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EXTRACTION OF ANTIOXIDANT FROM PROPOLIS OF STINGLESS BEE

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ABSTRACT

Propolis has constantly been utilized as useful food for its outstanding health effect. However, limited literature is available regarding the antioxidant activity of the stingless bee products in Malaysia especially for the propolis. This is due to its variability in species and chemical diversity of propolis produced by each stingless bee. In order to improve the efficiency of antioxidant extraction of propolis, an experiment was carried out to optimize the extraction conditions by varying the amplitude, cycle and extraction time of a sonication technique which by several antioxidant assays; DPPH Radical Scavenging Activity Assay (DPPH), Ferric Reducing Antioxidant Power (FRAP) and Total Phenolic Content (TPC) were analysed. The extraction process of bioactive compound from propolis was optimized by using central composite rotatable design (CCRD) of Response Surface Methodology (RSM) to evaluate the effect of factors investigated. DPPH Radical Scavenging Activity, Ferric Reducing Antioxidant Power (FRAP) and Total Phenolic Content (TPC) predicted by model for the propolis extract were 86.79%, 1015.63 mM FeSO4 /g and 13.66 mg GAE/g respectively. The suggested condition for extraction of bioactive compounds was obtained in higher levels: amplitude 52.40%, cycle 53% and extraction time 31. 37min. The validation experiments performed gave radical scavenging activity of DPPH values 78.52% which is 10.53% deviation, FRAP values of 722.76 mM FeSO4 /g which is 40.52% deviation and TPC values of 12.93 mg GAE/g which is 5.65% deviation respectively.

ABSTRAK

Propolis sentiasa digunakan sebagai makanan yang berguna untuk kesan kesihatannya yang luar biasa. Walau bagaimanapun, agak terhad untuk mendapatkan penulisan kajian mengenai aktiviti antioksidan produk lebah terutama untuk propolis di Malaysia. Ini disebabkan oleh kepelbagaian spesies dan kepelbagaian kimia propolis yang dihasilkan oleh setiap lebah kelulut. Untuk meningkatkan kecekapan pengekstrakan antioksidan propolis, satu eksperimen telah dijalankan untuk mengoptimumkan keadaan pengekstrakan dengan mempelbagaikan masa, amplitud, kitaran, menggunakan teknik pengekstrakan sonication yang diuji oleh beberapa ujian antioksidan; DPPH Aktiviti Pengendalian Radikal (DPPH), Kuasa Antioksidan Meremehkan Feruat (FRAP) dan Jumlah Kandungan Fenolik (TPC) analisis. Proses pengekstrakan kompaun bioaktif dari propolis dioptimumkan dengan menggunakan reka bentuk berputar komposit pusat (CCRD) dari Response Surface Methodology (RSM) untuk menilai kesan faktor yang diselidiki. Aktiviti Pengendalian Radikal (DPPH), Kekuatan Antioksidan Meremehkan Ferit (FRAP) dan Total Kandungan Fenolik (TPC) yang diramalkan oleh model untuk ekstrak propolis adalah masing masing 86.79%, 1015.63 mM FeSO4/g dan 13.66 mg GAE/g. Keadaan pengekstrakan yang dicadangkan untuk pengambilan sebatian bioaktif diperolehi pada tahap yang lebih tinggi: amplitud 52.40%, kitaran 53% dan masa pengekstrakan 31.37min. Eksperimen pengesahan yang dilakukan memberikan Aktiviti Pengendalian Radikal DPPH dengan nilai 78.52% iaitu sisihan sebanyak 10.53%, nilai FRAP 722.76 mM FeSO4/g yang mempunyai nilai sisihan 40.52% dan nilai TPC 12.93 mg GAE /g dengan sisihan sebanyak 5.65%.

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CHAPTER 1

INTRODUCTION

1.1 Background

In Malaysia, stingless bee is widely known as 'Lebah Kelulut'. They have been inhabited for over fifty million years in tropical forests and stingless bee honey is used by old folks as a traditional remediation for disease and sickness. Stingless bee is a huge monophyletic class of highly eusocial bees ordinarily found in abundance in warm and humid climate forests around the world. They belong to the family Apidae, subfamily Apinae and tribe Meliponini. Stingless bees can be found in most tropical or subtropical parts of the world, such as Australia, Africa, Southeast Asia and parts of Mexico and Brazil with over 600 recorded species and yet still numerous undescribed species (Rasmussen & Cameron, 2010). Malaysia is believed to have the most diverse Trigona species in this region and two well-known cultured species in Malaysia are *Heterotrigona itama* and *Geniotrigona thoracica* (Baharuddin, & Fisheries, 2014). To date, there are more than 35 variety of Trigona species were recorded in Malaysia and *Heterotrigona itama* is the largely abundant species found in Southern part of Malaysia (Akhir et al., 2017).

1.2 Characteristics of Stingless Bee

Stingless bees are biologically different from honeybees in various significant ways. Different from any other bee honey, stingless bees store pollen and honey in

large egg-shaped pots made of beeswax, normally mixed with a variety types of plant or called 'propolis' (Zabaiou et al., 2017). Besides, it is easy to distinguish between stingless bees from the honeybee in terms of their sizes. In general, stingless bees are slightly smaller in size compared to honeybees. With their body size scale from 3mm to 13mm long, they are capable to sip nectar from most profound space inside tiny flowers. Due to this benefit, their produced honey contains higher vitamins and minerals compared to any other sorts of honey produced by other bees. Stingless bees are active the entire year around, even though they are less active in cooler climate. Dissimilar from other eusocial bees, they do not sting but will protect by biting if their nest is disturbed. Despite being unable to sting, stingless bees may have very huge colonies made frightening by way of numerous defenders (Roubik et al., 2006). Stingless bees commonly nest in hollow trunks, tree branches, or rock crevices but they have also been encountered in wall cavities, old rubbish bins, water metres and storage drums (Norowi & Fahimie, n.d.). Stingless bee honey, propolis and bee bread are the three products originated from stingless bee hives (Akhir et al., 2017).

1.3 Propolis

The word propolis is derived from Greek, in which pro stands for "at the entrance to" and polis for "city", signifying this natural product is used in hive protection (Sabir, Hasanuddin, & Sabir, 2016). Propolis is a resinous substance collected by honeybees to seal honeycomb, which has been used in folk medication due to its antimicrobial and antioxidant properties (Miguel et al., 2014). Propolis or identified as bee glue is a sticky dark coloured material that honeybees collect from living plants. Propolis, is a natural complex matrix that is synthesized by honey bees from products collected in tree buds, saps, resins, mucilage, lattices and other plant

sources. Due to its waxy nature and mechanical properties, propolis is used by honey bees as cement to retain moisture and temperature balanced in the hive all year around, and to close up cracks or open spaces. At elevated temperatures, Propolis is soft, pliable and very sticky; conversely, when cooled, and particularly when frozen or at near freezing, it becomes solid and brittle. It will remain brittle after such handling even at higher temperatures (Sforcin, 2016). Figure 1.1 show the example of propolis of stingless bee hive.

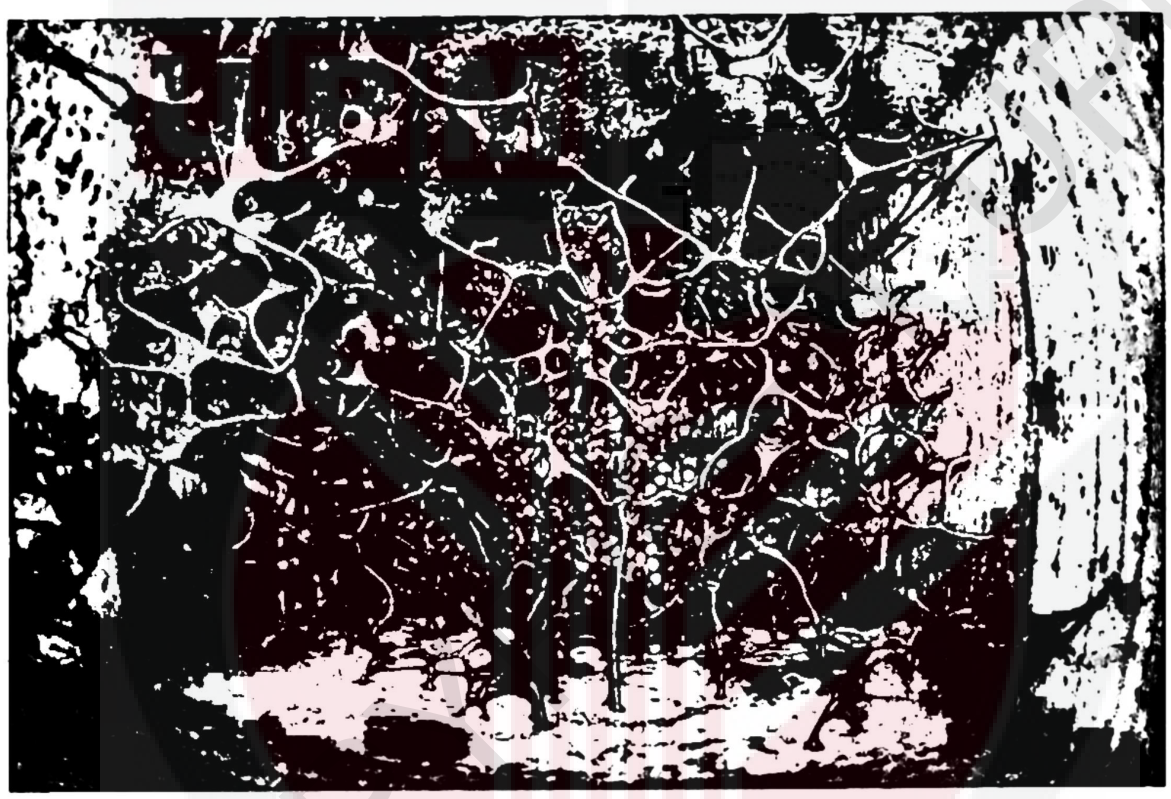


Figure 1.1: Propolis

Propolis has numerous functions in beehives for example exclude draught, protect against external invaders, and mummify their carcasses. In addition to these functions, propolis is too essential for bees in the prevention of growth and decomposition of microorganisms (Fitch, 2002). Propolis has been extensively used by several civilizations to treat colds, wounds and ulcers because of its antiseptic and local anaesthetic properties and have been used in complementary medicine because of its antimicrobial, anti-inflammatory, antitumor, immuno- modulatory and antioxidant activities, among others (Sforcin, 2016).

1.4 Problem Statement

Propolis are one of the products obtained from stingless bee hives other than honey. As we all know, numerous amount of study has been done regarding the antioxidant properties of Honey by various researchers. Besides, the chemical composition and biological activities of propolis of numerous countries have been studied widely by various scientific research groups. However, there are very limited information available in the literatures regarding the antioxidant activity of the stingless bee products in Malaysia especially for the propolis due to its variability in species and chemical diversity of propolis produced by each stingless bee. Therefore, the present study was conducted to applied modern extraction process which will give the highest antioxidant activity in the propolis extracts of stingless bee in an efficient way.

Thus, several extraction processes have to be designed in order to determine the antioxidant properties of propolis from stingless bee's (sp. *heterotrigona itama*). Solvent extraction is most commonly used method for isolation of antioxidant compounds. In this project, ethanol is used as the main solvent for the extraction of antioxidants due to its capability to extract antioxidant compounds from numerous food-based product. There is great prospective and opportunity for the determination of antioxidant from propolis in order to develop new product as well as for future research use and development purposes. However, the preparation of production of product from propolis is still a challenging issue as the availability are very limited in the Malaysian market besides the cost are quite pricey.

1.5 Objectives

The purposes of this research are:

- To evaluate antioxidants capacity from propolis of stingless bee's (sp. *heterotrigona itama*).
- To optimize sonication extraction technique by using Response Surface Methodology (RSM) to obtain maximum antioxidant activity from propolis extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Antioxidant

In general, antioxidants can be divided into soluble in water or we called as hydrophilic and in lipids as hydrophobic. Water-soluble antioxidants react with oxidants in the cell and the blood plasma while lipid-soluble antioxidants inhibit lipid peroxidation in cell membranes (Sies, 1997). Certain of these molecules can be synthesized within the body or provided with food (Vertuani et al., 2004)

Antioxidants are compounds that capable to either slow down or reduce the oxidation processes which occur under the influence of atmospheric oxygen. Antioxidants are involved within the defence mechanism of the organism in opposition to the pathologies associated to the attack of free radicals (Pisoschi & Negulescu, 2012). There is rising awareness in antioxidants, especially in those aiming to avoid the harmful effects of free radicals in the human body, as well as the deterioration of fats and other constituents of goods. (Molyneux, 2004).

Antioxidants lower the oxidative stress in cells and therefore effective in the treatment of various human illnesses, including cancer, cardiovascular diseases and inflammatory diseases.

Benefit in getting antioxidants through food is that there are approximated thousands of numerous antioxidants in the human diet and they are abundant in

chemical types as shown in Table 2.1 (Mbata, 2005) . Antioxidants, including phenolic compounds which mainly consist of flavonoids, phenolic acids and tannins contain various biological effects, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects, as a consequence of their antioxidant action. The antioxidant extracts were assessed in terms of their total phenols (TP), total flavonoids (TFA), total flavanols (TFO), phenolic acids, catechins, lignans and tannins (Litescu & Eremia, 2011).

Table 2.1: Antioxidant Components in Food

Components	**Compounds**	**Food sources**
Vitamins	**Vitamin C** (ascorbic acid)	Citrus fruit, berries, papaya
	Vitamin E (tocopherol and tocotrienols)	Seed-like cereal grains, nuts and oils derived from plants
	Beta carotene and other carotenoids	Orange pigmented, and green leafy vegetable
Elements	**Copper** (as part of superoxide dismutase)	Cocoa, wheat bran, yeast
	Selenium (as part of glutathione peroxidase	Grains, meats.
Macronutrient – derived	Peptides e.g. glutathione	Whey protein
Phytochemicals (food components of plant origin)	**Isoflavone** e.g. genistein and daidzen	Soy
	Flavonols e.g. quercetin and kaempferol.	Tea, red wine, onions, apples.
	Polyphenols e.g. rosmarinic acid	Herbs, oregano, thyme.
	Catechins e.g. epigallocatechin gallate	green tea, meats
Zoochemicals (food components of animal origin	**Ubiquinone** (coenzyme Q10)	Meats especially meat organs, fish

2.2 Composition of Propolis

The composition of propolis depends on the source of the numerous plants used by stingless bees. Chemical of propolis is simply understandable as it is a complex combination of compounds derived from variety of plants and prepared by salivary enzymes of bees. Therefore, composition of propolis depends of the flora, the seasons resins are collected, as well as the bee species. This chemical differences brings a key question of standardization, yet bees, by themselves not change its chemical composition. (Zabaiou et al., 2017). Crude propolis is normally composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances (Bankova, 2005).

The chemical composition of propolis can be classified into various which include flavonoids, phenolics, terpenes, aromatic compounds, volatile oils and bee wax. It is believed that the first 3 groups of compounds are the most important contributors to the biological attributions shown by propolis. After a number of noteworthy researches, researchers started to realize that the chemical composition is vastly uneven and dependent upon some factors for example the season, lighting, height above sea level, collector type and food accessibility through propolis development.. (Yeo, 2015)

Propolis sample analyses from different parts of the globe have been collectively reported to contain more than 300 different chemical compounds. By means of the use of most recent separation and purification methods, additional compounds have been identified in propolis including flavonoids, terpenes, phenolics along with their esters and mineral elements. Nevertheless, common phytochemicals compounds such as alkaloids, and iridoids have not been reported. 241 compounds

have been reported for the first time from propolis since years of 2000 and 2012(S. Huang et al., 2014).

