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JUDUL : EXTRACTION OF ANTIOXIDANT ACIVITY, PHENOLIC CONTENT AND MINERAL CONTENT FROM GUAVA PEEL

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EXTRACTION OF ANTIOXIDANT, PHENOLIC CONTENT AND MINERAL CONTENT FROM GUAVA PEEL

SITI ZUULAIKA BINTI REJAL

A thesis submitted in fulfillment
of the requirements for the award of the Degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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DECEMBER 2010

I declare that this thesis entitled “Extraction of antioxidant, phenolic content and mineral content from Guava peel” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature :

Name : Siti Zuulaika Binti Rejal

Date : 3 December 2010

Special Dedication of This Grateful Feeling to My…

Beloved father and mother;
Mr. Rejal Bin Jaafar and Mrs. Rohanah Binti Hanip

Beloved Friend;
Shaifulnizam Bin Sharif

Loving brother and sister;
Roziana Binti Rejal and Mohamad Kameel Bin Rejal

For Their Love, Support and Best Wishes.

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ABSTRACT

Guava peel contains high amount of antioxidant activity and phenolic content and also the employment of peels would result in the waste to wealth application. The objective of this research is to investigate the extraction on antioxidant activity, phenolic content and mineral contents (Mg, Ca and Na) in guava peel. Guava peels were freeze dried and grounded before mixing it with different type of organic solvent (methanol, ethanol, acetone and ethyl acetate) in various solvent ratio (20, 40, 60, 80, 100%). After that, the extraction time (20, 40, 60, 80, 100, 120, 150, 180 minutes) and temperature (25, 35, 45, 55, 65 ºC) were investigated to obtain the optimum condition for extraction. Then the extracts obtained were evaporated using rotary evaporator to remove solvent and were analyzed using Ultraviolet-Visible Spectrophotometer for analysis of antioxidant activity and phenolic content, Atomic Absorption Spectrophotometer for analysis of mineral content and High Performance Liquid Chromatography to verify ascorbic acid existence. It was found that methanol with 60% solvent/water ratio at temperature 55ºC for 120 minute gave the highest yields of antioxidant activity and phenolic content which were 1021.00 μmol/L and 596.67mg/L. After the analysis using AAS, it was prove that peel of guava contained 206.65, 17.31 and 2.04 ppm of Mg, Ca and Na respectively. From analysis of HPLC, it was determined that there were significant amount of ascorbic acid in the extracts.

ABSTRAK

Kulit jambu batu mengandungi kuantiti antioksida dan fenolik yang tinggi. Penggunaan kulit ini dapat menukarkan hasil sampingan kepada hasil yang lumayan. Tujuan kajian ini dilaksanakan adalah untuk menyiasat ekstrak aktiviti antioksida, kandungan fenolik dan kandungan mineral (Mg, Na, Ca) di dalam kulit jambu batu. Kulit jambu batu di kering beku dan dihancurkan sebelum mencampurkannya dengan pelbagai jenis pelarut organik (metanol,etanol,asetone dan etil asetat) di dalam pelbagai nisbah pelarut air (0,20,40,60,80 dan 100%). Selepas itu, masa ekstrak (20, 40, 60, 80, 100 dan 120 minit) dan suhu ekstrak (25, 35, 45, 55 dan 65 º C) disiasat untuk mendapatkan keadaan yang paling optimum untuk proses ekstraki. Cecair yang diekstrak akan disejatkan dengan menggunakan penyejat berputar untuk menyingkirkan pelarut dan dianalisis dengan menggunakan Spektrofotometer Ultra-Ungu untuk analisis aktiviti antioksida serta kandungan fenolik, Spektrofotometer Penyerapan Atom untuk analisis kandungan mineral dan Kromatografi Cecair Berprestasi Tinggi untuk mengesahkan kehadiran asid askorbik. Didapati bahawa metanol pada nisbah 60% pada suhu 55ºC selama 120 minit memberikan hasil tertinggi untuk aktiviti antioksidan dan kandungan fenolik iaitu 1021.00 μmol/l dan 596.67 mg/l. Setelah analisis menggunakan Spektrofotometer Penyerapan Atom dilakukan, terbukti bahawa kulit jambu biji mengandungi 206.65, 17.31 dan 2.04 ppm mineral magnesium, kalsium dan natrium masing-masing. Daripada analisis yang dibuat menggunakan Kromatografi Cecair Berprestasi Tinggi, dipastikan bahawa ada sejumlah besar asid askorbat dalam ekstrak.

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LIST OF ABBREVIATIONS

K	-	Potassium
Na	-	Sodium
Fe	-	Iron
I	-	Iodin
Zn	-	Zinc
Ca	-	Calcium
Mg	-	Magnesium
Cu	-	Cuprum
Cr	-	Cromium
Uv	-	UltraViolet
AAS	-	Atomic Absorption Spectrometer
HPLC	-	High Performance liquid chromatography
ha	-	Hectare
MT	-	Metric tonne
FRAP	-	Ferric Reducing Antioxidant Power
RM	-	Ringgit Malaysia
DNA	-	Deoxyribonucleic acid
GAE	-	Gallic acid equivalent
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
w/v	-	Weight/volume
TPC	-	Total Phenolic Content
TFC	-	Total Flavonoid Content
ABTS	-	2,2'-azino-bis
ORAC	-	Oxygen Radical Absorbance Capacity
TPTZ	-	2,4,6-Tris(2-pyridyl)-s-triazine
FC	-	Follin Ciocalteu
HCl	-	Hydrocloric Acid

$FeCl_3.6H_2O$	-	Ferrous Chloride
$NaCO_3$	-	Sodium Carbonate
K_2HPO_4	-	Potassium Phosphate
Rpm	-	Rotation per minute
M	-	Molarity
TP	-	Total Phenolic

CHAPTER 1

INTRODUCTION

1.1 Research Background

1.1.1 Guava Peel

Guava (*Psidium guajava* L.), also known locally as *jambu batu*, is grown commercially and in many home gardens in Malaysia. Guava contains a high level of ascorbic acid (50–300mg/100 g fresh weight), which is three to six times higher than oranges. Phenolic compounds such as myricetin and apigenin, ellagic acid, and anthocyanins are also at high levels in guava fruits. (Thaipong *et al.*, 2006)

Ascorbic acid is the main constituent of the peel, secondly in the firm flesh, and a little content in the central pulp varies from 56mg to 600mg and may range between 350mg and 450mg in nearly ripe fruit (Charles et al., 2006). Canning or other heat processing destroys about 50% of the ascorbic acid. The strong odour of the fruit is attributed to its carbonyl compounds (Gutierrez *et al.*,2008).

1.1.2 Antioxidant Activity

Antioxidant compounds can be found in food such as whole grains, fruits and vegetables. It plays an important role as a health protecting factor. Antioxidant definition has been defined as substances that have the ability to trap free radicals that will oxidize

nucleic acid, proteins that can initiate degenerative diseases such as cancer and heart disease,

Thus, antioxidant can inhibit the oxidative mechanisms that lead to degenerative diseases. Several epidemiological studies suggest that a high intake of food rich in natural antioxidants increases the antioxidant capacity of the plasma and reduces the risk of some, but not all, cancers, heart diseases, and stroke (Hassimotto, Maria and Franco, 2005). Lim, Lim and Tee (2007) states that, the most abundant antioxidants in fruits are polyphenols and Vitamin C, Vitamins A, B and E and carotenoids are present to a lesser extent in some fruits.

1.1.3 Phenolic Content

Phenolic is one of the antioxidant which can be found in fruits. Phenolic is also a hydrophilic antioxidants which was the major constituent in antioxidant activity and this was agreed by Thaipong *et al.,* (2005). Furthermore, phenolic is also one of the plentiful antioxidant that can be found in most fruits.

Lim, Lim and Tee (2007) states that the most abundant antioxidants in fruits are polyphenols and Vitamin C, Vitamins A, B and E and carotenoids are present to a lesser extent in some fruits. Dietary intake of plant phenolics are inversely related to coronary heart disease (Hertog *et al.,* 1997) and act as anti-ulcer, antispasmodic, antisecretory, or antidiarrhoeal agents in the gastrointestinal tract (Carlo *et al.,* 1999).

1.1.4 Mineral Content

Human as well as animal studies originally showed that optimal intakes of elements such as sodium, potassium, magnesium, calcium, manganese, copper, zinc, and iodine could reduce individual risk factors, including those related to cardiovascular disease (Mertz, 1982). Well-defined deficiencies of public health importance have been described for some elements, e.g., Fe, I, Zn, and Ca, whereas other elements have been clearly linked to deficiencies when patients receive intravenous therapy with inadequate solutions for

long periods of time, e.g., Mg, Cu, Cr, and Mn (Fairweather-Tait and Hurrell, 1996; Castillo *et al*., 1998, 340–356).

1.2 Problem statement

Traditionally, guava was prepared by washing, dicing and blending freshly before being extracted by solvent. Another alternative for sample preparation is by freeze drying the guava before extracting it by using solvent to obtain the antioxidant activity, phenolic contents and minerals content. Interestingly, the peel and seed fractions of some fruits possess higher antioxidant activity than the pulp fractions.

Therefore, the peel and seed fractions of fruits may potentially contain more antioxidants quantitatively or qualitatively than the pulp fractions.(Guo *et al.,* 2003). Usually, the investigation of antioxidant activity, phenolic contents, and mineral contents in guava is using the pulp; alternatively guava peel may be used as the raw material because it contains more antioxidants quantitatively or qualitatively than the pulp fractions. Besides, by using guava peel as raw material, the employment of peels that is considered to be a by product would result in the waste to wealth application.

1.3 Objective

The objective of this research is to investigate the extraction of antioxidant activity, phenolic content and mineral contents which are magnesium, calcium and sodium in guava peel.

1.4 Scope

In order to achieve the objective, the following scopes were identified:

Firstly, is to discover the most effective solvent to extract antioxidant activity, phenolic content and mineral contents in guava peel. Types of solvent use are polar protic solvent which are methanol and ethanol, polar aprotic which are acetone and ethyl acetate. Methanol has higher polarity compared with ethanol and acetone has higher polarity compare with ethyl acetate. The equipment will be using is Uv-Visible Spectrophotometer at 765 nm for analysis of phenolic content and 593 nm for analysis of antioxidant activity.(wavelength).

Secondly, is to obtain the most effective solvent concentration to extract antioxidant activity and phenolic content in guava peel. The solvent concentration ratios are 20, 40, 60, 80, 100%. The equipment used was Uv-Visible Spectrophotometer at 765 nm for analysis of phenolic content and 593 nm for analysis of antioxidant activity.(wavelength).

Next, is to obtain the most effective extraction time in order to extract antioxidant activity and phenolic content in guava peel. The extraction time ratios are 20, 40, 60, 80, 100, 120 minutes. The equipment used was Uv-Visible Spectrophotometer at 765 nm for analysis of phenolic content and 593 nm for analysis of antioxidant activity.(wavelength).

Then, the scope is to obtain the most effective extraction temperature in order to extract antioxidant activity and phenolic content in guava peel. The extraction temperature ranged of 25, 35, 45, 55 and 65 ºC. The equipment used was Uv-Visible Spectrophotometer at 765 nm for analysis of phenolic content and 593 nm for analysis of antioxidant activity.(wavelength).

Lastly, the scope of study is to study the Magnesium (Mg), Sodium (Na) and Calcium (Ca) content in guava peel by using Flame atomic absorption spectrophotometer(AAS) and also to study the ascorbic acid content in the guava peel by using High Performance Liquid Chromatography (HPLC).

1.5 Rationale and significance

Traditionally, the extraction on antioxidant activity and phenolic content used guava pulp; lack of study was done on the potential ability of extracting antioxidant activity and phenolic content from the peels. Other source of raw material would increase the utilization of the guava and not just the pulp only is valuable. Besides, the employment of peels that is considered to be a by-product would result in the waste to wealth application hence reducing the amount of waste.

Furthermore, the study on mineral contents in guava will add the benefits of guava peel to be applied in cosmetic or food supplement. Nowadays, the food industry tries to minimise the usage of synthetic antioxidants in food products due to potential health hazards. The consumers, authorities and food industry producers that concerned about food have created a need to produce natural antioxidant from fruit by products such as guava peels.

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CHAPTER 2

LITERATURE REVIEW

2.1 Guava

2.1.1 Origin

The guava has been cultivated and distributed by man and animals for so long that its place of origin is uncertain, but it is believed to be an area extending from southern Mexico to Central America. It was soon adopted as a crop in Asia and in warm parts of Africa. In India, guava cultivation has been estimated at 125,327 acres yielding 27,319 tons annually. It is common found in warm climates area because it can survive only a few degrees of frost (Morton, 1985).

Generally, it is a home fruit tree or planted in small groves, except in India where it is a major commercial resource. Brazil's modern guava industry is based on seeds of an Australian selection grown in the botanical garden of the Sao Paulo Railway Company at Tatu. Plantations were developed by Japanese farmers at Itaquera and this has become the leading guava-producing area in Brazil. The guava is one of the leading fruits of Mexico where the annual crop from 36,447 acres (14,750 ha) of seedling trees totals 192,850 tons (175,500 MT). Only in recent years has there been a research program designed to evaluate and select superior types for vegetative propagation and large-scale cultivation.

