

Full Length Research paper

Physico-chemical assessment of natural sweeteners steviosides produced from *Stevia rebaudiana bertonii* plant

A. Esmat Abou-Arab*, A. Azza Abou-Arab and M. Ferial Abu-Salem

Department of Food Technology, National Research Centre, Dokki, Cairo, Egypt.

Accepted 14 May, 2010

Stevia plants are a good source of carbohydrates (61.93% d.w.), protein (11.41% d.w.), crude fiber (15.52% d.w.), minerals (K, 21.15; Ca, 17.7; Na, 14.93 and Mg, 3.26 mg/100 g d.w. and Cu, 0.73; Mn, 2.89; Fe, 5.89 and Zn, 1.26 mg/100 g d.w.) also essential amino acids were found in amounts higher than those recommended by FAO and WHO for adults as well as non-essential amino acids. Stevioside was extracted and purified from the dried *Stevia* leaves by three methods. The first one, extraction by hot water (65°C) at different ratios of leaves to water (1:15 - 1:75). The optimum ratio was 1:35 in which the maximum stevioside content was obtained (7.53%), recovery of stevioside was 80.21% and purity related to depigmentation was 97.56%. The second method, extraction by methanol at ratio 4:1 methanol/leaves and purification, recovery of stevioside was 94.90% and purity was 81.53%. The third method, extraction by mixture of methanol/water (4:1) and purification, recovery of stevioside and purity were 92.34 and 79.81%, respectively. Physical and chemical characteristics of the extracted stevioside were studied. Stevioside extractions were analyzed by HPLC in which the highest amount of stevioside (23.20%) was obtained in methanol extract. Also, organolyptic characteristics of extractions indicate that this sweetener has been applied as substitutes for sucrose in different drinks and baking products.

Key words: *Stevia*, stevioside, physico-chemical properties, extraction, natural sweeteners.

INTRODUCTION

The plant *Stevia rebaudiana* Bertonii (compositae) has been widely cultivated in the world for the sweet diterpene glycosides that are mainly contained in its leaves. In Egypt, the gap between sugar production (1.757 million tons) and consumption (2.6 million tons) represents a serious problem, since it was estimated to be 0.843 million tons (Alaam, 2007). Nowadays, attention is concentrated upon using *Stevia* in food industries, in order to close the gap between the production and consumption.

The *Stevia* plant was recently introduced to Egyptian agriculture in order to produce a natural sweetener than can cover some of the lack of sugar production in Egypt (Alaam, 2007). *Stevia* cultivation in different places of the world; it is expected that in the Egyptian agricultural environment; one feddan of *Stevia* may produce up to 400 kg of *Stevia* sugar, annually. Taking the sweetening

powder of the *Stevia* sugar into consideration; these 400 Kg of *Stevia* sugar are equivalent to about 80,000 sweetening units. Note that one feddan of "Sugar cane" produces about 5,000 sweetening units and one feddan of "Sugar beet" produces about 3,500 sweetening units. A sweetening unit is equivalent to the sweetness of one kilogram of sucrose (Alaam, 2007). *Stevia* sweeteners and extracted products which are commonly used as non-nutritive and high-intensity sweeteners in beverages, foods and medicines. The leaves naturally contain a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudiosides (A, B, C, D, E) and dulcoside A (Dossier, 1999).

The increasing consumption of sugar (sucrose) has resulted in several nutritional and medical problems, such as obesity. Therefore, low caloric sweeteners have been investigated to substitute sugar. An important class of low caloric sugar substitutes is known as high intensity sweetener, this is at least 50 - 100 times sweeter than sucrose (Jaroslav et al., 2006). Nowadays, the most common high intensity sweeteners in the world market

*Corresponding author. E-mail: eabouarab@yahoo.com.

are made of synthetic compounds. A frequent metallic aftertaste of such synthetic sweeteners does not provide the realistic taste of sugar as well as some types of synthetic sweeteners such as saccharin is associated with the potential risk of cancer of bladder when they are used heavily (Jaroslav et al., 2006).

Stevioside is a diterpene glycoside present in *S. rebaudiana* Bertoni. *Stevia* has been used by the Guarani Paraguayan Indians and Mestizos as a general sweetening substance for centuries in which part of the plant are used as consumption sweetener. Nowadays, *Stevia* plant and stevioside are being used as sweetener in South America, Asia, Japan, China, and some countries in Europe (Jaroslav et al., 2006). Toxicological studies have shown that stevioside does not have mutagenic, teratogenic or carcinogenic effects and no allergic reaction have been observed when it is used as a sweetener. So, *Stevia* and stevioside have been applied as substitutes for sucrose, for treatment of diabetes mellitus, obesity, hypertension, and caries prevention (Jaroslav et al., 2006).

Stevioside is extracted from the leaves of *S. rebaudiana* Bertoni, a plant from north-eastern Paraguay. It is a white, crystalline, odourless powder which is approximately 300 times sweeter than sucrose (Allam et al., 2001). Structurally, stevioside (13-[2-O-β-D-glucopyranosyl-χ-glucopyran-osyl] oxy] kaur-16-en-19-oic-acid β-D- glucopyranosyl ester) is a glycoside with a glucosyl and a sophorosyl residue attached to the aglycone steviol, which has a cyclopentanone hydrophenanthrene skeleton.

Midmore and Rank (2006) classified the refining methods of stevioside to solvent partition extraction mainly methanol or water extraction and solvent partition extraction incorporating a de-colonizing agent (mainly calcium hydroxide precipitation to remove impurities). They reported different methods of purification that is, adsorption column chromatography ion-exchange or plasmide gel or adsorbed by the activated carbon; electrolytic technique; isolation procedures for rebaudioside A, rebaudioside C, and dulcoside A can be removed. Hot water extraction appeared to be the preferred medium for extraction since the better-tasting rebaudioside A was more soluble than stevioside in water. However, some patents claimed the advantages from the use of solvents, such as ethanol, methanol/chloroform or glycerin, sorbitol or propylene glycol. Liu et al. (1997) extracted stevioside from the dried leaves of *S. rebaudiana* with hot methanol for 7 h, and then concentrated it to dryness. A suspension of the residue in water was washed with ether and then extracted with butanol. The organic phase was evaporated, and the residue was re-crystallized from methanol, giving the stevioside. They also, studied the extraction of steviol glycosides that is, rebaudioside A, rebaudioside C, and dulcoside A by subcritical fluid extraction (Sub FE). A simple efficient Sub FE method

was developed and more than 88% extraction efficiency was obtained by using methanol as a modifier.

In Egypt there is a large interest on the strategical agriculture plan for new crops which can be suitable for cultivation on new reclaimed areas. It may reduce food gap especially in the area of sugar and sweeteners. Recently there is a great effort for natural sweeteners production. Some trials are in the beginning for *Stevia* cultivation in Egypt. This study was conducted to reveal and shed more light on the effect of some technological process on the production of natural sweeteners steviosides from *S. rebaudiana* Bretoni, besides physico-chemical and organolyptic properties of produced steviosides as substitute of sugar (sucrose) were studied

MATERIALS AND METHODS

Materials

Stevia plant leaves

The *Stevia* leaves (*S. rebaudiana* Bretoni), were obtained from *Stevia* International Company from Agro-industry Product (SICAP), Cairo, Egypt.

