SOXHLET EXTRACTION OF ASCORBIC ACID FROM GUAVA

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I declare that this thesis entitled "Soxhlet extraction of ascorbic acid from guava" 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."

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ABSTRACT

Natural antioxidant has gained interest among consumers and the scientific community due to their benefits as inhibitor cardiovascular disease and cancer. In this research, the extraction of antioxidants from guava with soxhlet extraction process has been studied. The effect of drying temperature, solvent polarity, and particle sizes of guava on extraction yields were investigated. Analysis of ascorbic acid content was performed using HPLC. The drying temperatures used were 32°C, 50°C, 60°C and 70°C. The solvents utilized were methanol, ethanol, hexane and pentane. In terms of particle, it was set at 1.0 mm, 3.0 mm, 5.0 mm and 10.0 mm. The results showed that the drying temperature at 50°C gave the highest yield of ascorbic acid which is 38.353mg/l, while using methanol as solvent yield 34.123 mg/l. It indicates that the relation between solvent polarities and yield of ascorbic acid were directly proportional. The particle size of guava was inversely proportional with the extraction yield of ascorbic acid where using 1.0 mm particle size gave 23.546 mg/l amount of ascorbic acid. This study indicated that natural antioxidant from guava has a big potential as an alternative source of antioxidant in order to replace the synthetic antioxidant in current market.

ABSTRACT

Antioksida semulajadi telah menarik perhatian di kalangan pengguna dan ahli-ahli sains kerana kebaikannya sebagai penghalang penyakit kardiovaskular dan kanser. Dalam kajian ini, pengekstrakan antioksida semulajadi daripada jambu batu dengan menggunakan pengekstrakan soxhlet telah dijalankan. Kesan perbezaan suhu pengeringan, kepolaran pelarut dan saiz partikel terhadap jambu batu melalui hasil pengekstrakan telah dikaji. Analisa asid askorbik telah dijalankankan menggunakan HPLC. Suhu pengeringan yang digunakan adalah 32°C, 50°C, 60°C dan 70°C. Pelarut yang digunakan adalah methanol, etanol, heksana dan pentana. Dari segi partikel, saiz yang telah ditetapkan adalah 1.0 mm, 3.0 mm, 5.0 mm dan 10.0 mm. Keputusan menunjukkan bahawa suhu pengeringan pada 50°C memberikan hasil asid askorbik yang tertinggi iaitu 38.353 mg/l, semasa menggunakan methanol sebagai pelarut, hasilnya adalah 34.123 mg/l. Ini menunjukkan bahawa hubungan antara kepolaran pelarut dengan kuantiti askorbik asid adalah berkadar langsung. Saiz partikel jambu batu adalah bekadar songsang dengan hasil pengektrakan askorbik asid di mana menggunakan partikel bersaiz 1.0 mm memberikan 23.546 mg/l kuantiti askorbik asid. Kajian ini menunjukkan bahawa jambu batu mempunyai potensi yang besar sebagai sumber alternatif bagi antioksida untuk menggantikan antioksida tiruan di pasaran semasa.

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DNA	-	Deoxyribonucleic acid
BHT	-	Butylated hydroxytoulene
BHA	-	Butylated hydroxy anisole
HPLC	-	High performance liquid chromatography
UV-Vis	-	Ultra-violet visible spectrocopy
AAC	-	Ascorbic acid
FRAP	-	Ferric reducing antioxidant power
AEAC	-	Ascorbic acid equivalent antioxidant capacity
Fe (III)	-	Ferrum (III) ion
ROS	-	Reactive oxygen species
CNS	-	Central nervous system
AIDS	-	Acquired immune deficiency syndrome
DPPH	-	1, 1-diphenyl-2-picrylhydrazyl

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Guava with the scientific name Psidium guajava is one of the species of tropical shrubs in the myrtle family Myrtacea. It have different name in every country. In Malaysia it known as jambu batu, in India it called amrood and in Sri Lanka it called pera. It is the one of the most grown in Malaysian garden beside the other plant. The guava can easily grow at any soil such as on heavy clay, marl, light sand, gravel bar near stream or on limestone. There are many types of guava in Malaysia and every type has different size of fruit and taste. It is small tree that have high until 33 feet and the trunk can achieved until 10 inch in diameter.

