EXTRACTION OF PROPOLIS BY USING WATER AND EVALUATION BY TLC, HPLC AND UV VIS SPECTROPHOTOMETRY

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ABSTRAK

Objektif penyelidikan ini adalah untuk melaksanakan ekstraksi propolis dengan menggunakan air sebagai pelarut ekstraksi dan menganalisis atau mengevaluasi ekstrak propolis dengan menggunakan Kromatografi Lapisan Tipis (TLC), Kromatografi Cair Prestasi Tinggi (HPLC) dan Analisis Spektrometri Ultraviolet. Kesan nisbah proplis dengan jumlah pelarut ekstraksi dikaji dengan peningkatan jumlah nisbah dari. 1:5, 1:10 hingga ke 1:15 di mana hasil ekstrak masing-masing adalah 5.95%, 9.05% dan 7.16%. Nisbah yang lebih tinggi akan meningkatkan hasil ekstrak, namun, hasil ekstrak pada nisbah lebih besar dari 1:10 akan menurun. Spektrometri UV Vis dilakukan untuk menentukan jumlah kandungan flavanon dan flavanols dan jumlah kandungan fenolik dalam Propolis Ekstrak Air (WEP) yang mempunyai kepekatan tertinggi dalam nisbah 1:5, iaitu 3.33 mg Quercetin Equivalent (QE) / g dan 6.12 mg Equivalent Acid Gallic (GAE) / g masing-masing. Analisis TLC telah mengesan bahawa ekstrak tersebut memiliki komponen asid kafein pada nilai R_f 0.22. Namun, dalam analisis HPLC, asid kafein tidak dikesan dalam ekstrak pada waktu retensi 3,82 minit seperti rujukan kromatogram asid kafein standard. Perpecahan puncak dan aliran baseline juga diperhatikan dalam ekstrak semasa analisis HPLC dan memerlukan proses penggambaran masalah. WEP menunjukkan jumlah flavon, flavonol dan jumlah kandungan fenolik yang lebih rendah berbanding dengan Propolis Ekstrak Etanol (EEP) dan Propolis Ekstrak Zaitun (OEP). Bagi TLC, komponen chrysin dikesan dalam EEP manakala tiada komponen yang dikesan dalam OEP. Untuk HPLC, asid kafein tidak dapat dikesan dalam ketiga-tiga ekstrak propolis.

ABSTRACT

The objective of this research was to perform extraction of propolis using water as extraction solvent and analyze the propolis extract by using Thin Layer Chromatography (TLC), High Performance liquid Chromatography (HPLC) and Ultraviolet–Visible (UV-Vis) Spectrophotometry. The effect of propolis to solvent ratio was determined by increasing the ratio from 1:5, 1:10 to 1:15 where the extraction yield of extract were 5.95%, 9.05% and 7.16% respectively. Larger ratio increases extract yield, however, extract yield at ratio larger 1:10 will not further increase percentage. Analysis by UV-Vis Spectrophotometry was carried out to determine the total flavone, flavonols and total phenolic contents in Water Extracted Propolis (WEP). Both contents showed a highest concentration in the 1:5 ratio, which were 3.33 mg Quercetin Equivalent (QE)/g and 6.12 mg Gallic Acid Equivalent (GAE)/g respectively. TLC analysis detected caffeic acid components in WEP at R_f value of 0.22. However, in HPLC analysis, the caffeic acid was not detected in sample at the retention time of 3.82 minutes as per reference of the standard caffeic acid chromatogram. Split peak and baseline drift were also observed in WEP during HPLC analysis and requires troubleshooting process. WEP showed lower amount of total flavone, flavonols and total phenolic contents as compared to ethanolic extracted peopolis (EEP) and olive oil extracted propolis (OEP). For TLC, chrysin components was detected in EEP whereas none of the components were detected by OEP. For HPLC, caffeic acid were not detected all three propolis extracts.

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LISTS OF SYMBOLS

&	And
%	Percentage
℃	Degree Celcius

LIST OF ABBREVIATIONS

TLC	Thin Layer Chromatography
HPLC	High Performance liquid Chromatography
UV-VIS	Ultraviolet–Visible
RSM	Response Surface Methodology
TPC	Total Phenolic Content
GAE	Gallic Acid Equivalent
MAE	Microwaved–assisted extraction
G	Gravitational constant
EEP	Ethanolic Extracted Propolis
SFE	Supercritical Fluid Extraction
PEG	Polyethylene Glycol
WEP	Water Extracted Propolis
GC-MS	Gas Chromatography- Mass Spectrometry
DAD	Diode Array Detector
PDA	Photodiode Art Detector
RP	Reversed Phase
XDB	Extra Stable Bond
μg	Microgram
mg	Milligram
g	Gram
μL	Microliter
mL	Millilitre
L	Litre
gdm	Gestational Diabetes Mellitus
v	Volume
ID	Internal Diameter
nm	Nanometer
QE	Quecertin Equivalent
Pe	Weight of Propolis Extract (g)
Pm	Weight of Raw Propolis (g)
RM	Ringgit Maslaysia

LIST OF ABBREVIATIONS

R_f	Retention Factor
rpm	Revolutions per minutes
mT	Millitorr
OEP	Oil Extracted Propolis
MS	Mass Spectrometry

CHAPTER 1

INTRODUCTION

1.1. Background of Study

Apitherapy uses traditional medicine in terms of bee products for disease prevention and treatment purposes (Trumbeckaite et al., 2015). In apitherapy, bee products such as honey, pollen, propolis, royal jelly and bee venom has been investigated to promote healing (Hellner et al., 2008). Propolis is a main component in bee hives that is actively being involved in recent research. It is a sticky substance that binds the hive together applied by honey bees *Apis mellifera* L (Popova, Bankova, and Trusheva, 2016). and is known to have medicinal properties (Fratellone, Tsimis and Fratellone, 2015).

Propolis primarily consists of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) (Gomez-Caravaca et al., 2006). Propolis has been confirmed to have beneficial pharmacological properties by recent research which it is antimicrobial, antiviral, antioxidant, anticancer, anti-inflammatory. immumodulatory, and agents anticaries (Wagh, 2013). Furthermore, Zhao et al. (2016) studied that the Brazilian green propolis is effective in improving antioxidant function in Type 2 Diabetes Mellitus patients.

Figure 1.1 Raw Bee Propolis (Conrad, 2016)

Stingless bee is less favored as it produces less amount of honey, but it produces propolis in a higher quantity than other bees in terms of potency (Zakaria et al., 2016). In Malaysia, study on propolis is rarely conducted compared to other countries. In fact, preliminary investigations on the composition and biological activity of propolis derived from Malaysian stingless bees are still incomplete and lacking of reliable evidences (Ibrahim et al., 2016). This study was aimed to identify the most ideal solvent for extraction and to analyse the beneficial chemical composition such as flavonoids and phenolics in Malaysian stingless bees *Trigona Thoracica species* propolis.

1.2. Problem Statements

Propolis has been cultivated and commercialized into medical devices, over the counter preparations, health supplements, cosmetics and other health related items due to its beneficial biological properties and hence increasing its market demand. However, crude propolis cannot be utilised as the main components which is known for biologically active components are the flavonoids and other phenolic derivatives.

Propolis must undergo extraction and purification to obtain the desired components. Many extraction methods are time consuming with low yield. Studies were conducted on the effect of different extraction solvents to identify the selectivity of desired components to extraction solvent for higher yield. Hence, extraction methods

are researched to obtain the most optimal extraction method and the least time consuming method.

Knowledge on the chemical composition of propolis is limited. Propolis differs in each location due to the specific climatic and phyto-geographic conditions of each plant sources causing its chemical composition to alter. The standardization and quality control of propolis products is hard to be achieved. Therefore, serious efforts have been made to overcome this obstacles resulting in slight improvement. For further development of propolis in the pharmaceutical industry, further efforts must be carried out to evaluate the propolis components.

1.3. Research Objectives

To resolve the problems stated, the objectives are defined as follows:

a. To perform extraction of propolis using water as extraction solvent.
b. To analyse or evaluate propolis extract by using Thin Layer Chromatography (TLC), High Performance liquid Chromatography (HPLC) and Ultraviolet–Visible UV-VIS Spectrometry analysis.

1.4. Scope of the study

This research studies and compares the yield of propolis extracted when using different extraction solvent. Furthermore, this study further evaluates and analysed the composition of reactive and desirable chemical components in the obtained propolis extract with using different analytical evaluation method. The respective extraction solvent may also provide propolis extract with different yield of chemical components. Hence, the study determines the most suitable extraction method and analytical method for evaluation.

This research allows further knowledge to be gain on the affinity of the flavonoids and phenolic components towards various extraction solvents. Different extraction methods are used to obtain the optimal condition of the extraction process.

These findings might be able to provide improvement by reducing extraction time and increase total yield of propolis extract. Furthermore, propolis extract may be evaluated to identify its total components by caring out different types of analysis methods. Physical and chemical properties and behaviour obtained may provide better understanding on the chemical, biological and therapeutic action. These data provides information and data for medical and pharmaceutical developments.

CHAPTER 2

LITERATURE REVIEW

2.1 Propolis

Propolis also known as bee glue (Bogdanov, 2016) is a natural, sticky resinous, balsamic, dark yellow to brown colored and strongly adhesive bee product (Saricoban and Yerlikaya, 2016). Generally, it serves as a substance to protect the bee hive from invaders (Park et al., 2004).

Propolis are commonly made up of 50% of resin consisting of flavonoids and phenolic acids, 30% of waxes, 10% of essential oils, 5% of pollen and 5% of more than 300 volatile organic compounds such as iron, zinc, vitamins, sugars and etcetera (Farré, Frasquet and Sánchez, 2004; Juliano, Pala and Cossu, 2007; Pastor et al., 2010). As compared to other compounds, flavonoids are widely investigated and is believed to have vital protective effect against oxidation reactions (Saricoban and Yerlikaya, 2016). There are several types of propolis such as Poplar type propolis, Aspen type propolis, Mediterranean type propolis, Brazilian green propolis and etcetera. Figure 2.1 shows some of the chemical compounds detected in poplar propolis from Europe.

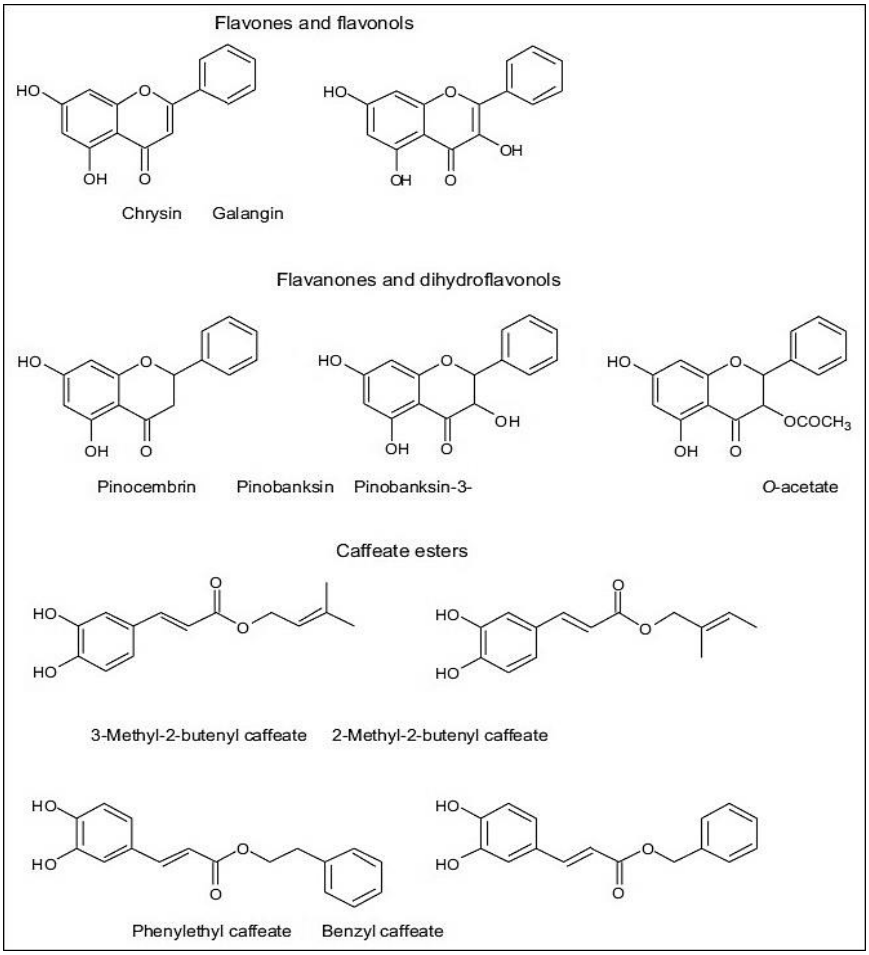

Figure 2.1 Some Chemical Compounds Detected in Poplar Propolis from Europe (Miguel, M.C.A. and Antunes, M., 2011)

Propolis shows significant biological activities that are health beneficial regardless of their different geographical origin. However, their chemical composition may differ (Kujumgiev et al., 1999; Seidel et al., 2008). Hence, it is important to identify and classify the types of propolis existing to distinguish its chemical components and biological activities.

2.1.1 Poplar Type Propolis

Poplar type propolis is the most common propolis to be researched (Silva-Carvalho, Baltazar and Almeida-Aguiar, 2015). They are obtained primarily from Europe, North America, New Zealand, and temperate zones of Asia and are mostly

composed of flavonoids, phenolicacids, and their esters (Bankova at el, 2000; Falcão et al., 2009; Sun et al., 2012), noticeably varies from other types. It originates from the bud exudates of the black poplar Populus nigra (Bankova, de Castro and Marcucci, 2000). The presence of the taxonomic markers of the black poplar confirms the poplar propolis.

2.1.2 Aspen Type Propolis

Aspen propolis originated from the northern European aspen plant source, Populus tremula (Popravko, Sokolov and Torgov, 1982; Bankova et al., 2002; Isidorov et al., 2014). Glycerol esters of substituted cinnamic acids (phenolic glycerides) acts as the trivial then again discriminant markers aspen bud exudates. (Bankova et al., 2016).

2.1.3 Mediterranean Type Propolis

Bankova.et al., (2016) implies that recent researches have revealed a new type of European propolis: Mediterranean propolis. These propolis samples originate from the Mediterranean region and its major components are diterpenes typical for the resin of the cypress tree, *Cupressus Sempervirens* (Popova et al., 2010; Popova et al., 2012). The only phenolic compounds in typical cypress propolis are the phenolic diterpenes totarol and totarolone. It usually does not contain flavonoids and phenolic acids.