Reagents

Ion exchange resin (Amberlite IR-4B), organic acids and solvents were obtained from Sigma Chemical Co. (St. Louis, Mo).

Sugar standards

The standard of stevioside was obtained from M.B. Bio medical USA.

Mineral standards

Standard solution (1000 ppm) of macro elements; potassium (K), calcium (Ca), sodium (Na), and magnesium (Mg) as well as micro elements; copper (Cu), manganese (Mn), iron (Fe) and zinc (Zn) were provided by Merck (Darmstadt, Germany). The standards were prepared from the individual 1000 mg/L (Merck). Working standards were prepared from the previous stock solutions.

Preparation of *Stevia* leaves

The *Stevia* plants that reached the maximum growth stage (mature stage before flowering) were harvested by cutting the plant at 5 - 10 cm from the ground. The brown and yellow leaves were removed from the plants then, washed in clean water and spread on trays covered with cheese-cloth to remove the excess of water. The plants were also dried in direct sunlight at temperature ranged from to 25 - 30 °C for 24 - 48 h.

Methods

The prepared *Stevia* leaves were analyzed chemically to determine the gross composition, amino acids content and mineral levels as well as total chlorophylls and carotenoids as follows:

Chemical analysis of stevia plants

The sample materials were blended to powder form with a high-speed blender (Braun KMM 30 mill), type 3045, CombiMax (Germany). This was kept in polyethylene bags and stored at $4 \pm 1^\circ\text{C}$ in a refrigerator until used.

The prepared samples were analyzed for moisture, protein, fat, crude fiber and ash according to the methods described in the AOAC (2000). The carbohydrate content was determined by subtracting the total crude protein, crude fiber, ash and fat from the total dry weight (100 g) of the food sample differences. Reducing sugars were estimated by 3, 5- dinitrosalicylic acid (DNS) method using D (-) fructose (Fluka) as standard (Miller, 1959). Total sugars were determined by the phenolsulphoric acid method according to Dubois et al. (1956). Non- Reducing sugars content was calculated by difference of total soluble sugars and reducing sugars.

Amino acids determination

After hydrolysis of Stevia leaves corresponding to 40 mg protein with aliquot (7.5 ml) of 6 N HCL at 110°C for 24 h, the HPLC apparatus (Waters Assoc, USA) was used for identifying the amino acids of the tested samples according to Millipore Cooperative (1987) modified PICO-TAG method.

Minerals determination

Mineral contents, that is, copper (Cu), magnesium (Mg), manganese (Mn), iron (Fe) and zinc (Zn) were determined on aliquots of the solutions of the ash were established according to the method of AOAC (2000) using Atomic Absorption Spectrophotometer, Perkin-Elmer Model 2380 manufacture (USA). The flame photometer was applied for calcium (Ca), potassium (K) and sodium (Na) determination according to the method described by Pearson (1976).

Determination of total chlorophylls and carotenoids

Both chlorophylls (A and B) and carotenoids were determined in Stevia leaves before and after dehydration with different methods according to Wettstein (1959) as follow: Five grams of each sample were mixed with 30 ml of 85% acetone in dark bottle and left at room temperature for 15 h, then filtered on glass wool into a 100 ml volumetric flask, and made up to volume by 85% acetone solution. The absorbance of the solution was then measured at 440, 644 and 662 nm using spectrophotometer. A blank experiment using acetone (85%) was carried out. The contents of total carotenoids and chlorophylls were calculated using the following equations:

$$\begin{aligned} \text{Chlorophyll A (mg/L)} &= (9.784 \times E662) - (0.99 \times E664). \\ \text{Chlorophyll B (mg/L)} &= (21.426 \times E664) - (4.65 \times E662). \\ \text{Total carotenoids (mg/L)} &= (4.695 \times E440) - 0.369 (\text{chl. A} + \text{chl. B}). \end{aligned}$$

Extraction and purification of stevioside from stevia plant

Stevia sweetener (stevioside) was extracted from the dried ground leaves of Stevia plant by using water, methanol and methanol/water (4:1) extraction. Using water with extraction efficiencies up to 98% achievable removed of stevioside and so produce "natural product" (Nishiyama, 1991). Methanol appears to be used in extraction process, presumably to improve extraction efficiency and facilitate the separation of individual steviosides (Brandle, 1998).

Extraction with water

The dried ground leaves of stevia plant were extracted by water according to Nishiyama et al. (1992). The dried ground leaves were mixed with hot water (65°C) at different percentage of powder leaves/water ratio of 1:15, 1:25, 1:35, 1:45, 1:55, 1:65 and 1:75 (w/v). Stevia leaves were extracted by using hot water for 3 h as follow: The crude extract containing stevioside was filtered through Whatman No. 4 filter paper (filtrate A) and purified by addition of 5% Ca (OH)₂ (based on wt. of dried leaves). The addition of Ca (OH)₂ was repeated twice (filtrate B and C) and the filtrates were collected, passing through ion exchange column (packed with Amberlite IR-4B resin) to remove the undesirable colors at a rate of 1 ml/sec at 25°C . The elute (clear and colourless solution) containing stevioside was collected (in which pigments were adsorbed on resin) and then concentrated by using rotary evaporator at 45°C to the maximum concentration value. At each step total soluble solids (TSS), pH, Stevioside depigmentation and recovery were determined.

Extraction with methanol

Dried ground Stevia leaves were extracted by using methanol according to the method of Nikolai et al. (2001). Methanol was added to ground leaves at ratio (4:1 v/w) and remained for 7 h, then filtered through Whatman No. 4 filter paper. The filtrate containing solvent was evaporated to dryness by using rotary evaporator at 45°C . The residue was washed with ether and then extracted with butanol (three times). The organic phase was evaporated, and the residue was recrystallized at -5°C overnight and purity during extraction and purification steps were determined by determination of pigments.

Extraction with methanol and water (4:1)

Dried ground *Stevia* leaves were extracted by using methanol and water according to Kinghorn et al. (1984). Methanol and water were added to ground leaves (4:1 v/w) and remained for 7 h, then filtered through Whatman No. 4 filter paper. The filtrate containing solvent and water was evaporated to dryness by using rotary evaporator. The residue was washed with ether and then extracted with butanol (three times). The organic phase was evaporated. Percent recovery (yield) and purity during extraction and purification steps were determined by determination of pigments. The stevioside solution was prepared by water, methanol and methanol/water for further studies of their physical and chemical characteristics. Methanol/water extraction was much easier and simpler than water extraction. However, in respect to safety, it is of great interest economically to use water for extraction than methanol.

Physical and chemical characteristics of stevioside

Physical and chemical characteristics, that is, colour, viscosity, total soluble solids (TSS), melting point, optical rotation, stability at different pH and temperatures and organic acids as well as solubility of the purified sweetener of stevioside which were obtained after extraction by the three methods (water, methanol and methanol/water) as follow:

Color measurement

The colour of solution containing stevioside was determined during the extraction and purification steps by measuring the absorbance at 420 nm (as indicator an carotenoids) and 670 nm (as an indicator

an chlorophylls) using a Spectrophotometer (model T80 x UVNIS Spectrometer PG Instruments Ltd) according to the method described by Nguyen and Nguyen (1993).

