzymatic modification has resulted in the hydrolyzation of phenolic compounds like flavonoid heterosides to free flavonoid aglycones and sugars, which resulted in products with enhanced pharmacological action (Najafi et al., 2007). Propolis collected by *Apis mellifera* and *Trigona carbonaria* were officially studied (Fernandes Jr et al., 2001), but the earlier one was studied and marketed more extensively.

Propolis possesses a wide range of medical attributions such as antioxidant (Bonvehí and Gutiérrez, 2011), antibacterial (Dias et al., 2012), anticancer (Kimoto et al., 2001), antifungal (Koc et al., 2005), anti-inflammatory (Bueno-Silva et al., 2013) and antiviral (El–Deen et al., 2013). Such attractive attributions have made it a popular ingredient in various health products for both internal and external applications which are marketed extensively to appeal to a wide range of consumers.

Raw propolis contain approximately 50% of resins and balsams, 30% of wax, 10% of essential and aromatic oils, 5% of pollen and 5% of impurities. The impurities include vitamins, minerals, sugars, enzymes aldehydes, ketones, alcohols and steroids (Farooqui and Farooqui, 2012). The understanding of propolis's proximate composition allows a quick assessment of the quality of propolis, especially the resins and balsams and the wax contents. The resins portion of propolis is the fraction that most propolis researchers are interested in as they are the polyphenolic fraction which contains the flavonoids and related phenolic acids. Meanwhile, the wax fraction is the fraction containing waxes and fatty acids.

The chemical composition of propolis can be grouped into classes of flavonoids, phenolics, terpenes, aromatic compounds, volatile oils and bee wax (Toreti et al., 2013). It is believed that the first 3 groups of compounds are the major contributors to the biological attributions shown by propolis. After a number of significant researches, researchers began to understand that the chemical composition is highly variable and dependent upon several factors like the season, illumination, altitude, collector type and food availability during propolis exploitation. Thus, investigations on propolis had never failed to surprise propolis researchers, as the aforementioned factors are all highly influential. These high permutations allow the production of propolis with unique chemical composition. Even though the chemical composition of propolis varies from the aforementioned factors, it all possessed biological attributions as mentioned earlier but with varies extend of performance.

The variation on chemical composition of propolis was understood to be contributed by the aforementioned factors but it was mainly influenced by differences in vegetation, collecting bee species and season. At a least influencing level, the differences may also be contributed by the contamination in wax, different extraction method and to certain degree on the sensitivity of the quantification methods. These differences may be responsible for the different extend in biological activities due to the presence/absence or concentration variability in constituents, and synergism or counteracting effect with other polyphenols (Farooqui and Farooqui, 2012).

2.1.1 Variation in Propolis Properties from Different Geographical Origins

Propolis of different region will yield propolis of completely different chemical composition and extend in their biological activities. Several researcher has conducted detailed reviews to summarize the variation in propolis properties from different regions (Banskota et al., 2001, Sforcin and Bankova, 2011).

The occurrence of individual characteristic of propolis from different regions can be easily explained. For example, the main source of bee glue for bees in the temperate zone is the resinous exudate of the buds of the poplar trees. However, these trees do not grow in the tropical or the subtropical regions. The bees in tropical or the subtropical regions will have to find alternative source for their propolis. Such phenomena have caused the chemical composition of propolis of the tropical and subtropical regions to be different from that of the poplar type propolis. The main source of Brazilian propolis is the leaf resin of *Baccharis dracunculifolia*, while Cuban propolis is originated from the floral resins of *Clusia rosea* (Bankova, 2005). Such theory was further verified by the study done on Venezuela propolis (Tomás-Barberán et al., 1993) and Brazilian propolis (Aga et al., 1994) whereby the polyphenols from the poplars were totally absent. Such observations clearly showed that the biological activities shown by the propolis from different areas are also different. Miyataka et al. (1998) reported that the Brazilian and Chinese propolis differ in their abilities to inhibit hyaluronidase and to release histamine from rat peritoneal mast cells induced by compound 48/80 or concanavalin A. It was suggested that such phenomenon might be contributed by a non-flavonoid compound with anti-allergic action, which is poorly water-soluble. Hegazi et al. (2000), discovered that the German propolis are more effective against *Staphylococcus aureus* and *Escherichia coli* while the Austrian propolis possessed a higher antibacterial activity against *Candida albicans*.