In many parts of the world, the guava runs wild and forms extensive thickets called "guayabales" in Spanish and it overruns pastures, fields and roadsides so vigorously in

Hawaii, Malaysia, New Caledonia, Fiji, the U.S. Virgin Islands, Puerto Rico, Cuba and southern Florida that it is classed as a noxious weed subject to eradication. Nevertheless, wild guavas have constituted the bulk of the commercial supply. In 1972, Hawaii processed, for domestic use and export, more than 2,500 tons (2,274 MT) of guavas, over 90% from wild trees. During the period of high demand in World War II, the wild guava crop in Cuba was said to be 10,000 tons (9,000 MT), and over 6,500 tons (6,000 MT) of guava products were exported. (Morton, 1987)

2.1.2 Phytochemistry of guava

Guava is a berry tropical fruits that consists of fleshy pulp and numerous small seeds. The fruit is small which only 3 to 6 cm long. It is pear-shaped fruit that turn from green to reddish-yellow when it is ripe. There are several fraction of guava that potentially nutritious which are pulp, peel, leaf and seed.

2.1.2.1 Pulp of guava

Pulp of guava is rich with nutritional value. The nutritional values from previous study were summarized in the Table 2.1.

Table 2.1 Nutritional contents in guava pulp (Gutierrez *et al.*, 2008).

Nutrient	Value	Reference
Carbohydrates	13.2%	Medina and Pagano, 2003
Fats	0.53%	
Proteins	0.88%	
Water Contents	84.9%	
Calories	36–50 kcal	Conway, 2002
moisture	77–86 g	
crude fibre	2.8–5.5 g	
ash	0.43–0.7 g	
calcium	9.1–17mg	
phosphorus	17.8–30mg	
	*value per 100g	

iron	0.30–0.70mg	Iwu,1993
Vitamin A	200–400 I.U	Fujita *et al.*,1985;
thiamine	0.046mg	Hernandez,1971;
riboflavin	0.03–0.04mg	Conway,2002
niacin	0.6–1.068mg	
ascorbic acid	100mg	
Vitamin B3	40 I.U	
Manganese	-	Nadkarni and Nadkarni, 1999

The unripe fruit is indigestible, causes vomiting and feverishness. It changes in chemical composition and the activities of hydrolytic enzymes (the activities of amylase and amylase decreased significantly with ripening), chlorophyll, cellulose, hemicelluloses, and lignin content increased while carotenoid content decreased. The unripe fruit is high in tannins, is astringent and has a tendency to cause constipation, but it is sometimes employed in diarrhoea (Gutierrez *et al.*, 2008).

2.1.2.2 Peel of guava

Previous study had shown that, the peel and seed fractions of some fruits possess higher antioxidant activity than the pulp fractions. Guo *et al* (2003) states that most of fruit peel and seed fractions were stronger than the pulp fractions in antioxidant activity based on their FRAP values. The contribution of vitamin C to the FRAP value of fruit pulps varied greatly from fruit to fruit as calculated. Therefore, the peel and seed fractions of fruits may potentially contain more antioxidants quantitatively or qualitatively than the pulp fractions.

Besides, Nurliyana *et al.*, *(*2010) stated that previous studies have determined the antioxidant levels and activities in tropical fruits such as mangosteen, papaya and star fruit, covering various parts of fruits. However, investigations on the antioxidant activities for fruit peels and seeds are lacking due to their low popularity and commercial application (Soong and Barlow, 2004). According to Caro and Piga (2007), seeds and peels of Italian fresh fig fruits cultivars showed higher antioxidant capacity and phenolic content than the

edible portions. That is why, guava, like other tropical fruits, is believed to be rich in antioxidants but information specifically on antioxidant levels and properties in its peels are still lacking.

2.1.3 Applications of guava

Guava has wide of traditional and modern applications. Traditionally, guava was used in food crop and folk medicine around the world. Nowadays, many pharmacological studies have demonstrated the ability of this plant to exhibit antioxidant, hepatoprotection, anti-allergy, antimicrobial, antigenotoxic, anti-plasmodial, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic, anti-inflamatory and antinociceptive activities, supporting its traditional uses as stated by Giuterrez *et al.,* (2008). Table 2.2 illustrates the commercial application of guava in some countries.

Table 2.2 The commercial application of guava (Giuterrez *et al.*,2008)

Fraction	Application	Country	Reference
Fruit	Food, juice, jelly nectar,stuffed of candies, tinned products, confectionery	All the countries	Jimenez-Escrig*et al.*,2001
Wood	spinning tops	Guetamala	Morton (1987)
Wood	Hair combs	El Salvador	Morton (1987)
Wood	Construction of house	Nigeria	Lucas *et al.,* (2006)
leaves	Employed to give black colour to cotton	South-East Asia	Rodarte (1994)
leaves	Serve to dye matting	Indonesia	Rodarte (1994)
Bark	Dyes, stains, ink, tattoos and mordants	Africa	Burkil (1985)

This fruit produces wide range of food products. The fruit is sweet and edible and is eaten raw or cooked cooking to eliminate the strong odour, but are preferred seeded and served sliced as dessert or in salads. It also makes good jam or made into jellies. The fruit is rich in vitamin C and source of antioxidant dietary fibre. The juice is used as a refreshing

drink. Guava powder containing 2,500-3,000 mg ascorbic acid was commonly added to military rations in World War II (Morton, 1987).

A standard dessert throughout Latin America and the Spanish-speaking islands of the West Indies is stewed guava shells (*cascos de guayaba*), that is, guava halves with the central seed pulp removed, strained and added to the shells while cooking to enrich the syrup. The canned product is widely sold and the shells can also be quick-frozen. They are often served with cream cheese. Sometimes guavas are canned whole or cut in half without seed removal. It is also made into syrup for use on waffles, ice cream, puddings and in milkshakes (Morton, 1987).

The roots, bark, leaves and immature fruits, because of their astringency, are commonly employed to halt gastroenteritis, diarrhea and dysentery, throughout the tropics. Crushed leaves are applied on wounds, ulcers and rheumatic places, and leaves are chewed to relieve toothache. (Morton, 1987).

The leaf decoction is taken as a remedy for coughs, throat and chest ailments, gargled to relieve oral ulcers and inflamed gums; and also taken as an emmenagogue and vermifuge, and treatment for leucorrhea. It has been effective in halting vomiting and diarrhea in cholera patients. It is also applied on skin diseases. A decoction of the new shoots is taken as a febrifuge. (Morton, 1987)

The leaf infusion is prescribed in India in cerebral ailments, nephritis and cachexia. An extract is given in epilepsy and chorea and a tincture is rubbed on the spine of children in convulsions. A combined decoction of leaves and bark is given to expel the placenta after childbirth (Morton, 1987).

2.1.4 Availability of guava

Guava was grown in Malaysia for fresh and export market and also for food processing. Guava consists of seedless and with seed. The seedless variety was not strong recommended for commercial growing due to its unstable characteristic such as **Clone GU15.** Guava with seed was more popular such as **Clone GU8, Clone GU9** and **Clone**

GU 10. Total area of guava in Malaysia was 1,440 hectare with an annual production of 18,880 mt in 2009. Area with most guava was in Daerah Muar Johor (213 ha), Daerah Batang Padang Perak (185 ha) and Daerah Segamat Johor (68 ha) (Hosnan, 2010).

The fruits matures 90 to 150 days after flowering. Generally, there are 2 crops per year, September/October period and March/April period. However in Malaysia, the fruits could generally be obtained all year round. The *market price of guava in Malaysia* was between RM6 to RM9 per kg for seedless guava and for farm price is around RM3 to RM4 per kg depending on sizes and fruits quality (Malaysian fruits. Worpress, 2009).

2.2 Antioxidant activity

2.2.1 Definition

Antioxidant has the ability to trap highly reactive free radicals and oxygen species which are present in biological systems. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease such as heart disease and cancer. Antioxidant compounds such as phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.(Prakash *et al.*)

Antioxidants can be classified into two classes which are hydrophilic and lipophilic. Vitamin C and phenolic are known as hydrophilic antioxidants, while carotenoidsare known as lipophilic antioxidants.(Thaipong *et al.*, 2005)

2.2.2 Antioxidant activity in guava peel

The amount of antioxidant activity in the guava fractions had been determined from the previous study. Table 2.3 shows that the value of total phenolic content by using Folin Ciocalteu method and antioxidant activity values by using FRAP assay in guava peel and

pulp and Table 2.4 below illustrates the FRAP values for pulp, peel and seed for some fruits. In Table 2.3 and Table 2.4, the value of FRAP and total phenolic content in peel are higher compare with pulp. It is proven that, the value of antioxidant activity and phenolic content in the peel posses the highest value compared with pulp and also seed. It is an advantage to use peel as raw material because of its high amount of antioxidant activity and phenolic content.

Table 2.3 Total Extractable Phenol Content (TEP) Expressed as Gallic Acid Equivalents (GAE), and Ferric Reducing Antioxidant Power (FRAP) in Two Fractions of the Fruit of Psidium guajava. (Escrig *et al.*, 2001)

Psidium guajava	DPPH					
	TE (g GAE kg^{-1} d m)	EC_{50} (g d m/g DPPH)	T_{EC50} (minutes)	AE ($i/EC_{50}T_{EC50}$)	FRAP 30 min (µmol Trolox/g d m)	CLT_{50} (µg of d m mL^{-1})
peel	58.7±4.0	1.92±0.08	54.74±2.05	0.007±0.002	462±51	1.65±0.05
pulp	26.3±0.8	3.7±0.06	30.75±264	0.009±0.002	238±7	2.75±0.15

Table 2.4 FRAP values of peel, pulp and seed fractions of 28 fruits (mmol/100g wet weight) (Guo *et al.*, 2003)

Fruits	Pulp	Peel	Seed	Total
Date	6.98 ± 0.29	16.69 ± 0.55	1.77 ± 0.13	43.10
Kiwi fruit	4.38 ± 0.20	11.13 ± 0.23	-	15.51
Longan	0.94 ± 0.05	3.98 ± 0.30	24.26 ± 2.79	29.18
Banana	0.73 ± 0.11	3.16 ± 0.16	-	3.89
Pineapple	0.80 ± 0.008	2.01 ± 0.03	-	2.81
Orange	1.89 ± 0.19	5.69 ± 0.26	-	7.58
Strawberry	3.29 ± 0.30	-	-	3.29

2.2.3 Phenolic content

Phenolic compounds widely distributed in the medicinal plants, spices, vegetables, fruits, grains, pulses and other seeds are an important group of natural antioxidants with possible beneficial effects on human health. They can participate in protection against the harmful action of reactive oxygen species, mainly oxygen free radicals. Free radicals are produced in higher amounts in a lot of pathological conditions and are involved in the development of the most common chronic degenerative diseases, such as cardiovascular disease and cancer. (Stratil *et al.* 2007)

2.3 Pre-treatment of raw material

There are many methods for pre-treatment of raw material before undergo the extraction for example freeze dried sample and fresh sample. The pre-treatment plays an important role in determines the value of antioxidant in fruits and also determines the capability for sample storage.

Fresh sample is easy to prepare but cannot be store for some time. The sample should be prepared continuously along the experiment to gain the most accurate result. This is aligned with the study by Guo *et al.,* (2003) that stated that, fresh fruits sample were flushed by tap water and then washed in distilled water for three times before the peel, pulp and seed fractions were carefully separated. A portion of 1/5 gram was weighed and ground in a mortar after addition of distilled water (1:9 w/v). The homogenate was centrifuged at 6000g for 10 min. The supernatant was recovered and used directly for FRAP assay without storage.

On the other hand, freeze dried sample is easier to prepared even though it consume longer time in preparing it, but once the sample was completely dried, the sample can be stored in certain temperature without affecting the natural antioxidant in the fruit, this is agreed by Escrig *et al.,* (2001). Freeze-drying did not alter composition and antioxidant property of extracts. (Spigno *et al.,* 2007)

2.4 Effect of extraction process

The aim of an extraction process is to provide the maximum yield of antioxidant activity and phenolic content. The variables investigated were solvent ratio, type of solvent, temperature of extraction and time of extraction.

2.4.1 Extraction solvent

In previous study, Spigno *et al.,* (2007) states that type of solvent has been the most investigated factor. There are many types of solvent can be used for extraction,but the most commonly used is alcoholic solvent. Alcoholic solvents have been commonly employed to extract phenolics from natural sources: they give quite high yield of total extract even though they are not highly selective for phenols. Particularly, mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than the corresponding mono-component solvent system. Ethanol and methanol are commonly alcoholic solvent used in extraction. Spigno *et al.,* (2007) was stated that the use of ethanol (a dietaryalcohol) may be preferable than methanol in view of a food application of the extracts, in the present paper the effect of different aqueous ethanol mixture on extraction from grape marc was investigated. In fact, ethanol, a polar solvent, effectively extracts flavonoids and their glycosides, catecols and tannins from raw plant materials, but solubility of these compounds can be enhanced using a mixed solvent over a limited compositional range.

