STABILITY OF STEVIOSIDE IN STEVIA EXTRACTION

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ABSTRACT

Stevia leaves have sweet taste more than 300-400 times sweeter than sugar. There are many researches have been done to show the benefits of stevia to our health. Producers use many types of preservation techniques to increase the stevia shelf life. One of methods is by addition of food additives. Thus, the aims of this research is to determine the stability of the sweet components in stevia, stevioside and also effect of additives to the stevioside. In this research we use fresh and dried stevia leaves. The leaves are cut into small pieces and will be extracted in 1L of water at 100^{o}C for 5 minutes. The stevia extractions are filtered to remove the leaves. Then the stevia extractions are divided into two categories, sample with additive and without additives. About 3g of solid citric acid is added into samples with additive. The sweetness and the acidity of the samples are analyzed for 21 days. Based on the data we found that the stevioside more stable in stevia extraction samples without additives. Addition of additives caused increased in the acidity of the samples and the stevioside undergo the hydrolytic breakdown into steviol glycosides. Thus the sweetness was decreased in 21 days. Fresh stevia extraction had capability to resist from microbial activities, since pH decreased as the protection from the microbial activities.

ABSTRAK

. Daun stevia mempunyai rasa yang manis, melebihi 300-400 kali lebih manis daripada gula. Khasiat stevia telah dibuktikan melalui banyak kajian telah dilakukan. Oleh kerana mempunyai khasiat yang banyak, ramai pengeluar menghasilan produk kesihatan atau makanan berasaskan stevia. Untuk memanjangkan jangka hayat daun stevia berbagai cara telah digunapakai, contohnya melalui penambahan bahan pengawet. Oleh itu, kajian ini dijalankan untuk mengakaji keasan penambahan bahan pengawet terhadap kestabilan stevioside iaitu komponen utama dalam stevia dan kesan bahan pengawet keatas stevioside.. Dalam kajian ini, daun stevia yang segar dan yang kering yang dipotong kecil digunakan. Kedua-dua jenis daun ini diekstrak dalam 1L air pada suhu 100^{o}C selama 5 mints. Daun stevia akan dikeluaran dari larutan tersebut, dan larutan stevioside akan dibahagikan kepada dua bahagian, iaitu larutan stevia dengan bahan pengawet dan larutan stevia tanpa pengawet. Untuk larutan stevia dengan pengawet, 3 g asid sitrik akan dicampurkan. Kemudian, kedua-dua larutan stevia dengan pengawet dan tanpa pengawet dianalisis untuk menguji tahap kemanisan, stevioside and sifat asid larutan. Analisis tahap kemanisan dan sifat asid diuji selama 21 hari. Berdasarkan data yang diperolehi larutan stevia tanpa pengawet mampu mengekalkan tahap kemanisan. Penambahan bahan pengawet menyebabkan sifat berasid larutan meningkat dan menyebabkan stevioside diuraikan menjadi steviol glkosida. Penguraian molekul stevioside menyababkan tahap kemanisan larutan berkurang. Selain itu, Daun Stevia yang segar memonunyai daya ketahan yang tinggi untuk menghalang tindakan microorganism di dalam larutan kerana sifat asid meninggat sedikit sahaja pada hari ke 21.

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LIST OF SYMBOLS/ABBREVIATIONS

$\%_t$	-	Percentage
°C		Degree Celsius
cm		Centimeter
g		gram
kg		Kilogram
L		Litter
LC-ESI-MS-MS		Liquid chromatography electrospray ionisation tandem mass spectrometry
m		Meter
mg		Milligrams
Min		minutes
mmHg		Millimeter Mercury
pH		Potentiometric hydrogen ion concentration

CHAPTER 1

1.0 INTRODUCTION

1.1 Backgrounds of Study

Along time ago, our ancestors more concern to their health quality. It was something normal when they ate many types of herbs as supplements or medicine to maintain their health. They prepared all the tonics by them selves based on their knowledge and experiences. So at that time lack of people who are suffering chronic diseased.