2.1.4 Brazilian Green Propolis

Brazilian green propolis is another widely examined propolis type. Its main bioactive constituents include phenolic acids, prenylated phenolic acids and flavonoids which are characteristic for the *Baccharis dracunculifolia species*, the most important botanical source of Southeastern Brazilian propolis (Bankova et al., 1999; Kumazawa et al., 2003)

2.1.5 Pacific Type Propolis

There are also Pacific type propolis found from Pacific islands such as Taiwan, Okinawa and Indonesia (Huang et al., 2007; Kumazawa et al., 2008; Trusheva et al., 2011). Macaranga tanarius is the typical plant source for Pacific propolis. In this plant, identification of the prenylated flavanones (propolins) propolin C, propolin D and propolin F as major peaks in Total Ion Current chromatogram as researched by Bankova et al. (2016).

2.1.6 Mixed Type Propolis

Propolis which comes from more than one plant sources is known as mixed propolis (Bankova et al., 2016). Characteristic markers of the particular source plants can be detected by GC-MS. A more detailed and specific analysis of the total ion chromatogram is necessary, in order to consider the abundant prominent peaks. Aspen-poplar, Cupressus- poplar (Bankova et al., 2002) and Pacific (Macaranga) – Mangifera indicia propolis (Trusheva et al., 2011).

2.1.7 Malaysian Propolis

In Malaysia, the number of stingless bee species varies between 17 to 32 species depending on the study areas (Schwarz, 1939; Mohd et al., 2010; Salim et al., 2012). Five species of stingless bee and unidentified species were found when sampling was done in a bee farm located in the state of Kelantan, Malaysia (Kelly et al., 2014). Five species were *Trigona* (*Geniotrigona) thoracica*, *Trigona (Heterotrigona) itama*, *Trigona* (*Lepidotrigona) terminata*, *Trigona* (*Lisotrigona) scintillans* and *Trigona* (*Tetragonula) laeviceps*.

Zakaria et al. (2016), examined the Malaysian propolis produced by two different species of stingless bees, *Heterotrigona itama and Geniotrigona thoracica* collected from AGROPOLIS Apiary, Agriculture Production and Food Innovation

Research Institute (AGROPOLIS UniSZA), Universiti Sultan Zainal Abidin (Zakaria et al., 2016). They found out sensitivity Malaysian propolis of *H. itama* species plant is a more potent antioxidant with higher antidiabetic activity compared to *G. thoracica* species. The antioxidant activity is usually influenced by flavonoid content of propolis extracts which are potential antioxidants. Flavonoid compounds have been infer to be able to lower serum glucose levels through the inhibition of oxidative stress by improving insulin receptor signaling in insulin resistant conditions to increase insulin sensitivity (Fuliang et al., 2005; Zakaria et al., 2016) .

Jacob et al. (2015) studied on the effects of Malaysian propolis and Brazilian red propolis on connective tissue fibroblasts in the wound healing process. They found out Malaysian propolis indicated a total positive effect for cell migration and cell proliferation following a concentration dependent curve. However, Brazilian red propolis displays only a trivial increase in fibroblast migration.

Ahmed, R. et al. (2017) infers that Malaysian propolis is rich source of reducing sugars with a significant amount of protein when investigating cardioprotective mechanism of the propolis in rat's bodies. The study also affirms that Malaysian propolis expressively altered nearly all biochemical parameters associated with isoproterenol-induced myocardial injury.

2.2 Extraction Methods

Crude propolis is a solid resinous compound and cannot be consumed directly or used as an active ingredient (Saricoban and Yerlikaya, 2016). It must be break down and undergo purification and extraction to obtain the desired pure components (Saricoban and Yerlikaya, 2016) which are flavonoids and phenolic compounds. (Ghisalberti, 1979; Burdock, 1998). The most collective extraction method is solid-liquid extraction involving the use of extraction solvent such as ethanol, water and oil (Pujirahayu et al., 2014). The extraction solvent influences the composition and consequently the biological activities (Pujirahayu et al., 2014).

Extraction is an important process for the discovery of bioactive constituents from plant materials. Suitable extraction technique is essential for the standardization of herbal products as it extracts the desirable soluble constituents, eliminate excessive

waste with the aid of the solvents (Dhanani et al., 2013). Extraction is the separation of biological active portions of plant using selective solvents through standard procedures (Handa et al., 2008). The main purpose of all extraction is to separate the soluble plant metabolites from insoluble cellular marc known as residue as initial crude some need further processing (Handa et al., 2008).

Extraction process should be optimized to maximize extraction efficiency (Peng et al., 2015). Extraction process can be influence by several factors such as extraction temperature, extraction time, solvent concentration and solid to solvent ratio (Fan et al., 2008).

Peng et al. (2015) did a research on "Anli" Pear extract by using response surface methodology to optimize their extraction process. From their research, it was found out that Total Phenolic Content (TPC) increased with the ethanol concentration from 40 to 60% (v/ v), and reached an apex of 8.1 mgGAE/gdm at 60% (v/v) and decline sharply with increased ethanol concentration. Yang et al. (2009) affirmed that the solvent polarity and molecular movement declined with the increasing of ethanol concentration leading to dissolution of phenolic compounds with lower diffusion coefficient and decreased solubility when extracting *Phyllanthus emblica L Species*.

Yang et al. (2009) also found out that the solvent to solid ratio increase the extraction yield when this ratio increased from 20:1 to 50:1 but consequently decreased after exceeding this ratio. Bendahou et al. (2007), states this is due to the increase of the driving force for the mass transfer of the TPC at the 50:1 ratio. The research indicates that recovery of TPC mainly depends on the ethanol concentration and solvent/solid ratio.

Furthermore, a study was done by Margeretha et al. (2012) for the optimization factors in three different extraction methods which are maceration, reflux and microwave assisted extraction. Ethanol concentration and extraction time by maceration method gives comparatively similar maximum yield of total phenolic and flavonoid. The optimal extraction was performed on the same optimum conditions of ethanol concentration at approximately 70% and extraction time at around 50 hours. This is also shown by Cunha. et al. (2004) with an increase in the efficiency of phenolic compounds in propolis extract when using ethanol concentration equal or greater than 70%. However, Margeretha et al. (2012) also concluded that both factors show difference

influence in reflux and microwave assisted extraction due to presence of heat stimulation (reflux) and especially microwave irradiation.

Karacabey and Mazza (2010) observed the influence of ethanol concentration and extraction temperature of compounds with antioxidant activity of grape waste. Higher temperature favors the extraction of bioactive compounds with increased rate of diffusion and solubility of analyte (Bachirbey et al., 2014). Nevertheless, according to González-Montelongo, Lobo and González (2010), excessive temperature may cause degradation or volatilization of some compounds decreasing process efficiency.

Conventional extraction methods of propolis are hydro-distillation and organic solvent extraction like soxhlet method, maceration method, reflux method and Microwave Assisted Extraction (MAE). The recent extraction technique used is the supercritical fluid extraction in which the extraction parameters such as temperatures and pressure are controlled (Paviani et al., 2013). Furthermore, ethanolic extraction and aqueous extraction method are used to purify propolis. This process normally requires stirring or shaking, filtration and lastly the drying process (Azwanida, 2015). Figure 2.2 shows the setup of the soxhlet extraction method done by Bankova et al. (2016).

Figure 2.2 Soxhlet Extraction Method (Bankova, V. et al., 2016)

Trusheva, Trunkova, and Bankova. (2007) describe the maceration method as an extraction method in which the propolis is left in the dark after addition of solvent with propolis to solvent ratio of 1 to 20 and 1 to 10 in an Erlenmeyer flask for 72 hours. Margeretha et al. (2012) modified this method by adding shaking and agitation to the mixture for a period of 14 to 72 hours. The extract is then purified by centrifugation at

1500G for 5 minutes and was washed for 2 times with extracting solution which is ethanol. Then, it is further centrifuged and combined with the initial supernatant. Samples are then preserved at 4 ℃ before analysis (Margeretha et al., 2012).

Margeretha et al. (2012) carried out the reflux extraction of propolis described by Alencar et al. (2007), similar to the maceration method but by refluxing the propolis solvent – complex at 70 ℃ water bath for 10 to 140 minutes instead of maceration in the dark. The extract was then centrifuged, washed twice with ethanol, centrifuged again and combined with initial supernatant similar to the maceration method.

They also carried out the MAE of propolis in similar procedures to maceration method but by using extraction vessel and by irradiating the proplolis solvent – complex with microwaves at different power levels (420 – 600 watts) over different periods of time (5-30 min). The extract was the purified in similar steps as the maceration method.

Then, they investigated the yield by comparison of the maceration method, reflux reaction and Microwaved–assisted extraction (MAE). They confirmed that the maceration method and reflux method provides similar yield of 0.2% and 4% of flavonoid and total phenolic, respectively whereas MAE gives a higher yield of 0.4% and 5.8% of flavonoids and total phenolic, respectively. On the basis of yield, extraction time and solvent consumption, MAE method is more efficient and selective in extracting flavonoid and total phenolic compared to the two other methods. The results is similar to which obtained by Trusheva, Trunkova and Bankova (2007) where MAE provides higher yield compared to maceration method.

Paviani et al. (2013) performed modified maceration method with ethanol solvent by magnetic stirrer for 1 day at room temperature. The extract was then evaporated by using vacuum oven at 60 ℃. For maceration method using water solvent, the raw propolis was grounded and sieved. After addition of water solvent, the propolis mixture was heated in water bath and then centrifuged. Then, extract is dried by vacuum oven.

Cunha et al. (2004) described the Soxhlet extraction method as a method extracting propolis in a Soxhlet extractor for 24 hours at 60 ℃ for alcohol and 100 ℃ for water. Paviani et al. (2013) performed this method with modification of the Soxhlet extraction time to 6 hours. The extract were refrigerated overnight and filtered. Then,

extract were evaporated in rotary evaporator with vacuum control followed by thermostatic bath for drying.

Maceration extraction method is the most suitable as it is simple and less costly method even compared to MAE, Soxhlet and reflux method. Hence it is used for small and medium enterprises (SMEs) compared to other modern extraction methods. For small scale experiment and study, it is convenient even if there is lack of equipment resources and sufficient time.

Stahl, Quirin and Gerard (2012) used supercritical carbon dioxide at 600 bar and 40 ℃ to extract the wax from raw propolis and retain the insoluble flavonoids. Catchpole et al. (2004) used supercritical carbon dioxide both as an antisolvent to precipitate high-molecular mass components and to extract the ethanol and soluble components of the liquid form ethanolic propolis extract. Paviani et al. (2010) performed the supercritical fluid extraction of dried ethanolic extract from green Brazilian propolis, investigating the fractionation of components of interest present in the propolis extract and the results indicated higher selectivity at low density resulting lower extraction yields.

Paviani et al. (2013) perform the supercritical fluid extraction experiment in which dry Ethanolic Extracted Propolis (EEP) was mixed with glass balls and packed inside the extractor. Then, carbon dioxide was pumped into the extractor bed for static period of 30 min to allow contact between the samples and the supercritical solvent to stabilized temperature and pressure. The samples were collected and all the tubing in the process line was washed with ethanol to recover the extract deposited in it.

Paviani et al. (2013) compared the conventional methods such as maceration by water, maceration by ethanol, and soxhlet extraction with the modern supercritical fluid extraction (SFE) technique. Their findings conclude that high extraction yield was shown when ethanol solvent was used in conventional extraction methods. For the supercritical extract, the flavonoids concentration show higher yield than the conventional ethanolic extract of propolis. This results infers that the supercritical method focus on flavonoids which has high biological activity.

Paviani et al. (2012) experimented on the effect of ethanol as a co-solvent in the supercritical carbon dioxide extraction method. The results indicate that maximum total extraction yields were 7.3% for SFE with no co-solvent which is exceedingly low

compared to 51% for SFE with 15% ethanol as co- solvent. This infers that the addition of co-solvent and the selectivity of supercritical carbon dioxide could be manipulated so as to obtain extracts with the yields and concentrations of interest.

Kubiliene et al. (2015) examined and compared the effect of different extraction solvent such as water; water and polyethylene glycol (PEG) mixture; olive oil; PEG, olive oil and water mixture and 70% ethanol on the composition and biological activities of propolis extract by maceration. Non-ethanolic solvent complex with addition of PEG allows higher yield of propolis extract as compares with extract containing water or oil only. Ethanolic propolis extract consists of similar concentration of total phenolic compounds compared to nonethanolic extract. Non-ethanolic extract also consists of higher antimicrobial effect compared to ethanolic propolis in which it demonstrates significant inhibition of growth and multiplication of all tested microorganisms.

According to Pujirahayu et al. (2014), the use of ethanol solvent in maceration extraction method of propolis showed the highest yield which is 18.33% whereas the lowest yield is by using olive oil as extraction solvent. Ethanolic extract obtained is solid sticky, water extract is gummy sticky and dark brown color whereas propolis produced from the solvent virgin coconut oil, olive oil and propylene glycol were gummy oily and yellowish brown color. Propylene flavonoid of the solvent is higher than other solvents that propylene glycol can be used to make propolis extract oil that has equivalent or better properties than ethanol extract. Silva et al. (2010) showed similar results that the oil extract of propolis which uses canola oil has anti-potency against *Aspergillus fumigates* better than ethanol extract (Silva et al., 2010).

Studies and researches on EEP are abundant with little knowledge and information of biological activities water extracted propolis (WEP) (Rocha et al., 2013). This is due to the fact that extractable matter such as flavonoid content will be obtained from direct extraction using water compared to other extraction solvent (Park and Ikegaki, 1998) . However, EEP is found to have advantages such as a strong taste and adverse reactions or intolerance to alcohol (Mello, Petrus and Hubinger, 2010).

Furthermore, alcohol in cosmetic, food and pharmaceutical preparations causes disadvantages to consumer or customer in terms of halal use. Hence, industries are

recently interested in developing non-alcoholic products which in this case would like to obtain WEP with similar chemical composition to EEP (Rocha et al., 2013).

An experiment was carried out by Rocha et al. (2013) to evaluate the efficacy and safety characterization of WEP and comparison with EEP. The results showed that a higher phenolic content was obtained in WEP. It was also shown that WEP exhibited a higher genotoxic effect antioxidant activity and was more efficacious in most microorganisms compared to EEP. Besides, no genotoxic effect which is carcinogenic or cause hereditary mutations was revealed in the micronucleus assay.

However, Park and Ikegaki. (1998) showed contradictions in which he water extract has also been studied, but in comparison with ethanol extract, it expressed weaker antioxidant and antimicrobial activities due to lower concentration of extracted flavonoids. This is because phenolic acids as polar substances easily soluble in water were mainly contained water extracts. Hence, further studies were encouraged on water extraction of propolis to justify its biological activity and efficacy from the extracted compound.

2.3 Evaluation Methods

2.3.1 Spectrophotometric Evaluations

The propolis extract should be evaluated in terms of total phenolic compounds and total flavonoid compounds. According to Kubiliene et al. (2015), spectrophotometric evaluations are used to determine the total content of phenolic compounds such as Folin-Ciocalteu method and total radical scavenging activity. In Folin-Ciocalteu method, chemical reduction involving reagents containing tungsten and molybdenum occurs (Stalikas, 2007). The presence of phenolic compounds in the sample will cause a blue color product under a broad light absorption spectrum (Stalikas, 2007). This method requires developing of color for 2 h at room temperature followed by measurement of absorbance at 760 nm wavelength. A standard calibration curve of gallic acid with concentration range 16 – 1,040 mg/mL was used to estimate total phenolics content.

