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EFFECT OF SOLVENT: SOLID RATIO, SOLVENT, TEMPERATURE, TIME AND METHOD ON EXTRACTION ON TOTAL PHENOLIC COMPOUND FROM PROPOLIS (*Trigona Thoracica*)

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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Dedication

Special Dedication of This Thankful Feeling to My

Beloved father and mother;

Mr. Hew Sai Kaw and Mrs. Koo Swee Leng

Beloved Friend;

Wong Sze Theng

Loving sisters;

Hew Shee Chin and Hew Poh Chin

For Their Love and Support.

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To all my friends, thank you for your understanding and encouragement in my many moment of crisis. Your friendship makes my life a wonderful experience. All of you are always on my mind.

Finally, Thank you, Buddha, for always being there for me.

ABSTRACT

Propolis is a resinous substance that is collected by bees from different plant sources. Propolis has been attracting the attention of researchers because it has several biological functions, such as antioxidant and antimicrobial effects, both conferred by phenolic compounds. The aim of this study was to identify a suitable solvent, solid to solvent ratio, temperature and method for extraction of propolis. Maceration method was conducted by using ethanol and water with solid to solvent ratio of 1:10 and 1:20, respectively to identify the most effective solvent and its composition. Next, maceration method with specific solvent and ratio was conducted at 25°C and 50°C to identify the most effective temperature. The ultrasound extraction was conducted with duty cycle of 10% and 20% to identify the most effective duty cycle. Then maceration method was further compared with ultrasound extraction method at specific temperature. The total polyphenol content of propolis extract was analysed by Folin-Ciocalteu method. In conclusion, ultrasound assisted extraction with ethanol (1:10) at 50°C is the most effective way to obtain the highest total phenolic content (53.96 mg GAE/g) from extracts. Additionally, the ultrasound should be set with 20% duty cycle with the extraction time of 150 minutes.

ABSTRAK

Propolis adalah bahan resin yang dikumpul oleh lebah dari pelbagai jenis tumbuhan. Propolis telah menarik perhatian penyelidik kerana ia mempunyai beberapa fungsi biologi, contohnya antioksidan dan kesan anti-mikrob, kedua-dua fungsi ini adalah sebab fenolik. Tujuan kajian ini adalah untuk mengenal pasti kaedah yang sesuai untuk ekstrak fenolik daripada propolis. Kaedah maceration telah dijalankan dengan menggunakan etanol dan air dengan nisbah 1: 10 dan 1: 20 untuk mengenalpasti pelarut yang paling berkesan dan komposisinya. Seterusnya, kaedah maceration dengan pelarut dan nisbah tertentu telah dijalankan pada 25 ° C dan 50 ° C. Ultrasonik telah berbanding dengan kitar tugas 10% dan 20%. Selepas itu, kaedah maceration berbanding dengan kaedah ultrasonik dekat suhu tertentu. Jumlah kandungan fenolik dalam propolis ekstrak akan dianalisis dengan kaedah Folin-Ciocalteu. Kesimpulannya, kaedah ultrasonik pada 50 ° C dan menggunakan etanol dengan nisbah 1: 10 telah memberikan keputusan tertinggi (53.96 mg GAE/g) sebatian fenolik dalam ekstrak propolis. Selain itu, kaedah ultrasonik mesti dengan kitar tugas 20% dan selama 150 minit.

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LIST OF ABBREVIATIONS

°C	celsius
CAS No	Chemical Abstracts Service) Registry Number
DNA	Deoxyribose Nucleic Acid.
e.g.	Exempli gratia
et al.	et alibi
g	gram
g/L	gram per litre
GAE	Gallic acid equivalent
h	hour
Hz	Hertz
I	Intensive of light
LDL	low-density lipoproteins
mg	milligram
min	minute
mL	millilitre
mM	milli-Molarity
nm	nanometers
ROS	Reactive oxygen species
R^2	Correlation coefficient
TPC	Total Phenolic Content
UV/ VIS	Ultraviolet-visible spectrophotometry

CHAPTER 1

INTRODUCTION

1.1 Background

Propolis is bee glue and is a resinous hive product. Propolis contains more than 160 of constituents (Greenaway et. Al, 1991). Phenolic compound which may be found in propolis can be used for many biological and pharmacological activities for example, anti-inflammatory and antimicrobial, antioxidant effects. So, propolis and propolis extract have long been used for the prevention and treatment for various type of diseases.

Generally, the biological component of propolis which is phenolic compound are prepared with solvent extraction method (Yang et al, 2011). The advantages of solvent extraction method are simple operation and low energy consumption. There are two types of extraction solvents; nonpolar and polar solvent. Among extraction solvents, nonpolar organic solvents are mainly ethyl acetate, chloroform, and n-butanol and polar solvents are water, methanol, and ethanol. The ethanol extraction is the most popular technique for the production of propolis extract. This method is suitable for gaining the propolis extract rich in biologically active compounds (Piete et al, 2002). However, according to Laskar et al., water extracts of propolis have a higher phenolic content than ethanol extract of proplis. Thus, these inconsistent results seem to imply that different extraction solvents can affect phenolic content propolis extracts.

Furthermore, the extraction method of phenolic compounds differs from phenolic sources and an ideal extraction method for a particular phenolic source has to be individually designed and optimized (Tan et al., 2011). Extraction efficiency is commonly a function of process conditions. Researchers have reported the influence of some variables on the extraction of phenolic compound from diverse natural product and, that are temperature, solvent-to-solid ratio and time. The positive or negative role

of each factor in the mass transfer of the process is not always obvious; the chemical characteristics of the solvent and the diverse structure and composition of the natural products ensure that each material-solvent system shows different behaviour, which cannot be predicted (Pinelo, 2004).

The tradition extraction method, such as maceration is time consuming, as it takes more than two days for the extraction. Recently, there are some modern extraction methods that have been developed to gain the fast and efficient extraction of organic compounds from solid matrices. Ultrasound assisted extraction is proved to be one of the most promising extraction method for natural product (Huie, 2002). The advantages of ultrasound are thought to be due mainly to the mechanic effect of acoustic cavitation. So, it will has the potential to increase the yield of phenolic compound in the studies on propolis. (Liu and Wang, 2004)

1.2 Motivation

Propolis, a natural component collected by bees from buds and exudates of selective trees and plants, and it is to be used in the beehive as a protective barrier to defend their enemies and repair the honeycomb. Propolis usually contains many chemical compounds, for example polyphenols, terpenoids, steroids, and amino acids. The composition of propolis depends on the vegetation at the place of collection. Propolis has been used in folk medicines in many regions around the world (Ghisalberti, 1979). Many researches have shown that propolis has a variety of biological roles, for example antioxidant and antimicrobial activity, this is because of propolis containing the group of phenolic compounds, which make propolis an important compound of study for the most diverse pharmaceutical applications, for example anti-aging and anti-acne cosmetics. Current applications of propolis include formulations for cold syndrome , which are upper respiratory tract infections, common cold, and flu-like infections, as well as dermatological preparations useful in wound healing, treatment of burns, acne, herpes simplex and genitals' and neurodermatitis. (Cabral et al., 2009). Besides that, propolis is a natural remedy that has been included at many health food stores in different forms for topical use. It is commercially available in the form of capsules, mouthwash solutions, creams, throat lozenges, powder, and also in many purified

products from which the wax was removed. (Vijay, 2013). Based on the propolis's benefits, so this research is focus on the formulation of suitable method and condition form for propolis extract which have the high rich of phenolic content.

1.3 Problem statement

After sample preparation, a complete extraction of phenolic compounds is the next critical step. The most common techniques to extract phenolics employ solvents, either organic or inorganic. There are various parameters may influence the yield of phenolics, for example temperature, solvent-to-sample ratio, time as well as solvent type. Furthermore, the optimum recovery of phenolics is different from one sample to the other and relies on the type of sources and its active compounds. The choice of extraction solvents such as water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol) and their mixtures (Garcia-Salas et al, 2010) will influence the yields of phenolics extracted. The choice of solvent could be depend on the properties of the phenolic components of the sources concerned. In this study, ethanol and water were chosen because water and ethanol are common solvent for extraction of biological compound. Furthermore, to selecting the optimal extraction solvent, there are two other important parameters that affect the yield of phenolic extracted, which are time and temperature. Generally, if increasing time and temperature, it will improve the analyte solubility. However, phenolics are generally degraded or undergo undesirable reactions such as enzymatic oxidation by extended extraction times and high temperatures (Biesaga and Pyrzynska, 2013). Besides that, the solvent-to-sample ratio also affect the recovery of phenolics. Increasing the solvent-to-sample ratio promotes phenolic extraction from samples but determining the optimum ratio is advisable so that solvent input and saturation effects of solvent by the phenolics are minimized (Ali Khoddami et al, 2013). So, there must be further study all the parameters that will influence the optimum extraction of propolis to ensure to get the high rich phenolic extract.

1.4 Objective

The following are the objective of this research:

- To investigate the effective solvent, solid to solvent ratio, temperature, time and method for extraction of antioxidant components of propolis.

1.5 Scope

The following are the scopes of this research:

i) To identify the effective extraction solvent (ethanol and water) and solid to solvent ratio (1:10 and 1:20).
ii) To study the best temperature for extraction method between 25°C and 50°C.
iii) To compare two different extraction methods between maceration and ultrasound extraction.
iv) To identify the effective extraction time for the extraction of propolis.
v) To analysis antioxidant component (polyphenols) of propolis and the total amount of phenolic by using Folin-Ciocalteau method.

