QUANTIFICATION OF BIOACTIVE COMPOUNDS FROM *AVERRHOA BILIMBI*

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A thesis submitted in fulfilment
of the requirements for the award of the degree of
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SUPERVISOR DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering

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I declare that this thesis entitled "Quantification of bioactive compounds from *Averrhoa Bilimbi*." is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Special Dedication of This Grateful Feeling to My Beloved father and mother;

Mr. MUHMEDB. ALI and Mrs. HABSAH BT. AB. KADIR

Loving siblings;

SALIHAH KAMILAH, NOR NAZIFAH,

MOHD IHSAN ADLI and MOHD SALAHUDDIN

For Their Love, Support and Best Wishes.

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QUANTIFICATION OF BIOACTIVE COMPOUNDS FROM *AVERRHOA BILIMBI*

ABSTRACT

Averrhoa bilimbi has been widely used in traditional medicine, thus, this fruits have received much attention because of its nutritional and antioxidant properties. The purposes of this study are to determine the bioactive compounds in *averrhoa bilimbi* and to study their optimum parameters during extraction. Antioxidant activity of the *averrhoa bilimbi* extract was determined based on 2,2-diphenyl-1-picrylhydrazyl freeradical (DPPH·), total phenolic content was measured using Folin–Ciocalteu reagent, while flavonoids is determined spectrophotometrically. Solvent extraction selected is methanol (50%) where this condition has yielded total flavonoids content is about 568.75 µg/ml QE. The highest antioxidant capacities measured is 80.02 % and total phenolic content shows about 175.3 mg/mL GAE.In this study, 60 minutes extraction time generally showed the highest effect on total flavonoids which is 496.25 µg/ml QE. *Averrhoa bilimbi* extraction had total phenolic about 164.7 mg/mL GAE and antioxidant activity is around 74.5%. 70° C extraction temperature shown the best extraction for total phenolics at 127.7 mg/ml GAE, while achieved highest total flavonoids at 656.25 µg/ml QE and the highest for DPPH radical scavenging activity is found about 70.05%. From the effect of agitation speed, 300 rpm achieved the highest value for total phenolics, total flavonoids and antioxidant capacity, DPPH which is about 193.3 mg/ml GAE, 717.75 µg/ml QE, and 77.03% respectively. Overall, based on the ideal extraction conditions chosen, optimum level of TPC, TFC and antioxidant capacity were obtained in *averrhoa bilimbi* fruit extract. The selected extraction conditions could be used for further studies and functional food product development

KUANTIFIKASI SEBATIAN BIOAKTIF DARIPADA *AVERRHOA BILIMBI*

ABSTRAK

Averrhoa bilimbi telah digunakan dengan secara meluas dalam perubatan tradisional dan secara tidak langsung, buah ini mendapat perhatian disebabkan kandungan nutrisi dan ciri-ciri antioksidannya. Objektif kajian ini adalah untuk menentukan sebatian bioaktif dalam *averrhoa bilimbi* dan mengkaji parameter optimum semasa pengekstrakan. Aktiviti antioksidan dalam *averrhoa bilimbi* ditentukan dengan 2,2-difenil-1-picrylhydrazyl radikal bebas (DPPH·), jumlah kandungan fenolik diukur menggunakan reagen manakala flavanoid ditentukan dengan. Pelarut untuk pengekstrakan yang dipilih ialah methanol (50%) dimana keadaan ini telah menghasilkan jumlah kandungan flavonoids sebanyak 568.75 µg/ml QE. Kapasiti antioksidan yang tertinggi dicatatkan pada 80.02% dan jumlah kandungan fenolik ialah sebanyak 175.3 mg/mL GAE. Dalam kajian ini, 60 minit masa pengekstrakan mencatatkan jumlah flavonoid yang paling tinggi iaitu 496.25 µg/ml QE. Pengekstrakan *averrhoa bilimbi* mempunyai jumlah fenolik sebanyak 164.7 mg/mL GAE dan aktiviti antioksidan adalah diantara 74.5%. 70° C suhu pengekstrakan menunjukkan pengekstrakan terbaik untuk jumlah fenolik iaitu 127.7 mg/ml GAE, mencapai jumlah flavonoid tertinggi pada 656.25 µg/ml QE dan yang paling tinggi untuk DPPH dicatatkan sebanyak 70.05%. Daripada kesan kelajuan pergolakan, 300 rpm mencapai nilai tertinggi bagi jumlah fenolik jumlah flavonoid dan kapasiti antioksidan masing-masing sebanyak 193.3 mg/ml GAE, 717.75 µg/ml QE, dan 77.03%. Secara keseluruhan, berdasarkan keadaan pengekstrakan ideal yang dipilih, tahap optimum jumlah kandungan fenolik, jumlah kandungan flavonoids dan kapasiti antioksidan diperoleh dalam pengekstrakan buah *averrhoa bilimbi*. Keadaan pengekstrakan yang dipilih boleh digunakan untuk kajian lanjut dan pembangunan produk makanan.

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LIST OF ABBREVIATIONS

AC	-	Antioxidant Capacity
$AlCl_3$	-	Aluminium Chloride
CE	-	Catechin equivalent
DPPH	-	2,2-diphenyl-1-picrylhydrazyl freeradical scavenging assay
FC	-	FolinCiocalteu's
GAE	-	Gallic Acid equivalent
MeOH	-	Methanol
$NaCO_3$	-	Sodium Carbonate
$NaNO_2$	-	Sodium Nitrate
NaOH	-	Sodium Hydroxide
QE	-	Quercetin equivalent
TFC	-	Total Flavonoids Content
TPC	-	Total Phenolics Content

LIST OF SYMBOLS

%	-	percentage
°C	-	degree Celsius
atm	-	atmospheric pressure
C_A	-	molar concentration of solute *A* in the solution (kg mol A/m^3)
C_{AS}	-	saturation solubility of the solute *A* (kg mol/m^3)
cm	-	centimeter
D_{BA}	-	diffusivity of solute in the solvent (m^2/s)
Ft	-	feet
g	-	Gram
In	-	Inches
k_L	-	mass transfer coefficient (m/s)
L	-	Litre
m	-	Metre
M	-	Molarity (mol/m^3)
mg	-	miligram
mL	-	mililiter
mm	-	milimeter
N_A	-	rate of dissolution of the solute *A* in the solution (kg mol/s)
nm	-	nanometer
rpm	-	revolutions per minute (r/min)
t	-	Time (h or min)
w/v	-	weight / volume (kg/m^3)
z	-	Distance inside the porous of the solid matrix (m)
µg	-	microgram

CHAPTER ONE

INTRODUCTION

1.1 Research background

Averrhoa bilimbi Linn (Oxalidaceae) is a small–sized tree growing up to 15 m tall and 30 cm diameter. The chemical constituents of *A.bilimbi* that have been identified include amino acids, citric acid, cyanidin–3–O–β–D–glucoside, phenolics, potassium ion, sugars and vitamin A (Tan et al., 2005). It is used as antibacterial, antiscorbutic, astringent; post–partum protective medicine; treatment of fever, mumps, pimples, inflammation of the rectum and diabetes (decoction of the leaves); treatment of itches, boils, rheumatism, cough and syphilis (paste of leaves); treatment of scurvy, bilious colic, whooping cough, hypertension and as a cooling drink (juice of preserved fruits); treatment of children's cough (syrup of flowers); treatment of stomach ache (fruits) (Tan et al., 2005). Besides that, *A. bilimbi*, has been widely used in traditional medicine for cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough and hypertension (Abas et al., 2006).

Figure 1.0 *Averrhoa Bilimbi* fruit (Sources: Sugeesh, 2008)

An increase in the consumption of fruits and vegetables is related with a decrease in the rate of cardiovascular disease and reduce risks of certain cancers. Fruits and derived products have a beneficial effect on the human health. Thus, *A. bilimbi* fruits have received much attention because of its nutritional and antioxidant properties. This is due mainly to the contribution of antioxidant compounds including vitamin C, phenolic compounds and carotenoids (Cano et al., 2008). The content of vitamin C in fruits and vegetables can be influenced by various factors such as genotypic differences, climatic conditions and cultural practices. Flavonoids contents in fruits mainly rely on genetic characteristics. Some analytical methods are applied to qualitative and quantitative flavonoid determination, especially by HPLC in conjunction with diode array detection and mass spectrometry for their identification and characterization.

Bioactive compounds evaluate from *Averrhoa bilimbi* are flavonoids, antioxidant capacity and phenolic. Flavonoids constitute the most common group of secondary plant metabolites that play important roles in the interactions of plants with their environments. The type, amount and localisation of flavonoids vary according to plant species and the developmental stage of the tissues, and may be modulated by environmental signals (Jeng et al., 2010). Ascorbic acid content is a powerful anti-

oxidant present in food and beverages, and it is also used as a marker chemical in evaluating food deterioration and product quality. Among the various bioactive substances, phenolic compounds which are plant secondary metabolites and have been proven to exhibit many health protective effects, have received most attention.

1.2 Problem statement

Results on quantification of bioactive compounds in *averrhoa bilimbi* will be varies due to different parameters on extraction. Optimum parameters on extraction are necessary to obtain the highest value in quantification of bioactive compounds which are total phenolics, total flavonoids and antioxidants capacity. Parameters that have been study in extraction includes effects on type of solvents with different concentration, effects of times, effects of temperatures and lastly effects of agitations. Basically, type of solvents with different concentration effects on extraction yield where the more polar the organic solvent, the more it is miscible or soluble in water hence resulting in a good extraction. Many authors established that the extraction yield of bioactive compounds is greatly depending on the solvent polarity (Turkmen et al., 2006; Lapornik et al., 2005). The difference in polarities of extracting solvents might influence the solubility of chemical constituents in a sample and its extraction yield. While for the concentration solvents, (Rødtjer et al., 2006) reported that quantification of the total amount of phenolics in the extracts showed that 50% solvent-water mixtures extracted the phenolics more efficiently and contained more complex mixtures of phenolic compounds than the pure solvent extracts did. The difference in polarities of extracting

solvents might influence the solubility of chemical constituents in a sample and its extraction yield.

On top of that, bioactive compound would be higher if optimum time is used for the extraction. The time must be not too long or too short because it will absolutely effect on the amount of extraction. Prolongation of extraction duration potentially increases the loss of phenolics, flavonoids and antioxidants by exposure to light and oxygen. An increase in extraction time might give rise to possible degradation (Garcia-Sales et al., 2010). However, a short extraction time might yield a small amount of bioactive compounds. Thoo et al. (2010) revealed that excess extraction time lead to reduction of phenolic and antioxidant yields. Besides that, to achieve higher bioactive compound, optimum temperature is a crucial factor that is necessary to be observed. For higher temperature, the phenolic, flavonoids and antioxidants capacity will be degrades and become unstable and this resulting to the lower of extraction while for low temperature, the amount of extraction on the fruit cannot be done greatly and lead to small amount recorded. Lastly, effect of agitation on extraction is one of the important parameters to be focus. By considering all the optimum parameters, higher quantification of bioactive compounds can be achieved.

1.3 Research objective

The purpose of this study is to determine the bioactive compounds in *averrhoa bilimbi* and to study their optimum parameters during extraction.

1.4 Scope of research

The following are the scopes of this research to support the above mentioned objectives:

i) To analyze the exact amount and accurate data on how much the capacity of bioactive compounds which are flavonoids, phenolic, and antioxidant content in *A. bilimbi*.

ii) To study optimum parameters such as effects on type of solvents with different concentrations, effects of times, effects of temperatures and effects of agitations.

1.5 Rationale and significance

This study will provide a better understanding on the quantification of bioactive compounds in *A. bilimbi* by using different methods in testing each of bioactive compounds include determination of flavonoids, phenolic, and antioxidant activity with the optimum parameters on extraction where accurate result will be identify showing the right content of bioactive compound in *A. bilimbi* which is expected to be achieved upon the completion of this study.

1.6 Thesis outline

In this research, it will divide into five chapters. Firstly, Chapter 1 is an overview about this research. It consists of the introduction on *A. bilimbi* which gives a brief idea on what are bioactive compounds content in it. The problem statement, objective and the scope of the study also are included in this chapter.

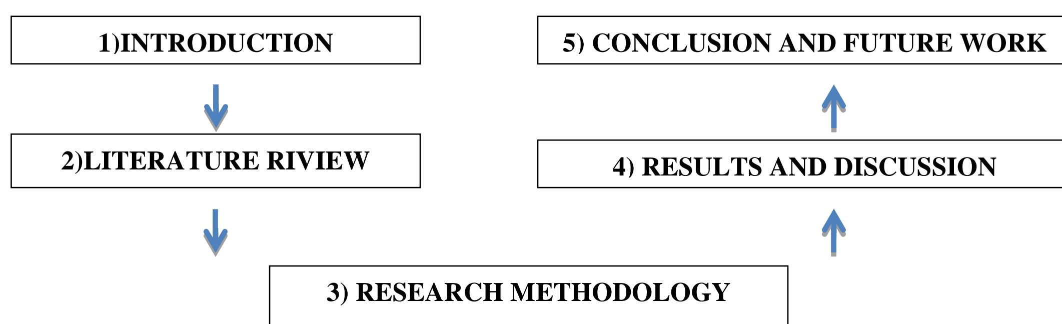


Figure 1.2 The road map for thesis

Chapter 2 is about literature review on *A. bilimbi* fruit, list of method apply in quantifying bioactive compounds and what is the finding in each of the method. In this chapter, all the relevant technical paper, journals, and books taken from those researches will be studied and discussed.

Then, Chapter 3 will be covered the parts of experimental set up and will be explained more details on methodology and operating procedures. The techniques and the algorithms that will be used in performing this study will be applied. The method and techniques used for this system is described in detail. In addition, in this chapter also explained the material used in this experiment and the method use to analysis the data.

Chapter 4 will be covered on the results and discussion of the research during the operation process. All the experimental result and data will be discussed in details which are including the capacity each of the bioactive compounds contain in *A. bilimbi* based on the different parameters. The detailed report on the product quality analysis was evaluated. Implementation of process that is involved during development of this analysis is explained in detail in this chapter.

Chapter 5 will be discussed on the conclusion can be made for the study and some recommendations can be taken.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction and Historical Background

Food engineering is a multidisciplinary field of applied physical sciences which include science, microbiology, and engineering education for food and related industries. Food engineering includes, but is not restricted to, the application of agricultural engineering, mechanical engineering and chemical engineering principles to food materials. Food engineers offered the technological knowledge transfer essential to the cost-effective production and commercialization of food products and services. This type of technology is being used not only for production but in fact it can provide a good result in identifying and quantifying some compound in certain fruits, vegetables and others. The quantification process is determined by some methods present to determine certain bioactive compound in the fruit for example. We look into our material world through the methods and the tools of analytical chemistry. Those analytical techniques existing now are always being required to improve by analytical chemists to meet the

arising requirements for better chemical measurement from our society. Usually one or more standard specific procedures are accessible for determination of an analyte in a provided sample. However, it is not accurately can be accepted by the other analyte. The analyst needs to depend on his experience and knowledge to carry out and analytical method for a sample. Since in *A. bilimbi*, there are some kinds of bioactive compounds, some methods with a right reagent should be apply to determine each of bioactive compounds contain in it.

2.2 Averrhoa bilimbi

The bilimbi, *Averrhoa bilimbi,* L., (Oxalidaceae), is intimately allied to the carambola but quite dissimilar in appearance, behaviour of fruiting, flavour and uses. The only strictly English names are "cucumber tree" and "tree sorrel", bestowed by the British in colonial times. "*Bilimbi*" is the common name in India and has become extensively used. In Malaysia, it is called *belimbing asam, belimbing buloh, b'ling,* or *billing-billing* while in Indonesia, it is known as *belimbing besu, balimbing, blimbing,* or *blimbing wuluh;* in Thailand, it is famous with *talingpling,* or *kalingpring.*In Haiti, it is called *blimblin;* in Jamaica, *bimbling plum;* in Cuba, it is *grosella china;* in El Salvador and Nicaragua, *mimbro;* in Costa Rica, *mimbro* or *tiriguro;* in Venezuela, *vinagrillo;* in Surinam and Guyana, *birambi;* in Argentina, *pepino de Indias.* To the French it is *carambolier bilimbi,* or *cornichon des Indes.* Filipinos normally call it *kamias* but there are about a dozen other native names (Morton, 1987).

Figure 2.1 Averrhoa Bilimbi fruit (Sources: Sugeesh, 2008)

2.2.1 Description of Averrhoa Bilimbi

Description of this fruit such as the tree is attractive, long-lived, reaches 16 to 33 ft (5-10 m) in height; has a short trunk soon separating into a number of upright branches. The leaves, very alike to those of the Otaheite gooseberry and mainly clustered at the branch tips, are alternate, imparipirmate; 12 to 24 in (30-60 cm) long, with 11 to 37 alternate or sub opposite leaflets, ovate or oblong, with rounded base and pointed tip; downy; medium-green on the upper surface, pale on the underside; 3/4 to 4 in (2-10 cm) long, 1/2 to 1 1/8 in (1.2-1.25 cm) wide. Small, fragrant, 5-petalled flowers, yellowish-green or purplish marked with dark-purple, are borne in small, hairy panicles emerging directly from the trunk and oldest, thickest branches and some twigs, as do the clusters of curious fruits. The *bilimbi* is ellipsoid, obovoid or just about cylindrical, faintly 5-sided, 1 1/2 to 4 in (4-10 cm) long; capped by a thin, star-shaped calyx at the stem-end and tipped with 5 hair-like floral remnants at the apex. The fruit is crisp when unripe, turns from bright-green to yellowish-green, ivory or nearly white when ripe and

falls to the ground. The outer skin is glossy, very thin, soft and tender, and the flesh green, jelly-like, juicy and extremely acid. There may be a few (perhaps 6 or 7) flattened, disc-like seeds about 1/4 in (6 mm) wide, smooth and brown.

