IDENTIFICATION OF CHEMICAL CONSTITUENTS IN DIFFERENT PROPOLIS EXTRACTS AND THEIR *IN-VITRO* ANTIMICROBIAL ACTIVITIES AGAINST *Propionibacterium acnes* AND *Staphylococcus epidermidis*: TOWARDS THE DEVELOPMENT OF A NEW FORMULATION

By

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LIST OF ABBREVIATIONS

AAFS	American Academy of Forensic Sciences
AED	Atomic emission detector
ANOVA	Analysis of variance
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BA	Blood agar
BF_3	Boron trifluoride
BP	British Pharmacopeia
BSA	Bistrimethylsilylacetamide
BSTFA	Bistrimethylsilyltrifluoroacetamide
C_2H_2	Ethyl group
CAMHB	Cation-adjusted Mueller-Hinton broth
CAPE	Caffeic acid phenethyl ester
CE	Capillary electrophoresis
CFU	Colony-forming unit
CLSI	Clinical Laboratory Standard and Institute
EAT	Ehrlich ascites tumour
ECD	Electron capture detector
EDTA	Ethylenediaminetetraacetic acid
EEP	Ethanolic extract of propolis
EI	Electron ionisation

EM	Electron multiplier
ESI	Electrospray ionisation
FID	Flame ionisation detector
GC-MS	Gas chromatography and mass spectrometry
HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
HSV	Herpes simplex virus
ICP OES	Inductively coupled plasma optical emission spectrometry
ID	Internal diameter
IR	Infrared
LC	Liquid chromatography
LOD	Limit of detection
MTBSTFA	N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
MIC	Minimum inhibitory concentration
MHA	Mueller-Hinton agar
MHBA	Mueller-Hinton blood agar
MS	Mass spectrometry
MSD	Mass selective detector
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide.
MTT	[3-(4,5-dimethylthizol-2-yl)-2,5- diphenyltetrazolium bromide]
NPD	Nitrogen phosphorus detector
NIST	National Institute of Standards
NMR	Nuclear magnetic resonance

O/W	Oil in water
OH	Hydroxyl group
$PFBB_r$	Pentafluorobenzyl bromide
PDA	Photo-diode-array
RT	Retention time
SIM	Selected ion monitoring
TBH	Tetrabutylammonium hydroxide
TCD	Thermal conductivity detector
TIC	Total ion chromatogram
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TMSI	Trimethylsilylimidazole
TMS-DEA	Trimethylsilyldiethylamine
UV	Ultraviolet
W/O	Water in oil
WEP	Water extract of propolis

IDENTIFIKASI BAHAN KIMIA DALAM EKSTRAK PROPOLIS YANG BERBEZA DAN AKTIVITI ANTIMIKROBIAL *IN-VITRO* TERHADAP *Propionibacterium acnes* DAN *Staphylococcus epidermidis*: KE ARAH PEMBANGUNAN FORMULASI BARU

ABSTRAK

Pengenalan: Propolis adalah bahan resin yang dikutip oleh lebah madu daripada pelbagai sumber tumbuhan. Komposisi propolis bergantung kepada sumber kawasan tumbuh-tumbuhan dan kaedah pengekstrakan. Setakat ini, tiada laporan mengenai bahan kimia propolis *Apis mellifera* dari Malaysia dan aktivitinya terhadap bakteria penyebab jerawat masih lagi kurang. Jerawat adalah penyakit kulit yang sangat biasa dan boleh menyebabkan parut jerawat kekal dan kesan psikologi seperti kemurungan, kurang keyakinan diri dan pengangguran. Rawatan sedia ada tidak mencapai hasil yang optimum dan menyebabkan kerintangan bakteria atau kesan yang tidak diingini.

Objektif: Kajian ini bertujuan untuk mengenal pasti 1) bahan kimia dalam propolis *Apis mellifera* daripada ekstrak air (WEP) dan ekstrak etanol (EEP) dari kawasan selatan dan utara Semenanjung Malaysia 2) aktiviti antimikrob *in-vitro* terhadap bakteria yang menyebabkan jerawat dan 3) juga untuk menyediakan rumusan topikal untuk jerawat.

Metod: WEP dan EEP disediakan dan sebatian silylated telah dikenal pasti dengan menggunakan kromatografi gas dan spektrometri jisim. Sebatian yang diperoleh

dibandingkan dengan perpustakaan spektra jisim. Sebatian yang hadir dalam semua ekstrak dikuantifikasi. Kemudian, ekstrak dinilai untuk aktiviti antimikrob terhadap *Propionibacterium acnes* dan *Staphylococcus epidermidis* menggunakan asai resapan telaga agar dan asai broth microdilution resazurin.