Viscosity

The stevioside samples were prepared for all samples under identical condition as follows: 10 gm of stevioside powder was dispersed in 100 ml distilled water stirred for 10 min. While stevioside solution were prepared by 100 ml solution, and then cooked for 10 min to permit complete starch gelatinization. Viscosity of the stevioside samples was measured at $27 \pm 1^\circ\text{C}$ by using Brookfield viscometer, using No. 4 spindle at 20 rpm (Model DV III Programmable Rheometer U.S.A) for stevioside, at different shear rates (Hayta et al., 2002).

Total soluble solids (TSS)

The total soluble solids (TSS) of stevioside samples were determined according to AOAC (2000) at room temperature ($25 \pm 1^\circ\text{C}$) expressed as °Brix (0 - 90), was determined with a land Refractometer (ATAGO, Japan).

Optical rotation

Optical rotation was determined by Disk Polarimeter using the Operating Instructions from Stevia sweeteners solution concentration (5.7% in water) according to Morita et al. (1978).

Melting point

The melting point of *Stevia* sweeteners was determined on a Keffer hot stage apparatus according to Nishiyama et al. (1992).

Solubility

The purified of *Stevia* sweeteners were dissolved in acetone, chloroform, ethanol, methanol and water and solubility was determined according to Soejarto et al. (1983).

Stability of stevioside at elevated temperatures

Fifty milligrams of solid stevioside were incubated in a sealed glass vial at different temperatures from 40 - 200°C for 1 h for evaluate stevioside degradation at the specific temperatures according to the method of Chang and Cook (1983).

Stability of stevioside at different pH at elevated temperatures

Aqueous solutions of stevioside 0.5 g/L water were heated in a sealed glass vial at different temperatures of 60 and 80°C for time periods of 1 and 2 h at different pH values of 1 - 10, which were individually adjusted by appropriate buffer systems. Losses in stevioside content were determined according to the method of Chang and Cook (1983).

Stability of stevioside in organic acids

Stevioside was dissolved in aqueous solutions (10 g/L) of the each (acetic acid, citric acid and tartaric acid) and determination of the

pH for different organic acids systems. The samples were stored in sealed glass vials at room temperature in the dark for different time periods of up to 4 months to determine the stability of stevioside in organic acids according to Sanyude (1990).

Determination of stevioside content

Total stevioside in dried powder leaves extracted by the three methods (water, methanol and methanol and water) were determined by Anthrone-sulphoric acid method as described by Wei (1984).

Determination of stevioside by HPLC

The stevioside obtained by water, methanol and water/ methanol extract analyzed by High-Performance Liquid Chromatography (HPLC) as described by Nishiyama et al. (1992). The HPLC system was a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, quaternary pump and a diode array detector. The analytical column was Nuclosil 100 C18 column (25 cm × 4.6 mm I.D., 5 µm, Germany). Separation was performed with a Waters and methanol -water (63: 35 v/v) as the elution solvent at flow rate of 2 ml/min and the detection wavelength was 219 nm. Under these analytical conditions, the typical retention time (t_R) of stevioside was 9.35 min (Figure 1) and the detection limit was 0.1 µg.

Organoleptic characteristics of the purified stevioside

The purified *stevia* sweetener (stevioside) was organoleptically evaluated for sweet taste, bitter taste, other taste and sweet/ bitter according to DuBois and Stephenson (1985) as shown in Table 12.

Statistical analysis

The results were statistically analyzed by analysis of variance and least significant difference (L.S.D.) at 0.05 levels according to the method described by Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Analysis of Stevia plants

Stevia plants that dried by sun light at temperature ranged between 25 - 30°C for 24 - 48 h were analyzed as follows:

Chemical composition of dried Stevia plants

The results obtained by the chemical analysis of dried *Stevia* plants showed that moisture, protein, fat, crude fiber, ash, and carbohydrates were 5.37, 11.41, 3.73, 15.52, 7.41, and 61.93%, respectively (Table 1). These results are in agreement with those reported by Gisleine et al. (2006), Manish and Rema (2006) and Savita et al. (2004). They reported that protein, fat, crude fiber, ash, and carbohydrates were (6.2 - 20.42%), (2.5 - 5.6%), (13.56 - 18.5%), (8.48 - 13.12%) and (35.2 - 52.8%), in

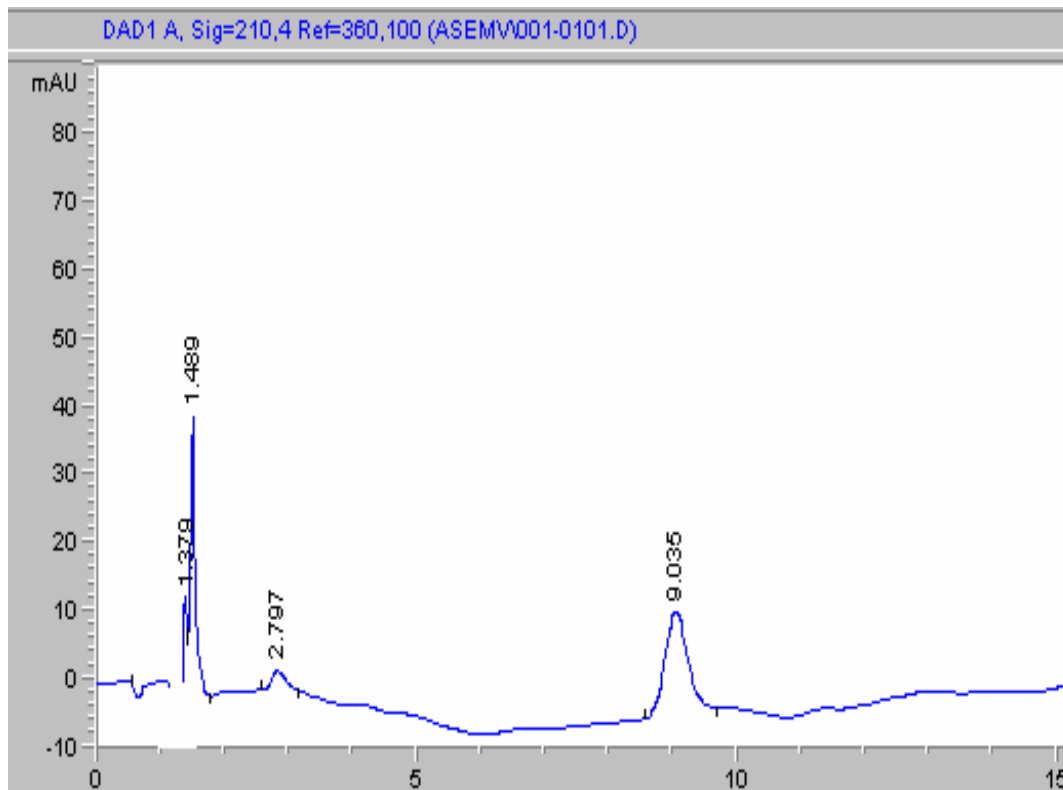


Figure 1. HPLC Chromatogram of stevioside standard Rt of stevioside was 9.35 min under separation methanol/water (63.35 v/v) as the elution solvent at flow rate of 2 ml/min and the detection wavelength 219 nm, column was Nucleosil 100 C18 (25 cm × 4.6 mm I.D., 5 μm).

Table 1. Chemical composition of dried *Stevia* leaves (*S. rebaudiana*).