2.2.2 Origin and Distribution

Perhaps a native of the Moluccas, the *bilimbi* is grown throughout Indonesia; is cultivated and semi-wild everywhere in the Philippines; is much grown in Ceylon and Burma. It is very familiar in Thailand, Malaysia and Singapore, common in gardens across the plains of India, and has run wild in all the warmest areas of that country. It is much planted in Zanzibar. Introduced into Queensland about 1896, it was readily adopted and commercially spread to growers. In 1793, the *bilimbi* was passed from the island of Timor to Jamaica and, after some years, *bilimbi* was planted in Cuba and Puerto Rico, Trinidad, the lowlands of Central America, Venezuela, Colombia, Ecuador, Surinam, Guyana and Brazil, and even in northern Argentina, and it is very famous among the Asiatic residents of those countries as it must be in Hawaii. Still it is grown only as an occasional interest in southern Florida.

2.2.3 Uses and applications

The *bilimbi* is usually regarded as too acid for eating raw, but in Costa Rica, the green, uncooked fruits are set up as a relish which is served with rice and beans.

Sometimes it is an accessory for fish and meat. Ripe fruits are often added to curries in the Far East. They yield 44.2% juice having a pH of 4.47, and the juice is famous for making cooling beverages on the order of lemonade. Mostly, the *bilimbi* is used in place of mango in making of chutney, and it is much preserved. To decrease acidity, it may be first pricked and soaked in water overnight, or soaked in salted water for a shorter period; then it is boiled with much sugar to make a jam or an acid jelly. The latter, in Malaysia, is putting to stewed fruits that are oversweet. Half-ripe fruits are salted, expose to the sun, and pickled in brine and can be thus kept for 3 months. A quicker pickle is made by place the fruits and salt into boiling water. This product can be kept only 4 to 5 days. The flowers are occasionally preserved with sugar.

For medical uses and application, the leaves are function as a paste or poultice on itches, swellings of mumps and rheumatism, and on skin eruptions in the Philippines. In another place, they are applied on bites of poisonous creatures. Malayans make used the leaves fresh or fermented as a treatment for venereal disease. A leaf infusion is a therapy for coughs and is taken after childbirth as a tonic. A leaf decoction is used to relieve rectal inflammation. A flower infusion is said to be helpful against coughs and thrush. In Java, the fruits combined with pepper are eaten to provide sweating when people are feeling "under the weather". A paste of pickled *bilimbis* is smeared all over the body to hurry the recovery after a fever. The fruit conserve is used as a treatment for coughs, beriberi and biliousness. Syrup prepared from the fruit is taken as a therapy for fever and inflammation and to stop rectal bleeding and alleviate internal haemorrhoids. It is used as antibacterial, ant scorbutic, astringent; post–partum protective medicine; treatment of fever, mumps, pimples, inflammation of the rectum and diabetes (decoction of the leaves); treatment of itches, boils, rheumatism, cough and syphilis (paste of leaves);

treatment of scurvy, bilious colic, whooping cough, hypertension and as a cooling drink (juice of preserved fruits); treatment of children's cough (syrup of flowers); treatment of stomach ache (fruits) (Tan et al., 2005). Besides that, *A. bilimbi*, has been widely used in traditional medicine for cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough and hypertension (Abas et al., 2006).

2.3 Method in quantifying of bioactive compounds in *A. bilimbi*

Some methods through analytical technique are applied in order to analyse and determine bioactive compounds contain in *A. bilimbi*. Choosing a right reagent is compulsory to be added in procedure leading an accurate data for result. Each of the bioactive compounds has their own identification method, reagent, conditions and technologies used. Below are the method selected for quantification of bioactive compound in *averrhoa bilimbi.*

2.4 Antioxidant capacity

Antioxidants can be classified within two classes as synthetic and natural. Among the synthetic types, the most commonly used to preserve food are butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), propyl gallate (PG) and tertbutyl hydroquinone (TBHQ). For the same function, tocopherol and ascorbic acid can be broadly used as natural antioxidants. Several reports reveal that BHA and

BHT could be toxic (Moure et al., 2001). In addition, the use of natural antioxidants such as ascorbic acid and tocopherols is limited because of higher manufacturing costs and lower efficiency of them (Moure et al., 2001). For these reasons, with the increasing awareness of consumers with regard to food additive safety, a need for identifying alternative natural and probably safer sources of food antioxidants are necessary. The rising interest in the substitution of synthetic food antioxidants by natural ones has focused research on vegetable sources and the screening of raw materials for identifying new antioxidants.

Carotenoids, a class of natural fat-soluble compounds mainly denovo synthesised by plants, are one class of major food micronutrients in human diet (Maiani et al., 2009). In plants, they have probable antioxidant properties due to their chemical structure. Carotenoids required in protecting plants against photo oxidative processes. They are efficient antioxidants, e.g. in scavenging singlet molecular oxygen and peroxyl radicals (Müller et al., 2011). Certain convenient methods were established for a quick, simple and reliable quantification of the antioxidant capacity. Beside the antioxidant capacities of them, several studies have been carried out in order to investigate their potential health effects (Moure et al., 2001). In addition to antioxidant activity, other biological properties such as anticarcinogenicity, antimutagenicity, antiallergenicity and antiaging activity have been reported for phytochemicals. Their use as chemo preventive agents by inhibiting radical generation has been an optional since free radicals are responsible for DNA damage and radical scavengers are probably important in cancer prevention (Moure et al., 2001).

In general, the methods to determine the total antioxidant capacity were divided into two major groups assays based on the single electron transfer (SET) reaction,

displayed through a change in colour as the oxidant is reduced, and assays based on a hydrogen atom transfer (Müller et al., 2011), which quantify the activity of the antioxidant to scavenge peroxyl radicals, such as the total radical trapping antioxidant parameter (TRAP) assay, the oxygen radical absorbance capacity (ORAC) assay and the luminol-chemiluminescence based peroxyl radical scavenging capacity (LPSC) assay. The ferric reducing antioxidant power (FRAP), the α-tocopherol/Trolox equivalent antioxidant capacity (αTEAC/TEAC) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays include electron transfer reaction. The antioxidant activity of a *A. bilimbi* may be determined using by various methods such as ferric thiocyanate method, thiobarbituric acid method, free radical-scavenging activity (2,2 –diphenyl-1-picrylhydrazyl) DPPH, and β-Carotene bleaching method. For antioxidant capacity measured using β-Carotene bleaching method, ferric reducing antioxidant potential (FRAP) and (2,2 –diphenyl-1-picrylhydrazyl) DPPH assays showed percentage of antioxidant capacity content is 91.89 % and from the order of pometia, *averrhoa*, syzyqium, antioxidant capacity showed the percentage is more than 70% (Ikram et al., 2009). Besides that, method used to determine the antioxidant activity by using ferric thiocyanate method, thiobarbituric acid method, free radical-scavenging activity (1,1–diphenyl-2-picrylhydrazyl) DPPH, showed the result where *A. bilimbi* and *C. mangga* contain moderate antioxidant activities (Abas et al., 2006).

2.5 Flavonoids

The flavonoids are members of a class of natural compounds that lately has been the subject of considerable scientific and therapeutic interest. The flavonoids played a major role in successful medical treatments of ancient times, and their use has persisted up till now. Flavonoids are ubiquitous among vascular plants and occur as aglycones, glucosides and methylated derivatives. More than 5000 flavonoids have been described so far within the parts of plants normally consumed by humans and approximately 650 flavones and 1030 flavanols are known (Pretorius, 2003). Small amount of aglycones (i.e., flavonoids without attached sugar) are regularly present and occasionally represent a considerably important proportion of the total flavonoid compounds in the plant. There is strong evidence from observational epidemiology that fruits and vegetables containing flavonoids in the diet are associated with a lower incidence of various cancers (Collins, 2005). Because of this, the idea has come out that it is the antioxidants in these foods that are the effective preventive agents. This is an interesting hypothesis; it is known that free radicals released during respiration can damage DNA, which results in mutation and consequently leads to cancer. This damage can be banned by the consumption of fruits and vegetables, which contain substantial amounts of various natural compounds with antioxidant properties. These natural compounds scavenge free radicals and as a result decreasing the degenerative effects of them. The most abundant flavonoids in fruits, herbs and vegetables are given in Table 2.1.

Table 2.1 Common flavonoids and content in variety foods

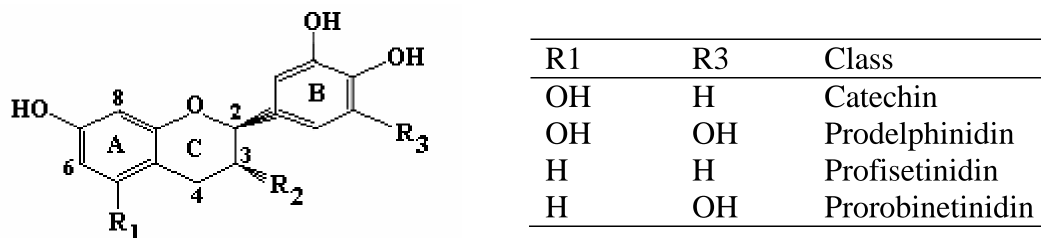
Flavonoids	Foods, oil and beverages
Quercetin	Onion, red apple, lettuce, broccoli, cranberry, berries, olive oil, tea, red wine
Myricetin	Cranberry, grapes, red wine
Kaempferol	Endive, leek, broccoli, radish, grapefruit, tea
Catechin, Epicatechin, their gallate forms	Grapes, green and black tea, red wine
Cyanidin	Cherry, raspberry, strawberry, grapes
Proanthocyanidin	Red wine, red grapes
Hesperidin, naringin	Citrus fruits
Apigenin	Apple skins, celery, parsley
Resveratrol	Fruit skins, berries

(Sources: Altiok, 2010)

Flavonoids contain the most common group of secondary plant metabolites that provide the interactions of plants with their environments. The type, amount and localisation of flavonoids vary according to plant species and the developmental stage of the tissues, and may be modulated by environmental signals (Jeng et al., 2010). Flavonoids defend plants from UV radiation, provide flower pigmentation to attract pollinators, protect plants against pathogens and act as signal molecules in plant–microbe interactions. They are also good to human health. Most of the beneficial health effects of flavonoids are credited to their antioxidant and chelating abilities. Dietary intakes of these compounds are important to human health because flavonoids are not synthesised by the human body. Flavonoid-rich diets have been shown to decrease the risk of heart disease and some hormone-related cancers. Flavonoids have characteristic $C_6C_3C_6$ chemical structure as shown in Figure 2.2.

The number of hydroxy groups and the presence of a 2,3-double bond and orthodiphenolic structure enhance antiradical and antioxidative activity of flavonoids

(Tapas et al., 2008). The glycosylation, blocking the 3-OH group in C-ring, lack of a hydroxy group or the presence of only a methoxy group in B-ring has a decreasing effect on antiradical or antioxidative activity of these compounds (Tapas et al., 2008). Tannins show strong antioxidative properties due to the higher number of OH groups. Some



R1	R3	Class
OH	H	Catechin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 2.2 The basic units of flavonoids
(Sources: Altiok, 2010)

tannin in red wine or gallate esters was proved to have antioxidative effect in vivo. The numbers of hydroxy groups connected with the aromatic ring, in ortho or para position relative to each other, enhance antioxidative and antiradical activity of phenolic acids (Tapas et al., 2008).

Figure 2.3 represents major flavonoids' structures. The six-membered ring condensed with the benzene ring is either-pyrone (flavones (1) flavonols (3)) or its dihydroderivative (flavanones (4) and flavan-3-ols (5)). The position of the benzenoid substituent classified the flavonoids into two classes: flavonoids (1) (2-position) and isoflavonoids (6) (3-position). Most flavonoids take place naturally associated with sugar in conjugated form and, within any one class, may be characterized as monoglycosidic, diglycosidic, etc. The glycosidic linkage is usually located at position 3

or 7 and the carbohydrate unit can be L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose (Tapas et al., 2008).

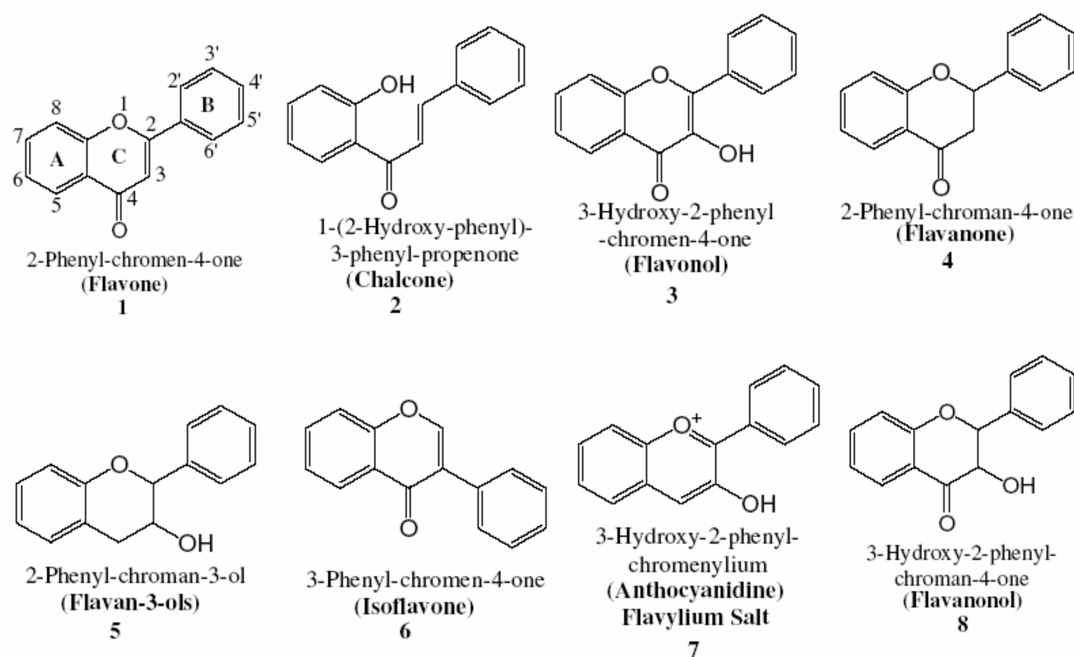


Figure 2.3 Chemical structures of some representative flavonoids.
(Sources: Altiok, 2010)

Total flavonoids contents were measured spectrophotometrically based on the formation of a complex flavonoid-aluminum, with some modifications where an aliquot of the extract solution was mixed with distilled water and subsequently with $NaNO_2$ solution (Guimarães et al., 2010). Total flavonoids contain in four types of fruit tested which are grapefruit, lemon, lime and orange, lemon shown the highest flavonoids content, 15.96 ± 0.24 mg CE/g extract for peel polar fraction, lime shown the highest flavonoids content, 2.36 ± 0.04 mg CE/g extract for juice polar fraction and orange

shown the highest flavonoids content, 0.62 ± 0.09 mg CE/g extract for crude juice (Guimarães et al., 2010).

2.6 Total phenolic content

Natural Phenolics and polyphenols are two related classes of natural compounds found in plants. At present, the natural bioactive compounds like phenolics, carotenoids, phytates, isothiocyanates, phytosterols, phytoesterogens, organosulphur and others have been reported to demonstrate many health benefits including excellent antioxidant property. Among the various bioactive substances, phenolic compounds which are plant secondary metabolites and have been reveal to exhibit many health protective effects, have gathered most attention total phenolic content (TPC) which are reducing component was estimate using the Folin – Ciocalteu reagent assay. The TPC was expressed as gallic acid equivalent (GAE) mg/100g edible portion. Total phenolic content in *A. Bilimbi* showed the value of 1261.63 ± 31.41 mg GAE/100g edible portion (Ikram et al, 2009).

2.7 Extraction Technique

Extraction is a necessary step in isolating these bioactive compounds. The most commonly used technique for the isolation of plant bioactive compounds is solvent extraction that is, maceration and percolation. Conversely, the extract yields and

resulting antioxidant activities of the plant materials are strongly dependent on the type of extracting solvent, due to the presence of different antioxidant compounds of diverse chemical characteristics and polarities that may or may not be soluble in a particular solvent. Mostly, polar solvents are used for the recovery of bioactive compounds from a plant matrix. Commonly used extraction solvents are ethanol, methanol, hexane and acetone whilst inorganic extraction solvents included water (Wang et al., 2008). The extraction efficiency can be enhanced by selecting the optimum solvent-to-water ratio, which therefore can increase the extraction yield. Extraction of phenolic compounds and antioxidants would be restricted if some other components are being extracted due to certain solvent-to-water ratio. This is because of the specific solvent-to-water ratio that favours extraction of most analytes from the sample matrix other than the desired ones. Therefore, the solvent-to-water ratio is inconclusive and varies from one research to another (Wang et al., 2008).