According to Yusoff and Leo, (2017), the flavonoids content was estimated using the modified aluminum chloride colorimetric method (Chia-Chi et al., 2002). The chemical reaction of this method is based on the formed complex, which provides a bathochromic displacement and the hyperchromic effect (Soares, Silva and Pezzini, 2015). The flavonoid content was conveyed as mg quercetin equivalent per gram of dry extract (mg QE/g). The sample extract (500 μL) was mixed with 250 μL of 5% aluminum chloride and 4.25ml methanol. The absorbance was read at 425nm upon 30 min of incubation .The total flavonoid content was compared and determined by plotting against the quercetin calibration curve from 0 to 100 μg. Absorbance was measured at 515 nm wavelength.

2.3.2 Chromatographic Evaluations

According to Bankova et al. (2016), the typical chromatographic techniques used for analysis of components in propolis extract are Thin Layer Chromatography (TLC), Gas Chromatography–Mass Spectrometry (GC–MS) and High Performace Liquid Chromatography (HPLC) .

TLC is used for separation of non-volatile mixture. (Harwood and Moody, 1990). TLC uses the affinity of sample for the mobile phase (solvent) and stationary phase for separation of the sample to produce retention factor so unknowns can be compared to known materials. Retention factor (Rf) is equal to the distance migrated over the total distance covered by the solvent and is affected by layer thickness, moisture on the TLC plate, vessel saturation, temperature, depth of mobile phase, nature of the TLC plate, sample size, and mobile phase parameters The R_f formula is:

$$R_f = \frac{distance\ traveled\ by\ sample}{distance\ traveled\ by\ solvent} \text{.....}(2.1)$$

TLC is suitable for observation under a UV light source of short (254nm) and long (365nm) wavelength UV light (USP Pharmacists' Pharmacopeia, 2017). In TLC, the choice of stationary phase as well as a suitable solvent depends upon the polyphenolic structures being studied (Gomez-Caravaca et al., 2006).

According to Medic-Saric et al. (2013) TLC uses a standard stationary phase of silica gel to separate more non-polar flavonoids (such as flavonols and isoflavonoids). Silica gel plates containing fluorescent dye (F254) of aluminium base are most extensively used (Handa et al., 2008). Besides, the nature of the compounds defines the choice of adsorbent layer; a stronger adsorbent (aluminium oxide) is used for weakly adsorbed compounds and a weak adsorbent (cellulose) is used for strongly adsorbed compounds.

Single mobile phase with 1-3 components is favored over a multicomponent mobile phase. The polarity of the compounds of interest is the key to selection of a mobile phase. Varieties of different mobile phases are applied as eluent: ethanol/water (55:45 by volume), petroleum ether/ethyl acetate (70:30 by volume), petroleum ether/acetone/formic acid (70:20:10 by volume), chloroform/ethyl acetate (60:40 by volume), toluene/chloroform/acetone (40:25:35 by volume), *n*-hexane/ethyl acetate/acetic acid (58:39:3 by volume) and chloroform/methanol/formic acid (89:6:5 by volume) (Medic-Saric et al., 2013).

Furthermore, TLC results are sensitive to temperature and humidity variations. All operations during which the plate is exposed to the air should be carried out at a relative humidity of 50%-60% under controlled room temperature of 20℃- 30℃ (Handa et al., 2008).

Nevertheless, according to Sawaya, Cunha & Marcucci. (2012), if the number of components in propolis is too great for sufficient separation, TLC may not be efficient for propolis characterization. Identification and quantification of components directly on TLC plates is further restricted by the fact that commercial standards are not identified for many propolis components. Hence, further research is to be carried out to confirm the reliability of this analysis technique.

Gas Chromatography- Mass Spectrometry (GC- MS) is broadly used for chemical analysis of complex compound such as propolis, complex metabolites, petroleum fractions, biological fluids, polymer and etcetera (Bankova et al., 2016). It is a combination of gas chromatography and mass spectrometry for detection of different substances (Bankova et al., 2016). Mass spectrometry provides the acquisition of molecular mass data and structural information together with the identification of compounds (Gomez-Caravaca et al., 2006).

However, in GC – MS derivatization method is needed to be done prior to GC analysis (Greenaway, Scaysbrook and Whatley, 1987). Derivatization process transforms an analyte for to a modified and detectable analyte known as the derivative in GC or other instrumental analytical methods (Orata, 2012). It is done by adding derivatization to increase volatility of propolis extract to be analyzed (Greenaway, Scaysbrook and Whatley, 1987). This hindered the search for compounds in data bases (Sawaya, 2006).

According to Greenaway, Scaysbrook and Whatley (1987), higher molecular weight flavones such as quercetin do not transmit well through a GC column and their percentage occurrence may be seriously underestimated by GC-MS analysis.

According to Gomez-Caravaca et al. (2012), HPLC is the most efficient and reliable analytical technique for the characterization of polyphenolic compounds. Phenolics and flavonoids absorb in the UV-VIS range and can easily be detected with a diode array detector (DAD) of HPLC (Marcucci et al., 2001; Marcucciet et al., 2000; Salomao et al., 2008). HPLC-DAD has also been successfully applied to the identification and quantification of components in fractions of propolis obtained by open column chromatography (Nakamura et al., 2010; Fischer et al., 2010). However, terpenes absorb poorly and sometimes go undetected unless a different detection method such as mass spectrometry is used (Sawaya, Cunha and Marcucci, 2011).

HPLC is preferred in the separation and quantification of phenolic compounds (Naczk and Shahidi, 2004). HPLC analysis of phenolics and is affected by various factors including sample purification, mobile phase, column types and detectors (Stalikas, 2007). Reversed phased C18 column, photo diode art detector (PDA) and polar acidified mobile phase are normally use in quantification of phenolics (Ignat, Volf and Popa, 2011). Reverse phase is an elution procedure used in liquid chromatography where the mobile phase is significantly more polar than the stationary phase and vice versa for normal phase (Handa et al., 2008).

A critical factor in identifying phenolics is the appropriate column selection (Khoddami, Wilkes and Roberts, 2013). Generally, based on the polarity, different classes of phenolics can be detected using a normal phase C18 or reversed phase (RP-C18) column 10–30 cm in length, 3.9–4.6 mm ID and 3–10 mm particle size (Robbins, 2003; Lopes-Lutz et al., 2010).

Acetonitrile and methanol, or their aqueous forms, are the dominant mobile phases utilized in HPLC quantification of phenolics (Qin et al., 2010; Zarena and Sankar, 2011; Diagone et al., 2012) The pH of the mobile phase should be maintained in the range pH 2–4 to avoid the ionization of phenolics during identification (Khoddami, Wilkes and Roberts, 2013). HPLC assays of phenolics are carried out at ambient column temperature (Handa et al., 2008). HPlC is also affected by the retention time and flow rate which elute the peak area.

HPLC seems to be a more suitable technique for the analysis of flavonoids from propolis. However, some minor flavonoids such as pinobanksin-3-methyl ether, pinobanksin-3-propanoate and pinobanksin-3-iso-butanoate were only detected in the GC-MS analysis (García-Viguera, Ferreres and Tomás-Barberán, 1993). Meanwhile, in the HPLC chromatogram some minor flavonoids were observed with the diode-array detector. Chalconoids which are aromatic ketones are not detected in the HPLC analysis but was observed in the GC-MS. This shows the advantage of GC-MS provides an overall view of propolis composition, identifying flavonoids, phenolic acid derivatives, acids, sugars and etcetera. Hence, GC-MS is a more convenient technique for pharmaceutical applications, because the qualitative and quantitative variations of propolis from the same area may be considerable. On the other hand, for the specific analysis of flavonoids from propolis, HPLC with a diode-array detector is proven to be more useful.

In the research of Thirugnanasampandan, Raveendran and Jayakumar (2012), GC-MS analysis revealed the presence of fatty acids, alcohols, and quercetin. However, they use the HPTLC and HPLC methods to identify and quantify the extract amount of quercetin detected in GC- MS.

HPLC analysis on phenolics profile of propolis extract typically uses reverse phase column due to the polarity of the phenolic compounds (Sawaya et al., 2002; Bruschi, Franco and Gremião, 2003; Sun et al., 2015). Flavonoids compounds were analyse and evaluated by Coneac et al. (2008), using HPLC with a stable bonded C18 column which are packed for high speed and high resolution reversed phase HPLC.

Natanela Croci et al. (2009) did a study on the HPLC evaluation of phenolic and polyphenolic acids from propolis. A Zorbax Eclipse XDB- C18 column Agilent Technologies (250 x 4.6 mm, 5µm) and a Hypersil BDS C18 (250 x 4.6 mm, 5µm)

Thermo – Fisher were used in place of Discovery RP-Amide (C16) column (250 x 4.6 mm i.d., 5 µm particle) for improvement of separation method. Flowrate of 1ml/min with injection volume of 20 µl was used at column temperature of 25 ℃. Acetonitrile and aqueous proportion was adjusted to pH between 2 to 3 for better separation efficiency. The results indicated that the experimental conditions will affect the determination of free phenolic and polyphenolic acids from propolis extract.

Zorbax Eclipse XDB –C18 column is a densely covered, deactivated, column which can be used for acidic and neutral samples but is most compatible for separation of acidic, basic, other highly polar compunds by reversed-phase liquid chromatography.

According to Handa et al. (2008), TLC is favored for generating a chemical profile where HPLC is preferred in estimating the quantity of the markers generates by TLC. Purification, degassing, and filtration of sample essential to protect expensive columns from deterioration is not required in TLC analysis. It can accommodate up to 18 different on a single 20 x 20 cm plate. A fresh plate may be use to avoid leftover from previous analysis to interfere with the results.

However, HPLC analysis is superior to TLC as it produces better separations due to its efficient flow kinetics. TLC fails to match the sensitivity of HPLC due to continuous development of HPLC. TLC is also an open system and accelerates the degradation of compounds sensitive to light and air, which in the case of HPLC pass through an enclosed environment. Detection of the analyte in HPLC occurs in solution, permitting high sensitivity, whereas in TLC the solid phase interaction makes detection less sensitive (Handa et al., 2008).

The comparison concludes that TLC is fast, adaptable and economical, whereas HPLC is more precise and accurate. Hence, both TLC and HPLC should be utilized in terms of conditions and needs.

Table 2.3 summaries the extraction methods and evaluation methods done by researchers using different extraction solvents for the propolis previous extraction studies.

2.4 Table of Extraction and Analysis Method

Table 2.1 Comparison of Some Researchers Based on Extraction Method, Extraction Solvent, Analysis Method and Sample

RESEARCHERS/ YEAR	EXTRACTION METHOD	EXTRACTION SOLVENT	ANALYSIS METHOD	SAMPLE
Najafi et al. (2007)	WEP	Ethanol & Water	Paper chromatography	Propolis from Mashhad area in Iran
Rocha et al. (2013)	Dynamic Maceration	Ethanol & Water (7 : 3)	Reversed Phase –HPLC	Brazillian Propolis
Kubiliene et al. (2015)	Maceration	Water, Water & PEG, Olive oil, Olive oil & PEG, Ethanol	Folin-Ciocalteu method & HPLC	Propolis was collected in Lithuania
Agarwal, Vemanaradhya and Mehta (2012)	EEP	Ethanol	Folin–Ciocalteu method, modified aluminum chloride colorimetric method, and 2.4-dinitrophenylhydrazine (2,4-DNP) method.	Propolis of Chinese origin was obtained from Ecuadorian Rainforest, New Jersey, USA
Al-Abbadi, Ghabeish & Ateyyat (2015)	WEP	Water	Antimicrobial test: Serial Dilution Method	Jordan propolis, Chinese, Turkish and Tablet propolis samples

RESEARCHERS/ YEAR	EXTRACTION METHOD	EXTRACTION SOLVENT	ANALYSIS METHOD	SAMPLE
Bankova et al. (2016)	EEP	Ethanol	GC-MS, LC- MS, MS. ESI-MS, NMR, Folin–Ciocalteu method, modified aluminum chloride colorimetric method, and 2.4-dinitrophenylhydrazine (2,4-DNP) method & Antimicrobial test.	Poplar type propolis, Aspen type propolis, Brazillian green propolis, South American red Propolis, Mediterranean type propolis, Pacific type propolis, Mangifera indica type propolis and Mixed propolis type
Bertrams et al. (2013)	Reflux method	Ethanol	TLC & TLC –MS	German propolis
Sawaya et al. (2004)	Maceration Soxhlet Method	Mixture of Ethanol & Water, Ethanol	TLC, HPLC	Brazilian Propolis

CHAPTER 3

METHODOLOGY

3.1 List of Chemicals and Reagents

3.1.1 Extraction Process

The chosen propolis sample of *Trigona Thoracica Species* was obtained from a stingless bee farm in Kelantan Biotech Corporation. Ultra-pure water acted as an extraction solvent was obtained from the Integral Water Purification System (Milli-Q, France).

3.1.2 Evaluation Process

Folin - Ciocalteu reagent, 7% sodium carbonate, gallic acid, 95% ethanol, 10 % aluminum chloride, 5 M potassium acetate and quercetin standard were purchased from Sigma Aldrich, USA. These materials were used in UV-Vis spectrophotometry for analysis. In the Thin Layer Chromatography (TLC) analysis technique, toluene: ethyl acetate: formic acid (8:2:0.1) was purchased from Sigma Aldrich, USA and prepared as mobile phase. Silica gel TLC plate of 20 cm × 20 cm was purchased from Sigma Aldrich, USA and standards reagent such as aluminum chloride was purchased from Sigma Aldrich, USA. High Performance Liquid Chromatography (HPLC) analytical technique used phosphate buffer saline solution: methanol in (60:40) for the mobile phase. Standard reagent for HPLC used was caffeic acid which was purchased from Sigma Aldrich, USA. Phosphate buffer saline solution was purchased from Essen-Haus Sdn. Bhd. and methanol solvent was obtained from laboratory.

3.2 List of Equipment and Apparatus

3.2.1 Extraction Process

An incubator shaker (Infors Ht Ecotron, UK) was used during extraction of propolis by water. Mortar and pestle was used for reducing propolis particles size. Measuring balanced, measuring cylinder and 300 mL volumetric flask were used in the mixing process. The Centrifuge 5810 R (Eppendorf, Permula Chemicals Sdn. Bhd.) was used for further separation of propolis extract. Whatman 1 filter paper (180mm diameter) was used for filtration process and freeze dryer (SP Scientific, USA) was occupied for drying process.

3.2.2 Evaluation Process

Incubator shaker (Infors Ht Ecotron, UK) was used for the well- mixing in extraction of sample. UV - Vis spectrometer (UV – 1800 Shimadzu, Japan) was used for analysis for total phenolic and total flavanones and flavanols content of propolis extract. The UV chambers were (Quanti-Tray Sealer, Model 2X) used in TLC analysis. HPLC (Agilent Technologies, USA) was used with 1260 Diode Array Detector (DAD), 0.45µm filter and column Zorbax Eclipse Plus – C18. Ultrasonic bath (Bandelin Sonorex Digitec, Berlin) was used for degassing process before injecting sample and standard vials for HPLC analysis.

