DEVELOPMENT OF PROPOLIS POWDER FOR ENCAPSULATION VIA FREEZE DRYING METHOD: EXTRACTION OF PROPOLIS, COMPONENTS IDENTIFICATION AND AN ANTIOXIDANT ACTIVITY STUDY

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PARVATHI NANDINI A/P BALAH KRISHNAN

Thesis submitted in fulfillment of the requirements

for the award of the degree of

Bachelor of Manufacturing Engineering Technology (Pharmaceutical)

Faculty of Engineering Technology

UNIVERSITI MALAYSIA PAHANG

January 2018

ACKNOWLEDGEMENT

First and foremost, I am very much obliged and indebted to the Almighty who has chosen me as His Divine instrument for the task of writing down this thesis. Thanks too for easing my pathway towards completion of this research and thesis in success.

I would like to acknowledge and express my deepest sense of appreciation to my research supervisor, Dr. Wan Nurul Huda Binti Wan Zainal, for her unwavering guidance, unfailing encouragement and incessant assistance throughout this research. Without her boost and dedicated involvement in every step throughout the process, this research and thesis would have never been accomplished. My project is nothing but an inspiration from Dr Wan Nurul Huda. Thank you Dr.

Most importantly, none of this could have happened without my family. To my grandparents, parents, uncles, aunties, sisters and brothers, it would be an understatement to say that I am grateful to you all very much. This dissertation stands as a testament to your unconditional love and encouragement.

Furthermore, sincere thanks to the staffs of Pharmaceutical Engineering Technology Department, Universiti Malaysia Pahang, especially Mr. Mohd Fazli Bin Ismail, Mr. Mohammad Hashsiim Bin Nawok Kani, Mr. Ikram Bin Safiee, Mr. Razhan Bin Hassan and Mr. Mohd Anuar Bin Hj. Ramli for providing detailed supervision and excellent technical assistance throughout this research.

Not forgetting my groupmates, Enidran Loganathan and Thavakumara Ganesan who have always been there throughout this research. Thank you for your guidance, advices and words of encouragement which spirits up my enthusiasm. Credits to my other classmates who willingly offered help when in need.

In a nut shell, I wish to express my appreciation to all those people, men, women, who contributed advices, precious time and endeavours towards me. I call upon God to shower His Divine grace on all for helping in making this work a success.

ABSTRAK

Propolis adalah produk yang diekstrak dari lilin lebah yang berfungsi sebagai penghalang dan pelindung dalam sarang lebah. Kajian ini dijalankan untuk mengatasi masalah propolis melekit. Objektif kumpulan adalah menghasilkan serbuk propolis menggunakan kaedah pengeringan beku. Objektif individu adalah mengekstrak propolis melalui kaedah pengekstrakan air dan etanol. Tujuan seterusnya adalah untuk menentukan komponen-komponen propolis dan menilai aktiviti antioksidannya. Propolis diekstrak menggunakan kaedah pengekstrakan air dan etanol. Penentuan komponen propolis seperti polifenol dan flavonoid dilakukan menggunakan spektrofotometer UV Vis pada panjang gelombang yang berlainan. Aktiviti antioksidan propolis juga ditentukan untuk menilai degradasi DPPH antara ekstrak etanol propolis (EEP) dan ekstrak air propolis (EAP). Kaedah pengekstrakan etanol menghasilkan lebih banyak hasil berbanding kaedah pengekstrakan air. Di samping itu, jumlah kandungan polifenol dan flavonoid dalam EEP terbukti lebih tinggi berbanding dengan EAP. Aktiviti antioksidan EEP diperhatikan lebih tinggi daripada EAP kerana degradasi kimia DPPH lebih cepat dalam EEP daripada EAP. Secara ringkas, kaedah pengekstrakan etanol menghasilkan EEP yang mengandungi komponen polifenol dan flavonoid serta aktiviti antioksidan yang lebih tinggi daripada kaedah pengekstrakan air.

ABSTRACT

Propolis is an extracted product from bees wax which functions as protective barrier in bee hives. This study was conducted to overcome the problem of stickiness and gummy characteristic of propolis. The group objective was to develop propolis powder using freeze drying method. The individual objectives of conducting this research were to extract propolis by water and ethanol extraction method. Next aim was to determine components of propolis and evaluate its antioxidant activity. Propolis was extracted by using water and ethanol extraction method. The determination of propolis compounds for instances, polyphenol and flavonoids was carried out using UV Vis Spectrophotometer at different wavelengths. An antioxidant activity of propolis was also determined by evaluating degradation of DPPH and comparing with control. The ethanol extraction method produced more yield compared to water extraction method. In addition, total polyphenol and flavonoid content in ethanol extract of propolis (EEP) was proven to be much higher in comparison to water extract propolis (WEP). The antioxidant activity of EEP was observed to be higher than WEP as the degradation of DPPH chemical was faster in EEP than WEP. In a nutshell, ethanol extraction method produced EEP with higher polyphenol, flavonoid content and higher antioxidant activity than water extraction method.

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LIST OF SYMBOLS

%	percentage
°	degree
°C	Degree centigrade or Celsius
µg	Microgram
μgmL^{-1}	microgram per millilitre
µm	micrometre
g	gram
M	Molar
mg	milligram
mL	MilliLitre
mm	millimetre
nm	nanometre

LIST OF ABBREVIATIONS

CE	Capillary Electrophoresis
DPPH	1,1-diphenyl-2-picrylhydracyl
EAP	Ekstrak Air Propolis
ECM	Extracellular Matrix
EEP	Ethanol Extract Propolis
EEP	Ekstrak Etanol Propolis
GAE	Gallic Acid Equivalents
GC-MS	Gas Chromatography–Mass Spectrometry
HD	Hydro-Distillation
HPLC	High Performance Liquid Chromatography
Mac	Maceration
MAE	Microwave Assisted Extraction
NaOH	Sodium Hydroxide
Sox	Soxhlet
TGF-β	Transforming Growth Factor-B
TLC	Thin Layer Chromatography
UE	Ultrasonic Extraction
USA	United States of America
USDA	United States Department of Agricultural
vs	Versus

CHAPTER 1

INTRODUCTION

1.1 Background Study

Propolis or bee glue is a natural resinous mixture produced by honey bees from substances collected from parts of plants, buds and exudates (Wagh, 2013). Bees use this glue to repair their hives by sticking any openings or cracks because of its waxy characteristic and mechanical properties. Propolis also acts as a shielding barrier against foreign intruders such as snakes and lizards. More than 300 constituents have been identified in different propolis samples (Bankova et al., 2000). Flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis samples (Silici and Kutluca, 2005). Propolis has various biological activities such as antibacterial (Grange and Davey, 1990), antiviral, anti-inflammatory and anti-cancer properties (Kumazawa, Hamasaka and Nakayama, 2003). Hence, it is believed that adding propolis in food and beverages can develop health and counteract diseases like inflammation and diabetes.

Propolis is a lipophilic in nature, hard and brittle material and it becomes soft, pliable, gummy and very sticky when heated. It possesses a characteristic and pleasant aromatic smell and varies in colour from yellow, green, red and dark brown depending on its source and age (Wagh, 2013).

Chemical compounds that are contained in propolis are polyphenols, terpenoids, steroids, and amino acids. The composition of propolis depends on the vegetation at the

site of collection (Kumazawa, Hamasaka and Nakayama, 2003). For example, propolis from Asia contains many kinds of flavonoids and phenolic acid esters meanwhile the main constituents in Brazilian propolis are terpenoids and prenylated derivatives of pcoumaric acids. Based on the examples, biological activities of propolis from different regions are also distinctive because of chemical configuration variances. At 25°C to 45°C, propolis is in the form of soft and sticky. On the other hand, in frozen condition, it's hard and brittle. Above 45°C, it will become more sticky and gummy. Propolis will become a liquid at 60°C to 70°C, but for some samples the melting point may be as high as 100°C (Wagh, 2013). Propolis cannot be used directly because of its complex structure. Hence, it has to be extracted by using water, alcohol, oil, ether or acetone. Many of the bactericidal components are soluble in water or alcohol which should remove the inert material and preserve the desired compounds (Wagh, 2013).

Propolis is a natural remedy which is beneficial in many fields. It is used in medicine for self-treatment of various diseases and in cosmetic production. Recently, propolis is used to formulate for cold and dermatological preparations useful in wound healing, treatment of burns, acne and neurodermatitis. Propolis is also used in mouthwashes and toothpastes to prevent caries (Sforcin and Bankova, 2011). Due to its antibacterial, antimicrobial, antiviral and antioxidant properties, propolis is widely used in human and veterinary medicine and pharmacology (Ehsani et al., 2013). Ethanolic extracts of propolis samples showed a high antibacterial activity against Gram-positive cocci (*Staphylococcus aureus*), but had a weak activity against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and yeast (*Candida albicans*) (Silici and Kutluca, 2005).

1.2 Problem Statement

While conducting this research, the main problem faced is stickiness of raw propolis. The stickiness and gluey condition are due to the presence of Balsam in propolis. Thus, stickiness problem of raw propolis causes the propolis difficult to be processed into capsule. Adding on, physical instability is found to cause a problem in the manufacturing of the propolis capsule (Swarbrick, 1996). Packing the propolis powder in capsules was better choice than tableting because capsules are stable and have an accurate dosing (Qureshi, 2007). Besides that, the process of encapsulation of

the propolis is also found to be hard as the propolis does not have good flowability properties. Due to this most capsules are not filled completely. This problem tends to affect the quality of the propolis capsules that is being manufactured.

1.3 Research Objectives

The group objective in conducting this project is to develop propolis powder for encapsulation via freeze drying method.

The individual objectives of conducting this research are as follow:

i. To extract propolis by water and ethanol extraction method.
ii. To determine components of propolis extract and evaluate its antioxidant activity.

1.4 Scope of Study

The propolis was collected from a hive of *Trigona Thoracica* bee species. In this study, propolis is extracted by using water and ethanol extraction method. Water extraction method was opted because this extraction method is time saving in producing propolis extract compared to ethanol extraction. By incubating the propolis in water and ethanol for one day and seven days respectively, liquid extractant of propolis was obtained. This research had focused on determining flavonoid and polyphenol components and antioxidant activity of propolis. These major propolis properties were determined by using UV Vis Spectrophotometer to find out whether EEP or WEP has higher amount of components. In addition, the antioxidant activity of propolis is tested with free radical scavenging testing utilizing 1,1-diphenyl-2-picrylhydracyl (DPPH) chemical.

CHAPTER 2

LITERATURE REVIEW

2.1 Bee species

The distributions of bees are closely correlated to many factors like disturbance in the forest and resource abundance. The variables can also be the density of big trees which have diameter measurement at breast height ranging from 30 to 40 cm, temperature and flowering intensity of trees and shrubs. Many more stingless bees, for example, *Trigona* species were identified where trees were larger and have constant ambient conditions but have diminished flowering intensities (Liow et al., 2001).

According to Liow et al., (2001) the most abundant species of bees in their research sites were *Trigona* (*Tetragonula*) *geissleri Friese*, and *Trigona* (*Tetragonula*) *melina* Gribodo with 412, 202 and 546 individuals collected, respectively, while seven species, *Trigona (Lepidotrigona*) *ventralis*, *Trigona (Geniotrigona) thoracica*, two species of *Nomia (Maculonomia)*, two species of *Lipotriches* and one species of *Halictidae* were collected only once each during the collection period.

Apidae, especially the genus *Trigona*, are abundant in rain forest (Appanah, Willemstein & Marshall 1986). Besides that, these species are said to be crucial pollinators (Sakai et al., 1999). Approximately, 13 out of 27 species of *Trigona* and *Apis* are found in Sumatra and Brunei, but were never encountered despite an extensive collection period that comprised months with a greater flowering intensity in the lowland forests (Medway, 1972). This situation is maybe due to some species are becoming so unique and rare that probabilities of encounters are very low.