However, the major chemical groups present in propolis resin consist of phenolic acids or their esters, flavonoids, terpens, aromatic aldehydes and alcohols, fatty acids, stilbens and b-steroids (Watanabe et al., 2011).

2.3 Antioxidant Properties of Propolis

Basically, propolis bioactivities were primarily as an effect of substances belonging to the phenolic group, especially flavonoids, which create propolis as a crucial purpose of study for the majority various food and pharmaceutical applications. Flavonoids are the fundamental compounds of propolis as they are responsible for the main pharmacological. Surrounded by them it might be found flavones, flavonols, flavanones, flavanonols, chalcones and dihydrochalcones, isoflavones, isodihydroflavone, flavans, isoflavans and neoflavanoids (Bankova, 2005).

Others, it is found that bee items such as honey, propolis and pollen possess active biological ingredients as well as antimicrobial, anti-inflammatory and antioxidant activities. Studies showed that propolis and honey have a defensive effect in aluminium toxicity (Bakour et al., 2017).

According to Gómez's (2006), more than 180 compounds, primarily polyphenols, have been identified as constituents of propolis. The concentration of phenolic compounds might be differ significantly based on the origin of the samples and such differences are likely to influence its biological activities and as a result its

clinical properties. For that reason, the assay and evaluation of these components is of great value.

Antioxidant capacity is one of the mainly valuable properties of propolis. In spite of the fact that there are some studies on the topic of the potential antioxidant activity of propolis, there is no strong facts on the safe dosage in people. Therefore, there is necessary for clinical studies using propolis and its biologically active compounds, including studies of safety and bioavailability.

2.4 Solvent Extraction

Plant cells synthesize a wie range of natural compounds which are useful to human beings. Even though the improvement in recent chemistry nowadays is able to synthesize the compounds, it is still not straightforward to synthesis several compounds as efficiently as mother nature does. Therefore, plant material is yet still the ideal resource of these bioactive compounds. Extraction methods have been extensively explored to gain such precious compounds from plant life for commercialization.

Propolis cannot be utilized as a crude material where it must be purified by extraction through solvents. This procedure has to eliminate the inert material along with keep the polyphenolic fractions. A multi-step extraction with ethanol is mostly suitable to attain dewaxed propolis extracts rich in polyphenolic components (Fitch, 2002).

Ethanol is mainly common solvent used in propolis extraction, but it results with concentrated leftover flavor. Even if methanol as well showed exceptional

extraction results, it is not possible to be practical in the production of safe to eat propolis for the reason that its high level of harmfulness (Yeo et al., 2015).

A dual solvent system of water–ethanol is typically applied in the extraction of propolis, mostly ethanolic solution containing 70% of ethanol. According to Gómez-Caravaca et al.(2006), extraction with ethanol is mostly suitable to attain dewaxed propolis extracts rich in polyphenolic compound in addition this is the main used solvent, mainly at concentrations of 70% and 80%. Compared with absolute ethanol, extraction with aqueous ethanol produced wax-free tinctures, containing higher amounts of phenolic substances.

Park and Ikegaki (1998) study used a range of concentrations of ethanol as solvent and measured the absorption spectra of the several extracts. Based on the result obtained, the 80% ethanolic extract showed highest absorption at 290 nm, which means that the highest concentration of flavonoids was recorded from the propolis when using this solvent concentration.

The ethanolic extract of propolis is one of the richest sources of phenolic acids as well as flavonoids. Ethanolic extract of propolis and its phenolic compounds have shown numerous biological activities, including antitumor effects, immunomodulatory and chemo preventive (Watanabe et al., 2011). The most active ingredients appeared to be soluble in ethanol and propylene glycol solvent. A small number of constituents were soluble in water, but still water extracts showed slightest certain bactericidal and fungicidal effects, as well as wound-healing properties. The choice of the solvent depends on the purpose of the extract (Sforcin, 2016)

Sun et al (2015) study found that water extract of propolis result in the smallest yield at the same time extracts with ethanol-water solvents got higher yields

by increase in ethanol concentrations. In addition, the highest Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) value were both observed in ethanol extract of propolis. Therefore, based on the results, water and low ethanol-water concentration solvent seemed to be ineffective in extracting phenolics compounds than those extracted with high ethanol-water solvents.

On the other hand, according to study conducted by (Woo et., al 2015) reported that summary of the major results of the extraction of propolis by ethanol tends to raise in extract yield and total flavonoid content when increase in ethanol concentration used. Total phenolic content exhibited the highest value in the ethanol concentration range between 50% -60%. Still, when the ethanol concentration is high, there is a tendency to lower the total phenolic content.

2.5 Ultrasound assisted extraction

Ultrasound is a key-technology in accomplishing the aim of economical green chemistry and extraction. Ultrasound is well-known to obtain an important outcome on the rate of assorted processes in the chemical and food production. By ultrasound, full extractions can currently be completed in minutes with high reproducibility, lessening the utilization of solvent, giving higher purity of the final result compared with other regular conventional extraction method. A number of classes of food components such as pigments, antioxidants, aromas, and other pure and mineral compounds have been extracted, examined and formulated well from a range of networks (Chemat et al., 2017).

Ultrasound has been demonstrated to aid solvent extraction and have to verify to be a potent tool for the phytopharmaceutical extraction industry as well as a

flexible method that can be used both on small and huge scale. It is significance noticing that after high frequency ultrasound is in use, the extraction yield did not rise drastically conversely the degradation of the constituents was reduced. In the case of small frequency sonication, degradation become valuable, especially when alkaloids are being extracted (Vinatoru, 2001)

Annegowda (2012) study found that sonication methods notably improved numerous antioxidants in the methanolic extracts of the starfruit compared to aqueous extracts. The results also emphasize the significance of the solvent system used for ultrasound extractions. On the other hand, according to study by Woo et al., (2015), they found that the extraction of propolis by sonication showed similar tendency as in extraction by ethanol concentration where the extract yield and total flavonoid content are higher by increasing the ethanol concentration.

The advantage of ultrasound extraction is thought to be due primarily to the mechanic impacts of sound cavitation. This technique has illustrated the possible to decrease extraction times extensively and boost extraction yields in a number of studies on therapeutic plants. Several extraction techniques consist of maceration extraction, ultrasound extraction, and microwave assisted extraction were done in order to clarify purely active constituents of poplar type in the propolis. Based on the finding, ultrasound methods provided high extraction yield, requiring short timeframes and less workforce. Ultrasound extraction was as well revealed to be the most effective technique based on proportion of extracted phenolics (Trusheva et al., 2007).

In conclusion, sonication methods being a physical form of food processing that holds high prospective to be investigated for industrial applications as an

efficient environment-friendly method for improving the extractability of natural antioxidants, and thus might successfully play an important role in avoiding some physiological and degenerative diseases in consumers.

2.6 Response Surface Methodology (RSM)

Conventionally, optimization in diagnostic chemistry has been carried out by observing the effect of one factor at a time on an experimental response. Its main weakness is that it does not comprise the interactive impacts between the variables studied. As a result, this method does not show the complete effects of the factor on the response. Another disadvantage of the one-factor optimization is the raise in the number of experiments essential to conduct the study, which leads to an increase of time and costs as well as an increase in the consumption of reagents and resources (Khoo et al., 2013)

Response surface methodology (RSM) is a collection of numerical and statistical techniques effective for analysing the impacts of several independent variables. In various processes, the correlation among the response and the independent variables is commonly unknown, for that reason, the first action in RSM is to estimate the response in terms of independent variables. This deal with employing a low-order polynomial equation in a predetermined area of the independent variables, which is later on examined to find the optimum values of independent variables for the best response (Yuan et al., 2013).

The response surface methodology has been successfully optimized the phenolic content and extract yield in fruit sample (Prasad et al.,2012). The use of RSM also has effectively optimized the extraction conditions. Based on RSM

optimized extraction conditions of Propolis Flavonoids Liposome, additional use of sonication-assisted extraction has helped demonstrated the stronger immunoenhancement activity. (Yuan et al., 2013).

Optimal extraction conditions were derived using response surface methodology for bee pollen exerted tyrosinase inhibition and antioxidant activity was well-matched with predicted values from optimization of RSM thus can be used to optimize extraction process of bee pollen. These results provide useful information about bee pollen as cosmetic therapeutics to reduce oxidative stress and hyperpigmentation (Kim et al., 2015). It can obtain the most favourable condition efficiently, particularly in the case of a number of variables. Hence, RSM is extensively used for optimization of extraction conditions in many fields of study.

2.7 Antioxidant Assay

2.7.1 DPPH Radical Scavenging Activity Assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, appear to colourless ethanol solution. The use of the DPPH assay provides a simple and quick way to assess antioxidants by spectrophotometry, where it be practical to assess different products at a time. The molecule of 1,1-diphenyl-2-picrylhydrazyl is characterised as a stable free radical by asset of the delocalisation of the extra electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals (Garcia et al., 2012)

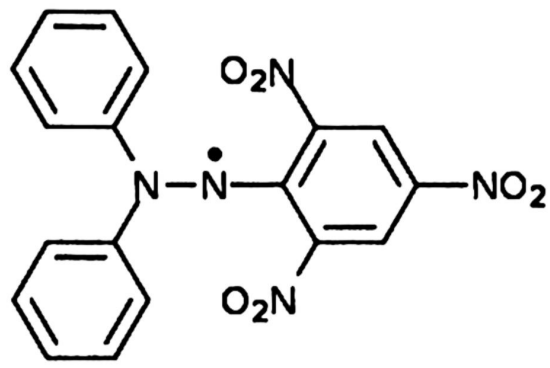


Figure 2.1: Structure of 2,2-diphenyl-1-picrylhydrazyl

The delocalisation as well gives rise to the deep purplish-blue colour, characterised by an absorption band in ethanol solution centred at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, in that case this gives rise to the reduced form with the loss of this purplish-blue colour.

2.7.2 FRAP (Ferric Reducing Antioxidant Power)

The FRAP assay has been useful broadly in food science field. Apart from measuring the total antioxidant content of various foods, the FRAP assay has been used also to discover absorption of antioxidants from foods to examine the consequence of processing and cooking on the antioxidant content of foods.

FRAP (ferric reducing antioxidant power) technique fundamentally relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)1,3,5-triazine). Reduction of a ferric tripyridyltriazine complex to ferrous-(2,4,6-tripyridyl-s-triazine), Fe(III) to Fe(II) can be checked by measuring absorbance at 593nm. The absorbance can be measured to test the total of iron reduced and can be linked with the total value of antioxidants where Trolox or ascorbic acid are commonly being used as references (Pisoschi & Negulescu, 2012).

2.7.3 TPC (Total Phenolic Content)

Total Phenolic Content (TPC) is one of the antioxidant assay commonly used to find out the antioxidant value of certain types of food. The mechanism principally based on reduction capability of phenolic functional group. Oxidation and reduction reaction of phenolate ion takes place at base condition. The reduction of Folin–Ciocalteu reagent, is reduced to blue oxides of tungstene and molybdene during phenol oxidation. The reduction of complex will raise when the extract contains more phenolic compounds. The concentration of blue colour reflects the amounts of phenolic compounds, which can be measured using spectrophotometer. Thus, the colour that display darker and higher absorbance showing higher antioxidant activity.

2.8 Summary

There has been rising concern in study into the function of antioxidants in food and human health. The valuable effect of many foodstuffs and beverages on human health has been recently recognized to originate from their antioxidant activity. Propolis has become the subject of intense in pharmacological and chemical studies for the last three decades but quite new in Malaysia environment. Numerous studies have proven its versatile pharmacological capability such as antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, antitumor and others. There are many methods to extract antioxidants from food-based product. However, extraction yield and antioxidant activity not only depend on the extraction method but also on the solvent used for extraction. The existence of various antioxidant compounds with various chemical characteristics and polarities may or may not be soluble in a certain solvent. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate. Ethanol has been known as a good solvent for

antioxidant extraction and is harmless for human consumption. Several study on the positive effects of ultrasound extractions have been published and the main reported enhancements have been found to be improved efficiency and cut of extraction time. A mainly mechanism for ultrasonic extraction is better mass transfer, the enhanced penetration of the solvent into the molecular cell due to cell disruption. Response Surface Methodology (RSM) offer cost-effective method of collecting data. The strength of the method lies in capturing precise efficient smooth approximations for accurate data gathered from numerical or functional experiments at discrete data points.

CHAPTER 3

METHODOLOGY

3.1 Introduction

In this chapter, the sample preparation, experimental work, antioxidant analysis identification is explained in detail. The propolis was extracted via sonication extraction technique where the type of solvent used were 70% aqueous ethanol concentration. The analysis was done by using Response Surface Methodology (RSM) which comprise of three independent variables involve (amplitude, cycle and time) with 5 levels range of reading with 20 experiments including 6 replicates at the centre point. After the extraction process was done, the sample was analysed for its antioxidant activity. The result obtained was optimized by using Response Surface Methodology (RSM) software, Design Expert where the best parameter of the optimized extraction conditions for extraction of propolis were determined. The research was conducted based on studies done from the related journal, book, manual and website.

3.2 Materials

Samples of the stingless bee propolis obtained from the *Heterotrigona itama* species were collected from a bee farm located at Seksyen U10 Alam Budiman, 40170 Shah Alam Selangor. Harvesting process was done by removing the samples

from the hive, separating between the bee bread and propolis. As shown in the figure 3.1, raw samples of propolis were stored in the bottle and placed in the freezer at -20°C for future use.

Figure 3.1: Types of Propolis used

3.3 Equipment

The equipment that will be use in this project are ultrasonic sonicator (ultrasonic processor UP400S (400 watts, 24kHz)), and UV Spectrophotometer (spectrophotometer UV/VIS Varian 50), weighing scale, stopwatch, thermometer, spatula, scissor, beaker, pipette and freezer.

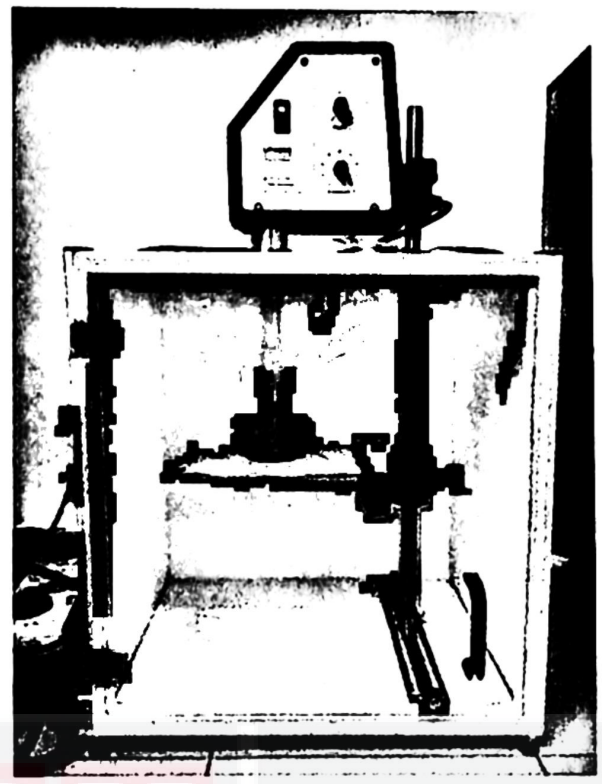


Figure 3.2: Types of Ultrasonic Sonicator used

3.3 Experimental Work

The experimental works, antioxidant analysis and optimization process are explained in detail. Basically, propolis was cut into small pieces in order to increase the surface area exposed with the solvent extraction. The sample were extracted by using 70% aqueous ethanol solvent with sonication extraction technique are applied. The extract obtained was analysed for its antioxidant activity. Based on the antioxidant analysis, optimization process was done by using the Response Surface Methodology (RSM) software, Design Expert to determine the best optimized extraction condition that gives the highest antioxidant value from propolis. Lastly, the verification process was done with another set of experimental work in order to determine the highest antioxidant value from propolis based on the optimized extraction condition obtained.