3.3 Extraction Process

Water extract of propolis was obtained by using modified maceration method by Paviani et al. (2013) as follow: Firstly, crude propolis *Trigona Thoracica Species* was grinded to increase surface area by using mortar and pestle. Figure 3.1 shows the appearance of crude propolis before grinding into smaller pieces.

Figure 3.1 Crude Propolis

Then, 25 g of grounded propolis pieces was weighed by using electronic weighing balance thrice for 3 propolis samples. After that, 125 mL, 250 mL and 375 mL of distilled water were measured respectively and poured into separate conical flask with 25 g of grounded propolis forming 1:5, 1:10 and 1:15 propolis to solvent ratios respectively. The conical flasks were placed into an incubator shaker (Infors Ht Ecotron) at approximately 27 ℃ or room temperature at 80 rpm for 7 days continuously as modified from Paviani et al. (2013).

Figure 3.2 Different ratio of propolis to water after incubation (A = 1:5; B = 1:10; C = 1:15)

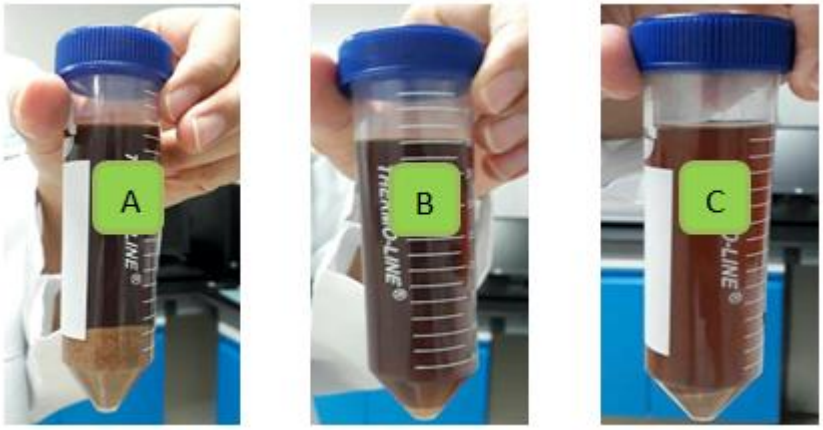

Figure 3.3 Centrifuged propolis extract to solvent with ratio of A (1:5), B (1:10) and C (1:15)

The incubated propolis extracts were then centrifuged at 4000 rpm for 20 minutes in the Centrifuge 5810 R (Eppendorf, Permula Chemicals Sdn. Bhd). The supernatant were then filtered by using filter paper to obtain first extract. The residue was then filtered again for the second extract. The filtrate residue then undergone sublimation in the freeze dryer (SP Scientific, USA) at optimized temperature at approximately -80 ℃ and 200 mT. The freeze drying process occurs for 7 days discontinuously for cleaning of equipment and to prevent equipment from overheating.

Figure 3.4 Freeze drying process of propolis extract

Each ratio powder extract was weighed to obtain the yield of extract and stored in 10 ℃ in chiller prior to analysis. The observed data and variables were recorded and tabulated.

The yield of extraction which is yield percentage was calculated using the formula:

$$\text{Percentage Yield} = \frac{Pe}{Pm} \times 100\% \text{.....} \quad (3.3)$$

Where: P_e is weight of propolis extract (g) and P_m is weight of raw propolis (g).

After measuring the weight of propolis extract, each ratio of propolis extract was then dissolve in 75% ethanol. 0.1 g of each ratio of propolis extract was dissolve in 10 ml of 75% ethanol to form 1% propolis extract as shown in Figure 3.5.

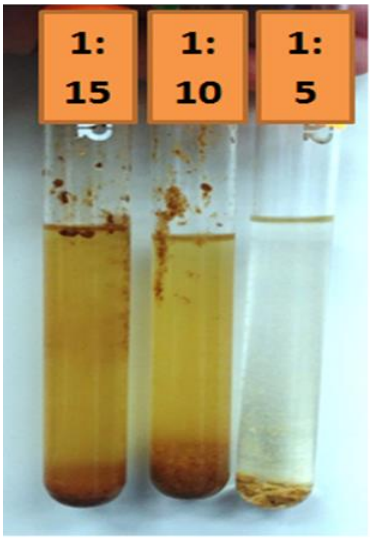

Figure 3.5 Different Propolis to Solvent Ratio of WEP Dissolved in 70% Ethanol

3.4 Optimization Process

There are several factors that will influence the extraction process and yield of extract that can be used for optimization in this study. These independent variables for optimization process are concentration of extraction solvent, temperature of extraction process and extraction time. This study optimized concentration of extraction solvent by altering propolis to extraction solvent ratio and investigated the feasibility of the obtained optimal extraction process. Table 3.3 shows the propolis to extraction solvent ratios that were used in this study.

Table 3.1 Propolis Sample to Extraction Solvent Ratio Selected for Optimization

Conical Flask	Amount of propolis (g)	Amount of extraction solvent (mL)	Ratio (Propolis : Extraction Solvent)
A	25	125	1:5
B	25	250	1:10
C	25	375	1:15

3.5 Evaluation Process

3.5.1 Analysis of Total Phenolic Contents (TPC) by UV –VIS Spectrometry

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method as referring to Ibrahim et al. (2016). 0.2 mL of diluted extract 1% propolis sample was transferred to a 25 mL screw-cap tubes. The Folin–Ciocalteu reagent (1 mL) was then added and mixed. 1 mL of sodium carbonate (10 %) was added after 3 minutes and the volume was adjusted to 10 mL by topping up with ultra-pure water. The same procedures were repeated for blank sample by using 1 mL of ultra-pure water in the place of the 1 mL extract. The mixture was incubated in the dark for 90 minutes. 3 mL of the mixture was transferred by micropipette into the cuvette where the absorbance was then measured at 725 nm in a UV Vis spectrophotometer , and the results were calculated using gallic acid calibration curve (50, 100, 150, 250 and 500 ppm, r^2= 0.98879). These procedures are repeated for 1% extract samples of all ratios. The results expressed as equivalents of gallic acid (mg GAE/g dry extract) by comparison with gallic acid calibration curve.

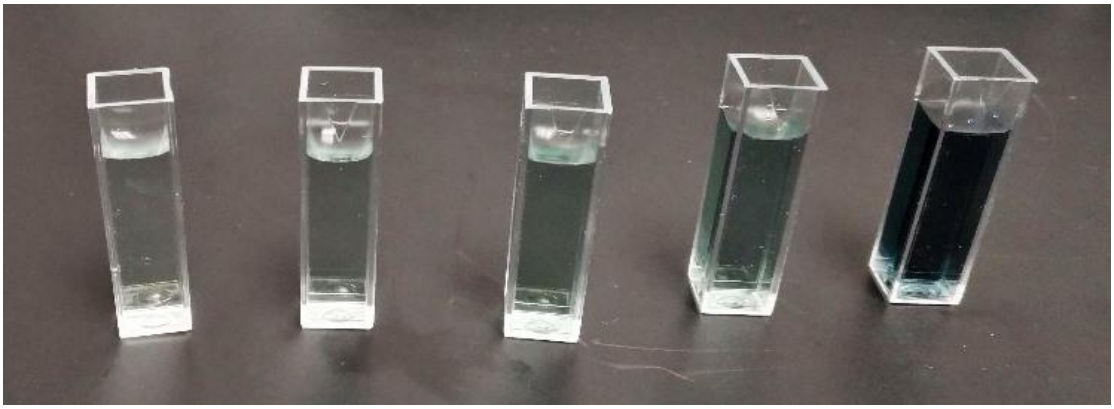

Figure 3.6 Five Gallic Acid Standards at Concentration Range from 50 – 500 ppm for TPC Quantification

3.5.2 Analysis of Total Flavanones and Flavanols Content by UV –VIS Spectrometry

The total flavanones and flavanols in 1% propolis were determined by the modified aluminum chloride colorimetric method as referring to Vemanaradhya, Agarwal and Mehta (2012). 0.5 mL of 1% propolis extract was mixed with 1.5 mL of 95% ethanol, 0.1 mL 10% aluminum chloride, 0.1 mL of 1 mol/L potassium acetate, and 2.8 mL ultra-pure water. A volume of 10% aluminum chloride was substituted by the same volume of distilled water in blank. Then, 3 mL of mixture was transferred into the cuvette after incubation at room temperature for 30 minutes. The absorbance of the reaction mixture was then measured at 415 nm wavelength in a spectrophotometer (UV – 1800 Shimadzu, USA). The obtained values were compared with a standard calibration curve of quercetin (25, 50, 100, 150 and 250 ppm, $r^2 =$ 0.99777) as a reference.

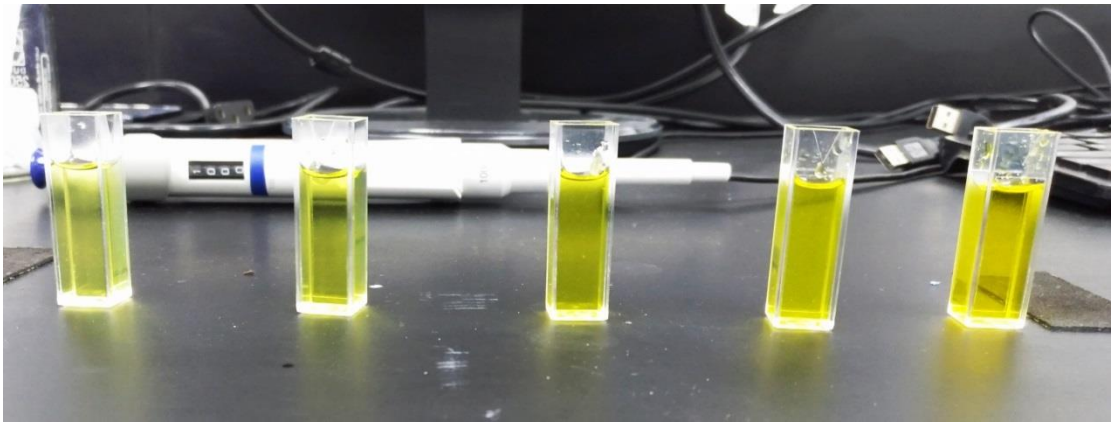

Figure 3.7 Five Quercetin Standards at Concentration Range from 25 – 250 ppm for Total Flavanones and Flavanols Content Quantification

3.5.3 Analysis of Components in Propolis by Thin Layer Chromatography (TLC)

TLC analysis of propolis was performed on silica gel plate with mobile phases systems toluene:ethyl acetate:formic acid in (8:2:0.1) by using modified TLC analysis from Ibrahim et al. (2016), to determine the components in the extract. The mobile

phase system was prepared and poured in beaker after mixing well and covered to allowed to saturate for about 1 hour in the fumehood. Then, 3 mL of 0.5 g of propolis extract was dissolve with 70% ethanol to form solution 16.67% propolis extract. 10 µL each of three different ratio of 16.67% propolis samples were used and were applied on a 8 cm × 5 cm plates and 1.5 cm from the bottom of the plate (Alemu et al, 2016). The silica gel plates were then placed vertically in each beakers primarily saturated with each of the solvent systems with the beakers covered and left until the mobile phase reached 1 cm from the top. The spots were visualized in long and short wavelength.

Then, three diluted standards reagent which are caffeic Acid, chrysin and pinocembrin are applied on a plate and allowed to air dry. The plates were then placed vertically in each beaker saturated with solvent systems in the fumehood and left until the mobile phase reached 1 cm from the top. The spots were visualized under 365 nm UV light wavelength in the UV viewing cabinet (Quanti-Tray Sealer, Model 2X) and their retention factor (R_f) values were calculate using the Equation 2.1.

3.5.4 Analysis of Components by High Performance Liquid Chromatography (HPLC)

HPLC analysis were performed using a Agilent HPLC equipped with quaternary pumps VL, an automatic controller of flow, an Ultraviolet-Visible Photo Diode Array spectrophotometric detector module (1260 DAD). A Bio-inert Auto Injector was used to carry 20 µl of the 1% and 5% propolis extract onto the column as refered to Yang et al. (2013). Chromatographic separation was accomplished using a stainless steel analytical column Zorbax Eclipse Plus C18 (ZORBAX Eclipse Plus C18, 250 x 4.6 mm, 5µm,USA) with phosphate buffer saline: (pH=4.5) in water and methanol (40:60, v/v) in isocratic mode as refering to Yang et al. (2013), previously filtered through a 0.45 µm membrane filter (Whatman, Germany) and degassed using an ultrasonic bath (Bandelin Sonorex Digitec, Berlin). Gradient separation was performed at a flow rate of 1.0 mL/min, with the column temperature set at 25 ℃. UV detection was performed at 260nm for quecertin (Yang et al., 2013) and 325 nm for caffeic acid (Spagnol et al., 2015). Standard calibration curves were first obtained by using quecertin and caffeic acid which were filtered through 0.45 µm syringe filter. The sample peaks were

compared with the standard calibration curve to determine and quantify the identified compound. Preparation of mobile phase, standard solutions and samples were prepared as refering to Yang et al. (2013).

Preparation of mobile phase

Mobile phase solution were prepared by mixing 400mL of phosphate buffered saline (pH=4.5) and 600 mL of methanol in the ratio (40/60, v/v) in a 1L Schott Bottle. The mobile phase solution were then checked for its pH by using benchtop pH meter (Mettler Teledo, USA) to ensure it remained at pH 4.5 and was adjusted by using phosphoric acid if pH increases due to ambient factors. Then, the mobile phase was filtered through a 0.45 µm membrane filter (Whatman, Germany) and degassed using an ultrasonic bath (Bandelin Sonorex Digitec, Berlin).

Preparation of standard solutions

Each 0.032 g pure standard substance of quercetin and caffeic acid was accurately weighted transferred to a 50 mL volumetric flask and made up to 10 mL with methanol. Then, 0-3200 ppm solutions were prepared by stepwise dilution 32 µg/ml mixed standard stock solution with phosphate buffered saline (pH=4.5) and methanol (40/60, v/v). These standard solutions were stored at 4 ℃. Each concentration of quercetin and caffeic acid standards solution were filtered through a 0.45 µm syringe filter into 2 mL HPLC vial respectively and left to degas for 30 minutes by using an ultrasonic bath (Bandelin Sonorex Digitec, Berlin). The vials were then injected into HPLC system.

Preparation of samples

0.50 g of WEP was dissolved in 5 ml of 70% ethanol to produce 1% WEP and 2.50 g of WEP was dissolved in 5 ml of 70% ethanol to produce 5% WEP. Then, each

propolis to solvent ratio of 1% WEP and 5% WEP were filtered through a 0.45 µm syringe filter into 2 mL HPLC vials respectively and left to degas for 30 minutes by using an ultrasonic bath (Bandelin Sonorex Digitec, Berlin). The vials were then injected into HPLC system.