Besides that, even baits were used to attract the bee species which are the honey bees (*Apis*) and stingless bees (*Trigona*).

2.2 Propolis

The historic evolutions incessantly utilised bee products as exquisite therapeutic assets in their medicinal preparations. The medicine history of the Chinese, Inca, Assyrian, Tibetan, Egyptian, and the Greco-Roman civilizations is very rich and possess records of formulations even comprising propolis as remedy or to prevent diseases (Berretta et al., 2017). On top of that, Egyptians also gained from the anti-putrefactive characteristics of propolis in order to preserve the deceased. Propolis was utilised as an antiseptic the Greek and Roman physicians (Sforcin, 2011).

The physical characteristics of propolis are, it is a dense brownish resin material and has reduced solubility in water compared to ethanol (Ehsani et al., 2013). It used as a sealant for unwanted open gaps in the hive and has plant substances, beeswax, and other bee secretions which are sticky (Silici and Kutluca, 2005) causing the propolis to be sticky.

Propolis is used in the bee hive to conceal the inner walls and to shield from the appearance of pests, such as snakes and lizards. Propolis protects bee hive against wind and rain. Furthermore, propolis prevents fungi and bacteria growth (Burdock, 1998). On top of that, propolis is not only an aid in colony level immunity but plays important role for direct defence against parasites and pathogens (Simone et al., 2009).

2.3 Propolis Products

The appliance of propolis in food industry is still not highlighted because propolis is made soluble in alcohol to obtain its extract hence, there will be ethanol residue in the final product too. Furthermore, propolis has strong taste and aroma which not many consumers prefer (Nori et al., 2011). For countless years, microencapsulation has been used in the pharmaceutical industry for controlled release and to improve stability of product and flavour masking. Moreover, microencapsulation protects products from environmental conditions and extends shelf-life (Alencar et al., 2008).

The applications of propolis include over-the-counter dermatological items where it has been claimed useful in wound healing, tissue regeneration, treatment of burns, neurodermatitis, leg ulcers and psoriasis. It has been marketed as a treatment for rheumatism and sprains. In dental medicine, propolis was used as anaesthetic which is five times stronger than cocaine. Propolis is also used in toothpaste and mouthwash preparations treating gingivitis, cheilitis and stomatitis. Meanwhile, in pharmaceutical and cosmetic field, propolis is used in face creams, ointments, lotions and solutions. It is marketed in tablets, powder and chewing gum (Wagh, 2013).

Propolis is safe to consume at low doses. Adverse effects are normal when patients consume doses over 15 g/day. The adverse effects most commonly experienced are allergic reactions, as well as skin or mucous membrane irritations. Caution should be used in the treatment of asthmatics and in patients with eczema and nettle-rash (Castaldo et al., 2002).

2.4 Characteristics of propolis

Propolis cannot be used directly because of its complex structure. Hence, it has to be extracted by using water, alcohol, oil, ether or acetone. Many of the bactericidal components are soluble in water or alcohol which should remove the inert material and preserve the desired compounds (Kumar et al., 2008). Propolis also possesses a characteristic and pleasant aromatic smell and varies in color from yellow green to red and to dark brown depending on its source and age (Bankova et al., 2000).

Propolis is a sticky material which is mixed by the honeybees to be utilized to protect their hive (Hausen et al., 1987). Biological activities of propolis from different regions are also distinctive because of chemical configuration variances (Wagh, 2013). On top of that, type of solvent used will influence the solubility of the components present in the raw propolis as the bactericidal components are soluble in water and alcohol (Cowan, 1999).

2.5 Benefits of Propolis to Humankind

Propolis ensures strong immunity among consumers which describes avoidance, control or elimination of parasitic infections (Cremer et al., 2007). An immune system

boosts the individual immune system especially the colony by cutting down the risk of microbe contact and disease spread among individuals (Meunier, 2015).

Other advantages of propolis utilised as a local anaesthetic, reducing spasms and healing gastric ulcers. In addition, consumption of isoflavonoids which is a component of propolis has been linked with lower chances of hormonally dependent cancers, relief from symptoms of postmenopausal problems, and a reduction in the risk of osteoporosis and cardiovascular disease (Wagh, 2013).

Another capability of propolis is as a detoxifying agent that could influence increased resistance to oxidative stress (Trusheva et al., 2007). In addition, propolis has been shown to lower blood pressure and cholesterol levels (Castaldo et al., 2002).

2.6 Extraction of Propolis

Due to propolis complex structure as mentioned by Kumar et al. (2008), raw propolis cannot be used directly. Therefore, propolis must undergo extraction with solvents. Extraction process is to eradicate the inert material and sustain the polyphenolic portions. The traditional method which is known as maceration is time consuming and requires from two to ten days to complete. Thanolic extraction of propolis is useful to acquire dewaxed propolis extracts which contains higher polyphenolic components (Pietta et al., 2002). Extraction by using 70% ethanol has become the most suitable extraction for the extracting biologically active components of propolis (Trusheva, Trunkova, and Bankova, 2007).

Besides traditional maceration and ethanol extraction, there are other well-established conventional extraction methods, such as hydro-distillation (HD) and organic solvent extraction like Soxhlet (Sox) (Reverchon et al., 2006). However, the drawbacks of conventional methods are that these processes costs high energy, more solvent use, high temperatures, impact to thermolabile substances, and solvent residue in the solute (Weinhold et al., 2008).

On the other hand, developments of modern extraction methods are useful for fast and efficient extraction of organic compounds from solid matrices. Examples of modern extractions are microwave assisted extraction (MAE) and ultrasonic extraction (UE). MAE consumes microwave energy to heat solvents with a sample to separate

some chemical components from the matrix into the solvent. These methods promise good quality of natural products extraction (Liu and Wang, 2004). MAE was very rapid but led to the extraction of a large amount of non-phenolic and non-flavonoid material (Popova et al., 2004).

UE gave the highest percentage of extracted phenolics. It has been demonstrated that the concentration of compound groups in propolis extracts correlates much better with the levels of antibacterial activity and is more informative than the concentration of individual components (Popova et al., 2004; Bonvehi, Coll and Jorda, 1994). The benefits of UE are thought to be due mainly to the mechanic effects of acoustic cavitation (Liu and Wang, 2004).

Both UE and MAE have demonstrated the potential to reduce extraction times significantly and increase extraction yields in studies related to medicinal plants. Although both these extractions are very efficient, the cost for these methods is much higher because they need expensive equipment and chemicals (Liu and Wang, 2004).

2.6.1 Ethanol Extraction of Propolis (EEP)

The solvent that is mostly used for propolis preparation is aqueous ethanol, ethyl ether, water, methanol and chloroform (Sun and Ho, 2005). The 70% of ethanol was found to extract most of the active components of propolis and give the highest yield (Sun et al., 2015).

Ethanol may dissolve 50–70% of propolis weight, depending on the wax amount (Trusheva et al., 2007). The appearance of EEP and WEP were gummy sticky whereas oil extract were gummy oily. The samples had changes in colour from yellowish-brown to dark brown. (Pujirahayu, Ritonga, Uslinawaty, 2014).

The organic solvent can dissolve the different chemical compounds according to a polarity of substance. Ethanol was used to extract propolis to generate the fatty acid and flavonoids (Khacha-ananda et al., 2013). Research of Paviani et al. (2013) showed that the results of the extraction of raw propolis with ethanol solvents showed high extraction yields. Alkane, alcohol and bee wax were found in hexane fraction of propolis (Prytzyk et al., 2003). Besides, the extraction time, light and temperature affected propolis extraction (Cunha et al., 2004). Although ethanol extraction is said to

produce higher yield as mentioned by Paviani et al., (2013), this method is time consuming and final product may have residue of alcohol.

2.6.2 Water Extraction of Propolis (WEP)

Water has also been used on many occasions to extract propolis. Water dissolves a small part of propolis constituents, about 10% of its weight (Trusheva et al., 2007). The water extract of propolis (WEP) has greater anti oxidative effects, greater inhibitory activity against some enzymes, and greater absorbency than ethanol extraction propolis (Matsui et al., 2004).

Water extract of propolis (WEP) and its main constituents, caffeoylquinic acid derivatives, can defend retinal ganglion cell line (RGC-5) from oxidative stress-induced cell death. In addition, these neuroprotective effects were paralleled by the same agents' antioxidant effects (Nakajima et al., 2009).

Besides that, water extract of propolis has ability to treat innumerable diseases such as cancer, cardiovascular diseases, and diabetes (Nagai et al., 2003). This is proven by an antioxidant activity which was measured by lipid peroxidation model system. The model system expressed very strong activity higher than that of ascorbic acid. In addition, the scavenging activity against superoxide anion radical of WEP was high (Nagai et al., 2003).

2.7 Chemical Composition of Propolis

Chemical compositions of propolis are qualitatively and quantitatively varying, relying on the season, bee species, vegetation, and collection area (Simone et al., 2009). Propolis which is being collected from temperate zones like West Asia, Europe and North America possesses a comparable chemical composition. The main constituent found was phenolic compounds such as flavonoids, cinnamic acids and derivatives. In Asia, due to difference in vegetation, the chemical composition of propolis is very different. Its colour differs depending on botanical source. The most regular colour of propolis is being dark brown, but red propolis has been observed in tropical countries

(Burdock, 1998; Simone et al., 2009). Figure 2.1 shows representative of chemical components in brown propolis (Berretta et al., 2017).

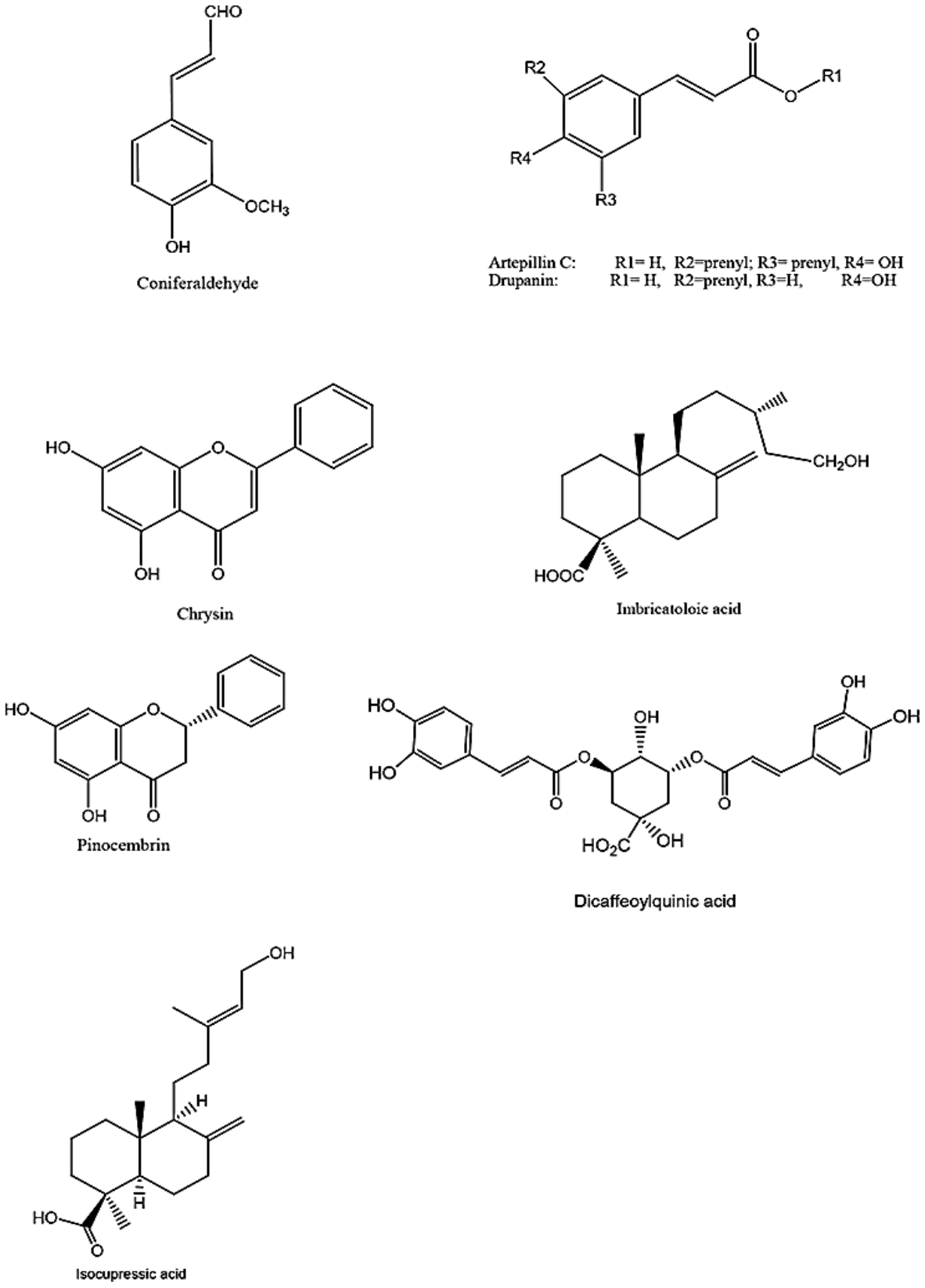


Figure 2.1: Representative of chemical components in propolis.