In this research, four solvent systems were used (methanol,ethanol,acetone and ethyl acetate) at five different concentration (20%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) and with 100% distilled water. Earlier, Alothman *et al*, (2009) studied extraction by using three solvent systems (methanol, ethanol and acetone) at three different concentrations (50%, 70% and 90%) and with 100% distilled water. According to Alothman *et al.,* (2009), the recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvent used for the extraction process. Furthermore, solvent polarity will play a key role in increasing phenolic solubility. Therefore, it is hard to develop a standard extraction procedure suitable for the extraction

of all plant phenols. Usually, the least polar solvents are considered to be suitable for the extraction of lipophilic phenols unless very high pressure is used.

The polarity of the solvent gives effect to the yield of the extraction. As solvent polarity increased, accordingly the relative extractions of Total Phenolic Content (TPC) and Total Flavoid Content (TFC) changed; their maximum yield was at different ethanol concentrations. Thus, there is no single ethanol concentration able to recover all of the individual phenolic compounds from a sample. It was also observed that the antioxidant capacity of the crude extracts was sensitive to the solvent polarity. In particular, DPPH radical-scavenging capacity of the crude extracts extracted with a high ethanol concentration decreased considerably after reaching 80% ethanol. Thus, it is believed that the effective phenolic compounds in the crude extract, to which are attributed the antioxidant capacities, were intermediately polar and their solubility was very sensitive to the solvent polarity. By compromising between the high extraction of phenolic compounds and high antioxidant capacity, a moderate ethanol concentration (40%) was selected as the most appropriate solvent to optimise the subsequent extraction parameters.(Thoo *et al.,* 2010).

Addition of water to ethanol improved extraction rate, but too high water content brought an increased concomitant extraction of other compounds, and, then to lower phenols concentrations in the extracts. Phenols extracted with different water content revealed the same antioxidant activity, suggesting that only different amounts but not different compounds were recovered. (Spigno *et al.,* 2007)

2.4.2 Extraction time

One of the important parameter that affects the extraction yield is extraction time. By study the effect of time range, the cost will be saved by not wasting time and able to gain high yield of extraction. Silva *et al.,* (2007) was studied that extraction time is crucial in solvent extraction for phenolic compounds, where phenolic compounds may be governed by the equilibrium concentrations for phenolic compounds reached before their corresponding apparent reduction. Hence, excess extraction time indeed reduced the yield of phenolic compounds. Overall, the experimental results showed that extraction time (20–

120 min) had significant effect on TPC, ABTS and DPPH but not on TFC.The difference in optimum extraction times for TPC and TFC may be due to different degrees of phenolic polymerisation, solubility of phenolics and interaction of phenolics with other food constituents which then leads to the difference time needed to reach equilibrium between the solution in the solid matrix (M.citrifolia) and in the bulk solution (ethanol).

It is also observed that the optimum extraction time for antioxidant compounds varies with antioxidant capacity. This phenomenon has been postulated that the estimation of ABTS and DPPH radical-scavenging capacities are not solely dependent on a single group of antioxidant compounds; indeed it is based on the ability of any compounds present that could scavenge ABTS or DPPH radicals. By taking consideration into practical and economic aspects as well in optimising the recovery of phenolic compounds and their antioxidant capacity, 80 min was chosen, as a compromise to the best extraction time for phenolic compounds and antioxidant capacity (Thoo *et al.*, 2010).

More contradictory are the data available for extraction length:some authors chose quite short extraction times (Bonillaet al., 1999; Pinelo *et al.*, 2005b; Yilmaz & Toledo,2006); other quite long times (Pekic ´ *et al.*, 1998; Pinelo *et al.*, 2005a; Lapornik, Prosek, & Wondra, 2005; Jayap-rakasha, Singh, & Sakariah, 2001; Spigno & De Faveri,2007). (Spigno *et al.*, 2007).

Beyond 20 h of extraction there was an apparent reduction in the amount of extracted phenols, but it is under investigation whether this was due to a real degradation or to polymerization reactions bringing new compounds with a different response to analytical measurements. (Spigno *et al.*, 2007)

From the study by Maisuthisakui and Pongsawatmanit, (2004) yield of the extract and total phenolic content were almost constant after 3 hours of extraction time at room temperature. However, extraction time from 4.5 to 6 hours gave the lowest EC_{50} value, comparing with those from other extraction times.

2.4.3 Extraction Temperature

Extraction temperature can give effect to extraction yield. By study the effect of temperature range, the cost will be saved by not wasting energy and able to gain high yield of extraction.

From the previous study, it was studied that many authors agree in the fact that an increase in the working temperature favours extraction enhancing both the solubility of solute and the diffusion coefficient, but also that beyond a certain value phenolic compounds can be denatured.

Spigno and De Faveri (2007) concluded that phenols yields at 60 ºC were higher than at 28 ºC, but an intermediate temperature of 45 ºC was selected for the next trials in order to verify if it could be possible to obtain the same result as for 60 ºC (or even better in case a certain degree of thermal degradation occurred at 60 ºC) with reduction of energy costs. More contradictory are the data available for extraction length: some authors chose quite short extraction times (Spigno *et al.*, 2007).

Furthermore, previous works reported opposing trend of phenols yield from grape marc: none significant difference between 5 and 24 h, at both 28 and 60 ºC (Spigno & De Faveri, 2007), significant increase from 12 to 24 h at room temperature (Lapornik *et al.*, 2005). That is why we decided to study extraction kinetics at both 60 ºCand 45 ºC. (Spigno *et al.*, 2007)

2.5 Analysis methods

2.5.1 Antioxidant activity

Antioxidant capacities have several assay that have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables and their products and foods for clinical studies including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)

(ABTS), 2,2- diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and the oxygen radical absorption capacity (ORAC) assay.

2.5.1.1 FRAP assay

FRAP assay is commonly used to study the antioxidant capacity of plant materials. The antioxidant capacity of fruits extracts is determined by the ability of the antioxidants in these extracts to reduce ferric iron to ferrous in FRAP reagent, which consists of2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) prepared in sodium acetate buffer, pH 3.6. The reduction of ferric iron in FRAP reagent will result in the formation of a blue product (ferrous – TPTZ complex) whose absorbance can be read at 593 nm (Alothman *et al.*, 2009).

Guo *et al.*, (2003) states that there are many different antioxidants contained in fruits and it is very difficult to measure each antioxidant component separately. Therefore, several methods have been developed to evaluate the total antioxidant activity of fruits or other plants and animal tissues. Among them, Trolox equivalent antioxidant capacity, total radical absorption potentials, oxygen radical absorption capacity assays are the representative methods frequently used in various investigations. However, none of the methods mentioned above can be treated as a total antioxidant capacity assay because what they really measure is the capacity of antioxidants in scavenging specific radicals, inhibiting lipid peroxidation or chelating metal ions.

The commonly used method for determining antioxidant activity is FRAP assay. This is based on several reasons that were first, the FRAP assay treats the antioxidants in the samples as reductants in a redox-linked colorimetric reaction. Second, the procedure of FRAP assay is relatively simple and easy to be standardized. One possible disadvantage with FRAP assay is the fact that this assay does not react fast with some antioxidants, such as glutathione. However, we consider that FRAP assay is still suitable for assessment of antioxidant activity of fruit samples because only limited amounts of plant glutathione are absorbed by humans. (Guo *et al.*, 2003)

Thaipong *et al.*, 2006 affirms that the FRAP technique showed high reproducibility, was simple, rapidly performed and showed the highest correlation with both ascorbic acid

and total phenolics compared to the other methods. Therefore, it would be an appropriate technique for determining antioxidant in guava fruit extract.

2.5.1.2 DPPH assay

The values of antioxidant activities determined with DPPH method are lowest, despite that this DPPH method gives the same values as the TEAC method. The standard Trolox is most often used. The DPPH method gives several times lower
values for extracts than TEAC. This significant difference in values could be explained by a relatively higher stability of the DPPH radical what may result in significantly lower reactivity. This radical will evidently react only with the more reactive phenolic substances. Therefore, it will not detect the less reactive phenolic substances, which still could have antioxidant activity in the human organism.(Stratil *et al*., 2007)

2.5.1.3 ORAC assay

The ORAC assay is said to be more relevant because it utilizes a biologically relevant radical source. These techniques have shown different results among crop species and across laboratories. There was no correlation of antioxidant activity between the FRAP and ORAC techniques among most of the 927 freeze-dried vegetable samples, whereas these methods revealed high correlation in blueberry fruit.(Thaipong *et al.*, 2006)

2.5.2 Folin Ciocalteu Assay

The FCM actually measures a total reducing capacity of a sample. Determination correlates well with redox and antioxidant ability of phenolic compounds. Dissociation of phenolic proton leads to phenolate anion, which is capable of reducing FC reagent. FCM is non-specific to phenolic compounds. Many non-phenolic compounds, in fruits above all ascorbic acid and saccharides can reduce the reagent. (Stratil *et al.*, 2007)

Phenols were measured colorimetrically using the Folin Ciocalteu reagent with catechin as the standard after correction for ascorbic acid contribution. On a fresh weight

basis, cranberry had the highest total phenols, and was distantly followed by red grape. Free and total phenol quality in the fruits was analyzed by using the inhibition of lower density lipoprotein oxidation promoted by cupric ion. (Vinson *et al.,* 2001). Thaipong *et al.,* (2006) affirms that total phenolics content was determined by the Folin–Ciocalteu method, which was adapted from Swain and Hillis (1959).

2.5.3 Mineral contents

Human as well as animal studies originally showed that optimal intakes of elements such as sodium, potassium, magnesium, calcium, manganese, copper, zinc, and iodine could reduce individual risk factors, including those related to cardiovascular disease (Mertz, 1982). Well-defined deficiencies of public health importance have been described for some elements, e.g., Fe, I, Zn, and Ca, whereas other elements have been clearly linked to deficiencies when patients receive intravenous therapy with inadequate solutions for long periods of time, e.g., Mg, Cu, Cr, and Mn (Castillo *et al*., 1998).

The mineral contents of seeds and different plant parts of beach pea (Lathyrus maritimus L.) were studied by Shahidi *et al.,* (1999). In that study, Potassium (K) was the most abundant macroelement present, ranging from 627mg/100 g in mature pod shells to 451mg/100 g in seeds, followed by calcium (Ca) which was present from 1630mg/100 g in leaves to 139mg/100 g in seeds. The content of phosphorus (P) in seeds (434mg/100 g) and magnesium (Mg) in leaves (393mg/100 g) were highest as compared to other plant parts. Sodium (Na) content was highest in branches plus stems (355mg/ 100 g) and lowest (113mg/100 g) in seeds.

2.5.4 Ascorbic acid

Ascorbic acid content was determined using the 2, 6-dichlorophenol-indophenol titration method described in Association of Office Analytical Chemists (1996). L-ascorbic acid was used to prepare a standard solution (1mg/mL). The ascorbic acid concentration was calculated by comparison with the standard and expressed as mg/100 g fresh mass. (Thaipong *et al.,* 2006).

In the previous study by Stratil *et al.*, (2007), the ascorbic acid was measured bu using HPLC. The high-performance liquid chromatographic method with mass spectrometric detection (HPLC/MS) was selected for assessment of ascorbic acid concentration in extracts. Standard solution of ascorbic acid in aqueous methanol (1:1, v/v) was prepared. Ascorbic acid was determined using HP 1100 liquid chromatography equipped with HP MSD 1100 (Hewlett-Packard).

CHAPTER 3

METHODOLOGY

3.1 Introduction

In this research, the methods for extraction of antioxidant activity, phenolic content, mineral content and ascorbic acid are separated into four main parts which are sample preparation, extraction of samples, purification of extracts and analysis.

3.2 Chemicals

Organic solvents that were used for solvent extraction was methanol, ethanol, acetone and ethyl acetate. Methanol and ethanol were polar protic solvent. Acetone and ethyl acetate were polar aprotic solvent. Polar protic solvents solvate anions by hydrogen bonding, while aprotic solvents that have dipole moments solvate positive charge via their negative dipole.

For FRAP assay, the chemicals used were 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ), Acid hydrochloric (HCl), ferrous Chloride ($FeCl_3.6H_2O$), acetate buffer (glacial acetic acid and sodium acetate trihydrate) and ferrous sulphate ($FeSO_4.7H_2O$). For FC assay, Folin-Ciocalteu reagent, sodium carbonate ($NaCO_3$) and gallic acid were used. For determination of mineral content, the chemical used were sodium, calcium, magnesium standard solution and nitric acid. For determination of L-ascorbic acid, the chemical used were potassium dihydrogen phosphate (K_2HPO_4), phosphoric acid and L-ascorbic acid

3.3 Equipment

Equipment used during this research were freeze dryer for sample preparation; water bath and refrigerated centrifuge during extraction; rotary evaporator for purification process and Atomic Absorption Spectrophotometer, Ultra-Violet Visible Spectrometer and High Performance Liquid Chromatography for analysis. Details explanations are provided in Section 3.3.1 until 3.3.7.

