

## PHYSICO-CHEMICAL ASSESSMENT OF NATURAL SWEETENERS STEVIOSIDES PRODUCED FROM STEVIA *Reboudiana bertonii* PLANT

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### ABSTRACT

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 & Mg, 3.26 mg/100g d.w. and Cu, 0.73; Mn, 2.89; Fe, 5.89 & Zn, 1.26 mg/100g 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 to 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. Physical, chemical and organolyptic characteristics of extractions indicate that this sweetener has been applied as substitutes for sucrose in different drinks and baking products.

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

### INTRODUCTION

The plant *Stevia rebaudiana Bertoni* (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 Kilograms of Stevia sugar, annually.

Taking the sweetening powder of the Stevia sugar into consideration; these 400 Kilograms 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. Nowadays, the most common high intensity sweeteners on the world market 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. Stevioside is a diterpene glycoside present in *Stevia rebaudiana Bertoni*. Stevia has been used by the Guarani Paraguayan Indians and Mestizos as a general sweetening substance for centuries which part of the plant they use to consumption sweetener. Nowadays, Stevia plant and stevioside are being used as sweetener in South America, Asia, Japan, China, and some countries in Europe. 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 *Stevia 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-glucopyranosyl) 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 i.e. 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 *Stevia 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 i.e., 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. In view of the present study was conducted to reveal and shed more light on the effect of some technological process on the production of natural sweeteners steviosides from *Stevia rebaudiana Bretoni*, Besides, physico-chemical and organolytic properties of produced steviosides as substitute of sugar (sucrose) were studied.

## MATERIALS AND METHODS

### Materials:

- 1- **Stevia plant leaves:** The *Stevia* leaves (*Stevia rebaudiana Bretoni*), were obtained from *Stevia International Company* from Agro-industry Product (SICAP), Cairo, Egypt.
- 2- **Reagents:** Ion exchange resin (Amberlite IR-4B), organic acids and solvents were obtained from Sigma Chemical Co. (St. Louis, Mo).
- 3- **Sugar standards:** The standard of stevioside was obtained from M.B. Bio medical USA.
- 4- **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 25 – 30 °C for 24 - 48 hrs.

**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:

**1- 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 A.O.A.C (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.

**2- Amino acids determination:**

After hydrolysis of Stevia leaves with 6 N HCL at  $110^{\circ}\text{C}$  for 24 hrs, 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.

**3- Minerals determination:**

Mineral contents, i.e. copper (Cu), magnesium (Mg), manganese (Mn), iron (Fe) and zinc (Zn) were determined on aliquots of the solutions of the ash by established according to the method of A.O.A.C (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).

**4-Determination of total chlorophylls and carotenoids:**

Both chlorophylls (A&B) and carotenoids were determined in Stevia leaves before and after dehydration with different methods according to Wettstein (1959).

**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.

**1- 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 ( $65^{\circ}\text{C}$ ) 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 hrs 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)<sub>2</sub> (based on wt. of dried leaves). The addition of Ca (OH)<sub>2</sub> was repeated twice (filtrate B and C) and the filtrates were collected to 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 & colorless solution) containing stevioside was collected (in which pigments were adsorbed on resin) then concentrated by using rotary evaporator at 45 °C to the maximum concentration value. At each step total soluble solids (T.S.S), pH, Stevioside depegmentation and recovery were determined.

**2-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 hrs, 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.

**3- 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 hrs, 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) & purity during extraction and purification steps were determined by determination of pigments. The stevioside solution was prepared by water, methanol & methanol/ water for further studies of their physical and chemical characteristics. 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.

#### **Physical and chemical characteristics of stevioside:**

Physical and chemical characteristics, i.e. color, viscosity, total soluble solids (T.S.S), melting point, optical rotation, stability at different pH & 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 & methanol/water) as follow:

##### **1- Color measurement:**

The color 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).

**2- Viscosity:**

Viscosity of the stevioside solution was measured at  $27 \pm 1$  °C by using Brookfield viscometer, using No. 4 spindle at 20 rpm (Model DV. III Programmable Rheometer U.S.A) for stevioside syrup, at different shear rates (Hayta *et al.*, 2002).