3.3.1 Sample Preparation

The propolis from species of Heterotrigona itama species were collected from bee farm located at Seksyen U10 Alam Budiman, Shah Alam, Selangor. Raw propolis were kept in laboratory fridge at a temperature of -20^{0}C prior the experiment. 2g of propolis sample was weighed by using a weighing scale. As shown in the figure 3.3, the raw propolis sample were cut into small pieces by using a scissors in order to increase the surface area exposed to the solvent during extraction process. 100ml of 70% aqueous ethanol solvent was measured and mixed with the propolis in a glass beaker.

Figure 3.3: Small cut of propolis

3.3.2 Sonication Extraction of Propolis.

In this experiment, the independent variables involved were amplitude (%), cycle (%) and time (min) with five factorial levels. Amplitude and cycle variable parameter was set directly on the sonicator while time was set by using a stopwatch.

The complete design consisted of 20 experimental points, and the experiment was carried out in a random order.

The samples were labelled with (SE1 – SE20) which stands for Sonication with Ethanol. The beaker consists of mix of propolis and solvent was placed in the sonicator. The beaker was placed constantly at the centre of the sonicator probe and the depth of the probe are set to be 1.5cm from the top surface of the solvent. Each set of samples was extracted based on the parameter of independent variable in the Table3.1.

Table 3.1: Independent variables parameter and their levels used in the response surface design

Sample	Amplitude (%)	Cycle (%)	Time (min)
SE1	30.0	30.0	20.0
SE2	70.0	30.0	20.0
SE3	30.0	70.0	20.0
SE4	70.0	70.0	20.0
SE5	30.0	30.0	40.0
SE6	70.0	30.0	40.0
SE7	30.0	70.0	40.0
SE8	70.0	70.0	40.0
SE9	20.0	50.0	30.0
SE10	83.64	50.0	30.0
SE11	50.0	20.0	30.0
SE12	50.0	84.0	30.0
SE13	50.0	50.0	13.18
SE14	50.0	50.0	46.82
SE15	50.0	50.0	30.0
SE16	50.0	50.0	30.0
SE17	50.0	50.0	30.0
SE18	50.0	50.0	30.0
SE19	50.0	50.0	30.0
SE20	50.0	50.0	30.0

The summary from the independent variables parameter and their levels used in the response surface design are shown table 3.2:

Table 3.2: Summary of independent variables parameter and their levels used in the response surface design

	Parameter	Factor Levels	
		Low Level	High Level
(A)	Amplitude (%)	20.0	83.64
(B)	Cycle (%)	20.0	84.0
(C)	Time (min)	13.18	46.82

Thereafter extraction process, the extract obtained was filtered through a Whatmann No. 1 filter paper and the samples were stored in tube and placed in the freezer at -20°C until used.

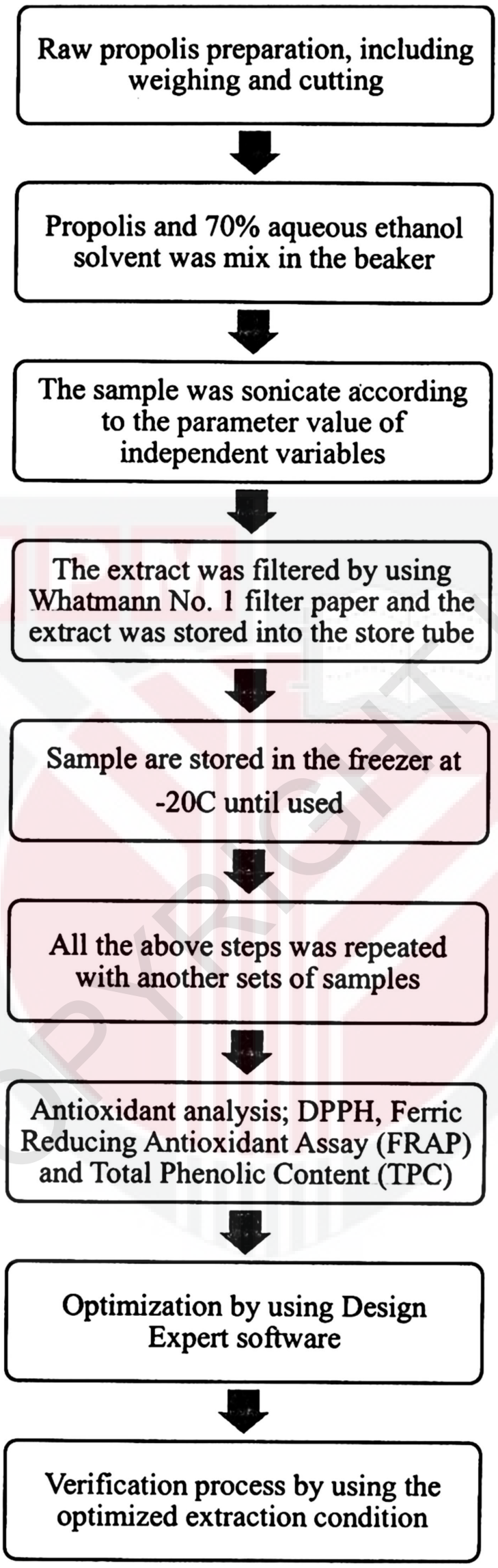


Figure 3.4: Flow chart of the whole process

3.3.3 Antioxidant Activity

The extract of propolis were analysed for antioxidant analysis. Three types of antioxidant assay were chosen in this study to analyses the antioxidant activity in the propolis extract which comprised; DPPH Radical Scavenging Activity Assay, Ferric Reducing Antioxidant Assay (FRAP) and Total Phenolic Content (TPC). All procedure was carried out based on studies from related journal with slight modification.

3.3.3.1 DPPH Radical Scavenging Activity Assay

The antioxidant activity propolis extract were determined using 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical base on the electron transfer reaction between DPPH reagent and the plant extracts. The DPPH free radical of the propolis was measured according to the standard method by with some modifications. Stock solution (24 mg DPPH/100ml of ethanol) and antioxidant standard (gallic acid) was prepared and then diluted to get five different concentrations. A 150µL of sample extract was added into the bottle and 2850µL of DPPH working solution are mix in the bottle tube. It was then left in the dark at room temperature for 30 minutes. All of the sample were done triplicate. After 30 minutes, the absorbance of the mixture was determined at 515nm by using the spectrophotometer (spectrophotometer UV/VIS Varian 50). The ability of extract to scavenge DPPH free radical was calculated using the following equation.

The DPPH value expressed as percentage scavenging of the DPPH value synthetic antioxidant depending on the following calculation:

$$RSA\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ os\ sample}{Absorbance\ of\ control} \times 100$$

Each test was performed in triplicate and the mean percentage value was expressed as radical scavenging activity (%).

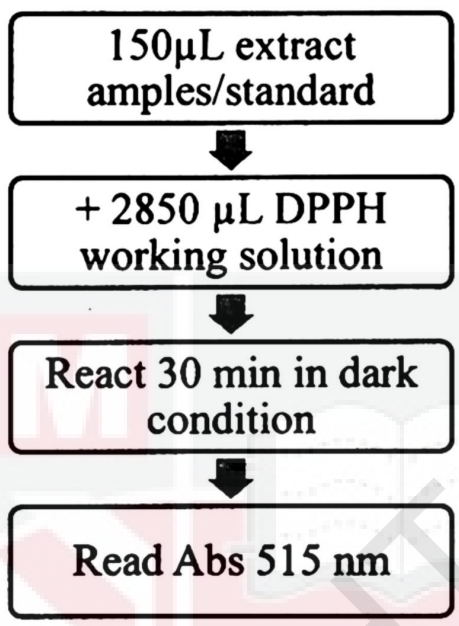


Figure 3.5: Flow chart of DPPH antioxidant analysis

3.3.3.2 Ferric Reducing Antioxidant Power Assay (FRAP)

The reducing capacity of the propolis sample were determined by using ferric reducing antioxidant power (FRAP) assay according to the procedure from the previous experiment with slight modifications. The principle of this method is based on the reduction of a ferric 2,4,6- tripyridyl-s-triazine complex (Fe^{3+} -TPTZ) to its ferrous, coloured form (Fe^{2+} -TPTZ) in the presence of antioxidants. FRAP reagent was prepared at 37 °C from 300 mmol/L acetate buffers (pH 3.6), 10 mmol/L 2,4,6-tri[2-pyridyl]-*s*-triazine (TPTZ) in 40 mmol/L HCl, and 20 mmol/L FeCl3 in the ratio of 1:1:10. Ferrous sulfate heptahydrate (FeSO4.7H2O) was used as a standard and TPTZ working reagent used as a blank solution.

Each experiment was conducted in triplicate. A total of 150 µL of samples extract and 2850 µL working solution were added to the bottle and vortex. The sample extract was left in the dark at room temperature for 30 minutes. The absorbance of the mixture was determined at 593nm by using the spectrophotometer.

A standard curve of FRAP values of each standard versus its concentration was plotted and the final result was expressed as mM ferric iron reduction to ferrous iron in samples extract (mM $FeSO_4$/g). The final result was presented as the concentration of antioxidant having a ferric reducing activity.

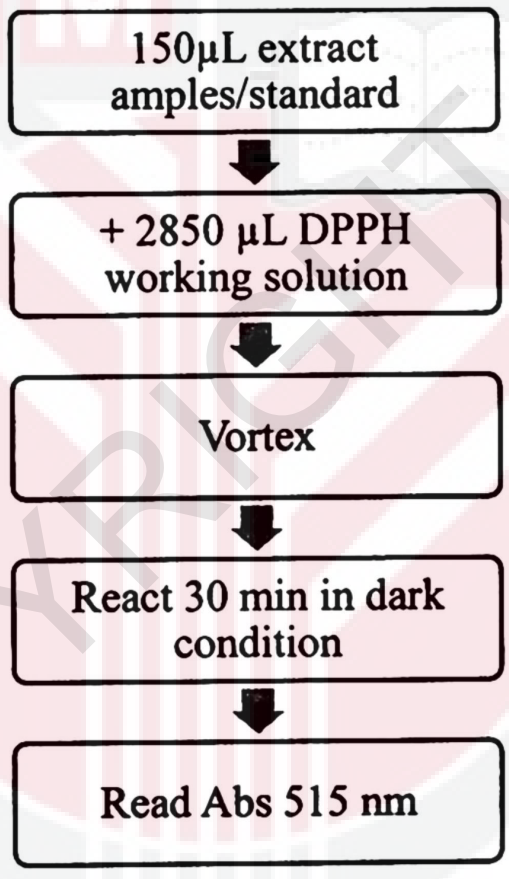


Figure 3.6: Flow chart of FRAP antioxidant analysis

3.3.3.3 Total Phenolic Content (TPC)

The total phenolic compounds of propolis extracts were determined by the Folin-Ciocalteu method with slight modifications. Samples was inserted into test tube and mixed thoroughly with 5 ml Folin-Ciocalteu reagent. After 5 mins, 4 ml of 7.5% sodium carbonate (Na_2CO_3) was added and allowed to react for 2 hrs at room

temperature. The absorbance was measure at 765 nm using microplate reader spectrophotometers. Samples were measured in triplicates. Standard curve of gallic acid solution (0,20,40,60,80,100,120,140,160,180 and 200 ppm) was prepared using the similar procedure. The results were expressed as mg GAE/ g extract sample.

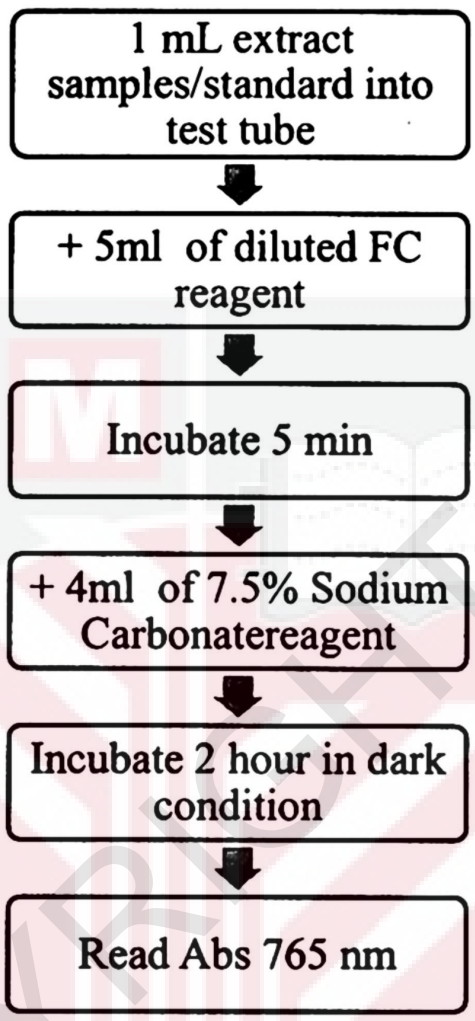


Figure 3.7: Flow chart of TPC antioxidant analysis

3.4 Optimization by using Response Surface Methodology (RSM)

Response surface methodology (RSM) is an efficient tool for optimizing the process, especially when many factors and interactions influence response. It has been used for optimizing complex procedure, extraction technology, conditions and so on. Amongst the benefit of RSM is that it can cut the quantity of experimental trials and assess the relations between multiple parameters, which are less laborious and time-consuming than other optimizing process, and more effective and accurate than other approaches (Tang et al, 2011).

The methodology includes three steps which firstly are experimental design, in which the independent variables and their experimental levels are set using well-established statistical experimental designs. Secondly, response surface modelling is done through regression analysis and lastly optimization of process by using the response surface models.

Design-expert software (Design Expert 6.0.6, Stat-Ease Inc) was used for the experimental design and statistical analysis of the data. Optimization of extraction conditions based on three responses; DPPH, FRAP and TPC was performed using factorial design and analysed according to response surface methodology (RSM). Independent variables of the process were amplitude (A), cycle (B) and extraction time (C). The factorial design was selected for optimization process variable with 5 levels, 20 experiments including 6 replicates at the centre point. The levels of the independent variables and also their encoded values are presented in Table 3.1.

The fitted polynomial equation is expressed as surface and contour plots in order to visualize the correlation among the response and experimental levels of each variable and to figure out the optimum conditions. The regression coefficients were then used to make numerical calculation to generate dimensional and contour plots. Three-dimensional surface response contour plots were generated by varying two variables within the experimental range and holding the other constants at the central point.

3.5 Sample storage

All of the samples are consisting of ethanol solvent which is it was easily volatile and condense into the environment. Because of this, the samples produced should be stored in an appropriate storage system as the storage condition will affect the properties of the extract. There are many factors needed to be considered in propolis extract such as type of storage container, presence of strong odour or sunlight due to the reason that the quality of extract can be affected by physical and chemical reaction and then affect the powder properties.

For this research, the propolis extract produced were stored in the closed plastic bottles purposely for storage of extract right after the extract was taken out from the sonicator and being filtered through filter paper. For secondary protection, the sample extract was then put in the freezer at temperature approximately -20^{0}C and ensured not overexposing with sunlight in order to maintain its storage temperature and the other effects on sample properties.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

Antioxidants properties of propolis extracts are affected by the amplitude, cycle and extraction time of sonication treatment.

4.2 Propolis Extraction

Propolis of stingless bees obtained from species of *Heterotrigona Itama* were extracted by using ethanol solvents. Ethanol is chosen as a bipolar solvent that is highly volatile. Volatile solvents have higher penetration susceptibility. Several studies have been conducted on the several solvents, and they predicted that ethanol obtains a higher amount of crude extract due to its bipolar capacity (Kumar, Maheshwari, & Singh, 2008).