The various types of propolis and its origin are listed in Table 2.1 (Wagh, 2013). Propolis collected from many countries such as China and Korea (Bogdanov, 2016), showed chemical composition similar to poplar. Propolis from the tropical area, such as Brazilian green and red propolis, are rich respectively in isoflavonoids that are different from the ones found in poplar propolis (Trusheva et al., 2006).

Table 2.1: Propolis distribution around the world and its' main bioactive compounds

No.	Geographic Origin	Plant Source	Main Bioactive Compound
1	Europe, North America and nontropic regions of Asia	*Populus spp.*	Polyphenols
2	Russia	*Betula verrucosa.*	Polyphenols
3	Brazil	*Baccharis spp.*	Prenylated p-coumaric acids, diterpenic acids
4	Cuba, Venezuela	*Chusia spp.*	Polyprenylated benzophenones
5	Pacific region (Okinawa, Taiwan)	Unknown	C-prenylflavanones Furofuran lignans
6	Canary Islands	Unknown	Furofuran lignans
7	Kenya	Unknown	Polyphenols
8	Greece and Cyprus	Unknown	Flavonoids, terpenes

Propolis contains around 50% of balsam resin, 30 % of wax, 10 % of essential and aromatic oils, 5 % of pollen, and 5 % of other substances, including wood fragments (Monti et al., 1983). More than 300 different compounds have been characterized so far in propolis, including aliphatic acids, esters, aromatic acids, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins, and inorganic substances. Of all, flavonoids are the compounds that possess greater research interest (Marcucci, 1995). Propolis contains extremely high bioflavonoid content that contribute to the antioxidant, antibacterial, antifungal, antiviral and anti-inflammatory properties (Pujirahayu, Ritonga, and Uslinawaty, 2014).

Propolis is basically containing balsamic and non- balsamic components. The main part is of plant derived substances and minor part is of bee and pollen derived substances. Compositions of raw poplar propolis are as summarised in Table 2.2.

Table 2.2 Composition of raw poplar propolis (Bogdanov, 2015)

Components	**Substances**
BALSAM 40-70% ethanol soluble Poplar Origin	Phenolics: Phenols, Phenolic acids, esters, flavonons, dihydroflavonons, flavons, flavonols, phenolic glycerides. Others: Aliphatic, acids, alcohols, esters, aldehydes, ketones, benzoic acid and esters
NON BALSAM Wax: 20-35% ethanol insoluble Beeswax origin	Beeswax components

Enigmatically, propolis exhibits anti-inflammatory properties because of its large content of polyphenol compounds. Propolis contains active compounds which can stimulate cell proliferation or apoptosis. Among them, there are caffeic acid, caffeic phenyl ester, artepillin C, quercetin, resveratrol, galangin, and genistein (Zhang et al., 2011).

Flavonoids are valuable because they can avoid swift blood sugar increase and are able to shelter diabetics from the complications of this metabolic disorder. Propolis has greater antibacterial activity to gram-positive bacteria than to gram-negative ones due to the synergistic activity of the many compounds (Grange and Davey, 1990).

2.7.1 Flavonoids in Propolis

Largest group of compounds identified from propolis are flavonoid pigments, which are ubiquitous in the plant kingdom (Burdock, 1998). The flavonoids in propolis were also confirmed as having botanical origins (Kumazawa et al., 2003).

Many different methods for instances spectrophotometry (Alencar et al., 2008), thin layer chromatography (TLC), gas chromatography–mass spectrometry, high performance liquid chromatography (HPLC) (Kumazawa et al., 2004; Pietta et al., 2002), and capillary electrophoresis (CE) have been developed to analyse propolis flavonoids.

Spectrophotometry is often operated to evaluate the total content of flavonoids. TLC is especially helpful for the fast screening of propolis for bioactive components before detailed instrumental analysis (Jiang et al., 2011).

2.7.2 Polyphenols in Propolis

Phenolic is the major propolis compounds which directly impacts the antioxidant property. The antioxidant activity of polyphenols is due to donation of hydrogen atoms from an aromatic hydroxyl group to the free radical which leads to stabilization of the radical (Berretta et al., 2017; Gardner et al., 2003).

Researchers identified a series of phenylpropanoid derivatives in Brazilian propolis. Meanwhile, some caffeic acid derivatives and isoferulic acid derivative were also identified in poplar propolis by Gas chromatography–mass spectrometry (GC-MS) (Pereiraa et al., 2003). Quinic acid derivatives were identified in propolis as well. In 2010, Petrova et al. identified two geranylstilbenes in propolis. Macaranga schweinfurthii is the only plant source of these two geranylstilbenes to this date (Petrova et al., 2010). In 2012, another stilbene, 5-farnesyl-3'-hydroxyresveratrol was identified in Solomon Island propolis, which is also present in Macaranga plants (Shimamura et al., 2012). These results show that Macaranga is might be the plant source of the propolis from Kenya and Solomon Island. However, many stilbenes especially prenylated stilbenes, were identified in Australian Kangaroo Island propolis, which makes this type of propolis a stronger scavenging activity towards DPPH free radical than Brazilian propolis (Koolaji et al., 2012).

Lignans are main chemical compounds in tropical propolis which are identified Brazilian propolis. Nemorosone is the exclusive and principal component of Clusia rosea floral resins which indicates that *Clusia spp*. is the plant origin of brown propolis (Camargo et al., 2013).

2.8 Antioxidant Property of Propolis

The propolis of various geographic origins had different antioxidant activities. This variability is due to the amount of the compounds like flavonoids and phenols which are also related to antioxidant activity of propolis (Kumazawa et al., 2004).

EEP from Argentina, Australia and China exhibits strong antioxidant activities which are related to the total polyphenol and flavonoid contents. Propolis with strong antioxidant activity contained antioxidative compounds like kaempferol and phenethyl caffeate (Kumazawa et al., 2004). Water extracts of Chinese propolis has stronger 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity than the methanol extracts (Banskota et al., 2000).

The antioxidant activity of flavonoid is expressed due to presence of o-dihydroxy phenyl ring. The antioxidant activity seemed to be related with total flavonoid contents of propolis extract. Flavonoids are rich and most effective antioxidant in propolis. Besides that, propolis with antioxidant activity has DPPH free radical scavenging activity (Kumazawa et al., 2004).

The naturally occurring polyphenols which is one of the factor in expression of antioxidant activity are expected to help reduce the risk diseases like cancer and cardiovascular diseases. Thus propolis with antioxidant activity may protect humans from deleterious oxidative processes (Kumazawa et al., 2004). Banskota et al. (2001) also mentioned in journal that phenolic constituents also possess antitumour and antihepatotoxic activities (Banskota et al., 2001).

2.9 Freeze Drying

Lyophilization or freeze drying process is one of the very usual methods applied in food and pharmaceutical industry. This process happens by removal of water or other solvent from a frozen pharmaceutical product (Labconco, 2010). Due to the absence of

liquid water and the low temperatures required for the process, most of deterioration and microbiological reactions are halted which gives a final product of excellent quality in the form of powder. The process could be divided into three parts which consist of freezing, primary drying and secondary drying. The freeze drying process starts with water being frozen first and then will be removal of the water which has undergone the freezing (Nireesha et al., 2013). This method is utilised to shield the primary structure of the product and increases the longetivity of product (Ratti, 2012).

2.10 Capsules

Capsules offer an alternate to tablets for oral delivery of therapeutic compounds. One advantage of capsules over tablets is their amenability to deliver not only solids but also nonaqueous liquids and semisolids as a unit dose solid dosage form. Besides, those tablets dosage forms are hard to swallow by the children and elderly people (Kathpalia and Kishon, 2014). Capsules are consisting of two types which are soft shelled capsule and hard shelled capsules. These two types of capsules are used for different type of drug ingredient, dry powders for hard capsules and oil for soft capsules (Swarbrick, 1996). Shell component is an essential part of capsule dosage forms. Capsule shells, available as hard or soft shells, are formulated from gelatin or a non-gelatin polymeric material such as hypromellose and starch, water, and with or without a nonvolatile plasticizer. The capsule shells may also be formulated to modify the release of their fill contents in a site-specific manner in the gastrointestinal tract.

CHAPTER 3

METHODOLOGY

The extraction of propolis was done using 70% of ethanol and water as solvent (Pujirahayu, Ritonga, Uslinawaty, 2014). After incubation of one week for ethanol extraction and one day for water extraction, the extracts were filtered with Whatman size 1 filter paper. The obtained liquid propolis extract was tested for total flavonoid content. After addition of aluminium chloride solution and other chemicals, the absorbance at 510nm was measured using UV Vis Spectrophotometer. The results obtained were compared with Quercetin standard calibration curve. Total polyphenol content determination was carried out using Folin- Ciocalteu reagent and other chemicals where the mixture was incubated in the dark to avoid disintegration of propolis components due to sunlight. The absorbance was measured using UV Vis Spectrophotometer at 750nm. The results obtained were compared with prepared gallic acid calibration curve. Antioxidant testing on propolis was carried out by using DPPH chemical. Absorbance was measured using UV Vis Spectrophotometer at 514nm. Results obtained were compared with standard Quercetin calibration curve. The DPPH degradation was evaluated. The summary of the entire process was shown in the flowchart as shown in Figure 3.1.