Keputusan: Empat puluh satu sebatian telah dikenal pasti dan empat bahan telah dikenal pasti buat pertama kalinya dari propolis iaitu asid m-salisilik, β-panasinsene, mannose-6-deoxy dan β-DL-lyxopyranose. Sebatian utama yang dikenal pasti daripada WEP adalah sebatian fenolik manakala daripada EEP adalah sebatian terpenoids. Di antara sebatian-sebatian tersebut, asid galik didapati hadir dalam WEP dan EEP dari kawasan selatan dan utara. Kuantifikasi asid galik dilakukan dengan menggunakan mod pemantauan ion terpilih dengan kelinearan R^2> 0.995. EEP dari kawasan utara mempunyai kandungan asid galik tertinggi [327.51 (SD = 14.58) µg/g, CV(%) = 4.5, n=3]. Ujian saringan antimikrob menunjukkan bahawa kedua-dua mikroorganisma yang diuji adalah sensitif kepada semua ekstrak propolis dan asid galik. Asai broth microdilution resazurin menunjukkan bahawa nilai kepekatan perencatan minimum (MIC) terhadap bakteria penyebab jerawat bagi EEP dari kedua-dua kawasan pada umumnya adalah lebih rendah daripada WEP. *Propionibacterium acnes* adalah lebih sensitif kepada EEP (MIC = 0.3 hingga 0.6 µg/ml) berbanding *Staphylococcus epidermidis* (MIC = 156 hingga 625 µg/ml). Kepekatan WEP yang lebih tinggi diperlukan untuk menghalang bakteria penyebab jerawat (MIC > 1 mg/ml). Aktiviti asid galik menunjukkan hasil yang memberangsangkan terhadap *Staphylococcus epidermidis* tetapi tidak sebaik EEP pada *Propionibacterium acnes*. Menariknya, formulasi baru

menunjukkan zon perencatan yang lebih besar berbanding dengan perencatan oleh 10% benzoyl peroksida.

Kesimpulan: WEP dan EEP *Apis mellifera* propolis dari Malaysia mempunyai sebatian kimia yang berharga dan menunjukkan potensi antimikrob. Dengan demikian, WEP boleh dipertimbangkan sebagai alternatif kepada EEP terutamanya apabila penggunaan ekstrak air diperlukan. Ujian makmal dan kajian klinikal yang selanjutnya adalah perlu untuk memastikan penggunaan formulasi propolis baru sebagai rawatan alternatif untuk jerawat.

IDENTIFICATION OF CHEMICAL CONSTITUENTS IN DIFFERENT PROPOLIS EXTRACTS AND THEIR *IN-VITRO* ANTIMICROBIAL ACTIVITIES AGAINST *Propionibacterium acnes* AND *Staphylococcus epidermidis*: TOWARDS THE DEVELOPMENT OF A NEW FORMULATION

ABSTRACT

Introduction: Propolis is a resinous material collected by the honeybees from various plant sources. The composition of propolis depends on the vegetation area and method of extraction. To date, there is no report on the chemical constituents of *Apis mellifera* propolis from Malaysia and their activities against acne-inducing bacteria is still lacking. Acne vulgaris is a very common skin disease and may cause permanent acne scarring and psychological morbidities such as depression, low self-esteem and unemployment. Current treatments do not achieve the optimal results and are associated with bacteria resistance or adverse effects.

Objectives: This study was aimed to 1) identify the chemical constituents of water (WEP) and ethanolic extracts (EEP) of *Apis mellifera* propolis from the southern and northern regions of Peninsular Malaysia, 2) determine their *in-vitro* antimicrobial activities against acne-inducing bacteria, and 3) prepare a new topical formulation for acne.

Methods: WEP and EEP were prepared and silylated compounds were identified by gas chromatography and mass spectrometry analysis. The compounds were characterised by

comparison with library searches. The compounds that were present in all extracts were quantified. The extracts were then evaluated for their antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis* using agar well diffusion and broth microdilution resazurin assay.

Results: Forty one individual compounds were identified and four compounds were identified for the first time from propolis which are m-salicylic acid, β-panasinsene, mannose-6-deoxy and β-DL-lyxopyranose. The main compounds identified from WEP and EEP were phenolic compounds and terpenoids, respectively. Gallic acid was found to be present in WEP and EEP from both regions. Quantification of gallic acid was performed using selected ion monitoring mode with linearity $R^2 > 0.995$. It was found that the EEP from the northern region has the highest content of gallic acid [327.51 (SD = 14.58) µg/g, CV (%) = 4.5, n = 3]. The antimicrobial screening test showed that both microorganisms were sensitive to the all propolis extracts and gallic acid. The broth microdilution resazurin assay demonstrates that MIC values of EEP from both regions were generally lower than WEP against both acne-causing bacteria. *Propionibacterium acnes* was more susceptible to EEP (MIC = 0.3 to 0.6 µg/ml) when compared to *Staphylococcus epidermidis* (MIC = 156 to 625 µg/ml). Higher concentration of WEP is needed to inhibit acne-causing bacteria (MIC > 1 mg/ml). Gallic acid activity showed promising result against *Staphylococcus epidermidis* but not as good as EEP on *Propionibacterium acnes.* Interestingly, a new formulation showed greater zone of inhibition compared to benzoyl peroxide 10%.

Conclusion: The WEP and EEP of *Apis mellifera* propolis from Malaysia have valuable chemical compounds and demonstrate good antimicrobial activity. The WEP may be considered as an alternative to EEP, especially when water extract application is more desirable. Further laboratory and clinical studies are needed to ascertain the use of the new propolis formulation as an alternative treatment for acne vulgaris.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Propolis is one of the important bee products and consists of a mixture of resinous substances collected by the honeybees from various plant sources, mainly from flowers and leaf buds, to be mixed with their saliva (Bankova *et al.*, 2000). The main function of propolis is to defend the bee colony (Marcucci, 1995; Bankova *et al.*, 2000). It is used to patch small holes in the beehives, to strengthen the borders of combs, to line all cells inside and balance the humid conditions or extreme drought. In addition, propolis is also used to embalm dead animals or insects that cannot be expelled by the honey bees (Ghisalberti, 1979).