3.6 Flowchart of Study

Figure 3.8 shows the flowchart of study for the extraction and evaluation of propolis by using water as an extraction solvent.

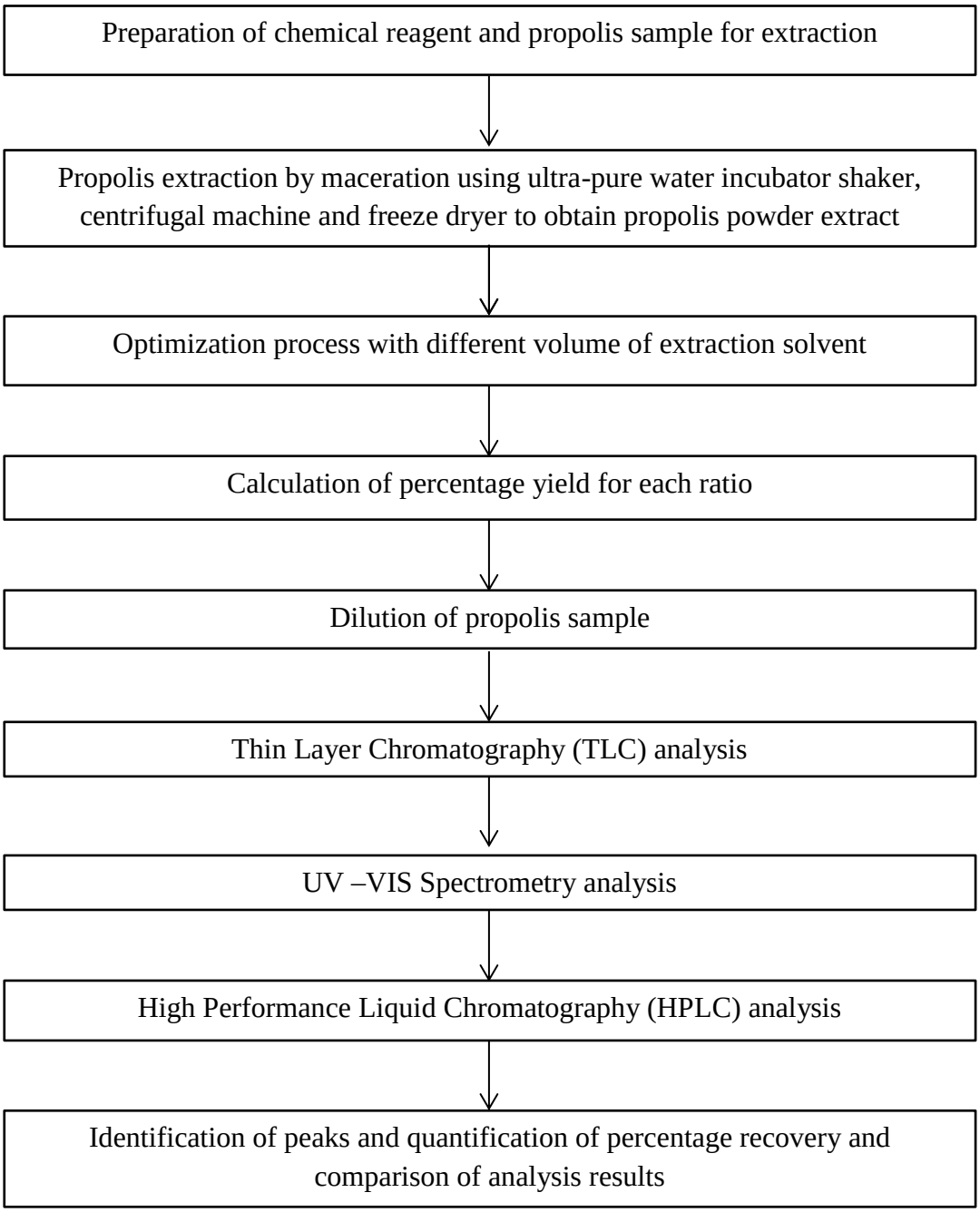

Figure 3.8 Flow Chart of Study

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Extraction Process

In the extraction process, the propolis was first dissolved in the extraction solvent which is water in three different propolis to solvent ratio of A (1:5), B (1:10) and C (1:15) and left incubated for 7 days. After incubation, it was centrifuged and filtered. Significant changes can be observed in the propolis extract during the filtration process in terms of color changes.

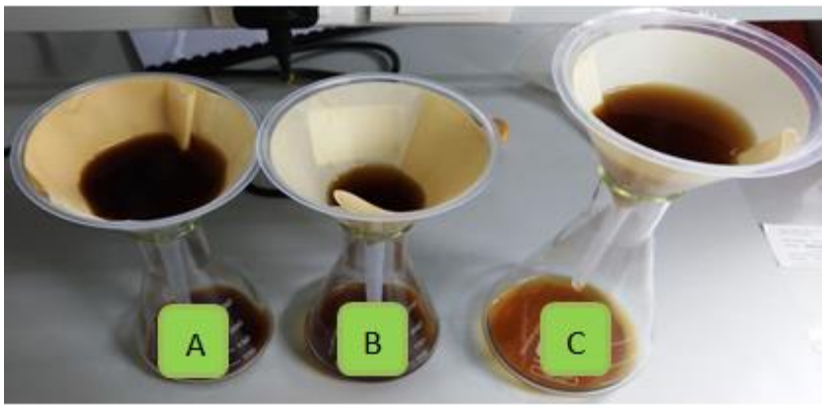

Figure 4.1 Filtration of Propolis Extract after Centrifugation Process

Propolis filtrate residue of ratio C shows a lighter color change followed by ratio B and lastly ratio A. This infers that the propolis content may be more concentrated in the less amount of water. Low solid to liquid ratio will promote higher concentration

gradient between sample and solvent to accelerate mass transfer of particles (Pinelo et al., 2006).

The propolis filtrate samples are then freeze dried under optimized condition to obtain propolis powder where the yield is calculated.

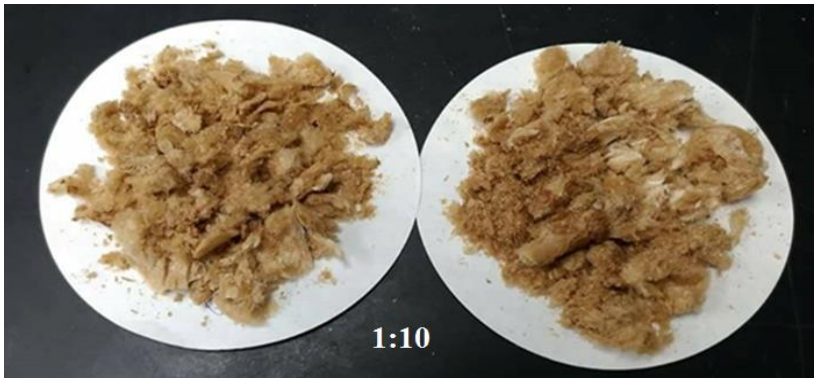

Figure 4.2 Propolis Powder Extract after Freeze Drying Process

Table 4.1 Percentage Yield (%) According to Propolis to Solvent Ratio When Using Water and Ethanol as Extraction Solvent

Extraction Solvent	Propolis to Solvent Ratio	Percentage Yield (%)
Water	1:5	5.95
	1:10	9.05
	1:15	7.16
Ethanol	1:5	5.79
	1:10	10.67
	1:15	4.20

Percentage yield of propolis presented in Table 4.1 was used to compare between the extraction by ethanol and water under the same extraction. From Table 4.1, this study shows that when water was used to extract crude propolis, propolis to solvent ratio, 1:10, gives the highest percentage yield of 9.05% followed by the ratio 1:15 which gives 7.16% and ratio 1:5 which gives 5.95%. Statistical tool ANOVA used in a previous research proof that volume of extraction solvent used does not significantly influence the extraction yield of propolis. (Trusheva and Bankova, 2007). This finding

also establishes that using propolis to solvent ratios larger than 1:10 (w/v) will only cause solvent and energy losses. In fact, the results from this study also follow the same outcome as the previous study and show the reproducibility of the discovery.

Furthermore, Ethanolic Extracted Propolis (EEP) has a higher percentage yield of 10.67% compared Water Extracted Propolis (WEP) which is 9.05% when propolis to solvent ratio is 1:10. Ethanol is acknowledged as an organic solvent capable of dissolving most of the content of propolis. However, the propolis content and extraction yield may also differ due to differences in the origin of propolis, bees types, food resources and harvest time (Sabir, 2005; Kalogeropoulos et al., 2009; Tylkowski et al., 2010).

Water gives a lower extraction yield compare to ethanol because it is a polar solvent whereas the ethanol, propyl alcohol and acetone are included as less polar solvents (Mageretha et al., 2012). Water dissolves a small part of propolis components, approximately 10% of its weight, whereas 70% ethanol may dissolve 50-70% of it, depending on the wax amount (Rebiai et al., 2014).

However, from the results, WEP has a higher percentage yield of 5.95% and 7.16% respectively than EEP with a percentage yield of 5.79% and 4.20% when propolis to solvent ratio is 1:5 and 1:10 respectively. Assumption can be made that the WEP have higher moisture content compared to EEP and this contribute to the higher weight of the powder extract. In fact, EEP had undergone sublimation of volatile ethanol by using rotary evaporator. This process was not performed by WEP as the heating solution in rotary evaporator is not suitable for WEP.

4.2 Evaluation Process

4.2.1 Analysis of Total Phenolic Content (TPC) by UV–Vis Spectrophotometry

For this analysis, 0.1 g of WEP was dissolved in 10 mL of 70% ethanol to form 1% ethanol. Then, the 1% WEP was analyzed by UV-Vis Spectrophotometer to identify and determine the Total Phenolic Content.

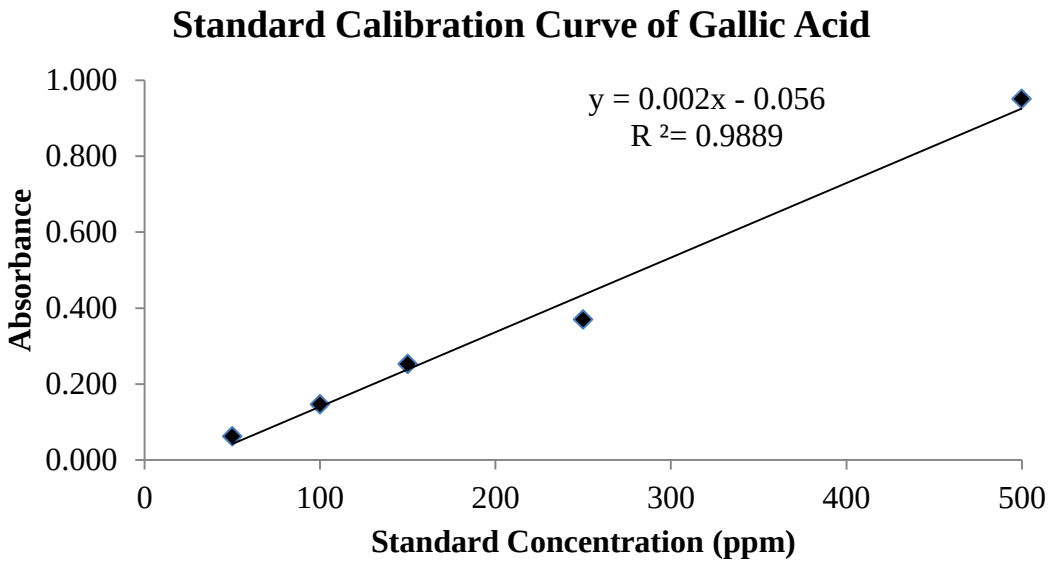

Figure 4.3 Standard Calibration Curve of Gallic Acid

From Figure 4.3, it is shown that the standard calibration curves of gallic acid at a wavelength of 725 nm showed a good correlation coefficient of 0.9888 and hence can be used for analysis of the sample to determine Total Phenolic Content (TPC) in gallic acid equivalent.

Table 4.2 Total Phenolic Content (TPC) of WEP according to Propolis to Solvent Ratio by Using Gallic Acid

Extraction Solvent	Propolis to Solvent Ratio	Gallic Acid Concentration C(µg/mL)	Gallic Acid Concentration C(mg/mL)	Total Phenolic Content (mg GAE/ g)
Water	1:5	61.22	0.06	6.12
	1:10	57.72	0.06	5.77
	1:15	53.34	0.05	5.33

From Table 4.2, the TPC concentration of WEP decreases with the increasing propolis to solvent ratio form 1:5, 1:10 and 1:15. However, in the extraction of date seeds, Al-Farsi and Lee (2018) confirmed phenolics extracted with water increased

from 2.5 g to 100 g with increasing solvent ratio to the 60:1. This is consistent with mass transfer principles in which the driving force during mass transfer is the concentration gradient between the solid and the liquid, which is greater when a higher solvent to solid ratio is used. Similar results about the effect of solvent to solid ratio on the extraction of phenolic compounds were reported for *Phyllanthus niruri* species by Wong, Tan and Ho (2013). These finding shows the contrast of the TPC values obtained in term of the mass transfer concept.

The TPC of WEP is shown to be higher compared to their total flavanones and flavanols content contents. The origin of the raw material may influence the composition and characteristics of the WEP extract (Cottica et al., 2015). The Total Phenolic Content (TPC) was the highest which was 6.12 mg Gallic Acid Equivalent (GAE)/g when the propolis to solvent ratio was 1:5 followed by 5.77 mg GAE/g and 5.33 mg GAE/g when the propolis to solvent ratio were 1:10 and 1:15 respectively.

In comparison with the TFC of propolis from a study done by Zakaria et al. (2016) on the species of *Heterotrigona itama (MHI)* and *Geniotrigona thoracica (MGT)*, which are 56.90 µg/mL and 29.10 µg/mL, the *Trigona Thoracica Species* propolis in this study is 61.20 µg/mL and considered as relatively high. In this study, the extracted propolis of the *stingless bees Trigona Thoracica Species* was obtained by using water as extraction solvent. Two species of propolis in the previous research were extracted by using methanol which could lead to an even higher TPC (Zakaria et al., 2016).This proves that the *Trigona Thoracica Species* propolis has comparatively higher TPC compared to *(MHI)* and *(MGT)* propolis.

Table 4.3 Total Phenolic Content (TPC) of WEP, EEP and OEP According to Propolis to Solvent Ratio by Using Gallic Acid

Extraction Solvent	Propolis to Solvent Ratio	Total Phenolic Content (mg GAE/ g)
Water	1:5	6.12
	1:10	5.77
	1:15	5.33
Ethanol	1:5	28.09
	1:10	26.07
	1:15	25.73
Olive Oil	1:1	16.07
	1:5	14.77
	1:10	12.50

From the Table 4.3, it was shown that WEP have a lower TPC compared to EEP by approximately fivefold. In the ratio of 1:5 which WEP has the highest TFC of 6.12 mg GAE/g, the TFC for EEP is fivefold higher which is 28.09 mg GAE/g. Lower polarity of ethanol to dissolve and extract less polar phenolic components (Oldoni et al., 2015). WEP have a lower TPC compared to OEP as whole. OEP has a higher TPC and it may be due to the impurities obtained during extraction or dilution process. Kubiliene et al. (2015) have proven that water and oil extraction of propolis is similar or slightly different nevertheless lower than ethanol extraction of propolis.