Temperature of all the sample has been recorded right after the sonication extraction process takes place. The results are presented in Table 4.1:

Table 4.1 Temperature of samples after extraction

Sample	Amplitude (%)	Cycle (%)	Time (min)	Temperature (°C)
SE1	30.0	30.0	20.0	46.0
SE2	70.0	30.0	20.0	58.0
SE3	30.0	70.0	20.0	60.0
SE4	70.0	70.0	20.0	71.0
SE5	30.0	30.0	40.0	54.0
SE6	70.0	30.0	40.0	63.0
SE7	30.0	70.0	40.0	68.0
SE8	70.0	70.0	40.0	72.0
SE9	20.0	50.0	30.0	61.0
SE10	83.64	50.0	30.0	71.0
SE11	50.0	20.0	30.0	45.0
SE12	50.0	84.0	30.0	73.0
SE13	50.0	50.0	13.18	58.0
SE14	50.0	50.0	46.82	69.0
SE15	50.0	50.0	30.0	67.0
SE16	50.0	50.0	30.0	67.0
SE17	50.0	50.0	30.0	67.0
SE18	50.0	50.0	30.0	67.0
SE19	50.0	50.0	30.0	67.0
SE20	50.0	50.0	30.0	67.0

Based on the value recorded, the highest temperature of the sample after extraction is sample from SE12 with value recorded are 73^0C while the lowest temperature recorded are SE 11 with only 45^0C. Based on the result, we can analyse the findings according to the parameter for each sample. As shown in the table, the highest temperature recorded was extracted with the highest cycle which is 84 % while the lowest temperature was extracted with 20 % cycle while others variable; amplitude and time have the same value. According to this finding, we can conclude that the highest temperature is primarily affected by the value of the cycle (%) and followed by the time (min) and amplitude (%) of sonication extraction.

This is due to the cycle during the extraction will create ultrasonic vibrations effect very well into solids and liquids where they can produce ultrasonic cavitation. Ultrasonication cause the transmission of power into a liquid where mechanical motions lead to friction within the liquid. For this reason, ultrasonication generates significant heat during processing.

4.3 Antioxidant Activities

4.3.1 DPPH Radical Scavenging Activity Assay

DPPH radical was used as a stable free radical to determined antioxidant activity of many natural compounds. The antioxidant activity of propolis extracts containing polyphenol components is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. Thus, the purple color of 2,2-diphenyl-1- picryl hydrazyl (DPPH) will reduce to α, α-diphenyl- β-picrylhydrazine (yellow colored). DPPH free radical scavenging activity (RSA) ethanol extracts of propolis were assessed to determine their antioxidant properties. The results are presented in Table 4.2.

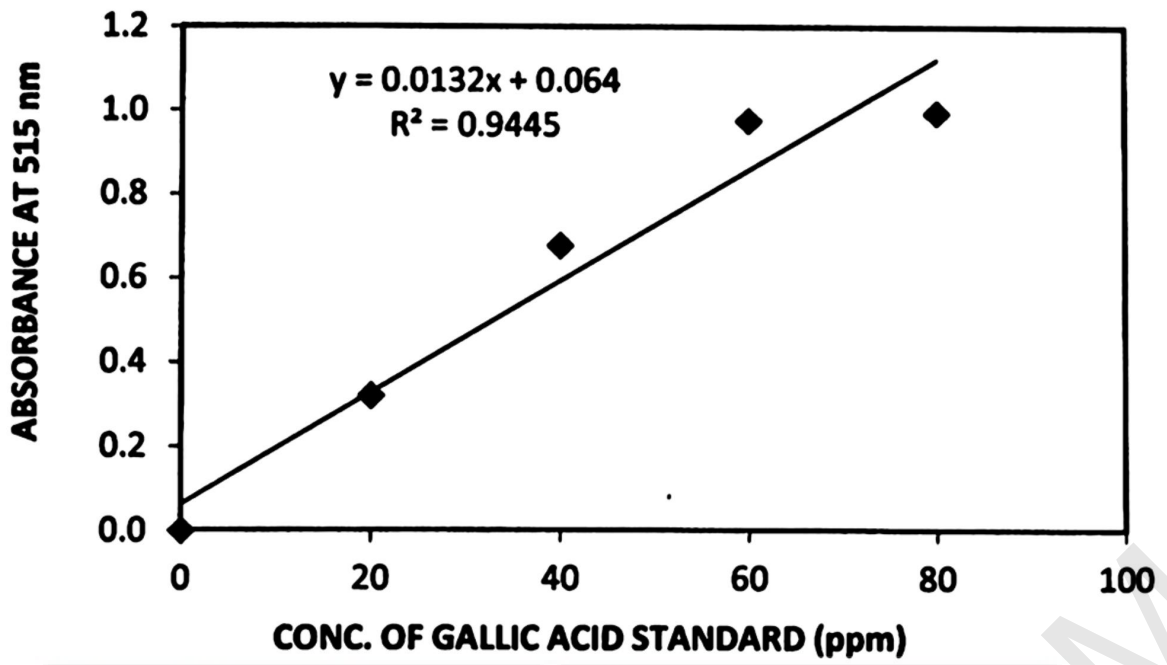


Figure 4.1: Standard Calibration Curve of DPPH

Table 4.2: The radical scavenging activity (RSA) value of propolis extract

Samples	Radical Scavenging Activity (RSA) %
SE1	57.33
SE2	65.34
SE3	67.59
SE4	68.48
SE5	65.05
SE6	70.41
SE7	73.23
SE8	68.03
SE9	68.25
SE10	69.78
SE11	73.94
SE12	74.61
SE13	70.07
SE14	74.28
SE15	84.43
SE16	85.60
SE17	88.33
SE18	85.31
SE19	89.29
SE20	86.01

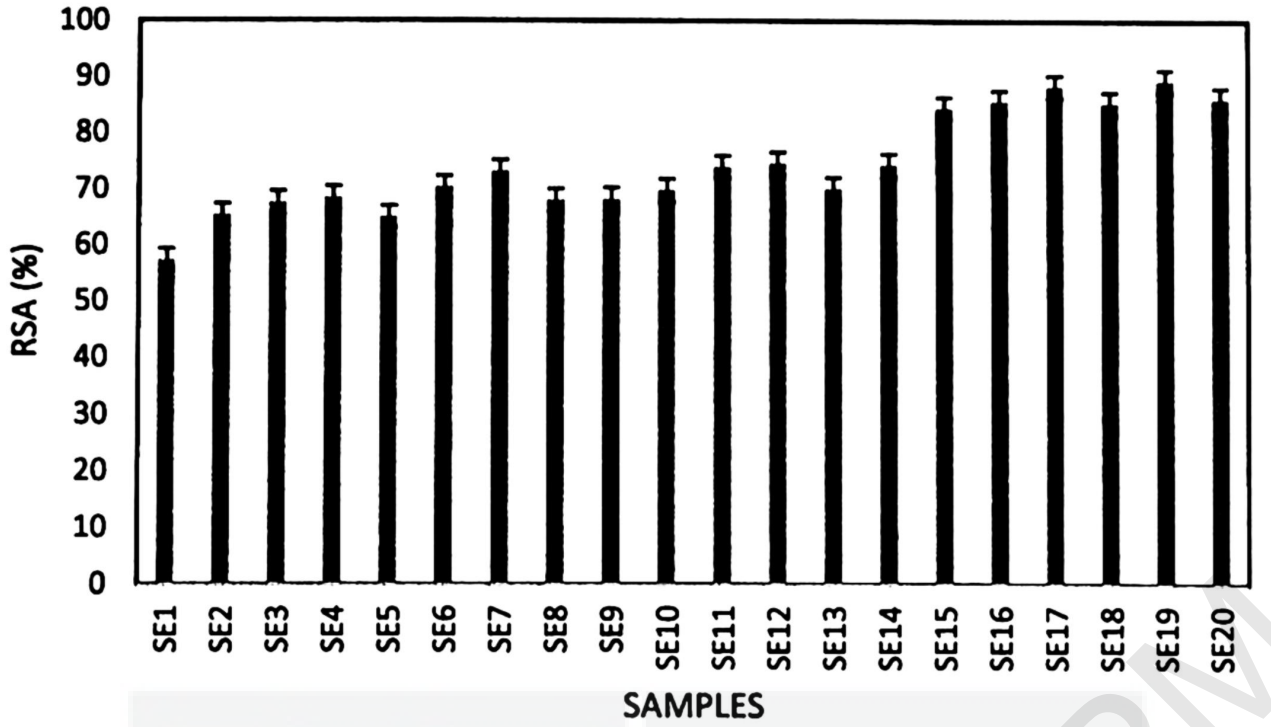


Figure 4.2: Radical Scavenging Activity (RSA) of propolis extract

Antioxidant activity propolis extract determined by DPPH method varied from 57.33% to 89.29%. Based on the results presented in table 4.2 and figure 4.2 show the DPPH value of ethanol extract of propolis tested with this method have higher value in sample between (SE15-SE20) compared to others. The RSA value of sample (SE15-SE20) ranging from 84.43% to 89.29 % compared to other types sample (SE1-SE14) which ranged from 57.33% to 74.61%. This is due to the sample of propolis between (SE15-SE20) are extracted with the same value of parameter for all the independent variable involved. However, these differences with the other samples may due to the different value parameter for three independent variable of extraction process applied during the sonication process. However, this result is not sufficient enough to prove the parameter of extraction process between (SE15-SE20) are the best parameter for the extraction process until the optimization process is done and discuss in the following parts of this thesis.

4.3.2 Ferric Reducing Antioxidant Power Assay (FRAP)

The ferric reducing antioxidant power assay (FRAP) is directly predicts the reducing potential of an antioxidants in a sample based on the capability of an antioxidant that react with a ferric tripyridyltriazine (Fe3+ - TPTZ) complex to give a coloured ferrous tripyridyltriazine (Fe2+-TPTZ). In general, the reducing properties are linked with the presence of compounds which exert their action by breaking the free radical through the donation of a hydrogen atom.

Figure 4.3 show the standard calibration curve of Ferric Reducing Antioxidant Power Assay (FRAP) and Table 4.3 show the FRAP value of ethanol extract of propolis.

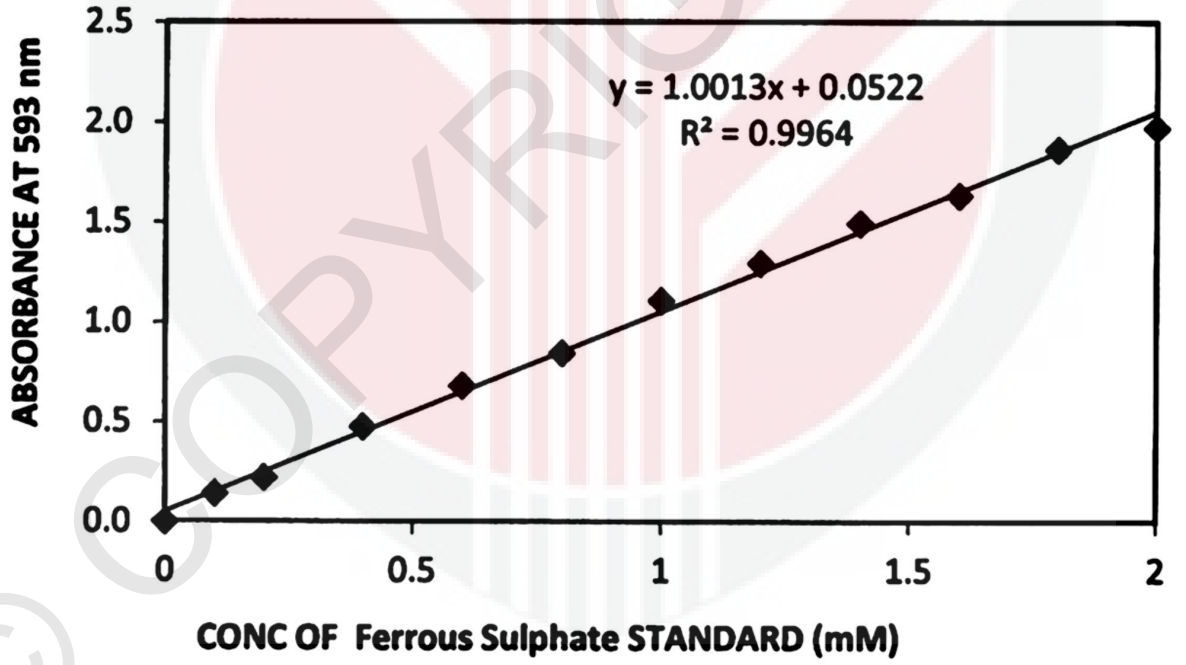


Figure 4.3: Standard Calibration Curve of FRAP

Table 4.3: FRAP value of propolis extract

Samples	FRAP per gram sample (mM FeSO4 /g)
SE1	200.87
SE2	397.56
SE3	469.92
SE4	551.02
SE5	432.11
SE6	484.20
SE7	476.19
SE8	483.55
SE9	437.48
SE10	572.74
SE11	237.28
SE12	430.04
SE13	522.78
SE14	632.01
SE15	1036.86
SE16	1065.69
SE17	1099.76
SE18	1000.53
SE19	1023.11
SE20	836.30

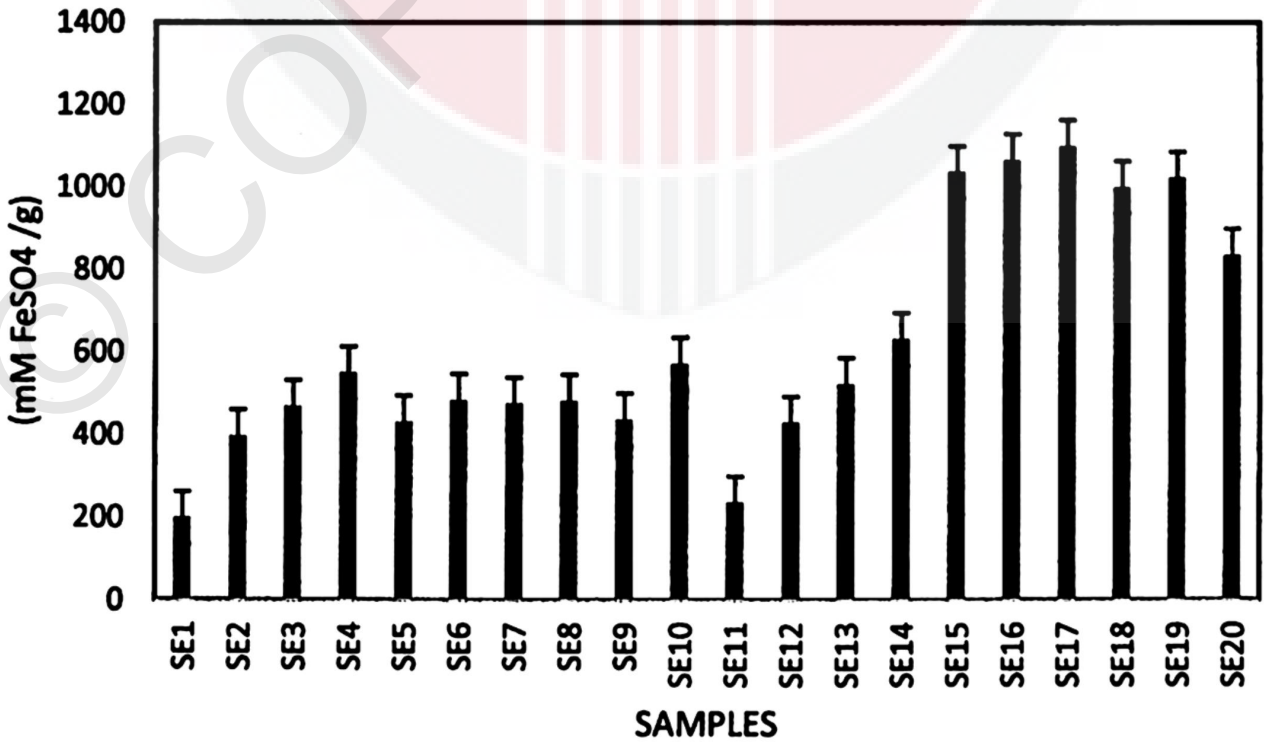


Figure 4.4: FRAP value of propolis extract

The concentration of FRAP ranged from 200.87 to 1099.58 mM FeSO4 /g. The results presented in table 4.3 and figure 4.4 also show the same trends of results as DPPH assay where the FRAP value of ethanol extract of propolis tested in this study have higher value in sample (SE15-SE20) compared to others. In fact, all propolis samples exhibited the reducing power. However, the FRAP value of sample (SE15-SE20) ranging from 836.30 mM FeSO4 /g to 1099.76 mM FeSO4 /g are higher compared to other samples (SE1-SE14) which ranged from 200.87 mM FeSO4 /g to 632.01 mM FeSO4 /g. This result need to be compared with another antioxidant assay which is Total Phenolic Content (TPC) in order to conclude and find out the best optimized condition for the extraction of propolis that gives the highest antioxidant activity of propolis extract.