Technically, three different types of solvent extraction can be differentiate. During a maceration the plant material is soaked in the solvent where the extraction time can vary from several minutes up to weeks. Other variables that can be considered include the amount of agitation, plant-solvent ratio, moisture content, temperature and the number of times that the extraction is repeated. Acknowledge maceration is the preparation of a cup of tea. Also the spectacular influence of stirring and the temperature on the extraction rate is well illustrated by this example. In a percolation-type extraction the plant material is keep flushed with fresh solvent. The extraction is sustained until sufficient compound is extracted. If needed the same material can be re-extracted with a second solvent. A coffee machine is an example of this type of extraction, in a Soxhlet extraction which is comparable to a percolation where the plant materialis continuously

Table 2.2 Advantages and disadvantages of maceration, percolation and Soxhlet type of extraction

Type of extraction	Advantages	Disadvantages
Maceration	1.Simple and cheap 2.Limited use of solvents 3.Can give good and selective extraction	Analytes must be sufficiently soluble without stirring and or heating slow
Percolation	1.Exhaustive extraction possible 2.Mild technique	1.Some supervision necessary 2.Selectivity can be a problem 3.Can be slow 4.Large amounts of solvent necessary
Soxhlet	1.Low solvent consumption 2.No supervision necessary	1.Harsh technique, thermal decomposition 2.Water and electricity needed 3.Extraction not as good as sometimes thought 4.Less suitable for large scale application

(Sources: Walton and Brown, 1999)

flushed with fresh solvent. This fresh solvent is formed by boiling the solvent containing the extracted analytes. Thus in contrast to a percolation the total amount of solvent is inadequated. Beyond of what is sometimes thought, a Soxhlet extraction can be far from complete due to chanelling or the presence of air in the semi-permeable thimble containing the plant material. The stong and weak points of the three main types of extraction have been summarised in Table 2.2. As a general guideline, one can say that a maceration is the most important for the extraction of compounds accuring in low concentration which are strongly bound and or diffuse slowly. A percolation is more necessary for extracting loosely bound compounds which take place in high

concentration or have a low solubility in the solvent. A Soxhlet is practical for certain quantitative extractions of thermally stable compounds.

In recent times introduced fully-automsated new variant of percolation-type extraction is the Accelerated Solvent Extractor. This extractor is installed with an autosampler for 24 steel extraction vials (11-33 ml) and can extract with any solvent up to 200° C and 200 atm. Due to the high pressure the solvent does not boil whilst the high temperature results a more efficient extraction. Claimed advantages are automation of the extraction process, time gain and low solvent used. No reports in the field of natural products have been made so far potentially there are plenty of applications for routine analysis of thermally stable secondary metabolites.

2.8 Solid Liquid Extraction

Solid-liquid extraction or leaching occurs with the selective dissolution of one or more solutes from a solid matrix by a liquid solvent. Most of the bulk of the biomass exists as fairly inert, insoluble, and often polymeric material, such as cellulose of plants. The first step of the extraction is therefore to discharge and solubilise the smaller secondary metabolites in the matrix, resulting in the initial extract. Since there are many plant secondary metabolites in different plants, to select proper extraction method and solvent to be used and optimizing operating parameters become to be critical points. From the industrial aspects, there are some factors that should be considered, because they affect the rate of extraction:

i) Preparation of the solid: In plant, the cell structure is the most significant factor that needs to be measured. Although the solute can be on the surface of the cell, in most of the cases it is stored on intracellular spaces, capillaries, or cell structures where the success of the solvent extraction strongly relies on the solid condition. One of the pre-treatment steps is the grinding of the sample. Grinding before solvent extraction cause an increase of the contact area between the solvent and solute by breaking the cell structures.

ii) Diffusion rate: Due to the complexity of the cell structure and the existence of variety and porous compartments in the cell, the diffusivity of biological materials has to be evaluated as effective diffusivity. The effective diffusivity of also relied on the composition and on the position of the solute in the solid material.

iii) Temperature: Usually, elevated temperature is attractive in terms of extraction process developments. Higher temperatures lead an increase of the solute's solubility in the solvent, increasing the solute diffusion rate into the solvent bulk, promote to a higher mass transfer rate. However for the extraction of bioactive compounds from plants, the use of elevated temperatures can create undesirable reactions such as degradation compounds and losing their biological activities.

iv) Solvent choices: Although the selection of extraction method may have a significant effect on the quality of the extract, the solvent used promotes the most obvious means of influencing the qualitative composition of the extract.

v) Solid material humidity: The water in the solid material can compete with the extraction solvent for the solute's dissolution, influencing the mass transfer.

Furthermore, it contributes detoriation, spoilage and consequently biological activity loss. Thus, in most of the cases the material is dried under conditions that do not leads degradation of the compounds

vi) Solvent-solid ratio: The solvent-to-solute ratio is always a necessary variable for the extraction of compounds in general. This is the parameter that measures the amount of solvent used, and it is always linked to economic aspects.

2.9 Solvent extraction

Some of the common properties of the solvent that should be considered when selecting the most suitable extraction solvent include the ability of the solvent to dissolve the target compounds, ease of removal, cost, inertness, toxicity and flammability. Table 2.3 shows the solvent characteristics that should be considered for the extraction from natural matrices. As expected, the matrix and the target compounds have possibly the most significant effect on the selection of suitable extraction solvent. Low polarity solvents yield more lipophilic compounds, whereas alcohols extract both non-polar and polar compounds, and water extracts only polar components from the sample. Because of the toxicity of some organic solvents, there are some limitations to their use in industry.

In terms of human consumption, the existence of some solvents, such as acetone, ethanol, ethyl acetate, 1-propano, 2-propanol, methanol and propyl acetate are acceptable in small residue percentages, referring to good manufacturing practice

Table 2.3 Solvent characteristics for natural product extraction

Characteristics	Effect in the process
Selectivity	Solvent selectivity guarantees the extract purities and solubilises the target compounds
Compatibility with solute	The solvent should not react with the target compounds
Chemical and thermal stability	The stability of the solvent at operating extraction conditions must be guaranteed not to alter the final extract
Low viscosity	To keep the extraction rate higher, lower viscosity is needed to increase the diffusion coefficient
Ease of recovery	Economic aspects must be considered, and lower boiling point solvents are easily recovered and reused
Low flammability	According to the process needs and safety aspects, flammable solvents must be prevent
Low toxicity	Natural products require the absence of solvent traces and toxicity, besides the worker exposition
Regulatory issue	According to the pharmaceutical and food industries, environmental regulations should be considered so as to avoid process irregularities.
Low cost	Economic aspects can promote to the final product quality

(Sources: Altiok, 2010)

(Meireles, 2009). Thus, the deduction of solvent from the extract is very important to obtain the limits indicated in national legislation and it should be easily removed in economic point of view.

Several factors have been used in evaluating the effectiveness of the extraction method and the suitability of a solvent for particular extraction procedures. The most common encountered criterion is extraction yield which is the total yield or the yield of a creating target compounds or compounds. In the bioactivity tests, the solvent provide the highest recoveries is chosen and the extract is further purified to isolate the bioactive compounds

2.10 Mass transfer: Equations and Kinetics

Solvent extraction is the extraction of the soluble material inside the solid matrix that uses a specific solvent. The extraction mechanism can be elaborated in the following steps: First, the solvent must transferred onto the solid surface. After that, the solvent diffuses into the solid matrix by diffusion. The solute is dissolved until a solubility restriction depending on the type and nature of the solvent. It is significant to notice that the solute plus solvent from a diluted in any practical application. The solution containing the solute penetrates to the surface by effective diffusion. At the end, the solution is transferred from the surface to the bulk solution by natural or forced convection. The rate of dissolution of a solute in the solvent of extraction is related to the rate of mass transfer of the solute from the solid matrix to the liquid. The transfer of the solute inside the solid particle take place because of the concentration gradient in the solid-liquid interface, and it can be characterized by the effective diffusion. The equation that describes this phenomenon is explained on the Fick's law and is given by;

$$\frac{N_A}{A_m} = -D_{BA}\frac{dC_A}{d_z} \qquad 2.1$$

where N_A is the rate of dissolution of the solute A in the solution (kg mol/s), A_m is the area of the solid-liquid interface (m^2), D_{BA} is the diffusivity of solute in the solvent (m^2/s), C_A molar concentration of solute A in the solution (kg mol A/m^3), and z is the distance inside the porous of the solid matrix (m).

It is assumed that, on the surface of the solid particle, the transfer of solute occurs by molecular transport and there is essentially no resistance in the solid phase like as a pure material (Geankoplis, 1993). In this step, the rate of mass transfer can be expressed by the following equation and it can be used for the case when the diffusion in solid is very rapid

$$N_A = A_M k_L (C_{AS} - C_A) \tag{2.2}$$

where C_{AS} is the saturation solubility of the solute A (kg mol/m^3) and k_L is mass transfer coefficient (m/s). By a material balance, the rate of accumulation of A in the solution is equal to the mass flux of A times the area.

$$\frac{V dC_A}{dt} = N_A = A_m k_L (C_{AS} - C_A) \tag{2.3}$$

Integration Eq. (2.3) with the initial condition, $C_A = C_{AO}$ at $t = 0$ to at $t = t$ $C_A = C_A$,

$$\int_{C_{AO}}^{C_A} \frac{dC_A}{C_{AS} - C_A} = \frac{A_m k_L}{V} \int_{t=0}^{t} dt \tag{2.4}$$

$$u = C_{AS} - C_A, du = -dC_A$$

$$\int \frac{dC_A}{C_{AS} - C_A} = -\int \frac{du}{u} = -\ln u = -\ln(C_{AS} - C_A) = -\ln \frac{C_{AS} - C_A}{C_{AS} - C_{AO}}$$

$$-\ln\frac{C_{AS}-C_A}{C_{AS}-C_{AO}} = \frac{A_m k_L}{V}t$$

$$\frac{(C_{AS}-C_A)}{(C_{AS}-C_{AO})} = e^{-(k_L A_m/V)t} \qquad 2.5$$

The solution approaches a saturated condition exponentially. Mass transfer may change depending on;

i) If external surface very irregular, surface area will increase

ii) If the soluble material forms a high proportion of the total solid, disintegration of the particle may occur

iii) If the solid completely dissolving, the interfacial area changes markedly.

Plant material generally consists of cellulose, sugars, oily parts with lower amount of complex mixture of bioactive compound nor the high proportion of soluble material in plants

2.11 UV Spectroscopy (Ultraviolet–visible spectroscopy)

Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption or reflectance in the visible

range directly influenced the perceived colour of the chemicals involved. In this section of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectoscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption determined transitions from the ground state to the excited state.

This is the most generally used detector for liquid chromatography. Its popularity is due to its robustness, selectivity (1-10ng range for many compounds), tunable selectivity, good baseline stability (relatively insensitive to temperature fluctuations and refractive index effects) and large dynamic range. It can be used with gradients. Disadvantages are the need for solvents to be transparent at the detection wavelength and the sometimes large (>1000-fold) variations in relative response factors at a given wavelength. With UV spectoscopy, double bonds are detected. Thus UV detection is not the method of selection for compounds lacking double bonds, such as saturated hydrocarbons and sugar. When only isolated double bonds or carbonyls are present in a molecule (e.g many essential oil constituents), detection have to take place at low wavelengths 200 – 210 nm and the sensitivity can be relatively poor. The selection of solvents is in this case limited to water, acetonitrile, methanol and other alcohols, and saturated hydrocarbons like hexane. When conjugated double bonds or aromatic rings, are exist the wavelength shifts to higher values and the sensitivity increases. Many more solvents can then be used.

In general, detection occurs at the wavelength which promotes the best ratio in absorption between compounds of interest and impurities. Thus when a compound shows a maximum at 225 nm and a slightly lower maximum at 285 nm it may be better to determine at 285 nm since there will be less interference from impurities. When UV

detection is used for controlling the evaluate in preparative applications there are two possibilities to overcome saturation of the detector; one can install a cell with a shorter path length (e.g. 0.2 instead of 5mm) or, more cheaply, one can measure at a wavelength different from the absorption maximum (e.g. 320 nm instead of 280 nm)

Figure 2.4 UV-vis spectrophotometer

2.12 Conclusion

From this research there are a lot of methods in quantifying the bioactive compound in *A. bilimbi* and technique of extraction. From the methods provided, optimum parameters will be selected for the accurate and good result in determination the bioactive compound in each of the test. Research methodology will be explained each of the method selected in quantifying the flavonoids, total phenolic content and antioxidant capacity for each of the extraction parameters.

CHAPTER THREE

RESEARCH METHODOLOGY

3.1 Introduction

In this part, some methods will be used to identify bioactive compounds content in *A.bilimbi* depends on different parameters during extraction such as type of solvents and concentration, temperature, pressure and time. Before applying the methods, preparation of sample and extract should be done. Then, method for quantifying the bioactive compound can be applied. List of methodology apply are antioxidant capacity test, where determination of free radical scavenging activity (DPPH) will be apply, for flavonoids determination, spectrophotometrically based on the formation of a complex flavonoid-aluminum, with some modifications where an aliquot of the extract solution was mixed with distilled water and subsequently with $NaNO_2$ solution will be used (Guimarães et al., 2010). Besides that, for total phenolic content (TPC) which are reducing component was estimate using the Folin – Ciocalteu reagent assay and lastly ascorbic acid test will be determined.

3.2 Sample and chemicals

Table 3.1 List of materials

Test	Materials
Preparation of sample and extract	Fruit powder Distilled water 50% and 96% methanol 50% and 96% ethanol 50% and 96% propanol 96% hexane
Antioxidant capacity test (DPPH)	2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·) scavenging assay Methanol
Flavonoids determination	Distilled water Sodium Nitrate solution Aluminium Chloride Sodium Hydroxide solution
Total phenolic content (TPC)	Folin–Ciocalteu reagent Distilled water Sodium carbonate solution

3.3 Equipment / apparatus

1. Shaking incubator

2. UV-vis spectrophotometer

3. Refrigerated centrifuge

4. Freeze dryer

3.4 Experiment procedure

3.4.1 Preparation of sample and extract

The whole fruit was washed under running tap water to remove dirt and other foreign materials and wiped with tissue paper. The edible portion was then cut into small pieces and stored at -80^{0}C before lyophilization using a bench-top freeze dryer. The lyophilized fruits were ground into powder form, and kept at -20^{0}C prior to analysis. Methanol, ethanol, propanol and hexane solution extract was prepared by mixing 1 g of the lyophilized fruit powder with different type of solvent and concentration. The mixture was placed in a conical flask (wrapped with an aluminium foil) and agitated at different speeds varies from 50 to 300 rpm, different temperatures from 30° C to 70° C, with the aid of an incubator shaker for 15 minutes to 4 hours. The mixture was then filtered through a filter paper (Whatman No. 4) to obtain a clear solution. The extract was used for determination of AC and TPC (Ikram et al., 2009).

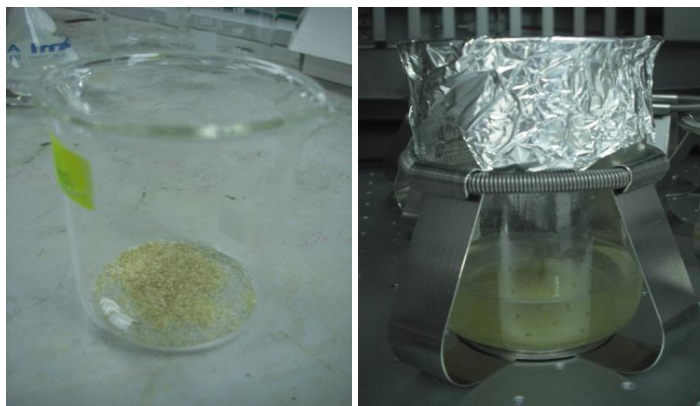


Figure 3.1: Sample before extraction and during the extraction

3.4.2 Extraction parameters

Four different extraction media were selected for initial study. The extraction media were methanol, ethanol, propanol and hexane. Those extraction mediums have different in concentration which are methanol 50%, methanol 96%, ethanol 50%, ethanol 96%, propanol 50%, propanol 96% and hexane. Extraction medium with different concentration that yielded the highest total phenolic, antioxidants activities and flavonoids content was further extracted based on different extraction that are varies from 15 minutes to 4 hours. Result from the best extraction time will lead to determine the parameters on extraction temperature. Lastly, extraction effect on agitations (50 rpm to 300 rpm) was found out.

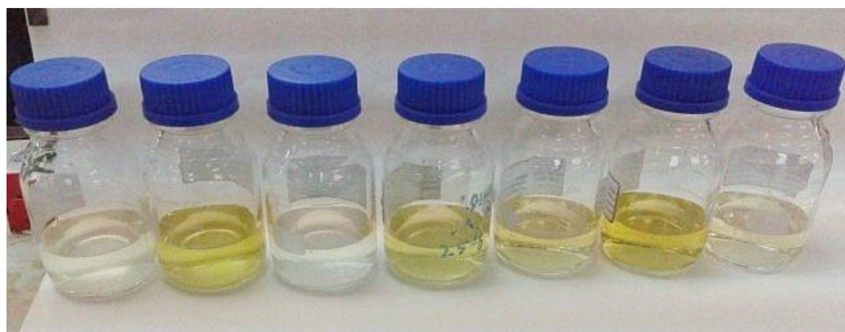


Figure 3.2 Different types of solvent extraction. From left, ethanol 50%, ethanol 96%, methanol 50%, methanol 96%, propanol 50%, propanol 96% and hexane

Figure 3.3 Different extraction time. From left 15 minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes and 240 minutes.