4.2.2 Analysis of Flavonoids Content: Total Flavones and Flavonols by UV-Vis Spectrophotometry

For this analysis, 0.1g of WEP was dissolved in 10mL of 70% ethanol to form 1% WEP. Then, the 1% WEP was analyzed by UV-Vis Spectrophotometer to identify and determine the Total Total Flavones and Flavonols and Total Phenolic Content.

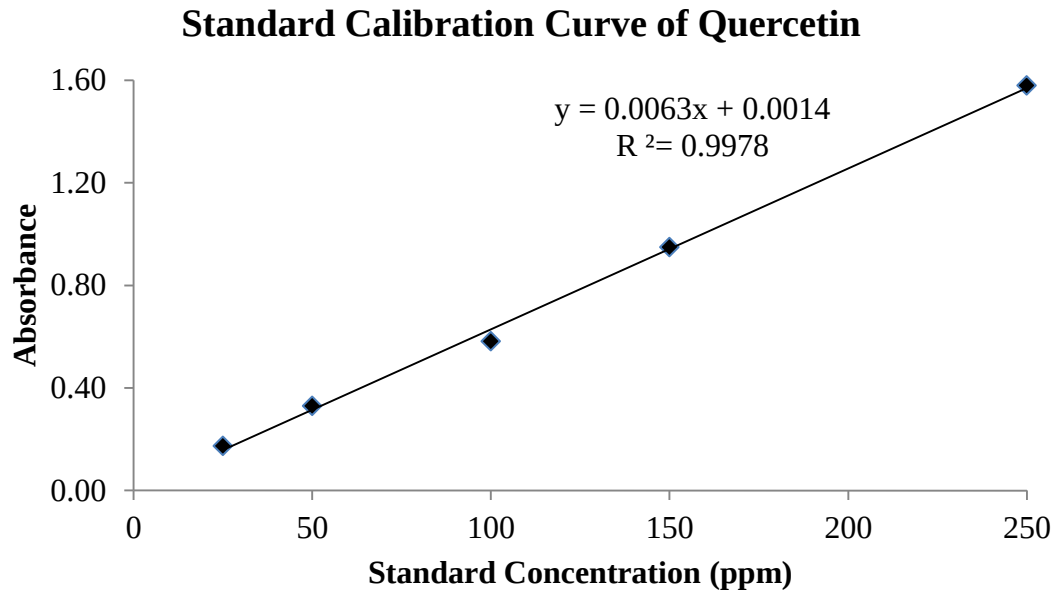

Figure 4.4 Standard Calibration Curve of Quercetin

From Figure 4.4, it is shown that the standard calibration curves of quercetin at a wavelength of 415 nm showed a strong correlation coefficient of 0.9978 and hence can be used for analysis of the sample to determine total flavones and flavonols content in quercetin equivalent.

Table 4.4 Total Flavonoids Content (TFC) of WEP According to Propolis to Solvent Ratio by Using Quercetin

Extraction Solvent	Propolis to Solvent Ratio	Quercetin Concentration C(µg/mL)	Quercetin Concentration C(mg/mL)	Total Flavones and Flavonols (mg QE/g)
Water	1:5	33.34	0.03	3.33
	1:10	28.12	0.03	2.81
	1:15	23.56	0.02	2.36

From Table 4.4, it is shown that the highest of total flavones and flavonols content was 3.33 mg Quercetin Equivalent (QE)/g when the propolis to solvent ratio

was 1:5 followed by 2.81 mg QE/g and 2.36 mg QE/g when the propolis to solvent ratio were 1:10 and 1:15 respectively. Water may not be an efficient extract solvent as it is a polar compound which does not favors the extraction of less polar compounds such as phenolic compounds, affecting the composition and quantity of extracted (Oldoni et al., 2015).

In comparison with the total flavones and flavonols content of propolis from a study done by Zakaria et al. (2016) on the species of *Heterotrigona itama (MHI) and Geniotrigona thoracica (MGT)*, which are 163.9 μg/mL and 61.5 μg/mL respectively, the *Trigona Thoracica Species* propolis in this study is 33.3 μg/mL and considered as low total flavanones and flavanols content. Nonetheless, in this study, the extracted propolis of the *stingless bees Trigona Thoracica Species* is obtained by using water as extraction solvent. The two species of propolis in the previous research were extracted by using methanol which could lead to higher total flavones and flavonols content (Zakaria et al., 2016).

Table 4.5 Total Flavones and Flavonols Content of WEP, EEP and OEP According to Propolis to Solvent Ratio by Using Quercetin

Extraction Solvent	Propolis to Solvent Ratio	Total Flavones and Flavonols Content (mg QE/g)
Water	1:5	3.33
	1:10	2.81
	1:15	2.36
Ethanol	1:5	7.85
	1:10	7.25
	1:15	7.56
Olive Oil	1:1	6.48
	1:5	2.50
	1:10	1.83

From Table 4.5, it was shown that WEP have a lower total flavones and flavonols compared to ethanol by estimated for two to three fold. In the ratio of 1:5 which WEP has the highest total flavones and flavonols content of 3.336 mg QE/g. The

total flavones and flavonols content for EEP is doubled which is 7.8487 mg QE/g. This may be due to the lower polarity of ethanol to dissolve and extract less polar flavonoids components (Oldoni et al., 2015). However, WEP have a slight higher total total flavones and flavonols content compared to OEP (Olive oil Propolis Extract) as whole. In the ratio 1:10, OEP has a high total flavones and flavonols content and it may be due to the impurities obtained during extraction process. Kubiliene et al. (2015) have proven that water and oil extraction of propolis is similar or slightly different nevertheless lower than ethanol extraction of propolis. EEP results were obtained from project group member, H'ng Sin Hooi and OEP results were obtained from project group member Shankri Jayaraman for comparison purposes.

4.2.3 Analysis of Components by Thin Layer Chromatography (TLC)

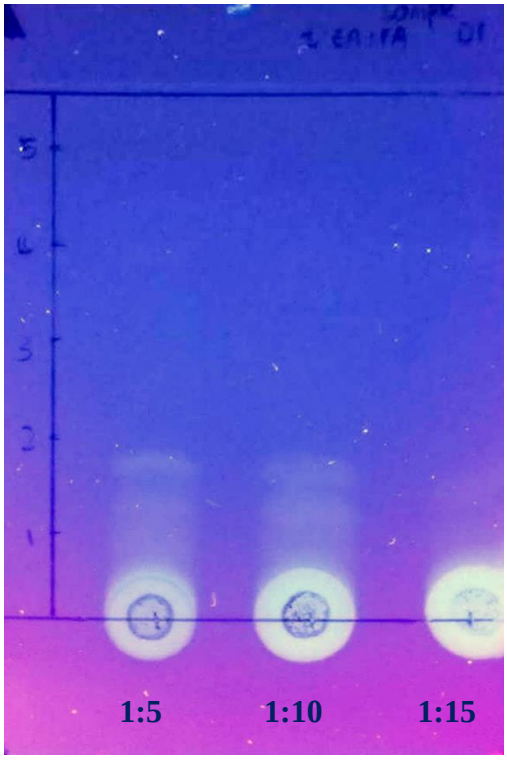

Figure 4.5 Silica Gel Plate for WEP Sample

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Table 4.6 Retention factor (R_f) Value of WEP According to Propolis to Solvent Ratio

Extraction Solvent	Propolis to Solvent Ratio	R_f Values
Water	1:5	0.22
		0.31
	1:10	0.22
		0.31
	1:15	Not Clear
		Not Clear

Table 4.6 tabulated the R_f value of WEP according to their ratio. This value was obtained by dividing the distance travelled by the spots over the solvent front. From Figure 4.5 and Table 4.6, it was shown that the silica gel plate showed several bands of WEP sample under the UV light at the wavelength 365 nm at a retention time of 0.22 and 0.31. The first sample on the most right hand side of Figure 4.5 shows the band of the propolis to solvent ratio of 1:5, two clear separations can be observed close to the sample spots as WEP is more polar and will elute less in less polar mobile phase ratio , 8: 2: 0.1 of toluene: ethyl acetate: formic acid. However, the two separations became less clear followed by the propolis to solvent ratio of 1:10 and 1:15 respectively. On the 1:10 ratio, the separation bands are still visible. However, on the 1:15 ratio, separation bands are no longer clear and cannot be defined.

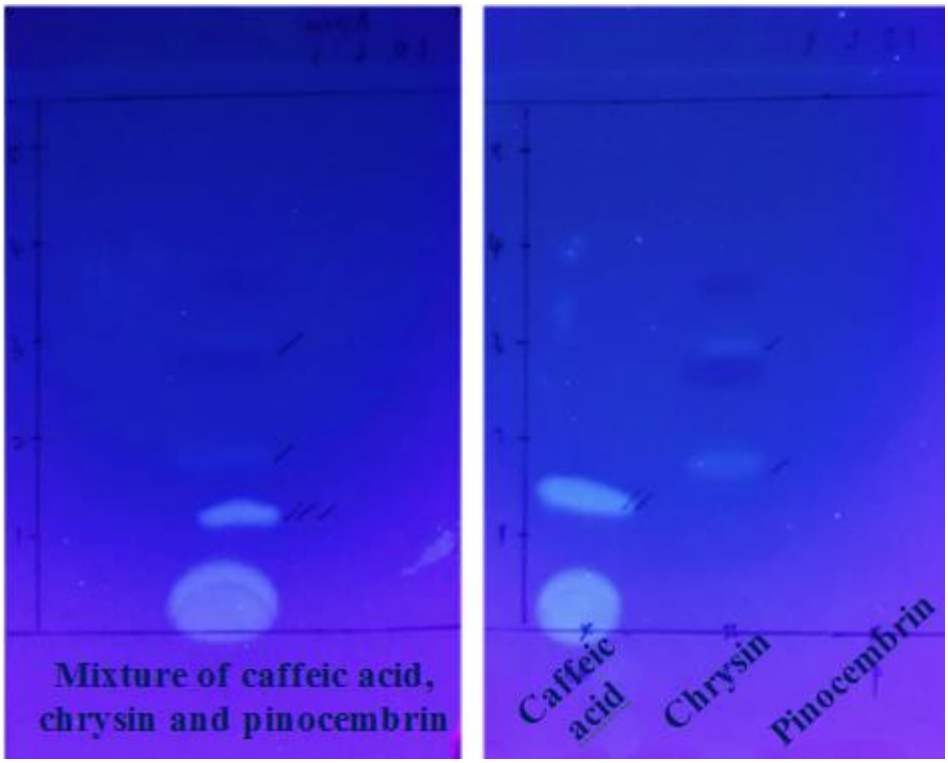

Figure 4.6 Silica Gel Plate for Caffeic Acid, Chrysin and Pinocembrin Standards

Table 4.7 Comparison of Retention factor (R_f) Value of Caffeic Acid, Chrysin and Pinocembrin Standards

Standards	R_f Value
Caffeic Acid	0.22
Chrysin	0.33
Pinocembrin	Not Visible

From Table 4.6, R_f value of the separation band of WEP at 0.22 was similar to that of standard caffeic acid components as in Figure 4.6 and Table 4.7. Hence, it can be confirmed that the one of the separation band indicates the caffeic acid components. Pinocembrin standard are not detected in this wavelength as it is normally detected under wavelength of 292 nm. (Alimkhodzhaeva et al., 1994)

Table 4.8 Comparison of Retention factor (R_f) Value of WEP and EEP According to Propolis to Solvent Ratio

Ratio	Water (R_f Values)	Ratio	Olive Oil (R_f Values)
1:5	0.22 0.31	1:1	0.90
1:10	0.22 0.31	1:5	0.87
1:15	Not Visible Not Visible	1:10	0.85

From Table 4.8, it was shown that EEP have four separation bands in each propolis to solvent ratio. Hence, four components can be detected in the EEP. Chrysin components detected as it eluted near the sample spots at R_f value of 0.33. EEP is less polar compared to WEP and hence components can elute later in less polar mobile phase.

Table 4.9 Comparison of Retention factor (R_f) Value of WEP and OEP According to Propolis to Solvent Ratio

Ratio	Water (R_f Values)	Ethanol (R_f Values)
1:5	0.22, 0.31	0.11, 0.15, 0.29, 0.33
1:10	0.22, 0.31	0.11, 0.15, 0.29, 0.33
1:15	Not Visible	0.11, 0.15, 0.29, 0.33

From Table 4.9, in comparison with WEP, OEP elute later compared to WEP and EEP at which all the R_f values are near 1 which is 0.9, 0.87 and 0.85. This is due to the non-polarity of the OEP. Hence, the standards components were not detected in the OEP. Less polar compound moves higher up the plate resulting in a higher R_f value which is more than 0.8 (Bele and Khale, 2011) which shows that there contain reduce amount of polar phenolic content.

4.2.4 Analysis of Components by High Performance Layer Chromatography (HPLC)

In this study, caffiec acid at concentration of 200 ppm elutes at 3.82 minutes at which the highest peak was identified when using the mobile phase phosphate buffer saline: methanol (60:40) and the Zorbax Esclipse Plus C-18 column at detection wavelength of 325 nm as shown in Figure 4.7.

A set of four standard solutions with different concentrations were prepared to carry out a calibration curve of caffeic acid standard. As depicted in Figure 4.8, the calibration curve was made to show the correlation between the response of the method and the different concentration of the standard analyte. Peak area of caffeic acid was plotted against concentration of caffeic acid to form the calibration graph. The correlation coefficient of the caffeic acid standard calibration graph is 0.9539, which indicates an acceptable correlation between the concentration and peak area of caffeic acid.

The standard calibration graph of caffeic acid was able to act as a reference for identifying the concentration of caffiec acid in the WEP samples. Peak area of each samples can be compared with the standard calibration curve to calculate the concentration of caffeic acid when the peak is eluted at approximately 3.82 minutes.