Propolis comes from the ancient Greek words; "*pro*" and "*polis*". *Pro* is before and *polis* is city, meaning before the city or defender of the city or beehive. The colour of propolis is variable from yellow to dark brown, depending on the resins of plant source. In some regions, the propolis is reported to be red (Daugsch *et al.*, 2008) or green (Teixeira *et al.*, 2005) in colour. Propolis is soft, pliable and very sticky at room temperature, but become hard and brittle when frozen.

Propolis has been widely used in ancient remedies as early as 300 BC. The Egyptians used propolis to embalm cadavers due to its anti-putrefactive properties (Abdel-Maksoud and El-Amin, 2011). The Greek and Roman physicians, Aristotle, Dioscorides, Pliny and Galen used propolis to promote healing and as an antiseptic in wound treatment (Castaldo and Capasso, 2002). They also used propolis as a mouth disinfectant and these practises had been continued in the Middle Ages and among Arab physicians. The ancient Georgian used propolis to cure some diseases and put propolis cake on the belly button of a newborn baby (Lotfy, 2006). In Central America, the Incas used propolis as an anti-pyretic agent (Castaldo and Capasso, 2002). In the World War I, propolis was used for wound treatment (Mindell, 2000). In World War II, propolis was used as an antibiotic when the availability of penicillin was limited (Mindell, 2000) and for the treatment of tuberculosis by the Soviet clinicians (Wagh, 2013). In the seventeenth century, propolis was listed as an official drug in the London pharmacopoeias (Wagh, 2013).

Nowadays, propolis use is perpetuated in the modern medicine and is commonly used to treat various diseases such as respiratory problems (Carr and Nahata, 2006), gastrointestinal diseases (Pizzorno and Murray, 2012), cardiovascular diseases (Fuliang *et al.*, 2005; Nader *et al.*, 2010) preventing cancer (Watanabe *et al.*, 2011) and urinary tract infection (Lavigne *et al.*, 2011). Propolis is widely used for dental purposes to control plaque, gingivitis (Pereira *et al.*, 2011), dental caries (Hayacibara *et al.*, 2005), pulp capping agent (Parolia *et al.*, 2010) and aphthous stomatitis ulcer (Samet *et al.*, 2007).

Propolis is also used to treat genital herpes (Vynograd *et al.*, 2000), vaginal infection (Dota *et al.*, 2011) and treatment of burns and wounds (Han *et al.*, 2005; McLennan *et al.*, 2008)**.** The use of propolis has been expanded in cosmetics industry because of its excellent UV protective and antioxidant properties (Gregoris *et al.*, 2011). It is used as an active ingredient for the skin care products such as facial cleanser and creams (Baumann, 2007), eye cream, soap, shampoo, lipstick and toothpaste (Botushanov *et al.*, 2000).

Chemical compounds of propolis are different based on the vegetation at the area from which it was collected (Bankova, 2005). This special characteristic of propolis has attracted many researchers to conduct new studies, especially from unexplored regions. Researches on propolis on its chemical compounds, biological activities, and medicinal uses have been extensively performed in the last the 30 years. Currently, more than 300 chemical compounds of propolis has been identified worldwide (Toreti *et al.*, 2013). However, the content of chemical compounds of Malaysian propolis is still unknown.

Generally, propolis contains resins which comprised of flavonoids and phenolic acids, waxes and fatty acids, volatiles compounds which comprised of terpenoids and aromatic acids, pollen and minerals (Krell, 1996). Propolis compounds include water and lipid soluble constituents. The various chemical compounds of propolis thus affect their method of extraction. Since ethanol has a polar end, the hydroxyl group (OH) and a non-polar end, the ethyl group (C_2H_5), it can dissolve both polar and non-polar compounds. Water soluble compounds are also found in propolis, but in lower concentrations. Significant biological activities of the water extract of propolis have been reported by

various authors since a decade ago (Nagai *et al.*, 2003; Yildirim *et al.*, 2004; Nakajima *et al.*, 2007; Urushisaki *et al.*, 2011). Therefore, it is important that solvents for identification of propolis chemical constituents should be able to extract both polar and non-polar compounds.

Propolis possess various biological activities such as antimicrobial (Kartal *et al.*, 2003; Schnitzler *et al.*, 2009), antiviral (Schnitzler *et al.*, 2009), antifungal (Oliveira *et al.*, 2006), anti-inflammatory (Naito *et al.*, 2007), antioxidant (Ahn *et al.*, 2004), antitumour (Sobocanec *et al.*, 2011), hepato-protective (Banskota *et al.*, 2001), anti-ulcer (Barros *et al.*, 2008), UV protection (Cole *et al.*, 2009) and local anaesthetic properties (Paulino *et al.*, 2003). Antibacterial studies of propolis showed activity against a wide range of Gram positive bacteria (Uzel *et al.*, 2005) such as *Staphylococcus aureus* and *Streptococcus pyogenes*, but no activity against Gram negative bacteria (Stepanovic *et al.*, 2003). However, to date, no studies have been performed on acne-inducing bacteria.

Acne vulgaris is the most common skin disease that commonly occurs among adolescent (Uslu *et al.*, 2008; Hanisah *et al.*, 2009). It involves multiple mechanisms including androgen rise during puberty, increased sebum production, ductal hyperkeratinisation, increased cohesiveness of keratinocytes, bacterial hypercolonisation and release of inflammatory mediators into the skin (Toyoda and Morohashi, 2001). The exact sequence of events and how each factors interact remains unclear (Williams *et al.*, 2012). The most common microorganisms involved in the pathogenesis of acne vulgaris are *Propionibacterium acnes* and *Staphylococcus epidermidis* (Nishijima *et al.*, 2000; Pawin *et al.*, 2004).