The understanding of types of propolis and the compounds present have proven crucial as it allows further understanding and discovery of potential biological attributions of the studied propolis. Besides, it allows the possibility of tailoring the specific extraction method for the specific propolis.

Table 2.1 Most widespread propolis type, geographical origins and their plant sources (Sforcin and Bankova, 2011, Toreti et al., 2013)

Propolis type	**Geographical origins**	**Plant source**
Poplar	Europe, North America, Bulgaria, Albania, South Brazil, Argentine, Uruguay, Mongolia non-tropic regions of Asia, New Zealand	*Populus* spp. of section Aigeiros, *P. italic*, *P. tremula*, *P. suaveolens*, *P. fremontii*, *P. euramericana*, *P alba*, most often *P. nigra* L.
Green (alecrim) Brazilian	Brazil	*Baccharis* spp., predominantly *B. dracunculifolia* DC.
Type 6 Brazilian	Northeast Brazil	*Hyptis divaricate*
Birch	Russia, Hungary and Poland	*Betula verrucosa* Ehrh.
Red propolis	Cuba, Brazil (type 13 Brazilian, northeast Brazil), Mexico	*Dalbergia spp.,* specifically *Dalbergia ecastaphyllum* (for Brazilian propolis)
Mediterranean	Sicily, Greece, Crete, Malta	*Cupressaceae* (species unidentified)
"Clusia"	Cuba, Venezuela and other equatorial regions	*Clusia spp.* or *Clusia minor*

Table 2.1 Continued

Propolis type	Geographical origins	Plant source
"Pacific"	Pacific region (Okinawa, Taiwan, Indonesia)	*Macaranga tanarius*
"Delchampia"	Equatorial regions	*Delchampia spp.*
"Plumeria"	USA (Hawaiian island)	*Plumeria acuminate, Plumeria acutifolia*
"Xanthorrhoea"	Australia	*Xanthorrhoea*

The type of propolis found in the Asia Pacific region are categorized under Pacific propolis and is believed to be produced from *Macaranga tanarius L.* (Kumazawa et al., 2008). Rhe study by Wiryowidagdo et al. (2009) on Javanese propolis, which drawn the botanical origins of Javanese propolis towards *Ceiba petandra*, *Euphoria longan* and *Hevea brasiliensis*. The study has also highlighted that propolis sample with high aromatic acids were coming from area with *C. petandra* and *E.longan* as the dominant vegetation. The sample which contains low aromatic acids is from the area with relatively high number of *C. petandra* and *H. braziliensis*. Such proposal has shown that Javanese propolis is distinctively different from that produced from the Northeastern Asia. With the richness in Malaysia floras, about 15,000 species of vascular plants (Mohd et al.), it is believed that the propolis plant source and the chemical composition will also be different from propolis of other regions.

2.1.2 Bees Species and Races

There are 2 species of bees which have been reported in the studies of propolis, that is the propolis produced by the *Apis mellifera* and *Trigona carbonaria*. The propolis of the honey bee, *Apis mellifera* is being studied far extensively compared to the propolis produced by the stingless bees, *Trigona carbonaria*. In this study, propolis produced by *Trigona itama* is also being studied for the first time in propolis research.

Apis mellifera and *Trigona carbonaria* are both closely related to each other. The defense mechanism is the most significant differentiating factor between these 2 species. The defense mechanism of *Apis mellifera*, e.g. the honey bee is stinging while the *Trigona carbonaria*, e.g. the stingless bee, is through biting. *Apis mellifera* is native to the continent of Europe, Asia and Africa. While *Trigona carbonaria* is mostly found in the tropical and subtropical region of the world, but only a few produce honey and propolis on the scale that can be farmed by humans. Since stingless bees are mostly found in the tropical region of the world, it is active all year round compared to the honeybee from other region with 4 seasons.