3.3.1 Freeze Dryer

Model of freeze dryer used was Cleanvac 8 Freeze Dryer as shown in Figure 3.1. Freeze dryer is the equipment used to remove moisture by freezing the substance at low temperature. First, the freeze dryer was in cold trap mode in order to freeze the substance at temperature -23ºC with pressure at 0.005 torr. After the substance was completely frozen, the mode freeze dyer was change to vacuum mode for complete drying process. The usage time duration was dependable on quantity of substance, physical phase and also type of substance.

Figure 3.1 Cleanvac 8 Freeze dryer

3.3.2 Shaking Water Bath

Model of water bath used was BS-21 water bath as illustrated in Figure 3.2. Water bath is the equipment that uses water as medium for heating purpose and also has adjusted shaking function. The temperature range is from 25 to 120ºC and the shaking range is from 20 to 180 rpm.

Figure 3.2 Shaking Water Bath

3.3.3 Refrigerated Centrifuge

Model of refrigerated centrifuge used was Eppendorf centrifuge 5810 R as shown in Figure 3.3. Refrigerated centrifuge is the equipment that utilizes the density difference between the solids and surrounding fluid. (Belter *et al.*, 1988). The maximum capacity is 6 x 125g and maximum speed is 12 000 rpm respectively. This equipment was used in this research to separate mixture of sample with solvent into the unwanted pellet and supernatant.

Figure 3.3 Refrigerated Centrifuge

3.3.4 Rotary Evaporator

Rotary evaporator was used to remove extraction solvent by evaporation process in reduced pressure. Rotary evaporator consists of evaporating flask (A), heated water bath (B), rotor motor (C), mechanical mechanism (D), condenser (F), receiving flask (G), vapour duct (H) as illustrated in Figure 3.4. The function of rotor motor is to rotate the evaporation flask that containing the sample. The vapour duct was used as vacuum-tight conduit that used to draw off the vapour of the sample. To reduce the pressure within the evaporator system, the vacuum system was needed and condenser which uses a coil through for coolant (water) passes. The function of condensate-collecting flask at the bottom of the condenser was to collect the distilling solvent after re-condenses and a mechanical mechanism to lift the evaporation flask from the heating bath. The temperature set is depending with the boiling point of the solvent that need to be separated.

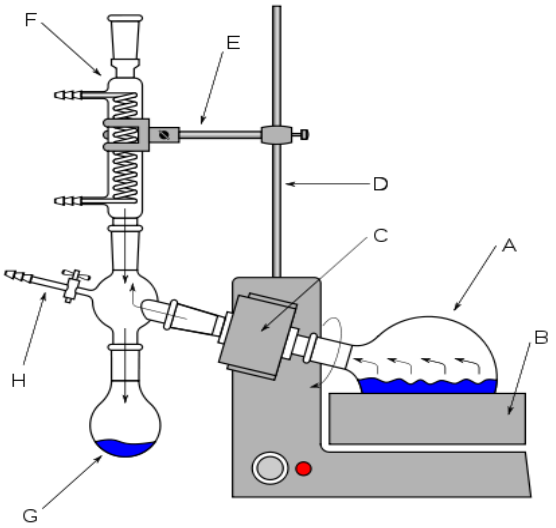

Figure 3.4 Rotary evaporator
(http://commons.wikimedia.org/wiki/File:Rotary_Evaporator.svg)

3.3.5 Atomic Absorption Spectrometer (AAS)

Z 5000 Atomic Absorption Spectrometer was the model used in this research to analyze mineral contents as illustrated in Figure 3.4. In flame AAS, a pneumatic nebulizer convert solution into an aerosol mist. The aerosol mist mixes with combustion gases in spray chamber before passing to the burner where the flame's thermal energy desolvates the aerosol mist to dry aerosol particles. Thermal energy in flame atomization is provided by combustion of fuel (air) and oxidant (acetylene). The burner provides a long path length for monitoring absorbance. (Harvey, 2000).

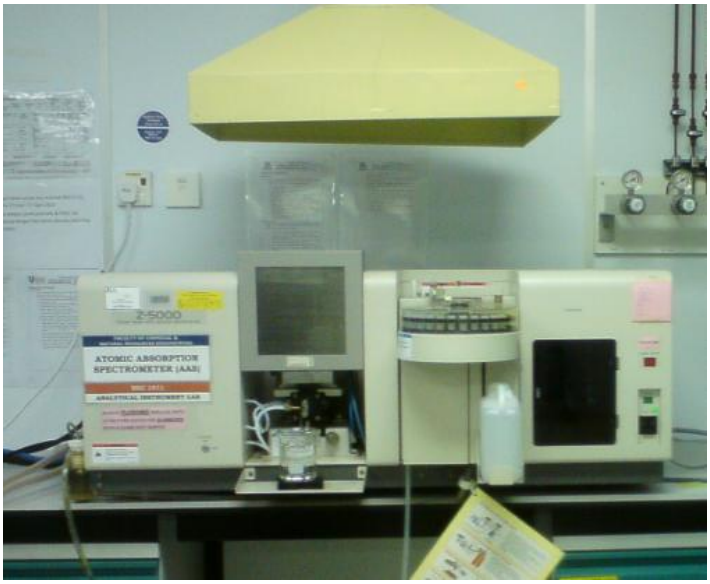

Figure 3.5 Atomic absorption Spectrometer

3.3.6 Ultra-Violet Visible Spectrometer

Ultra-Violet Visible Spectrometer as shown in Figure 3.6 is the instrument that measures the intensity of light passing through a sample (I), and compares it to the intensity of light before it passes through the sample (I_o). This device was used in the quantitative determination of antioxidant activity and phenolic content in the extracted sample.

Figure 3.6 UV -Visible Spectrometer

3.3.7 High Performance Liquid Chromatography (HPLC)

In this research, HPLC as illustrated in Figure 3.7 was used to identify, quantify and purify the ascorbic acid in the extracts. HPLC consist of stationary phases, mobile phase, pump, and a detector. Stationary phase is a liquid film coated on a packing material consisting of 3- 10µm porous silica particles. (Harvey, 2000). The pump used to move the mobile phase and analyte through the column and the detector provides a characteristic retention time for the analyte.

Figure 3.7 High Performance Liquid Chromatofraphy at Bio-scale Analytical Laboratory

3.4 Sample preparation

Guava fruits were bought from Tunas Mart, Kuantan and only their peels were taken for research purpose. The wet and fresh peels were weighed using analytical balance to obtain its initial weigh (52g). The peels were freeze dried for 4 days at temperature -23ºC, pressure 0.005torr by using Cleanvac 8 Freeze Dryer. The dried peels were blended

by using blender and the final weight was weighed (26 g) before placed in the plastic bag as shown in Figure 3.8 below and stored it in -20ºC freezer until used.

Figure 3.8 Dried guava peel after blended

3.5 Extraction

The variables investigated to obtain the optimum condition for extraction process are solvent ratio, type of solvent, time and temperature of extraction.

3.5.1 Extraction by using different types of solvent

0.5 g of sample was weighed and mixed with 50mL of solvent (methanol, ethanol, acetone and ethyl acetate) in 40 % solvent/water ratio. The mixture was placed in water bath at 50ºC for 120 minutes.

3.5.2 Extraction by using different solvent ratio

0.5 g of sample was weighed and mixed with 50mL of ethanol in different ethanol ratio (0, 20, 40, 60, 80 and 100 %). The mixture was placed in water bath at 50ºC for 120 minutes.

3.5.3 Extraction by using different extraction time

0.5 g of sample was weighed and mixed with 50mL of 60% ethanol ratio. The mixture was placed in water bath at 50ºC for various time ranges (20, 40, 60, 80, 100, 120, 150 and 180 minutes)

3.5.4 Extraction by using different type of extraction temperature

0.5 g of sample was weighed and mixed with 50mL of 60% ethanol ratio. The mixture was placed in water bath at various temperature (25, 35, 45, 55, 65 ºC) for 120 minutes.

3.6 Purifications of extracts

After the extraction, the extracts must be purify by separating them to obtain clear liquid by undergo several steps of solid liquid separation which are filtration and centrifugation.

3.6.1 Filtration

After extraction, the extracts were filter by using muslin cloth to separate the solid and liquid. The clearer solution was obtained and used for further purification step.

3.6.2 Centrifugation

The solution was centrifuged by using Centrifuge 5810R at condition 4750 rpm, 25 ºC for 15 minutes. After that, the supernatant was taken for further process and the pellet was disposed.

3.6.3 Solvent separation

The supernatants obtained in previous process then introduced to rotary evaporator to separate the solvent from the extracts and also to make the solution become more concentrated. The operating condition (temperature) for rotary evaporator was depending on the boiling point of the solvents that need to be separated.

3.7 Analysis

3.7.1 Antioxidant activity

3.7.1.1 Preparation of Standard Calibration curve of FeSO4.7H2O for FRAP Assay

A calibration curve was prepared, using FeSO4.7H2O aqueous solution (200, 400, 600, 800 and 1000 μM, R=0.997). A stock solution for FeSO4.7H2O was prepared by mixing 0.27802 g of FeSO4.7H2O in 100 ml volumetric flask that contained distilled water. The dilutions for every concentration were performed. Then, the absorbance was determined at 593nm against blank prepared using distilled water by using UV visible spectrometer. The graph of Optical Density (abs) against Concentration of FeSO4.7H2O (μM) was plotted.

3.7.1.2 FRAP Assay

FRAP assay was performed by mixing 40μL guava extracts with 3mL FRAP reagent. Then the mixture was incubated at 37 ºC for 4 min. Then, the absorbance was determined at 593nm against blank prepared using distilled water by using UV visible spectrometer. FRAP reagent was always fresh prepared by mixing 2.5 ml of a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with 2.5 ml of 20 mM FeCl3.6H2O and 25 ml of 0.3 M acetate buffer (glacial acetic acid 16 ml, sodium acetate trihydrate 3.1g and distilled water 16ml) at pH 3.6 with ratio 1:1:10 respectively.

3.7.2 Total Phenolic Content

3.7.2.1 Preparation Standard Calibration Curve of Gallic Acid for Folin- Ciocalteu Assay

A calibration curve using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l, R=0.998) was prepared. A stock solution for gallic acid was prepared by mixing 0.15 g of gallic acid in 250 ml volumetric flask that contained distilled water. The dilutions for every concentration were performed. Then, the absorbance was determined at 765 nm against blank prepared using distilled water by using UV visible spectrometer. The graph of Optical Density (abs) against Concentration of Gallic acid (mg/L) was plotted.

3.7.2.2 Folin- Ciocalteu Assay

Total phenolic contents (TP) of the extracts were determined using Folin Ciocalteu assay. 40 µl extract solution was mixed with 1.8 ml of FC reagent which was pre-diluted, 10 times, with distilled water before using it. After the reaction standing for 5 min at room temperature, 1.2 ml of (7.5% w/v) sodium carbonate solution was added. Then, the solutions were allowed to stand at room temperature for 1 hour. Then, UV visible spectrophotometer was used to measure the absorbance at 765 nm.

3.7.3 Analysis of Mineral Content (Magnesium (Mg), Calcium (Ca) and Sodium (Na) by using AAS

The purify sample was analyzed by using Atomic Absorption Chromatography (AAS) to obtain the Magnesium (Mg), Calcium (Ca) and Sodium (Na) content in guava. The 20 ml sample was filtered by using vacuum pump through 0.45µm nylon membrane filter/glass fibre filter. The stock solution of 30 mg/L was prepared for each element. Ultrapure water was used for dilution to prepare standard working curve (0, 1, 5, 10, 30 mg/l) for each element. A small amount of nitric acid was added to preserve the solution.

3.7.4 Analysis of Ascorbic Acid by using HPLC

3.7.4.1 Preparation of mobile phase potassium dihydrogen phosphate (K_2HPO_4)

25mM potassium dihydrogen phosphate (K_2HPO_4) with pH 3.5 with phosphoric acid was the mobile phase. This solution was prepared by mixing 2.17725g of K_2HPO_4 in 500ml ultra pure water. The K_2HPO_4 solution and 800ml of ultrapure water was filtered by using vacuum pump through 0.45µm nylon membrane filter/glass fibre filter.

3.7.4.2 Analysis of ascorbic acid by using HPLC

The purify sample was analyzed by using High Performance Liquid Chromatography in order to prove the existence of L-ascorbic acid in the sample. The HPLC properties for L-ascorbic acid were first determined. A calibration curve was prepared, using a standard solution of L-ascorbic acid (2.5, 5.0, 10.0, 15.0 and 20.0 µg/ml). L-ascorbic acid stock solution of 1000 µg/ml was prepared and was diluted to obtain each of the desired concentration for standard working curve.

CHAPTER 4

RESULT & DISCUSSION

4.1 Introduction

In this chapter, the results and discussions were divided into three main sections. First section is the result and discussion for parameters that affect the efficiency of the extraction of antioxidant activity and phenolic content in guava peels. The second section is the result and discussion of mineral contents in the guava peels after the optimum condition from all parameters was obtained. Lastly, the result and discussion to prove the existence of the ascorbic acid in the guava peels.