1.6 Main contribution of this work

Propolis has a huge potential as an alternative source of antioxidant. The phenolic compound which found in propolis can be used for many biological and pharmacological activities for example, anti-inflammatory, antimicrobial, antioxidant effects, et al. However, propolis cannot be used as raw material, and it must be purified by extraction with solvents. The extraction technique used to obtain aggregate value compounds from natural raw materials. So, the antioxidant in propolis extract can be make as cream or other and commercialized to replace the synthetic antioxidant in current market. Furthermore, antioxidant based on propolis extract will give extra value to propolis and give benefit to the bee framer.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Phenolic compound which found in propolis which collected by stingless bee can be used for antioxidant effects. So, propolis extract can be used in cosmetic products. (Ramos and Miranda, 2007) This paper presents of the study for identify a suitable method for extraction of stingless propolis. Maceration method will be conducted by using ethanol and water as solvent. Ultrasound extraction method will compare with maceration method. The parameters like temperature, solid to solvent ratio, and time also were analysed. The Folin-Ciocalteu method currently used to determine the total polyphenol contents. (Liu et al., 2002; Luximon-Ramm et al., 2002).

2.2 Propolis

2.2.1 Introduction of propolis

The Figure 1 is shown the propolis. Propolis with CAS No. 9009-62-5 also called as bee glue is the generic name for the resinous product collected by bees from different plant sources (CHEMID, 1996). The word propolis is derived from Greek word, pro- with the meaning of for or in defence, and polis- with the meaning of the city, therefore propolis is defined as defence of the city or the hive (Ghisalberti, 1979). Propolis is strongly adhesive, so it is utilised by bees to seal holes in their honeycombs, smooth out the internal walls and prevent intruders enter their hive.

The bees are collected resin from the cracks in the bark of trees and leaf buds. This resin will be masticated and added with salivary enzymes. Furthermore, the partially digested material is mixed with beeswax and utilised in the hive (Ghisalberti, 1979; Marcucci,

1995). The composition of raw propolis is different with the source. In general, propolis is composed of 50% of resin and vegetable balsam, 30% of wax, 10% of essential and aromatic oils, 5% of pollen and 5% of various other substances, which including organic debris (Cirasino et al., 1987; Monti et al., 1983).

Figure 1 : Propolis

2.2.2 Historical and current uses of propolis

With the long history of bee domestication, it has led to a thorough exploitation of bee products. Besides that, the many affirmative properties of propolis lead to its application in many human pursuits. The use of propolis is started from at least 300 BC (Ghisalberti, 1979) and continues until today function as home remedies and personal products. This is because propolis is reputed to have the function of antiseptic, antimycotic, bacteriostatic, astringent, choleric, spasmolytic, anti-inflammatory, anaesthetic and antioxidant properties. Besides that, propolis has been claimed effective in wound healing, tissue regeneration, treatment of burns, neurodermatitis, leg ulcers, psoriasis, morphoea, herpes simplex and genitalis, pruritus ani and activity against dermatophytes. Furthermore, propolis has been offered for sale as a treatment for rheumatism and sprains; and in dental medicine, since it is claimed to be an anaesthetic five times as efficient as cocaine. Propolis has also used in toothpaste and mouthwash preparations to treat gingivitis, cheilitis and stomatitis. Next, propolis has also function in pharmaceutical and cosmetic products such as face creams (vanishing creams and

beauty creams), ointments, lotions and solutions. (Ayala et al., 1985; Bankova et al., 1983; Bjorkner, 1994; Dobrowolskiet al., 1991; Esser, 1986; Ghisalberti, 1979; Hausen et al., 1987a; Marcucci, 1995).

2.2.3 Chemical compounds of propolis

Propolis is typically composed of resin and vegetable balsams (50-70%), essential and aromatic oils and beeswax (30-50%), pollen (5-10%) and other constituents which are amino acids, minerals, vitamins A, B complex, E and the highly active bio-chemical substance known as bioflavenoid (Vitamin P), phenols and aromatic compounds. The Table 1 below is showing the chemical compounds that are found in the propolis.

Table 1 : Chemical Compounds of Propolis (Marcucci, 1995)

Basic Structure	Compound
Alcohol	Benzene methanol; cinnamy alcohol; glycerol; α-glycerophospahte; hydroquinone; isobutanol; phenethyl alcohol; prenyl alcohol
Aldehydes	Benzaldehyde; caproic aldehyde; *p*-hydroxybenzaldehyde; isovanillin; protocatechualdehyde; vanillin
Aliphatic acids and aliphatic esters	Acetic acid; angelic acid; butyric acid; crotonic acid; furaric acid; isobutyric acid; methylbutyric acid; isobutyl acetate; isopentyl acetate; isopentenyl acetate
Amino acids	Alanine; β-alanine; α-amino butyric acid; δ-amino butyric acid; arginine; asparagine; aspartic acid; cystine; cystein; glutamic acid; glycine; histidine; hydroxyproline; isoleucine; leucine; lysine, methionine; ornithine; phenyalanine; proline; pyroglutamic acid; sarcosine; serine; threonine; tryptophan, tyrosine; valine
Aromatic acids	*p*- Anisic acid; benzoic acid; caffeic acid; cinnamic; coumaric(-o,-m,-p) acid; 3,4-dimethoxycinnamic acid; ferulic acid; gallic acid; gentistic acid; hydroxycinnamic acid; *p*-

	hydroxyl benzoic acid; isoferulic acid; 4-methoxy cinnamic acid; protocatechuic acid; salicylic acid; vanillic acid; veraric acid
Aromatic esters	Benzyl acetate; benzyl benzoate; benzyl caffeate; benzyl coumarate; benzyl-3,4-dimethoxycinnamate; benzyl ferulate; benzyl isoferulate; benzyl salicylate; buteny; caffeate; butyl caffeate; cinnamyl benzoate; cinnamyl caffeate; butyl caffeate; cinnamyl coumarate; cinnamyl isoferulate; ethyl benzoate; ehyl caffeate; methyl benzoate; 2-methyl-2-butenyl caffeate; 3-methyl-2-butenylcaffeate; 3-methy-2-butenylferulate; 3-methyl-3-butenyl ferulate; 2-methyl-2-butenyl isoferulate; 3-methyl-3-butenylisoferulate; methyl salicylate; phenyl ethyl caffeate; phenyl ethylcoumarate; phenylethylisoferulate; pentyl caffeate; pentenyl caffeate; pentenyl ferulate; prenylcaffeate; prenyl coumarate; prenyl ferulate; prenyl isoferulate
Chalcones and dihydrochalcones	Alpinetin chalcone; naringenin chalcone; pinobanksin chalcone pinobanksin-3-acetate chalcone; pinocembrin chalcone; pinostrobin chalcone; sakuranetin chalcone; 2',6',a-trihydroxy-4'-methoxy chalcon; 2',6'-dihydroxy-4'-methoxydihydro chalcone; 2',4',6'-trihydroxydihydro chalcone
Flavanones	Naringenin; pinabanksin; pinobanksin-3-acetate; pinobanksin-3-butyrate; pinobanksin-3-hexanoate; pinobanksin-3-methyl ether; pinobaksin-3-pentanoate; pinocembrin; pinistrobin; sakuranetin; 3,7-dihydroxy-5-methylflavanone; 2,5-dihydroxy-7-methoxyflavanone
Flavanes and flavonols	Acacetin; apigenin; apigenin-7-methyl ether; chrysin; fisetin; galangin; galangin-3-methyl ether; izalpinin; isorhamnetin; kaempferol-7-methyl ether; kaempferol-7,4-dimethyl ether; pectolinarigenin; quercetin; quercetin-3,7-dimethyl ether; ramnetin ramnocitrin tectocrisin
Hydrocarbons ester	Heneicosane; hentriacontane; heptacosane; hexacosane

ethera, hydroxyl and keto waxes	nonacosane; pentacosane; tricosane; tripentacontane; tritriacontane; dotriacontylhexadecanoate; ; dotriacontyl-[(Z)-octadec-9-enoate] tetracosyl-hexadecanoate;
Waxy acids	Arachid acid; behenic acid; cerotic acid; lauric acid; linoleic acid lignoceric acid; montanic acid; myristic acid; oleic acid; palmitic acids; stearic acid
Ketones	Acetophenone; *p*- acetophenolacetophenone; dihydroxy-acetophenone; methyacetophenone; hept-5-en-2-one; 6-methylketone
Terpenoids and other compounds	α-Acetoxibetulenol; β-bisabolol; 1.8-cineole; α-copaene; cymene; limonene; pterostilbene; styrene; xanthorreol; xylitol; naphthalene; 4-hexanolactone; sesquiterpene alcohol; sesquiterpene alcohol; sesquiterpene diol
Steroid	Calinasterol acetate; β-dihydrofucosterol acetate; ucosterol acetate; stigmasterol acetate
Sugar	Fructofuranose-1; fructofuranose-2; α-D-glucopyranose; β-D-glucopyranose

2.2.4 Melting point

At 25°C to 45°C, propolis is soft, pliable, and sticky substance. Particularly, it becomes hard and brittle when in frozen condition. Above 45°C, it will become more and more sticky and gummy. Propolis will become liquid at 60°C to 70°C, however for some samples the melting point may be as high as 100°C. (Vijay, 2013)

2.2.5 Antioxidant activity of propolis

Polyphenols that contain in the resinous part of raw propolis have been verified to inhibit specific enzymes, stimulate some hormones and neurotransmitters, scavenge free radicals, and stop the multiplication of micro-organisms. According to numerous scientific report, observed antioxidant activity was due to the content of carotenoids, polyphenols and flavones and flavonols which is a group of phenolic compounds.