4.3.3 Total Phenolic Content Analysis

The results obtained showed that the Total Phenolic Content (TPC) value determined by the modified Folin-Ciocalteu method have the same trends as DPPH and FRAP value previously. As show in Table 4.4 and Figure 4.6, the TPC value have a higher value in sample (SE15-SE20) compared to another sample (SE1-SE14).

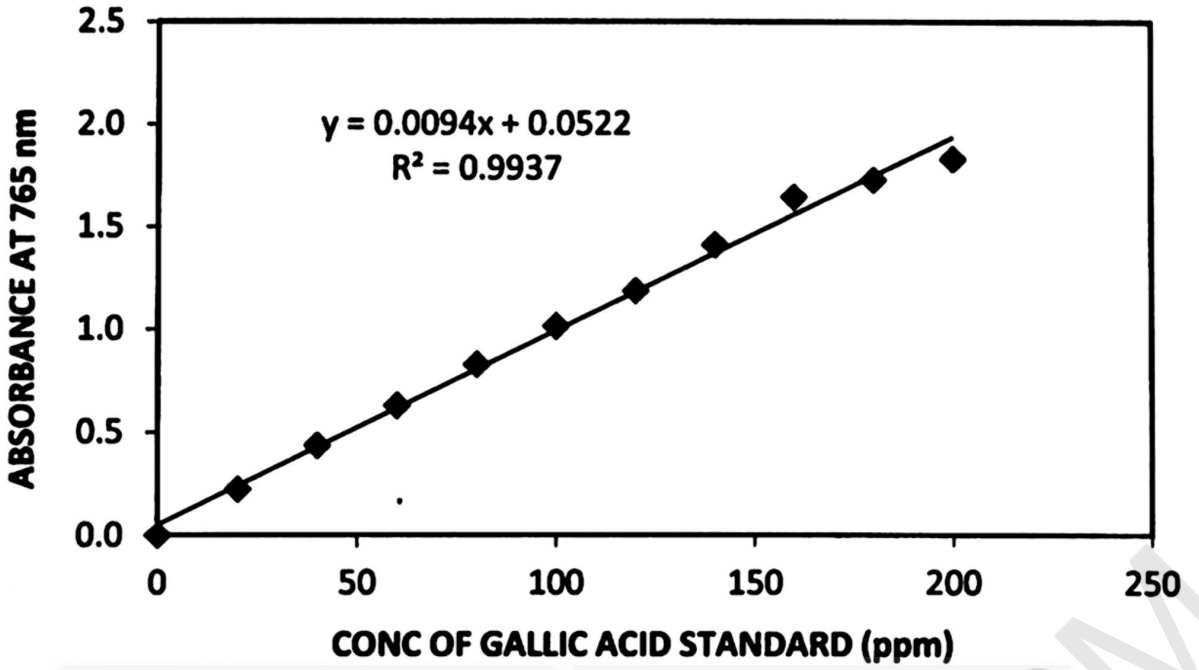


Figure 4.5: Standard Calibration curve of Total Phenolic Compound

Table 4.4: TPC value of propolis extract

Samples	TPC (mg GAE/g)
SE1	7.28
SE2	7.74
SE3	8.77
SE4	10.41
SE5	6.96
SE6	11.12
SE7	9.84
SE8	10.40
SE9	8.93
SE10	11.04
SE11	5.27
SE12	10.92
SE13	10.68
SE14	12.03
SE15	14.20
SE16	13.13
SE17	12.91
SE18	12.84
SE19	13.65
SE20	13.63

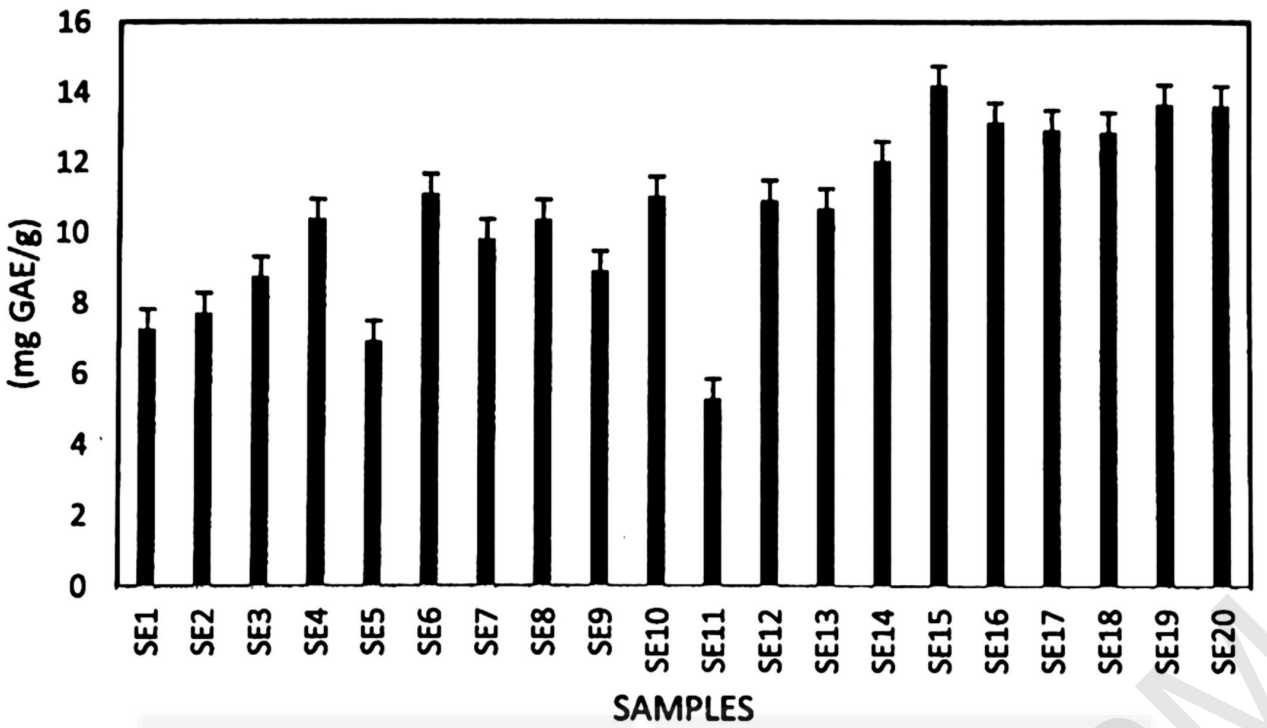


Figure 4.6: TPC value of propolis extract

The results presented in Table 4.4 and Figure 4.6 show samples from (SE15-SE20) significantly have higher total phenolic content ranging from 12.91 mg GAE/g to 14.20 mg GAE/g compared to samples from (SE1-SE14) samples extract which value ranged between 5.27 mg GAE/g and 12.03 mg GAE/g.

However, samples extract from SE11 show a significant low value of total phenolic content compared to the previous antioxidant assay where the lowest antioxidant value are obtained from sample SE1 for both DPPH and FRAP. These results may due to its lower value of extraction variable parameter during sonication takes place.

4.4.4 Summary

Based on the antioxidant assay conducted on ethanol extract of propolis by using sonication extraction technique with three independent variables involved, the results showed sample from (SE15-SE20) have a significant higher antioxidant activity for all antioxidant assay conducted which include DPPH, FRAP and TPC compared to sample from (SE1-SE14). From all of the sample, SE1 show a very low

value of antioxidant activity for two from three assays conducted on it. However, samples that gives the highest value of antioxidant are not constant for all three types of assay. Based on the result obtained from DPPH assay, sample from SE19 have the highest RSA value which is 89.29%, while SE17 show the highest FRAP value which is 1099.76 mM FeSO4 /g followed by 14.20 mg GAE/g for SE15 form TPC assay.

Table 4.5: Experimental data of the responses investigated of the propolis extract

Sample	Amplitude (%)	Cycle (%)	Time (min)	Radical Scavenging Activity (RSA) %	FRAP per gram sample (mM FeSO4/g)	Total Phenolic Content (mg GAE/g)
SE1	30.0	30.0	20.0	57.33	200.87	7.28
SE2	70.0	30.0	20.0	65.34	397.56	7.74
SE3	30.0	70.0	20.0	67.59	469.92	8.77
SE4	70.0	70.0	20.0	68.48	551.02	10.41
SE5	30.0	30.0	40.0	65.05	432.11	6.96
SE6	70.0	30.0	40.0	70.41	484.20	11.12
SE7	30.0	70.0	40.0	73.23	476.19	9.84
SE8	70.0	70.0	40.0	68.03	483.55	10.40
SE9	20.0	50.0	30.0	68.25	437.48	8.93
SE10	83.64	50.0	30.0	69.78	572.74	11.04
SE11	50.0	20.0	30.0	73.94	237.28	5.27
SE12	50.0	84.0	30.0	74.61	430.04	10.92
SE13	50.0	50.0	13.18	70.07	522.78	10.68
SE14	50.0	50.0	46.82	74.28	632.01	12.03
SE15	50.0	50.0	30.0	84.43	1036.86	14.20
SE16	50.0	50.0	30.0	85.60	1065.69	13.13
SE17	50.0	50.0	30.0	88.33	1099.76	12.91
SE18	50.0	50.0	30.0	85.31	1000.53	12.84
SE19	50.0	50.0	30.0	89.29	1023.11	13.65
SE20	50.0	50.0	30.0	86.01	836.30	13.63

As shown in Table 4.5, independent variables of sonication extraction which is amplitude, cycle and extraction time from sample (SE15-SE20) showed a significant higher in antioxidant value for propolis extract compared to the others (SE1-SE14).

Based on this finding, optimization have been done in order to validate and proof the result from antioxidant value of DPPH, FRAP and TPC and will be discussed in the following section.

4.4 Optimization by Response Surface Methodology

The objective of the study is to analyse optimized extraction parameter that result in the highest antioxidant activity using sonication extraction method. The optimum combination of the influencing parameters for the DPPH, FRAP and TPC can be found using Response Surface Methodology (RSM). Twenty randomized analyses were determined based on a central composite rotatable design (CCRD). Determinations of DPPH, FRAP and TPC value were carried out using the optimized extraction conditions. Verification of the response surface model was performed by comparing experimental values to predicted values obtained from the optimized model. A mathematical model obtained for the independent variable for of DPPH, FRAP and TPC were:

$$DPPH = +86.62+0.85A +1.49B+1.83C - 7.02A^2 -5.17B^2 -5.91C^2 -2.21AB -1.09AC -0.95BC \quad (1)$$

$$FRAP = +1010.46+41.35A +57.86B +32.25C -179.16A^2 -239.78B^2 -153.60C^2 -20.04AB -27.29AC -47.39BC \quad (2)$$

$$TPC = +13.41+0.76A +1.16B +0.47C -1.32A^2 -1.99B^2 -0.83C^2 -0.30AB +0.33AC -0.25BC \quad (3)$$

Where:

A=Amplitude

B=Cycle

C=Time

A^2 = Amplitude^2

B^2 = Cycle^2

C^2 =Time^2

AB=Amplitude*Cycle

AC=Amplitude*Time

BC=Cycle*Time

According to the model (Equation 1 and 2), amplitude (A), cycle (B), extraction time (C), have positive influences on the antioxidant values of DPPH, FRAP and TPC . By contraries, the quadratic term of them (A^2, B^2, and C^2), the interaction between amplitude and cycle (AB), amplitude and time (AC) and cycle and time (BC) exhibited negative influences.

However, there is a slight difference in model (Equation 3) where amplitude (A), cycle (B), extraction time (C), and amplitude and time (AC) have positive influences on the antioxidant values of DPPH, FRAP and TPC . By contraries, the quadratic term of them (A^2, B^2, and C^2), the interaction between amplitude and cycle (AB), and cycle and time (BC) exhibited negative influences.

Table 4.6: Analysis of variance (ANOVA) table for response (DPPH)

Source	Sum of Squares	Degree of Freedom	Mean Square	f-Value	p-value	
Model	1482.25	9	164.69	18.04	< 0.0001	significant
A	9.94	1	9.94	1.09	0.3212	
B	30.20	1	30.20	3.31	0.0990	
C	45.95	1	45.95	5.03	0.0487	
A^2	710.99	1	710.99	77.90	< 0.0001	
B^2	384.46	1	384.46	42.12	< 0.0001	
C^2	502.75	1	502.75	55.08	< 0.0001	
AB	39.02	1	39.02	4.28	0.0655	
AC	9.57	1	9.57	1.05	0.3301	
BC	7.20	1	7.20	0.79	0.3953	
Residual	91.27	10	9.13			
Lack of fit	73.38	5	14.68	4.10	0.0738	not significant
			R^2=0.9420			

Table 4.7: Analysis of variance (ANOVA) table for response (FRAP)

Source	Sum of Squares	Degree of Freedom	Mean Square	f-Value	p-value	
Model	1.487E+006	9	1.652E+005	38.44	< 0.0001	significant
A	23351.54	1	23351.54	5.43	0.0420	
B	45713.09	1	45713.09	10.64	0.0086	
C	14199.92	1	14199.92	3.30	0.0991	
A^2	4.626E+005	1	4.626E+005	107.64	< 0.0001	
B^2	8.285E+005	1	8.285E+005	192.80	< 0.0001	
C^2	3.400E+005	1	3.400E+005	79.12	< 0.0001	
AB	3212.78	1	3212.78	0.75	0.4075	
AC	5958.10	1	5958.10	1.39	0.2663	
BC	17962.97	1	17962.97	4.18	0.0681	
Residual	42973.84	10	4297.38			
Lack of fit	662.95	5	132.59	0.016	0.9998	not significant
			R^2= 0.9719			

Table 4.8: Analysis of variance (ANOVA) table for response (TPC)

Source	Sum of Squares	Degree of Freedom	Mean Square	f-Value	p-value	
Model	110.54	9	12.28	15.41	< 0.0001	significant
A	7.89	1	7.89	9.90	0.0104	
B	18.31	1	18.31	22.97	0.0007	
C	3.00	1	3.00	3.76	0.0811	
A2	25.02	1	25.02	31.40	0.0002	
B2	56.79	1	56.79	71.26	< 0.0001	
C2	9.99	1	9.99	12.54	0.0053	
AB	0.74	1	0.74	0.93	0.3580	
AC	0.86	1	0.86	1.08	0.3238	
BC	0.50	1	0.50	0.63	0.4445	
Residual	7.97	10	0.80			
Lack of fit	6.59	5	1.32	4.77	0.0557	not significant
R^2= 0.9328						