Normally, the diameter of fruit is about 3 to 10 cm. On average, the fruit contain 74-78% moisture, 13-26% dry matter, 05-1% ash, 0.4-0.7% fat and 0.8-1.5% protein (Chin and Yong, 1980). In addition, it also contains a fair of amount of phosphorus, calcium, iron, potassium and sodium (Lim and Khoo, 1990). Nowadays, guava is grown commercially in many countries due to the demand in food industries. The product that based on guava such as drinks or juice, jelly, pickles and chips are made in most commonly traded domestically or internationally.

For the traditional folks, guava grown not only for their edible fruit but it has their own used in medicine. Guava leaves always used as a remedy for diarrhea and treats the diabetes. Beside that, it can be chew to relieve toothache, crushed leaves can be applied on wound or ulcer. Malaysian folks use the guava leaves to remove the smells of fish and use it as one of the materials to make black dye.

In the modern world, guava was classified as one of super fruit because of the rich of nutritious. The fruit is rich with ascorbic acid (vitamin C), fair amount of vitamin A (beta-carotene), vitamin B such as thiamin (B1), riboflavin (B2) and niacin. Vitamin A, B, C, E and phenolic compound that contain in guava are known as antioxidants. Antioxidants are chemicals compound that can destroy single oxygen molecules always known as free radical. It delaying oxidative damage or preventing from damaging healthy cells. The consumption of fruits and vegetables containing antioxidants has been reported to provide protection against a wide range of degenerative disease including ageing, cancer, diabetes and cardiovascular diseases (Vimala and Mohd Ilham Adenan, 1999; Cagaray, 1992).

Actually, free radical is naturally produced in our body by the metabolism of amino acids and fats. Oxidation is essential to many living organisms for the production of energy to fuel biological process. However the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid, arthritis, and arteriosclerosis as well degenerative processes associated with aging (Halliwel and Gutteridge, 2003). These free radicals are unstable and it can react with cells and destroy it. The bind of free radical with DNA structure will lead to mutation and it is cause of cancer. Free radicals formed in our body in several of type such as superoxide, hydroxyl, peroxyl and alkoxy. The antioxidant is needed to inhibit all the free radical from react. They scavenge radicals by inhibiting initiation and breaking of chain reaction, suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide and quenching superoxide and single oxygen (Shi *et al.*, 2001).

High concentrations of phytochemical in plant extracts are associated with strong antioxidant activity. Ascorbic acid and phenolic compounds including vitamins, pigments and flavonoids have been identified to be responsible for antioxidant properties in most plant (Tsai *et al.*, 2002). The antioxidant effect of polyphenols has been reported in many vitro studies including human low-density lipoprotein (Teissedre *et al.*, 1996). Nowaday, synthetic antioxidants is the most popular of antioxidant use in pharmaceutical and food industries. Synthetic antioxidant such as butylated hydrotoluene (BHT) and butylated hydroxyanisole (BHA) and natural antioxidant such as tocopherol and ascorbic acid are widely used in food industries due to their protecting ability against oxidation reduction reactions (Roberto *et al.*, 2000). Eventhough, synthetic antioxidant was produced to replace the natural antioxidant because the natural antioxidant is less commercialize. It is known that BHT and BHA retard lipid oxidation. Due to increasing consumer awareness of health aspect, their used is slowly replaced by alternative antioxidants which are without toxic effect (Saniah Kormin, 2005). The awareness of consumers caused the demand of natural antioxidant is increased.