4.2 Effect of types of solvent extraction on extraction of antioxidant activity and phenolic content

Effect of type of solvent extraction was determined to discover the most effective solvent to extract antioxidant activity and phenolic content in guava peels. Types of solvent used were polar protic solvent which were methanol and ethanol, polar aprotic which were acetone and ethyl acetate.

4.2.1 Result for type of solvent extraction on extraction of antioxidant activity and phenolic content

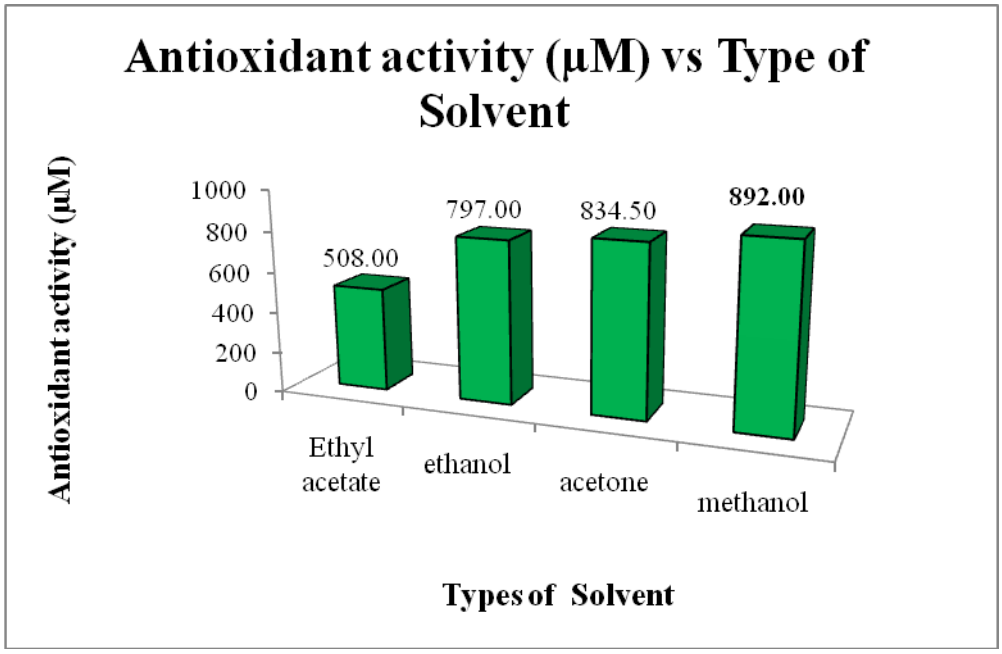

Figure 4.1 Effect of types of solvent extraction on extraction of antioxidant activity in peel of guava

Figure 4.1 shows the antioxidant activity in the peel of guava that was extracted by several types of solvents. Each sample was extracted at 50 ºC for 120 minutes. This initial extraction condition was obtained by referring from previous study. The antioxidant activity obtained for different type of solvent was 508.0, 797.0, 834.5 and 892.0 µM. The highest value of antioxidant activity obtained in this study was 892.0 µM by using methanol. The solvent extraction that gave the lowest value for antioxidant activity 508 µM was ethyl acetate.

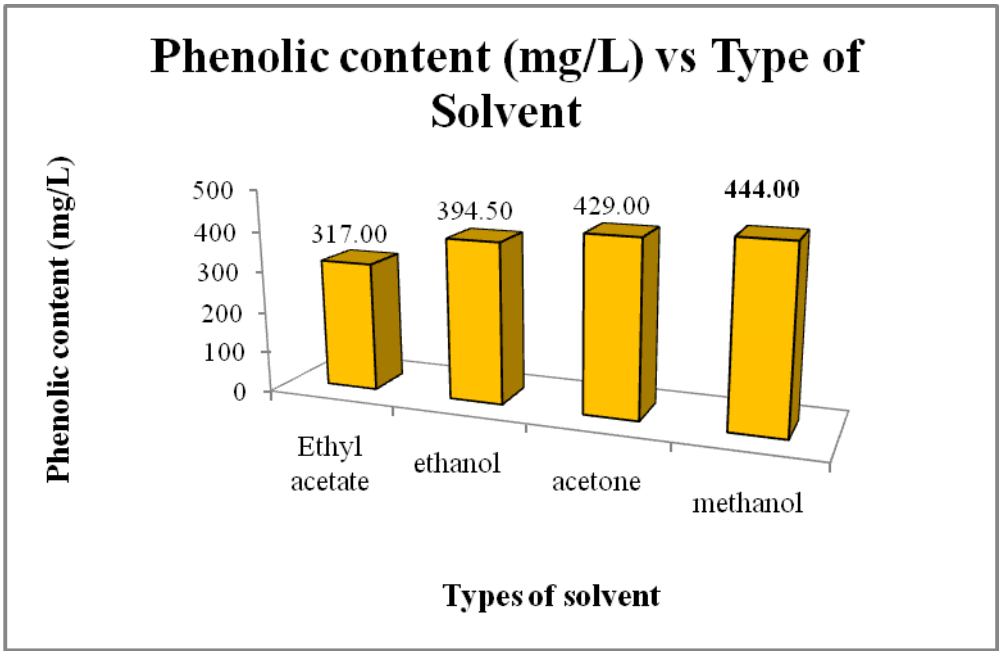

Figure 4.2 Effect of types of solvent extraction towards extraction of phenolic content in peel of guava

Figure 4.2 demonstrates the phenolic content in peel of guava for different type of solvent extraction. Each sample was extracted at 50 rpm and 50 ºC for 120 minutes in different types of solvent. The phenolic content obtained from 0.5g of peel in 50ml of different types of solvent was 317.0, 394.5, 429.0 and 444.0 mg/L for ethyl acetate, ethanol, acetone and methanol respectively. The highest phenolic content was 444.0 mg/L that was obtained by using methanol as the extraction solvent followed by acetone and ethanol. This is agreed with the study by Siddhuraju and Becker, (2003) that methanol was the most effective solvent for extraction of antioxidant in Drumstick Tree (Moringa oleifera Lam.) leaves. The ethyl acetate was the weakest solvent extraction for phenolic content compared to all the solvent used in this research that only yielded 317 mg/L phenolic content.

4.2.2 Discussion of effect on types of solvent extraction

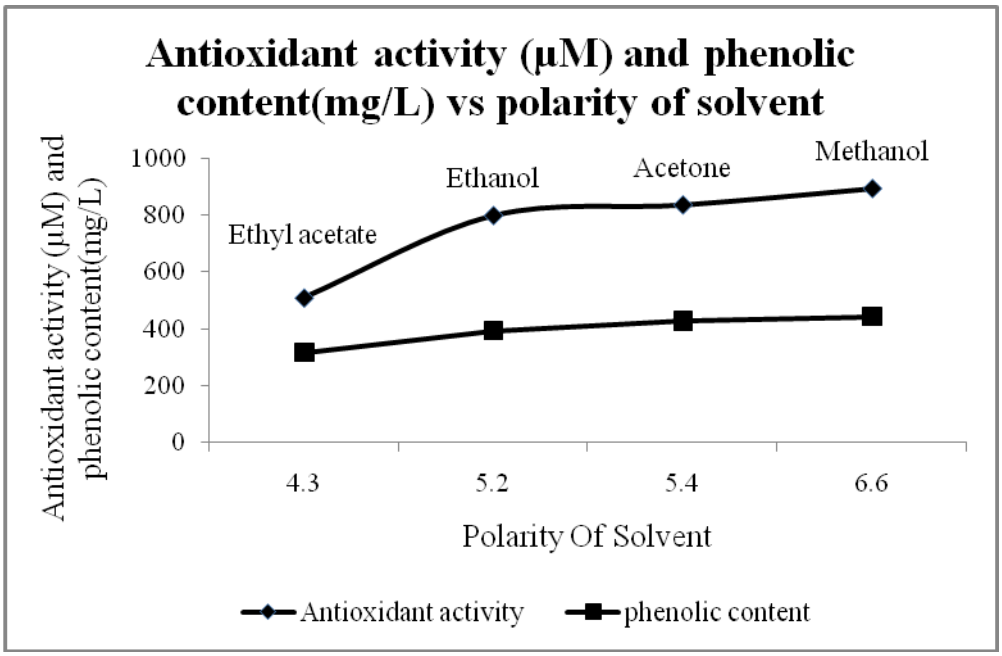

Figure 4.3 Effect of polarity of solvents with the extraction of antioxidant activity and phenolic content in peel of guava

Different types of solvent caused different amount of antioxidant activity and phenolic content that were extracted. Figure 4.3 illustrates the relationship between the polarity of solvent with the extraction of antioxidant activity and phenolic content in peel of guava. It was observed that, as the polarity of solvent increased the extraction of antioxidant activity and phenolic content also increased. From the result obtained for antioxidant activity and phenolic content, methanol was the best solvent for extraction; this is because methanol has the highest value of polarity compared with the other solvents.

Antioxidants composed of hydrophilic and lipophilic antioxidant. Ascorbic acid and phenolic are the example of hydrophilic antioxidant and caretonoids are the lipophilic antioxidants. Thaipong *et al*, (2005) affirms that hydrophilic antioxidants such as phenolic and ascorbic acid are the major contributors to the antioxidant activity in peel of guava. Hydrophilic has tendency to interact with polar molecules such as methanol since polar molecule dissolved in polar solvent. Therefore, the solvent that has higher polarity will have greater tendency to interact with hydrophilic antioxidant. In addition, high polarity increased the solubility of phenolic content and antioxidant contents in the solvent. With

the increased of solubility of phenolic content and antioxidant content, the antioxidant activity has also increased.

The solvent polarity has effects with the efficiency of the extraction of antioxidant activity and phenolic content was also agreed by Thaipong *et al*, (2009) and Alothman *et al.*, (2009) that states that the solvent polarity will play role in increasing the phenolic solubility.

4.3 Effect of different solvent ratio on extraction of antioxidant activity and phenolic content

Effect of different solvent ratio was investigated to obtain the most effective solvent ratio to extract antioxidant activity and phenolic content in guava peel. Furthermore, the effectiveness of absolute and aqueous each of the solvent was investigated. The solvent ratios that were studied were 0, 20, 40, 60, 80 and 100 %.

4.3.1 Result of different solvent ratio on extraction of antioxidant activity and phenolic content

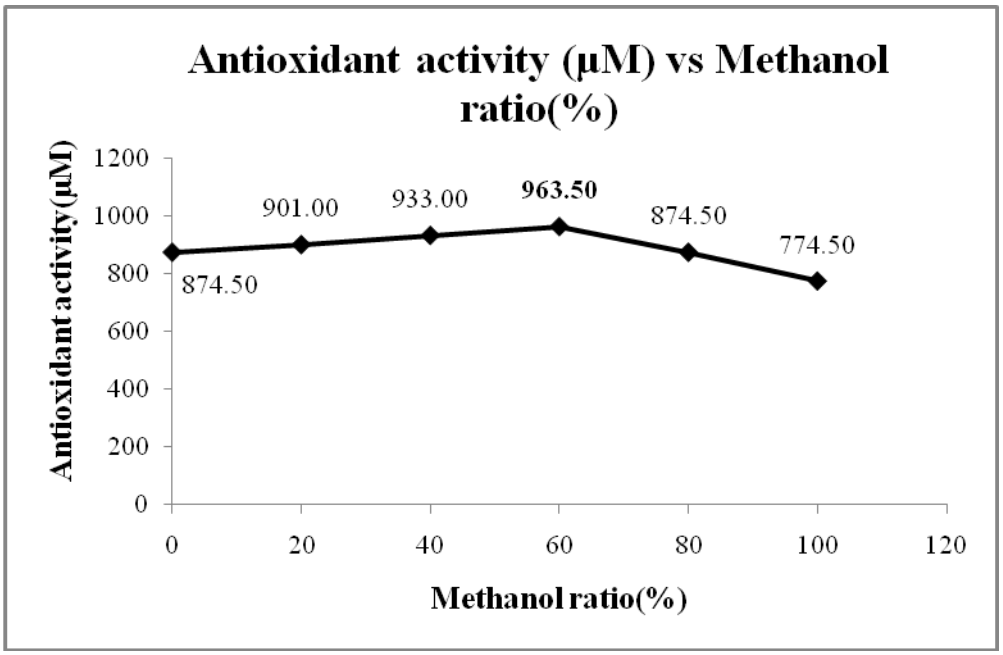

Figure 4.4 Effect of different solvent ratio on extraction of antioxidant activity

Figure 4.4 illustrates the result of antioxidant activity in different ratio of methanol used as extraction solvent. The antioxidant activity obtained was 874.5, 901.0, 933.0, 963.5, 874.5 and 774.5 µM. The antioxidant activity was increased progressively from 0 until 60% ratio and decreased gradually after that. The ratio of methanol that gave the highest antioxidant activity was at ratio of 60 % that obtained 963.5 µM of antioxidant activity. The lowest antioxidant activity was extracted by methanol with 100% ratio that yielded only 774.5 µM of antioxidant activity.