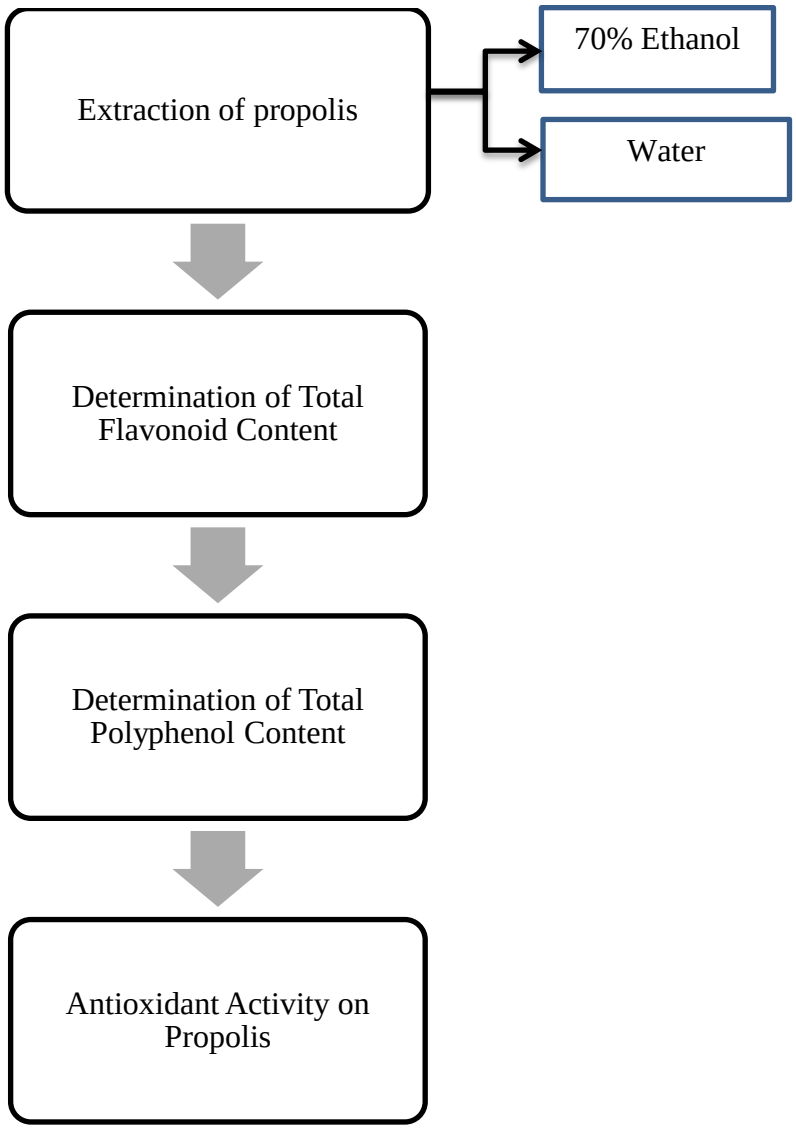


Figure 3.1 Flowchart of this whole study

3.1 Raw Materials and Chemicals

The raw propolis used in this study was supplied by the company, Kelantan Biotech Corp SDN. BHD. The list of chemicals used in this study are summarised in Table 3.1.

Table 3.1 List of Chemicals

Chemical	**Chemical Formula**	**Usage**	**Purity**	**Supplier**
Ethanol	C_2H_6O	Ethanolic extraction of propolis	95%	Sigma Aldrich
Folin–Ciocalteau reagent	C_6H_6O	Determination of total polyphenol content of propolis extract	-	Sigma Aldrich
Sodium carbonate solution	Na_2CO_3	Determination of total polyphenol content of propolis extract	20%	Sigma Aldrich
Aluminium chloride solution	$AlCl_3$	Determination of total flavonoids content of propolis extract	10%	Sigma Aldrich
Sodium hydroxide solution	NaOH	Determination of total flavonoids content of propolis extract	1M	Sigma Aldrich
DPPH (1,1-diphenyl-2-picrylhydracyl)	$C_{18}H_{12}N_5O_6$	Testing of antioxidant property	-	Sigma Aldrich

3.2 Equipment

The list of equipment used in this study are summarised in Table 3.2.

Table 3.2: List of Equipment

Equipment	Brand/ Country	Purpose of Use
UV-Vis Spectrophotometer	Shimadzu/ Malaysia	Determination of Total Flavonoid Polyphenol Content
Incubator	Infors HT Ecotron/ USA	Extraction Of Propolis
Chiller	Thermo Scientific/ USA	Extraction Of Propolis

3.3 Propolis and extracts

3.3.1 Crush propolis

Prior to use, course debris like twigs were removed. Then, the propolis was crushed and grinded into small pieces to increase the surface area of propolis with solvent for maximum extraction. Then, the grinded propolis was stored in 20 ^{o}C freezer until the point of use.

3.3.2 Ethanolic Extraction of Propolis

Propolis was dissolved in ethanol and extracted with ease in 70% ethanol. About 70% ethanol was prepared by adding 736.84 mL of 95% ethanol to 263.16 mL of ultra-pure water making up a stock solution of 1000mL. The calculation done using Equation 3.1;

$$C_1 . V_1 = C_2 . V_2 \quad (3.1)$$

Where: C_1 is initial concentration of ethanol, V_1 is volume of ethanol needed, C_2 is desired concentration of ethanol and V_2 is volume of stock solution.

The ethanol extraction was carried out at ratio of 1: 10 (propolis: ethanol). About 50 g of crushed propolis samples was added into a dark bottle and 500 mL of 70% ethanol was poured into. The top of the bottle was sealed and then was agitated at 80 rpm at room temperature for one week. The alcoholic extract was filtered using Whatman size 1 filter paper to discard the waxes. The EEP was stored in chiller until point of use to prevent growth of bacteria the yield of extraction was calculated using Equation 3.2;

$$\text{Yield} = (Pe / Pm) \times 100\% \quad (3.2)$$

Where: Pe was weight of propolis extract (g) and Pm is weight of raw propolis (g)

3.3.3 Water Extraction of Propolis

Water extraction of propolis was carried out at ratio of 1: 10 (propolis: water). About 100 g of powdered propolis samples was added into dark bottles covered with aluminium foil and 1000 mL of ultra-pure water was poured into. The top of the dark bottle was sealed and then was shaken at 70 rpm. The mixture was left at room temperature for one day of incubation. The water extract was then filtered using Whatman size 1 filter paper. WEP was then stored in chiller until point of use to prevent growth of bacteria. The percentage of yield of WEP was calculated according to Equation 3.2.

3.4 Total Flavonoid Content

The total flavonoid contents of WEP were determined using the aluminium chloride colorimetric method (Koksal and Gulcin, 2008; Chang et al., 2002). About 0.5 mL of EEP was mixed with 1.5 mL of 95% ethanol, followed by 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M of potassium acetate and 2.8 mL of ultra-pure water. After incubation at room temperature for 30 min, the absorbance of the mixture was measured at 415 nm with a UV- Vis spectrophotometer (Shimadzu, Malaysia). The flavonoid content was calculated in comparison with a standard calibration of quercetin solution and expressed as micrograms of quercetin equivalent (QE) per gram of sample. The content of total flavonoids in EEP was calculated from the standard calibration

curve calculation, Equation 3.3 which was prepared using quercetin and expressed as micrograms of quercetin equivalents (QE). The standard calibration curve of quercetin was shown in appendix. The procedure was repeated for WEP.

$$\text{Total flavonoid compound (mg quercetin/g extract)} = \frac{[(\text{Absorption at } 415\text{nm} + 0.007) \text{ x } 1000]}{6.061} \quad (3.3)$$

3.5 Total Polyphenol Content

About 1 mL of EEP was mixed with 2.5 mL of Folin- Ciocalteu reagent, 2 mL of 20% sodium carbonate solution in 25 mL volumetric flask. The volume was completed with ultra-pure water. The mixture was allowed to stand for 15 minutes at room temperature in the dark and then the absorbance was measured at 765 nm against blank. The blank was prepared using the same procedure with 1 mL of ultra-pure water in the place of the 1 mL extract. Gallic acid was used as the standard. The total phenolic content was expressed in µg of Gallic acid equivalents (GAE) per mL of the extract. The standard calibration curve of gallic acid was shown in appendix. The same procedure for determination of total polyphenol content was repeated for WEP.

3.6 Antioxidant Activity Assessment

About 100 µL of propolis extract was added to 0.5 mL of 300 µM DPPH and 3.7 mL of 95% ethanol. The mixture was shaken at 100 rpm for five minutes and left to stand at room temperature for 20 min in the dark. Then, absorbance at wavelength of 514 nm was measured using spectrophotometer. Ethanol was used as a blank. The degradation of DPPH was evaluated by comparing with a control. Control was formed by mixing 0.5 mL of DPPH solution and 1.5 mL of water. Results were expressed by the proportion of DPPH degradation compared with the control. Standard calibration curve DPPH was shown in appendix. The antioxidant activity was calculated using Equation 3.4:

$$\text{Antioxidant activity (\% inhibition)} = \left\{\left(\frac{\text{Abs C} - \text{Abs E}}{\text{Abs C}}\right) \text{ x100}\right\} \quad (3.4)$$

Where: Abs_C is the absorbance of the control and Abs_E is the absorbance in presence of extract samples.

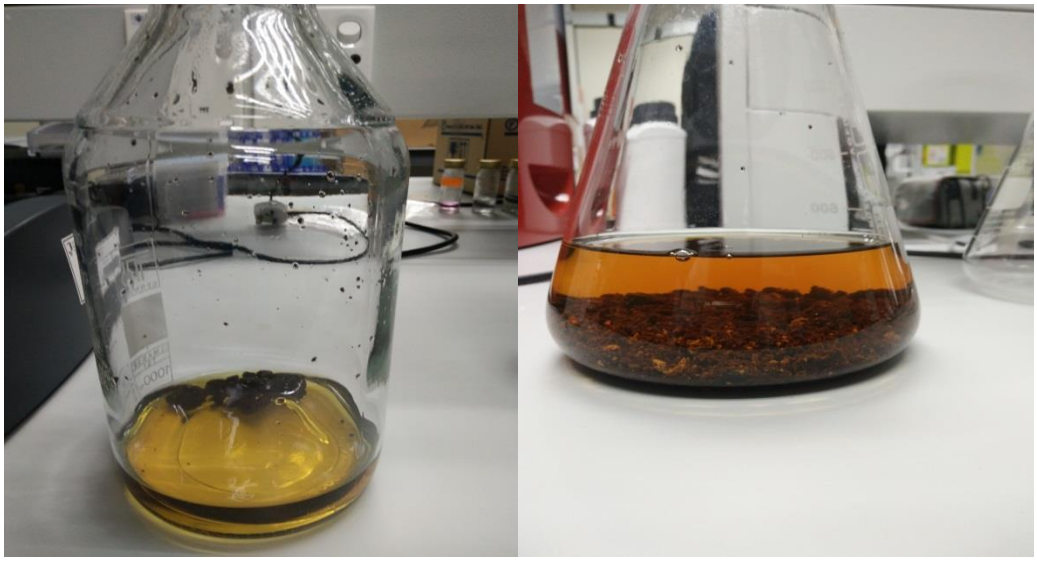
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of propolis

The extraction of propolis using solvents like water and ethanol was conducted by maceration technique. There was change in colour on both ethanol and water extract of propolis. For EEP, the initial colour was yellow. The extract produced was in brownish red after being incubated for one week duration at room temperature with 80 rpm agitation. On the other hand, the WEP change colour from green to greenish brown colour. The incubation of WEP was done for 1 day period with 80 rpm at room temperature.

The changes in colour were due to propolis dissolving in extracting solvents which were the ethanol and water. The change of colour of EEP and WEP are shown in Figure 4.1 (a) and Figure 4.2 respectively.



(a) (b)

Figure 4.1: Colour change of EEP after (a) 3 hours and (b) 1 week of incubation

Figure 4.2: WEP after one day of incubation

The purification of extracted propolis liquid was done to remove unwanted debris like wood and waxes. Comparing between EEP and WEP, EEP was being filtered at much faster rate than WEP. This was because at the end of incubation, the unwanted wax in EEP clumped together forming ball like lumps. Figure 4.3 shows the filtration method carried out for both EEP and WEP.