In modern lifestyle era, people always busy to fulfill their daily activities and seldom to think about their health quality. However, lately there many healthy life styles campaigns have been promoted in media. From the campaign, awareness of people about their health increase. As the result, we can see the demand of the supplements set can be found in the market today. The demands of the supplements getting increase by days.

There is a type of herb that has huge potential to be used as food supplements. In Malaysia, many people still do not know about this herb. But in others country this herb has been used for long time a go. The name of this herb is stevia.

Stevia or the full name, *Stevia rebaudiana* (Bertoni) Bertoni is a green herb plant of the Aster or Chrysanthemum family. Stevia leaves contain several chemical substances called glycosides. The glycosides have sweet taste but not contain any calories. The main glycoside is known as stevioside and it is 300-400 times sweeter than sugar. Stevioside content varies between 4 and 20% of the dry weight of the leaves depending on the cultivar and growing conditions. This herb has been around for a little over 120 years where Guarani Indians used it in Paraguay for centuries.

There are the advantages of stevioside as a dietary supplement for human being; it is stable in high temperature, it is calorie-free, it maintains good dental health which inhibits the formation of cavities and plaque by reducing the intake of sugar and opens the possibility for use by diabetic and phenylkenoturia patients and obese persons. Phenylketonuria (PKU) is a genetic disorder that is characterized by an inability of the body to utilize the essential amino acid, phenylalanine (Living with PKU, Clinic University of Colorado).

Since there are numerous of benefits, stevia has been used in many products. Stevia really started to catch on in American nutritional consciousness as a healthy alternative sweetener to sugar. Now it has been used by over 40% of the Japanese market. Besides that, it has been used as tonic to cure many diseases such as for diabetes, obesity, cavities, hypertension, fatigue, depression, sweet cravings, lower uric acid level and also infections (Stevia plant Summary, Raintree Nutrition, Inc., Carson City)

In normal condition, fresh food cannot be used after several days. Thus, to increase the shelf life there are some methods will be used as preservation to the food. Addition of food additive is an alternative way to increase shelf life of the stevia based products. All consumers need to know the self life of the product once it unsealed. So that, a research should be done to study the stability of the food components with and without out the preservative and also the shelf life without preservative.

1.2 Problem Statement

This research is proposed to determine the stability of stevia extraction to maintain its quality in term of acidity and sweetness. Acidity of the stevia extraction is very important to determine the ability of the stevia extraction resistant from the microbial activity. The sweetness is referred to the concentration of steviosides, the main sweetness in stevia extraction.

Nowadays there are many health based products can be found in the market. It comes in different types: tablets, cream, mixed herbs drinks pack and many more. So, it can be taken in fast and easy way. This is an easier way for those to get the health supplements and encourage people to maintain their health.

Stevia rebaudiana is a one of herbs which is high in demand nowadays. This herb has large potential of being a tonic and also as an alternative sugar. Since stevia has many benefits, the leaves have been used in many products.

Many preservation methods have been used to increase the products shelf life. So, the products can be stored in long period and in good quality. One of method, by addition of certain type food additives into the stevia based products.

Consumers used the stevia based products to gain the benefits from this herb. Thus, as the consumers we need to know what the effect of the food preservative to the stevia based products. Either we gain or we loss of the benefit if additive is added?.

1.3 Objectives

The objectives of this research are:-

i. To study the stability of the stevia extraction to maintain its quality; pH, sweetness
ii. To study affect of stevia leaves condition, fresh leaves and dried leaves
iii. Effect of additive to stevioside in stevia extraction

1.4 Scope of Study

To achieve the objectives, scopes have been classified in this research. The scopes for this research are listed as below:

i. Study on stability of extraction of stevia fresh leaves and dried leaves with/without additive

ii. To study the pH and the sweetness of the stevia extraction

iii. To compare the results

1.5 Significance of Study

Potential markets for stevia in future are expected to be huge, regarding the research and development afford by researchers. Besides that, it has been proved that stevioside has greats impact as a tonic. Dr. Paul Chanand friends (1998) in Division of Cardiovascular Medicine from Taipei Medical College and affiliated Taipei Wan Fang Hospital has done his research. The study showed that the hypotensive effect was maximum when using 200 mg/kg stevioside. The maximal decrease of mean systolic blood pressure was 31% (200 to 137 mmHg) whereas the maximal decrease of mean diastolic blood pressure was 33% (149 to 100 mmHg).