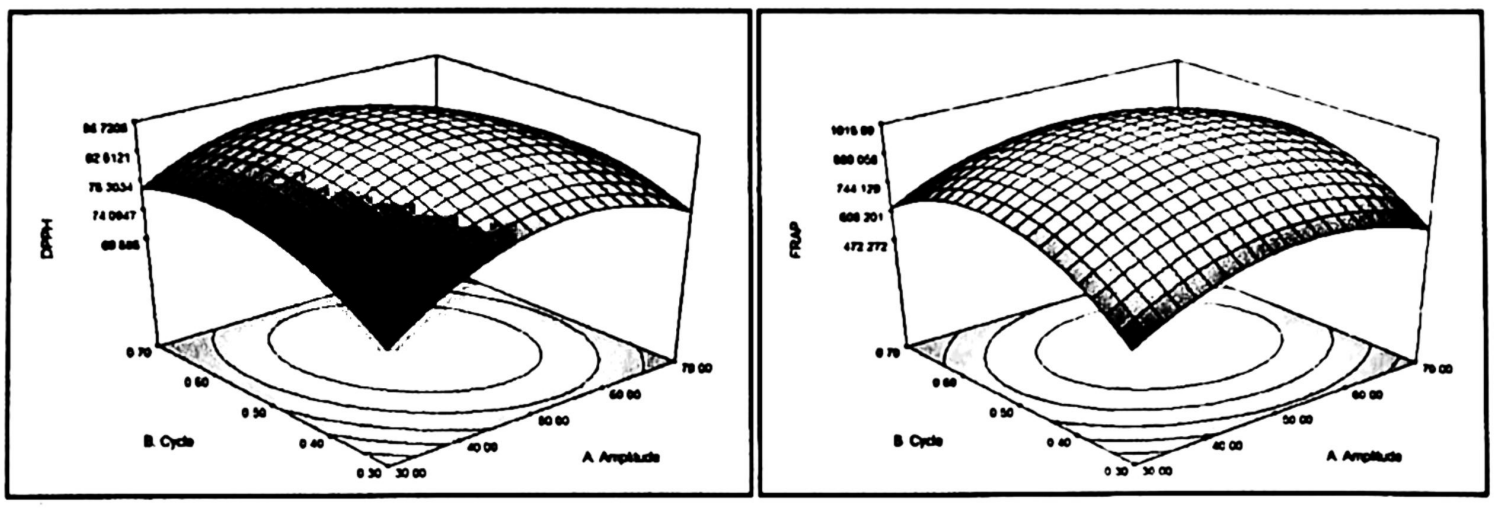
The fitness of the model is checked through Analysis of Variance. The model is found to be significant and is presented through the ANOVA table shown above. P values were used as a tool to check the significances of each coefficient where the smaller the P value, the more significant the corresponding coefficient was. In general, greater coefficients with smaller p-value ($p < 0.05$) indicated the considerable effect of these coefficients on respective responses. Based on the table above, all of the p-value for each response shown values ($p < 0.05$).

The result of the analysis of variance (ANOVA) for the antioxidant activity indicates that the fitted model is considered statistically significant and predictive, with the coefficient of determination (r^2) was of 0.9420 for DPPH and 0.9719 for FRAP demonstrating that the model is suitable for predicting the data obtained (equation 1&2). Besides, the analysis of variance (ANOVA) result obtained for TPC indicate that the fitted model is statistically significant and can be used for predictive

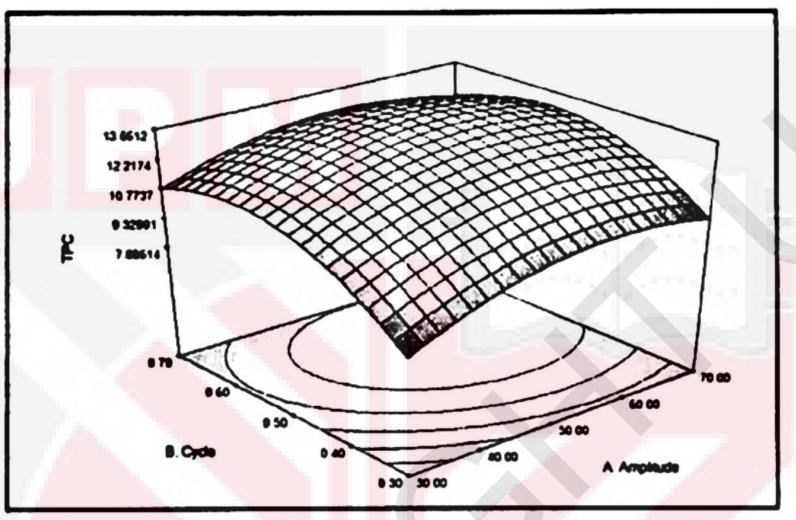
purposes, the coefficient of determination (r^2) was 0.9328, indicating that 93.3%% of the variability of the data can be explained by the proposed model (equation 3).

3D response surface were the graphical representations of regression function. They showed the sort of interactions among three tested variables and the relationship between responses and experiment levels of each variable. Different shapes of the contour plots indicated different interactions between the variables. Circular contour plot indicated that the interactions between the corresponding variables were negligible (Tang et al, 2011).

The 3D response surface curves were plotted to explain the results of amplitude, cycle and extraction time effect on total antioxidant activities of DPPH, FRAP and TPC under different circumstances. 3D response surface plots showed similar patterns for DPPH, FRAP and TPC with two variables involved while keeping the other variables at their respective central values as shown in Figure 4.7, Figure 4.7 and Figure 4.9.



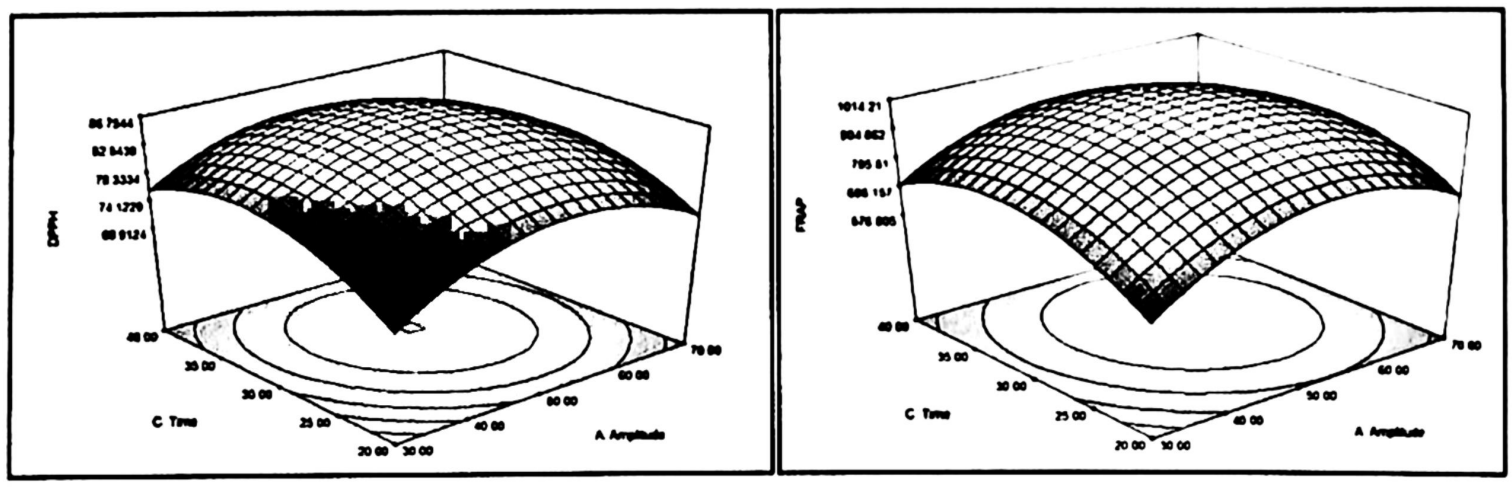
A B



C

Figure 4.7: Response surface plots show the effect of amplitude & cycle on A) DPPH, B) FRAP and C) TPC keeping time at their respective central value

Figure 4.7 show the response surface plot showing the effect of amplitude and cycle on DPPH, FRAP and TPC on propolis while keeping the time at the central 30 min, respectively. It can be observed that both amplitude and cycle have significant effects on antioxidant activity. Examination of the plots in Fig. 4.7 showed that there were optimal concentration levels for DPPH, FRAP and TPC. Under the mentioned conditions, the maximum antioxidant activity could be obtained with an amplitude at 50 % and cycle at 50%.

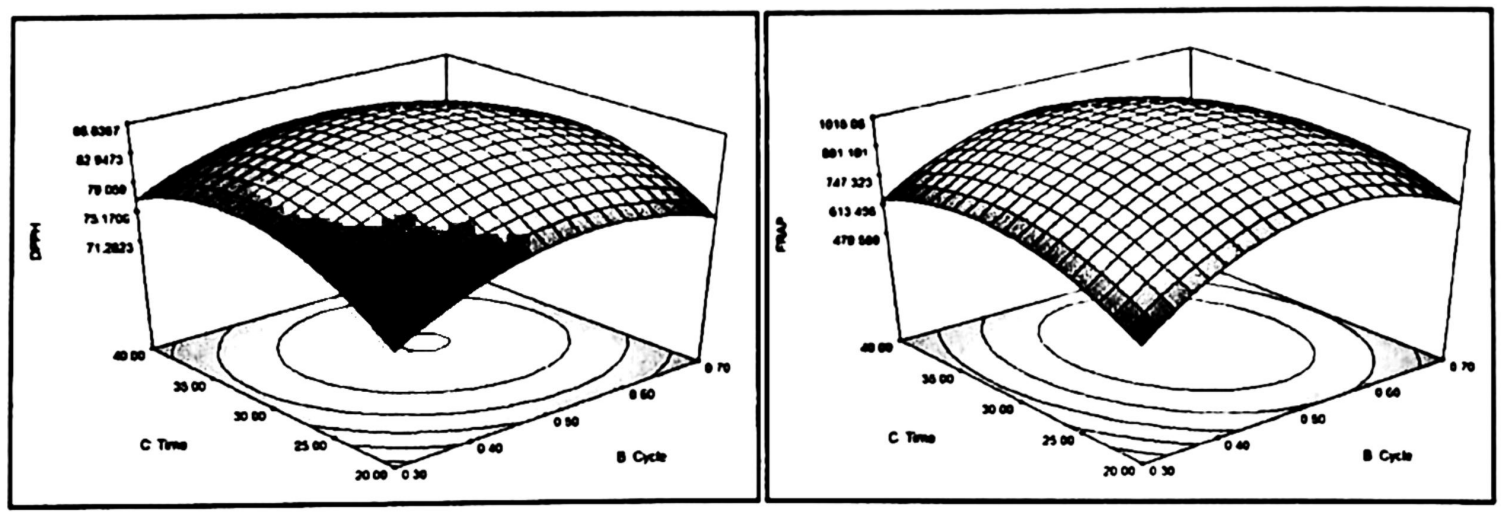


A B

C

Figure 4.8: Response surface plots show the effect of amplitude & time on A) DPPH, B) FRAP and C) TPC keeping the cycle at their respective central value

The antioxidant activity is also time-dependent, under the condition of ultrasonic-assisted extraction. Figure 4.8 shows the effect of amplitude and time on the antioxidant activity of propolis while keeping the cycle at the central values of 50 %, respectively. Under these conditions, the maximum antioxidant activity could be obtained with an amplitude at 50 % and extraction time for first 30 min than decrease. As the extraction time prolongs, the phenolics compounds was not constant throughout extended times at thermal process. The chemical degradation of bioactive compound existing in extract may occur, resulting in a reduction in extraction yield (W. Huang, et al., 2009).



A

B

C

Figure 4.9: Response surface plots show the effect of cycle & time on A) DPPH, B) FRAP and C) TPC keeping the amplitude at their respective central values

Figure 4.9, shows the effect cycle and extraction time on the antioxidant activity of propolis while keeping the amplitude at the central values of 50 % respectively. According to Fig. 4.9, it was observed that both extraction time and cycle influenced antioxidant activity of propolis extract. It was noticed that both factors time and cycle operate independently on antioxidant activity. Under the said conditions, the maximum antioxidant activity could be obtained with an amplitude of 50 % and extraction time at 30 min.

4.4.1 Determination and experimental validation of the optimal conditions

Based on results, an optimization for extraction condition for both responses was determined by RSM and verified by experiment. Based on the analysis conducted by design expert software, an optimal extraction condition of propolis extract for DPPH, FRAP and TPC was determined as amplitude 52.40%, cycle of 53.0%, and extraction time, 31.37 min from the optimization result, the value predicted for those responses was 86.79% for Radical Scavenging Activity (RSA), 1015.63 (mM FeSO4 /g) for FRAP and 13.66 (mg GAE/g).

An experiment was conducted to verified the result and showed propolis extract prepared under optimized condition have antioxidant value; 78.52% for DPPH Radical Scavenging Activity, 722.76 mM FeSO4 /g for FRAP and 12.93 mg GAE/g for TPC which was not well-matched with predicted value as shown in the table 4.9:

Table 4.9: Predicted and optimized values of DPPH, FRAP and TPC under optimized condition

Optimized Extraction Condition			DPPH (RSA%)		FRAP (mM FeSO4 /g)		TPC (mg GAE/g)	
Amplitude	**Cycle**	**Time**	**Predicted**	**Optimized**	**Predicted**	**Optimized**	**Predicted**	**Optimized**
52.40%	53.0%	31.37min	86.79%	78.52%	1015.63	722.76	13.66	12.93

Differences in the result between optimized value from the predicted value may be due to the prolonged storage of propolis samples from the beginning time of propolis was harvested from the farm until it is used for antioxidant analysis. Besides that, the sample are often exposed to sunlight when antioxidant assay was conducted is also one of the factors. It is reported in the literature on storage effect on

phenols and on the antioxidant activity of extract showed prolonged storage of samples has shown significant degraded in antioxidant value of the samples over a period of time. Besides, high temperature and exposure to light were the main factors that decreased antioxidant activity (Del-Toro-Sánchez et al., 2015)

CHAPTER 5

CONCLUSION

5.1 Conclusion

The results of this study showed that the objectives of this study have been achieved. The optimization of extraction conditions is significance to obtain extracts rich in antioxidant activity from propolis extract of stingless bee species. In addition, by using factorial design it was clearly noticed the relationship between the responses, the extraction conditions and the interactions between the different extraction conditions tested. Using Central Composite Rotatable Design (CCRD) combined with Response Surface Methodology (RSM), the optimum condition of amplitude, cycle and time was obtained. These optimum extraction conditions were of amplitude 52.40%, cycle 53% and 31.37min time of extraction had yielded optimal antioxidant value in propolis extract at which the values for DPPH, FRAP and TPC were 78.515%, 722.755 (mM FeSO4 /g) and 12.930 (mg GAE/g) respectively.

The optimum processing conditions were a combination of the central composite rotatable design followed for the present analysis that shows the validity of the experimental design. Based on RSM optimized extraction conditions, in term of antioxidant capacity of natural products, the optimization can be studied using other antioxidant methods in order to strongly prove the result. Taken together, these results provide useful information about propolis as bee product that have high

antioxidant content and can be explore in future studies. The experimental conditions allow a fast and cost-saving process in extraction of antioxidant from the propolis. And, at the same time, propolis showed obvious radical scavenging activities on DPPH, FRAP, and TPC, which indicated that propolis had antioxidant activity and could be developed in the near future.