The antioxidant activity due to the ability of phenolic compounds to donate hydrogen ions that can attack the free radicals to prevent the oxidation reactions in the cell. Besides that, phenolic compound can also prevent the oxidation and deterioration of food substances during storage. The high antioxidant activity of propolis makes it a good natural antioxidant that can function as a natural preservative. (Cao et al., 2004; Sforcin, 2007; Ćetković et al., 2004; Masteikova et al., 2009; Pieta, 2000.)

Due to its antioxidant activity, propolis can be used to treat or prevent free radicals that induced skin damages. Antioxidants can neutralize free radicals before they are touching the cell, as well as stop ongoing interior cellular damage. Antioxidants stop the cascade of free radical damage by donating an electron, thus ending the cycle of further free radical creation. It is important to have antioxidant protection at all cellular layers, because it is impossible to stop all free radicals at the surface. Many of them get through the initial skin barrier or come from inside the cell itself via cellular metabolism. Antioxidants will combine with free radicals can help to prevent a large cascade of damage. (Sehn et al., 2009)

2.3 Stingless bee

2.3.1 Introduction of stingless bee

Stingless bees are exist in all tropical regions of the world. There are hundreds of species have been described. Egg-laying queens are larger than the workers and clear-cut forms of division of labor and task occur among the members of stingless bee colonies. Furthermore, stingless bees have a system of mass-provisioning their brood cells. During short periods, a controlled number of bees deposit the food in the cell, after which the queen lays an egg on top of the food. In all the stingless bee species, this system is defined as a well-defined cycle: periods of cell-building behavior alternate with short bouts of intensive cell-provisioning behavior. (Marinus, 1999)

2.3.2 Stingless bee and propolis

The nests of most stingless bee's species are built within protective cavities such as hollow trees or in the ground. Few species build their nests in exposed positions. Propolis is a sticky dark-colored material that collected by stingless bees from living plants, a mixture of beeswax and plant resins and use in construction and adaptation of their nests (Bankova et al., 2000). Stingless bees use propolis not only as a building material but also as a means of maintaining low levels of bacterial and fungal concentrations in the hive. The Figure 2 is shown the hive of the stingless bee.

Figure 2 : Bee's Hive

2.4 Free Radical

2.4.1 Damage of Free Radical

A state of oxidative stress exists, when more free radical damage occurs than can be neutralized by internal defense system. Living in stressful environments overwhelms the body's natural defenses counter free radical damage. Ongoing oxidative stress will cause an increased rate of aging and eventual illness. Besides that, in-ammation, glycation, and DNA damage also are sources of process of aging. These additional causes of aging are arguably related to or subsets of oxidative stress.

Human skin is naked and is constantly exposed to the air, solar radiation, other

environmental pollutants, or other chemical insults (toxins and accumulated ions), which are capable of inducing the generation of free radicals as well as reactive oxygen species (ROS) of our own metabolism. When combined with the natural aging process, we experience dead lifeless skin, uneven tones, blemishes, pigmentation and wrinkles.

Superoxide dismutase, catalase and glutathione peroxidase are the enzymes for the main system that against oxidation. If the free radicals production becomes exceed than the capacity of enzymatic system, the second line of defence (vitamin C and vitamin E) will activated .Vitamins C and E against free radicals and become oxidized and inactive (Halliwell, 1994).

2.4.2 Sources of Free Radical Damage

There are 80% of free radical damage receive by skin is from exposure to the sun's rays. The solar rays are composed of packets of energy called photons, which are very high-energy particles and also called free radicals.

For tissues which except skin, the cell's own metabolism will produce in more than 85% of all free radical damage. Our cells take oxygen after inhaled by the lungs and use it in enzymatic reactions to burn fuel (glucose, fat, or even protein) and produce energy. Each cell uses its energy to carry out its own individual activities. However, each cell will produces extra energy. As energy is created, radicals are created. The extra radicals will function as free radicals. These extra packets of energy are called free radicals because they are not take part to any particular ongoing biochemical reaction. Free radicals penetrate into the interior of the cell, combining with whatever component they strike and damaging that structure.

Other than sun and internal cellular metabolism, skin, being the body's first line defense system, is exposed to other sources of free radical damage. Other sources of free radical damage to the skin include ozone, pollutants, applied substances (some sunscreens), alcohol, severe physical and emotional stress, poor nutrition, obesity, and toxins. Smoking is also claimed as critically damaging to cells and tissues, by delivering massive amounts of free radicals with every puff. (Dr. Charlene DeHaven, 2014)

2.5 Extraction Method

2.5.1 Maceration

Maceration is a popular and inexpensive way to get essential oils and bioactive compounds. Maceration normally consists of several steps for small scale extraction. Firstly, the sample is grinded into small size to increase the surface area for proper mixing with solvent. Next, the appropriate solvent named as menstruum is added in a closed vessel. Occasional shaking in maceration improve the extraction by two ways, which are increase diffusion and remove concentrated solution from the sample surface for bringing new solvent to the menstruum for more extraction yield. (Azmin et al, 2013). Figure 3 is shown the maceration was conducted in laboratory Universiti Malaysia Pahang.

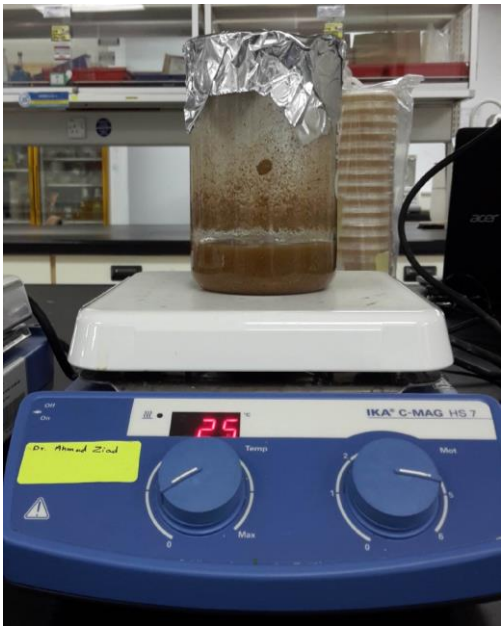

Figure 3: The maceration extraction

2.5.2 Ultrasound Extraction

Ultrasound is a special type of sound wave beyond the human hearing. Generally, ultrasound is from 20 kHz to 100 MHz. Ultrasound can passes through a medium by creating compression and expansion. This process will created a phenomenon called cavitation. Cavitation which means production, growth and collapse of bubbles. A large

amount of energy can produce from the conversion of kinetic energy of motion into heating the contents of the bubble. Only liquid and liquid containing solid materials have cavitation effect. The main benefit of ultrasound is ultrasound energy can facilitates the organic and inorganic compounds leaching from plant matrix (Herrera and Luque de Castro, 2004). This is because ultrasound enhanced the mass transfer and accelerated access of solvent to cell materials of plant parts. The extraction mechanism by ultrasound involves two main types of physical phenomena, which are the diffusion across the cell wall and rinsing the contents of cell after breaking the walls (Mason et al., 1996). Figure 4 is shown the ultrasound probe in propolis extraction.

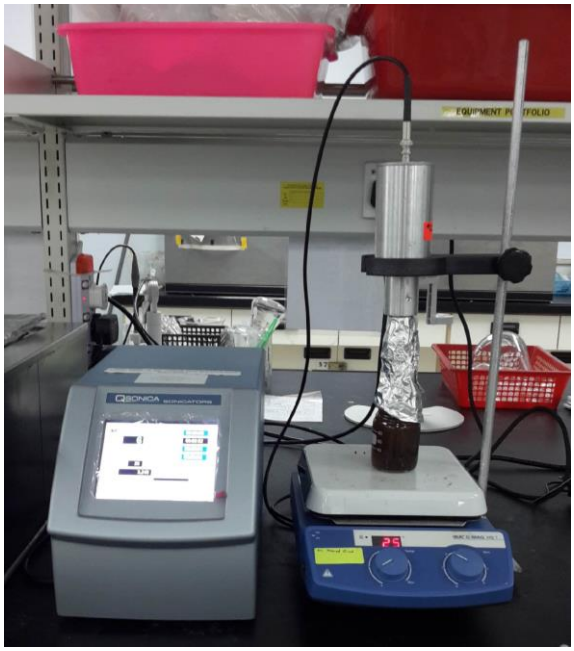

Figure 4: Maceration Assisted by Ultrasound

2.6 Effect of extraction process

The aim of an extraction process is to provide the maximum yield of antioxidant activity and phenolic content. The variables investigated is types of solvent, which between ethanol and water, the composition of solid to solvent (1:10 and 1:20) and the temperature. (25°C and 50°C).

2.6.1 Solvent System

Generally, water and organic solvents such as ethanol, methanol, acetone, and diethyl ether are used for the extraction of polyphenols or other bioactive compounds for example flavonol from plant materials. Furthermore, the type of solvent and the extraction methods being used will affect the percent of recovery. (Sun and Ho, 2005; Turkmen et al., 2006). Solvents with low viscosity will allows them to diffuse into the pores of the plant materials easily to extract out the bioactive compounds. This is because it have low density and high diffusivity. (Naczk and Shahidi, 2006). For the change of solvent polarity, vapour pressure and viscosity of antioxidant compound that are being dissolved in the solvent also change. As a result, the extract's antioxidant activity also change (Zhou and Yu, 2004; Turkmen et al., 2006; Alothman et al., 2009. The selection of solvent systems is decide based on the basis of the efficiency in extracting polyphenols and other antioxidant compounds from sample matrix (Luthria et al., 2006; Alothman et al., 2009).