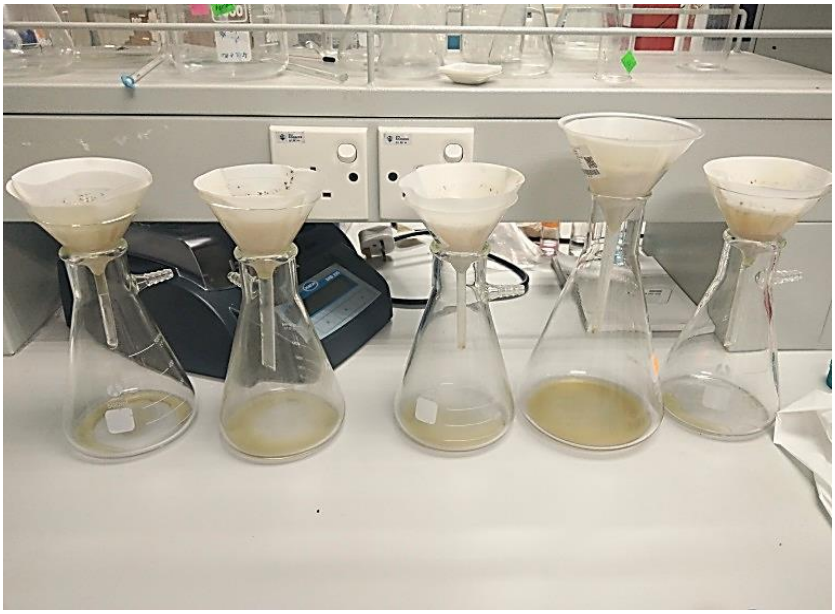


Figure 4.3: Filtration to remove unwanted waxes and debris.

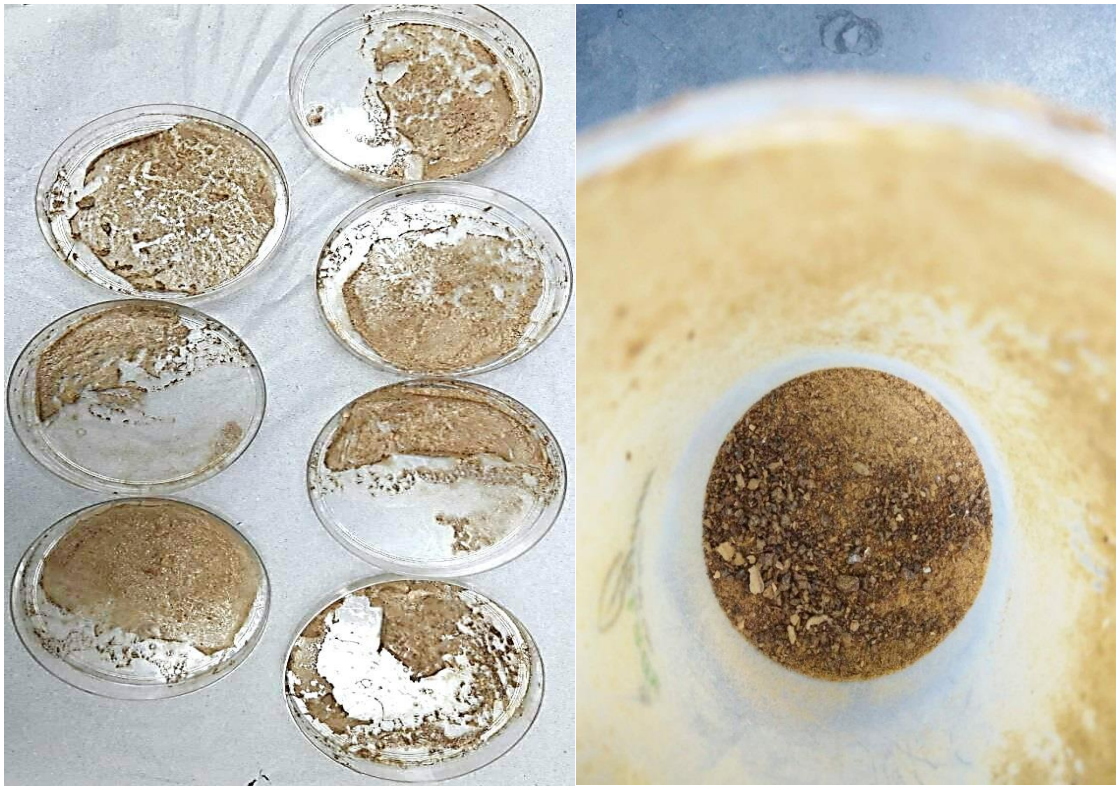
Subsequently after filtration, rotary evaporator instrument was utilised to remove ethanol residue from the EEP. This was to ensure that there was no ethanol residue which gives impact to consuming patients. Rotary evaporation was done at room temperature at 80rpm. Figure 4.4 shows the rotary evaporation of EEP at initial state and final state.

(a) (b)

Figure 4.4: Rotary evaporation of EEP (a) at initial state, (b) at final state

During rotary evaporation, there was drastic change in the volume and colour of EEP. The volume decreased from 10 mL to 3 mL at the end of evaporation. From observation, at initial state, EEP was less viscous but at the end of rotary evaporation the liquid turned to thick and viscous liquid. Moreover, colour changed from brownish, clear liquid to yellowish, opaque liquid as shown in Figure 4.4. When the EEP was rotary evaporated, the ethanol was evaporated. This was due to rotation of the flask which provided larger surface area allowing greater evaporation of ethanol from propolis extract.

Propolis powder was produced after freeze-drying process for two days continuously. The powder was formed due to removal of water as a vapour by sublimation from the frozen propolis extract which was froze in -80 °C refrigerator prior to freeze-drying commencement (Ratti, 2001). Figure 4.5 shows propolis powder after freeze drying process of (a) EEP and (b) WEP.



(a) (b)

Figure 4.5: Propolis powder of (a) EEP and (b) WEP after freeze drying

The final weight of the propolis powder obtained was used to calculate the percentage yield of the propolis extract using Equation 3.2. The results were tabulated in Table 4.1.

Table 4.1: The final weight of propolis acquired and yield percentage

Sample	**Composition (Propolis weight (g): solvent volume (mL))**	**Initial weight of Propolis (g)**	**Final Weight of propolis (g)**	**Yield (%)**	**Appearance of propolis after extraction**	**Colour of the obtained extract**
EEP	10g:100 mL of ethanol	10	1.137	11.37	Gummy and sticky	Brownish red
WEP	10g:100 mL of water	10	0.831	8.31	Gummy and sticky	Greenish brown

From the Table 4.1, the EEP produced more propolis powder compared to WEP, which were 11.37% and 8.31% respectively. The EEP had more yield because being an organic solvent, it dissolves the components of propolis easily compared to water in WEP. The difference in percentage of yield from EEP and WEP was due to characteristics of ethanol of having the ability to dissolve most content of propolis as an organic solvent as compared to water. Trusheva et al. (2006) had mentioned that EEP produces higher yield than that of WEP. Trusheva et al. (2007) mentioned that the greater percentage of total extract shows higher amounts of waxes have been extracted out. Paviani et al. (2013) quoted that the differences in extraction yield were influenced by characteristics of the raw propolis, such as the harvesting season and bee species.

4.2 Total Flavonoid Content

Figure 4.6 shows total flavonoid content of EEP and WEP. From Figure 4.6, it is shown that the EEP has total flavonoid content of 239.573 ug/ mL while WEP has 98.94 ug/mL. Total flavonoid content in EEP was much higher than in WEP. The flavonoid content in EEP was almost 2.5 times higher than in WEP. Authors Paviani et al. (2013) and Mello et al. (2010) had also mentioned that flavonoid content in EEP was higher than in WEP. This scenarios was due to the relationship between polyphenols and flavonoids that was strong, hence, when polyphenol contents was higher in EEP than WEP, the flavonoids have a tendency to become higher in EEP too (Mello et al., 2012).

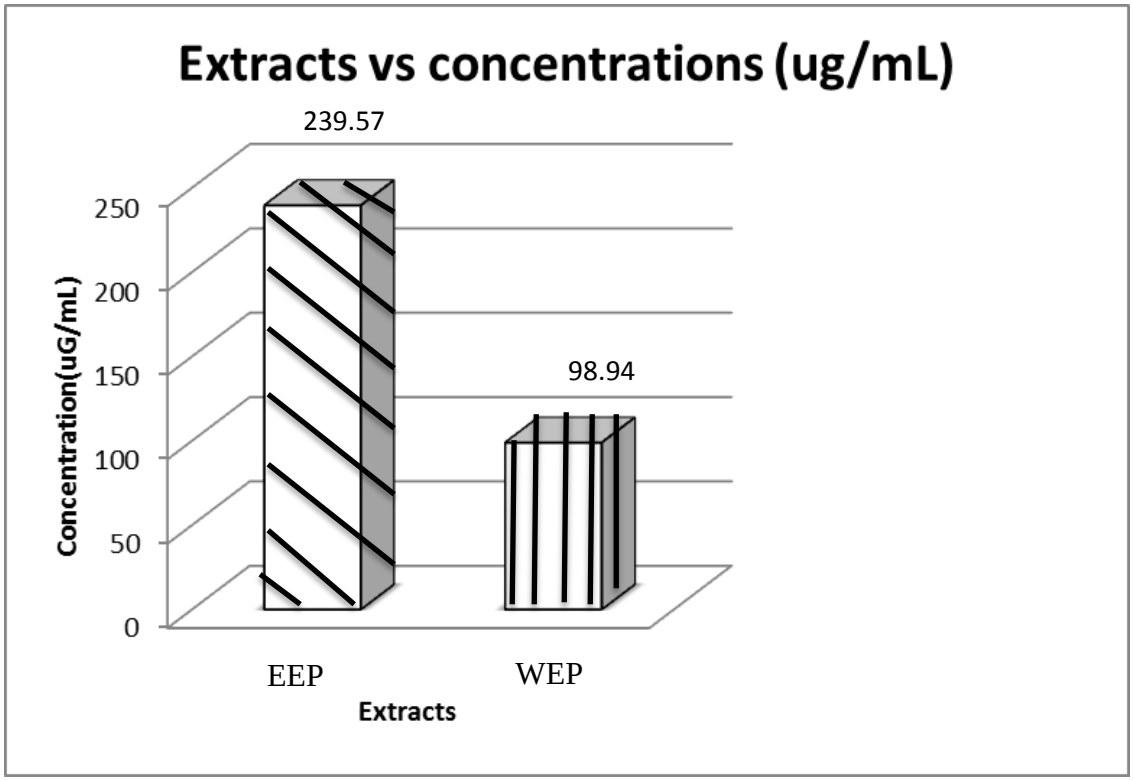


Figure 4.6: Total flavonoid content of EEP and WEP

4.3 Total Polyphenol Content

Figure 4.7 shows the total polyphenol content of EEP and WEP. From Figure 4.7, the results have shown that the EEP had concentration of polyphenol of 122.44 ug/mL and WEP had 12.60 ug/mL. The amount of polyphenol in EEP was 10 times higher than in WEP. According to Mello and Hubinger (2012), addition of gallic acid causes polyphenols hydrolysis which can influence phenols' lower solubility in WEP than in EEP. In addition, Kacha-Anandha et al. (2013) reported that polyphenolic compound dissolves at ease in EEP than in WEP.