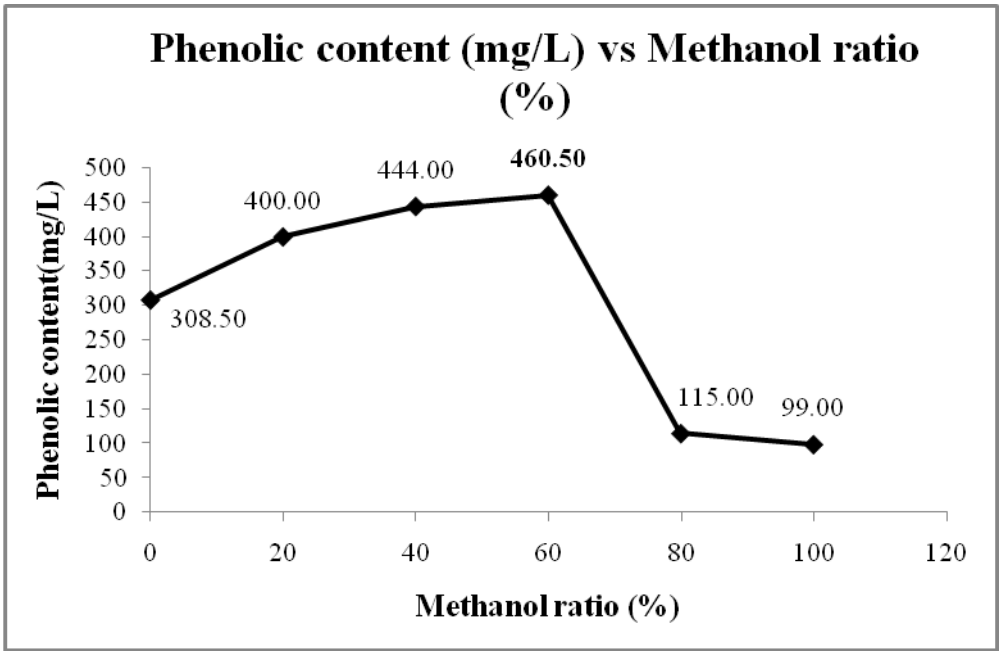

Figure 4.5 Effect of different methanol ratio on extraction of phenolic content in guava peel

Figure 4.5 shows the result of phenolic content in different ratio of methanol used as extraction solvent. The phenolic content obtained from the extraction was 308.5, 400.0, 444.0, 460.5, 115.0 and 99.0 mg/L. The phenolic content was increased steadily with the increased of methanol ratio from 0 until 60% and decreased rapidly from 60 to 80% and remained decreased until 100 %. The highest yield of phenolic content was at ratio of 60% that yielded 460.5 mg/L phenolic content. At ratio 100%, only 99 mg/L of phenolic content obtained, which was the lowest value compared with the other ratio.

4.3.2 Discussion

Based on the result obtained for effect of different solvent ratio onextraction of antioxidant activity and phenolic content, methanol at ratio 60% was the best ratio for extraction. Spigno *et al*, (2007) affirms that the mixtures of alcohols and water have revealed to be more efficient in extracting phenolic content constituents than the mono-component solvent system. This is due to the increasing of polarity of the solvent system that increases the polar interactions between antioxidant activity and phenolic content with solvent system. The aqueous methanol mixture enhanced the solubility of antioxidant

activity and phenolic content in the solvent system compared with the absolute solvent. Hence, the yield of extraction was increased.

Therefore, the extraction of phenolic content and antioxidant activity was increased with the increased of the ratio of methanol. Moreover, Thoo *et al,* (2010) states that the 100% ethanol (mono-component solvent) does not contribute to extract water soluble compound such as phenolic compounds. Therefore in this study, for mono-component solvent system which was methanol at ratio of 100% gave the lowest extraction of antioxidant activity and phenolic content due to its low polarity compared with the other methanol ratio.

4.4 Effect of different extraction time on extraction of antioxidant activity and phenolic content

Effect of different extraction time was investigated to obtain the most effective extraction time in order to extract antioxidant activity and phenolic content in guava peel. The extraction times that were investigated were at 20, 40, 60, 80, 100, 120, 150 and 180 minutes.

4.4.1 Result for effect of different extraction time on extraction of antioxidant activity and phenolic content

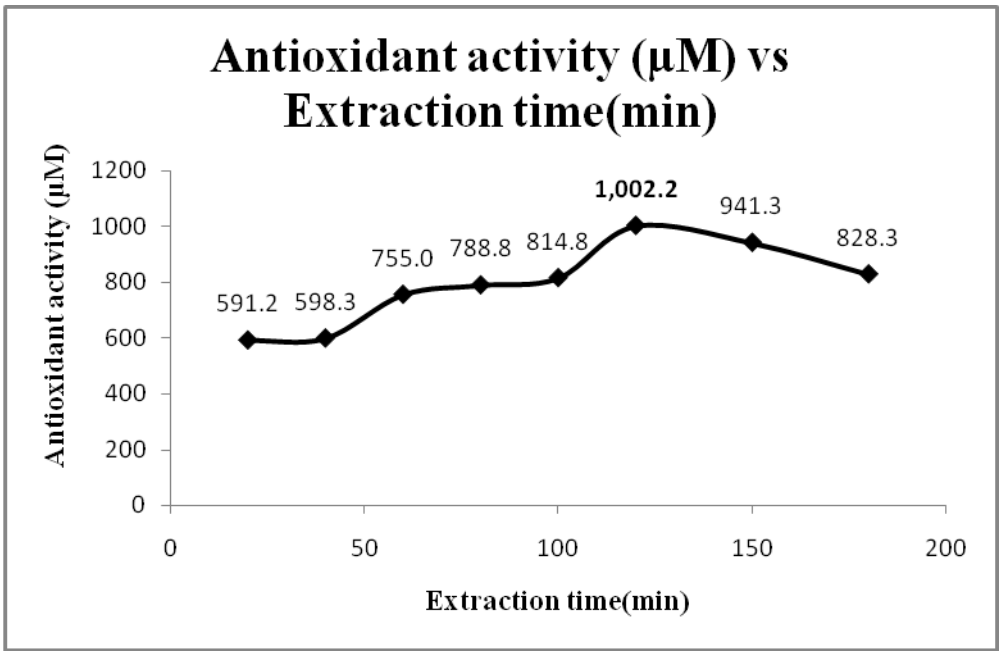

Figure 4.6 Effect of different extraction time on extraction of antioxidant activity in guava peel

Figure 4.6 above illustrates the effect of various extraction times for extraction of antioxidant activity and phenolic content in peel of guava. The sample was extract by using 60% aqueous methanol at 50 ºC. The antioxidant activity obtained from the extraction was 591.0, 598.3, 755.0,788.8, 814.8, 1002.2, 941.3 and 828.3 µM. The antioxidant activity was slightly increased at extraction time 20 to 40 minutes and progressively increased from 50 to 60 minutes, then continued to increase until reached 120 minutes. Afterwards, the extraction of antioxidant activity was decreased gradually. At time 120 minutes, the extraction of antioxidant activity which was 1002.2 µM reached the highest yield and at time 20 minutes, the extraction of antioxidant activity which was 591.2 µM was the lowest result.

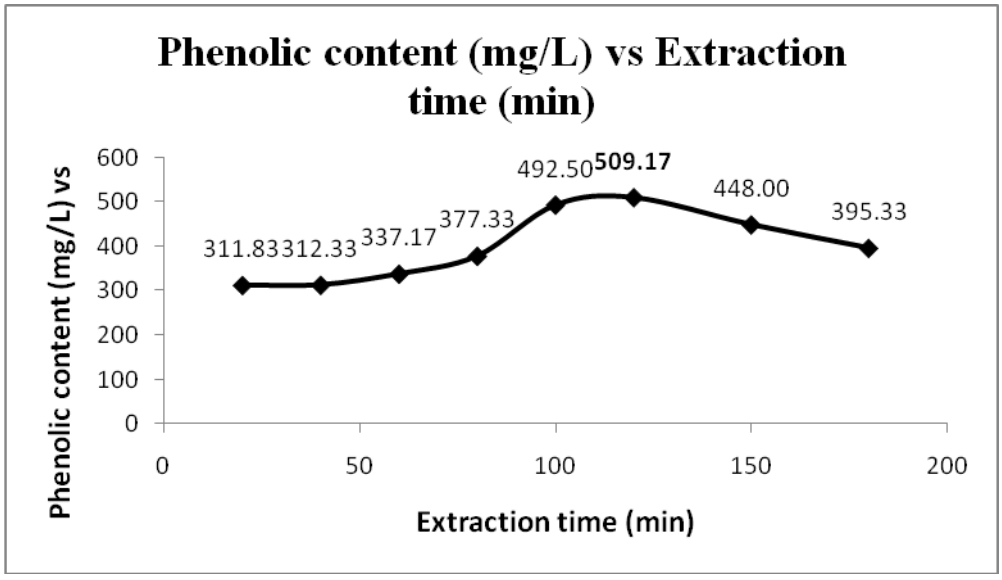

Figure 4.7 Effect of different extraction time towards extraction of phenolic content in guava peel

The result for effect of extraction time towards phenolic content shared a similar trend with the result for antioxidant activity. From Figure 4.7, the value of phenolic content was increased with extraction time of 0 to 120 minutes. Afterwards, the value of phenolic content was decreased towards extraction time. The highest value of phenolic content was 509 mg/L at extraction time of 120 minutes. The lowest value of phenolic content was 311.8 mg/L at extraction time of 20 minutes.

4.4.2 Discussion

Based on the result obtained for effect of extraction time towards extraction of antioxidant activity and phenolic content, the best extraction time was at time 120 minutes. The extraction of antioxidant activity and phenolic content was increased from 20 to 120 minutes. After 120 minutes, the extraction of antioxidant activity and phenolic content was decreased. From time between 20 until 120 minutes, the solvent not yet reach the equilibrium state with the antioxidant and phenolic content.

Therefore, not all the phenolic content and antioxidant were fully solubilised in the solvent. Thus, the phenolic content and antioxidant activity were gradually increasing until 120 minutes. This is because 120 minutes is the time needed to reach equilibrium state between the solute in the sample and the solvent.

Previous study states that extraction time is crucial in solvent extraction for phenolic compound, where phenolic compound may be governed by the equilibrium state of concentrations (Thoo *et al.*, 2010). Hence, the excess extraction time certainly reduced the yield of phenolic compounds and caused the extraction of antioxidant activity and phenolic content decreased when extraction time was longer than 120 minutes.

4.5 Effect of different extraction temperature on extraction of antioxidant activity and phenolic content

Effect of different extraction temperature on extraction of antioxidant activity and phenolic content was determined to obtain the most effective extraction temperature. Extraction temperature had effect on the depletion of antioxidant contents and also affected the antioxidant activity. The extraction temperatures investigated were at temperature of 25, 35, 45, 55 and 65 °C.

4.5.1 Result for effect of different extraction temperature on extraction of antioxidant activity and phenolic content

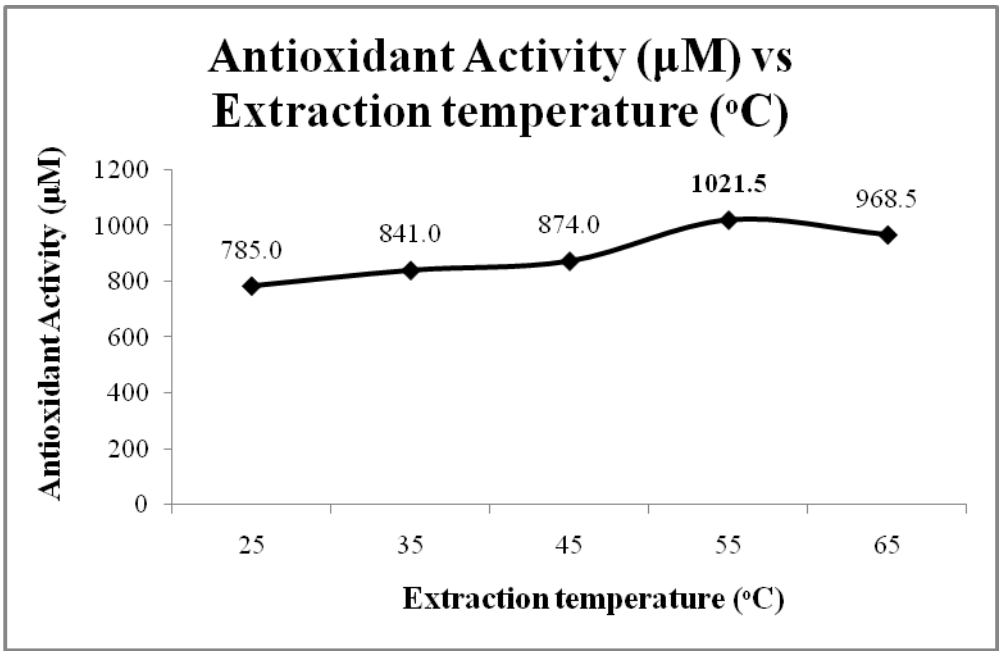

Figure 4.8 Graph of Antioxidant Activity (μM) against extraction temperature (°C)

From Figure 4.8, the value of antioxidant activity was increased from temperature of 25 ºC until 55 ºC. Afterwards, the value of antioxidant activity was decreased. The antioxidant activity obtained 785.0, 841.0, 874.0, 1021.5 and 968.5 μM. The highest value of antioxidant activity was 1021.5 μM at extraction temperature of 55 ºC. At extraction temperature of 25 ºC, the result obtained was 785.0 μM, which was the lowest value of antioxidant activity.