2.6.2 Temperature

Temperature is one of the parameter used in extraction. This parameter will affect the result of antioxidant yield from the extract because different extraction from phenolic source needed different temperature. The temperature has impact on solubility, diffusion coefficient (mass transfer rate) and stability of the phenolic compounds (Luthria, 2008). An increase in temperature and a decrease viscosity significantly increase the diffusion rate. However, high temperature may degrade the phenolic. (Durling et al., 2007). Thus, heating temperature is of much consideration during processing

2.6.3 Solid to Solvent ratio

One of the effects that influenced the extraction of antioxidant activity is the solid to solvent ratio. The driving force for the extraction is the concentration gradient within the particles, which is related to solvent to solid ratio. The rate of extraction increase with a larger concentration increase with a larger concentration gradient (Cacace &

Mazza, 2003). The solid to solvent normally expressed as the weight of extraction sample (gram) to the volume of solvent (millilitre).

2.6.4 Time

The extraction of phenolic compound from plant and fruit is affected by the time of extraction to maximize the yield of phenolic compound. Furthermore, it is also observed that the optimum extraction time for antioxidant compounds will different with antioxidant capacity. According to the study of Benhammou et al, (2008), it state that as the time of the extraction increase the antioxidant yield will increase. However, based on the study of Herodež et al., (2003) state that the active component yields will not continue to increase once equilibrium is reached. In the other hand, the number of antioxidant capacities will decreased as the maximum time for the extraction is reached. Therefore, to study the extraction time is very important and the maximum time should be identified to avoid reduction of yield of antioxidant compounds.

2.7 Analysis methods

2.7.1 Folin-Ciocalteau method

In the UV/VIS spectrophotometric method, colorimetric reactions are widely used. This is because colorimetric reaction is easy to perform, rapid and applicable in routine laboratory use, and low-cost (Pelozo et al. 2008). However, it is important that a reference substance is need to use in colorimetric assay. This method is measures the total concentration of phenolic hydroxyl groups in the plant extract. A specific redox reagents (Folin-Ciocalteu reagent) react with polyphenols in plant extracts to form a blue complex that can be quantified by visible-light spectrophotometry (Schofield et al, 2001).

The Folin-Ciocalteu method is described in several pharmacopoeias (Council of Europe, 2007). The reaction produces a blue chromophore constituted by a phosphotungstic-phosphomolybdenum complex (Schofield et al, 2001; Gülçin, I, 2004), where the maximum absorption of the chromophores depends on the alkaline solution and the

concentration of phenolic compounds (Schofield et al, 2001). However, this reagent will decomposes rapidly in alkaline solutions, and makes it necessary to utilise an enormous excess of the reagent to obtain a complete reaction. This excess can result in precipitates and high turbidity, making spectrophotometric analysis impossible. To prevent this problem occur, Folin and Ciocalteu included lithium salts in the reagent, which prevented the turbidity (Folin and Ciocalteu, 1927). The reaction generally provides accurate and specific data for several groups of phenolic compounds. This is because differences in unit mass (Glasl, 1983) and reaction kinetics (Folin and Ciocalteu, 1927) can makes many compounds change color differently.

2.8 Total Phenolic Content

In organic chemistry, phenols which also called phenolics, are a class of chemical compounds contenting of a hydroxyl group (—OH) bonded directly to an aromatic hydrocarbon group. Carbolic acid (C6H5OH) as shown in Figure 4 is the simplest of the class phenol.

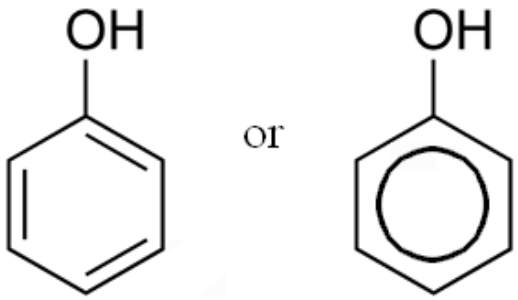

Figure 5 : Carbolic Acid

2.8.1 Flavonol

Flavonols are a non-nutrient, bioactive compound that found in the flavonoid family (Harnly et al, 2006). Flavonoids are 3-ringed polyphenolic compounds. (Harnly et al, 2006). Flavonoids may help prevent the oxidation of low-density lipoproteins (LDL) cholesterol which can be a precursor to atherosclerosis, the plaque buildup which can contribute to heart disease (Huxley and Neil, 2003). Flavonoids continue to be examined by scientists due to their association with beneficial health effects including

reduced risk of cardiovascular disease, protection against LDL cholesterol oxidation and a possible, but conflicting, link to cancer prevention.

Besides that, many studies have shown that propolis has a number of biological roles, including antioxidant and antimicrobial activity, both conferred mainly by substances belonging to the group of phenolic compounds, especially flavonoids, which make propolis an important object of study for the most diverse pharmaceutical applications, such as anti-aging and anti-acne cosmetics (Cabral et al., 2009).

2.9 Ultra-Violet Visible Spectrometer

Ultra-Violet Visible Spectrometer (UV/VIS) as shown in Figure 5 is the instrument used to measures the intensity of light passing through a sample (I), and compares it to the intensity of light before it passes through the sample (Io). This device was used in the quantitative determination of antioxidant activity, phenolic content and flavonol content in the extracted sample.

Figure 6 : UV - Visible Spectrometer

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview

This paper presents an effective condition for stingless propolis extraction. The parameters like solvent (ethanol and water), solid to solvent ratio (1:10 and 1:20), temperature (25°C and 50°C) and extraction method (maceration and ultrasound). Total polyphenol content and flavonoid content of propolis extract will be analysed by colorimetric method (Folin-Ciocalteu method).

3.2 Introduction

In this research, the methods for extract the propolis with antioxidant activity were separated into three main parts which are sample preparation, extraction of samples with different parameters and evaluation the content of extracts.

3.3 Chemicals

3.3.1 List of chemicals

Experiment was conducted in the Biological Laboratory of University Malaysia Pahang. Gallic acid and sodium carbonate were purchased from Sigma-Aldrich. The Folin-Ciocalteu reagent (Fluka) was purchased from R&M Chemicals. Distilled water and ethanol 70% were analytical grade and obtained from chemical store of University Malaysia Pahang (Gambang, Malaysia) and used without any further purification. Propolis (*Trigona Thoracica)* was obtained by AMS Stingless Bee Farm.

3.3.2 Description of chemical used

Propolis was art as active compound in extraction. Solvents that were used for solvent extraction were ethanol and water. For Folin Ciocalteu assay, Folin Ciocalteu reagent, sodium carbonate (NaCO3) and were used. Gallic acid was used to prepare the standard for calibration curve.

3.4 Method

3.4.1 Collection of Sample

Propolis sample was collected from *Trigona Thoracica* stingless bee hives at AMS Stingless Bee Farm. Samples were cut in small piece and were stored at - 20°C until use. (Cunha et al., 2004)

3.4.2 Preparation of the Extraction of Propolis by Using Maceration

Pulverized raw propolis (10 g) was placed inside a 500mL beaker and corresponding amount of solvent (1:10 or 1:20) was added. The sample and solvent was stirred with magnetic stirrer. For every 30 minutes, 1mL of propolis extract was collected to analysis the total phenolic content. The analysis was continued until the maximum extraction time was reached and shown decreasing in total phenolic content. The heating system of hot plate was turned on, if the temperature 50°C was needed. (Boryana Trusheva et al., 2007)

3.4.3 Preparation of Propolis Extract Using Ultrasound

Pulverized raw propolis (10 g) was placed inside a 500mL beaker and corresponding amount of solvent (1:10 or 1:20) was added. The ultrasound probe will set as 10% duty cycle or 20% duty cycle with the constant 6 amplitude. The sample and solvent were stirred with magnetic stirrer with the ultrasound assisted. For every 30 minutes, 1mL of

propolis extract was taken to analysis the total phenolic content. The analysis was continued until the maximum extraction time was reached and shown decreasing in total phenolic content. The heating system of hot plate was turned on, if the temperature 50°C was needed. (Boryana Trusheva et al., 2007)

3.4.4 Total Phenolic Content of Propolis Extract

Total phenolic content was determined by using Folin-Ciocalteu method. 0.1 mL extract was mixed with 2.5mL distilled water, 0.1mL of Folin-Ciocalteu reagent and 0.5mL of 20% sodium carbonate. The mixture was left at room temperature for 2 h until the colour was developed and the absorbance was then measured at 760 nm wavelength. Total phenolic content was estimated using calibration curve of gallic acid, concentration rang 0 – 1mg/mL. The total phenolic content was expressed in mg of gallic acid equivalent mg (GAE) per g of sample used. (Loreta Kubiliene et al, 2015)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter is about results and discussion of the research. In this chapter, it is consist of 6 subtopic, which are the calibration curve, the effect of solvent and solid to solvent ratio, the effect of temperature, the effect of duty cycle, the effect of method and the effect of extraction time. Each parameters will discussed based on the result obtained.