High temperature and exposure to light were the main factors that decreased antioxidant activity. Besides, prolonged storage of sample might cause a loss of phenolic compounds as well as antioxidant activity (Del-Toro-Sánchez et al., 2015). Furthermore, the results obtained from the factorial design indicate that the independent variables: amplitude, cycle and time of extraction are considered statistically significant in the extraction process of bioactive compounds in the propolis sample, being that the best response was obtained with the combination of the higher levels of the variables studied

5.2 Recommendation

Based on the result obtained, there are some improvement can be applied in order to improve the effectiveness of the experimental work and result obtained in the future. This model can be improved by conducting several times more optimization process from the beginning process and compare with the previous one in order to find the best optimize extraction process of propolis. Sonication has helped to remove oxygen that may potentially increase oxidation of phenolic compounds. However, as there are limitations for introducing ultra-sound in liquid extraction, further studies for optimization by using sonication assisted extraction are recommended. Therefore, the results obtained can be applied for extraction of many other types of food sample.

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APPENDIX A

Figure A1: Weighing Balance

Figure A2: Varying button of Sonicator

Figure A3: Position of Beaker during sonication

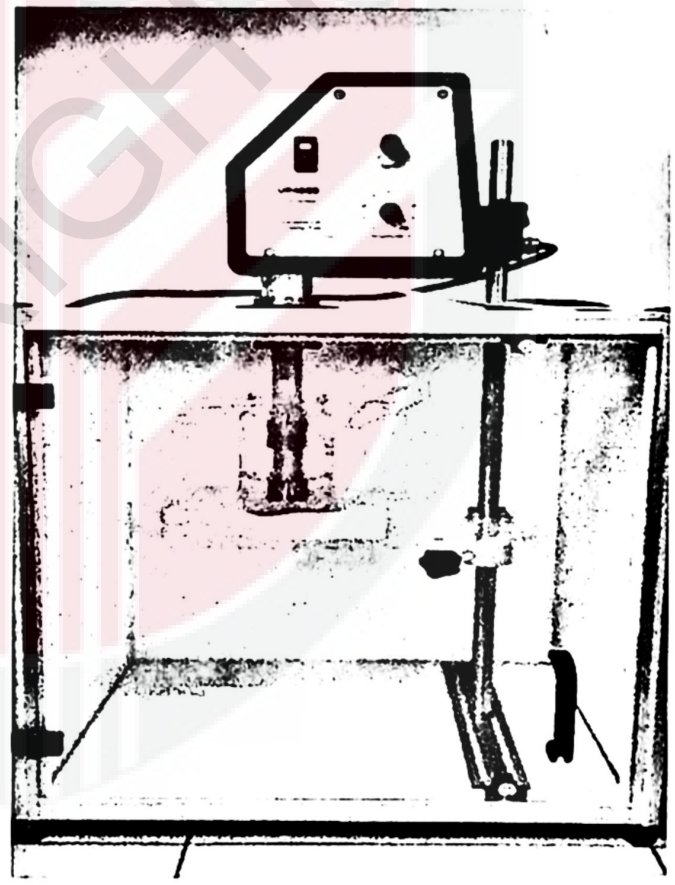

Figure A4: Ultrasonic sonicator

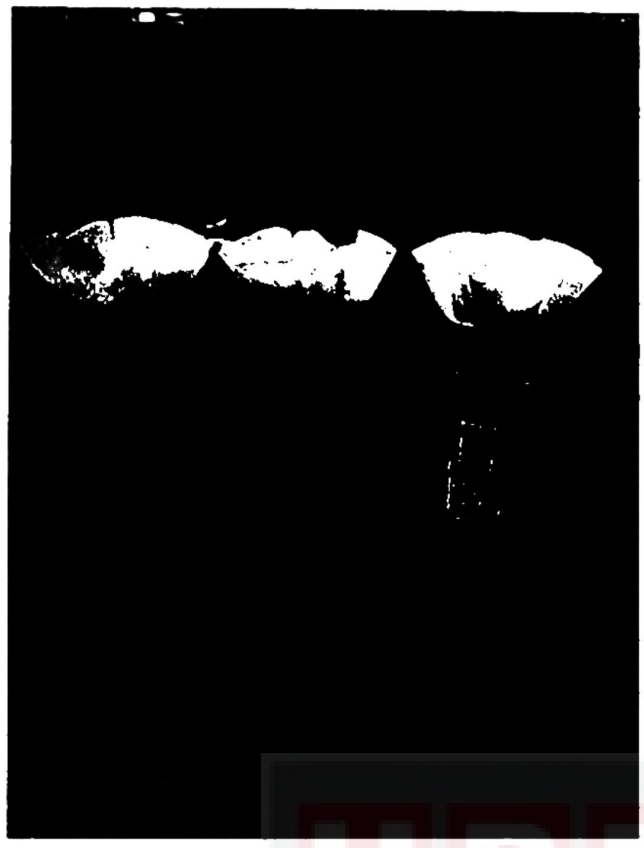

Figure A5: Filtration of sample

Figure A6: Storage Tube

APPENDIX B

SONICATE 30 AMP/0.3 CYCLE/20 MIN (ETHANOL 70%) - SE1

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.4454	0.6308	58.6136	42.94	0.043	2.147
2nd	2.00	0.4558	0.6204	57.6473	42.15	0.042	2.108
3rd	2.00	0.4763	0.5999	55.7424	40.60	0.041	2.030
AVERAGE		0.4592	0.6170	57.3344	41.90	0.04	2.09
SD		0.02	0.02	1.461	1.19	0.00	0.06

SONICATE 70 AMP/0.3 CYCLE/20 MIN (ETHANOL 70%) - SE2

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.35075	0.72545	67.4085	50.11	0.050	2.505
2nd	2.00	0.37535	0.70085	65.1227	48.25	0.048	2.412
3rd	2.00	0.39285	0.68335	63.4966	46.92	0.047	2.346
AVERAGE		0.3730	0.7032	65.3426	48.43	0.05	2.42
SD		0.02	0.02	1.965	1.60	0.00	0.08

SONICATE 30 AMP/0.7 CYCLE/20 MIN (ETHANOL 70%) - SE3

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.3919	0.6843	63.5848	46.99	0.047	2.350
2nd	2.00	0.31965	0.75655	70.2983	52.47	0.052	2.623
3rd	2.00	0.3352	0.741	68.8534	51.29	0.051	2.564
AVERAGE		0.3489	0.7273	67.5788	50.25	0.05	2.51
SD		0.04	0.04	3.534	2.88	0.00	0.14

SONICATE 70 AMP/0.7 CYCLE/20 MIN (ETHANOL 70%) - SE4

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.3445	0.7317	67.9892	50.58	0.051	2.529
2nd	2.00	0.33855	0.73765	68.5421	51.03	0.051	2.552
3rd	2.00	0.33455	0.74165	68.9138	51.34	0.051	2.567
AVERAGE		0.3392	0.7370	68.4817	50.98	0.05	2.55
SD		0.01	0.01	0.465	0.38	0.00	0.02

SONICATE 30 AMP/0.3 CYCLE/40 MIN (ETHANOL 70%) - SE5

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.3649	0.7113	66.0937	49.04	0.049	2.452
2nd	2.00	0.3669	0.7093	65.9078	48.89	0.049	2.444
3rd	2.00	0.3966	0.6796	63.1481	46.64	0.047	2.332
AVERAGE		0.3761	0.7001	65.0499	48.19	0.05	2.41
SD		0.02	0.02	1.650	1.34	0.00	0.07

SONICATE 70 AMP/0.3 CYCLE/40 MIN (ETHANOL 70%) - SE6

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.32735	0.74885	69.5828	51.88	0.052	2.594
2nd	2.00	0.30445	0.77175	71.7106	53.62	0.054	2.681
3rd	2.00	0.32345	0.75275	69.9452	52.18	0.052	2.609
AVERAGE		0.3184	0.7578	70.4129	52.56	0.05	2.63
SD		0.01	0.01	1.138	0.93	0.00	0.05

SONICATE 30 AMP/0.7 CYCLE/40 MIN (ETHANOL 70%) - SE7

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.3414	0.7348	68.2773	50.82	0.051	2.541
2nd	2.00	0.24175	0.83445	77.5367	58.37	0.058	2.918
3rd	2.00	0.2812	0.795	73.8710	55.38	0.055	2.769
AVERAGE		0.2881	0.7881	73.2283	54.85	0.05	2.74
SD		0.05	0.05	4.663	3.80	0.00	0.19

SONICATE 70 AMP/0.7 CYCLE/40 MIN (ETHANOL 70%) - SE8

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.34925	0.72695	67.5479	50.22	0.050	2.511
2nd	2.00	0.3328	0.7434	69.0764	51.47	0.051	2.573
3rd	2.00	0.3502	0.726	67.4596	50.15	0.050	2.508
AVERAGE		0.3441	0.7321	68.0279	50.61	0.05	2.53
SD		0.01	0.01	0.909	0.74	0.00	0.04

SONICATE 20 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE9

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.35215	0.72405	67.2784	50.00	0.050	2.500
2nd	2.00	0.33925	0.73695	68.4770	50.98	0.051	2.549
3rd	2.00	0.33375	0.74245	68.9881	51.40	0.051	2.570
AVERAGE		0.3417	0.7345	68.2478	50.79	0.05	2.54
SD		0.01	0.01	0.878	0.72	0.00	0.04

SONICATE 83.64 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE10

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.30805	0.76815	71.3761	53.34	0.053	2.667
2nd	2.00	0.30505	0.77115	71.6549	53.57	0.054	2.679
3rd	2.00	0.36255	0.71365	66.3120	49.22	0.049	2.461
AVERAGE		0.3252	0.7510	69.7810	52.04	0.05	2.60
SD		0.03	0.03	3.007	2.45	0.00	0.12

SONICATE 50 AMP/0.2 CYCLE/30 MIN (ETHANOL 70%) - SE11

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.30745	0.76875	71.4319	53.39	0.053	2.670
2nd	2.00	0.25875	0.81745	75.9571	57.08	0.057	2.854
3rd	2.00	0.2753	0.8009	74.4193	55.83	0.056	2.791
AVERAGE		0.2805	0.7957	73.9361	55.43	0.06	2.77
SD		0.02	0.02	2.301	1.88	0.00	0.09

SONICATE 50 AMP/0.84 CYCLE/30 MIN (ETHANOL 70%) - SE12

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.2703	0.8059	74.8839	56.20	0.056	2.810
2nd	2.00	0.2652	0.811	75.3577	56.59	0.057	2.830
3rd	2.00	0.2843	0.7919	73.5830	55.14	0.055	2.757
AVERAGE		0.2733	0.8029	74.6082	55.98	0.06	2.80
SD		0.01	0.01	0.919	0.75	0.00	0.04

SONICATE 50 AMP/0.5 CYCLE/13.18 MIN (ETHANOL 70%) - SE13

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.30615	0.77005	71.5527	53.49	0.053	2.674
2nd	2.00	0.3298	0.7464	69.3551	51.70	0.052	2.585
3rd	2.00	0.33025	0.74595	69.3133	51.66	0.052	2.583
AVERAGE		0.3221	0.7541	70.0737	52.28	0.05	2.61
SD		0.01	0.01	1.281	1.04	0.00	0.05

SONICATE 50 AMP/0.5 CYCLE/46.82 MIN (ETHANOL 70%) - SE14

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.29425	0.78195	72.6584	54.39	0.054	2.720
2nd	2.00	0.2625	0.8137	75.6086	56.80	0.057	2.840
3rd	2.00	0.27375	0.80245	74.5633	55.94	0.056	2.797
AVERAGE		0.2768	0.7994	74.2768	55.71	0.06	2.79
SD		0.02	0.02	1.496	1.22	0.00	0.06

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE15

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.1814	0.8948	83.1444	62.94	0.063	3.147
2nd	2.00	0.1616	0.9146	84.9842	64.44	0.064	3.222
3rd	2.00	0.15965	0.91655	85.1654	64.59	0.065	3.229
AVERAGE		0.1676	0.9087	84.4313	63.99	0.06	3.20
SD		0.01	0.01	1.118	0.91	0.00	0.05

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE16

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.16065	0.91555	85.0725	64.51	0.065	3.226
2nd	2.00	0.14295	0.93325	86.7172	65.85	0.066	3.293
3rd	2.00	0.16135	0.91485	85.0074	64.46	0.064	3.223
AVERAGE		0.1550	0.9212	85.5990	64.94	0.06	3.25
SD		0.01	0.01	0.969	0.79	0.00	0.04

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE17

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.1358	0.9404	87.3815	66.39	0.066	3.320
2nd	2.00	0.1182	0.958	89.0169	67.73	0.068	3.386
3rd	2.00	0.12275	0.95345	88.5941	67.38	0.067	3.369
AVERAGE		0.1256	0.9506	88.3309	67.17	0.07	3.36
SD		0.01	0.01	0.849	0.69	0.00	0.03

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE18

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.1993	0.8769	81.4811	61.58	0.062	3.079
2nd	2.00	0.1637	0.9125	84.7891	64.28	0.064	3.214
3rd	2.00	0.11125	0.96495	89.6627	68.25	0.068	3.413
AVERAGE		0.1581	0.9181	85.3110	64.71	0.06	3.24
SD		0.04	0.04	4.116	3.36	0.00	0.17

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE19

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.11315	0.96305	89.4862	68.11	0.068	3.405
2nd	2.00	0.1267	0.9495	88.2271	67.08	0.067	3.354
3rd	2.00	0.1059	0.9703	90.1598	68.66	0.069	3.433
AVERAGE		0.1153	0.9610	89.2910	67.95	0.07	3.40
SD		0.01	0.01	0.981	0.80	0.00	0.04

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE20

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.1598	0.9164	85.1515	64.58	0.065	3.229
2nd	2.00	0.16235	0.91385	84.9145	64.38	0.064	3.219
3rd	2.00	0.1297	0.9465	87.9483	66.86	0.067	3.343
AVERAGE		0.1506	0.9256	86.0048	65.27	0.07	3.26
SD		0.02	0.02	1.687	1.38	0.00	0.07

SONICATE 52.35 AMP/0.53 CYCLE/31.37 MIN (ETHANOL 70%) – OPT1

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.2008	0.8197	80.3234	62.51	0.063	3.126
2nd	2.00	0.1254	0.8951	87.7119	68.54	0.069	3.427
3rd	2.00	0.1164	0.9041	88.5938	69.26	0.069	3.463
AVERAGE		0.1475	0.8730	85.5430	66.77	0.07	3.34
SD		0.05	0.05	4.542	3.71	0.00	0.19

SONICATE 52.35 AMP/0.53 CYCLE/31.37 MIN (ETHANOL 70%) – OPT2:

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.2699	0.7506	73.5522	56.98	0.057	2.849
2nd	2.00	0.2065	0.814	79.7648	62.06	0.062	3.103
3rd	2.00	0.1886	0.8319	81.5189	63.49	0.063	3.174
AVERAGE		0.2217	0.7988	78.2786	60.84	0.06	3.04
SD		0.04	0.04	4.186	3.42	0.00	0.17

SONICATE 53.03 AMP/0.52 CYCLE/31.90 MIN (ETHANOL 70%) - OPT3

READING	WT OF SAMPLE (g)	ABSORBANCE		% RSA	DPPH (X) (µg GAE/mL)	DPPH (mg GAE/mL)	DPPH PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED				
1st	2.00	0.3181	0.7024	68.8290	53.13	0.053	2.656
2nd	2.00	0.2882	0.7323	71.7589	55.52	0.056	2.776
3rd	2.00	0.2594	0.7611	74.5811	57.82	0.058	2.891
AVERAGE		0.2886	0.7319	71.7230	55.49	0.06	2.77
SD		0.03	0.03	2.876	2.35	0.00	0.12