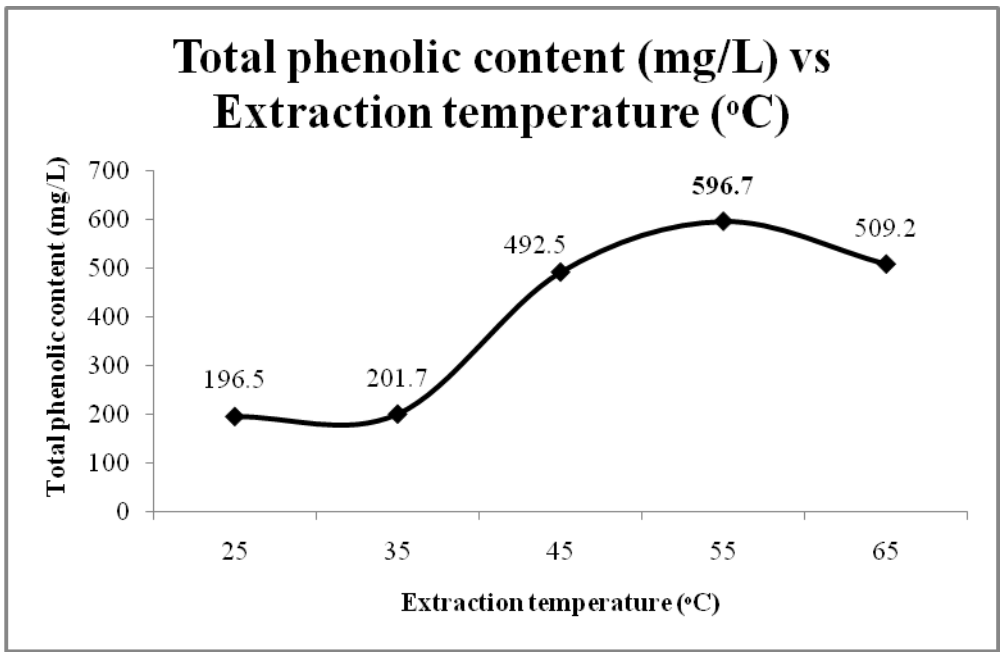

Figure 4.9 Graph of total phenolic content (mg/L) against extraction temperature (ºC)

The result for effect of extraction temperature towards phenolic content shared a similar trend with the result for antioxidant activity. From Figure 4.9, the value of phenolic content was slightly increased at temperature of 25 ºC until 35 ºC. After that, the value of phenolic content was rapidly increased until readecreased. The phenolic content obtained was 196.5, 201.7, 492.5, 596.7 and 509.2 mg/L. The highest value of phenolic content was 596.7 mg/L at extraction temperature of 55 ºC. The lowest value of phenolic content was 196.5 mg/L at extraction temperature of 25 ºC.

4.5.2 Discussion

Based on the result obtained for effect of extraction temperature towards extraction of antioxidant activity and phenolic content, the best extraction temperature was at 55 ºC. From 25 to 55 ºC, the extraction of antioxidant activity and phenolic content was increased and was decreased at temperature 65 ºC. Extraction temperature was very crucial in enhancing the efficiency of the extraction, because heat can boost the solubility of phenolic contents and antioxidant activity. Temperature at 55 ºC provides moderate heat to the extraction, hence, yielded the highest value compared with the other extraction temperature.

This is supported by Thoo *et al,* (2010) that studies that heat has been found to enhance the recovery of phenolic compounds. Increased of temperature promotes solvent extraction by enhancing both diffusion coefficients and the solubility of polyphenols content.

At temperature 65 ºC, the phenolic contents and other antioxidant compounds were denatured due to excess heat during the extraction. As the phenolic contents and other antioxidant compounds reduced, therefore the antioxidant activity was also reduced. This was agreed by Spigno *et al.,* (2007) that states that the increased in working temperature favours extraction enhancing both the solubility of solute and the diffusion coefficient, but also that beyond a certain value of phenolic compounds can be denatured. In addition, Montelongo *et al.,* (2010) affirms that high temperature bioactives can react with other components of plant material, thus impeding extraction.

4.6 Mineral contents

Mineral contents that were investigated in this research were calcium (Ca), magnesium (Mg) and sodium (Na). They were determined by using Atomic Absorption Spectrometer.

4.6.1 Amount of Ca, Mg and Na contents in guava peels

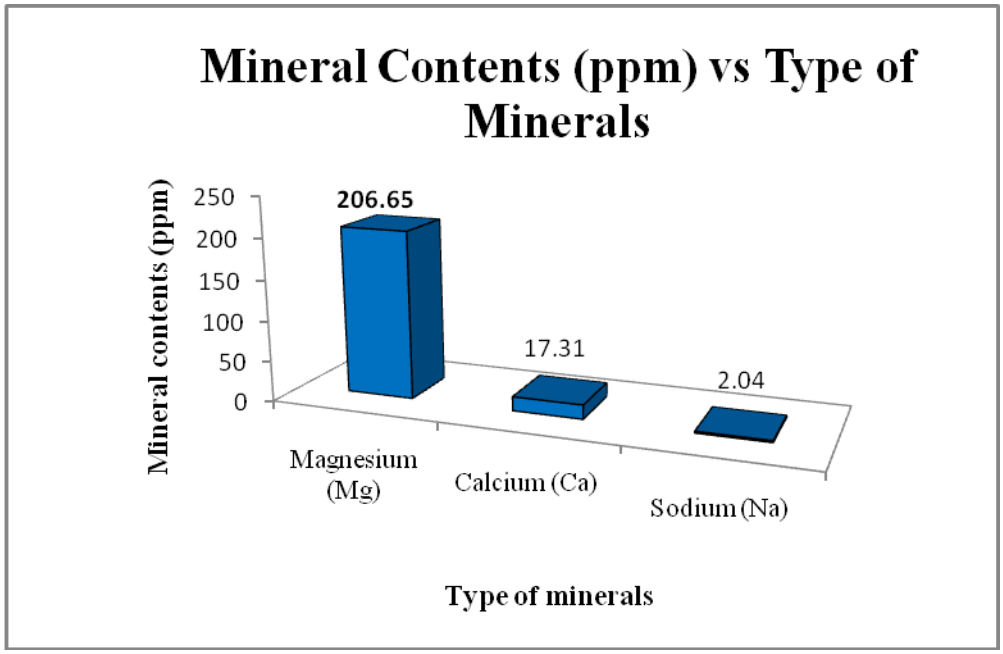

Figure 4.10 Graph of mineral contents against type of minerals

The results for each of mineral contents were shown below in figure 4.10. The highest concentration value of minerals was magnesium that has concentration of 206.65 ppm, compared with calcium and sodium that have values of 17.31 and 2.04 ppm respectively.

Mg, Ca and Na were the macro minerals that were presented in the peel of guava. After analyzed the extracts by using AAS, it was proven that peel of guava contained minerals such as Mg, Na and Ca rather than other antioxidant compound such as ascorbic acid. The accessible of these minerals in the peel of guava shows that peel of guava have high source of important nutrients.

4.7 Determination of ascorbic acid in extracts

Ascorbic acid was determined by using HPLC. The purpose to determine the ascorbic acid is to verify the antioxidant compound in the peels of guava.

4.7.1 Amount of ascorbic acid in extracts

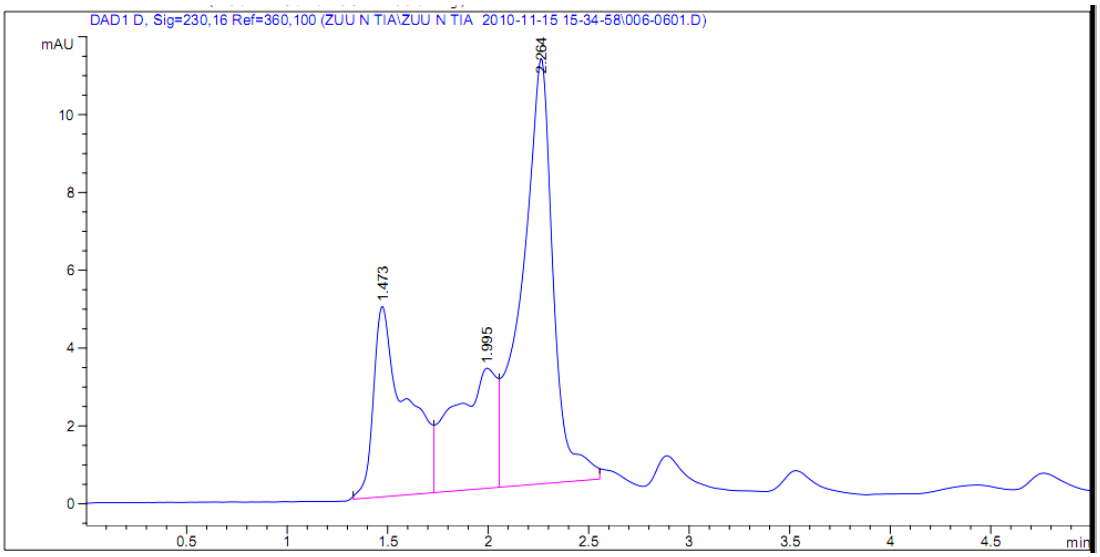

Figure 4.11 Graph of area (mAU.s) against time (s)

There was significant amount of ascorbic acid in the guava peels that was determined by using HPLC. Figure 4.11 shows that, ascorbic acid was the highest peak among the others in the sample. It was detected at time 2.264 minutes and from table B1.6 the area of the peak was 118.5939 mAU.s. After doing calculation, the amount of ascorbic acid in 0.1g guava peels was 95.5926 ng/ µl. The calculation to determine the amount of ascorbic acid was shown below:

$$Y = mX + c$$

Where:

Y = Area

X = Amount of ascorbic acid

m = gradient of L-ascorbic acid standard calibration curves

c = y axis intercept of L-ascorbic acid standard calibration curves

X = (118.59390 + 15.37246) ÷ 1.40143

= 95.5926 ng/µl ascorbic acid

4.7.2 Discussion

Ascorbic acid is one of the natural antioxidant compounds exists in fruits including guava. This statement was agreed by Thaipong *et al.*, (2006) that affirms guava contains a high level of ascorbic acid (50-300 mg/100 g fresh weight), which is six times higher than oranges. Moreover, Lim, Lim and Tee (2007) states that, the most abundant antioxidants in fruits are polyphenols and Vitamin C, Vitamins A, B and E and carotenoids.

After analyzed the sample by using HPLC, significant amount of ascorbic acid in the extracts was traced. Hence, the existence of ascorbic acid in guava peels proved that the antioxidants were exist in the peels and the activity that investigated earlier was antioxidant activity.

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

The extraction of antioxidant activity, phenolic content and minerals content in guava peel was investigated through this study The extraction of antioxidant activity, phenolic content and mineral contents was conducted by investigates the effects of types of solvent, solvent ratio, extraction time and extraction temperature to the performance of the extraction process.

It was found that the most optimum condition for extraction was by using methanol with 60% solvent ratio at temperature 55 ºC for 120 minutes. This optimum condition gave the highest yields of antioxidant activity and phenolic content which were 1021.00 μmol/L and 596.67mg/L respectively. After obtaining the optimum extraction condition, it was also discovered that peel of guava contained minerals which were 206.65, 17.31, 2.04 ppm of Mg, Ca, Na respectively. From analysis of HPLC, it was determined that there were significant amount of ascorbic acid in the extracts. Hence, it was confirmed the existence of antioxidants in the peels and the activity that investigated was antioxidant activity.

5.2 Recommendation

There are several recommendations that can be considered for improvement in further research. In this research, the method of extraction used was solvent batch extraction in water bath. There are other methods of extraction that can give higher yield such as Soxhlet extraction and microwave assisted extraction. Soxhlet extraction will give higher yield because; the sample will be wash repeatedly with solvents. The microwave-assisted extraction also will be given higher yield by using microwave energy to heat solvents and samples to increase the mass transfer rate of solutes from sample matrix into the solvents. (Duverney *et al.*, 2005)

Antioxidants consist of hydrophilic and lipophilic antioxidants. For further research, the comparative study on antioxidant activity should be performing in both types of these antioxidants. This is significant as to determine which one will contribute the higher antioxidant activity in the peel of guava.

In this research, antioxidant activity, mineral contents and phenolic content of guava peel was studied. There are other fractions of this fruit that have potentially abundant of antioxidants and minerals for instance leaf and seed. These fractions also considered as by product of guava so it can contribute to waste to wealth application.

Extraction time is important to be optimised in order to minimise energy cost. The range of extraction time should be expanded in order to study the effect of time consuming to the efficiency of the extraction.