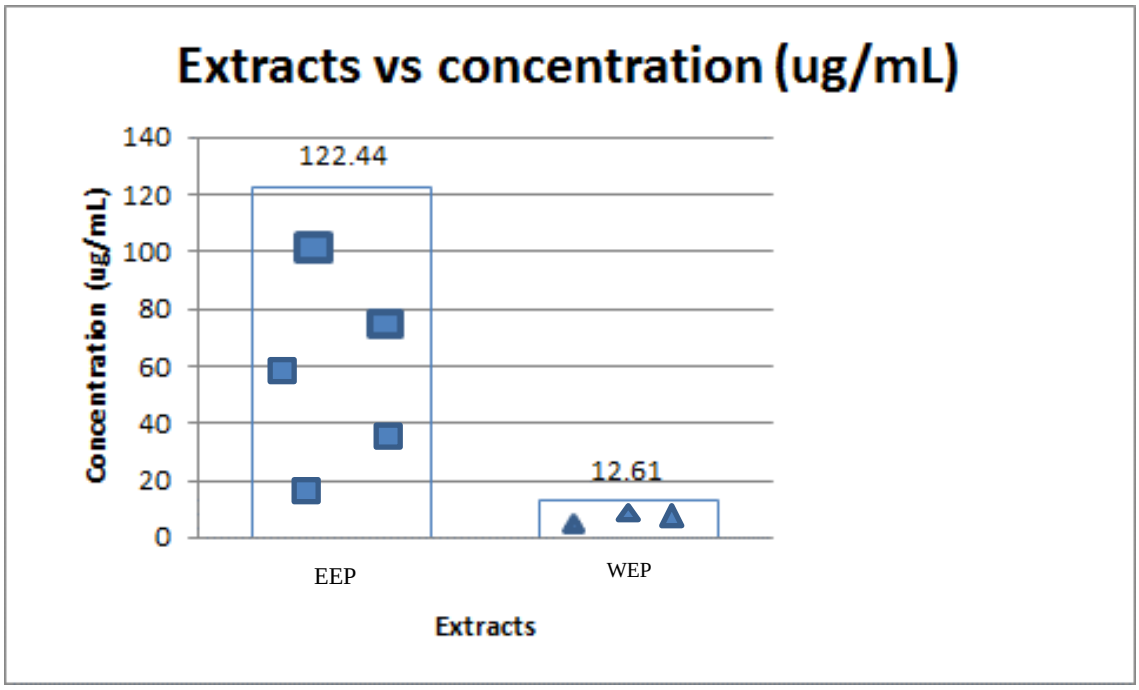
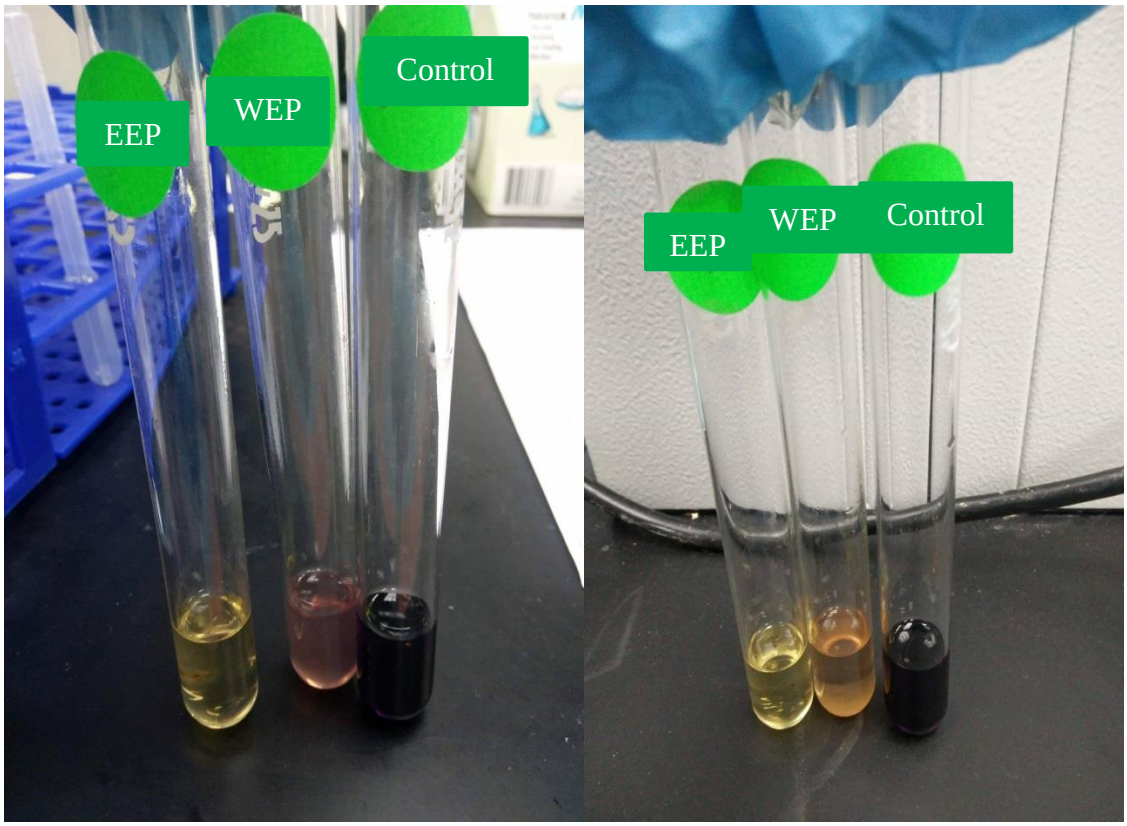


Figure 4.7: Polyphenol concentration of EEP and WEP

4.4 Antioxidant Activity of Propolis Extracts

The EEP and WEP which were prepared with DPPH had change in colour after incubation. For EEP, the color changed drastically from dark yellow to light yellow right after incubation started. For WEP, the colour changed from pinkish purple and to yellowish orange. The control remained purple throughout the testing. These colour change happened because there was degradation of DPPH happened due to addition of extracts, EEP and WEP. Figure 4.8 shows the change of colour of EEP, WEP and control before and after incubation for 20 minutes. The antioxidant activity of EEP, WEP and control against time was tabulated in Table 4.2.



(a) **(b)**

Figure 4.8: The change of colour of EEP, WEP and control solution (a) before and (b) after incubation of 20 minutes

Table 4.2: The antioxidant activity of EEP, WEP and control against time

Time (minutes)	**EEP (ug/mL)**	**WEP (ug/mL)**	**Control (ug/mL)**	**Scavenging activity of EEP (%)**	**Scavenging Activity of WEP (%)**
0	19.92	107.15	301.16	93.39	64.42
2	4.38	104.12	299.92	98.54	65.28
4	4.67	133.15	369.09	98.73	63.92
6	75.79	121.62	369.34	79.48	67.07
8	75.57	114.19	368.82	79.51	69.04
10	75.38	112.09	368.90	79.57	69.62
15	75.27	107.28	368.65	79.58	70.90
20	75.06	100.94	368.91	79.65	72.64

According to Table 4.2, the EEP had an antioxidant activity in the range of 79.48% to 98.73% while WEP showed values ranging from 63.92% till 72.64%. The EEP has higher antioxidant activity compared to WEP. The initial antioxidant activity for EEP and WEP was 93.39% and 64.42%, respectively. After four minutes, the EEP had showed its highest antioxidant activity at 98%. After six minutes, the antioxidant activity had reduced to 79% until minute of 20. Meanwhile, for the WEP, there was increase in antioxidant activity after six minutes of testing at 67%. The activity was increasing gradually till its maximum was reached after 20 minutes which was at 72%.

Kumazawa et al (2003) and Mello et al (2010) have mentioned that due to factors like different climate and geographic area, the antioxidant results were different. Nagai et al. (2003) have reported that WEP is a natural antioxidant and this explains the reason for WEP's increased antioxidant activity after 20 minutes. These also reported that properties of propolis were ascribed to the antioxidant effect which can be effects of flavonoid presence. Furthermore, Thaipong et al. (2006) reported that polyphenols are hydrophilic antioxidants. Hence, higher antioxidant activity in EEP compared to WEP is a contribution from polyphenols. In short, polyphenols and flavonoid played a vital role in determining antioxidant activity of propolis.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Introduction

This chapter presents the conclusion drawn from the results obtained and analysed from the propolis extraction, flavonoid and polyphenol content determination and antioxidant assessment. Recommendations for further research of development of propolis capsule using freeze drying method and improvements to this study are given.

5.2 Conclusion

Propolis is a natural antioxidant and exhibited high polyphenols and flavonoids content and high antioxidant activity as well. Extraction of propolis was successfully done using ethanol and water as solvent to dissolve the active components of propolis. Both EEP and WEP had sticky and gummy appearance after extraction period. EEP changed colour to brownish red colour while WEP changed to greenish brown colour. EEP had produced higher amount of yield as compared to WEP. The highest yield produced by EEP was 11.37% followed by WEP at 8.31%. The highest flavonoid and polyphenol content was determined in EEP at 239.573 ug/mL and 122.44 ug/mL, respectively. The WEP had lower flavonoid and polyphenol content of 98.94 ug/mL

and12.61 ug/mL, respectively. Furthermore, antioxidant activity was at its highest in the EEP ranging from 79.48% to 98.73% compared to the WEP whose antioxidant ranged from 63.92% till 72.64%. Ethanol is the most suitable solvent used to extract and determine the properties and components of propolis. However, due to time constraint and extra additional purification step in ethanol extraction method, water extraction method was chosen for bulk production.

Bulk propolis powder of 14.0 g was produced by carrying out water extraction of propolis in large volume since water extraction took less time to complete. One day of incubation was sufficient to extract propolis components. Instead of ethanol, water was opted as solvent in extraction to prevent ethanol residue in final product.

5.3 Recommendation

Recommendations for further research of this study are:

(a) Further investigations on other methods of propolis extractions like MAE, UE and DNA extraction.
(b) More studies on other properties of propolis like anti-tumour, antibacterial and anti-inflammation.
(c) Investigations on other factors which influences propolis characteristics and determination of components
(d) Investigations on applications of propolis in many other sectors like food industry.
(e) More studies on factors that can affect better extraction and components determination.
(f) More studies on conversion of propolis extract to powder form.
(g) More studies on how to change raw propolis which is complex to edible form.
(h) Should be conducted in more sterile area to produce more safe, pure and efficient product.

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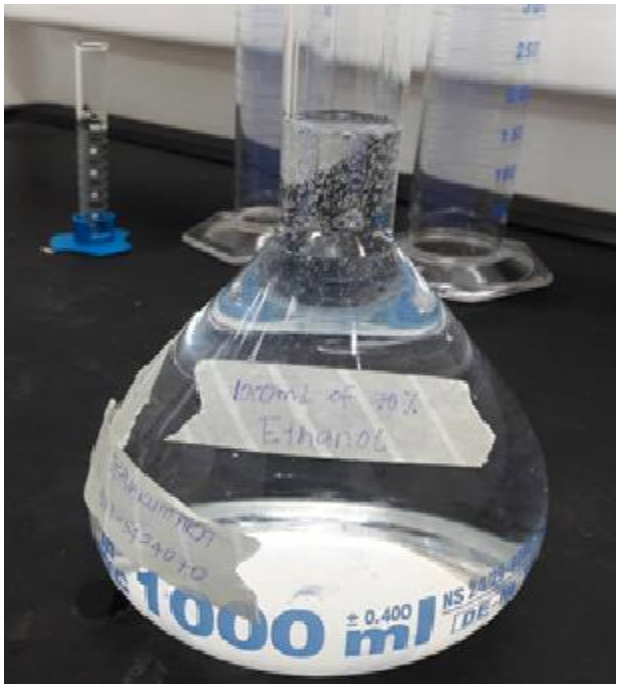
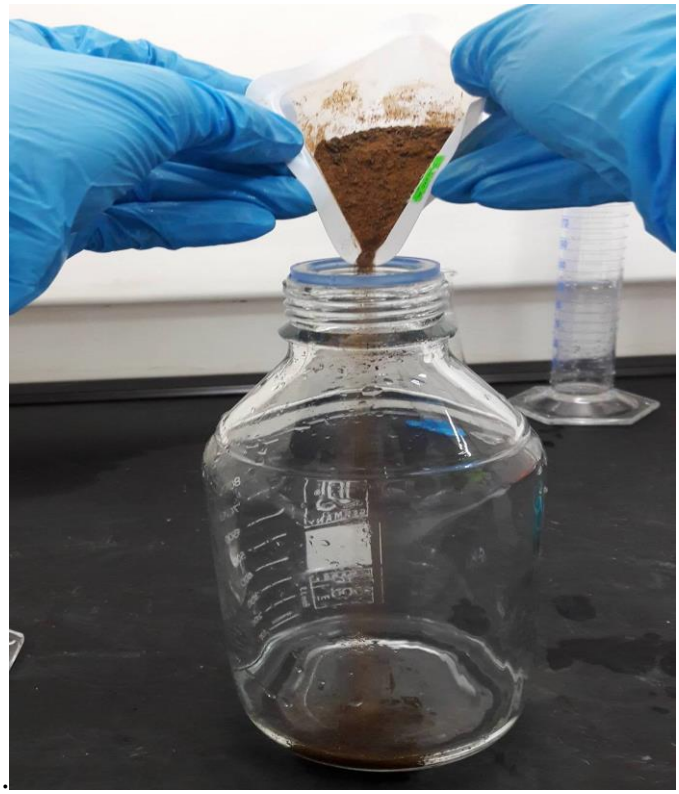
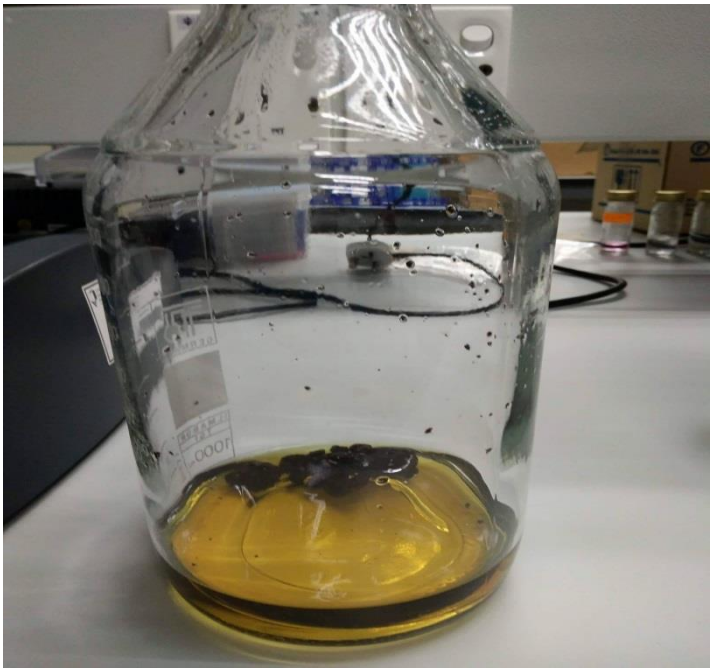
APPENDIX