Components	(% on dry weight basis)
Moisture	5.37 ^g ± 1.12
Protein	11.41 ^c ± 0.19
Fat	3.73 ^h ± 0.29
Crude fiber	15.52 ^b ± 0.19
Ash	7.41 ^e ± 0.14
*Carbohydrates	61.93 ^a ± 6.6
Reducing sugar	5.88 ^f ± 0.21
Non-reducing sugars	9.77 ^d ± 0.13
Total soluble carbohydrates	15.65 ^b ± 2.97
LSD at 5 %	0.28

* Calculated by difference.

-All values are means of triplicate determinations ± standard deviation (SD).

this order. *Stevia* leaves also contained 5.88, 9.77 and 15.65% for reducing sugars, non-reducing sugars and total soluble carbohydrates, respectively (Table 1).

From this study and other investigations, *Stevia* leaves are a good source of carbohydrates, protein, and crude fiber which are the essential factors for maintenance of

health. Also, the consumers benefited when the leaves of *Stevia* was used as substitutes of sugar in place of pure stevioside in different food preparations. On the other hand, high ash content indicates that the *Stevia* leaves are good source of inorganic minerals. Beside, a low fat value confirms that the leaves are not a good source of oil.

Amino acids composition

Regarding to amino acids composition, data in Table 2 showed that dried *Stevia* leaves contained essential amino acids (arginin, lysine, histidine, phenyl alanine, leucine, methionine, valine, threonine and isoleucine), the amounts is higher than those recommended by FAO and WHO (1985) for daily adults as well as non-essential amino acids (aspartate, serine, glutamic, proline, glycine, alanine, cystine and tyrosine). This indicates that the residue remained after the extraction of stevioside sweetener could be used as a good source of essential-amino acids which can be factors to maintain a good health. Similar results obtained by Mohammad et al. (2007) who reported that out of 20 amino acid, nine amino acids were identified as glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine

Table 2. Essential and non- essential amino acids content of Stevia leaves (*S. rebaudiana*) (g/100g dry wt. basis).

Amino acid	Essential-amino acids		Non essential-amino acids	
	Amino acid (g/100g)		Amino acid	Amino acid (g/100g)
	Stevia leaves	FAO/WHO		Stevia leaves
Arginin	0.45	0.35	Aspartate	0.37
Lysine	0.70	0.58	Serine	0.46
Histidine	1.13	0.18	Glutamic	0.43
Phenyl alanine	0.77	0.63	Proline	0.17
Leucine	0.98	0.66	Glycine	0.25
Methionine	1.45	0.25	Alanine	0.56
Valine	0.64	0.35	Cystine	0.40
Therionine	1.13	0.34	Tyrosine	1.08
Isolucine	0.42	0.28		
Total essential amino acids	7.67	3.62	Total non- essential amino acids	3.72

Table 3. Minerals content (mg/100 g) of dried Stevia leaves (*S. rebudiana*).

Elements	Mineral content (mg/100 g weight basis)
Macro minerals	
1. Potassium (K)	21.15 ^a ± 0.35
2. Calcium (Ca)	17.70 ^b ± 0.20
3. Sodium (Na)	14.93 ^c ± 0.05
4. Magnesium (Mg)	3.26 ^e ± 0.58
Micro (heavy metals)	
5. Copper (Cu)	0.73 ^g ± 0.002
6. Manganese (Mn)	2.89 ^e ± 0.58
7. Iron (Fe)	5.89 ^d ± 0.34
8. Zinc (Zn)	1.26 ^f ± 0.72
LSD at 5%	0.481

-All values are means of triplicate determinations ± standard deviation (SD).

- Means within columns with different letters are significantly different (P < 0.05).

and methionine in *Stevia* leaves.

Mineral contents

Data presented in Table 3 indicate the mean concentrations of macro minerals (potassium, calcium, sodium and magnesium) and micro elements, that is, heavy metals (copper, manganese, iron and zinc) determined in dried *Stevia* leaves. Potassium was detected at the highest content (21.15 mg/100 g) followed by calcium (17.70 mg/100 g) and sodium (14.93 mg/100 g). On the other hand, the lowest level of major minerals (magnesium) was detected at concentration 3.26 mg/100 g d.w. basis. Regarding to heavy metals, the leaves contained lower amount of Cu, Mn, Fe and Zn which recorded 0.73, 2.89, 5.89 and 1.26 mg/100 g, respectively. These results coincide with those reported

by Manish and Rema (2006).

Stevia contains substantial amounts of important nutrients. This further establishes *Stevia* as a mineral loaded ingredient required to protect the body, regulates and maintain the various metabolic processes. Potassium, calcium, magnesium, and sodium which are nutritionally important, were found in reasonable amount in *Stevia* leaves. The high concentration of these minerals could be an advantage (Choudhary and Bandyopadhyay, 1999).

Zinc and manganese are considered as antioxidant micro nutrients and their presence could therefore boost the immune system (Jimoh and Oladiji, 2005) and in prevention of free radical mediated diseases. Iron is an essential element for synthesis of hemoglobin. Higher amount of iron in *Stevia* leaves is again useful in maintenance of normal hemoglobin level in the body. Moreover, *Stevia* leaves could also be use to prepare

Table 4. Chlorophyll (A and B), carotenoids and stevioside content in fresh and sun dried *Stevia* plant (*S. rebudiana*).

Pigment properties	Fresh leaves*		Dried leaves		LSD at 5%
	Contents (mg/100 g)		Contents (mg/100g)	Reduction %	
	1	2			
Chlorophyll A	101.06	77.40 ^a ± 0.03	40.71 ^b ± 0.05	47.40	0.07
Chlorophyll B	60.57	46.39 ^a ± 2.71	27.22 ^b ± 3.83	41.32	7.08
Carotenoids	39.82	30.50 ^a ± 3.98	7.67 ^b ± 3.0	74.85	7.92
Total pigments	201.45	154.29 ^a ± 0.0	75.61 ^b ± 0.0	50.99	2.27
**Stevioside %	1.77	1.36 ^b ± 4.0	7.50 ^a ± 3.0	0.81	8.02

- All values are means of triplicate determinations ± standard deviation (SD).

- Means within columns with different letters are significantly different (P < 0.05).

* Moisture content (76.59%).

** Stevioside content (g/100 g).

1. Contents (mg/100 g) wet weight basis.

2. Contents (mg/100 g) dry weight basis.

Table 5. Effect of extraction ratio by hot water (65°C) on stevioside content and total soluble solids (TSS).

Extraction ratio (leaves/water w/v)	Stevioside %	Total soluble solids (TSS) %
1:15	6.75 ^e ± 0.99	9.0 ^a ± 1.0
1:25	7.13 ^d ± 0.99	7.5 ^b ± 0.20
1:35	7.53 ^c ± 0.99	6.6 ^b ± 0.20
1:45	7.59 ^b ± 0.99	5.5 ^c ± 0.10
1:55	7.60 ^{ab} ± 0.99	5.0 ^d ± 1.0
1:65	7.61 ^a ± 0.99	4.4 ^{de} ± 0.20
1:75	7.60 ^{ab} ± 0.17	3.6 ^e ± 0.20
L.S.D at 5 %	1.72	0.98

- All values are means of triplicate determinations ± standard deviation (SD).

- Means within column with different letters are significantly different (p < 0.05).

various sweet preparations for combating iron deficiency as anemia which is the major nutritional disorder of developing countries.