Propionibacterium acnes has been implicated in the pathogenesis of acne vulgaris in favourable conditions. It is a Gram positive, anaerobic bacteria and live deep within follicles of the skin. Excessive sebum production and clogging of follicles is the optimum environment for proliferation of *Propionibacterium acnes*. Hypercolonisation of *Propionibacterium acnes* induce a local follicular inflammatory response and rupture of follicular wall, resulting in marked inflammation. If the inflammatory response continues, it will lead to the formation of cysts and pustules, eventually leading to formation of scar.

Staphylococcus epidermidis is another Gram positive, aerobic pus-forming bacteria that is commonly detected in acne lesions (Nishijima *et al.*, 1994; Higaki *et al.*, 1997). It triggers inflammation in acne vulgaris by activating the complement cascade (Toyoda and Morohashi, 2001). It is part of the normal skin flora around the follicles and usually involved in superficial infection of acne (Nishijima *et al.*, 1994; Higaki *et al.*, 1997). Since the pathogenesis of acne is multifactorial, the best treatment for acne vulgaris should be focused towards these multiple pathogenic factors. The antimicrobial, anti-inflammatory and antioxidant properties of propolis could be beneficial for acne vulgaris.

Several anti-inflammatory, antibiotics and their combinations have been used for many years to treat acne vulgaris such as benzoyl peroxide, retinoid and erythromycin. Topical benzoyl peroxide has both anti-inflammatory and antibiotic activity and is commonly used to treat mild to moderate acne vulgaris. However, benzoyl peroxide do not achieve the optimum effect and cause skin irritation such as erythema, scaling, burning and

flaring up of acne (Gollnick and Krautheim, 2003). Retinoids such as retin A are effective (Thielitz and Gollnick, 2008) but teratogenic and can cause birth defect (Loureiro *et al.*, 2005). The used of topical antibiotics, primarily erythromycin exerts less skin irritation but has the potential for bacterial resistance (Eady *et al.*, 1994).

Therefore, combination products should to be used to increase the effectiveness and to create better tolerability than benzoyl peroxide, retinoid or antibiotic alone. Topical benzoyl peroxide is usually combined with erythromycin or with retinoids such as azelaic acid. Therefore, new formulations for acne with better anti-inflammatory and antibiotic properties and minimal adverse effects are needed. In this study, a new acne formulation using propolis extracts will be developed and tested.

1.2 Justification of this study

Chemical compounds of propolis differ and are dependent on many factors such as the source of plants, geographical origin and methods of extraction (Bankova, 2005). Since Malaysia is a tropical country and is blessed with a wide range of plant species, the chemical compounds of Malaysian propolis may differ with that obtained from other regions. Thus, the present study attempted to identify the chemical constituents of propolis derived from *Apis mellifera* bees cultured in the *Acacia mangium* forest from the southern and northern regions of Peninsular Malaysia (Johor and Penang).

Although ethanolic extracts of propolis have been reported to possess antimicrobial activity against a wide range of Gram positive bacteria, information on their activities on acne-inducing bacteria is still lacking. Furthermore, there are limited studies on the properties of its water extract since it has low water solubility compounds (Silva *et al.*, 2012). Water extract should be considered as an alternative to ethanolic extract since liquid or water-based formulation may be more desirable for certain people in some conditions such as oily skin problems. Malaysian propolis may have different chemical constituents and it is postulated that this difference may or may not affect its pharmacological properties. Thus, this study compared the *in-vitro* antimicrobial activities of water and ethanolic extracts of propolis against two microorganisms frequently involved in the pathogenesis of acne vulgaris, i.e. *Propionibacterium acnes* and *Staphylococcus epidermidis.*

Acne vulgaris is a very common skin disease among teenagers worldwide including Malaysia (Collier *et al.*, 2008; Uslu *et al.*, 2008; Hanisah *et al.*, 2009; Kubota *et al.*, 2010). Although it is not a life-threatening disease, it may cause permanent acne scarring and psychological morbidities such as depression and low self-esteem. Several treatments have been introduced to decrease the aesthetic and psychological problems caused by acne. However, the present treatments such as benzoyl peroxide and retinoid do not achieve the desired effects and are often associated with adverse effects. Alternative therapy with naturally-derived compounds have received considerable interest since they are also effective and with fewer adverse effects than synthetic agents. Since propolis has been reported to possess good anti-inflammatory (Shi *et al.*, 2012) and antimicrobial activities (Machado *et al.*, 2012), it may have potential against acne-inducing bacteria and thus, is an alternative to the existing treatments. Therefore, its effectiveness against acne-inducing bacteria should be properly investigated.

1.3 Research Hypothesis

1) Ethanolic extract of propolis has higher *in-vitro* antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis* than water extract of propolis.

2) A new propolis formulatios that display *in-vitro* antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis* based on the best extract as determined in 1) can be developed and tested against these microorganisms.

1.4 Research Questions

1) What are the chemical constituents of water (WEP) and ethanol extracts (EEP) of the Malaysian *Apis mellifera* propolis collected from *Acacia mangium* apiary?