World Health organization (WHO) has some data and statistics regarding to the chronic diseases risks, and the diseases:

- Heart attacks and strokes kill about 12 million people every year (7.2 million due to ischaemic heart disease and 5.5 million to cerebrovascular disease). In addition, 3.9 million people die annually from hypertensive and other heart conditions.
- An estimated 177 million people are affected by diabetes, the majority by type 2 diabetes. Two-thirds live in the developing world.
- More than one billion adults worldwide are overweight, and at least 300 million of these are clinically obese.

- Up to 80% of cases of coronary heart disease, 90% of type 2 diabetes cases, and one-third of cancers can be avoided by changing to a healthier diet, increasing physical activity and stopping smoking

The production of stevia based products should be encouraged, improved and developed because of the advantages to the health. So, the research about the effects of the food additives in stevioside seems to be a motivation for the stevia base products producers to increase their production, quality and quantity.

Consequently, it can give a hope for those who are suffering chronic health problems since stevia has huge potential to be used as medicine. This research can give knowledge for those who are involved in research about stevia. Hopefully this research can give some positives impact to promote and increases the opportunities for the stevia products to be sold the in Malaysia. .

CHAPTER 2

LITERATURE RIVIEW

2.1 Introduction

This herb has a sweet taste. The taste is 300-400 times sweeter than sugar. The name of this herb is *Stevia rebaudiana* (Bertoni) Bertoni or *Eupatorium Rebaudianum. Stevia rebaudiana* (Bertoni) Bertoni or the simplest name, stevia is found growing wild in the highlands of the Amambay and Iguacu district (a border area between Brazil and Paraguay). It has been used over 120 years by the Guarani Indians in Paraguay for centuries. In 1887, Antonio Bertoni, an American scientist, noted that the native Indians of Paraguay would grind up a particular herb and use it to sweet tea. Later in 1931, two French chemists, Bridel and Lavielle worked for the extraction process of making a sweetener from the leaves.

Stevia can grow up to 1 m tall and has leaves 2-3 cm long. It belongs to the *Asteraceae* family (Sunflower family). It is estimated that as many as 200 species of stevia are native to South America. *Stevia rebaudiana* (Bertoni) Bertoni, commonly

known as sweetleaf, sweet leaf, sugarleaf, or simply stevia, is widely grown for its sweet leaves. However no other Stevia plants have exhibited the same intensity of sweetness as *Stevia rebaudiana*. It is grown commercially in many parts of Brazil, Paraguay, Uruguay, Central America, Israel, Thailand and China. Below is the specific classification of stevia;

Table 2.1 Specific classification of Stevia

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Asteridae*
Order	*Asterales*
Family	*Asteraceae* – Aster family
Genus	*Stevia* Cav. - candyleaf
Species	*Stevia rebaudiana* (Bertoni) Bertoni- candyleaf
Sources: *Stevia rebaudiana* (Bertoni) Bertoni, National Plant Data Center .	

Figure 2.1 The flowers of *Stevia rebaudiana*

Figure 2.2 The leaves of *Stevia rebaudiana*

The leaves of stevia plant contain several chemical substances called glycosides, which have a sweet taste. The major glycoside is known as stevioside. The tastes of the stevioside in the leaves about 300 times sweeter than sucrose (0.4% solution) (Jan M.C. and Geuns, 2003). The structure of stevioside 3 mainly in leaves is given in Fig. 2.3 (Kinghorn, 2002). Their content varies between 4-20% of the dry weight of the dry weight of the leaves depending on the cultivar and growing conditions. Stevioside is the main sweet component. Other compounds present but in lower concentration are: steviolbioside2, rebaudioside A 4, B 5, C6, D7, E8, F9 and dulcoside A 10 (Kennelly, 2002; Starrat et al.,2002)