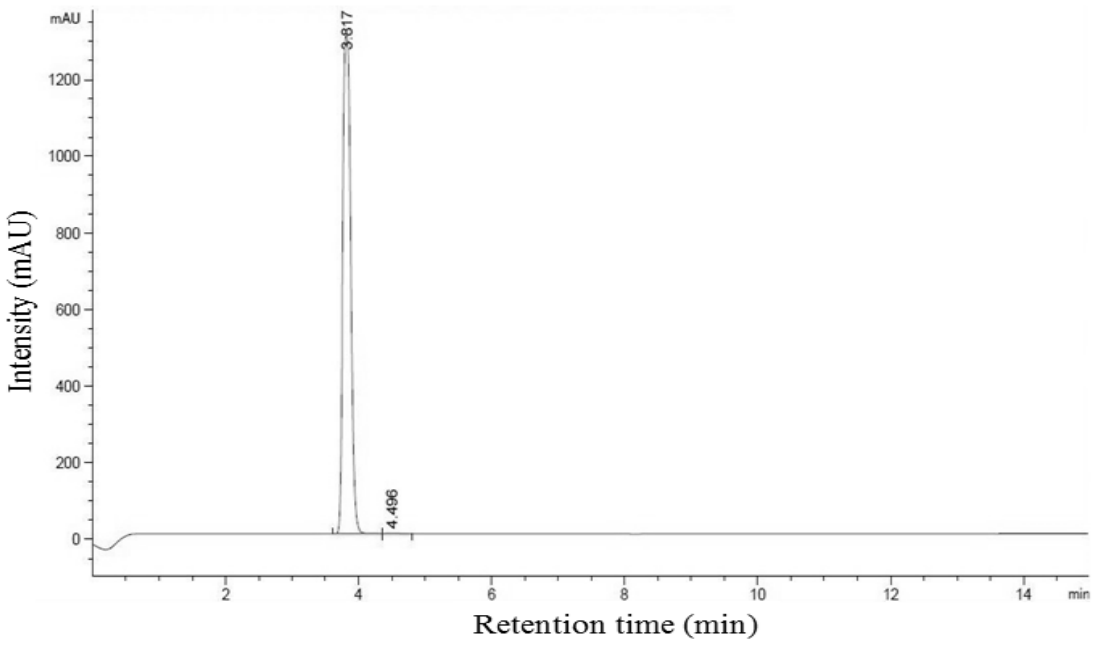

Figure 4.7 Chromatogram of Caffeic Acid (200 ppm)

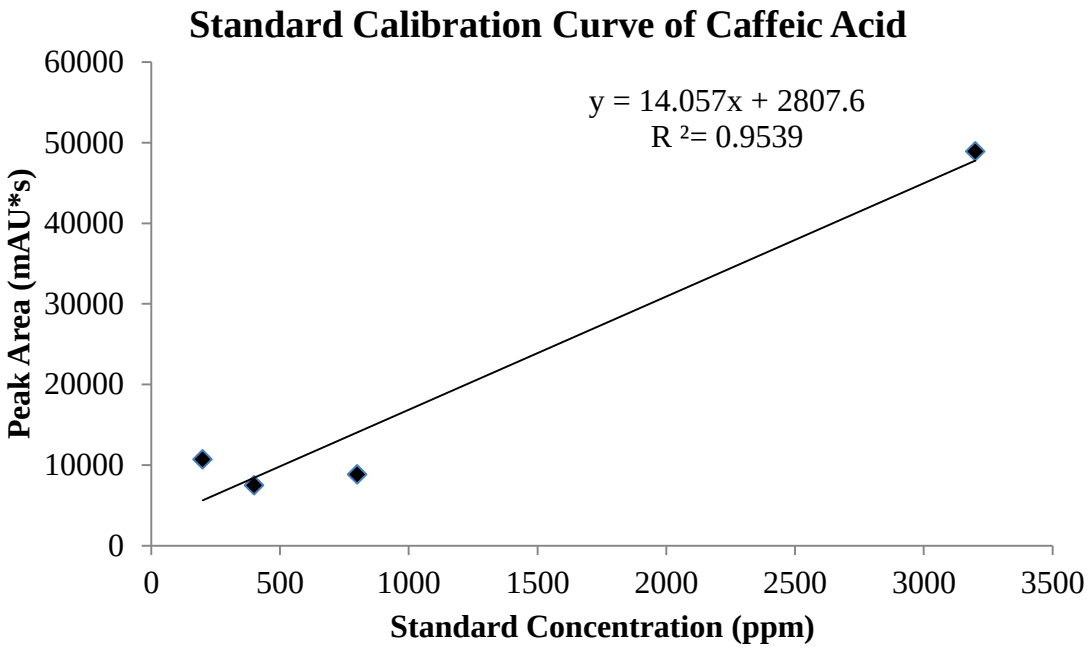

Figure 4.8 Standard Calibration Curve of Caffeic Acid

As shown in Figure 4.9, 4.10, 4.11, 4.12, 4.13 and 4.14, there was no caffeic acid peak detected at approximately 3.82 minutes in the sample, indicating the absence of the caffeic acid for all propolis sample for both ratio of 1% WEP and 5% WEP. Furthermore, the identification and confirmation of caffeic acid could be done if the HPLC system is coupled with mass spectrometry (MS) as a detector.

Mass spectrometry (MS) is applied for analyzing combinatorial libraries (Williams and Burinsky, 2001) sequence biomolecules (Kondrat, 2001) and helps explore single cells (Gohlke and McLafferty, 1993). Structure elucidation of unknown substances; environmental and forensic analytes; quality control of drugs, foods, and polymers all rely to a vastly on mass spectrometry (Blakley and Vestal, 1983; Hubschmann, 2015).There is a possibility of the retention time of the component to be shifted to different retention time.

Variation of the retention time causes the components to elute either faster or slower than the standard retention time of the method. Several other possible reasons are contamination buildup of the column, selective evaporation of mobile-phase components and column overloaded with sample (Sailaja et al., 2014). The column used in this research was used repeatedly by several groups of student without thorough flushing and cleaning process.

In chromatography, baseline is the part of chromatogram that indicates any time period during which only mobile phase passing through detector (Ravisankar et al., 2012). Noises of the chromatographic system are the only signal that is present in the baseline (Ravisankar et al., 2012). Baseline drift is which the baseline rises away from its base due to noises. Figure 4.9, 4.10, 4.11, 4.12, 4.13 and 4.14 showed that there is a minor baseline drifting to a positive direction. There is a possibility that there is contamination buildup in eluent causing changes in gradient elution (Sailaja et al., 2014). Column temperature fluctuation and non-homogenous mobile phase may also lead to baseline drifting (Thorat et al., 2013).

In a chromatogram, a peak shape is an important parameter as it is a reflection of the process occurring in the chromatographic system and indicates the chromatographic problems (Desai and Raskapur, 2012). Figure 4.9, 4.10, 4.11, 4.12, 4.13 and 4.14 also showed the highest peaks which are also split peaks at the retention time ranges from 2 to 4 minutes. The split peaks obtained may be due to several explanations which mainly are co-elution with unknown interference; contamination on column or guard inlet and sample solvent is too strong (Vinodrao Burghate et al., 2014). There may be many components that have similar retention time, causing the peaks to overlap with each other.

Figure 4.10, 4.12, 4.13 and 4.14 showed that there are many other peaks in addition to the highest peaks. The peaks may represent other component that exists in the WEP samples. For example, in Figure 4.14, the chromatogram showed numbers of unknown peaks in this sample (5% WEP) compared to other chromatograms. The peaks showed may also indicate impurities or contaminants in the WEP samples.