2) Do WEP and EEP have *in-vitro* antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis?*

3) Does the new propolis formulation display *in-vitro* antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis?*

1.5 Research Objectives

1.5.1 General objectives

This study was aimed to identify the chemical constituents of WEP and EEP of *Apis mellifera* propolis from the southern (Johor) and northern regions (Penang) of Peninsular Malaysia and to measure their *in-vitro* antimicrobial activities against *Propionibacterium acnes* and *Staphylococcus epidermidis.*

1.5.2 Specific objectives

1. To extract Malaysian *Apis mellifera* propolis using water and ethanol. Propolis sample was collected from *Acacia mangium* apiary in Kota Tinggi, Johor and Bukit Mertajam, Penang, Malaysia.

2. To identify the chemical constituents of water extract of propolis (WEP) and ethanolic extract of propolis (EEP) from both regions using GC-MS.

3. To evaluate and compare the *in-vitro* antimicrobial activity of WEP and EEP against *Propionibacterium acnes* and *Staphylococcus epidermidis.*

4. To prepare a new skin formulation containing propolis extracts for acne and *in-vitro* antimicrobial activity of the new propolis formulation against *Propionibacterium acnes* and *Staphylococcus epidermidis* will be determined.

CHAPTER 2

LITERATURE REVIEW

2.1 Propolis and its function in the bee hive

Propolis is a complex resinous materials collected by honey bees from various plant sources. The bees collect the resin from flowers and leaf buds using their mandibles and carry them to the hive on their hind legs or corbicula (Teixeira *et al.*, 2005). These resins are mixed with their saliva and other secretions as well as their wax to produce propolis.

The main function of propolis is to defend the bee colony, by using it as an architecture material and anti-infectious agents. The honey bees use propolis as a glue to seal the cracks, to restrain all structural elements, to balance humid condition or extreme drought, to omit external light, to smoothen the wall, to protect the wings from fraying, to inhibit bacterial and fungal growth, and to mummify the killed invaders that are too heavy to be shifted out from the bee hives (Grassberger *et al.*, 2013).

2.2 Physical characteristic of propolis

The physical appearance of propolis such as its colour, texture and odour vary widely, depending on many factors such as source of plant resins, climate, geographical origin and age (Burdock, 1998; Guo *et al.*, 2011). Studies on propolis from many regions in Europe, Africa and Asia reported that the colour of propolis varies from light yellow,

yellow, green, red, light brown to dark brown (Kumazawa *et al.*, 2004). Other researchers found that the Brazilian propolis was green (Teixeira *et al.*, 2005) and red in colour (Daugsch *et al.*, 2008).

Propolis is a soft, elastic and very sticky substance at 25°C to 45°C. Above 45°C it will become increasingly sticky and gummy. Typically, propolis will change to liquid at 60°C to 70°C. For some samples, the melting point may be as high as 100°C (Krell, 1996). However, it becomes hard and brittle at less than 15°C or when frozen.

Propolis odours can be fruity, sweet, fresh, aromatic, pungent, orange-peel like, grassy, floral, bitter or others depending on its aromatic compounds (Yang *et al.*, 2010). The characteristic odour of propolis is due to its terpenoids compounds (Yang *et al.*, 2010), which are important in differentiating good-quality propolis from lower quality or fake propolis.

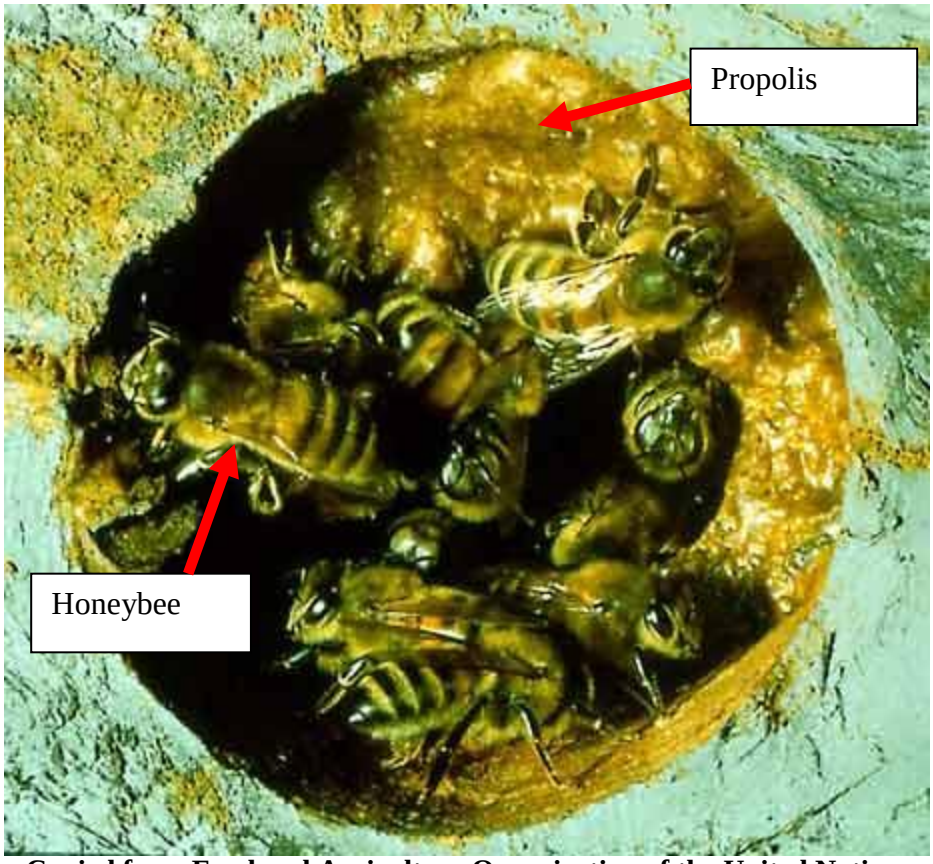

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Figure 2. 1 *Apis mellifera* propolis

2.3 Chemical compounds of propolis

Propolis generally contains 45-55% resins which comprise of flavonoids, phenolic acids and their esters, 25-35% waxes and fatty acids, 10% essential oils (volatiles), 5% pollens and 5% minerals (Krell, 1996). So far, more than 300 chemical compounds have been identified from propolis (Toreti *et al.*, 2013).