Fernandes Jr et al. (2001) had done study comparing antibacterial property of propolis produced from *Apis mellifera* L. and 8 different species of stingless bees, e.g. *Melipona scutellaris* (“Uruçu”), *Melipona sp* (“Manduri”), *Partamona sp* (“Cupira”), *Melipona mandaçaia* (“Mandaçaia”), *Scaptotrigona sp* (“Tiúba”), *Trigona spinipes* (“Arapuá”), *Nannotrigona testaceicornis* (“Irái”) and *Tetragonisca angustula* (“Jataí”). It was found that only propolis that are produced by a certain species of stingless bees, which are the “Cupira”, “Manduri”, “Uruçu”, and “Mandaçaia”, possessed superior antibacterial properties compared to that of the *Apis mellifera*. These propolis are produced from the stingless bees from the Northeast of Brazil (Pernambuco State). Farnesi et al. (2009), had carried out similar study comparing the antibacterial propolis produced by honey bee, *Apis mellifera* and 2 species of stingless bees, *Scaptotrigona sp* and *Melipona mandacaia*. However, the result obtained contradicted the finding of Fernandes Jr et al. (2001), they have concluded that the propolis produced by the honey bees is better than that of the stingless bees. From the comparison of these 2 literatures it was observed that there were inconsistent in finding on propolis quality produced by different bees.

Silici and Kutluca (2005) have carried out study on the propolis produced in the same area but of 3 different races of honeybees, e.g. *Apis mellifera caucasica*, *Apis mellifera carnica* and *Apis mellifera anatolica*. It was observed that there are already differences in the propolis collecting manner of these 3 races of honeybees. The most important characteristic observed through the *Apis mellifera carnica* is their minimum usage of propolis, while the *Apis mellifera caucasica* utilized propolis to the extend of building brace comb. The results of such study have also proven that even with the slight differences of races of honeybees, the propolis produced are different.

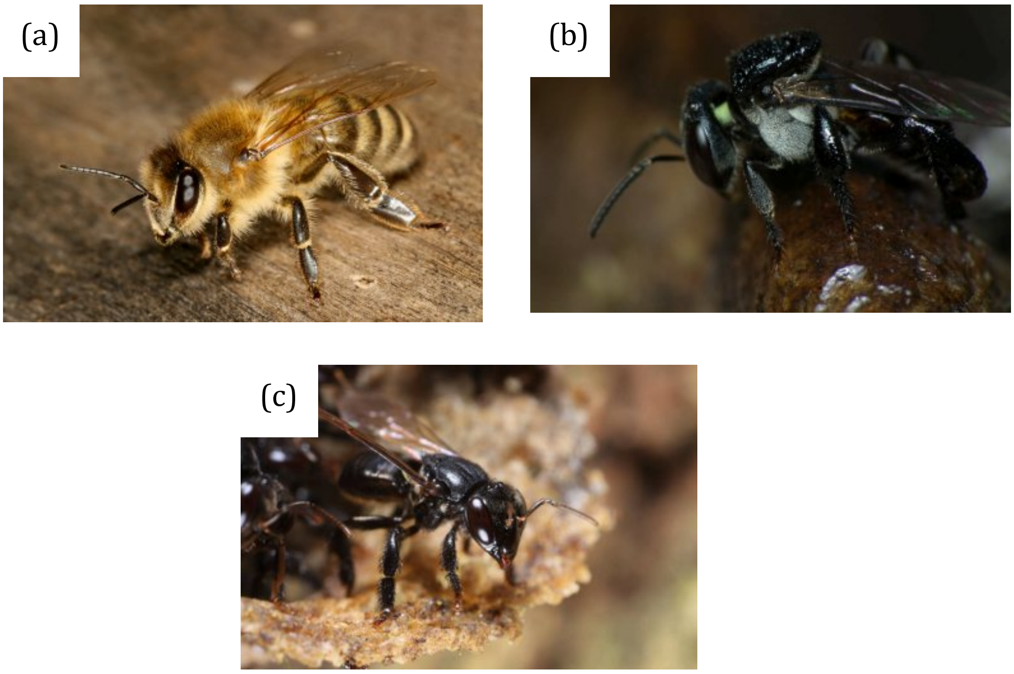

Fig. 2.1 (a) *Apis mellifera* (b) *Trigona carbonaria* (c) *Trigona itama* (adapted from Wikipedia)

2.1.3 Seasons

The manner of propolis collection of bees and phytogeography changes with the season, thus seasonal factor is one of the factors affecting the quality of propolis produced. This factor is less pronounced in countries with distinctive seasonal variation but more prominent in countries whereby propolis are produced throughout the year, like Brazil and Taiwan. The understanding of seasonal effect have on propolis is important as it allows beekeepers to know when propolis are best to be harvested, with the highest bioactive content.