Chlorophyll (A and B), carotenoids, total pigments and stevioside contents in stevia plants

Fresh and dried (sun drying method) *Stevia* plants were analyzed for chlorophyll (A and B), carotenoids, total pigments and stevioside (Table 4). It was found that sun dried plants showed reduction in the pigments content being 47.40, 41.32, 74.85 and 50.99% (on dry basis) with chlorophyll A and B, carotenoids and total pigments, respectively. These changes in the structure of these pigments turned the green colour into brown in sun drying. This also would affect the colour changes during extraction and purification of the sweetener. On the other hand, stevioside content was 1.77% in the fresh leaves and 7.50% in sun dried leaves. The reduction percent of stevioside after drying by sun was 0.81%.

Extraction and purification of stevioside sweetener from *Stevia* leaves

Water extraction and purification of stevioside

Table (5) shows that the more water used for extraction, the more stevioside extracted, but the concentration of stevioside in the extract decreased. Similar results obtained by Midmore and Rank (2006) who found that boiling water extraction can achieve 93 - 98% removal of stevioside.

The present investigation indicates that: There is an increase in amount of water added (1:15 - 1:75), an increase in stevioside content and a reduction in TSS were noticed. Regarding to the collected data, increasing the ratio to 1:35 gave yield of stevioside being 7.53%.

This level was increased by 11.56 and 5.61% of the extraction ratios 1:15 and 1:25 (leaves/water), respectively. However, after such ratio (1:35), slightly increase in stevioside content was noticed. Statistical analysis proved that slightly significant differences occur between the ratio of 1:35 and others (1:45 - 1:75).

Table 6. Effect of water extraction (1:35) and purification on the recovery and purity of stevioside produced.

Steps	pH	TSS %	Stevioside/ 100 g leaves	Recovery related to stevioside %	Depigmentation		Purity related to depigmentation %
					Carotenoids pre-moves at 420 nm %	Chlorophyll (A and B) pre-moves at 670 nm %	
A	5.8	6.6	7.53	-	-	-	-
B	9.6	4.6	7.31	97.08	84.65	41.92	63.29
C	8.7	4.0	6.62	87.92	90.18	64.30	77.24
D	6.4	3.6	6.16	81.81	93.42	77.51	85.47
E	6.1	3.0	6.04	80.21	97.86	97.26	97.56

A: Crude water extract (optimum ratio 1:35)

B: After Ca (OH)₂ filtration (1)

C: After Ca (OH)₂ filtration (2)

D: After ion exchange treatment (1)

E: After ion exchange treatment (2)

From the above results, it could be concluded that the optimum ratio was 1:35 in which the maximum stevioside content was obtained.

The water extracted stevioside at the optimum ratio (1:35) was purified by Ca (OH)₂ and ion exchange treatments. During and after purification, pH, TSS, stevioside, depigmentation and purity of stevioside were analyzed and the results reported in Table 6.

From the data presented in Table (6) it could be noticed that TSS stevioside content and the recovery of stevioside were decreased during the purification steps that is, after treatment with Ca (OH)₂ (B and C) and ion exchange treatment (D and E). Moreover, depigmentation as indication on the removal of carotenoids and chlorophylls was increased during such process.

Results showed that pigments present in the crude extract affected to great extent the purification process and also affected the purity of the produced stevioside. Therefore, the removal of carotenoids and chlorophylls A and B was increased as the purification steps preceded. The treatment with Ca (OH)₂ removed about 84.65,

90.18, 41.92 and 64.30% of carotenoids and chlorophylls A and B and increased to 93.42, 97.86 and 77.51, 97.26% after resin treatment. The purity of the stevioside as a factor of carotenoids and chlorophylls A and B removal was 63.29 and 77.24% after Ca (OH)₂ treatments and 85.47 and 97.56% after resin treatment, respectively. From the above results it could be concluded that the end of the purification steps, the recovery was 80.21% and purity was 97.56%.

Methanol extraction and purification of stevioside

From the results obtained, it could be notice that after methanol crude extract by (4:1 v/w), TSS was 3.6% declined to 1.5% after extracted by butanol (Table 7). Stevioside content was also decreased from 6.86 - 6.51% after butanol treatment. Meanwhile purity increased from 68.15 - 81.53% in methanol and butanol extract, respectively.

Methanol is preferable to extract stevioside and

showed better extraction ability for isolation of stevioside from *Stevia* leaves than water extraction. However, the purity of stevioside was lower than that produced by water extraction. These results are agreement with that reported by Jaroslav et al. (2007).

Methanol/water extraction and purification of stevioside

From the results showed in Table 8, it could be noticed that after methanol/water extract, TSS was 3.4% declined to 1.2% after butanol extract. Stevioside content was also decreased from 6.51 (after methanol/water extract) to 6.01% after butanol extract. Meanwhile purity increased from 67.29 - 79.81% after methanol/water extract and butanol extracted, respectively.

From the above results it could be concluded that the natural stevioside sweetener was extracted from the dried ground leaves by water, methanol and methanol/water. The highest recovery was detected with methanol extraction

Table 7. Effect of methanol extraction on the recovery and purity of stevioside produced.

Steps	pH	T.S.S%	Stevioside/ 100 g	Recovery related to stevioside %	Purity related to stevioside/ T.S.S%
1. Methanol extract	5.9	3.6	6.86	-	68.15
2. Butanol extract	5.6	1.5	6.51	94.90	81.53

Table 8. Effect of methanol /water extraction and purification on the recovery and purity of stevioside produced.

Steps	pH	T.S.S%	Stevioside/ 100 g	Recovery related to stevioside %	Purity related to stevioside/ T.S.S%
A. Methanol extract	5.5	3.4	6.51	-	67.29
B. Butanol extract	5.0	1.2	6.01	92.34	79.81

Table 9. Physical and chemical characteristics of stevioside syrup (water extract), stevioside powder (methanol extracts) and stevioside syrup (methanol and water extract).

Characteristics	Stevioside syrup (water extract)	Stevioside powder (methanol extracts)	Stevioside syrup (methanol and water extract)	L.S.D at 5 %
pH	5.90 ^a ± 0.51	5.72 ^b ± 0.02	5.50 ^c ± 0.03	5.06
Reducing sugar %	2.96 ^c ± 0.20	3.62 ^a ± 0.44	3.43 ^b ± 0.44	n.s
Non-reducing sugar %	1.16 ^c ± 0.56	3.08 ^a ± 0.31	2.28 ^b ± 0.45	0.64
Total sugars %	4.13 ^c ± 0.04	6.80 ^a ± 0.04	5.68 ^b ± 0.02	6.91
Viscosity (c.p)	4.04 ^a ± 0.48	-	3.53 ^a ± 0.17	0.59
Total soluble solids (T.S.S) %	69.70 ^b ± 0.20	-	70.50 ^a ± 0.03	0.23
Stevioside %	56.34 ^c ± 0.48	77.07 ^a ± 0.36	64.67 ^b ± 0.47	0.88
Purity related to T.S.S %	80.69 ^b ± 4.42	-	91.73 ^a ± 1.00	1.16
Optical rotation	-33 ^o	-36 ^o	-34 ^o	-
Melting point	-	195°C	-	-

- All values are means of triplicate determinations ± standard deviation (SD).

- Means within columns with different letters are significantly different (P < 0.05).