4.2 The calibration curve

The total phenolic concentration of the propolis extracts in terms of gallic acid equivalent (GAE) was calculated from the standard curve equation: y= 2.8323x with the R^2= 0.9968 which shown in Figure 7. The concentration range was 0 to 1.05mg/mL. Yield of total phenolic content was calculated using the Equation 1 shown below:

$$\text{Yield (mg/g)} = \frac{Concentration\ of\ total\ phenolic\ (mg/mL) \times volume\ of\ solvent\ used(mL)}{total\ weight\ of\ propolis\ sample\ used(g)} \quad \text{(Equation 1)}$$

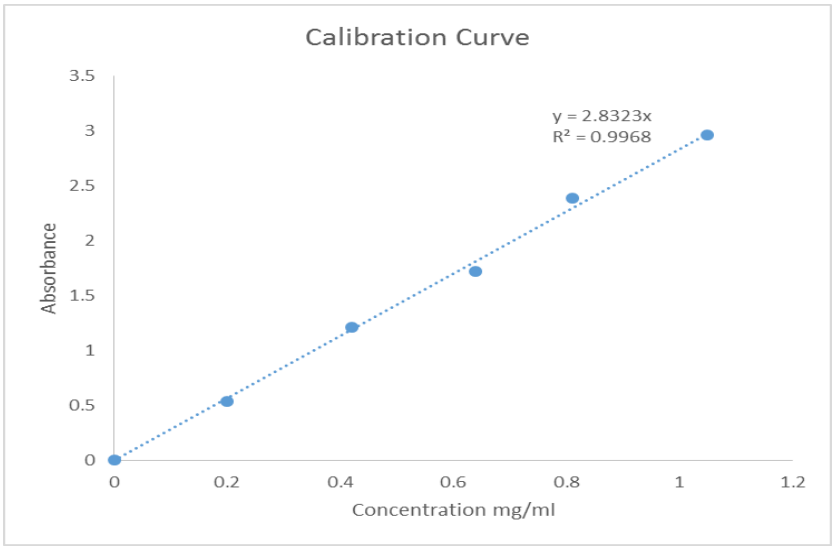

Figure 7: Standard calibration curve for the quantification of total phenolic content

4.3 The Effect of Solvent and Solid to Solvent Ratio

Based on Figure 8, the yield of total phenolic content of solid to solvent ratio 1:20 was slightly higher than 1:10 for both solvents. The yield for water extract with 1:10 ratio was 2.69GAEmg/g and for 1:20 ratio the yield was 2.71 GAEmg/g. Furthermore, the yield for ethanol extract with 1:10 ratio was 31.52 GAEmg/g and 32.80GAEmg/g for 1:20 ratio. ANOVA statistical test shows the value of $P > 0.05$ ($P = 0.3452$). Based on the ANOVA test, the 1:10 ratio and 1:20 ratio of ethanol extraction was no significantly influence the extraction yield. There is a physical process when dissolving of bioactive components into a solvent. So, the reasons for the yield of 1:20 solid to solvent ratio is slightly higher than 1:10 solid to solvent ratio were consistent with mass transfer principles where the driving force for mass transfer is considered to be the concentration gradient between the sample and the solvent. The higher solid-to-solvent ratio increase concentration gradient and as a increase diffusion rate that allows more extraction of solids by solvent (Al-Farsi and Chang, 2007). Furthermore, the chance of bio-active components coming into contact with solvent increased with increase amount of extraction solvent. This will leading to higher leaching-out rates (Zhang et al., 2007). Overall, the main effect of the solid-to-solvent ratio was to modify the solubility and equilibrium constant. Thus increase the total phenolic yields to a maximum at the highest solid-to-solvent ratio (Cacace and Mazza, 2003). However, the result showed only slightly increase of active component yields and did not much influence in the extraction yield. This is because the equilibrium is almost reached (Herodež et al.,

2003). According to Zhang et al., (2007), there is a possibility of bio-active component coming into contact with the extracting solvent when the amount of solvent is increase, however it will not increase as equilibrium is reached. This means that, the extraction of solvent will not increase the yield as all the solvent has reacted with the solid. The optimum yield of phenolic content can reach when the solid to solvent ratio reach it optimum condition. So, this shows that the solid to solvent larger than 1:10 is unnecessary. This approach will aid in efficient usage of solvent and solvent mixtures for extracting phytochemicals and avoidance of saturation effect, as well as reducing solvent waste disposal cost. Furthermore, use of high solid-to-solvent ratios would cause in dilute solutions. (Ho et al., 2008)

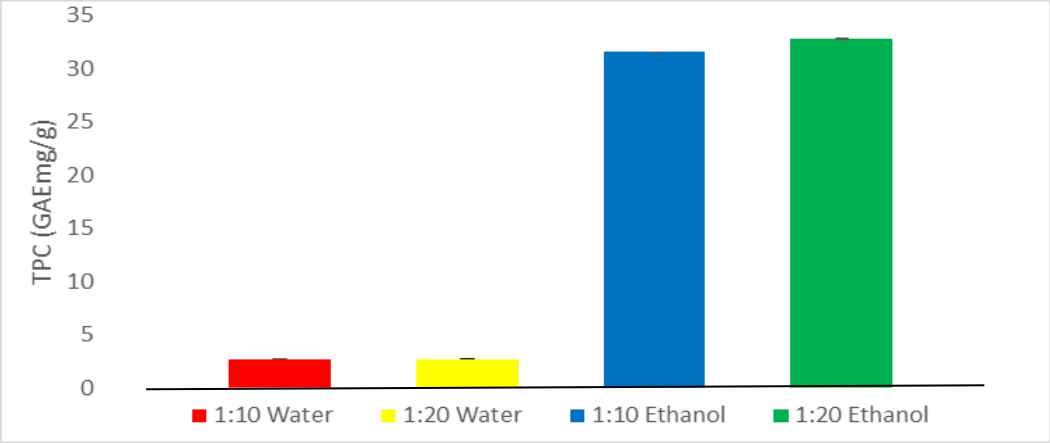

Figure 8: The effect of solid to solvent ratio (1:10 and 1:20) and solvent (water and ethanol) to yield of total phenolic content (GAEmg/g).The fixed parameters are temperature (25°C) and method (maceration)

Table 2: The summary table for the effect of solid to solvent ratio (1:10 and 1:20) and solvent (water and ethanol) to yield of total phenolic content (GAEmg/g). The fixed parameters are temperature (25°C) and method (maceration)

Type of Solvent	Solid to Solvent ratio	Yield (GAEmg/g)
Water	1: 10	2.69 ± 0.000
	1:20	2.71 ± 0.004
Ethanol	1:10	31.52 ± 0.021
	1:20	32.80 ± 0.041

The yield of extraction depends on the polarity of solvent. This shows that the extraction yield increases with increasing polarity of the solvent used in extraction. It

can be found that the yield of the water extract (2.69GAEmg/g) was much less than that of the ethanol extract (31.52GAEmg/g). This may be due to the fact that ethanol is an aqueous solvent which is suitable for extracting some bioactive compounds with strong polarity (Chunli Sun et al, 2015). Hydrogen bonding influences the solubility of one substance to another, and it has been shown that ethanol is better hydrogen bond donors and acceptors than water. (Nedić, 2011). According to Xu et al. (2006), the yield for water extraction is low also because the polyphenols present in plant tissues are often bonded with other molecules (e.g. proteins and polysaccharides) via hydrogen and hydrophobic bonds. Consequently, a good solvent for extraction must not only display high solvency but should also be able to break the hydrogen bonds. The ethanol solvent is suitable for extracting some bioactive compounds with broad range of polarity. Thus, these results imply that ethanol solvent may be appropriate to extract phenolic of propolis. (Chunli Sun et al, 2015). Polar solvents are frequently used for recovering polyphenols from plant matrices. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption. (Quy Diem Do et al, 2014). Besides that, water extraction is the low yield of phenolics with low polarity. So, water is an ineffective solvent for phenolic extraction of propolis and ethanol is shown the most suitable solvent for phenolic extraction.

4.4 The Effect of Temperature

Based on Figure 9, the yield of ethanol extract of total phenolic content at 50°C was higher than 25°C about 11%. The yield for ethanol extract at 50°C was 35.37GAEmg/g and for 25°C the yield was 31.52 GAEmg/g. Based on the ANOVA test, the P value was 0.02850 which was $P < 0.05$, so there was significantly influence the extraction yield.

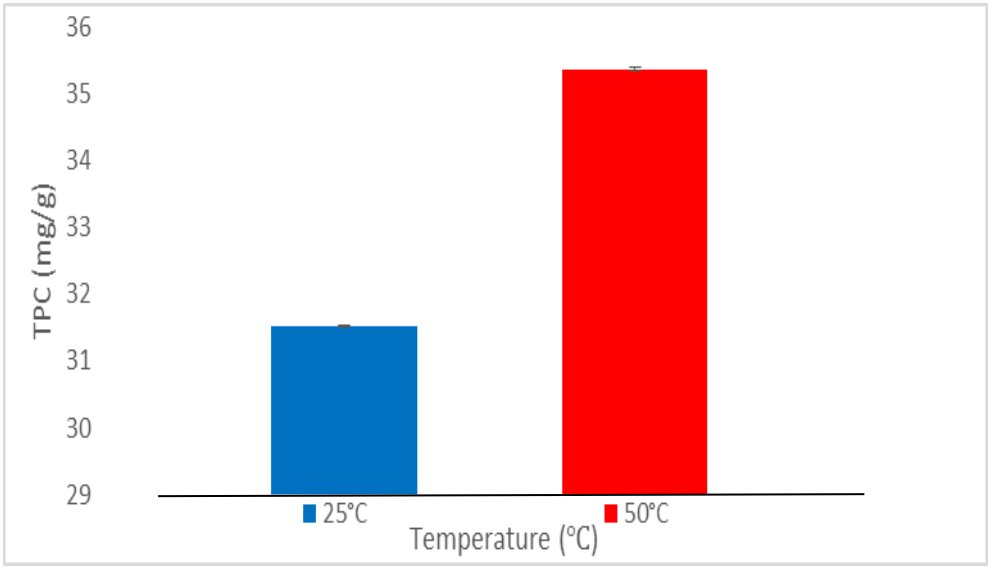

Figure 9: The effect of temperature (25°C and 50°C) on yield of total phenolic content (GAEmg/g). The fixed parameters are solid to solvent ratio (1:10) of propolis, solvent (ethanol) and method (maceration).