Chemical compounds of propolis varies depending on its plant sources, geographical origins, types of honey bees and methods of extraction (Bankova, 2005). This special characteristic of propolis has attracted many researchers to conduct new studies, especially from unexplored regions.

The main compounds of propolis from the temperate zone (Europe, North America and non-tropic Asia) were reported as flavonoids, aglycones, phenolic acids and their esters (Hegazi *et al.*, 2000; Maciejewicz *et al.*, 2001). In contrast, the main compounds of propolis originating from tropical regions (Southeast Asia, Central Africa and South America) especially Brazil were reported as artepillin C (Park *et al.*, 2004) and prenylflavonones (Myanmar, Indonesia). While the main compounds of propolis from Mediterranean were terpenoids and sugars (Popova *et al.*, 2011).

The differences of the propolis compounds are due to different botanical sources. The main plant sources of propolis from temperate zones were poplar and birch tree (Christov *et al.*, 2006). On the other hand, conifer species of the *Cupressaceae* family were the main botanical source of propolis from the Mediterranean regions (Popova *et*

al., 2012). Studies of propolis from tropical regions especially Brazil revealed that *Baccharis dracunculifolia* and *Dalbergia ecastaphyllum* were the main botanical sources (Park, 2004; Dausghch 2008). Investigation of propolis from Asia indicated *Macaranga tanarius* L. and *Mangifera indica* L. as the plant sources of the Indonesian propolis (Trusheva *et al.*, 2011).

Malaysia is a tropical country blessed with a wide range of plant species. Therefore, the chemical compounds of Malaysian propolis may be different with propolis obtained from other region. This study was designed to identify the various chemical constituents of propolis derived from *Apis mellifera* bees cultured in *Acacia mangium* forest from two states in Malaysia (Johor and Penang).

Methods of propolis extraction will also influence the chemical compounds of propolis. Many studies have reported the use of ethanolic extract (Kujumgiev *et al.*, 1999; Kartal *et al.*, 2003; Naito *et al.*, 2007; Sobocanec *et al.*, 2011; Bonveha and Gutiarrez, 2012; Dziedzic *et al.*, 2013) while limited studies are available on its water extract (Nagai *et al.*, 2003; Yildirim *et al.*, 2004; Nakajima *et al.*, 2007; Urushisaki *et al.*, 2011) since propolis has low water solubility. Interestingly, a study on the water extract of propolis detected caffeoylquinic acid derivatives which showed neuro-protective (Nakajima et al, 2007) and anti-influenza effects (Urushisaki *et al.*, 2011). This important finding from the water extract had attracted the interest of many researchers to conduct further investigations.

2.3.1 Flavonoids

Flavonoids are polyphenolic compounds that categorised into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. For analytical purpose, they are basically classified into three types which is the flavonoids glycoside, non-polar flavonoids (aglycones, methylated or alkylated) and anthocyanins (Sarker and Nahar, 2007).

Flavonoids are well known and possess remarkable biological activities such as potent antioxidant, antimicrobial and anti-inflammatory properties (Bankova *et al.*, 2002; Ahn *et al.*, 2004). Flavonoids aglycones like pinobanksin, pinocembrin, galangin and chrysin are the main compounds found in the propolis originating from the temperate zones including Europe and some regions of Asia, where the major plant source is poplar trees (Bankova *et al.*, 2002; Ahn *et al.*, 2004; Ahn *et al.*, 2007).

In contrast, propolis from the Mediterranean region, (Popova *et al.*, 2011) was found not to contain flavonoids aglycones, in very little amount in a single sample. This may be attributed to the occasional presence of *poplar* tree. The main flavonoids identified from Mediterranean propolis were flavonoids glycoside such as kaempferol and quercetin derivatives (Falcao *et al.*, 2012). Similarly, these flavonoids aglycones are also not identified in propolis from the tropical regions (Kumazawa *et al.*, 2004). The main flavonoids from tropical regions are prenylflavonones such as 5,7-dihydroxy-4-methoxy-8,3-diprenylflavanone, 5,7,4-trihydroxy-8,3-diprenylflavanone, dihydroxy-4-

methoxy-8-prenylflavanone, 5,7,4-trihydroxy-8-prenylflavanone and propolin C, D F and G (Li *et al.*, 2009; Trusheva *et al.*, 2011).

2.3.2 Phenolic acids

Phenolic acids and derivatives are aromatic compounds containing a carboxylic acid function and a phenolic ring, like benzoic and cinnamic acids derivatives (Sarker and Nahar, 2007). The phenolic acids seem to be another important class of propolis compounds with significant biological activities including anti-radical, antioxidant and anti-cancer properties (Sroka and Cisowski, 2003; Wu *et al.*, 2011).