Seasonal effect was studied by Simões-Ambrosio et al. (2010) by targeting at 3 phenolic compounds, e.g. the prenylated p-coumaric acid derivative, artepillin C and baccharin and flavonoids. From the study, the concentration of these 3 compounds varies along the year, such was also shown through the extend of bioactivity. It was observed that propolis produced during the fall season showed higher bioactivity in oxidative metabolism inhibition of the neutrophils. Such activity is correlated to the presence of baccharin which appear to be high in concentration in propolis collected during that period. Thus it was concluded that baccharin is the main compound contribute to the bioactivity of the propolis.

2.2 Biological attributions

As mentioned earlier, propolis was found to possess a lot of biological attributions. The antimicrobial, antioxidant and anti-cancer properties are among those claimed attributions which were widely studied. It was believed that the complex composition of propolis containing a wide range of pharmacologically active constituents, such as polyphenols, terpenoids, steroids and amino acids (Farooqui and A Farooqui, 2010), work in synergy contributing to the claimed attributions.

Table 2.2 Biological activities of propolis and contributing constituents (Farooqui and A Farooqui, 2010)

Activity	**Constituent**
Antimicrobial	Terpenes: diterpenes, triterpenes
Antibacterial	Chrysin, apigenin, pinocembrin and galangin
Antiviral (anti-influenze virus and anti-herpes simplex virus type2)	Polyphenols, flavonoids, phenyl-carboxylic acids, and esters of substituted cinnamic acids (caffeic acid, *p*-coumaric acid, benzoic acid, galangin, pinocembrin, chrysin)

Table 2.2 Continued

Activity	Constituent
Antioxidant	CAPE, caffeic acid, Quercitin, kaempferol, luteolin, chrysin Polyisoprenylated benzophenone Artepillin C Caffeoylquinic acid derivatives
Anti-inflammatory	CAPE, quercetin, chrysin
Immunomodulatory	CAPE
Hepatoprotective	CAPE, chrysin, diterpenes
Cardioprotective	CAPE, acacetin, chrysin, quercitin
Anticancer	CAPE, artepillin C, chrysin, quercetin, propolin C and D
Antitumor	Artepillin C, caffeic acid, CAPE, quercetin, cinnamic acid derivatives, baccharin, drupanin, propolins
Anti-ulcer	Caffeic, ferulic, p-coumaric and cinnamic acids, essential oil

2.2.1 Antioxidant activity

The antioxidant activity of propolis is due to the presents of phenolic acids and flavonoid compounds which have the ability to reduce free radical formation, scavenge free radicals and chelate metal ions (Kumazawa et al., 2004). These compounds were found in both water and ethanolic extracted propolis, but showing different extend of antioxidant activity. Evidences showed that both ethanol and water extracted propolis exert antioxidative effect through different mechanism (Amic et al., 2007).

2.2.2 Antibacterial activity

Propolis is highly known for its antibacterial properties thus many researchers investigated this particular bio-attribution. Through their findings, it was noticed that propolis had antibacterial activity against a wide range of Gram-positive bacteria but had limited activity against Gram-negative bacteria (Farooqui and Farooqui, 2012). Ethanolic extracted Brazilian propolis contains a high concentration of pinocembrin and galangin which are believed to cause the inhibition of the glucosyltranferase activity and reduction in the growth of *Streptococcus mutans*, implicating its potential application as remedy for dental cavities and oral diseases. Besides, propolis was found to be effective towards coping with *Salmonella* infection (*Salmonella enteritidis* in food contamination and *Salmonella typhimurium* in human infection), which caused serious health problem around the world, causing diarrhea, fever and abdominal cramps (Farooqui and A Farooqui, 2010). Its effectiveness towards Gram-positive bacteria is due to the richness in flavonoids.