- n.s: non-significant.

(94.90%) followed by methanol/water extraction (92.34%) and water extraction (80.21%). However, the highest purity was found in the stevioside extracted by water (97.56%) followed by methanol (81.53%) and methanol/water (79.81%).

The difference in purity according to TSS and depigmentation could be attributed to the ability of water as a solvent to extract more soluble solids than methanol and methanol/water extraction. In addition such effect was also noticed concerning extraction of more pigments water extraction in case of water compared to methanol and methanol/water extraction. Therefore it was found that the purification needed more steps in case of water extraction while it was finished by two steps in case of methanol and methanol/water extraction. This indicated that methanol and methanol/water extraction were much easier and simple than water extraction. However, in respect to safety and it is of economically a great interest

to use water for extraction than methanol followed by butanol.

Physical and chemical characteristics of the extracted stevioside

Stevioside syrup (water extract)

Results shows that stevioside syrup contained 2.96, 1.16 and 4.13% reducing, non-reducing and total sugars, respectively (Table 9). Meanwhile, stevioside syrup contained 4.04 centipoises (c.p), 69.70, 56.34, 5.9, 80.69%, -33° for viscosity, TSS, stevioside content, pH, and purity related to TSS and optical rotation, respectively. On the other hand, DuBios and Stephenson (1985) found that the optical rotation stevioside syrup was -39°. Beside, Nishiyama et al. (1992) reported that

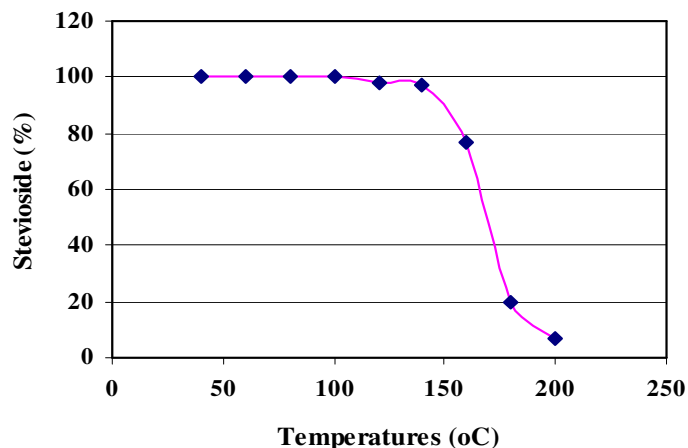


Figure 2. Stability and degradation rate of stevioside (50 mg) as a solid substance at elevated temperatures (40 - 200°C) for 1 h.

the crystals of stevioside were separated and purified until the formation of colourless crystals with $[\alpha]^{20}_D = -39^\circ$ (aqueous solution).

Stevioside powder (methanol extract)

Results shows that stevioside powder contains 3.62, 3.08 and 6.80% for reducing, non- reducing and total sugars respectively (Table 9). Also, stevioside powder had 77.07, -36° and 195°C for stevioside, optical rotation in water and melting point, respectively.

These results are in agreement with the previous results obtained by Soejarto et al. (1983) who found that the crystals melting point of stevioside was (196 - 202°C). On the other hand, Nishiyama et al. (1992) reported that melting point and optical rotation were markedly different from those of other sweeteners.

Stevioside syrup (methanol/water extract)

Results show that stevioside contained 3.43, 2.28 and 5.68 from reducing, non- reducing and total sugars, respectively (Table 9). Meanwhile stevioside contained 3.53 centipoises (c.p), 70.5, 64.67, 5.50, 91.73 and -34° for viscosity, (TSS), stevioside, pH, purity related to TSS and optical rotation, respectively. On the other hand, DuBios and Stephenson (1985) found that the optical rotation was -39° . However, Nishiyama et al. (1992) reported that the crystals of stevioside were separated and purified until the formation of colourless crystals with $[\alpha]^{20}_D = -39^\circ$ (aqueous solution).

So, it could be concluded that significant difference ($p < 0.05$) was observed between the three extracted methods of stevioside for physical and chemical characteristics. The highest stevioside percent (77.07%) was detected

with stevioside powder and the highest purity (91.73%) was found with stevioside syrup extracted by methanol and water. Methanol/water extraction was much easier and simple than water extraction. However, in respect to safety and it is of economically a great interest to use water for extraction than methanol.

Stability of stevioside at elevated temperatures

Incubation of the solid sweetener stevioside at elevated temperatures for 1 h showed good stability up to 120°C, whilst at temperatures exceeding 140°C forced decomposition was seen which resulted in total decomposition by heating at 200°C (Figure 2). As a consequence, the application of stevioside as a sweetening agent might not be suitable or recommended in baking or other processes requiring high temperatures. Crammer and Ikan (1987) expressed that since stevioside is stable at 95°C it is a suitable sweet additive to cooked or baked foods. On the other hand, Chang and Cook (1983) reported that Stevia sweeteners have high heat stability after one hour heating at 100°C. Beside, Tanaka (1988) who reported that Stevioside and rebaudioside A are reasonably thermally stable under the elevated temperatures used in food processing and do not undergo browning or caramelization when heated.

Stability of stevioside at different pH at elevated temperatures

In aqueous solution stevioside is remarkably stable over a wide range of pH values and temperatures. Under thermal treatment in a pH range of 1 - 10 over 2 h, practically no degradation of stevioside could be observed at 60°C and only slight losses up to 5% (pH 2 and 10) occurred on heating to a temperature of 80°C. Under strong acidic conditions (pH 1.0) forced decomposition of stevioside was observed which resulted in total decomposition after incubation at a temperature of 80°C for 2 h (Figure 3). These results are in accordance with that reported by Buckenhuskers and Omran (1997) who showed that the stevioside excellent heat stability is up to 100°C for 1 h at pH range 3 - 9, but rapid decomposition occurs at pH level greater than 9 under these conditions.

Stability of stevioside in organic acids

Stability of stevioside at room temperature in solutions of the organic acids (10 g/L), acetic acid, citric acid and tartaric acid over time periods of up to 4 months showed a tendency towards enhanced decomposition of the sweetener at lower pH values, depending on the acidic medium (Figure 4). In 10 g/L solution of acetic acid

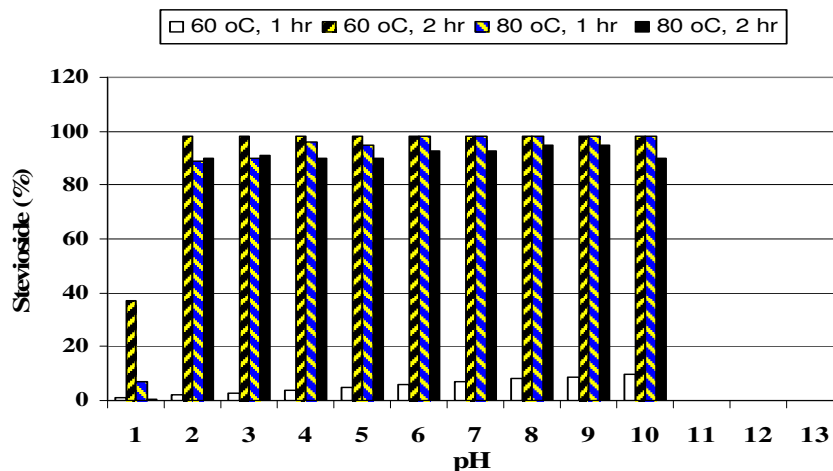


Figure 3. pH stability and degradation rate of stevioside (0.5 g/L water) in pH range of 1 - 10 under thermal treatment at 60 and 80°C for 1 and 2 h.