Table 3: The summary table of the effect of temperature (25°C and 50°C) on yield of total phenolic content (GAEmg/g). The fixed parameters are solid to solvent ratio (1:10) of propolis, solvent (ethanol) and method (maceration).

Temperature	Yield (GAEmg/g)
25°C	31.52 ± 0.021
50°C	35.37 ± 0.017

TFC yield increased with the increase of temperature maybe because at high temperature the propolis tissue softened and weakened the phenol–protein and phenol–polysaccharide interactions (Shi.et al. 2003) that resulted more polyphenols dissolving into the solvent. Furthermore, some studies reported that heat improved the extraction efficiency of phenolic compounds and enhanced antioxidant activity of phenolic extracts (Benmezianne, et al, 2014; Dorta, et al, 2012). This was probably due to increase in temperature increase phenolic solubility, faster diffusion rate, better mass transfer and extraction yield (Richter, et al, 1996). Besides that, higher extraction temperature may improve the recovery, because in hot condition weaken the cell wall integrity and some pectic polysaccharides from cell wall could be extracted. (Sun, et al, 2002). As a result, the solvent containing ethanol can easily get in contact with the phenolic materials, and the yield of phenolic content is improved. In other hand, increasing temperature extraction above certain values may promote possible concurrent degradation of phenolic compounds (Abderrahmane Mokrani and Khodir Madani,

2016) However, based on the result, at 50°C a good extraction yield of phenolic content was achieved without affecting the stability of phenolic compounds.

4.5 The Effect of Duty Cycle

Figure 10 shows that the extraction yield with increasing of duty cycle (DC). For the duty cycle of 20%, the highest yield was 51.22GAEmg/g and at 90 minute. However, for duty cycle 10%, the highest yield which 43.21GAEmg/g at 120minute. There was 18.54% increase of yield, when duty cycle increasing from 10% to 20%. Based on the ANOVA test, the P value was 0.0193 which was $P < 0.05$, so there was significantly influence the extraction yield.

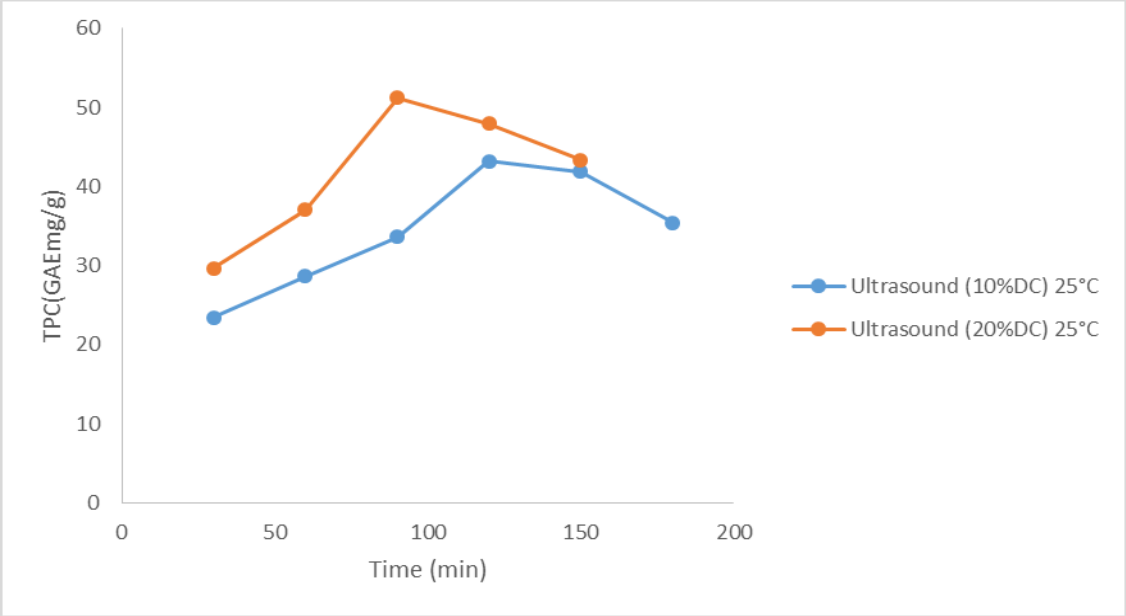

Figure 10: The effect of duty cycle on the yield of total phenolic content and extraction time. The fixed parameter are solvent (ethanol), solid to solvent ratio(1:10) and temperature(25°C).

Duty cycle is a measure of how long the cells are exposed to ultrasound waves. The higher the duty cycles the greater the damage of the cell walls and consequently facilitate the release of larger amounts of phenolic contents (Eleni Naziri, 2012). Herrera and Luque de Castro (2004) found that the duty cycle was a significant factor in the ultrasound extraction of phenolic compounds from strawberries. Besides that, Kobus (2008) also found that pulsed ultrasound effectively accelerated the extraction of

bioactive components from dried roots of valerian. In conclusion, optimum extraction of phenolic compounds from propolis were found at 20% duty cycle of ultrasound

4.6 The Effect of Type of Methods

Based on Figure 11, the yield of ethanol extract of total phenolic content with the ultrasound assisted extraction was higher (about 52.55%) than the maceration. The yield for ethanol extract with the ultrasound assisted extraction was 53.96 GAEmg/g and for the maceration was 35.37 GAEmg/g. Based on the ANOVA test, the P value was 0.04608 which is $P < 0.05$, so there was significantly influence the extraction yield.

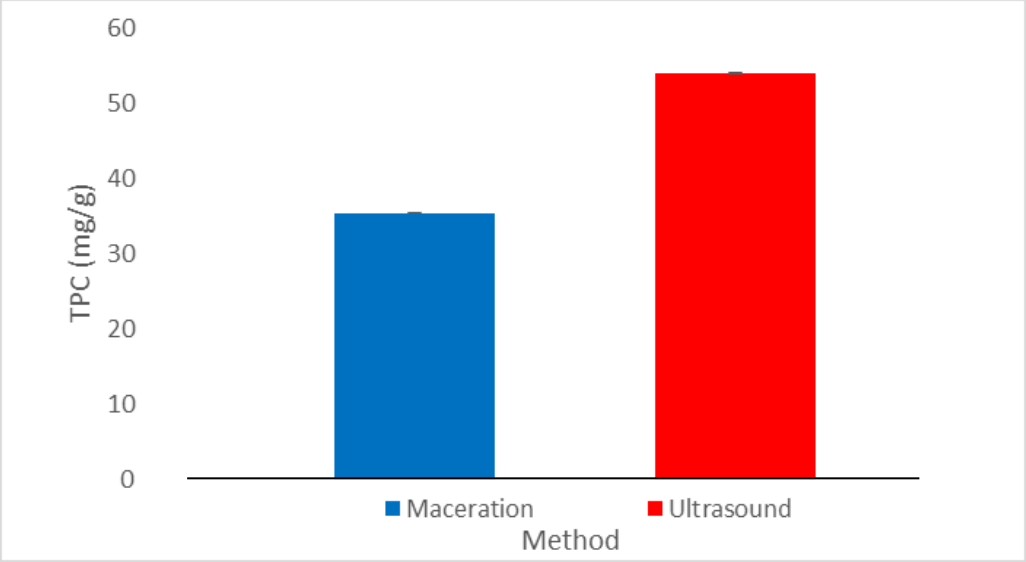

Figure 11: The effect of types of methods (maceration and ultrasound assisted extraction) on the yield of total phenolic content. The fixed parameters are temperature (50°C), solvent (ethanol) and solid to solvent ratio (1:10)

Table 4: The summary table of effect of types of methods (maceration and ultrasound assisted extraction) on the yield of total phenolic content. The fixed parameters are temperature (50°C), solvent (ethanol) and solid to solvent ratio (1:10)

Methods	Yield(GAEmg/g)
Maceration extraction	35.37 ± 0.017
Ultrasound assisted extraction	53.96 ± 0.008

Sound waves can create bubbles in a liquid and generate negative pressure. The bubbles form, grow and finally collapse. Close to a solid boundary, cavity collapse is asymmetric and generate high-speed jets of liquid. The liquid jets have strong impact on the solid surface of sample (Luque-Garcia and Luque de Castro, 2003). Furthermore, ultrasound can penetrate the matrix material, rupturing the cell walls, resulting more phenolic release from the matrix into the extraction medium (Wang, 2006) Besides that, ultrasound can enhance the extracting power of the solvent by driving solvent into the matrix to extract the targeted components and increase the extraction rate. According to Gabaldo´nLeyva et al. (2007), ultrasound increased mass transfer of some compounds from red bell pepper by increasing cell wall permeability. So, the controlling mechanism of ultrasound-assisted extraction is generally attributed to mechanical, cavitation, and thermal effects which can result in disruption of cell walls, particle size reduction, and enhanced mass transfer across cell membranes, which lead to target compounds dissolving in the solvent. (Shirsath, 2012)

Besides that, ultrasound-assisted extraction is an attractive alternative to conventional extraction techniques because it is easy, inexpensive and efficient. The main benefit of including ultrasound in an extraction procedure is that it increases yield of the propolis extraction process.