To get the natural antioxidants we should extract it from fruits or vegetables. For those who need the antioxidant they can get it trough the fruit when they eat. But for the food and pharmaceutical industries, the extract of antioxidants from fruits and vegetables is needed for their manufacturing process. The natural antioxidants can be extracted trough the fruits, vegetables, spies and herbs. Extraction is used when we want to separate substances. The process of extracting the antioxidant can be done by many method of extraction. One of the example, it can be done is by using solvent which a desired substance dissolves in and the undesired substance does not dissolve in. There are several ways to do extraction process such as soxhlet extraction, hydrodistillation, ultrasonic extraction and many more. Soxhlet extraction is one of the oldest method and most widely used approaches for conventional extraction of solid samples. The advantage of this method are: (a) the sample phase is always in contact with fresh solvent, thereby enhancing the displacement of target compound from the matrix and (b) the compound are not discomposed due to moderate extraction condition (Lee *et al.*,2000).

In this work, the application of soxhlet extraction method to extract the ascorbic acid from guava will be investigated. Guava was selected in this research because it rich with antioxidants especially ascorbic acid.

1.2 Identification of Problem

Natural antioxidant, particularly in fruits and vegetables have gained interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidant is associated with a lower risk of cardiovascular disease and cancer (Renaud *et al*., 1998). Natural antioxidants are perceived safe, less toxic and beneficial for human health. However it is very expensive and not widely commercialized. Source of antioxidants are spices and herbs, and such material have been used troughout history for flavouring and preservative (Kikuzaki and Nakatani, 1993). The alternative sources of natural antioxidants are needed to make sure the price is low and value to commercialize. The chosen of natural resources such as fruits and vegetable is importance because different fruit have different amount of antioxidant. Guava is considered a highly nutritious fruit because it contains a high level of ascorbic acid (50-300mg/100g fresh weight), which is three times higher than orange. Beside that, it is easy to get since the fruit can grow well in Malaysia and the price is cheap compared to herbs or spies. Solvents type such as pentane, hexane, methanol and ethanol, drying temperature are the input parameter that can affect the extraction yield.

Therefore, in this research an experiment works will be done to evaluate the effect of drying temperature, solvent polarity and size particles on extraction yield. The experiments will be done by using soxhlet extractor to get the antioxidants of guava. Then it will be analyzed by using high performance liquid chromatography (HPLC) to identify and measure the quantity of antioxidant.

1.3 Research Objective

The main objective of this research is to extract ascorbic acid from guava using soxhlet extraction method.

1.4 Research Scopes

In order to achieve the objective, the scope of work was identified as follows:

1. Different temperature of drying was used to dry the sample. The same amount of sample of guava at 10g will be dried under four different temperature; 40°C (A), 50°C (B), 60°C (C) and 70°C (D) for 24 hours. The solvent used at these trials will be ethanol.

2. The samples of dried guava were prepared in small shape by grind it. The sample was extracted using soxhlet extractor with four different solvents polarity; methanol (A), ethanol (B), pentane (C) and hexane (D). The other parameter such as drying temperature of sample, time of drying, amount of sample, amount of solvent and time of extraction was fixed.

3. The sample was extracted using soxhlet extractor with four different sizes. Different method was used to produce different size of samples (grind via electrical mill (A), crush with mortar (B), cut in cubic shape with dimension 5x5x5 mm and cut in cubic shape with dimension 10x10x10). The other parameter such as drying temperature of sample, time of drying, amount of sample, amount of solvent and time of extraction was fixed.

4. The extraction yield was analyzed using high performance liquid chromatography (HPLC) to identify and measure the quantity of antioxidant.

1.5 Rational and Significant

Guava has a huge potential as an alternative source of antioxidant since the price is cheaper and has high antioxidant contents compared to traditional source (spices and herbs). Therefore, the antioxidant in guava can be commercialized to replace the synthetic antioxidant in current market. Furthermore the antioxidant based on guava will give extra value to guava and give benefit to the farmer.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant Material

2.1.1 Plant Description

Guava (Psidium guajava L.), also known locally as jambu batu, is grown commercially and in many home gardens in Malaysia. The tree is very hardy and can grow to about 7-8 meters high with characteristic smooth, pale mottled bark that peels off in thin flakes. The fruits vary in size, shape and flavour depending on the variety. The better varieties are sweet while others may be astringent (Lim *et al.*, 2006). The guava has been cultivated and distributed by man, birds, and sundry 4-footed animals for so long that its place of origin is uncertain, but it is believed to be an area extending from southern Mexico into or through Central America (www.hort.purdue.edu).