The main phenolic acids present in propolis originating from temperate zone from which the *poplar sp.* are the main plant sources were benzoic acid, 4-pentenoic acid, 5-phenyl, 2-propenoic acid,3-phenyl, cinnamyl cinnamate, benzyl hydroxybenzoate, cinnamic acid and *p*-coumaric acid (Christov *et al.*, 2006). In contrast, the main phenolic acids of the Brazilian propolis in which the *Baccharis dracunculifolia* acts as the main plant source, contains artepillin C as the major compound (Park *et al.*, 2004).

Propolis originating from the tropic region of Asia like Indonesia from which the main plant sources of *Macaranga tanarius* L. and *Mangifera indica* L, have major phenolic acids such as benzoic acid, phenylic acid, dofuranuronic and D-glucofuranuronic acid (Trusheva *et al.*, 2011). In contrast, phenolic acids were not detected in propolis from Mediterranean such as in Greece (Popova *et al.*, 2010).

2.3.3 Terpenoids

Terpenoids make up the majority of propolis's volatile compounds and can be identified by GC-MS. They are compounds that have isoprene units including terpenes, diterpenes, and sesquiterpenes (Sarker and Nahar, 2007). Terpenoids become the important class in the propolis compounds due to their valuable biological activities. Aromadendre, erythritol, cedrol, eudesmol, lupenone and globulol are some examples of terpenoids in propolis (Melliou *et al.*, 2007; Vardar-Unlu *et al.*, 2008; De Castro Ishida *et al.*, 2011).

Propolis samples from the Mediterranean regions (Malta and Greece) contain the highest concentrations of terpenoids (Popova *et al.*, 2010; Popova *et al.*, 2011). Similar finding was observed from propolis samples from Southwest Asia (Siripatrawan *et al.*) which revealed that terpenoids were the major compounds identified (Popova *et al.*, 2013). On the other hand, terpenoids were not identified at all (Hegazi *et al.*, 2000) or were present in low amounts (Greenaway and Whatley, 1992) propolis samples from the temperate regions.

An investigation of propolis terpenoids compounds (14,15-dinor-13-oxo-8(17)-labden-19-oic acid and a mixture of labda-8(17),13*E*-dien-19-carboxy-15-yl oleate and palmitate, 3,4-seco-cycloart-12-hydroxy-4(28),24-dien-3-oic acid and cycloart-3,7-dihydroxy-24-en-28-oic acid) showed promising antimicrobial activities against both Gram positive and negative bacteria as well as fungus (Popova *et al.*, 2009).

2.3.4 Fatty acids

Fatty acid is a carboxylic acid with a long aliphatic tail, which can either be saturated or unsaturated (Sarker and Nahar, 2007). Oleic, myristic, stearic and palmitic acids were common fatty acids found in propolis sample (Markham *et al.*, 1996). Investigation on properties of fatty acids in propolis is still lacking, although a study on fatty acid isolated from other natural products such as sunflower oil indicated good antioxidant activity (Parthasarathy *et al.*, 1990). Myristic, stearic and palmitic acids are widely used in cosmetic industry as an ingredient in cleansing agent and as a moisturiser due to its high absorption rate via the skin.

2.3.5 Sugars

Other main class of propolis compounds was sugars. Sugar is a carbohydrate that is water-soluble and can be classified into monosaccharide, disaccharide, oligosaccharide and polysaccharide (Sarker and Nahar, 2007). Sugars and their derivatives can be identified by GC-MS. The high percentage of sugars indicates that the plant source has high mucilage (Bankova *et al.*, 2000). Propolis samples from the Mediterranean (Popova *et al.*, 2010; Popova *et al.*, 2011) and tropical regions in Asia (Wiryowidagdo *et al.*, 2009) have been reported to have significant amount of sugars and their derivatives (up to 37.2% of total ion chromatogram).

2.3.6 Trace element

Available literatures on trace element (mineral) from propolis are still inadequate. However, the mineral contents of propolis tend to influence the quality and the possible presence of pollutants (toxic minerals). In addition, similar with other compounds, mineral compounds are also dependent on the geographical and botanical origins. In recent studies on the mineral elements of propolis, Br, Co, Cr, Fe, Rb, Sb, Sm and Zn were detected in propolis from Argentina (Cantarelli *et al.*, 2011) while Ba, Ca, Cd, Cr, Co, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, and Zn were detected in samples from Brazil (Korn *et al.*, 2013). Both studies were conducted using neutron activation analysis (Samet *et al.*) and inductively coupled plasma optical emission spectrometry (ICP OES), respectively.

2.4 Biological activities of propolis

Propolis is well known to have various biological activities such as antimicrobial including antibacterial, antifungal and antiviral (Kartal *et al.*, 2003; Oliveira *et al.*, 2006; Schnitzler *et al.*, 2009; Boonsai *et al.*, 2014), anti-inflammatory (Naito *et al.*, 2007; Machado *et al.*, 2012), antioxidant (Ahn *et al.*, 2004; Ahn *et al.*, 2007; Mohammadzadeh *et al.*, 2007), antitumour (Sobocanec *et al.*, 2011; Xuan *et al.*, 2014), hepatoprotective (Banskota *et al.*, 2001; Paulino *et al.*, 2014), neuroprotective (Shimazawa *et al.*, 2005; Nakajima *et al.*, 2007), anti-diabetic (Zhu *et al.*, 2011), anti-amnesia (Chen *et al.*, 2008) local anaesthesia (Paintz and Metzner, 1979) antiulcer (Barros *et al.*, 2008) and UV light protection (Cole *et al.*, 2009; Gregoris *et al.*, 2011). Overall, these activities were

attributed to the presence of biologically active compounds such as phenolic compounds (flavonoids, phenolic acids and their esters) and terpenoids.