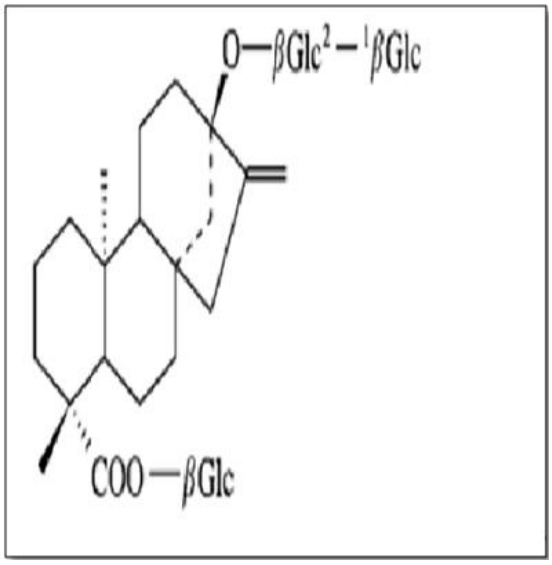
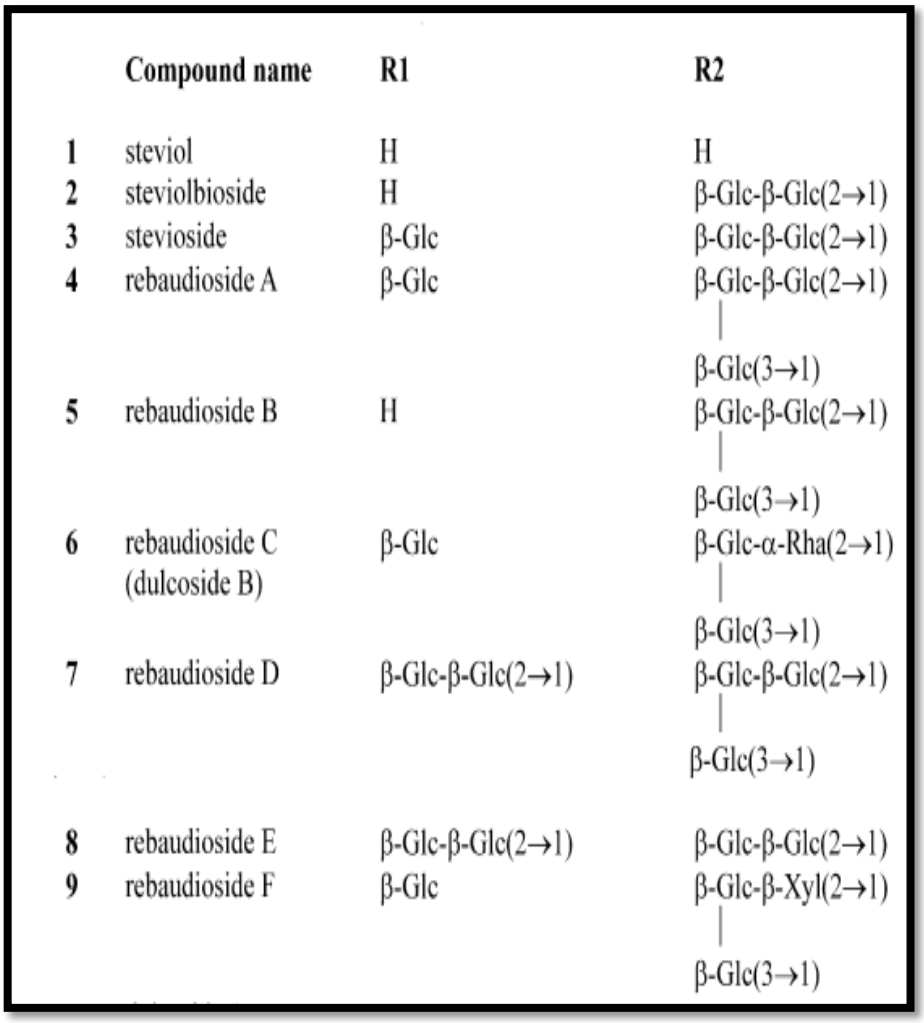