Figure 2.1: Guava, Psidium Guajava

2.1.2 Nutrient Composition of Guava

Food is composed of several groups of constituents including carbohydrate, protein, fat, inorganic mineral components and organic substances present in very small amount. The organic components generally functions as flavour, pigments, enzymes, emulsifier, acids, oxidant and antioxidants (Saniah Kormin, 2005). For guava, on average, the fruit contains 74–87% moisture, 13–26% dry matter, 0.5–1% ash, 0.4–0.7% fat and 0.8–1.5% protein (Chin and Yong, 1980). It considered a highly nutritious fruit because it contains a high level of ascorbic acid or vitamin C (50–300 mg/100 g fresh weight), which is three to six times higher than oranges (Kriengsak Thaipong *et al.*, 2006). Red-fleshed Brazilian guava has several carotenoids such as phytofluene, b-carotene, bcryptoxanthin, g-carotene, lycopene, rubixanthin, cryptoflavin, lutein, and neochrome (Mercadante *et al.*, 1999).

The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups: vitamins, phenolics, and carotenoids. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoids are known as lipophilic antioxidants (Halliwell, 1996). In recent years, there has been a considerable interest in finding natural antioxidants from plant materials. The antioxidant phytochemicals from plants, particularly flavonoids and other polyphenols, have been reported to inhibit the propagation of free radical reactions, to protect the human body from disease (Kinsella, Frankel, German, & Kanner, 1993; Terao & Piskula, 1997), and to retard lipid oxidative rancidity (Duthie, 1993). Table 2.1 shows the antioxidant content and activities extracts from tropical fruits. Both varieties of guava fruit contain relatively high quantity of antioxidants as shown by the high amount of (total phenolic compound) TPC and (ascorbic acid) AAC recorded. In the case of AAC, guava can contain as much as ten times that of other fruits such as banana, dragon fruit, starfruit and sugar apple. For antioxidant activities, this can be primary or secondary. Primary antioxidant properties are generally measured by DPPH assay (expressed as AEAC and IC50) and FRAP. The DPPH assay measures the ability of the fruit extract to donate hydrogen to the DPPH radical resulting in bleaching of the DPPH solution. The greater the bleaching action, the higher the antioxidant activity (AEAC value), and this is reflected in a lower IC_{50} value. FRAP on the other hand measures the ability of the extract to donate electron to Fe (III). The higher the FRAP value, the greater is the antioxidant activity (Lim *et al*., 2006).

Table 2.1: The Antioxidant Content and Activities Extracts from Tropical Fruits (Lim *et al*., 2006)

Fruits	TPC (mg GAE/100g)	AAC (mg/100g)	IC_{50} (mg/ml)	AEAC(mg AA/100g)	FRAP (mg GAE/G)
Guava (seeded)	138±31a	144±40	1.71±0.61f	218±79	2.09±0.18
Guava (seedless)	179±44a	132±46	2.11±0.63f,g	176±54	1.65±0.06
Banana (mas)	51±7c	4.9±0.6	13.4±2.5i	27.8±5.5	0.18±0.06
Dragon fruit	21±6d	8.0±1.6	27.5±3.9j	13.5±2.1	0.07±0.01
Star fruit	131±54a	5.2±1.9	3.8±2.1g,h	98±55	0.83±0.02
Sugar apple (brown)	175±36a	21.3±2.1	3.9±0.4g,h	82.1±6.9	0.62±0.10
Sugar apple (green)	165±18a	6.8±0.8	4.6±0.8h	71.4±11.8	0.58±0.04
Water apple	35±4e	4.1±2.1	12.0±3.8i	31±10	0.30±0.02
Orange	75±10b	67±9	5.4±1.3h	70±17	0.61±0.05