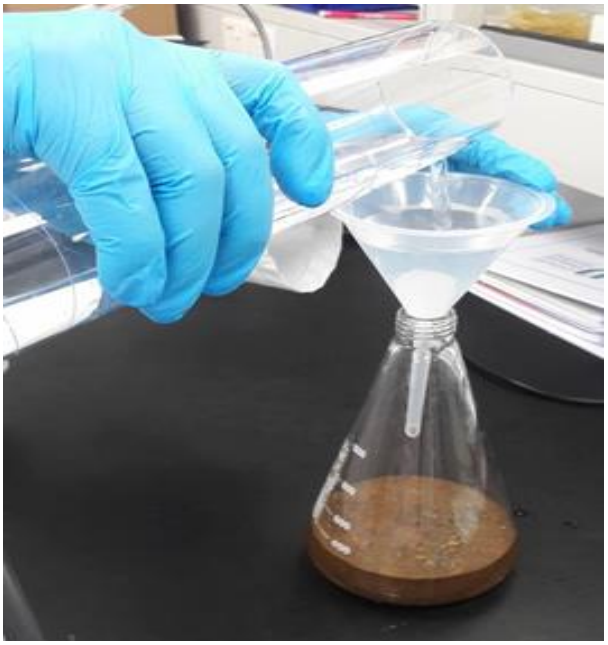
The picture shows ethanol stock solution was prepared for extraction of propolis.



The picture shows propolis powder was put in ethanol to be incubated for 1 week at room temperature.

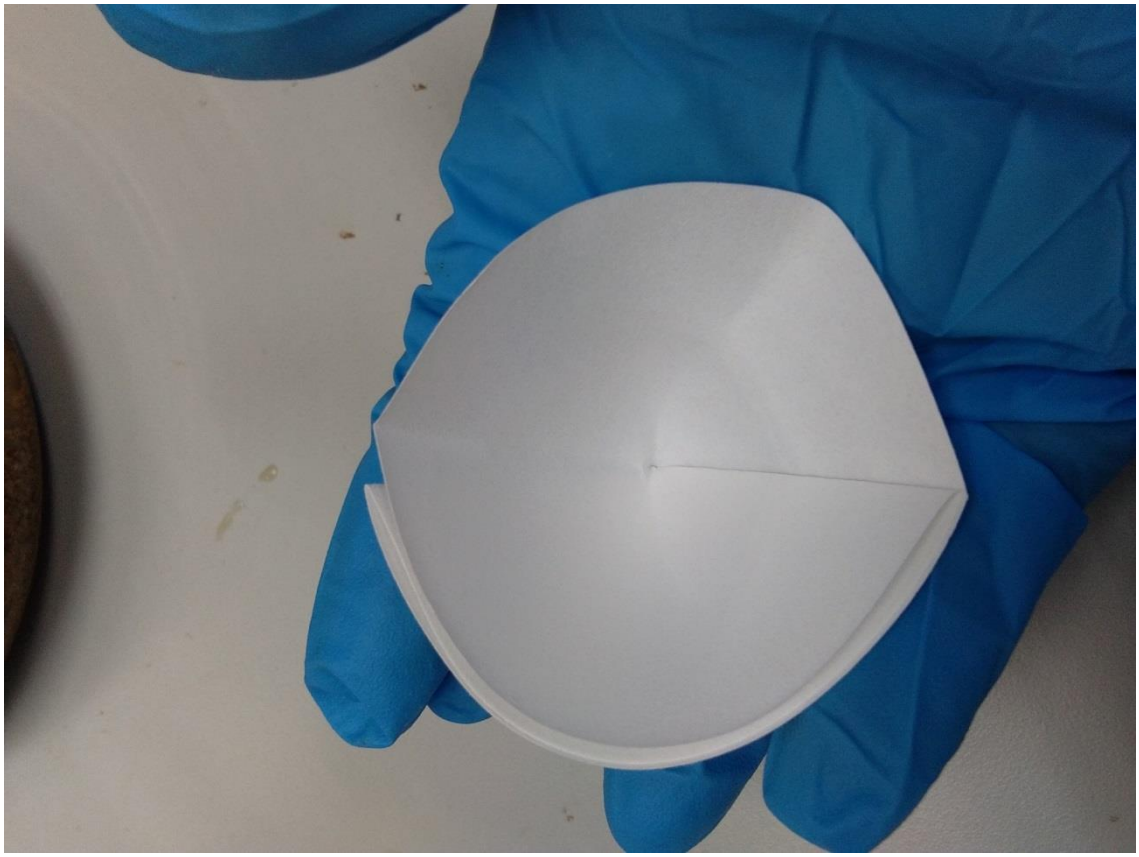


The picture shows ethanolic extraction of propolis that was about to be incubated at room temperature with agitation of 80rpm for one week.



The picture shows water extraction of propolis that was about to be incubated at room temperature with agitation of 80rpm for one day.

The picture shows process of filtration for WEP and EEP using Whatman size 1 filter paper.



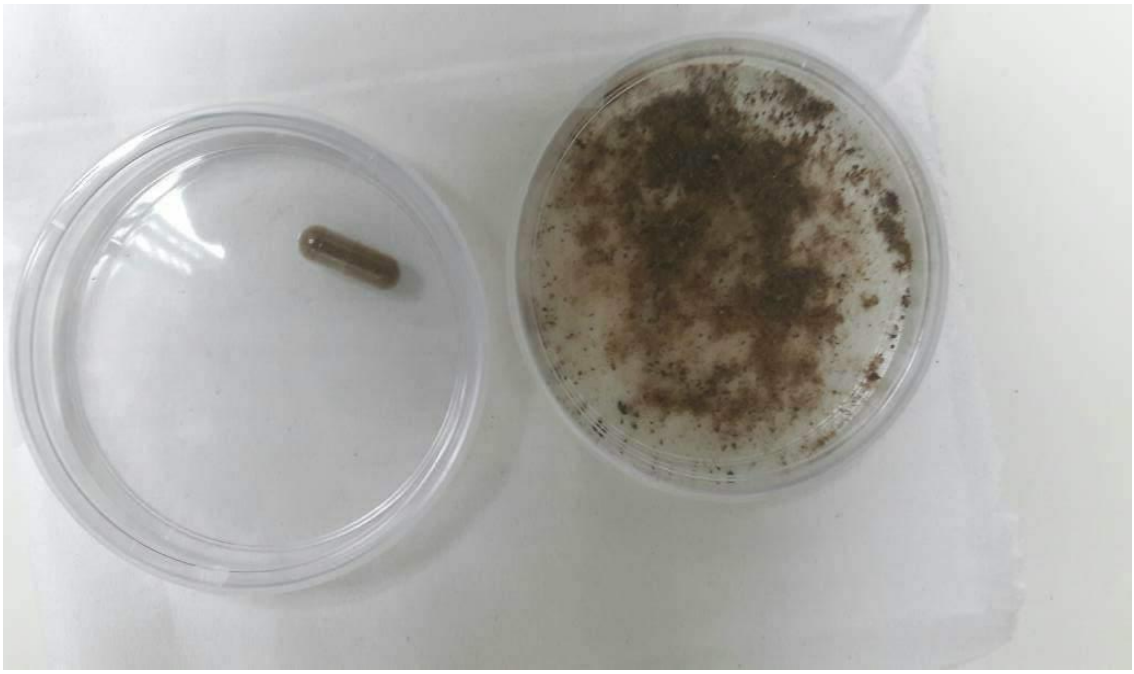
The picture shows size 1 filter paper of Whatman brand used for filtration and purification process of extracts.

The picture shows rotary evaporator which is used to remove ethanol residue in EEP.

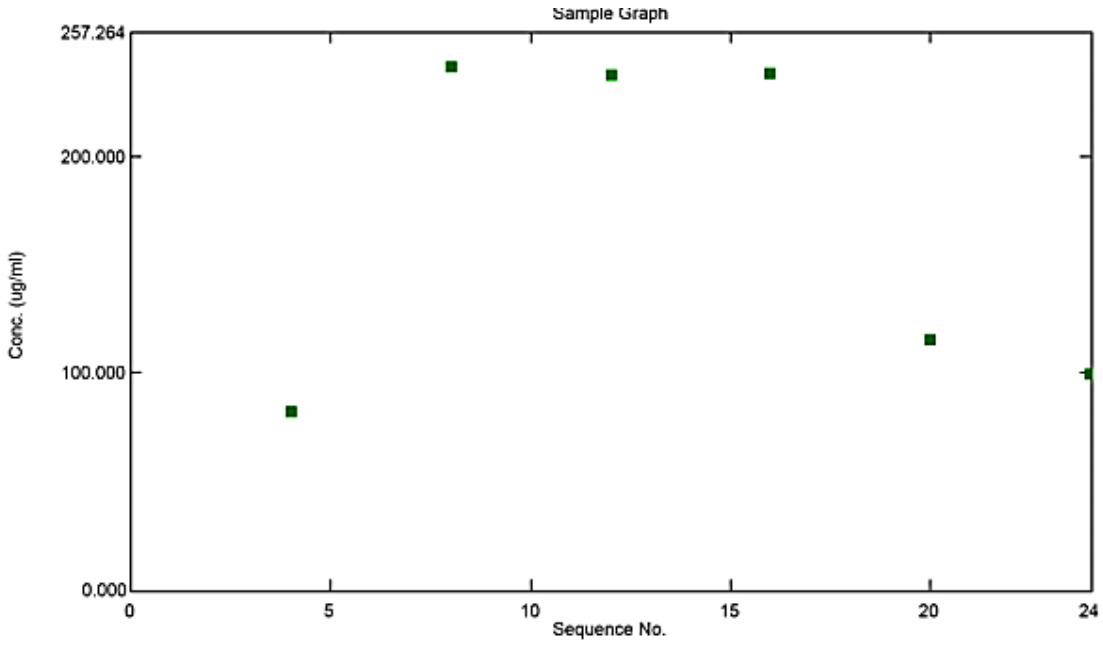
The picture shows the extract obtained after EEP was evaporated for 5 hours to remove ethanol residue.