**3- Total soluble solids (T.S.S):**

The total soluble solids (T.S.S) of stevioside samples were determined according to A.O.A.C (2000) at room temperature ( $25 \pm 1$  °C) expressed as °Brix (0 - 90), was determined with a land Refractometer (ATAGO, Japan).

**4- 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).

**5- Melting point:**

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

**6- 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).

**7- Stability of stevioside at elevated temperatures:**

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

**8- 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 hr. at different pH values from pH 1 to pH 10, which were individually adjusted by appropriate buffer systems. Losses in stevioside content were determined according to the method of Chang and Cook (1983).

**9- 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

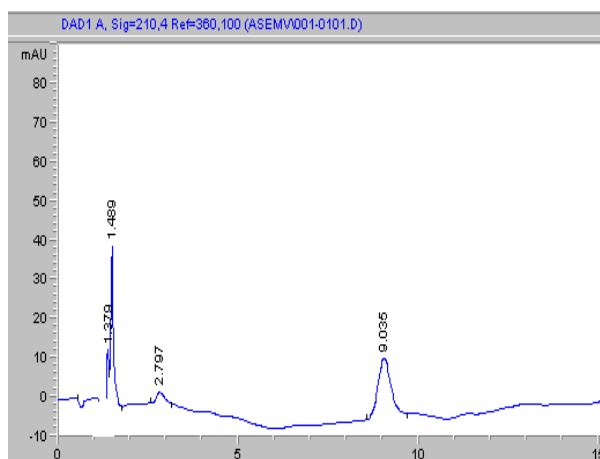
months to determine the stability of stevioside in organic acids according to Sanyude (1990).

**Determination of stevioside content:**

Total stevioside in dried powder leaves extracted by the three methods (water, methanol and methanol & 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 extracts was analyzed by High-Performance Liquid Chromatography (HPLC) as described elsewhere 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 x 4.6 mm I.D., 5  $\mu$ m, 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 (Fig.1.) and the detection limit was 0.1 $\mu$ g.



**Fig. 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 Nuclosil 100 C18 (25 cm x 4.6 mm I.D., 5  $\mu$ m).

**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 follow:

Properties	Score
Sweet taste	40
Bitter taste	40
Other taste	10
Sweet/ bitter	10
Total	100

**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 hrs were analyzed as follows:

**1- 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). Stevia leaves also contained 5.88, 9.77 and 15.65 % for reducing sugars, non- reducing sugars and total soluble carbohydrates, respectively They reported that protein, fat, crude fiber, ash, and carbohydrates were (6.2 to 20.42 %), (2.5 to 5.6 %), (13.56 to 18.5 %), (8.48 to 13.12 %) and (35.2 to 52.8 %), in this order. (Table 1).

**Table 1: Chemical composition of dried Stevia leaves (*Stevia rebusiana*)**

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

\* Calculated by difference.

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

**2- 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, therionine and isolucine) amounts 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 and methionine in Stevia leaves.

**Table 2: Essential and non- essential amino acid contents of Stevia leaves (*Stevia rebudiana*) (g/100g dry wt. basis).**

Essential-amino acids			Non essential-amino acids	
Amino acid	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
Isoleucine	0.42	0.28		
<b>Total essential amino acids</b>	<b>7.67</b>	<b>3.62</b>	<b>Total non-essential amino acids</b>	<b>3.72</b>

### 3- Mineral contents:

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).

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

Elements	Mineral content (mg/100 g weight basis)
<b>Macro minerals</b>	
1- Potassium (K)	21.15 <sup>a</sup> ± 0.35
2- Calcium (Ca)	17.70 <sup>b</sup> ± 0.20
3- Sodium (Na)	14.93 <sup>c</sup> ± 7.05
4- Magnesium (Mg)	3.26 <sup>e</sup> ± 4.58
<b>Micro (heavy metals)</b>	
5- Copper (Cu)	0.73 <sup>g</sup> ± 2.00
6- Manganese (Mn)	2.89 <sup>e</sup> ± 0.58
7- Iron (Fe)	5.89 <sup>d</sup> ± 0.34
8- Zinc (Zn)	1.26 <sup>h</sup> ± 4.72
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).