2.1.3 Medicinal Applications

Guava was also used as a hypoglycemic agent in folk medicine. The leaves and skin of the fruit have greater effects. Guava tea, the infusion of dried guava fruit and leaves, has recently become popular as a drink in Taiwan (Cheng and Yang, 1983). It proved that guava juice exhibited hypoglycemic effects in mice and interestingly, the decreased serum glucose level of infusions from the African mistletoe (Loranthus bengwensis L.). In other studies, the anti-diarrheal (Lutterodt, 1989) antipyretic (Olajide, Awe, and Makinde, 1999), antimicrobial (Jaiarj *et al*., 1999) and bio-antimutagenic (Matsuo, Hanamure, Shimoi, Nakamura, and Tomita, 1994) properties of guava leaf extract have been demonstrated. There is an important role of oxidative stress in the development of cancer and diabetes.

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. 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. 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 (www.hort.purdue.edu).

2.2 Antioxidant

2.2.1 Effect of Antioxidant to Free Radicals

Antioxidants, which can inhibit or delay the oxidation of an oxidisable substrate in a chain reaction, would therefore seem to be very important in the prevention of these diseases (Ames *et al*., 1993). They scavenge radicals by inhibiting initiation and breaking of chain reaction, suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen (Shi *et al*., 2001). It is well known that reactive oxygen species (ROS) formed in vivo, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. Tissue damage resulting from an imbalance between ROS-generating and scavenging systems has been implicated in the pathogenesis of a variety of disorders, including degenerative disorders of the CNS, such as Alzheimer's disease, cancer, atherosclerosis, diabetes mellitus, hypertension, AIDS and aging (Halliwell and Gutteridge, 1998; Mantle, Eddeb, and Pickering, 2000). High consumption of fruits and vegetables has been associated with a lowered incidence of degenerative diseases including cancer, heart disease, inflammation, arthritis, immune system decline, brain dysfunction and cataracts (Ames, Shiganaga, and Hagwn, 1993; Feskanich *et al*., 2000; Gordon, 1996; Halliwell *et al*., 1996).

2.2.2 Antioxidant in Guava

Guava, as in many other fruits and vegetables, is also rich in antioxidants that help to reduce the incidence of degenerative diseases such as arthritis, arteriosclerosis, cancer, heart disease, and inflammation and brain dysfunction. Guava fruit is considered a highly nutritious fruit because it contains a high level of ascorbic acid (50–300 mg/100 g fresh weight), which is three to six times higher than oranges In addition, antioxidants were reported to retard ageing (Feskanich et al., 2000; Gordon, 1996; Halliwell, 1996). The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups: vitamins, phenolics, and carotenoids. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoids are known as lipophilic antioxidants (Halliwell, 1996). Among the most abundant antioxidants in fruits are polyphenols and ascorbic acid. The polyphenols, most of which are flavonoids, are present mainly in ester and glycoside forms (Fleuriet and Macheix, 2003). In the case of guava, free elagic acid and glycosides of myricetin and apigenin are found to be present (Misra and Seshadri, 1968; Koo and Mohamed, 2001). Besides classical antioxidants including vitamin C, E and -carotene, phenolic compounds had been identified as important antioxidants contained in fruits. Some phenolic compounds are even more powerful as antioxidants than vitamin C, E in vitro and significantly bioavailable as demonstrated by animal and human studies (Bravo, 1998).

2.3 Soxhlet Extraction

2.3.1 The Usage and Benefits

Extraction techniques are widely employed for the isolation of bioactive substances from natural sources (Self, 2005). Soxhlet extraction is one of the oldest method and most widely used approaches for conventional extraction of solid samples. It is the most conventional of all methods and consists of a simple distillation process repeated a number of times. Soxhlet extraction (on which official methods are based) is straightforward and inexpensive (Luque-Garc´ıa and Luque de Castro, 2004). The advantage of this method are: (a) the sample phase is always in contact with fresh solvent, thereby enhancing the displacement of target compound from the matrix and (b) the compound are not discomposed due to moderate extraction condition (Lee *et al.*,2000). In Soxhlet extraction, both the solvent and the