3.4.3 Antioxidant Capacity Test

The scavenging activity of the extract was determined based on 2,2-diphenyl-1-picrylhydrazyl freeradical (DPPH·) scavenging assay described by Amatya and Tuladhar (2011). The plant extracts (0.5 g) was dissolved in methanol to make 10ml stock solution. The volumes of 0.1, 0.2, 0.4, and 1ml of stock solution were pipette out separately into 10ml volumetric flask and diluted further with methanol up to the mark to make four sample solutions corresponding to 0.5, 1, 2, and 5 mg ml^{-1} concentrations, respectively. Positive controls (BHA) of similar concentrations were also prepared by the same method.

The test was performed in 4ml reaction mixtures containing 3.9ml methanolic DPPH solution ($6x10^{-5}$ M) and 0.1 ml of sample solution of various concentrations (0.5, 1, 2, and 5 mg ml^{-1} corresponding to 50,100, 200 and 500 µg extract or BHA) or 0.1 ml of methanol (as blank). Three replicates were made for each concentration of each extract and BHA. After 30 minutes of incubation in the dark room temperature, absorbance of the reaction mixtures was measured at 517nm using MeOH as reference.

The blank reading was measured every time just before the experiment of each concentration of extract or BHA starts. The inhibitory effect of DPPH by the sample was calculated according to the formula:

$$\text{Percentage inhibition} = (A_{C\,(o)} - A_{S\,(t)} / A_{C\,(o)}) \times 100$$

Where:

$A_{C(o)}$ = Absorbance of the blank at t = 0 min

$A_{S\,(t)}$ = Absorbance of the sample at t

Figure 3.4 Extraction on antioxidant capacity test of DPPH scavenging assay

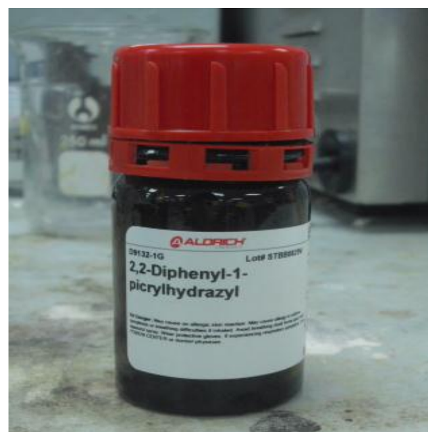


Figure 3.5 DPPH Reagent

3.4.4 Flavonoids determination

Total flavonoid contents (TF) of the fruit extracts were determined according to the colorimetric assay described by Alothman et al. (2009). One ml of properly diluted fruit extract was mixed with 4 ml of distilled water. At zero time, 0.3 ml of (5% w/v) $NaNO_2$ was added. After 5 min, 0.3 ml of (10% w/v) $AlCl_3$ was added. At 6 min, 2 ml of 1 M solution of NaOH were added. After that, the volume was made up to 10 ml, immediately by the addition of 2.4 ml of distilled water. The mixture was shaken vigorously and the absorbance of the mixture was read at 510 nm. A calibration curve was prepared using a standard solution of quercetin (20, 40, 60, 80 and 100 µg / l, R^2= 0.9989). The results were also expressed on a fresh weight basis as µg quercetin equivalents (QE) / 100 g of sample. Figure 3.4 showed the extraction of different temperature on total flavonoids content test.

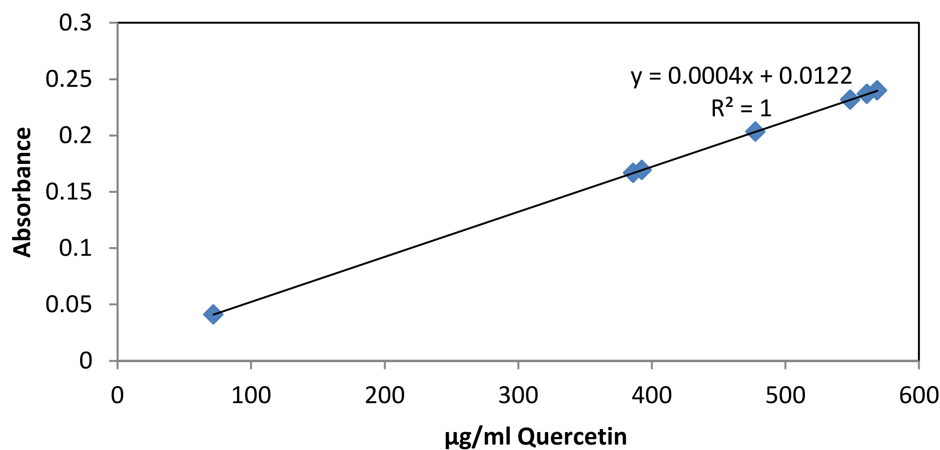


Figure 3.6 Calibration curve for absorbance vs. µg/ ml QE

Figure 3.7 Extraction of different temperatures on total flavonoids content test.

3.4.5 Total phenolic content (TPC)

Total phenolic contents (TP) of the fruit extracts were determined using FC assay which was described by Alothman et al. (2009). Briefly, 40µl of properly diluted fruit extract solution were mixed with 1.8ml of FC reagent. The reagent was pre-diluted, 10 times, with distilled water. After standing for five minutes at room temperature, 1.2 ml of 975% w/v) sodium carbonate solution were added. The solutions were mixed and allowed to stand for 1 h at room temperature. Then, the absorbance was measured at 765nm, using a UV-visible spectrophotometer. A calibration curve was prepared, using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l, r^2 = 0.999). Calibration curve is showed in figure 3.5. Results were expressed on fresh weight basis (fw) as mg gallic acid equivalents/ 100g of sample.

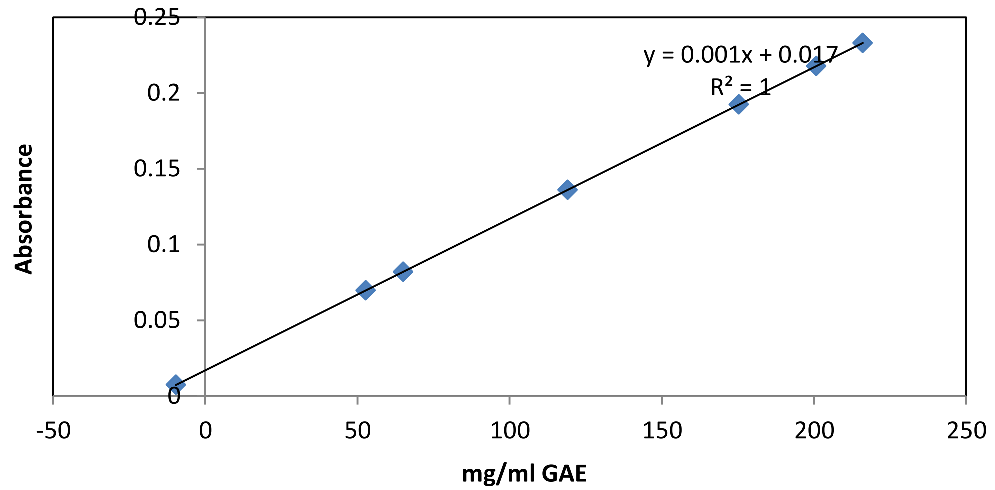


Figure 3.8 Calibration curve for absorbance vs. mg/ ml GAE

Figure 3.9 Extraction of different time on total phenolic contents using Folin Ciocateu's reagent

Figure 3.10 Folin Ciocateu's Phenol reagent

CHAPTER FOUR

RESULT AND DISCUSSION

4.1 Total phenolic, total flavonoids and antioxidant activity on extraction parameter

This study investigated different parameters of extraction in order to obtain the best extraction method for *averrhoa bilimbi* sample. The extraction yields which are total phenolic, total flavonoids and antioxidants activity from *averrhoa bilimbi* samples may be influenced by many factors, such as the types of solvents with different concentration, extraction time, extraction temperature and effect on agitation (Cacace and Mazza, 2003; Dar and Sharma, 2011).

4.2 Effects on solvent

In this study, the maceration technique of extraction is used where the concentration of extraction solvents was fixed at 60% (v/v) while the extraction time was 4 hours, with extraction temperature at 50° C and constant at agitation of 200 rpm. The effect of each extraction parameter was studied using three different bioactive compounds namely, total phenolic, total flavonoids and DPPH. The total phenolic, total flavonoids and antioxidants activity of *averrhoa bilimbi* fruits was extracted using seven different extraction solvents with different solvent to water ratio which are ethanol (96%), methanol (96%), propanol (96%), ethanol (50%), methanol (50%), propanol (50%) and hexane. The total phenolic, total flavonoids and DPPH of *averrhoa bilimbi* using seven different extraction solvent with different concentration are shown in Table 4.1.

The propanol (50%) extract of *averrhoa bilimbi* had the highest total phenolic as compared to other extraction solvent at all concentration studied. Methanol (50%) and ethanol (50%) also show a slightly different compared to propanol (50%) where there are no significant differences. The methanol (50%) showed the highest total flavonoids and DPPH scavenging activity Methanol (50%) extract is a best solvent because Methanol shows the highest polarity index with 0.762, followed by ethanol, 0.654 and propanol, 0.617 (Reichardt, 2003). Due to different in polarity index, those three extraction medium mark the difference for total flavonoids, total phenolic and DPPH percent of inhibition. The more polar the organic solvent, the more it is miscible or soluble in water hence resulting in a good extraction. The hexane extract *bilimbi* was

Table 4.1 Effect of extraction solvents with different concentration at 4 hours, 50° C and 200 rpm on the total phenolic, total flavonoids and antioxidants activities of *averrhoa bilimbi* fruit

Extraction of solvents	Total Phenolics (mg/ml GAE)	Total Flavonoids (µg/ml QE)	DPPH scavenging Activity (%)	Polarity Index
Methanol (50%)	175.3	568.75	80.02	> 0.762
Methanol (96%)	119	386.25	62.00	0.762
Ethanol (50%)	200.7	548.75	79.48	> 0.654
Ethanol (96%)	65	561.25	45.10	0.654
Propanol (50%)	216	477.75	78.96	> 0.617
Propanol (96%)	52.7	392.75	27.11	0.617
Hexane	-9.7	72	0.31	0.009

the least effective for all bioactive compound tests since it has the lowest relative polarity index which is 0.009. Many authors established that the extraction yield of phenols is greatly depending on the solvent polarity (Turkmen et al., 2006; Lapornik et al., 2005).

50% solvent extraction marked highest bioactive compound contents compared to 96% solvent extraction for each solvent. The increment of extraction yield increases as the water ratio increase. As the ratio of water to *averrhoa bilimbi* higher, the difference of concentration between the bulk solution and the solutes also higher. Therefore, more bioactive compounds can diffuse out if a higher volume of water is used. Cacace and Mazza (2003) obtained that extraction of anthocyanins from black currants using aqueous ethanol increased with ethanol concentration up to a maximum at about 60% and then decreased with further increase in solvent concentration. Since black currant has the same characteristic as *averrhoa bilimbi* which is they have a sour taste, hence the total amount of bioactive compounds in the 50% solvent-water mixtures extracted more

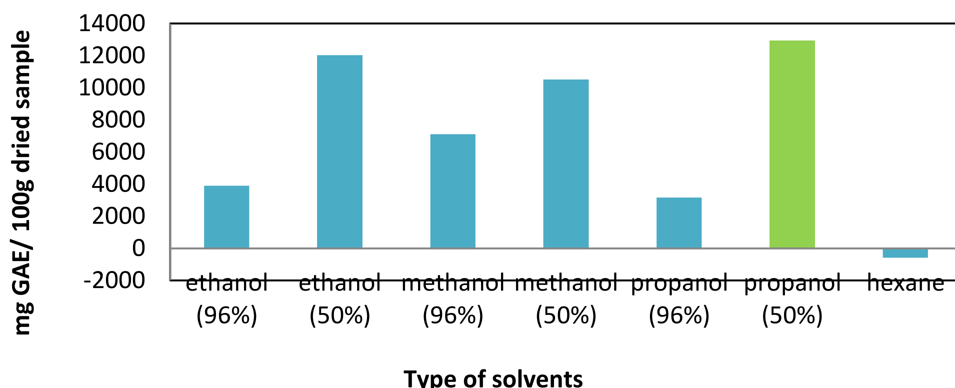


Figure 4.1 Total phenolics contents for different type of solvents, 4 hours, 50° C and 200 rpm

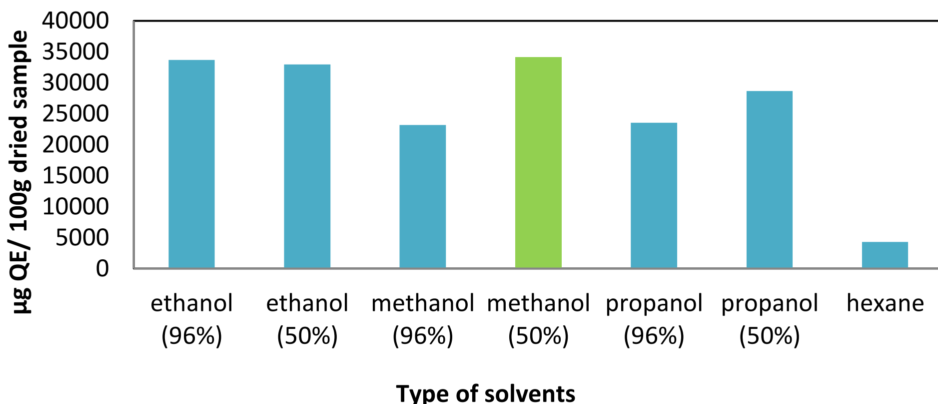


Figure 4.2 Total flavonoids content for different type of solvents, 4 hours, 50° C and 200 rpm

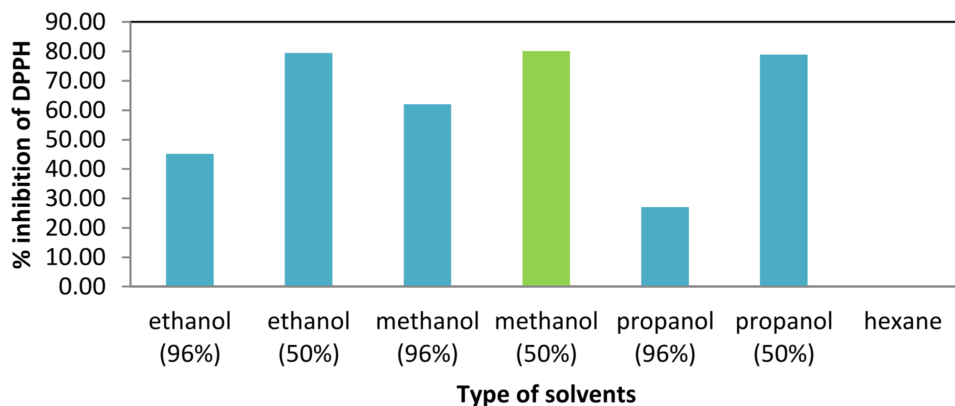


Figure 4.3 Percent inhibition of DPPH for different type of solvents, 4 hours, 50° C and 200 rpm

efficient and contained more complex mixtures of bioactive compounds than the pure solvent extracts did.

The difference in polarities of extracting solvents might affect the solubility of chemical constituents in a sample and its extraction yield. Therefore, the selection of an appropriate solvent system is one of the most relevant steps in optimizing the recovery of total phenolic content, total flavonoids content and other antioxidant compounds from a sample (Zhao et al., 2006). It is proved that the recovery of phenolic, flavonoid and antioxidant compounds from each fresh sample matrix is dependent on the extracting solvent used as well as species of plant. The results of the present investigation revealed that methanol (50%) solvent extracts of *averrhoa bilimbi*, prepared by simple maceration techniques, will be the best solvent compared to others.

4.3 Effect of time

Extraction time is one of the critical factors for extraction of phenolic, flavonoids and antioxidant compounds since these compounds are potentially prone to degradation if exposed to ambient conditions for long duration. The choice of the best extraction time was based on the highest radical scavenging activities, which depicted by DPPH and supported by total phenolic as well as total flavonoids. The total phenolic, total flavonoids and antioxidant activities of *averrhoa bilimbi* fruit extracted using 50% methanol at six different extraction times (15-240 min) are shown in Table 4.2.

The result showed that 180 minutes of extraction yielded the highest total phenolic with 186 mg/ml, an extraction time of 60 minutes had shown the highest total

Table 4.2 Effect of extraction time in methanol (50%) at 50° C, 200 rpm on the total phenolic, total flavonoids and antioxidants activities of *averrhoa bilimbi* fruit.

Extraction of time	Total Phenolics (mg/ml GAE)	Total Flovonoids (µg/ml QE)	DPPH scavenging Activity (%)
15 minutes	106.7	332.0	65.50
30 minutes	137.0	404.5	72.58
60 minutes	164.7	496.25	74.50
120 minutes	173.3	481.25	76.22
180 minutes	186.0	447.75	76.91
240 minutes	162.7	480.25	79.31

flavonoids content while for highest scavenging activity, DPPH assay for methanol (50%) is obtained by 240 minutes. Extraction time of 15 minutes had shown significantly lowest for all extraction yields which are total phenolic, total flavonoids and antioxidants activity, DPPH while for 30 minutes marked the second lowest for all extraction yield. As observed, 60 to 240 minutes extraction showed a slightly different from one and another. Increasing trend that was found for total phenolic in the following manner: 15 < 30 < 240 <60 <120 <180 minutes. Here, there is no significant difference for total phenolic showed starting from 60 minutes to 240 minutes. Antioxidants activity, DPPH keep increasing by the time and still there is just a small different recorded at 60 minutes onwards and the same things occurs while quantifying the total flavonoids in *averrhoa bilimbi*.