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

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

Fresh and dried (sun drying method) Stevia plants were analyzed for chlorophyll (A& 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 color into brown in sun drying. This also would affect the color 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 %.

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

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

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

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

**Extraction and purification of stevioside sweetener from Stevia leaves:  
1- 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.

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

Extraction ratio (Leaves/water w/v)	Stevioside %	Total soluble solids (T.S.S)%
1:15	6.75 <sup>e</sup> ± 0.99	9.0 <sup>a</sup> ± 1.0
1:25	7.13 <sup>d</sup> ± 0.99	7.5 <sup>b</sup> ± 0.20
1:35	7.53 <sup>c</sup> ± 0.99	6.6 <sup>b</sup> ± 0.20
1:45	7.59 <sup>b</sup> ± 0.99	5.5 <sup>c</sup> ± 0.10
1:55	7.60 <sup>ab</sup> ± 0.99	5.0 <sup>d</sup> ± 1.0
1:65	7.61 <sup>a</sup> ± 0.99	4.4 <sup>de</sup> ± 0.20
1:75	7.60 <sup>ab</sup> ± 0.17	3.6 <sup>e</sup> ± 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).

The present investigation indicates that the increasing of amount of water added (1:15 to 1:75) an increasing in stevioside content and a reduction in T.S.S. 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 to 1:75).

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)<sub>2</sub> and ion exchange treatments. During and after purification, pH, T.S.S, 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 T.S.S stevioside content and the recovery of stevioside were decreased during the purification steps i.e., after treatment with Ca (OH)<sub>2</sub> (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)<sub>2</sub> 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 steviosid as a factor of carotenoids and chlorophylls A and B removal was 63.29, and 77.24% after Ca (OH)<sub>2</sub> 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%.

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

Steps	pH	T.S.S %	Stevioside/ 100 g leaves	Recovery related to stevioside %	Depigmentation		Purity related to depigmentation %
					Carotenoids pre-moves at 420 nm %	Chlorophyll (A & 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)<sub>2</sub> filtration (1)

C: After Ca (OH)<sub>2</sub> filtration (2)

D: After ion exchange treatment (1)

E: After ion exchange treatment (2)

## 2- Methanol extraction and purification of stevioside:

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

**Table 7: Effect of methanol extraction on the recovery and purity of steviosid 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

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).

## 3- Methanol /water extraction and purification of stevioside:

From the results showed in Table (8) it could be noticed that after methanol/water extract, T.S.S was 3.4% declined to1.2% 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 to 79.81% after methanol/water extract and butanol extracted, respectively.

**Table 8: Effect of methanol /water extraction and purification on the recovery and purity of steviosid produced**

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

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 (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 T.S.S 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:**

##### **1- 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, T.S.S, stevioside content, pH, and purity related to T.S.S and optical rotation respectively. On the other hand, DuBios and Stephenson (1985) found that the optical rotation steviosid syrup was 39°. Beside, 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).

##### **2- 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.

### 3- 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, (T.S.S), stevioside, pH, purity related to T.S.S 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).