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APPENDIX A

PROPERTIES OF SOLVENTS

Table A1.1 Polarity index for different type of solvent

Type of solvent	Type of polar	Polarity Index
Ethyl Acetate	aprotic	4.3
Ethanol	protic	5.2
Acetone	aprotic	5.4
Methanol	protic	6.6
Water	protic	9.0

APPENDIX B

RESEARCH DATA

Table B1.1 Result of the effect of types of solvent extraction in extraction of antioxidant activity and phenolic content

Type of solvent	Antioxidant activity (µM)	Phenolic content (mg/L)
Ethyl acetate	508.00	317.00
Ethanol	797.00	394.50
Acetone	834.50	429.00
Methanol	892.00	444.00

Table B1.2 Result of the effect of different solvent ratio in extraction of antioxidant activity and phenolic content

Methanol/water ratio(%)	Antioxidant activity (µM)	Phenolic content (mg/L)
0	874.50	308.50
20	901.00	400.00
40	933.00	444.00
60	963.50	460.50
80	874.50	115.00
100	774.50	99.00

Table B1.3 Result of the effect of different extraction time in extraction of antioxidant activity and phenolic content

Extraction time (min):	Antioxidant activity (µM)	Phenolic content (mg/L)
20	598.33	311.83
40	591.17	312.33
60	755.00	337.17
80	788.83	377.33

100	814.83	429.00
120	1002.17	509.00
150	941.33	448.00
180	828.33	395.33

Table B1.4 Result of the effect of different extraction temperature in extraction of antioxidant activity and phenolic content

Extraction temperature (°C):	**Antioxidant activity (µM)**	**Phenolic content (mg/L)**
25	785.00	196.00
35	841.00	201.00
45	874.00	492.50
55	1021.00	596.67
65	968.00	509.17

Table B1.5 Result of Ca, Mg and Na concentration in guava peels

Mineral	Concentration (ppm)
Calcium (Ca)	17.31
Magnesium (Mg)	206.65
Sodium (Na)	2.04

Table B1.6 Result of ascorbic acid by using HPLC

Peak number	Retention time (s)	Peak area (mAU.s)
1	88.38	55.54211
2	119.70	46.18456
3	135.84	118.59390

Table of Each Element 11/25/2010 3:37 PM

Analysis Mode : Flame/Manual
Analysis Name : Standard analisys
Comment :
Description : Determination of Sodium (Na) and calcium(Ca) in fruits peel.

Meas. Date : 11/25/2010 3:36 PM
Element : Ca STD Unit : ppm UNK Unit :

Sample ID	Sample Name	Conc.	RSD(%)	ABS	REF
STD1		0.00	0.00	-0.0007	-0.0394
STD2		1.00	0.00	0.0537	-0.0507
STD3		5.00	0.00	0.1181	-0.0341
STD4		10.00	0.00	0.1664	-0.0137
STD5		30.00	0.00	0.4086	0.0076
UNK-001	Guava Peel	17.31	0.00	0.2528	0.0000
UNK-002	banana peel	3.21	0.00	0.0727	-0.0079

Coefficient : K3=--
K2=--
K1=1.277717E-002
K0=3.167001E-002
Corr.Coef. : 0.9913

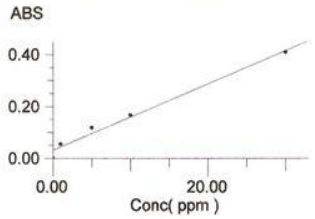


Figure B1.1 Amount of Calcium in guava peel by using AAS

Table of Each Element 11/25/2010 3:45 PM

Analysis Mode : Flame/Manual
Analysis Name : Standard analisys
Comment :
Description : Determination of Sodium (Na) and calcium(Ca) in fruits peel.

Meas. Date : 11/25/2010 3:45 PM
Element : Na STD Unit : ppm UNK Unit :

Sample ID	Sample Name	Conc.	RSD(%)	ABS	REF
STD1		0.00	0.00	0.0083	-0.0050
STD2		5.00	0.00	0.9412	0.1439
STD3		10.00	0.00	1.0920	0.2745
UNK-001	Guava Peel	2.04	0.00	0.3592	0.0354
UNK-002	Banana Peel	0.85	0.00	0.2311	0.0150

Coefficient : K3=--
K2=--
K1=1.083700E-001
K0=1.386500E-001
Corr.Coef. : 0.9231

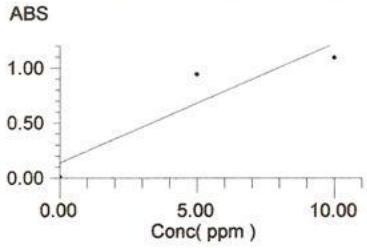


Figure B1.2 Amount of Sodium in guava peel by using AAS

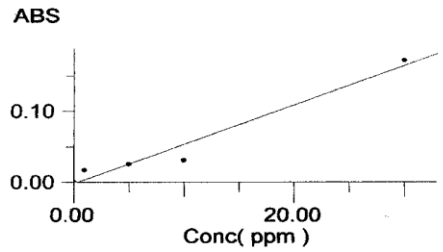
Table of Each Element 11/19/2010 10:57 AM

Analysis Mode : Flame/Manual
Analysis Name : Standard analisys
Comment :
Description : To determine Potassium(K), Sodium(Na) and Magnesium(Mg) in Fruits peel

Meas. Date : 11/19/2010 10:56 AM
Element : Mg STD Unit : ppm UNK Unit :

Sample ID	Sample Name	Conc.	RSD(%)	ABS	REF
STD1		0.00	0.00	0.0000	0.0020
STD2		1.00	0.00	0.0169	0.0065
STD3		5.00	0.00	0.0256	0.0106
STD4		10.00	0.00	0.0309	0.0133
STD5		30.00	0.00	0.1714	1.6881

Coefficient : K3=--
K2=--
K1=5.547014E-003
K0=-2.072528E-003
Corr.Coef. : 0.9806



UNK-001		206.65	0.00	1.1442	0.4052

Figure B1.3 Amount of Magnesium in guava peel by using AAS

APPENDIX C

STANDARD CALIBRATION CURVES

Table C1.1 Standard calibration curves of $FeSO_4.7H_2O$ for FRAP assay

Concentration (μM)	Optical Density(abs)
200	0.694
400	1.218
600	1.650
800	2.041
1000	2.468

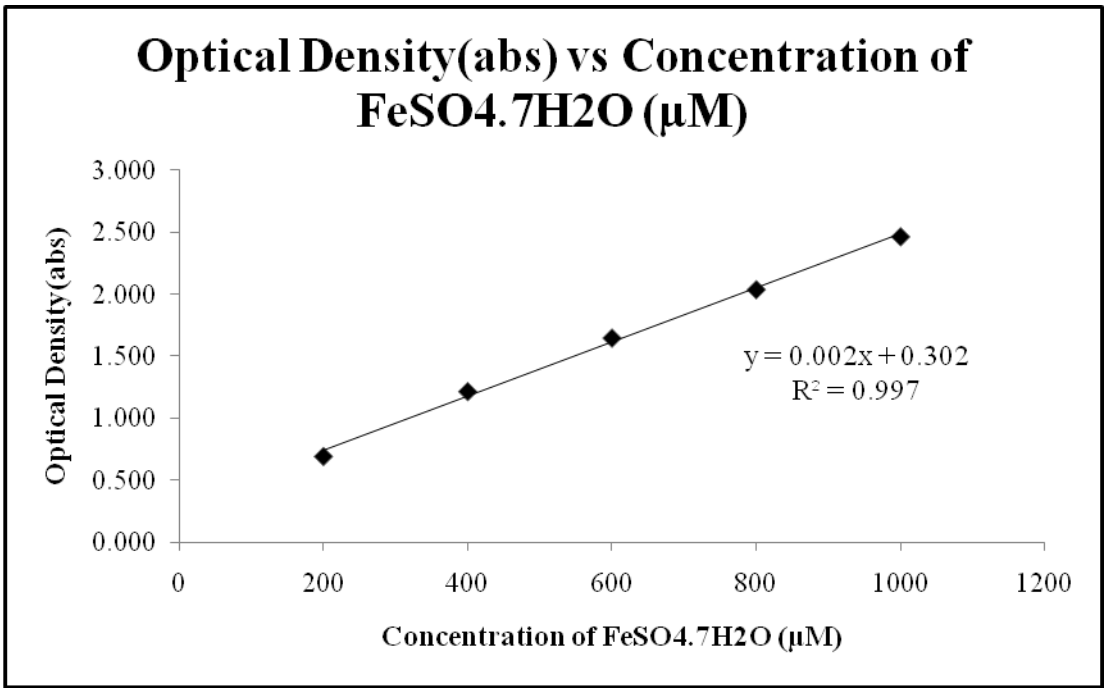


Figure C1.1 Graph of standard calibration curves of $FeSO_4.7H_2O$ for FRAP assay

Table C1.2 Standard calibration curves of gallic acid for Folin Ciocalteu Assay

Concentration (mg/L)	Optical density(Abs)
100	0.123
200	0.337
300	0.581
400	0.85
500	1.071
600	1.345

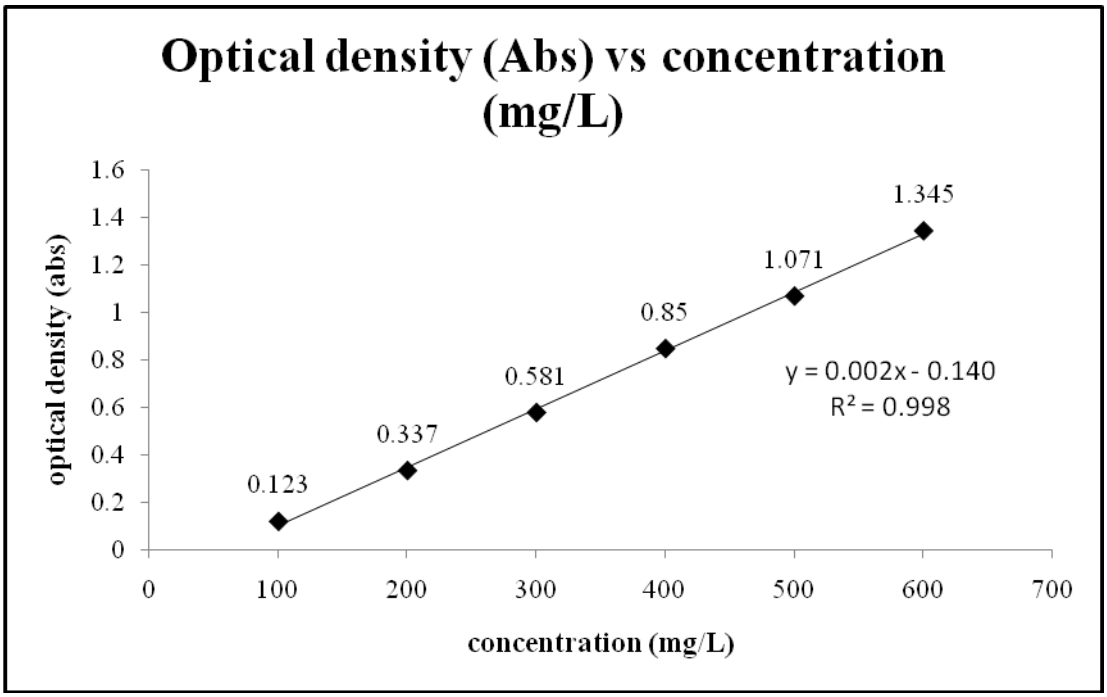


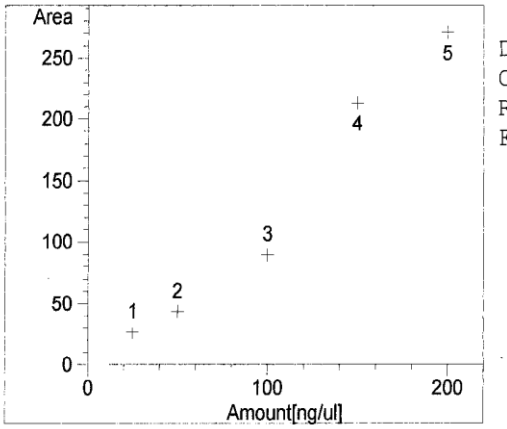
Figure C1.2 Graph of standard calibration curves of gallic acid for FC Assay

Table C1.3 Standard Calibration curve of L-Ascorbic Acid for determination of ascorbic acid by using HPLC

Amount (ng/µl)	Area	Amount/area
25	26.86117	9.30712×10^{-1}
50	43.23739	1.15641
100	89.52229	1.11704
150	213.04228	7.04086×10^{-1}
200	270.85394	7.38405×10^{-1}

Method C:\CHEM32\1\DATA\ZUU N TIA\ZUU N TIA 2010-11-15 15-34-58\ZUU N TIA.M

Calibration Curves



at exp. RT: 2.309
DAD1 D, Sig=230,16 Ref=360,100
Correlation: 0.98305
Residual Std. Dev.: 22.51926
Formula: y = mx + b
m: 1.40143
b: -15.37246
x: Amount
y: Area

Figure C1.3 Graph of Standard Calibration curve of L-Ascorbic Acid for determination of ascorbic acid by using HPLC