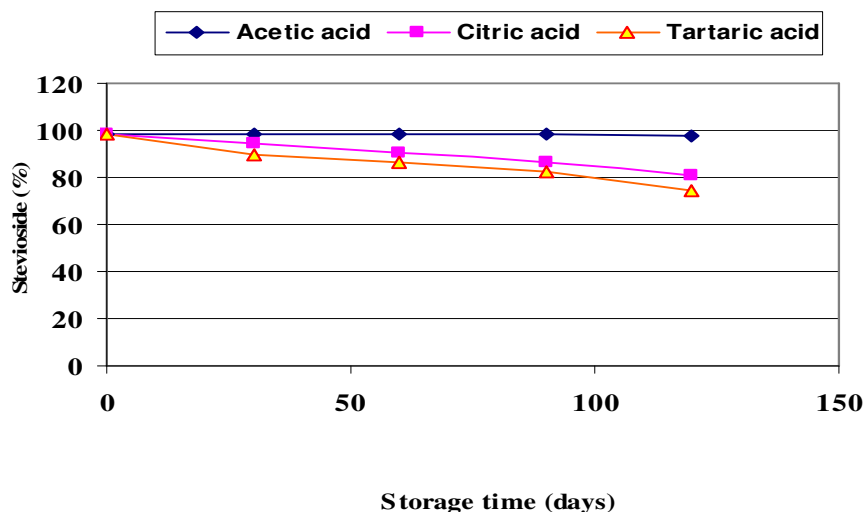


Figure 4. Degradation rate of stevioside of aqueous solution of organic acids (10 g/L) for storage periods of up to 4 months at room temperatures (25°C ± 2).

(pH 2.6), citric acid (pH 2.1) and tartaric acid (pH 2.1), losses in stevioside concentration of 2, 22, and 33% were observed after 4 months of storage, respectively. Similar results are observed by Buckenhuskers and Omran (1997) who showed that *Stevia* sweeteners in aqueous solutions are stable in acidic and neutral conditions but not in alkaline condition. Also stevioside seems to be relatively stable in food substances at slightly elevated temperatures. Considerable decomposition occurs at higher temperatures of storage (Shirokawa and Onishi, 1979). On the other hand, Chang and Cook (1983) reported that citric acid solutions (pH 2 - 4) of steviol glycosides are highly stable for at least 180 days at 20 °C. At elevated temperatures (80°C, in water, 8 h), however, the same samples showed 4 and 8% decomposition at pH 4.0 and 3.0, respectively. At 100°C, decomposition

was expectedly higher: 10 and 40%, respectively, pH 4.0 and pH 3.0. Also, at 100°C decomposition was 4% at pH 6.0, but increased to about 16% at pH 8.0. So in acid solutions, stevioside is highly stable. So far, it does not interact with other food components or to cause browning.

Determination of stevioside by HPLC

Samples of crude extraction stevioside syrup (water extraction), stevioside powder (methanol extraction) and stevioside extraction (methanol/water) were analyzed by HPLC as presented in Table 10. It is clear that the highest amount of stevioside 23.20% were obtained in methanol extract followed by methanol/water extract (4:1)

Table 10. Stevioside content determined by HPLC.

Item	Stevioside %
Water extract	15.82 ^c ± 1.0
Methanol extract	23.20 ^a ± 0.99
Methanol /water extract	18.62 ^b ± 1.0

-All values are means of triplicate determinations ± standard deviation (SD).

- Means within columns with different letters are significantly different ($p < 0.05$).

Table 11. Mean values of the organoleptic characteristics of stevioside sweetener.

Samples*	Organoleptic characteristics			
	Sweet acceptability	Bitter acceptability	Other acceptability	Bitter/sweet
Sucrose	19.82 ^a ± 3.29	2.10 ^c ± 0.03	0.55 ^c ± 0.05	0.87 ^c ± 0.58
Stevioside syrup	15.62 ^b ± 2.44	14.11 ^b ± 2.63	8.42 ^b ± 2.57	6.56 ^b ± 0.09
Stevioside powder	9.61 ^c ± 2.53	14.71 ^a ± 2.55	9.34 ^a ± 2.45	7.41 ^a ± 0.37
LSD at 5 %	2.52	2.34	2.32	2.78

* Samples were prepared at equal 5 % w/v sweeteners of sucrose.

- All values are means of triplicate determinations ± standard deviation (SD).

- Means within columns with different letters are significantly different ($P < 0.05$).

18.62% and water extract which gave the lowest amount of stevioside (15.82%). These results are in agreement with the reported of Liu et al. (1997), found that the dry leaves of *Stevia* with methanol extraction gave the highest yield of stevioside 27.5% followed by methanol/water extract and water extract (23.51 and 15.25%), respectively.

Solubility

The results obtained in this respect indicated that *Stevia* sweeteners were highly soluble in methanol and less soluble in water (about one gram *Stevia* sweeteners dissolved in 10.2 ml water). On the other hand, the *Stevia* sweeteners were slightly soluble in hexane, insoluble in acetone, chloroform and ether. The results obtained were in disagreement with the results reported of Crammer and Ikan (1987) and Soejarto et al. (1983). They found that the stevioside is weakly soluble in water and highly soluble in ethanol and insoluble in acetone, chloroform and ether. This is due to the *Stevia* sweeteners consisting of some different sweetener compounds.

Organolyptic characteristics of stevioside sweetener

The natural syrup and powder stevioside sweetener were organolyptically evaluated for sweet taste, bitter taste, other taste and bitter/sweet taste, and compared with sucrose. The mean values were statistically analyzed and presented in Table (11).

Results show a difference in the sweet acceptability

between syrup and powder stevioside and sucrose. This indicates that sucrose had the higher acceptability scores of sweet taste followed by syrup stevioside. Meanwhile, for bitter taste, the results showed no difference between the syrup and powder. However, a highly significant difference ($p < 0.05$) was found between the prepared sweetener and sucrose.

From the same table a highly difference was recorded in bitter/sweet between sucrose, syrup and powder stevioside. These results agreed with Yoshikawa et al. (1979) who found that stevioside had slight bitterness, some astringency, after taste and showed fairly low general acceptability among the 4 diterpene glycosides while rebaudioside A showed the lowest bitterness and astringency, and highest acceptability. Soejarto et al. (1983) also reported that the bitter taste common too many *Stevia* species was probably due to sesquiterpene lactones. It was also, suggested that volatile aromatic or essential oils, tannins and flavonoids, contribute to the unpleasant flavours associated with *Stevia*.

Conclusion

Reviewing above mentioned results obtained in this study, it could be concluded that *Stevia* plants are a good source of carbohydrates, protein, crude fiber, minerals, essential and non-essential amino acids which are vital for human nutrition and maintains a good health. Physical and chemical characteristics of the extracted stevioside as well as their stability at different temperatures and pH value, beside their organolyptic characteristics indicate that this sweetener could be applied as substitute of

sucrose in different drinks, beverages and bakery products.