2.3 Extraction Methods of Propolis

Plant cells synthesize a broad range of natural compounds which are beneficial to human beings. Although the advancement in modern chemistry today is able to synthesize the compounds, it is still not easy to synthesis some compounds as efficiently as mother nature does. Thus plant material is still the preferable source of these bioactive compounds. Extraction techniques have been widely investigated to obtain such valuable compounds from plants for commercialization.

Propolis originated from plant with the bioactive compounds is further modified by bees using enzymes. Even so, crude propolis still contains ash, wax, bioactive compounds and pollen, thus cannot be directly consumed. The crude propolis need to be pretreated by crushing it into powder and then extracted using solvent. Thus the bioactive compounds are extracted from the matrix of wax, ashes and other impurities. Extraction process is crucial to produce propolis extract for human consumption or therapeutic purposes. There are a number of extraction methods currently applied for the extraction of bioactive compounds from propolis, which are categorized into as the conventional extraction techniques and the advance extraction method.

The conventional techniques used for propolis extraction are the maceration and Soxhlet extraction methods but the main drawback of these methods is that they are time consuming. Other drawbacks of these conventional methods are high solvent consumption, high energy cost, high operating temperature, injurious for thermolabile substances and solvent residue in the solute causing lower quality extracts (Paviania et al., 2011). The advance extraction methods are referred to the ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction. The development of these advance extraction methods is to tackle those problems arising from the conventional extraction method.

Figure 2.2 depicts the extraction technologies explored for propolis extraction till date.

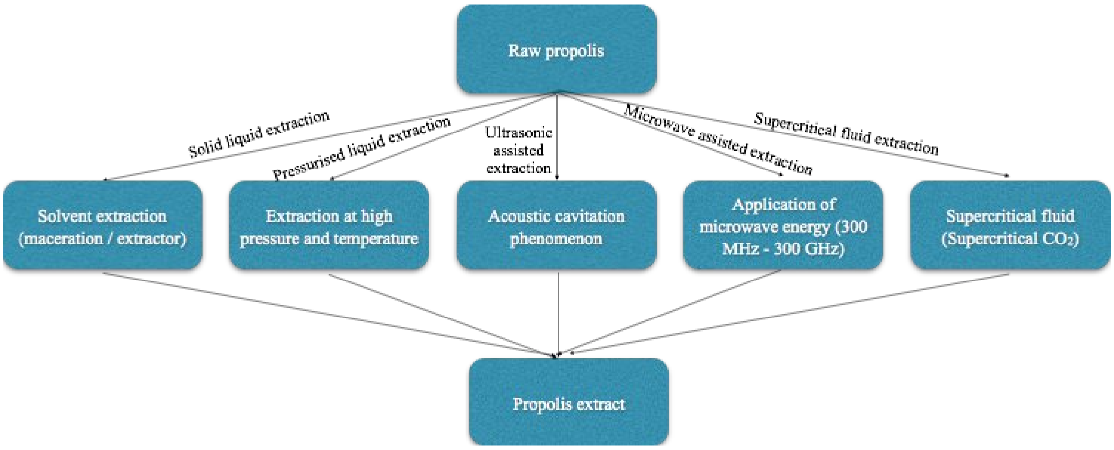

Figure 2.2 Schematic diagram representing the extraction methods available for propolis extraction

2.3.1 Conventional Extraction Methods

Presently, the most favorable extraction techniques used in propolis research and industry are still the conventional maceration or the Soxhlet extraction method.

Maceration is a process whereby the extraction material is placed in pieces or powder, depending on the extraction efficiency, in a container full of solvent and let it stand for an elongated period of time until complete extraction is achieved.

Soxhlet extraction is an advanced extraction technique compared to maceration method. This technique surpassed in performance of maceration except for, in limited field of application, the extraction of thermolabile compounds. Figure 2.4 shows the conventional Soxhlet system, the extraction material is placed in the