4.7 The Effect of Extraction Time

As shown in Figure 12, the extraction time for the maximum yield of total phenolic content obtained was between 90 to 150 min. The duration allowed extraction of 53.96 mg GAE/g of TPC from propolis for ultrasound assisted extraction at 50°C with ethanol as solvent, 1:10 solid to solvent and 20 duty cycle (DC). Next, for ultrasound assisted extraction with ethanol as solvent, 1:10 solid to solvent, 20 duty cycle (DC) and at room temperature, the duration for maximum yield of TPC (51.22mgGAE/g) was 90 min. Then, for ultrasound assisted extraction with ethanol as solvent, 1:10 solid to solvent, 10 duty cycle (DC) and at room temperature, the duration for maximum yield of TPC (43.21mgGAE/g) was 120 min. Furthermore, for maceration extraction with ethanol as solvent, 1:10 solid to solvent and 50°C, the duration for maximum yield of TPC (35.37mgGAE/g) was 150 min. Besides that, for maceration extraction with ethanol as

solvent, 1:20 solid to solvent and at room temperature, the duration for maximum yield of TPC (32.80mgGAE/g) was 120 min. Then, for maceration extraction with ethanol as solvent, 1:10 solid to solvent and at room temperature, the duration for maximum yield of TPC (31.52mgGAE/g) was 120 min. For both 1:10 and 1:20 water maceration extraction at the room temperature, the maximum yield were 2.69GAEmg/g and 2.71GAEmg/g respectively at 480 min. After the maximum yield of TPC, increasing the extraction time did not improve the recoveries.

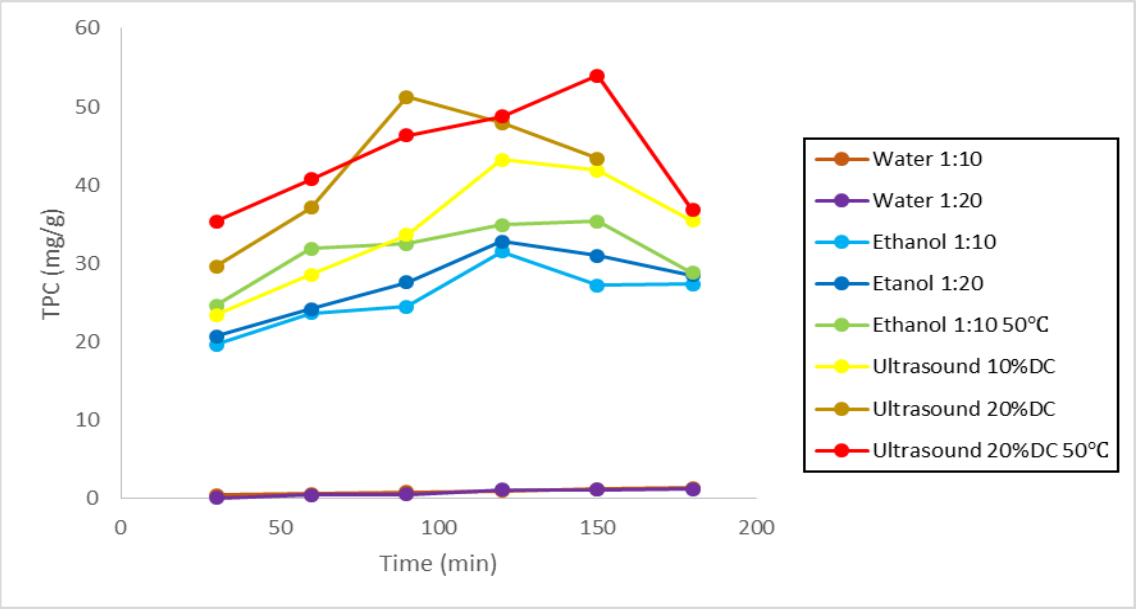

Figure 12: The effect of extraction time on yield of total phenolic content for all of the parameters (temperature, solvent, solid to solvent ratio, method and duty cycle)

Benhammou et al, (2008) stated that as the time of the extraction increase the antioxidant yield will also increase. However, according to Herodež et al., (2003) the active component yields will not continue to increase once equilibrium is reached. Besides that, the value of antioxidant capacities is significantly decreased as the maximum time for the extraction is reached. The result could be explained by the Fick's second law of diffusion which predicts a final equilibrium between the concentrations of solute in the solid matrix and in the bulk solution after a certain time. Therefore, a longer time is not required to extract more phenolic (Silva, 2007). Furthermore, longer extraction times increase the possibility of phenolic oxidation (Naczk, 2006). This oxidation might be prevented by addition of reducing agents to the solvent system (Khanna, 1968). Apart from environmental factor, reduction of phenolic content with longer extraction time could also be due to the endogenous enzymes in plant tissues

destroyed the phenolic compounds in propolis extract (Kuljarachanan et al., 2009). Therefore, to study the extraction time is definitely important to recognize the suitable parameter to be used in the extraction of propolis. According to Chew et al., (2011) extraction time is a crucial in solvent extraction because appropriate extraction time can result in time saving. In conclusion, the extraction time of 150 min was selected as the best extraction time for ultrasound assisted extraction at 50°C to extract the phenolic compounds of propolis.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

This Chapter is presented conclusion can be drawn from the result and discussion in the previous chapter, followed by recommendations.

5.1 Conclusion

In this research, the aim is to investigate the effective solvent, solid to solvent ratio, temperature, time and method for extraction of antioxidant components of propolis.

Based on the results, ultrasound assisted extraction with ethanol (1:10) at 50°C is the most effective way to obtain the highest total phenolic content (53.96 mg GAE/g) of extraction of propolis. Additionally, the ultrasound should be set with 20% duty with the extraction time of 150min.

These findings can be taken as consideration for the ways to future research in propolis extraction.

5.2 Recommendations

Based on the study in this thesis, a number of recommendations has been developed to the future of study:

- The propolis extract can further analysis phenolic antioxidant activity by using DPPH radical scavenging. This will ensure that, the phenolic content in propolis has high antioxidant activity.

- The propolis extract can further analysis it composition by High Performance Liquid Chromatography (HPLC). This will ensure the extract sample is contain the phenolic compound and the compounds are identified.
- The propolis extract can further purify and use it for producing type of product, for example cream with antioxidant function.

CHAPTER 6

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CHAPTER 7

APPENDIX

Calibration Curve Data

ABS = Absorbance

Concentration			ABS 1	ABS 2	ABS 3	ABS 4	ABS 5	Average
0	mg/mL	0ppm	0	0	0	0	0	0
0.2	mg/mL	20ppm	0.539	0.539	0.539	0.539	0.539	0.539
0.42	mg/mL	42ppm	1.208	1.208	1.208	1.208	1.21	1.2084
0.64	mg/mL	64ppm	1.715	1.715	1.715	1.715	1.715	1.715
0.81	mg/mL	81ppm	2.389	2.389	2.389	2.389	2.389	2.389
1.05	mg/mL	105ppm	2.958	2.958	2.958	2.958	2.958	2.958

Water Extraction (1:10, 25°C)

Time (minute)	ABS 1	ABS 2	ABS 3	ABS 4	ABS 5	Average	Gradient	Concentration (mg/mL)	Yield (mg/g)
30	0.121	0.121	0.121	0.121	0.121	0.121	2.8323	0.042721463	0.469936094
60	0.162	0.162	0.163	0.163	0.163	0.1626	2.8323	0.057409173	0.6315009
90	0.21	0.21	0.21	0.21	0.21	0.21	2.8323	0.074144688	0.815591569
120	0.255	0.255	0.255	0.255	0.255	0.255	2.8323	0.090032836	0.990361191
150	0.321	0.321	0.321	0.321	0.321	0.321	2.8323	0.113335452	1.246689969
180	0.346	0.346	0.346	0.346	0.346	0.346	2.8323	0.1221622	1.343784204
210	0.386	0.386	0.386	0.386	0.386	0.386	2.8323	0.136284998	1.499134979
240	0.388	0.388	0.388	0.388	0.388	0.388	2.8323	0.136991138	1.506902517

300	0.397	0.396	0.396	0.396	0.396	0.3962	2.8323	0.139886311	1.538749426
330	0.498	0.498	0.498	0.498	0.498	0.498	2.8323	0.175828832	1.934117149
360	0.562	0.562	0.562	0.562	0.562	0.562	2.8323	0.198425308	2.182678389
390	0.600	0.600	0.600	0.600	0.600	0.600	2.8323	0.211841966	2.330261625
420	0.575	0.575	0.575	0.575	0.574	0.5748	2.8323	0.202944603	2.232390637
450	0.616	0.616	0.616	0.616	0.616	0.616	2.8323	0.217491085	2.392401935
480	0.693	0.693	0.693	0.693	0.693	0.693	2.8323	0.244677471	2.691452177
510	0.506	0.506	0.506	0.506	0.506	0.506	2.8323	0.178653391	1.965187304
540	0.554	0.554	0.555	0.555	0.554	0.5544	2.8323	0.195741976	2.153161741
570	0.523	0.523	0.523	0.523	0.523	0.523	2.8323	0.18465558	2.031211383
600	0.509	0.509	0.509	0.509	0.509	0.509	2.8323	0.179712601	1.976838612