Prolongation of extraction duration potentially increases the degradation of phenolic, flavonoids and antioxidants by exposure to light and oxygen. From table we can observed that for phenolic content, after 180 minutes the phenolic start to degrades and also for flavonoids, where after 60 minutes extraction the compounds starts to unstable and lead to degradation. In this study, 60 minutes extraction generally showed

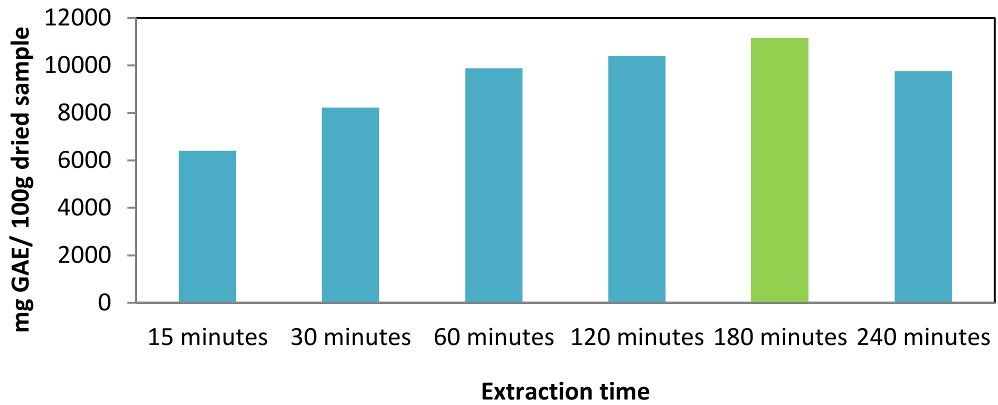


Figure 4.4 Total phenolic contents at different extraction time in methanol (50%) at 50°C and 200 rpm.

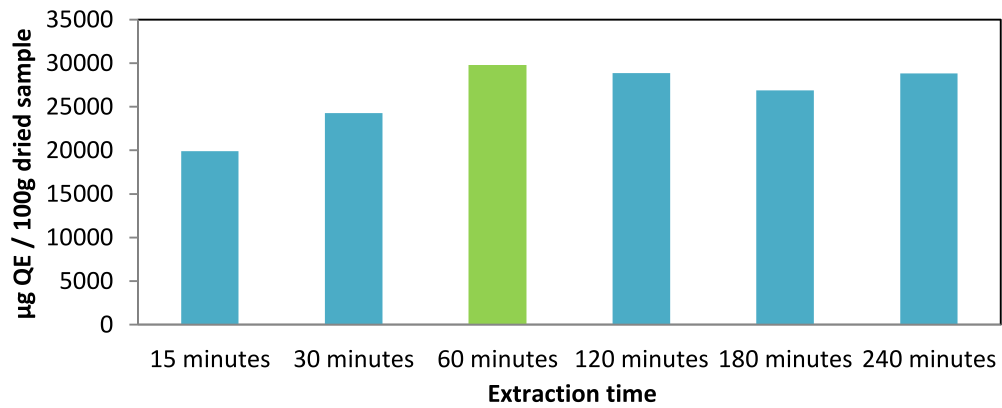


Figure 4.5 Total flavonoids content at different extraction time in methanol (50%) at 50° C and 200 rpm.

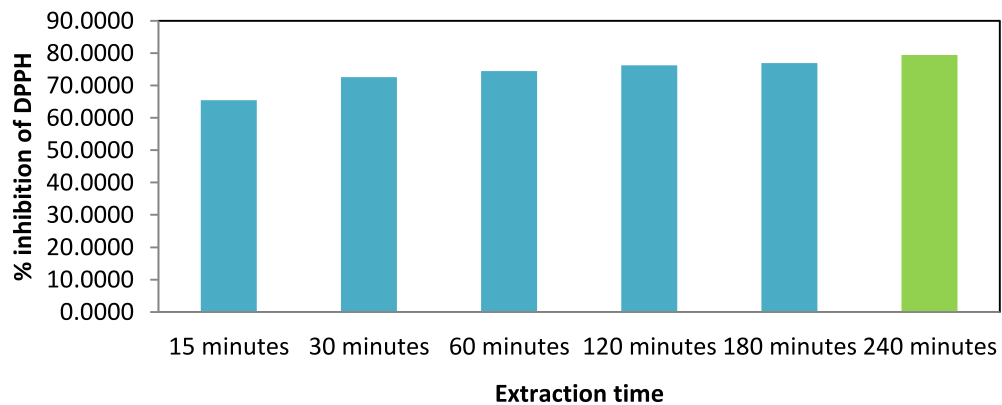


Figure 4.6 Percent inhibition of DPPH at different extraction time in methanol (50%) at 50° C and 200 rpm.

the highest effect on total flavonoids while for phenolic and antioxidants activity, it marked a significant difference from the highest one. Therefore, 60 minutes extraction was considered as economic and practical, which was also chosen as the best extraction time for the *averrhoa bilimbi* fruit sample. Conversely, 15 minutes appeared to be the least optimum extraction time for total phenolic, total flavonoids and antioxidants activities. An increase in extraction time might give rise to possible degradation. However, a too short extraction time might yield a small amount of bioactive compounds.

4.4 Effect of temperature

The impact of extraction temperature on the phenolic compounds, flavonoids compounds and antioxidant capacity were investigated in the range from 30° C to 70° C. Linear relationships were observed between extraction temperature and recovery of total phenolics, total flavonoids, and antioxidants activity. The total phenolics, total flavonoids and antioxidant activities of *averrhoa bilimbi* fruit extracted using 50% methanol at five different extraction temperatures (30-70) ° C are shown in Table 4.3. Based on the table 4.3, at all extraction temperatures, total phenolics, total flavonoids and antioxidants activity has increase with the temperature. Extraction temperatures at 70° C record the highest bioactive compounds. Heat treatment was performed to speed up the mechanism of the diffusion process when extracting from plants. The surface tension and viscosity of the solvent reduced and the solvent reached the active sites inside the matrix more simply at higher temperature. In addition, high temperature can

Table 4.3 Effect of extraction temperature in methanol (50%) at 60 minutes, 200 rpm on the total phenolic, total flavonoids and antioxidants activities of *averrhoa bilimbi* fruit

Extraction of temperature	Total Phenolics (mg/ml GAE)	Total Flovonoids (µg/ml QE)	DPPH scavenging Activity (%)
30° C	87	366.25	41.92
40° C	96.7	440.25	46.06
50° C	144.3	527.75	59.22
60° C	154.3	570.25	66.42
70° C	172.7	656.25	70.05

reduce the cell barrier by weaken the integrity of the cell wall and membrane which lead solvent to get easily contact with bioactive compounds. The temperature effect on the extraction yield came from its effect on diffusion phenomena. Li et al. (2006) confirmed that for temperature, especially high temperature (65° C or 80° C) affects the integrity of the cell wall, and results in a high total phenolic content recovery of in extraction of citrus peels (Yen Ben lemon, Meyer lemon, grape fruit, mandarin and orange). This was in agreement with Tagliazucchi et al. (2010) where temperature is one of the most critical variables affecting the release of phenols from grape skin which increase in the temperature favours extraction by enhancing both the solubility of polyphenols and the diffusion coefficient. Increased solubility of bioactive compounds contents by increasing temperature favored the release of bound polyphenol in a sample with the breakdown of cellular constituents of plant cells which leads to increased cell membrane permeability. In regard to the equilibrium principle, elevated temperature could increase the extraction rate and thus shorten the extraction time to reach maximum polyphenol content recovery. However, elevated temperature may not appropriate for all kinds of phenolic compounds. Sant'Anna et al. (2012) reported that in total polyphenol extraction from

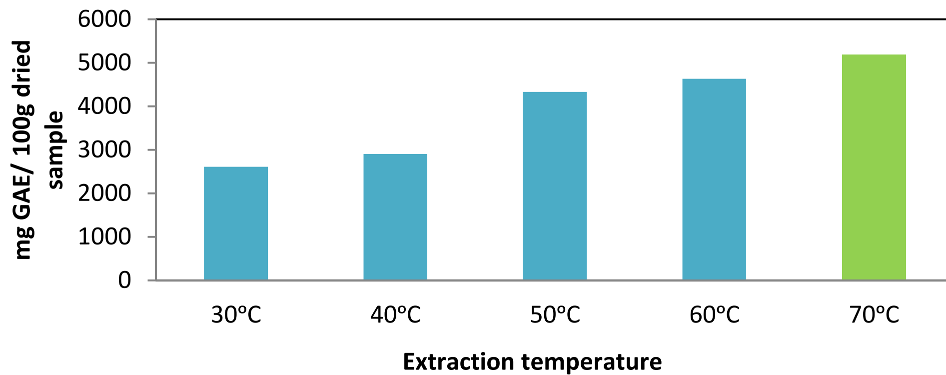


Figure 4.7 Total phenolics contents at different extraction temperature in methanol (50%) at 60 minutes and 200 rpm.

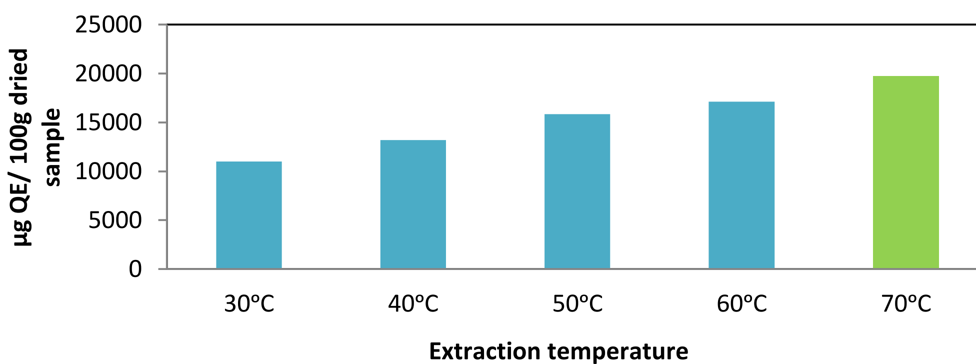


Figure 4.8 Total flavonoids content at different extraction temperature in methanol (50%) at 60 minutes and 200 rpm.

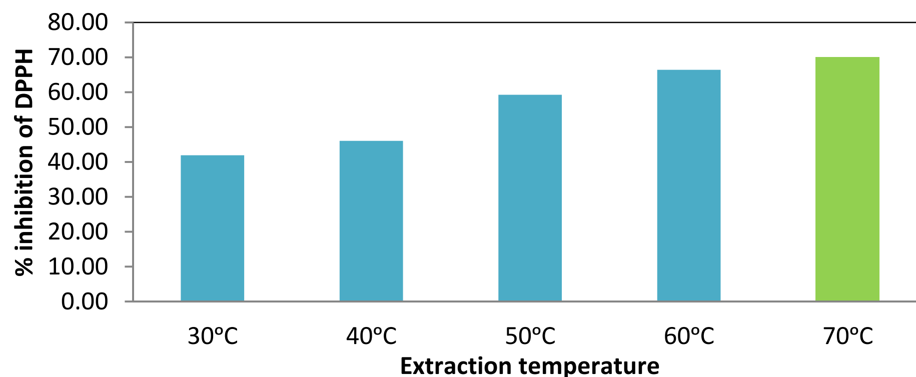


Figure 4.9 Percent inhibition of DPPH at different extraction temperature in methanol (50%) at 60 minutes and 200 rpm.

grape marc the processing temperature cannot be increased indefinitely, because bioactive compounds are relatively thermo labile, being susceptible to degradation at high temperatures.

In the present study, increasing extraction temperature had an encouraging effect to total phenolics, total flavonoids and antioxidants activity, DPPH. From this circumstance, it is believed that the total phenolic compounds, total flavonoids compounds and antioxidants activity, DPPH presents in *averrhoa bilimbi* are thermally stable and that the extraction time chosen in the second stage is suitable for both moderate to high temperature without leading to unfavorable degradation. It was found that by increasing extraction temperature, the higher solubility of solute resulted in the increase of diffusion rate and thus increases the diffusion coefficient. This has shortened the extraction time as shown in Arrhenius type of relationship. Taking into consideration the industrial requirement which is high extraction temperature for a shorter time as well as making a compromise between the recovery of total phenolic compounds, total flavonoids compounds and antioxidants activity, DPPH, a temperature of 70° C was selected as the best extraction temperature in this step.

4.5 Effect of agitation

Effect of agitation is one of the factors that determined how much the extraction occurs where leads to evaluate the value for total phenolics content, total flavonoids content and antioxidant capacity, DPPH in *averrhoa bilimbi* sample. When it comes to the final parameter, all those previous parameters that have been selected is now used up

Table 4.4 Effect of agitation in methanol (50%), 60 minutes, 70° C on the total phenolics, total flavonoids and antioxidants activities of *averrhoa bilimbi* fruit.

Effect of agitation	Total Phenolics (mg/ml GAE)	Total Flovonoids (µg/ml QE)	DPPH scavenging Activity (%)
50 rpm	130.7	502.75	68.96
100 rpm	153.7	567	74.18
150 rpm	168.3	608.75	74.62
200 rpm	174	659.5	74.89
250 rpm	182	670	75.55
300 rpm	193.3	717.75	77.03

to studied effect of agitation speed at 50 rpm, 100 rpm, 150 rpm, 200 rpm, 250 rpm, and 300 rpm on *averrhoa bilimbi* extraction. Table 4.4, showed the effect of agitation on the total phenolic, total flavonoids and antioxidants activities of *averrhoa bilimbi* fruit.

Based on the table summarized, 300 rpm achieved the highest value for total phenolics, total flavonoids and antioxidant capacity, DPPH which is about 193.3 mg/ml GAE, 717.75 µg/ml QE, and 77.03% respectively. When the solution is agitated, mass is transported by the bulk motion of the fluid which known as convective mass transfer. Convective mass transferred occurred at the surface when a fluid is outside the solid (Mohamad, 2010). Therefore, as the agitation speed increases, the Reynolds number is also increased. The Reynolds number is related to the mass transfer coefficient. Hence, the higher the reynolds number, the higher the mass transfer coefficient. This result leads to choose the best agitation rate at 300 rpm.

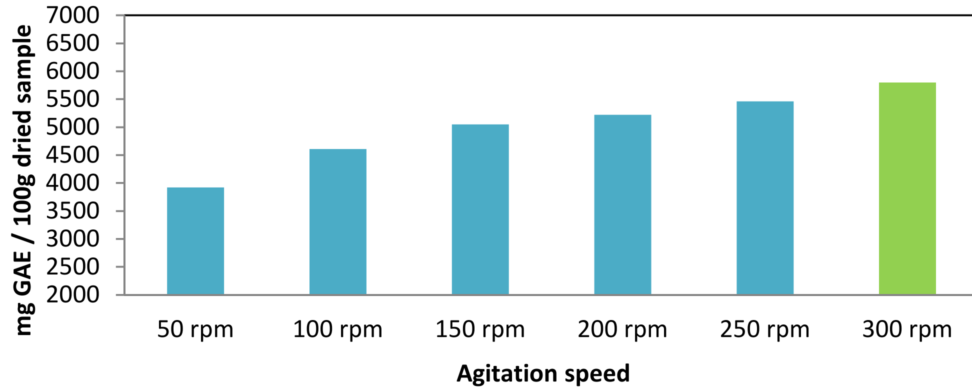


Figure 4.10 Total phenolics contents at different agitation speed in methanol (50%), 60 minutes and 70° C.

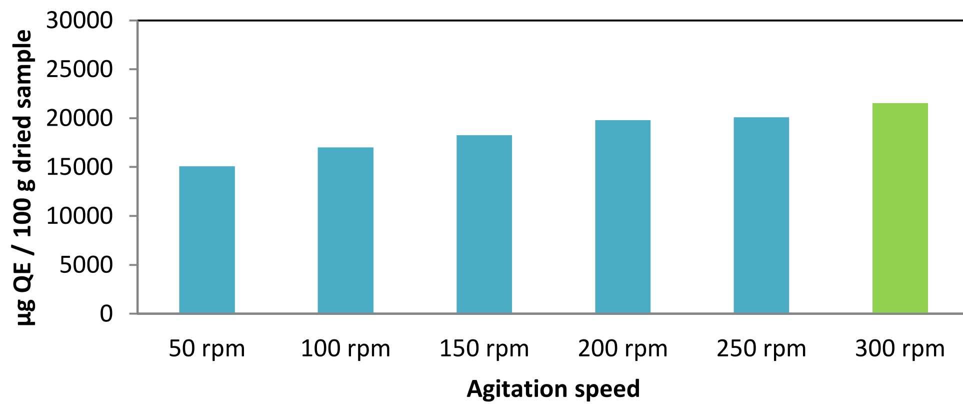


Figure 4.11 Total flavonoids content at different agitation speed in methanol (50%), 60 minutes and 70° C.