REFERENCES

- Alaam AI (2007). Sugar crops council: Future view. The Proceeding of Thirty-eight Annual Conference, Egyptian Sugar Expertese Society Hawamdia, Egypt.
- Allam AI, Nassar AM, Besheite SY (2001). Nitrogen fertilizer requirement of *Stevia rebaudiana Bertoni* under Egyptian condition. Egypt J. Agric. Res. 79: 1018.
- A. O. A. C. (2000). Official Methods of Analysis of the Association of Official Analytical Chemists International 17th Ed. Published by the Association of Official Analytical Chemists International, Suite 400, 2200 Wilson Boulevard, Arlington, Virginia 22201-3301. USA.
- Brandle JSA (1998). *Stevia rebaudiana*: Its agricultural, biological, and chemical properties (Review). Can. J. Plant Sci. 78: 527-536.
- Buckenhusers HJ, Omran HT (1997). *Stevia rebaudiana Bertoni* and stevioside. Sugar and S. Subst. in Food Proc. Nutr., Ismailia, Oct. pp. 157-178.
- Chang SS, Cook JM (1983). Stability studies of stevioside and rebaudioside A in carbonated beverages. J. Agric. Food Chem. 31: 409-412.
- Choudhary KA, Bandyopadhyay NG (1999). Preliminary studies on the inorganic constituents of some indigenous hyperglycaemic herbs on oral glucose tolerance test. J. Ethnopharmacol., 64: 179-184.
- Crammer B, Ikan R (1987). Progress in chemistry and properties of rebaudiosides. In: Development in Sweeteners. 3. Applied Science Publishers, London, New York pp. 45-64.
- Dossier A (1999). Applications for using stevioside, extracted are refined from *Stevia rebaudiana Bertoni* leaves, as a sweetener. SCF Dossier EC 161.01 (1997), submitted by Specchiasol SRL, Italy.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956). Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28: 350-356.
- DuBois DE, Stephenson RA (1985). Diterpenoid sweeteners. Synthesis and sensory evaluation of stevioside analogues with improved organolyptic properties. J. Med. Chem. 28: 93-98.
- AO, WHO (1985). Food and Agriculture Organization of the United Nations L. World Health Organization Energy and Protein Requirements. Reports a Joint FAO/ WHO Export Consolation. Technical Report Series No. 724.
- Gisleine EC, Abdol HA, Caudio CA, Letícia de AFF, Gilson T, Mirian HT, Wilson EF, Roberto BB (2006). Investigation of the tolerability of oral Stevioside in Brazilian hyperlipidemic Patients. Brazilian Archives of Biol. and Technol. An International J. 49(4): 583-587, July 2006 ISSN 1516-8913 Printed in Brazil.
- Hayta M, Alpaslan M, Baysar A (2002). Effect of drying methods on functional properties of tarhana: a wheat flour- yogurt mixture. J. Food Sci. 67: 740-744.
- Jaroslav P, Barbora H, Tuulia H (2006). Characterization of *Stevia rebaudiana* by comprehensive two-dimensional liquid chromatography time-of-flight mass spectrometry. J. Chromatogr. doi: 10.1016/J.Chroma.04.050.
- Jaroslav PI, Elena V, Ostr PI, Karsek MR, Karolnka B, Pavla K Josef C (2007). Comparison of two different solvents employed for pressurized fluid extraction of stevioside from *Stevia rebaudiana*: methanol versus water. Anal. Bioanalytical Chem. 388: 1847-1857.
- Jimoh FO, Oladiji AT (2005). Preliminary Studies on *Piliostigma thonningii* seeds: Proximate analysis, mineral composition and phytochemical screening. Africani. Biotechnol. 4: 1439-1442.
- Kinghorn AD, Soejarto DD, Nanayakkara NPD, Compadre CM, Makapugay HC, Hovanec BJ M, Medon PJ, Kamath SK (1984). Phytochemical screening procedure for sweet ent-kaurene glycosides in the genus stevia. J. Nat. Prod. 47: 439-444.
- Liu J, Ong CP, Li SFY (1997). Subcritical fluid extraction of stevia sweeteners from *Stevia rebaudiana*. J. Chromatogr. Science 35: 446-450.
- Manish T, Rema S (2006). Preliminary Studies on *Stevia rebaudianci* Leaves Proximal Composition, Mineral Analysis and Phytochemical Screening. J. Med. Sci. 6: 321-326.
- Midmore JD, Rank AH (2006). An intense natural sweetener-laying the ground work for a new rural industry; May 2006 RIRDC Publication No 06/020 RIRDC Project No UCQ-17A.
- Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 420-428.
- Millipore Cooperative (1987). Liquid Chromatographic Analysis of Amino Acids in Foods Using a Modification of the PICO-TAG Method.
- Mohammad MR, Mohammad UD, Sher MM, Habib AN, Iqbal AQ (2007). *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana Bertoni*. Pak. J. Bot. 39: 2467-2474.
- Morita T, Fjuita M, Morita E (1978). Stevioside sweetener composition. Japanese Patent. 61: 202-667.
- Nguyen TH, Nguyen TM (1993). Determination and extraction of sweeten substance from Vitnamese stevia reboniana. Top. Chi. HoaHoc. 31: 39-40.
- Nikolai B, Oxana R, Alexander N (2001). Peculiarities of diterpenoid steviol glycoside production *in vitro* cultures of *Stevia rebaudiana Bertoni*. Plant Sci. 161: 155-163.
- Nishiyama P (1991). Correlation between total carbohydrate content and stevioside content in *Stevia rebaudiana* leaves. Arquivos de Biologia e Tecnologia 34: 3-4.
- Nishiyama P, Alvarez M, Vieira LG (1992). Quantitative analysis of stevioside in the leaves of *Stevia rebaudiana* by near infrared reflectance spectroscopy. J. Sci. Food Agric. 59: 277-281.
- Pearson D (1976). The Chemical Analysis of Food. 7th ed. Churchill Livingstone, Edinburg London and New York p. 75.
- Sanyude S (1990). Alternative sweeteners. Canadian Pharm. J. 123: 455-460.
- Savita SM, Sheela K, Sunanda S (2004). Stevia Rebaudiana-A functional Component for Food Industry. J. Hum. Ecol.15: 261-264.
- Shirokawa T, Onishi T (1979). Quantitative analysis of stevioside in soy sauce and hydrolyzate of vegetable protein. Kagawa-ken Hakka Shokuhin Shitenjo Hokoku 71: 35-39.
- Soejarto DD, Compadre CM, Medon PJ, Kamath SK, Kinghorn AD (1983). Potential sweetening agents of plant origin. II. Field search for sweet-tasting stevia species. Economic Botany 37: 71-79.
- Snedecor GW, Cochran WG (1980). "Statistical Methods", 7th Ed. Oxford and IBIT Public. Co.
- Tanaka O (1988). Isolation of sweetener from *Stevia rebaudiana*. Jpn. Kokai. 63: 177-764.
- Wei Y (1984). A new method for the determination of total glycosides in *Stevia rebaudiana*. Shipin kexue (Beijing). 43: 25-27.
- Wettstein DV (1959). Chlorophyll- letate and Der supmkroskopisch fromwecksee Der plastiden. Exp. Cell Res., 17: 427.
- Yoshikawa S, Ishima T, Katayama O (1979). Taste of components of stevioside. Am. Chem. Soc., 177(1): 74.