Figure 2.3 Structure of stevioside



	Compound name	R1	R2
1	steviol	H	H
2	steviolbioside	H	β-Glc-β-Glc(2→1)
3	stevioside	β-Glc	β-Glc-β-Glc(2→1)
4	rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
5	rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
6	rebaudioside C (dulcoside B)	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
7	rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
8	rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
9	rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)

Figure 2.4 Structure of stevioside and related compounds. In rebaudioside D and E RI is composed of 2 b-Glc-b-Glc (2 → 1). In rebaudioside A,B,C,D,E and F in group R2 an additional sugar moiety is added on carbon 3 of the first b-Glv. In rebaudioside F one b-Clc is substituted for by-b-Xyl.

Stevia has many advantages as dietary supplements for human are manifold. It is because, heat- stable, non-caloric, assist dental health by reducing the intake of sugar, and open the possibility for use by diabetic and phenylketonuria patients and obese

persons (Geuns,2003). Other researchers found that stevioside was highly stable to both heat and acid. When heated to 100°C for one hour in pHs ranging from 3 to 9, over 98 percent of the stevioside remained unchanged (Fujita and Edahiro, 1979). It even remained stable when heated to 100°C at a pH of 3 for 5 hours (Abe and Sonobe, 1977).

Products based on stevia, can be found in many forms. For examples, in most health-food stores, and is also incorporated into drinks, teas and other items. One of the common products, stevia as a sweetener and as a replacement for potentially toxic, synthetic substitute sugars like saccharin. Compare to other high potency synthetic sweeteners such asoatame, saccharine, sucralose and acesulfame-K, stevioside is come from a natural plant product. Besides that, stevia more than just a natural low-calorie, low glycemic, super sweet supplement. But also, stevia is loaded with vitamins and minerals, including magnesium, niacin, riboflavin, zinc, chromium and selenium.

Several modern clinical studies suggest that stevia may have the ability to lower and balance blood sugar levels, support the pancreas and digestive system, protect liver, and combat infectious microorganisms (Suzuki et al., 1977). By 1921, stevia was being hailed by American trade commissioner George Brady as a "new sugar plant with great commercial possibilities." He was so convinced that it made "an ideal and safe sugar for diabetics," that he presented it to the United States Department of Agriculture. More recent studies have found stevioside to be not only safe, but also to reduce the incidence of breast tumor and kidney damage when consumed on a long-term basis. A high-quality study conducted by Dr. Toyoda of the National Institute of Health in Japan found that even when used in doses as high as 5 percent of the diet for two years, stevioside had no side effects on laboratory rats except for a slight loss of weight, reduced risk of cancer, and an improvement in kidney function (Toyoda et al., 1997).

2.2 Absorption and Metabolism of Glycosidic Sweeteners of Stevia Mixture and Their Aglycone, Steviol, in Rats and Humans

Our bodies need energy to do from moving to thinking to growing. The energy comes from food through metabolism. Metabolism is the chemical reactions in the body's cells that convert the fuel from food into the energy needed

A research has been done by Eriko Koyama and his friends in 2003. In this research, the researchers study about the steviol and stevia mixture in term of the absorption in rats and also about the hepatic metabolism in rats and humans.

Stevia sweeteners, crude extracts from its leaves, have been used for a many years to sweeten beverages and foods. Stevia sweeteners are glycosides of the diterpene derivative steviol (ent-13-hydroxykaur- 16-en-19-oic acid), consisting mainly of stevioside (triglucosylated steviol) and rebaudioside A (tetraglucosylated steviol), together with the other components rebaudioside C and dulcoside A.

Absorption was investigated both in vivo and ex vivo. In ex vivo experiments using the rat everted sac method, no absorption of stevia mixture was observed, but significant absorption of steviol was noted (equivalent to approximately 70% of the absorption reference- salicylic acid- value). In the in vivo experiment, rats received a single oral administration of either steviol or stevia mixture; a peak steviol concentration in plasma was observed 15 min after its oral administration, demonstrating rapid absorption.