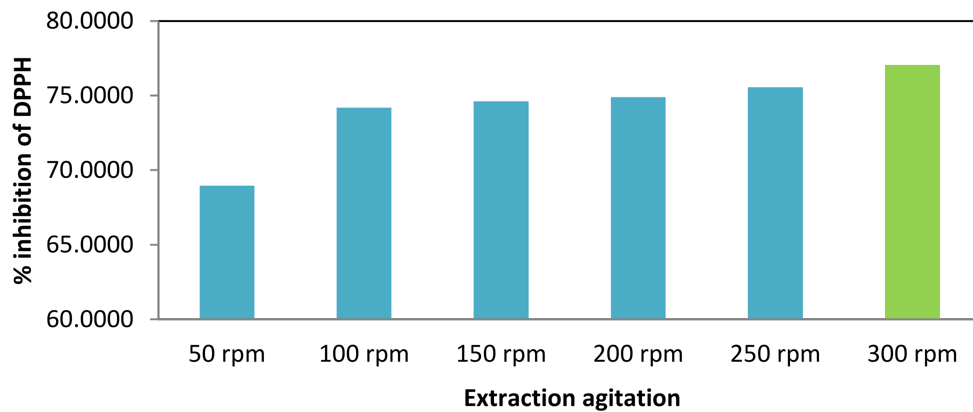


Figure 4.12 Percent inhibition of DPPH at different agitation speed in methanol (50%), 60 minutes and 70° C.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Averrhoa bilimbi is the potential source for bioactive compounds such as total phenolics content, total flavonoids and antioxidants capacity, DPPH. The aim of the present research was to get a better insight into optimization of the solvent extraction on bioactive compounds, perfect duration for extraction time, suitable extraction temperature and lastly optimum speed for agitation. Analysis of results brought to the following conclusions.

The best extraction condition for *averrhoa bilimbi* fruit for solvent extraction is 50% methanol. Addition of water to methanol improved extraction rate where methanol shows the highest polarity index. This condition have yielded total flavonoids content is about 568.75 µg/ml QE. The highest antioxidant capacities measured using DPPH assay is 80.02 % and total phenolic content shows about 175.3 mg/mL GAE. In this study, 60 minutes extraction time generally showed the highest effect on total flavonoids which is

496.25 μg/ml QE while for phenolics and antioxidants activity are slightly different from the highest one. *Averrhoa bilimbi* extraction had total phenolic about 164.7 mg/mL GAE and antioxidant activity is around 74.5%. Therefore, 60 minutes extraction was considered as economic and practical, which was selected as the best extraction time for the *averrhoa bilimbi* fruit sample.

In order to maximize recovery yield, 70° C is chosen as a suitable extraction temperature. From this study, it is believed that the total phenolic compounds, total flavonoids compounds and antioxidants activity, DPPH presents in *averrhoa bilimbi* are thermally stable and that the shorten extraction time chosen in the second stage is suitable for both moderate to high temperature without leading to unfavorable degradation. *Averrhoa bilimbi* sample had the best extraction for total phenolics at 127.7 mg/ml GAE, while highest total flavonoids value is 656.25 μg/ml QE and lastly highest DPPH radical scavenging activity is about 70.05%. Lastly, from the effect of agitation speed, 300 rpm achieved the highest value for total phenolics, total flavonoids and antioxidant capacity, DPPH which is about 193.3 mg/ml GAE, 717.75 μg/ml QE, and 77.03% respectively. This result leads to choose the best agitation rate at 300 rpm for *averrhoa bilimbi* extraction. Overall, based on the ideal extraction conditions chosen, optimum level of total phenolic content, total flavonoids content and antioxidant capacity were obtained in *averrhoa bilimbi* fruit extract. The selected extraction conditions could be used for further studies and functional food product development

5.2 Recommendations

The efficiency of the extraction greatly depends on solvent extraction where polarity in different solvents leads a different value in extraction of bioactive compounds. Water as one of the solvent can be used for further study to compare it efficiency in extraction since water has highest polarity among other solvents. Instead of using drying sample from grinding, juice sample can be used directly to quantify the bioactive compounds in *averrhoa bilimbi*. The detailed studies such as on what compounds that it gave the functions for bioactive compounds obtained will lead to the greater understanding of this research. In order to obtain accurate results, the sample of *averrhoa bilimbi* should be taken from the same sources which means from the same tree. Hence, the result for each of parameters can be compared fairly. During preparation of solvent extraction selected for second stage and onwards, its more preferred to be done simultaneously to get the similar value for concentration in each of the extraction before quantify bioactive compounds in sample

In this study some aspects should be improved to get better and accurate results. Following are the step of precautions that need to be considered during doing the research;

i) Handling equipment is one of the crucial factor to achieve good results where wrong handling the equipment for example did not set the right value of parameters such as temperature, time and agitation speed during each of the extraction may able to get inaccurate results. Other than that, the equipment condition need to be cleaned from any impurities in order to prevent any

contaminant of sample occurs. During run the experiment, follow the instruction properly.

ii) Apparatus used need to be cleansed thoroughly before being used again to avoid any sample contaminant.

iii) Handling chemical should be done correctly at the suitable place such as fume hood especially for volatile, hazardous and reactive chemical. Personal protective equipment (PPE) such as gloves, goggle, safety boats and others must be wearing to avoid any exposure from hazardous chemical and exposed to hazard if equipment fails.

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APPENDIX A

Total Phenolic Test Result

Blank = -0.000

Table A.1 Trial results of TPC for solvent extraction

Solvent extract	1st trial	2nd trial	3rd trial	Average / y
Ethanol (96%)	0.088	0.091	0.067	0.0820
Ethanol (50%)	0.2	0.217	0.236	0.2177
Methanol (96%)	0.183	0.11	0.115	0.1360
Methanol (50%)	0.183	0.18	0.214	0.1923
Propanol (96%)	0.062	0.071	0.076	0.0697
Propanol (50%)	0.217	0.244	0.238	0.2330
Hexane	0.009	0.006	0.007	0.0073

Calibration curve

$y = 0.001x + 0.017$, $R^2 = 0.999$

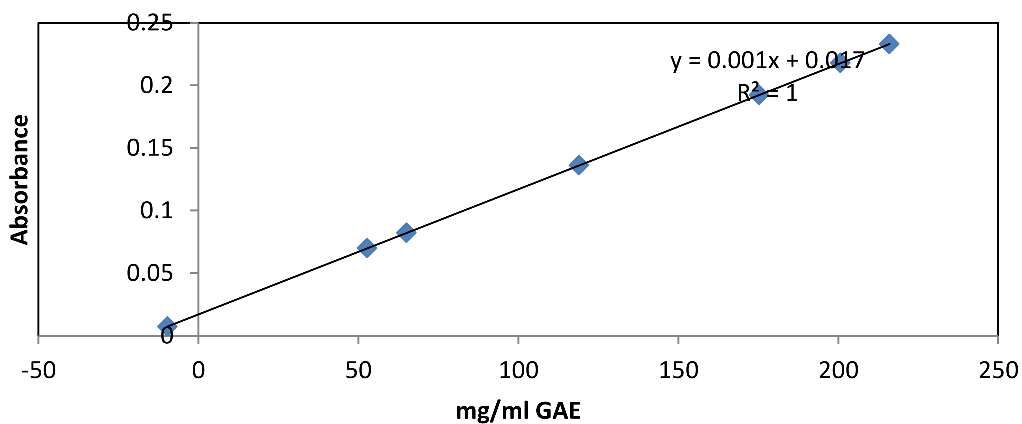

Figure A.1 Calibration curve for absorbance vs. mg / ml GAE

1g sample = 60ml

mg GAE	60ml	100g
ml	1g dried sample	100g

= mg GAE / 100g dried sample

Table A.2 Solvent extract in mg GAE / 100g dried sample

mg/mL GAE	mg GAE/ 100 g dried sample	Solvent extract
65	3900	Ethanol (96%)
200.7	12042	Ethanol (50%)
119	7120	Methanol (96%)
175.3	10518	Methanol (50%)
52.7	3162	Propanol (96%)
216	12960	Propanol (50%)
-9.7	-582	Hexane

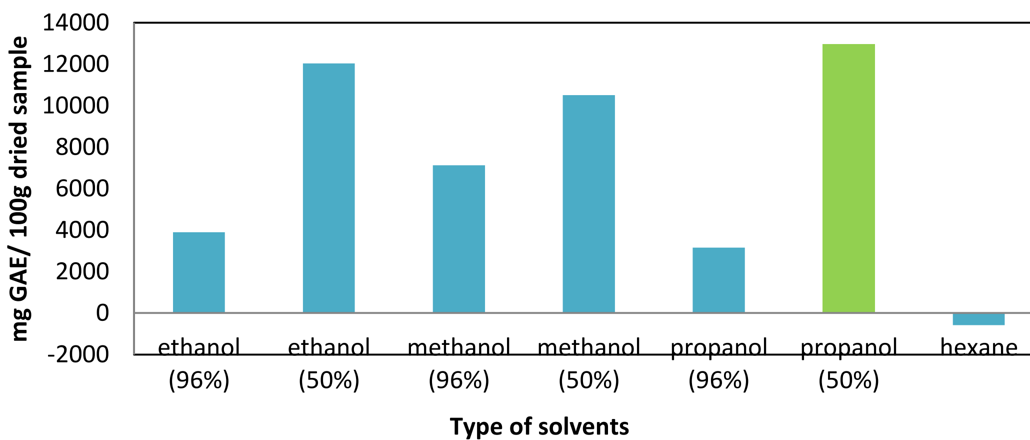

Figure A.2 Total phenolic content for different type of solvent

Total Flavonoids Test Result

Blank: -0.0000

Table A.3 Trial results of TPC for solvents extraction

Solvent extract	1st trial	2nd trial	3rd trial	Average
Ethanol (96%)	0.232	0.241	0.237	0.236667
Ethanol (50%)	0.194	0.221	0.28	0.231667
Methanol (96%)	0.154	0.188	0.158	0.166667
Methanol (50%)	0.23	0.24	0.249	0.239667
Propanol (96%)	0.162	0.172	0.174	0.169333
Propanol (50%)	0.18	0.22	0.21	0.203333
Hexane	0.043	0.041	0.039	0.041

Calibration curve

$y = 0.0004x + 0.0122$, $R^2 = 0.9989$

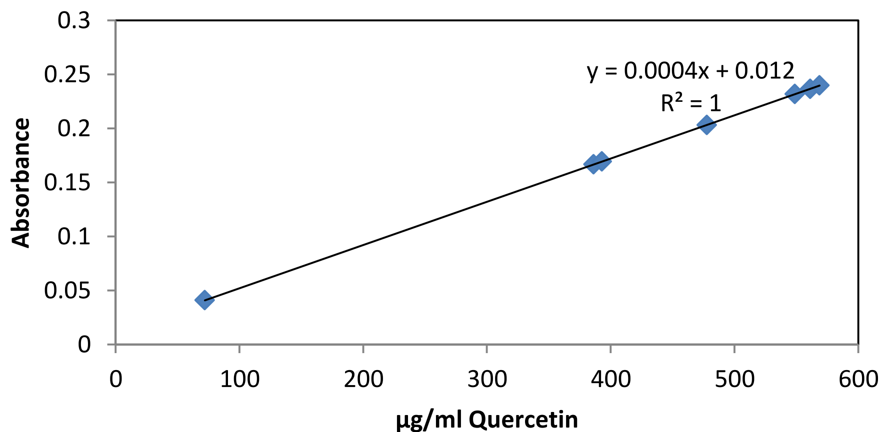

Figure A.3 Calibration curve for absorbance vs. μg/ ml Quercetin

1g sample = 60ml

μg QE	60ml	100g
ml	1g dried sample	100g

= μg QE / 100g dried sample

Table A.4 Solvent extract in μg QE / 100g dried sample

μg/ml Quercetin	μg QE/ 100 g dried sample	Solvent extract
561.25	33675	Ethanol (96%)
548.75	32925	Ethanol (50%)
386.25	23175	Methanol (96%)
568.75	34125	Methanol (50%)
392.75	23565	Propanol (96%)
477.75	28665	Propanol (50%)
72	4320	Hexane

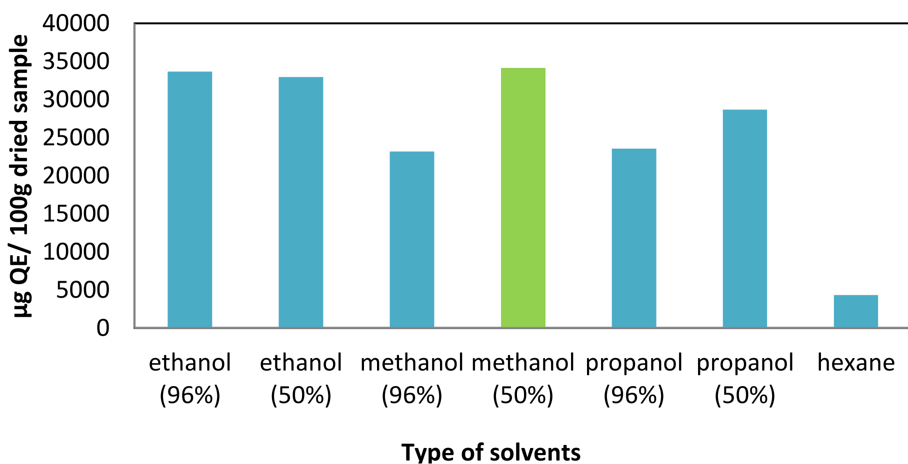

Figure A.4 Total flavonoids content for different type of solvents

DPPH Test Result

Blank = -0.000, Control = 0.637

Table A.5 Trial results of DPPH for solvents extraction

Solvent extract	1st trial	2nd trial	3rd trial	Average	% inhibition of DPPH
Ethanol (96%)	0.353	0.339	0.357	0.3497	45.10
Ethanol (50%)	0.084	0.111	0.197	0.1307	79.48
Methanol (96%)	0.227	0.214	0.285	0.2420	62.00
Methanol (50%)	0.14	0.104	0.138	0.1273	80.02
Propanol (96%)	0.455	0.461	0.477	0.4643	27.11
Propanol (50%)	0.136	0.13	0.136	0.1340	78.96
Hexane	0.645	0.635	0.637	0.6390	0.31

% inhibition of DPPH = ((Abs control - Abs sample)/Abs control) x 100

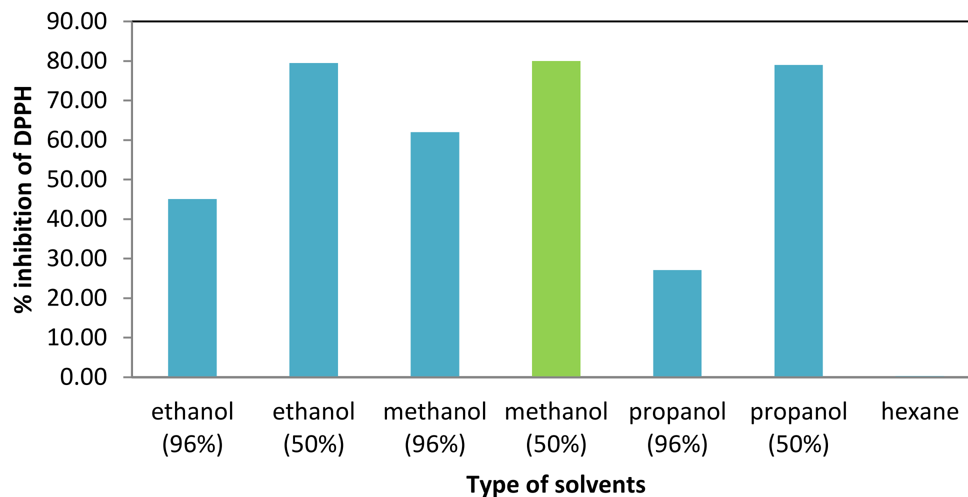

Figure: A.5 Antioxidant capacity, DPPH for different type of solvents

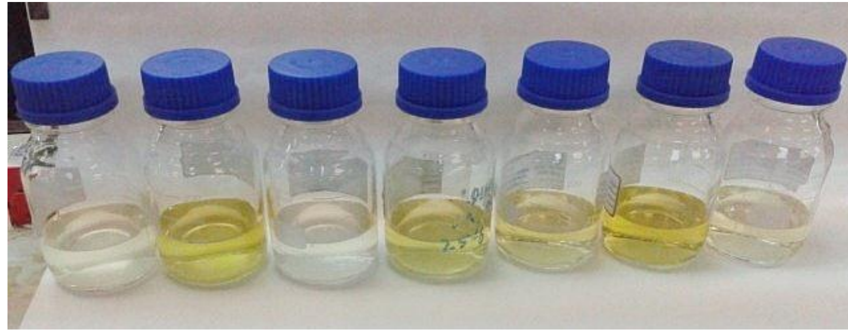

Figure A.6 Different types of solvent extraction. From left, ethanol 50%, ethanol 96%, methanol 50%, methanol 96%, propanol 50%, propanol 96% and hexane

Figure A.7 Total phenolic content test on different type of solvent

Figure A.8 Antioxidant capacity test on different type of solvent

APPENDIX B

Total phenolics test result

Blank: -0.000

Table B.1 Trial results of TPC for extraction time

Extraction time	1st trial	2nd trial	3rd trial	Average /y
15 minutes	0.119	0.119	0.133	0.1237
30 minutes	0.154	0.167	0.141	0.1540
60 minutes	0.179	0.189	0.177	0.1817
120 minutes	0.186	0.203	0.182	0.1903
180 minutes	0.197	0.218	0.194	0.2030
240 minutes	0.184	0.185	0.17	0.1797