**Table 9: hysical 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 <sup>a</sup> ± 2.51	5.72 <sup>b</sup> ± 2.00	5.50 <sup>c</sup> ± 3.0	5.06
Reducing sugar %	2.96 <sup>c</sup> ± 0.20	3.62 <sup>a</sup> ± 0.44	3.43 <sup>b</sup> ± 0.44	n.s
Non-reducing sugar %	1.16 <sup>c</sup> ± 6.56	3.08 <sup>a</sup> ± 0.31	2.28 <sup>b</sup> ± 0.45	0.64
Total sugars %	4.13 <sup>c</sup> ± 4.0	6.80 <sup>a</sup> ± 4.0	5.68 <sup>b</sup> ± 2.0	6.91
Viscosity(c.p)	4.04 <sup>a</sup> ± 0.48	-	3.53 <sup>a</sup> ± 0.17	0.59
Total soluble solids (T.S.S) %	69.70 <sup>b</sup> ± 0.20	-	70.50 <sup>a</sup> ± 0.03	0.23
Stevioside %	56.34 <sup>c</sup> ± 0.48	77.07 <sup>a</sup> ± 0.36	64.67 <sup>b</sup> ± 0.47	0.88
Purity related to T.S.S %	80.69 <sup>b</sup> ± 4.42	-	91.73 <sup>a</sup> ± 1.00	1.16
Optical rotation	-33°	-36°	-34 °	-
Melting point	-	195°C	-	-

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

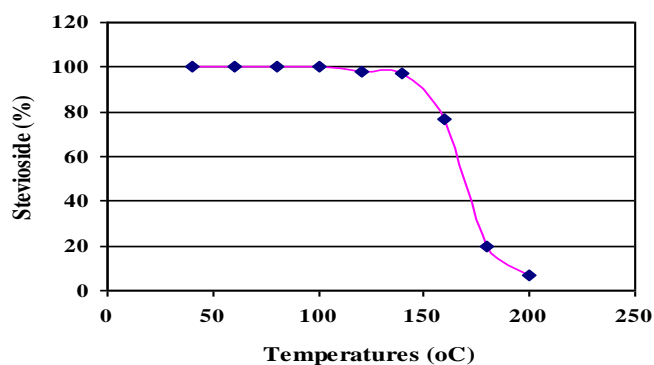
- Means within columns with different letters are significantly different (P < 0.05).

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 hr showed good stability up to 120 °C, whilst at temperatures exceeding 140°C forced decomposition was seen which resulted in total decomposition

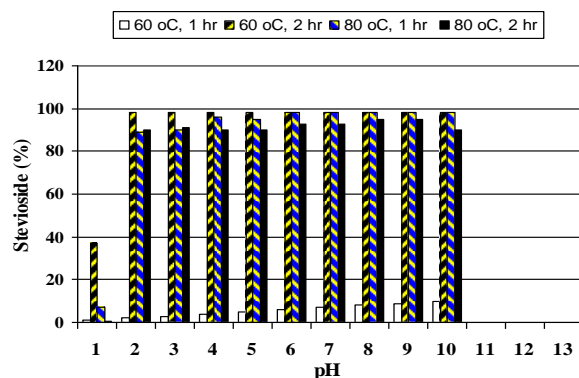
by heating at 200 °C (**Fig.2**). As a consequence, the application of stevioside as a sweetening agent might not be suitable or recommended in baking or other processes requiring high temperatures. Crammer and Ikan (1987) expressed that since stevioside is stable at 95 °C it is a suitable sweet additive 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.



**Fig. 2: Stability and degradation rate of stevioside (50 mg) as a solid substance at elevated temperatures (40 – 200 °C) for 1 hr.**

**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 hr 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 hr (Fig.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 hr at pH range 3-9, but rapid decomposition occurs at pH level greater than 9 under these conditions.

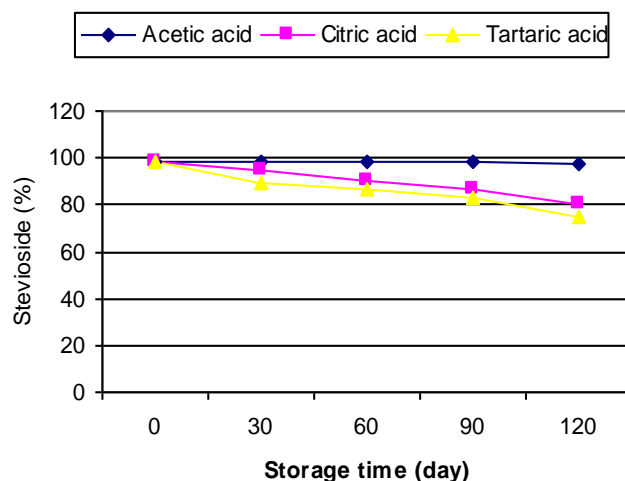


**Fig. 3. pH stability and degradation rate of stevioside (0.5 g/L water) in pH range of 1-10 under thermal treatment at 60 and 80 °C for 1 and 2 h.**