However, after oral administration of stevia mixture, the steviol concentration in plasma increased steadily over 8 h, suggesting that stevia mixture components are first degraded and then absorbed as steviol in the rat intestine.

Steviol metabolism in humans and rats was examined by incubating steviol with liver microsomes from the two species. Oxidative (monohydroxy and dihydroxy) metabolites of steviol were observed by LC-ESI/MS after incubation with both human and rat liver microsomes. The intrinsic clearance of steviol in human liver microsomes was 4-times lower than that found in rat liver microsomes.

This study suggests that there are no major species differences in steviol hepatic metabolism between rats and humans. Absorption from the human intestine can be predicted to occur in an analogous manner to that from the rat intestine.

2,3 The Effect of Stevioside on Blood Pressure and Plasma Catecholamines in Spontaneously Hypertensive Rats

Blood pressure is an amount of the force used to the walls of the arteries as the heart pumps blood through the body. The pressure is determined by the force and amount of blood pumped, and the size and flexibility of the arteries. Blood pressure is always changing depends on activity, temperature, diet, emotional condition, posture, physical state and also medication use. The systolic blood pressure shows the maximum pressure use when the heart contract. The diastolic blood pressure represent the minimum pressure in the arteries when the heart in rest situation. An ideal blood pressure for adult is 120 mmHg (systolic pressure) and less than 80 mmHg (diastolic pressure). When the blood pressure higher than normal, it can increase the potential of heart failure, heart attack, stroke and kidney failure.

Hypertensive heart disease is related to blood pressure. Hypertensive refers to coronary artery disease, heart failure, and enlargement of the heart that occur because of high blood pressure. High blood pressure can raise the pressure in blood

vessels. The heart need to work harder to pumps blood against the pressure. When the duration is too long, this causes the heart muscle to thicken and the left ventricle to become enlarged. As the result, amount of blood pumped by the heart each minute will be decreased. Hypertensive heart disease is the leading cause of illness and death from high blood pressure.

According to this situation, Pau Chan and friends in 1998 has performed a research to study about the effect of stevioside on blood pressure and hypertensive. The research for the effect on the stevioside on blood pressure was in spontaneously hypertensive rats (SHR). The hypertensive effect on systolic and diastolic blood pressure was dose-dependent for intravenous doses of 50, 100 and 200 mg/kg in conscious SHR. The study showed that the hypotensive effect was maximum when using 200 mg/kg stevioside, The maximal decrease of mean systolic blood pressure was 31% (200 to 137 mmHg) whereas the maximal decrease of mean diastolic blood pressure was 33% (149 to 100 mmHg). The hypertensive effect was sustained around 60 minutes. For 50 mg/kg, the hypertensive effect was statistically significant, but decrease the blood pressure is small. The maximal hypotensive effects occur at 10 minutes after stevioside was injected. The heart rate was not changing significantly even though different dosages of steviosides.

Catecholamine is any of a group of sympathomimetic amines, including dopamine, epinephrine and norepinephrine). The aromatic portion of whose molecule is catechol. The catecholamines act an important role in the body's physiological response to stress. Their release at sympathetic nerve endings increases the rate and force of muscular contraction of the heart. Thus increasing cardiac output which is constricts peripheral blood vessels, resulting in raised blood pressure. The blood glucose levels increase by hepatic and skeletal muscle glycogenolysis. It can promote an increase in blood lipids by increasing the catabolism of fats.

The plasma catecholamine levels were maintained at 60 minutes after dosage of 100 mg/kg. Even though levels of norepinehrine be liked to reduce, but it did not achieve statistical significant. Table below shows the effect of stevioside on plasma catecholamine.

Table 2.2 The effect of 100 mg/kg stevioside on plasma catecholamine level

Times	0 min	60 min
Norepinephrine	2.78 $\pm$ 0.20	2.48 $\pm$ 0.12
Epinephrine	3.94 $\pm$ 0.41	4.05 $\pm$ 0.62
Dopamine	2.20 $\pm$ 0.12	2.23 $\pm$ 0.43

The research showed that pure stevioside was effective in lowering blood pressure in conscious SHR.