Calibration curve

$y = 0.001x + 0.017$, $R^2 = 0.999$

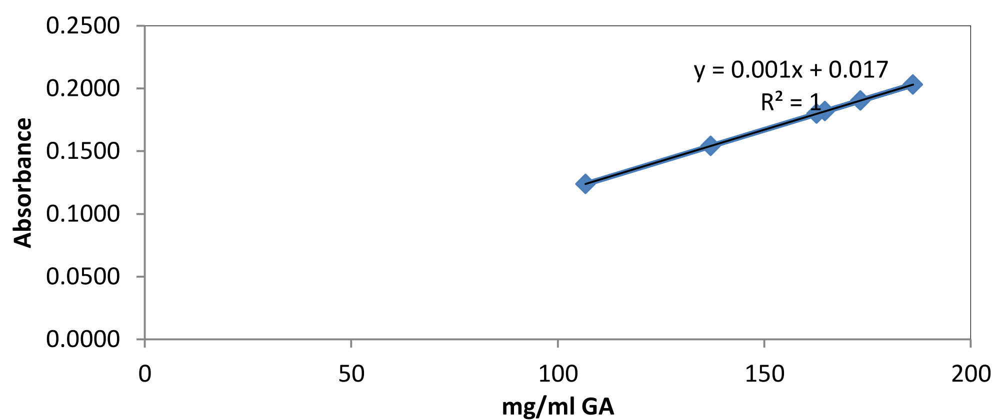

Figure B.1 Calibration curve for absorbance vs. mg/ml GAE

1g sample = 60ml

mg GAE	60ml	100g
ml	1g dried sample	100g

= mg GAE / 100g dried sample

Table B.2 Extraction time in mg GAE / 100g dried sample

mg/ml GAE	mg GAE/ 100g dried sample	Extraction time
106.7	6402	15 minutes
137	8220	30 minutes
164.7	9882	60 minutes
173.3	10398	120 minutes
186	11160	180 minutes
162.7	9762	240 minutes

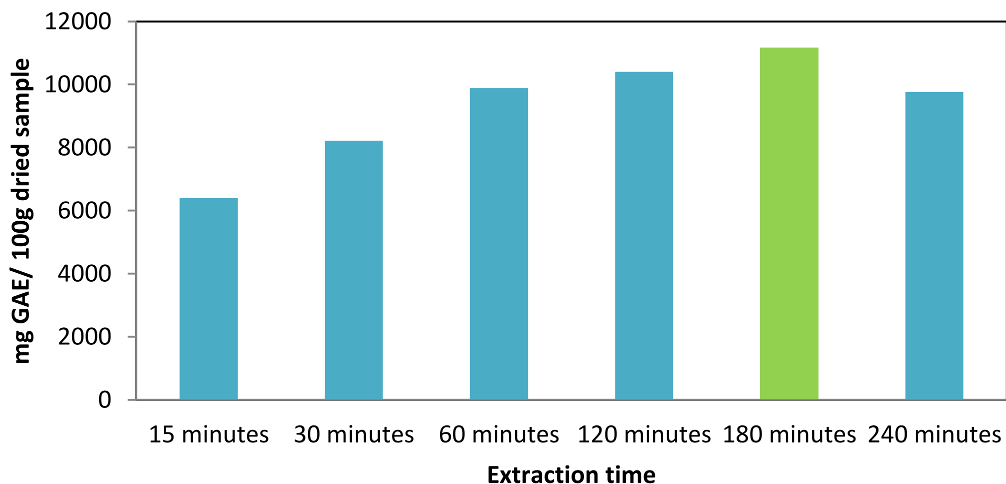

Figure B.2 Total phenolics content for different extraction time

Total Flavonoids Result

Blank = -0.000

Table B.3 Trial results of TFC for extraction time

Extraction time	1st trial	2nd trial	3rd trial	Average
15 minutes	0.155	0.132	0.148	0.1450
30 minutes	0.171	0.167	0.184	0.1740
60 minutes	0.256	0.186	0.19	0.2107
120 minutes	0.207	0.202	0.205	0.2047
180 minutes	0.194	0.193	0.187	0.1913
240 minutes	0.199	0.204	0.21	0.2043

Calibration curve

$y = 0.0004x + 0.0122$, $R^2 = 0.9989$

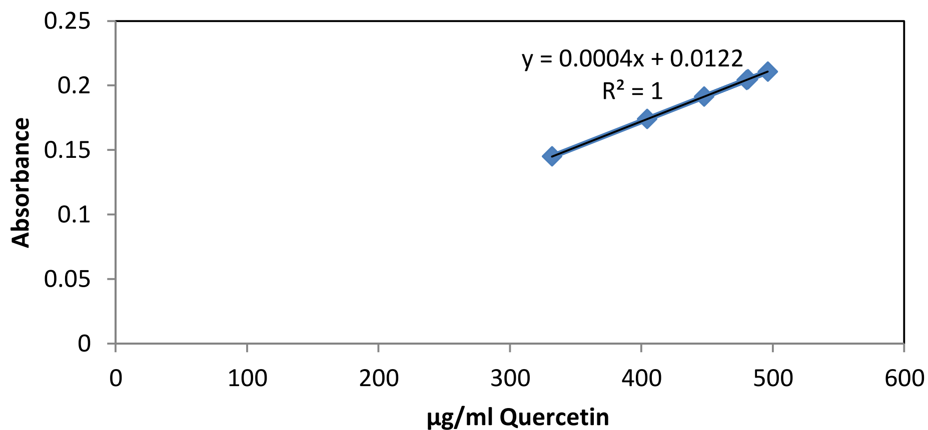

Figure B.3 Calibration curve for absorbance vs. µg/ ml Quercetin

1g sample = 60ml

µg QE	60ml	100g
ml	1g dried sample	100g

= µg QE / 100g dried sample

Table B.4 Extraction time in µg QE / 100g dried sample

µg/ml Quercetin	µg QE/ 100 g dried sample	Extraction time
332	19920	15 minutes
404.5	24270	30 minutes
496.25	29775	60 minutes
481.25	28875	120 minutes
447.75	26865	180 minutes
480.25	28815	240 minutes

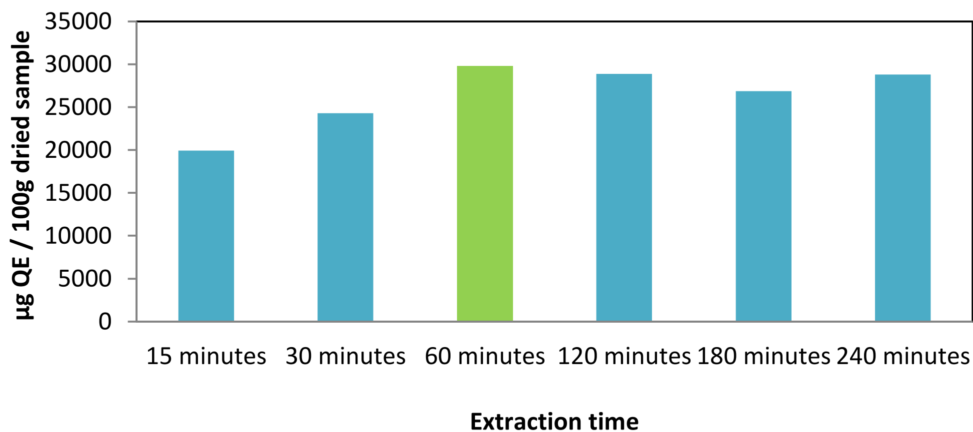

Figure B.4 Total flavonoids content for different extraction time

DPPH Test Result

Blank = -0.000, Control = 0.4850

Table B.5 Trial results of DPPH for extraction time

Extraction time	1st trial	2nd trial	3rd trial	Average	% inhibition of DPPH
reference	0.534	0.443	0.478	0.4850	
15 minutes	0.161	0.173	0.168	0.1673	65.4983
30 minutes	0.136	0.136	0.127	0.1330	72.5773
60 minutes	0.126	0.125	0.12	0.1237	74.5017
120 minutes	0.118	0.115	0.113	0.1153	76.2199
180 minutes	0.112	0.114	0.11	0.1120	76.9072
240 minutes	0.11	0.104	0.087	0.1003	79.3127

% inhibition of DPPH = ((Abs control - Abs sample)/Abs control) x 100

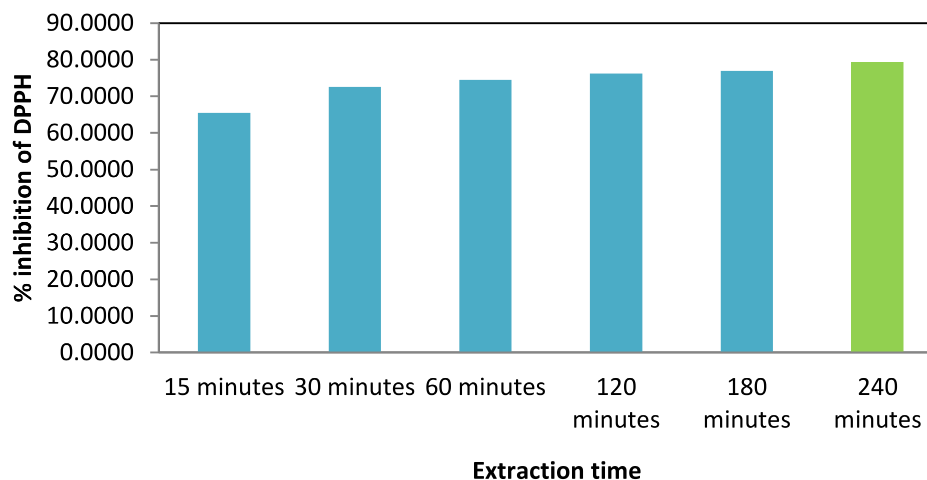

Figure B.5 Antioxidant capacity for different extraction time

Figure B.6 Different extraction time. From left 15 minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes and 240 minutes

Figure B.7 Total phenolics content test for different extraction time

Figure B.8 Total flavonoids content for different extraction time

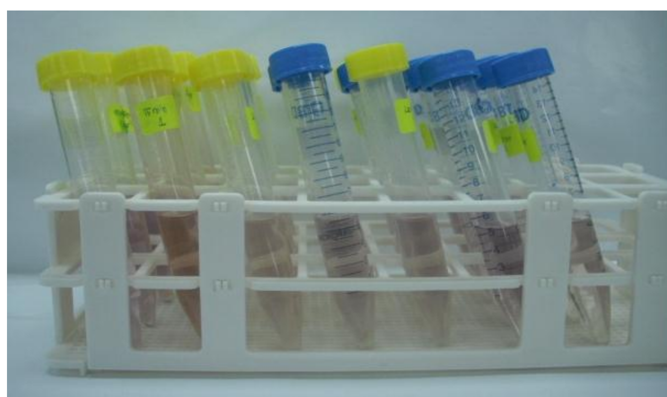

Figure B.9 Antioxidant capacity, DPPH for different extraction time

APPENDIX C

Total phenolic test

Blank = -0.000

Table C.1 Trial results of TPC for extraction temperature

Extraction temperature °C	1st trial	2nd trial	3rd trial	Average/y
30	0.11	0.107	0.095	0.1040
40	0.107	0.115	0.119	0.1137
50	0.166	0.166	0.152	0.1613
60	0.177	0.161	0.176	0.1713
70	0.204	0.155	0.21	0.1897

Calibration curve

$y = 0.001x + 0.017$, $R^2 = 0.999$

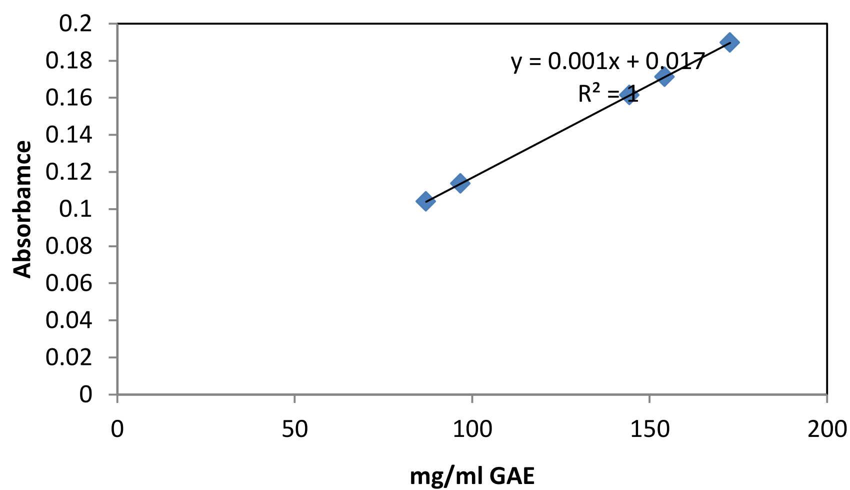

Figure C.1 Calibration curve for absorbance vs. mg/ml GAE

0.5g sample = 30ml

mg GAE	30ml	100g
ml	1g dried sample	100g

= mg GAE / 100g dried sample

Table C.2 Extraction temperature in mg GAE / 100g dried sample

mg/ml GAE	mg GAE/ 100g dried sample	Extraction temp.
87	2610	30°C
96.7	2901	40°C
144.3	4329	50°C
154.3	4629	60°C
172.7	5181	70°C

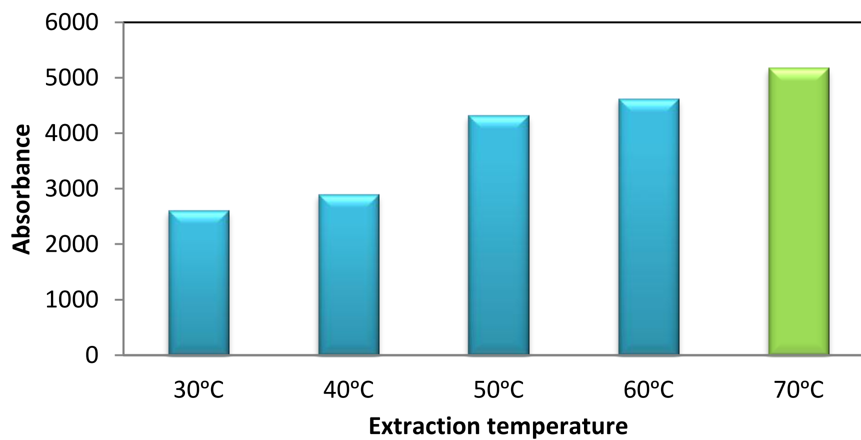

Figure C.2 Total phenolics content for different extraction temperature

Total flavonoids

Blank = -0.000

Table C.3 Trial results of TPC for extraction temperature

Extraction temperature °C	1st trial	2nd trial	3rd trial	Average/ y
30	0.163	0.156	0.157	0.1587
40	0.187	0.185	0.193	0.1883
50	0.235	0.218	0.217	0.2233
60	0.245	0.227	0.249	0.2403
70	0.282	0.269	0.273	0.2747

Calibration curve

$y = 0.0004x + 0.0122$, $R^2 = 0.9989$

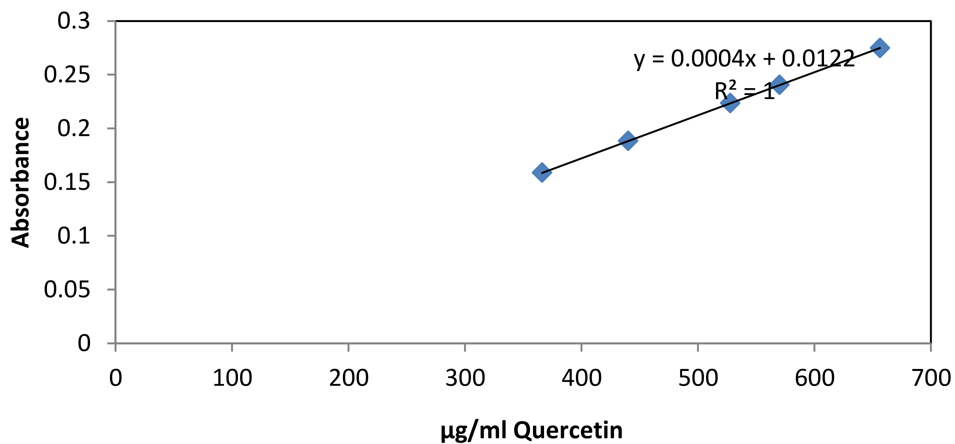

Figure C.3 Calibration curve for absorbance vs. µg/ml Quercetin

0.5g sample = 30ml

μg QE	30ml	100g
ml	1g dried sample	100g

= μg QE / 100g dried sample

Table C.4 Extraction temperature in μg QE / 100g dried sample

μg/ml Quercetin	μg QE/ 100 g dried sampl	Extraction temperature
366.25	10987.5	30°C
440.25	13207.5	40°C
527.75	15832.5	50°C
570.25	17107.5	60°C
656.25	19687.5	70°C