Example Calculation

For time 30 minute,

Average = (0.121 + 0.121 + 0.121 + 0.121 + 0.121)/5 =0.121

Concentration = 0.121/2.8323 = 0.042721463 GAE mg/mL

Yield = (0.042721463 mg/mL X 110mL of solvent)/ 10g of propolis = 0.469936094mg/g

Water Extraction (1:20, 25°C)

Time (minute)	Abs 1	Abs 2	Abs 3	ABS4	ABS 5	Average	Gradient	Concentration (mg/mL)	Yield (mg/g)
30	0.011	0.011	0.011	0.011	0.011	0.011	2.8323	0.003883769	0.081559157

60	0.061	0.062	0.061	0.061	0.061	0.0612	2.8323	0.021607881	0.453765491
90	0.07	0.07	0.07	0.069	0.07	0.0698	2.8323	0.024644282	0.517529923
120	0.151	0.151	0.151	0.151	0.151	0.151	2.8323	0.053313561	1.11958479
150	0.155	0.155	0.155	0.155	0.155	0.155	2.8323	0.054725841	1.149242665
180	0.168	0.168	0.168	0.168	0.168	0.168	2.8323	0.05931575	1.245630759
210	0.169	0.169	0.169	0.169	0.169	0.169	2.8323	0.05966882	1.253045228
240	0.196	0.196	0.196	0.196	0.196	0.196	2.8323	0.069201709	1.453235886
270	0.197	0.197	0.197	0.197	0.197	0.197	2.8323	0.069554779	1.460650355
300	0.231	0.231	0.232	0.231	0.231	0.2312	2.8323	0.081629771	1.714225188
330	0.315	0.315	0.315	0.315	0.315	0.315	2.8323	0.111217032	2.335557674
360	0.304	0.304	0.304	0.304	0.304	0.304	2.8323	0.107333263	2.253998517
390	0.324	0.324	0.324	0.324	0.324	0.324	2.8323	0.114394662	2.402287893
420	0.32	0.32	0.32	0.32	0.32	0.32	2.8323	0.112982382	2.372630018
450	0.366	0.365	0.365	0.366	0.365	0.3654	2.8323	0.129011757	2.709246902
480	0.333	0.333	0.333	0.333	0.333	0.333	2.8323	0.117572291	2.469018112
510	0.314	0.314	0.314	0.314	0.314	0.314	2.8323	0.110863962	2.328143205
540	0.263	0.263	0.263	0.263	0.263	0.263	2.8323	0.092857395	1.950005296
570	0.286	0.286	0.286	0.286	0.286	0.286	2.8323	0.100978004	2.120538079
600	0.274	0.274	0.274	0.274	0.274	0.274	2.8323	0.096741164	2.031564453

Ethanol Extraction (1:10, 25°C)

Time (minute)	ABS1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.505	0.505	0.505	0.506	0.506	0.5054	2.8323	0.178441549	1.784415493	19.62857
60	0.609	0.609	0.61	0.61	0.61	0.6096	2.8323	0.215231437	2.152314373	23.67546
90	0.631	0.631	0.631	0.631	0.632	0.6312	2.8323	0.222857748	2.228577481	24.51435

120	0.812	0.812	0.812	0.811	0.811	0.8116	2.8323	0.286551566	2.865515659	31.52067
150	0.701	0.701	0.702	0.702	0.702	0.7016	2.8323	0.247713872	2.477138721	27.24853
180	0.705	0.705	0.705	0.705	0.706	0.7052	2.8323	0.248984924	2.489849239	27.38834
210	0.678	0.678	0.678	0.678	0.678	0.678	2.8323	0.239381421	2.393814215	26.33196

Actual Concentration = concentration X 10 dilution factor

Ethanol Extraction (1:20, 25°C)

Time (minute)	ABS1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.279	0.279	0.279	0.279	0.279	0.279	2.8323	0.098507	0.985065	20.68637
60	0.326	0.326	0.326	0.326	0.326	0.326	2.8323	0.115101	1.151008	24.17117
90	0.372	0.372	0.372	0.372	0.372	0.372	2.8323	0.131342	1.31342	27.58182
120	0.442	0.443	0.443	0.442	0.442	0.4424	2.8323	0.156198	1.561981	32.80161
150	0.418	0.418	0.418	0.418	0.418	0.418	2.8323	0.147583	1.475832	30.99248
180	0.384	0.384	0.384	0.384	0.384	0.384	2.8323	0.135579	1.355789	28.47156
210	0.406	0.406	0.406	0.406	0.406	0.406	2.8323	0.143346	1.433464	30.10274
240	0.408	0.408	0.408	0.408	0.408	0.408	2.8323	0.144053	1.440525	30.25103

Actual Concentration = concentration X 10 dilution factor

Ethanol Extraction (1:10, 50°C)

Time (minute)	ABS 1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.635	0.636	0.635	0.636	0.636	0.6356	2.8323	0.224411256	2.244112559	24.685238
60	0.82	0.821	0.822	0.822	0.822	0.8214	2.8323	0.290011651	2.900116513	31.901282

90	0.838	0.837	0.838	0.838	0.838	0.8378	2.8323	0.295801998	2.958019984	32.53822
120	0.898	0.899	0.899	0.899	0.899	0.8988	2.8323	0.317339265	3.173392649	34.907319
150	0.91	0.911	0.911	0.911	0.911	0.9108	2.8323	0.321576104	3.215761042	35.373371
180	0.74	0.741	0.741	0.741	0.741	0.7408	2.8323	0.261554214	2.615542139	28.770964
210	0.713	0.713	0.713	0.713	0.713	0.713	2.8323	0.251738869	2.517388695	27.691276

Actual Concentration = concentration X 10 dilution factor

Ethanol Extraction (Ultrasound assisted, 10%duty cycle, 1:10, 25°C)

Time (minute)	ABS 1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.604	0.604	0.604	0.604	0.604	0.604	2.8323	0.213254246	2.132542457	23.45797
60	0.737	0.737	0.736	0.737	0.737	0.7368	2.8323	0.260141934	2.601419341	28.61561
90	0.864	0.865	0.866	0.866	0.866	0.8654	2.8323	0.305546729	3.055467288	33.61014
120	1.113	1.111	1.113	1.113	1.113	1.1126	2.8323	0.392825619	3.928256188	43.21082
150	1.079	1.079	1.079	1.079	1.079	1.079	2.8323	0.380962469	3.809624687	41.90587
180	0.912	0.912	0.912	0.912	0.913	0.9122	2.8323	0.322070402	3.220704021	35.42774

Actual Concentration = concentration X 10 dilution factor

Ethanol Extraction (Ultrasound assisted, 20% duty cycle, 1:10, 25°C)

Time (minute)	ABS 1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.762	0.763	0.763	0.763	0.764	0.763	2.8323	0.269392	2.693923666	29.63316
60	0.955	0.955	0.955	0.955	0.955	0.955	2.8323	0.337182	3.371817957	37.09
90	1.316	1.319	1.319	1.32	1.32	1.3188	2.8323	0.465629	4.65628641	51.21915
120	1.234	1.236	1.236	1.234	1.234	1.2348	2.8323	0.435971	4.359707658	47.95678

150	1.115	1.116	1.116	1.118	1.118	1.1166	2.8323	0.394238	3.942378985	43.36617

Actual Concentration = concentration X 10 dilution factor

Ethanol Extraction Trial 1 (Ultrasound assisted, 20% duty cycle, 1:10, 50°C)

Time (minute)	ABS 1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.968	0.969	0.969	0.97	0.97	0.9692	2.8323	0.342195	3.421953889	37.64149
60	1.063	1.064	1.064	1.064	1.065	1.064	2.8323	0.375666	3.756664195	41.32331
90	1.211	1.211	1.211	1.211	1.211	1.211	2.8323	0.427568	4.275677012	47.03245
120	1.245	1.246	1.247	1.247	1.246	1.2462	2.8323	0.439996	4.399957632	48.39953
150	1.526	1.527	1.527	1.527	1.527	1.5268	2.8323	0.539067	5.390671892	59.29739
180	1.009	1.01	1.011	1.011	1.012	1.0106	2.8323	0.356812	3.568124846	39.24937

Actual Concentration = concentration X 10 dilution factor

Ethanol Extraction Trial 2 (Ultrasound assisted, 20% duty cycle, 1:10, 50°C)

Time (minute)	ABS 1	ABS2	ABS3	ABS4	ABS5	Average	Gradient	Concentration (mg/mL)	Actual Concentration (mg/mL)	Yield (mg/g)
30	0.852	0.852	0.852	0.852	0.852	0.852	2.8323	0.300816	3.008155916	33.08972
60	1.034	1.034	1.034	1.034	1.034	1.034	2.8323	0.365074	3.650743212	40.15818
90	1.175	1.177	1.177	1.179	1.179	1.1774	2.8323	0.415705	4.157045511	45.7275
120	1.264	1.264	1.264	1.264	1.264	1.264	2.8323	0.44628	4.462804081	49.09084
150	1.252	1.252	1.252	1.252	1.252	1.252	2.8323	0.442044	4.420435688	48.62479
180	0.886	0.886	0.886	0.886	0.886	0.886	2.8323	0.31282	3.128199696	34.4102
210	0.816	0.816	0.816	0.816	0.816	0.816	2.8323	0.288105	2.881050736	31.69156

Actual Concentration = concentration X 10 dilution factor