TABLE A-1: AVERAGE VALUE OF DPPH AND RADICAL SCAVENGING ACTIVITY (RSA) FROM OPTIMZED CONDITION:

SAMPLE	DPPH (mg GAE/g)	RSA (%)
OPT1	3.340	85.54303
OPT2	3.040	78.27862
OPT3	2.770	71.72301
AVERAGE	3.050	78.515

SONICATE 30 AMP/0.3 CYCLE/20 MIN (ETHANOL 70%) - SE1

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.6826	0.6091	3.680	184.012
2nd	2	0.7905	0.7170	4.355	217.732
AVERAGE		0.7366	0.6631	4.017	200.872
SD		0.076	0.076	0.477	23.844

SONICATE 70 AMP/0.3 CYCLE/20 MIN (ETHANOL 70%) - SE2

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.3865	1.3130	8.080	403.994
2nd	2	1.3453	1.2718	7.822	391.118
AVERAGE		1.3659	1.2924	7.951	397.556
SD		0.029	0.029	0.182	9.105

SONICATE 30 AMP/0.7 CYCLE/20 MIN (ETHANOL 70%) - SE3

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.4146	1.3411	8.256	412.776
2nd	2	1.7803	1.7068	10.541	527.064
AVERAGE		1.5975	1.5240	9.398	469.920
SD		0.259	0.259	1.616	80.814

SONICATE 70 AMP/0.7 CYCLE/20 MIN (ETHANOL 70%) - SE4

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.9622	1.8887	11.678	583.911
2nd	2	1.7517	1.6782	10.363	518.126
AVERAGE		1.8570	1.7835	11.020	551.019
SD		0.149	0.149	0.930	46.517

SONICATE 30 AMP/0.3 CYCLE/40 MIN (ETHANOL 70%) - SE5

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.3705	1.2970	7.980	398.994
2nd	2	1.5824	1.5089	9.304	465.217
AVERAGE		1.4765	1.4030	8.642	432.105
SD		0.150	0.150	0.937	46.827

SONICATE 70 AMP/0.3 CYCLE/40 MIN (ETHANOL 70%) - SE6

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.8173	1.7438	10.773	538.627
2nd	2	1.4690	1.3955	8.596	429.777
AVERAGE		1.6432	1.5697	9.684	484.202
SD		0.246	0.246	1.539	76.969

SONICATE 30 AMP/0.7 CYCLE/40 MIN (ETHANOL 70%) - SE7

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.4990	1.4255	8.783	439.152
2nd	2	1.7360	1.6625	10.264	513.220
AVERAGE		1.6175	1.5440	9.524	476.186
SD		0.168	0.168	1.047	52.373

SONICATE 70 AMP/0.7 CYCLE/40 MIN (ETHANOL 70%) - SE8

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.6523	1.5788	9.741	487.062
2nd	2	1.6476	1.5741	9.601	480.037
AVERAGE		1.6500	1.5765	9.671	483.549
SD		0.003	0.003	0.099	4.967

SONICATE 20 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE9

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.5128	1.4393	8.869	443.465
2nd	2	1.4745	1.4010	8.630	431.496
AVERAGE		1.4937	1.4202	8.750	437.480
SD		0.027	0.027	0.169	8.464

SONICATE 83.64 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE10

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.9329	1.8594	11.495	574.755
2nd	2	1.9048	1.8465	11.414	570.723
AVERAGE		1.9189	1.8530	11.455	572.739
SD		0.020	0.009	0.057	2.851

SONICATE 50 AMP/0.2 CYCLE/30 MIN (ETHANOL 70%) - SE11

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.7404	0.6669	4.042	202.075
2nd	2	0.9657	0.8922	5.450	272.486
AVERAGE		0.8531	0.7796	4.746	237.280
SD		0.159	0.159	0.996	49.788

SONICATE 50 AMP/0.84 CYCLE/30 MIN (ETHANOL 70%) -SE12

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.3121	1.2386	7.615	380.743
2nd	2	1.6276	1.5541	9.587	479.342
AVERAGE		1.4699	1.3964	8.601	430.043
SD		0.223	0.223	1.394	69.721

SONICATE 50 AMP/0.5 CYCLE/13.18 MIN (ETHANOL 70%) - SE13

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	1.7015	1.6280	10.049	502.438
2nd	2	1.8317	1.7582	10.863	543.128
AVERAGE		1.7666	1.6931	10.456	522.783
SD		0.092	0.092	0.575	28.772

SONICATE 50 AMP/0.5 CYCLE/46.82 MIN (ETHANOL 70%) - SE14

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	2.0849	2.0114	12.445	622.258
2nd	2	2.1473	2.0738	12.835	641.759
AVERAGE		2.1161	2.0426	12.640	632.008
SD		0.044	0.044	0.276	13.789

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE15

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.7818	0.7083	21.501	1075.067
2nd	2	0.7329	0.6594	19.973	998.656
AVERAGE		0.7574	0.6839	20.737	1036.862
SD		0.035	0.035	1.081	54.031

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE16

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.7799	0.7064	21.442	1072.098
2nd	2	0.7717	0.6982	21.186	1059.285
AVERAGE		0.7758	0.7023	21.314	1065.692
SD		0.006	0.006	0.181	9.060

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE17

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.8320	0.7585	23.070	1153.510
2nd	2	0.7632	0.6897	20.920	1046.003
AVERAGE		0.7976	0.7241	21.995	1099.756
SD		0.049	0.049	1.520	76.019

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE18

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.7154	0.6419	19.426	971.311
2nd	2	0.7528	0.6793	20.595	1029.752
AVERAGE		0.7341	0.6606	20.011	1000.531
SD		0.026	0.026	0.826	41.324

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE19

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.7511	0.6776	20.542	1027.095
2nd	2	0.7460	0.6725	20.383	1019.126
AVERAGE		0.7486	0.6751	20.462	1023.111
SD		0.004	0.004	0.113	5.635

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE20

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.6120	0.5385	16.195	809.738
2nd	2	0.6460	0.5725	17.257	862.866
AVERAGE		0.6290	0.5555	16.726	836.302
SD		0.024	0.024	0.751	37.567

SONICATE 52.35 AMP/0.53 CYCLE/31.37 MIN (ETHANOL 70%) - OPTIMIZATION 1

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.5652	0.4750	14.075	703.752
2nd	2	0.5846	0.4944	14.721	736.043
AVERAGE		0.5749	0.4847	14.398	719.897
SD		0.014	0.014	0.457	22.833

SONICATE 52.35 AMP/0.53 CYCLE/31.37 MIN (ETHANOL 70%) - OPTIMIZATION 2

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.5322	0.4420	12.976	648.823
2nd	2	0.6073	0.5171	15.477	773.827
AVERAGE		0.5698	0.4796	14.227	711.325
SD		0.053	0.053	1.768	88.391

SONICATE 52.35 AMP/0.53 CYCLE/31.37 MIN (ETHANOL 70%) - OPTIMIZATION 3

READING	WT OF SAMPLE (g)	ABSORBANCE		FRAP (mM FeSO4 /mL)	FRAP PER GRAM SAMPLE (mM FeSO4 /g)
		RAW DATA	CALCULATED		
1st	2	0.5517	0.4615	13.626	681.281
2nd	2	0.6187	0.5285	15.856	792.803
AVERAGE		0.5852	0.4950	14.741	737.042
SD		0.047	0.047	1.577	78.858

TABLE B-2: AVERAGE VALUE OF FERRIC REDUCING ANTIOXIDANT POWER (FRAP) FROM OPTIMZED CONDITION:

SAMPLE	FRAP (mM FeSO4 /g)
OPT1	719.897
OPT2	711.325
OPT3	737.042
AVERAGE	722.755

SONICATE 30 AMP/0.3 CYCLE/20 MIN (ETHANOL 70%) - SE1

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	1.4254	1.4202	145.53	0.146	7.277
2nd	2.00	1.4252	1.42	145.51	0.146	7.276
AVERAGE		1.4253	1.4201	145.52	0.15	7.28
SD		0.00	0.00	0.02	0.00	0.00

SONICATE 70 AMP/0.3 CYCLE/20 MIN (ETHANOL 70%) - SE2

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.312	0.3068	135.43	0.135	6.771
2nd	2.00	0.3848	0.3796	174.15	0.174	8.707
AVERAGE		0.3484	0.3432	154.79	0.15	7.74
SD		0.05	0.05	27.38	0.03	1.37

SONICATE 30 AMP/0.7 CYCLE/20 MIN (ETHANOL 70%) - SE3

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.3625	0.3573	162.29	0.162	8.114
2nd	2.00	0.4118	0.4066	188.51	0.189	9.426
AVERAGE		0.3872	0.3820	175.40	0.18	8.77
SD		0.03	0.03	18.54	0.02	0.93

SONICATE 70 AMP/0.7 CYCLE/20 MIN (ETHANOL 70%) - SE4

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4242	0.419	195.11	0.195	9.755
2nd	2.00	0.4733	0.4681	221.22	0.221	11.061
AVERAGE		0.4488	0.4436	208.16	0.21	10.41
SD		0.03	0.03	18.47	0.02	0.92

SONICATE 30 AMP/0.3 CYCLE/40 MIN (ETHANOL 70%) - SE5

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.2881	0.2829	122.71	0.123	6.136
2nd	2.00	0.3499	0.3447	155.59	0.156	7.779
AVERAGE		0.3190	0.3138	139.15	0.14	6.96
SD		0.04	0.04	23.24	0.02	1.16

SONICATE 70 AMP/0.3 CYCLE/40 MIN (ETHANOL 70%) - SE6

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4529	0.4477	210.37	0.210	10.519
2nd	2.00	0.4983	0.4931	234.52	0.235	11.726
AVERAGE		0.4756	0.4704	222.45	0.22	11.12
SD		0.03	0.03	17.08	0.02	0.85

SONICATE 30 AMP/0.7 CYCLE/40 MIN (ETHANOL 70%) - SE7

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4683	0.4631	218.56	0.219	10.928
2nd	2.00	0.3863	0.3811	174.95	0.175	8.747
AVERAGE		0.4273	0.4221	196.76	0.20	9.84
SD		0.06	0.06	30.84	0.03	1.54

SONICATE 70 AMP/0.7 CYCLE/40 MIN (ETHANOL 70%) - SE8

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4167	0.4115	191.12	0.191	9.556
2nd	2.00	0.4798	0.4746	224.68	0.225	11.234
AVERAGE		0.4483	0.4431	207.90	0.21	10.39
SD		0.04	0.04	23.73	0.02	1.19

SONICATE 20 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE9

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.346	0.3408	153.51	0.154	7.676
2nd	2.00	0.4401	0.4349	203.56	0.204	10.178
AVERAGE		0.3931	0.3879	178.54	0.18	8.93
SD		0.07	0.07	35.39	0.04	1.77

SONICATE 83.64 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) -SE10

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4437	0.4385	205.48	0.205	10.274
2nd	2.00	0.5013	0.4961	236.12	0.236	11.806
AVERAGE		0.4725	0.4673	220.80	0.22	11.04
SD		0.04	0.04	21.66	0.02	1.08

SONICATE 50 AMP/0.2 CYCLE/30 MIN (ETHANOL 70%) - SE11

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.2537	0.2485	104.41	0.104	5.221
2nd	2.00	0.2576	0.2524	106.49	0.106	5.324
AVERAGE		0.2557	0.2505	105.45	0.11	5.27
SD		0.00	0.00	1.47	0.00	0.07

SONICATE 50 AMP/0.84 CYCLE/30 MIN (ETHANOL 70%) - SE12

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4374	0.4333	202.71	0.203	10.136
2nd	2.00	0.4974	0.4922	234.04	0.234	11.702
AVERAGE		0.4674	0.4628	218.38	0.22	10.92
SD		0.04	0.04	22.15	0.02	1.11

SONICATE 50 AMP/0.5 CYCLE/13.18 MIN (ETHANOL 70%) - SE13

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4287	0.4235	197.50	0.198	9.875
2nd	2.00	0.489	0.4838	229.57	0.230	11.479
AVERAGE		0.4589	0.4537	213.54	0.21	10.68
SD		0.04	0.04	22.68	0.02	1.13

SONICATE 50 AMP/0.5 CYCLE/46.82 MIN (ETHANOL 70%) - SE14

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5016	0.4964	236.28	0.236	11.814
2nd	2.00	0.5181	0.5129	245.05	0.245	12.253
AVERAGE		0.5099	0.5047	240.66	0.24	12.03
SD		0.01	0.01	6.21	0.01	0.31

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE15

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5834	0.5782	279.79	0.280	13.989
2nd	2.00	0.5991	0.5939	288.14	0.288	14.407
AVERAGE		0.5913	0.5861	283.96	0.28	14.20
SD		0.01	0.01	5.91	0.01	0.30

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE16

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.524	0.5188	248.19	0.248	12.410
2nd	2.00	0.5781	0.5729	276.97	0.277	13.848
AVERAGE		0.5511	0.5459	262.58	0.26	13.13
SD		0.04	0.04	20.35	0.02	1.02

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE17

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.4751	0.4699	222.18	0.222	11.109
2nd	2.00	0.6102	0.605	294.04	0.294	14.702
AVERAGE		0.5427	0.5375	258.11	0.26	12.91
SD		0.10	0.10	50.81	0.05	2.54

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE18

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5765	0.5713	276.12	0.276	13.806
2nd	2.00	0.5042	0.499	237.66	0.238	11.883
AVERAGE		0.5404	0.5352	256.89	0.26	12.84
SD		0.05	0.05	27.19	0.03	1.36

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE19

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5495	0.5443	261.76	0.262	13.088
2nd	2.00	0.5921	0.5869	284.41	0.284	14.221
AVERAGE		0.5708	0.5656	273.09	0.27	13.65
SD		0.03	0.03	16.02	0.02	0.80

SONICATE 50 AMP/0.5 CYCLE/30 MIN (ETHANOL 70%) - SE20

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5129	0.5077	242.29	0.242	12.114
2nd	2.00	0.6268	0.6216	302.87	0.303	15.144
AVERAGE		0.5699	0.5647	272.58	0.27	13.63
SD		0.08	0.08	42.84	0.04	2.14

SONICATE 53.03 AMP/0.52 CYCLE/31.90 MIN (ETHANOL 70%) - OPT 1

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.6198	0.6133	285.57	0.286	14.279
2nd	2.00	0.6426	0.6361	297.45	0.297	14.872
AVERAGE		0.6312	0.6247	291.51	0.29	14.58
SD		0.02	0.02	8.40	0.01	0.42

SONICATE 53.03 AMP/0.52 CYCLE/31.90 MIN (ETHANOL 70%) - OPT 2

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5519	0.5454	250.21	0.250	12.510
2nd	2.00	0.5356	0.5291	241.72	0.242	12.086
AVERAGE		0.5438	0.5373	245.96	0.25	12.30
SD		0.01	0.01	6.00	0.01	0.30

SONICATE 53.03 AMP/0.52 CYCLE/31.90 MIN (ETHANOL 70%) - OPT 3

READING	WT OF SAMPLE (g)	ABSORBANCE		TPC (X) (µg GAE/mL)	TPC (mg GAE/mL)	TPC PER GRAM SAMPLE (mg GAE/g)
		RAW DATA	CALCULATED			
1st	2.00	0.5266	0.5201	237.03	0.237	11.852
2nd	2.00	0.5316	0.5251	239.64	0.240	11.982
AVERAGE		0.5291	0.5226	238.33	0.24	11.92
SD		0.00	0.00	1.84	0.00	0.09

TABLE B-3: AVERAGE VALUE OF TOTAL PHENOLIC CONTENT (TPC) FROM OPTIMZED CONDITION:

SAMPLE	TPC (mg GAE/g)
OPT1	14.576
OPT2	12.298
OPT3	11.917
AVERAGE	12.930