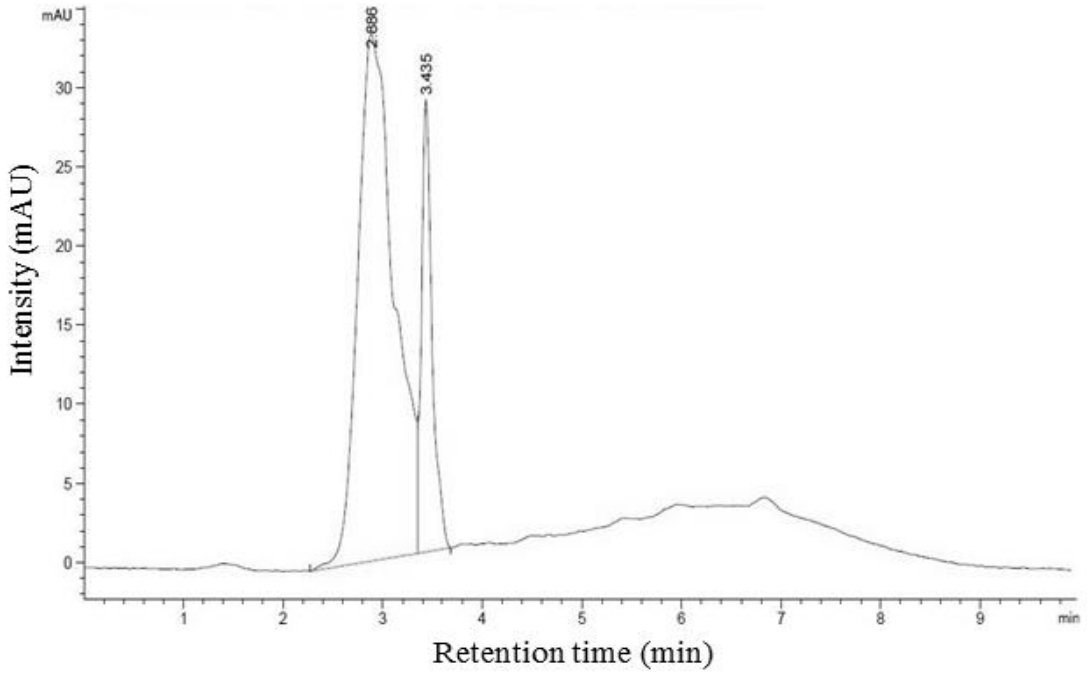

Figure 4.9 Chromatogram of 1% WEP with Ratio of 1:5

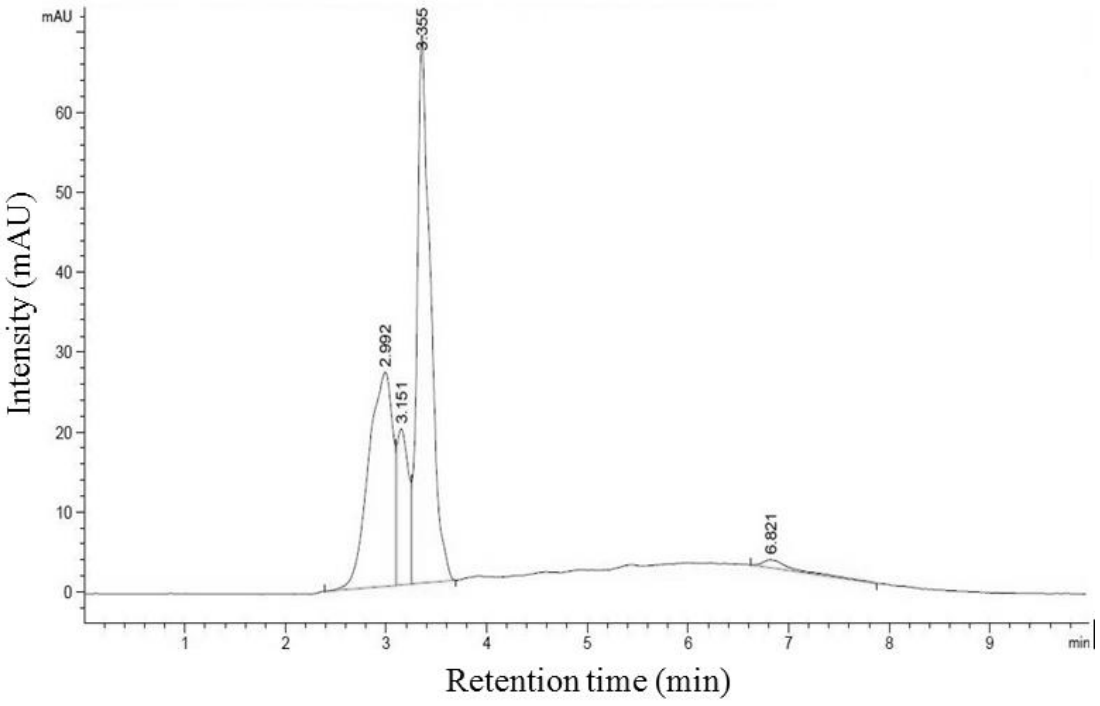

Figure 4.10 Chromatogram of 1% WEP with Ratio of 1:10

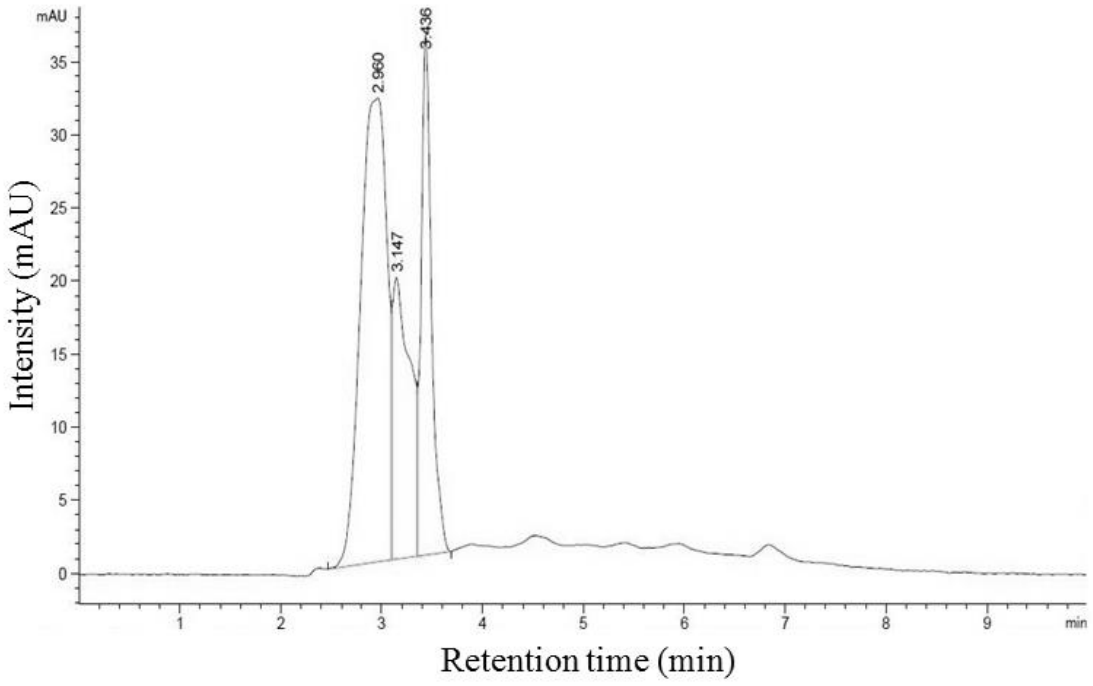

Figure 4.11 Chromatogram of 1% WEP with Ratio of 1:15

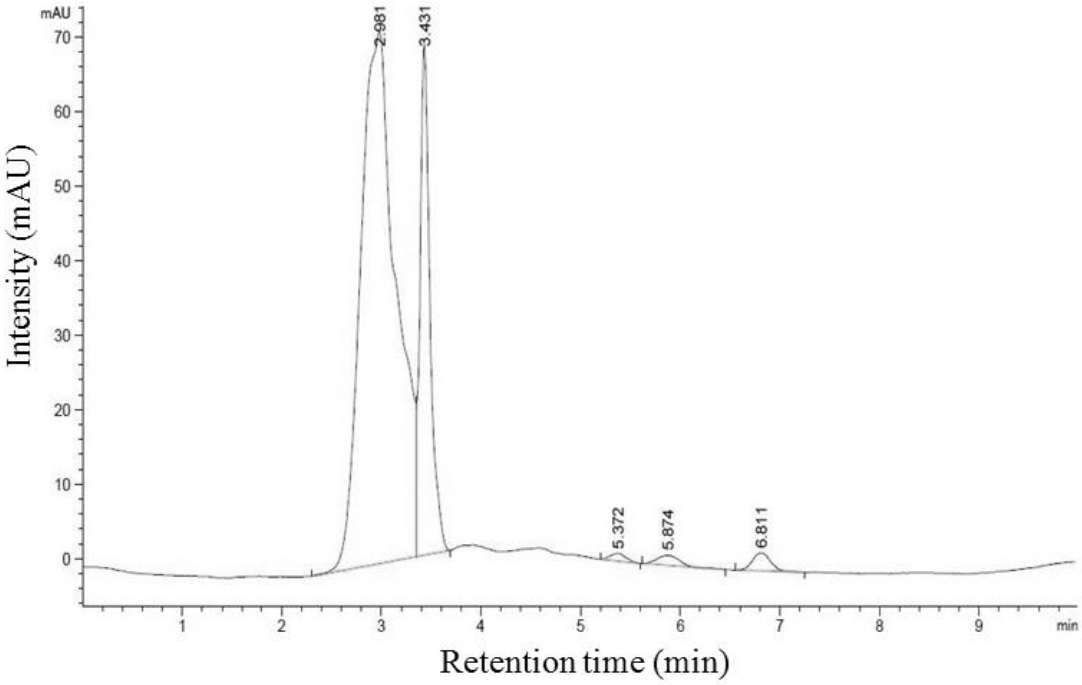

Figure 4.12 Chromatogram of 5% WEP with Ratio of 1:5

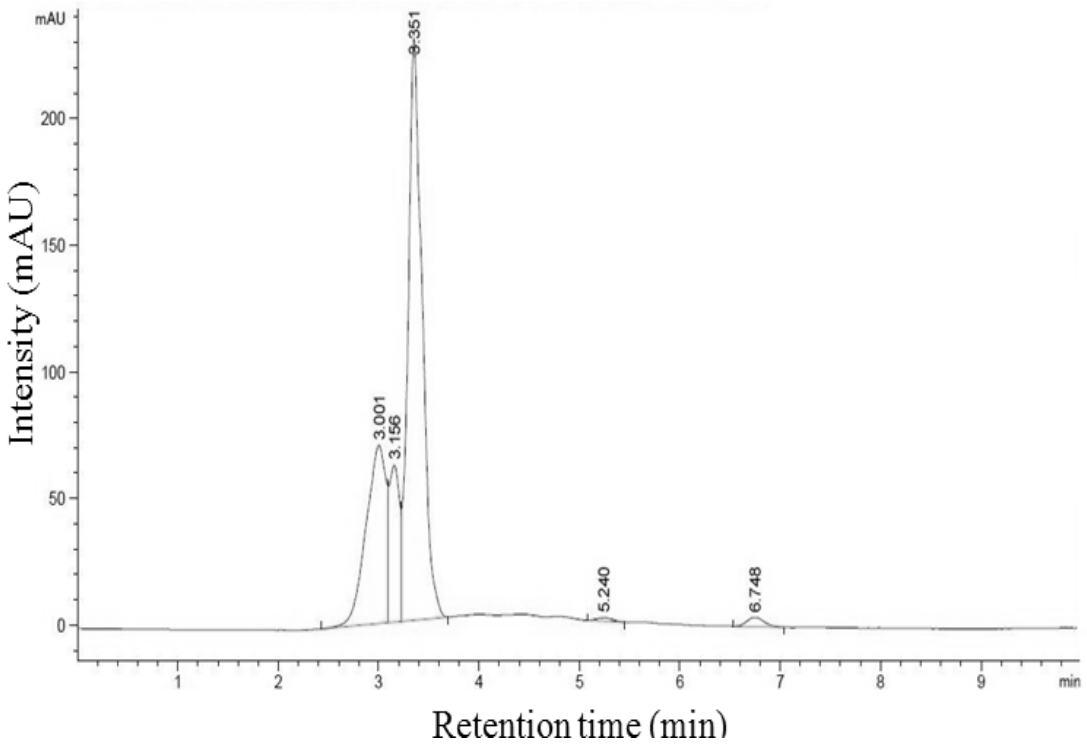

Figure 4.13 Chromatogram of 5% WEP with Ratio of 1:10

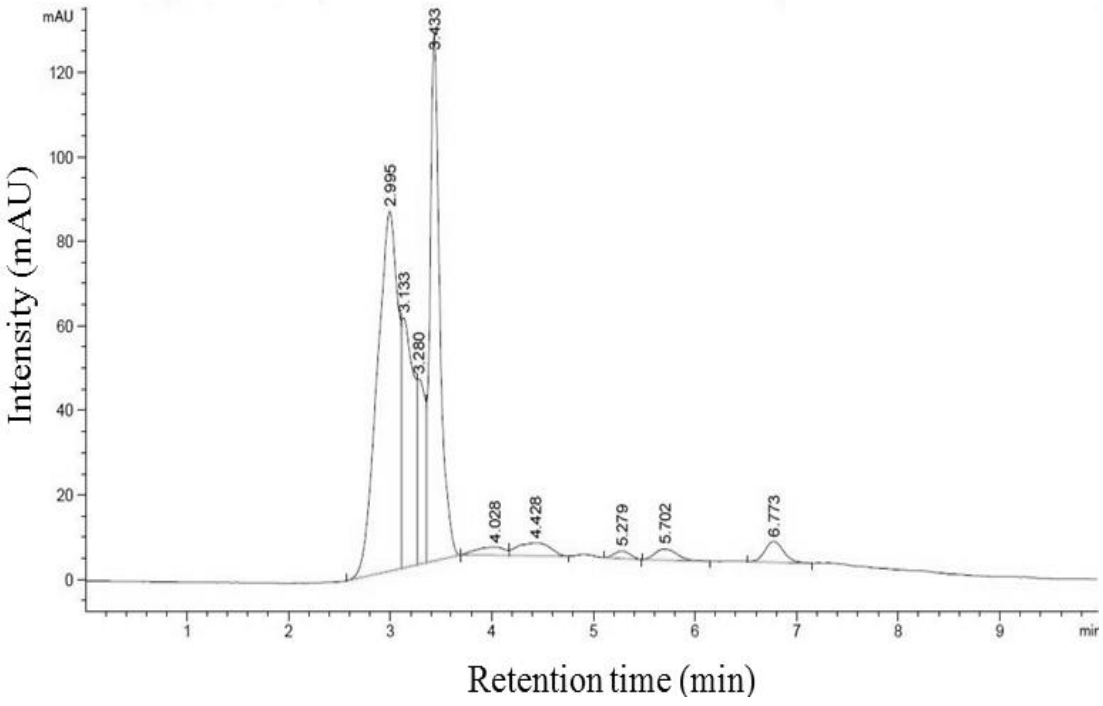

Figure 4.14 Chromatogram of 5% WEP with Ratio of 1:15

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

In the present study, modified maceration method by using water was successfully applied to extract propolis from crude propolis. Parameters affecting the percentage yield of Water Extracted Propolis (WEP) were studied by using several different ratios to optimize the extraction process. However, in terms of total flavanones and flavanols contents and total phenolics contents, the results obtained showed a decreasing concentration of both contents with an increasing sample to solvent ratio, which were contrast to previous studies done by researchers where the contents should increase with the ratio. This proves that the results should be further studied. In HPLC analysis of the WEP, no caffeic acid peak was identified at the targeted retention time and several complications such as split peaks and baseline drift were observed. Hence, the component was not detected in the WEP sample and showed that a further optimization steps and troubleshooting are needed.

Furthermore, WEP showed the least amount of concentration of total flavanones and flavanols contents and total phenolics contents in comparison with EEP and OEP. TLC analysis was able to detect caffiec acid and chrysin in EEP and WEP respectively whereas no components were detected in OEP. This may be due to the decreasing polarity in the WEP followed by EEP and OEP. Caffeic acid peak were no detected in all three extract at the targeted retention time.

It can be concluded that further studies with analytical method validation should be carried out to identify and overcome the complications that have occurred in this research.

5.2 Recommendations

In this study, several actions are recommended in order to obtain more accurate results. Firstly, optimization process should be applied in terms of extraction process by changing factors such as temperature and solvent type. HPLC analysis can also be optimized by selection of mobile phase, column types, column temperature and analyte used.

Besides, for each analytical method such as UV Vis spectrometry, TLC and HPLC, analytical method validation should be carried out to ensure that the method used is validated to enable reproducibility and reliability of the results obtained.

Nonetheless, the optimized process was only carried out marginally on the extraction process by altering one factor. Optimization process and method validation were not carried out on the analytical evaluation method due to the lack of resources such as equipment availability; lack of chemical resources and ultimately due to the lack of time period to complete the research. Therefore, it is suggested that further studies should be implemented with sufficient amount of time.

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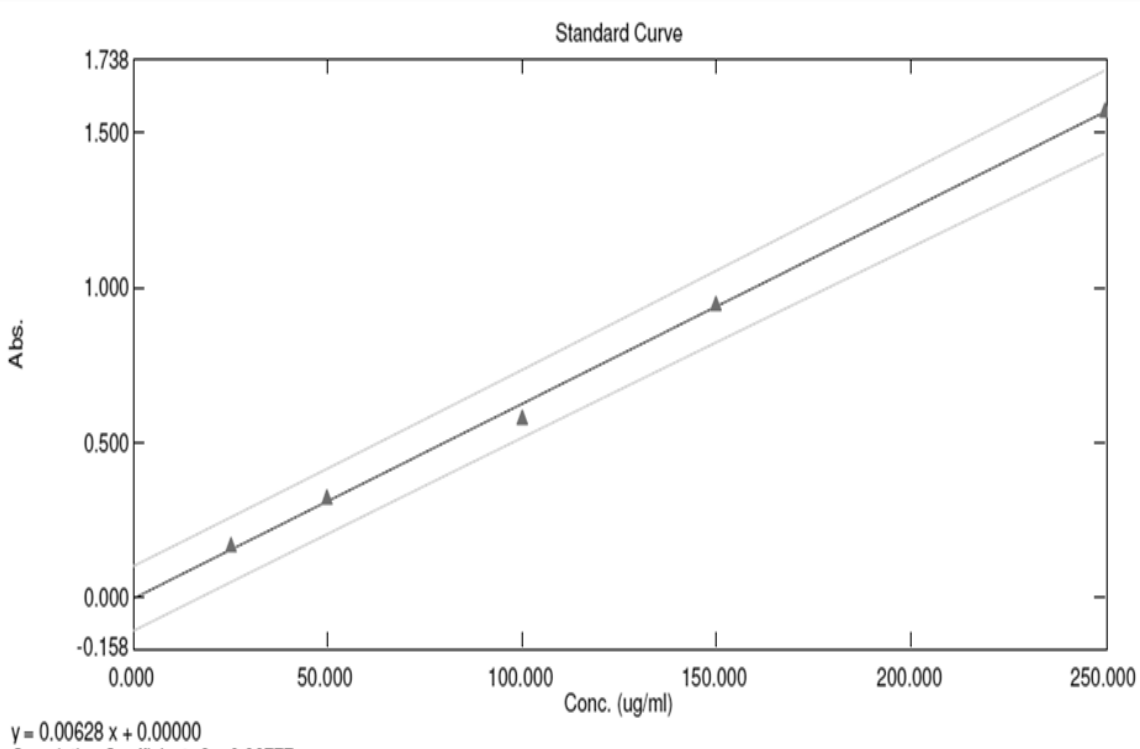
APPENDIX

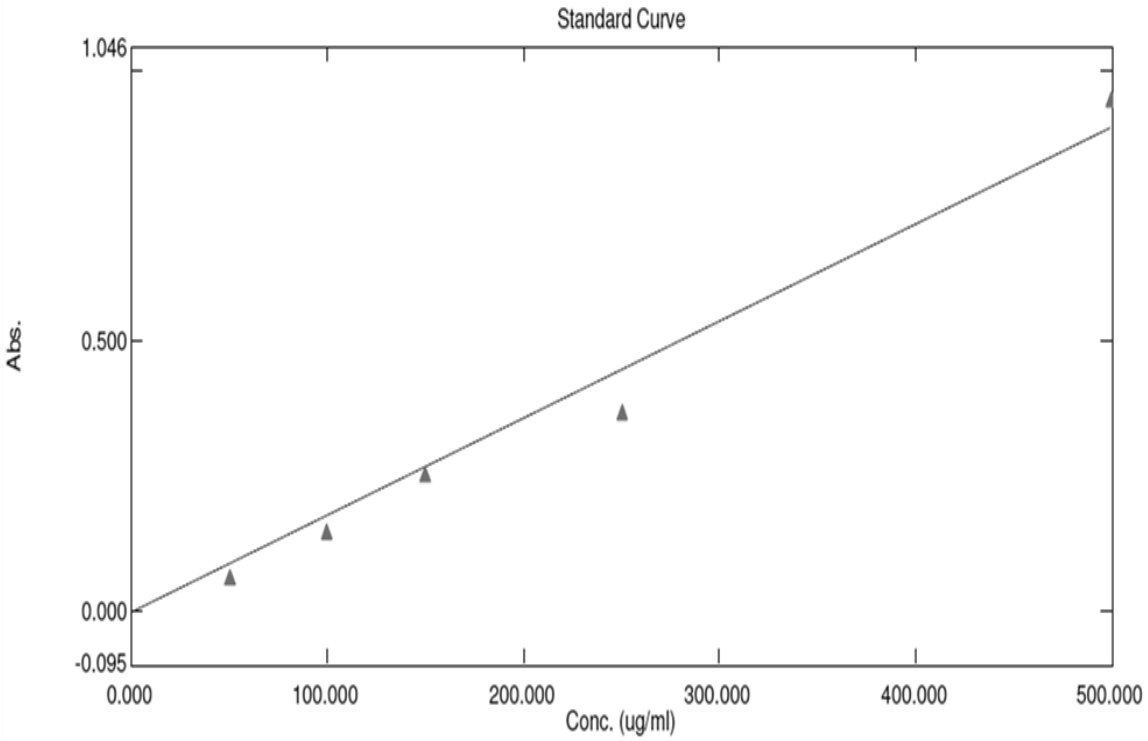
APPENDIX A

1(a) Standard calibration Curve of Quercetin generated by UV-Vis Spectrophotometry system



y = 0.00628 x + 0.00000
Correlation Coefficient r2 = 0.99777
Chi Square = 0.00619
Standard Error of Estimate = 0.02656
Residual Standard Deviation = 0.02656
Multiple Correlation Coefficient r2 = 0.99777

1(b) Standard Calibration Curve of Gallic Acid Generated by UV-Vis Spectrophotometry system



y = 0.00179 x + 0.00000
Correlation Coefficient r2 = 0.98879
Chi Square = 0.03261
Standard Error of Estimate = 0.05304
Residual Standard Deviation = 0.04788
Multiple Correlation Coefficient r2 = 0.98879