2.4.1 Antimicrobial activity

Antimicrobial properties of propolis in the bee hive had been recognised since ancient time. The combination of humid condition, small space and suitable temperatures make the hives a good condition for bacterial growth (Vojvodic *et al.*, 2011). However, this does not happen because of the antimicrobial activity of propolis. The special properties of propolis had attracted many researchers' interest. Investigations on the antimicrobial properties of propolis including their activity against bacteria, fungal and viral have been conducted.

2.4.1.1 Antibacterial activity

Many researchers have investigated the antibacterial properties of propolis and found that it has antibacterial activity against a wide range of Gram positive bacteria *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Enterococcus faecalis Enterococcus faecium* and *Bacillus subtillis* (Kartal *et al.*, 2003; Vardar-Unlu *et al.*, 2008) but no activity against Gram negative bacteria *Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumonia* (Kartal *et al.*, 2003).

Some authors reported that propolis is active only against certain Gram positive bacteria, others found that propolis also had some activity against Gram negative bacteria in higher concentration (> 4 mg/ml). These include *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Salmonella typhimurium* and *Enterobacter aerogenes* (Uzel *et al.*, 2005; Katircioglu and Mercan, 2006; Vardar-Unlu *et al.*, 2008). Antibacterial study of propolis against anaerobic bacteria especially against oral pathogen (cariogenic bacteria) include *Streptococcus mitis, Streptococcus mutans,* and *Streptococcus salivarius* (De Castro Ishida *et al.*, 2011) and periodontopathic bacteria *Prevotella intermedia, Prevotella* melaninogenica*, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Capnocytophaga gingivalis* and *Fusobacterium nucleatum* indicated that they were susceptible to propolis activities (Gebara *et al.*, 2002).

Studies on antibacterial properties of propolis had been expanded to include antibiotic resistant microorganism such as *Enterococcus faecalis* and *Salmonella spp.* Propolis has been found to have antibacterial activities against these bacteria and when combined with selected antibiotics revealed some synergistic effects (Stepanovic *et al.*, 2003). However, the mechanism of action of propolis on bacterial is complex and a simple correlation cannot be made to elucidate the exact mechanism of action.

The possible mode of antibacterial action of propolis confirmed using microcalorimetric and electron microscopic indicated that propolis inhibits bacterial growth by preventing cell division and inhibited protein synthesis (Takaisi-Kikuni and Schilcher, 1994).

2.4.1.2 Antiviral activity

The antiviral properties of propolis have been thoroughly investigated *in vitro* and *in vivo*. A pharmacological activity of propolis against several types of viral such as herpes simplex virus (HSV), human immunodeficiency virus (HIV), influenza A and B, vaccinia virus, hepatitis virus and poliovirus showed that propolis may be a potential medicine for viral diseases.

A study on *in vitro* antiviral activity of propolis revealed good anti-herpetic effects against herpes simplex virus type 1 (HSV-1). The findings indicated that plaque formation was significantly reduced with the mode of inhibitory effect probably due to prevention of virus adsorption to the host cells (Schnitzler *et al.*, 2009).

The *in-vitro* anti-HIV activity of isolated compounds of propolis terpenoids (moronic acid, anwuweizonic acid, betulonic acid and aromatic compounds) indicated that moronic acid has significant anti-HIV activity (Ito *et al.*, 2001). A comparative multi-centre study of the efficacy of propolis, acyclovir and placebo in the treatment of genital herpes type 2 showed propolis to be more effective than both acyclovir and placebo ointments in healing genital herpetic lesions, and in reducing local symptoms (Vynograd *et al.*, 2000).

Another study on the mode of antiviral activity of propolis demonstrated that the activity was different from that of classic antiviral drug (acyclovir). Acyclovir inhibited the viral DNA polymerase during the intracellular replication cycle when new viral DNA is

synthesised. However, the antiviral activity of propolis is probably due to prevention of virus adsorption to host cells (Schnitzler *et al.*, 2009).

2.4.1.3 Antifungal activity

The *in vitro* antifungal activity of propolis demonstrated promising results against a wide range of fungal (Stepanovic *et al.*, 2003; Koc *et al.*, 2005; Dota *et al.*, 2011). Stepanovic *et al.* (2003) investigated the antifungal properties of ethanolic extract of propolis against resistant or multiresistant yeast to antifungal drug (nystatin) which indicated the synergistic effects between antifungal drug and propolis. The results revealed propolis alone had significant antifungal activity and when combined together displayed ability to enhance antifungal effect. Koc *et al*. (2005) found that propolis activity against 29 strains of dermatophytes (*Trichopyton rubrum* and *Trichopyton mentagrophytes*) isolated from dermatophytoses patients were as good as using a standard antifungal drugs (terbinafine, itraconazole, ketoconazole, and fluconazole). In another study, Dota *et al*. (2011) found that propolis has good inhibition against 89 strains of *Candida spp* isolated from vulvovaginal candidiasis patients.

The *in vivo* antifungal activity of propolis against *Candida* yeast obtained from denture stomatitis patients revealed significant reduction of *Candida* in the saliva (Ota *et al.*, 2001). Nevertheless, although the antifungal properties of propolis had been studied extensively, the specific mechanism of action that involved in antifungal susceptibility still unknown.