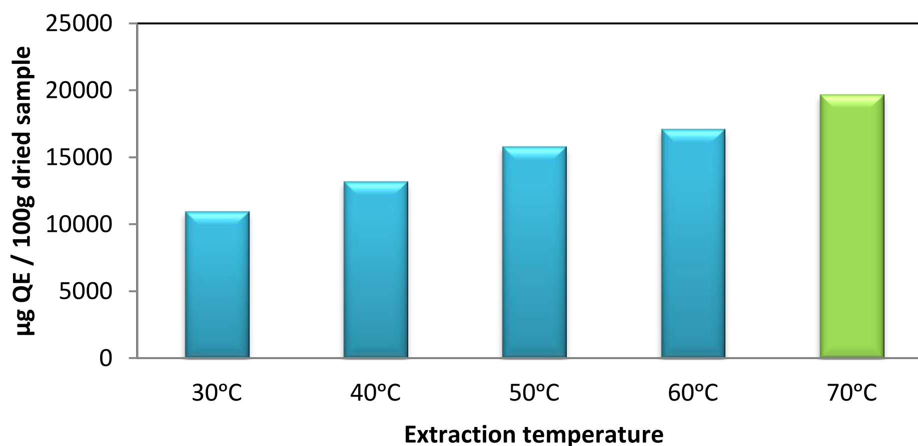

Figure C.4 Total flavonoids content for different extraction temperature

DPPH Test Result

Blank = -0.000

Table C.5 Trial results of DPPH for extraction temperature

Extraction temperature	1st trial	2nd trial	3rd trial	Average	% inhibition of DPPH
control	0.582	0.585	0.596	0.5877	
30°C	0.339	0.346	0.339	0.3413	41.92
40°C	0.323	0.3	0.328	0.3170	46.06
50°C	0.259	0.22	0.24	0.2397	59.22
60°C	0.201	0.202	0.189	0.1973	66.42
70°C	0.182	0.171	0.175	0.1760	70.05

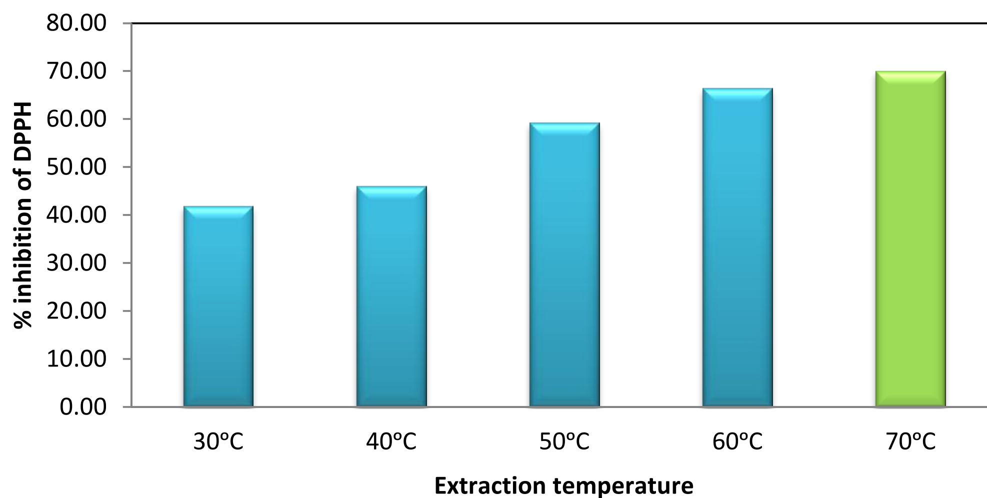

Figure C.5 Antioxidant capacity, DPPH for different extraction temperature

Figure C.6 Different extraction temperature. From left is 30° C, 40° C, 50° C, 60° C and 70° C

Figure C.7 Total phenolic content test for different extraction temperature

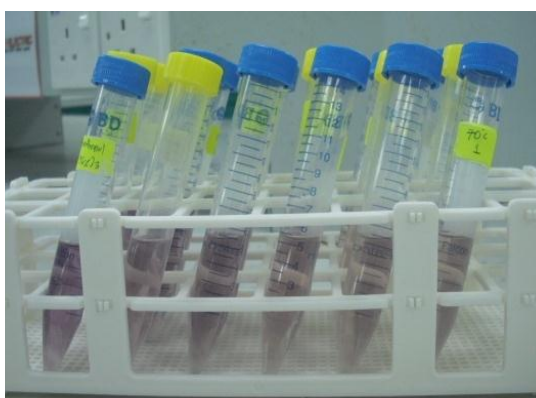

Figure C.8 Antioxidant capacity, DPPH test for different extraction temperature

Figure C.9 Total flavonoids content for different extraction temperature

APPENDIX D

Total Phenolics Test

Blank = -0.000

Table D.1 Trial results of TPC for effect of agitation

Effect of agitation	1st trial	2nd trial	3rd trial	Average /y
50 rpm	0.167	0.161	0.115	0.1477
100 rpm	0.169	0.163	0.18	0.1707
150 rpm	0.186	0.191	0.179	0.1853
200 rpm	0.182	0.212	0.179	0.1910
250 rpm	0.193	0.219	0.185	0.1990
300 rpm	0.204	0.237	0.19	0.2103

Calibration curve

$y = 0.001x + 0.017, R^2 = 0.999$

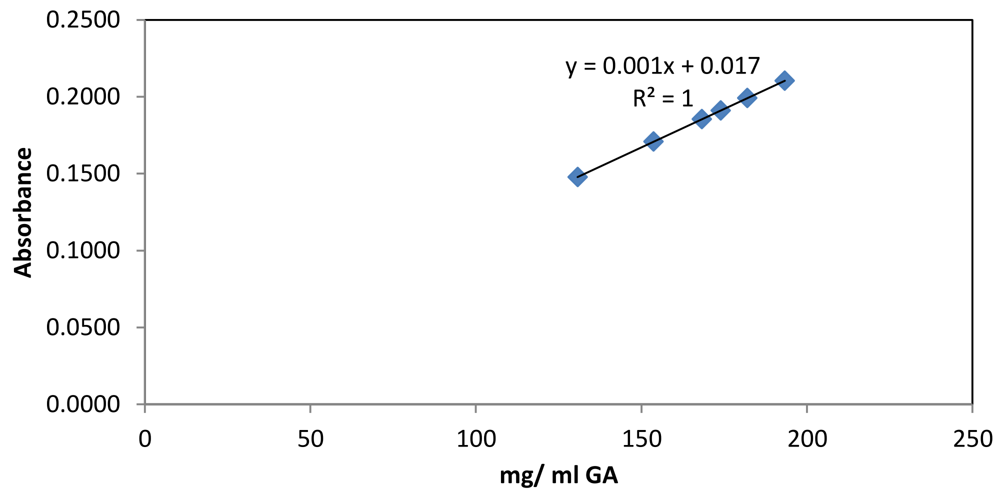

Figure D.1 Calibration curve for absorbance vs. mg/ml GAE

0.5g sample = 30ml

mg GAE	30ml	100g
ml	1g dried sample	100g

= mg GAE / 100g dried sample

Table D.2 Agitation speed mg GAE / 100g dried sample

mg/ml GA	mg GAE/ 100g dried sample	Agitation speed
130.7	3921	50 rpm
153.7	4611	100 rpm
168.3	5049	150 rpm
174	5220	200 rpm
182	5460	250 rpm
193.3	5799	300 rpm

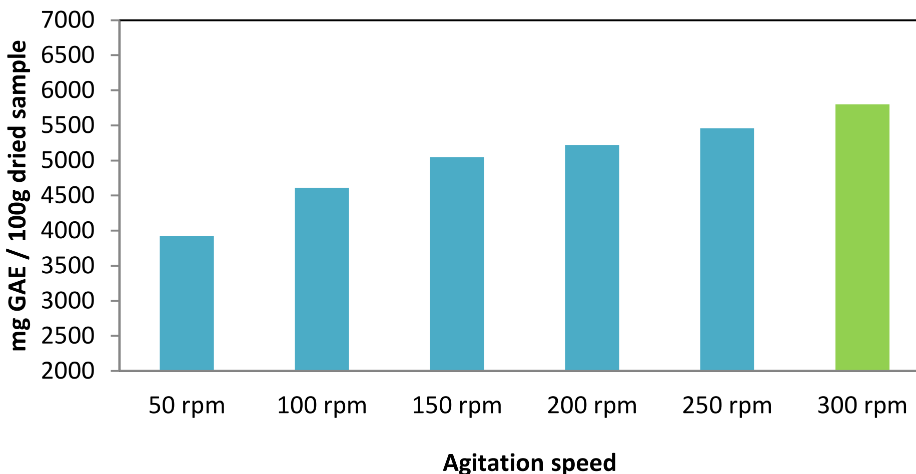

Figure D.2 Total phenolics content for different agitation speed

Total Flavonoids Result

Blank = -0.000

Table D.3 Trial results of TFC for effect of agitation

Effect of agitation	1st trial	2nd trial	3rd trial	Average/y
50 rpm	0.22	0.213	0.207	0.2133
100 rpm	0.235	0.245	0.237	0.2390
150 rpm	0.258	0.254	0.255	0.2557
200 rpm	0.262	0.303	0.263	0.2760
250 rpm	0.268	0.268	0.305	0.2803
300 rpm	0.317	0.292	0.289	0.2993

Calibration curve

$y = 0.0004x + 0.0122$, $R^2 = 0.9989$

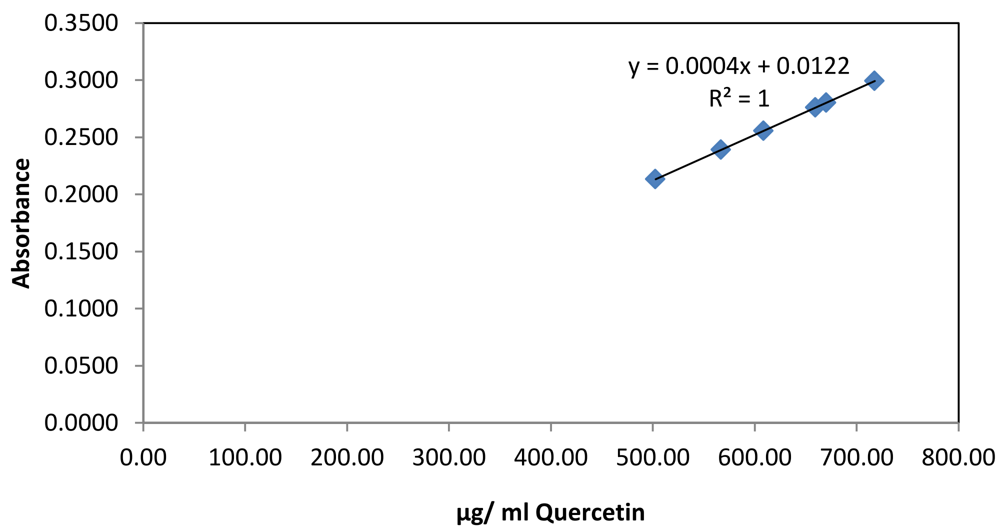

Figure D.3 Calibration curve for absorbance vs. µg/ml Quercetin

0.5g sample = 30ml

µg QE	30ml	100g
ml	1g dried sample	100g

= µg QE / 100g dried sample

Table D.4 Agitation speed extraction in µg QE / 100g dried sample

µg/ml Quercetin	µg QE/ 100 g dried sample	Agitation speed
502.75	15082.5	50 rpm
567	17010	100 rpm
608.75	18262.5	150 rpm
659.5	19785	200 rpm
670	20100	250 rpm
717.75	21532.5	300 rpm

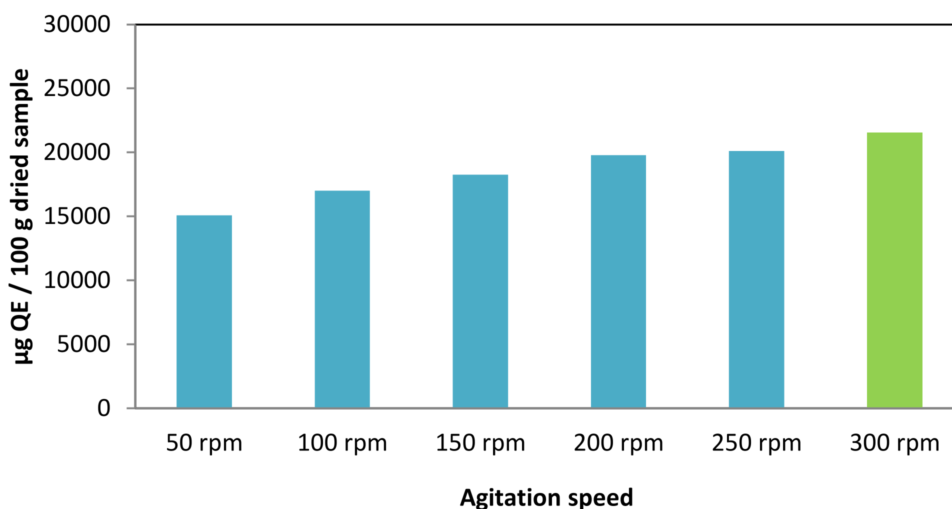

Figure D.4 Total flavonoids content for different agitation speed

DPPH Test result

Blank = -0.000, Control = 0.6067

Table D.5 Trial results of DPPH for effect of agitation

Effect of agitation	1st trial	2nd trial	3rd trial	Average	% inhibition of DPPH
reference	0.608	0.606	0.606	0.6067	
50 rpm	0.194	0.191	0.18	0.1883	68.9577
100 rpm	0.157	0.159	0.154	0.1567	74.1772
150 rpm	0.157	0.155	0.15	0.1540	74.6168
200 rpm	0.15	0.153	0.154	0.1523	74.8915
250 rpm	0.147	0.15	0.148	0.1483	75.5508
300 rpm	0.143	0.136	0.139	0.1393	77.0342

% inhibition of DPPH = ((Abs control - Abs sample)/Abs control) x 100

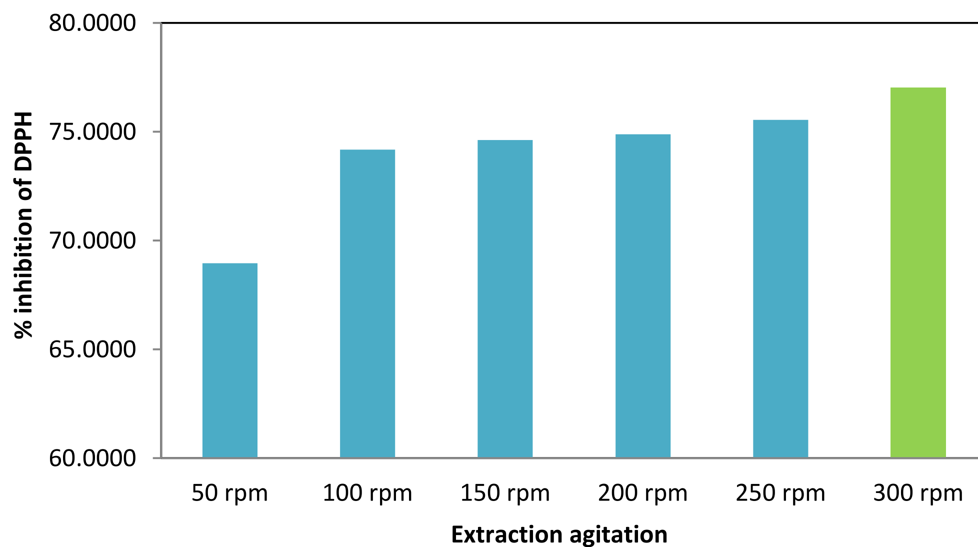

Figure D.5 Antioxidant capacity, DPPH for different agitation speed

Figure D.6 Different agitation speed. From left is 50 rpm, 100 rpm, 150 rpm, 200 rpm, 250 rpm, and 300 rpm

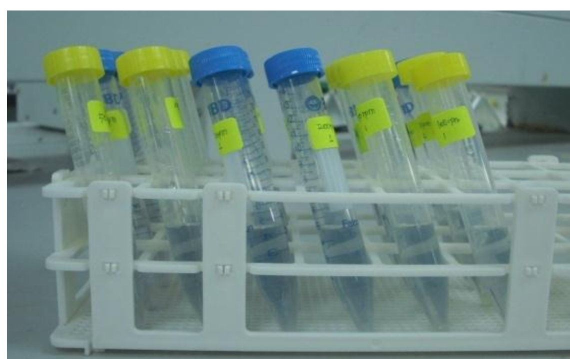

Figure D.7 Total phenolic content test for different agitation speed

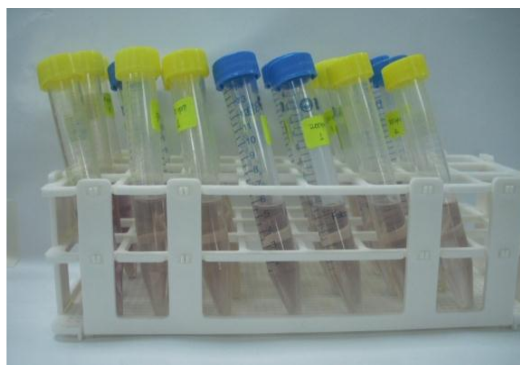

Figure D.8 Antioxidant capacity, DPPH test for different agitation speed

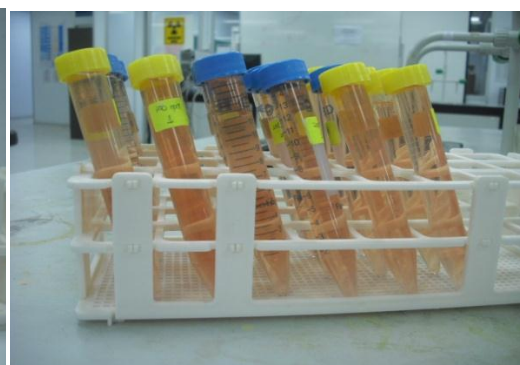

Figure D.9 Total flavonoids content for different agitation speed