The pictures show the WEP were freeze dried to be changed in to powder. The pictures are the final product of 2 days of freeze drying.

The picture shows the propolis powder of WEP after being freeze dried.



The picture shows propolis powder was encapsulated in hard gelatin capsule.



The figure above was generated to show total flavonoid content in EEP and WEP.

Sample Table

	Sample ID	Type	Ex	Conc	WL415.0	Comments
1	water	Unk-Repeat			0.515	
2	water-2	Unk-Repeat			0.516	
3	water-3	Unk-Repeat			0.516	
4	water-Avg	Average		82.125	0.516	Avg of preceding 3 Samples
5	ethanlol	Unk-Repeat			1.516	
6	ethanlol -2	Unk-Repeat			1.515	
7	ethanlol -3	Unk-Repeat			1.516	
8	ethanlol -Avg	Average		241.342	1.516	Avg of preceding 3 Samples
9	ethanol	Unk-Repeat			1.493	
10	ethanol -2	Unk-Repeat			1.494	
11	ethanol -3	Unk-Repeat			1.494	
12	ethanol -Avg	Average		237.804	1.493	Avg of preceding 3 Samples
13	EEP	Unk-Repeat			1.498	
14	EEP-2	Unk-Repeat			1.497	
15	EEP-3	Unk-Repeat			1.495	
16	EEP-Avg	Average		238.318	1.497	Avg of preceding 3 Samples
17	wep	Unk-Repeat			0.724	
18	wep-2	Unk-Repeat			0.723	

The table shows results of total flavonoid content in EEP and WEP.

	Sample ID	Type	Ex	Conc	WL415.0	Comments
19	wep-3	Unk-Repeat			0.723	
20	wep-Avg	Average		115.215	0.724	Avg of preceding 3 Samples
21	wep2	Unk-Repeat			0.624	
22	wep2-2	Unk-Repeat			0.624	
23	wep2-3	Unk-Repeat			0.624	
24	wep2-Avg	Average		99.407	0.624	Avg of preceding 3 Samples
25						

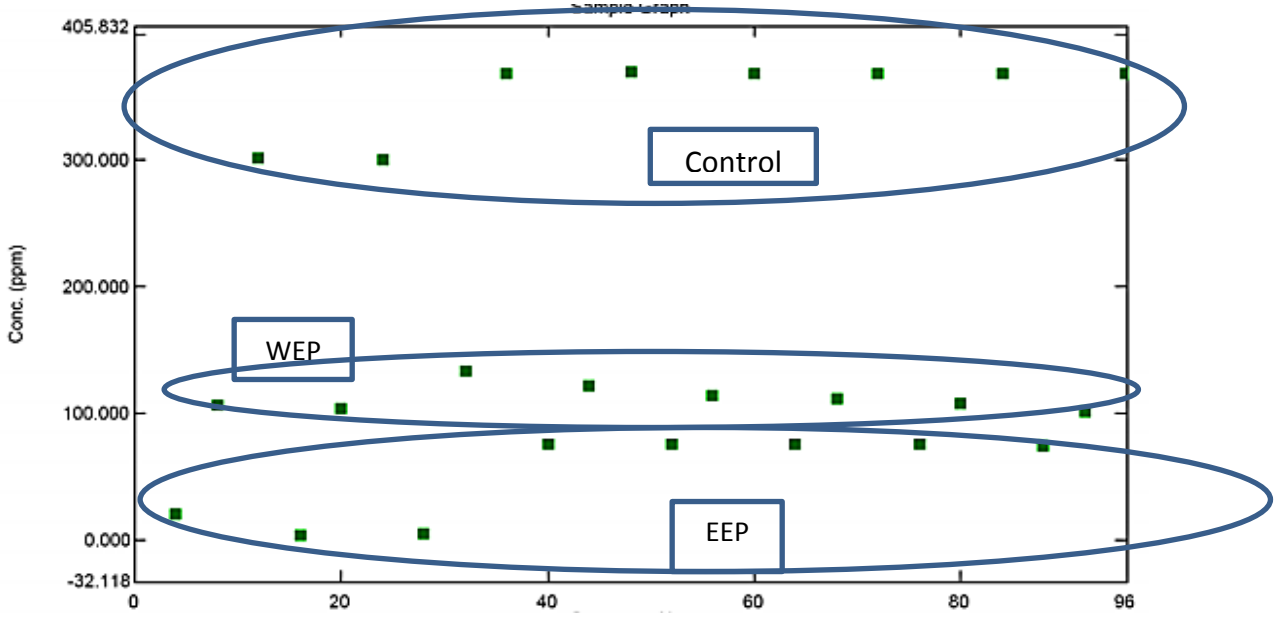
The table shown is results of total flavonoid content in WEP.

	Sample ID	Type	Ex	Conc	WL725.0	Comments
1	Ethanol	Unk-Repeat			0.216	
2	Ethanol-2	Unk-Repeat			0.217	
3	Ethanol-3	Unk-Repeat			0.216	
4	Ethanol-Avg	Average		120.489	0.216	Avg of preceding 3 Samples
5	ethanol 1	Unk-Repeat			0.223	
6	ethanol 1-2	Unk-Repeat			0.222	
7	ethanol 1-3	Unk-Repeat			0.222	
8	ethanol 1-Avg	Average		123.796	0.222	Avg of preceding 3 Samples
9	ethanol 2	Unk-Repeat			0.221	
10	ethanol 2-2	Unk-Repeat			0.221	
11	ethanol 2-3	Unk-Repeat			0.221	
12	ethanol 2-Avg	Average		123.031	0.221	Avg of preceding 3 Samples
13						

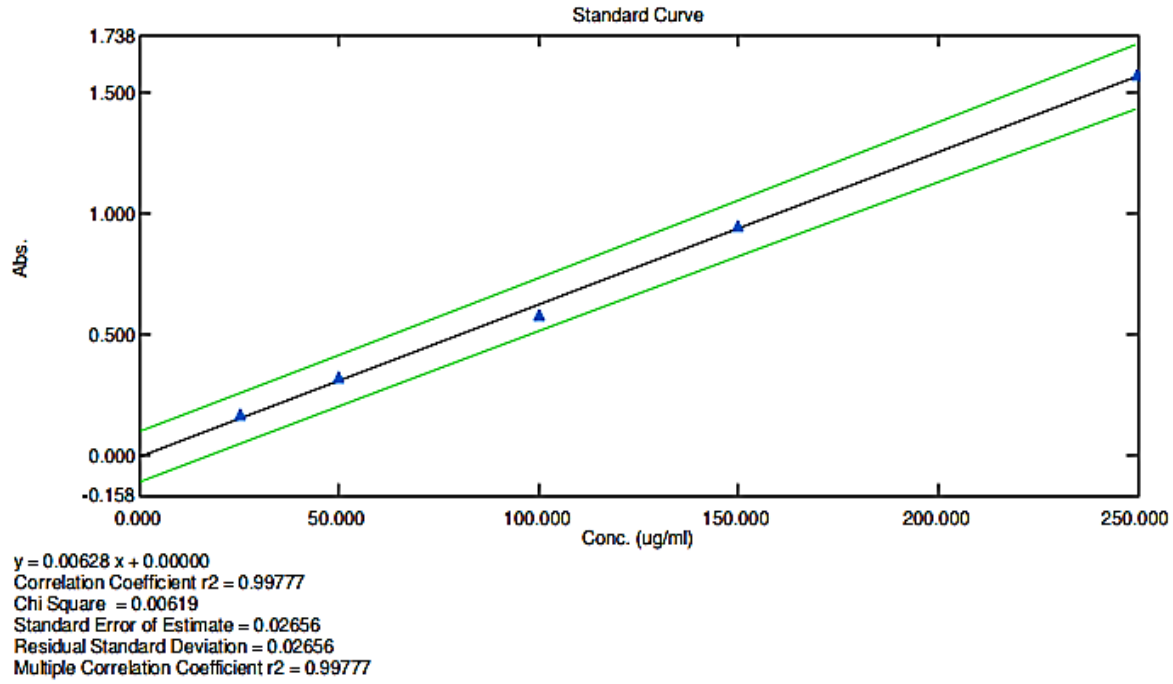
The table shows total polyphenol content in EEP

	Sample ID	Type	Ex	Conc	WL725.0	Comments
1	WEP 1:10	Unk-Repeat			0.104	
2	WEP 1:10-2	Unk-Repeat			0.103	
3	WEP 1:10-3	Unk-Repeat			0.103	
4	WEP 1:10-Av	Average		57.723	0.104	Avg of preceding 3 Samples

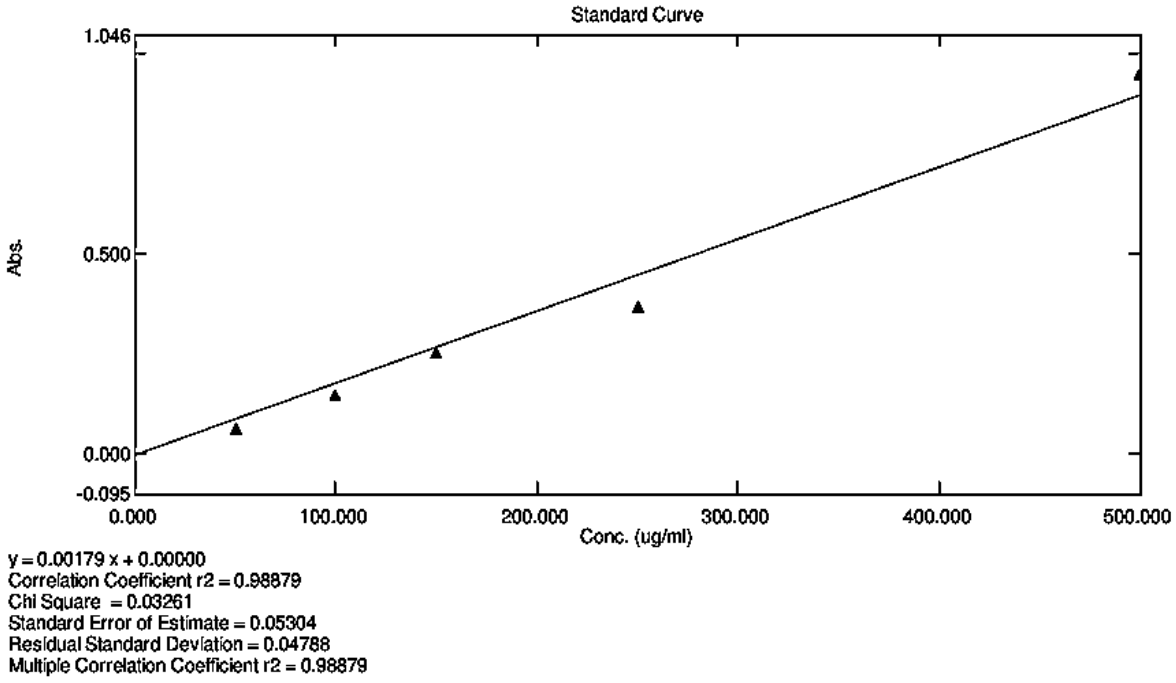
The table shows total polyphenol content in WEP.



The figure shows antioxidant activity of EEP, WEP and control for 20 minutes.



The figure shows Quercetin standard calibration curve for total flavonoid content determination.



The figure shows the standard calibration curve of gallic acid for determination of total polyphenol content.