#### 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 (Fig. 4). In 10 g/L solution of acetic acid (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 is not interact with other food components or to cause browning





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

#### **Determination of stevioside by HPLC:**

Samples of crude extraction stevioside syrup (water extraction), stevioside powder (methanol extraction) and stevioside extraction with (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) 18.62 % and water extract which gave the lowest amount of stevioside (15.82 %). These results are agreement with those reported by (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.

**Table 10: Stevioside content determined by HPLC**

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

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

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

#### **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 by 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. These due to the Stevia sweeteners consist 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.

**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 <sup>a</sup> ± 3.29	2.10 <sup>c</sup> ± 0.03	0.55 <sup>c</sup> ± 2.58	0.87 <sup>c</sup> ± 3.58
Stevioside syrup	15.62 <sup>b</sup> ± 2.44	14.11 <sup>b</sup> ± 2.63	8.42 <sup>b</sup> ± 2.57	6.56 <sup>b</sup> ± 3.09
Stevioside powder	9.61 <sup>c</sup> ± 2.53	14.71 <sup>a</sup> ± 2.55	9.34 <sup>a</sup> ± 2.45	7.41 <sup>a</sup> ± 3.37
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 ( $P < 0.05$ ).

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.

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### تقييم الخواص الطبيعية والكيميائية للمحليات الطبيعية (الأستيوسيد) المنتجة من نبات الاستيفيا

عصمت أنور أبو عرب وعزة أنور أبو عرب و فريال محمد أبو سالم  
قسم الصناعات الغذائية- المركز القومي للبحوث- الدقى- القاهرة- مصر

نبات الاستيفيا هو مصدر جيد للكربوهيدرات (٦١,٩٣٪ وزن جاف) والبروتين (١١,٤١٪ وزن جاف) والألياف الخام (١٥,٥٢٪ وزن جاف) والمعادن (بوتاسيوم ٢١,١٥، الكالسيوم ١٧,٧، الصوديوم ١٤,٩٣ والماغنيسيوم ٣,٢٦ ملجم/١٠٠ جم وزن جاف والنحاس ٠,٧٣، المنجنيز ٢,٨٩، الحديد ٥,٨٩ والزنك ١,٢٦ ملجم/١٠٠ جم وزن جاف) وأيضاً يحتوى على الأحماض الأمينية الأساسية حيث توجد بكميات أكبر من الموصى عليها بواسطة الـ FAO/ WHO وكذلك تحتوى على الأحماض الأمينية غير الأساسية. تم استخلاص الأستيوسيد وتنقيته من أوراق نبات الاستيفيا الجافة بواسطة ثلاث طرق: الأولى عن طريق الإستخلاص بالماء الساخن (٦٥ درجة مئوية) بنسب مختلفة من الأوراق: الماء (١:١٥) إلى (١:٧٥). فكانت النسبة المثلى للإستخلاص ١: ٣٥ والذي كان الحد الأقصى لمحتوى الأستيوسيد التى تم الحصول عليها كانت (٧,٥٣٪)، كان نسبة استرجاع الأستيوسيد ٨٠,٢١٪ وكانت النقاوة المتعلقة بإزالة الصبغات ٩٧,٥٦٪. الطريقة الثانية: عن طريق الإستخلاص بالميثانول بنسبة ١:٤ الميثانول /الأوراق وبعد الإستخلاص والتنقية، كان نسبة استرجاع الأستيوسيد ٩٤,٩٠٪ ونقاوة ٨١,٥٣٪. الطريقة الثالثة: الإستخلاص بواسطة خليط من الميثانول / الماء (٤: ١) وبعد الإستخلاص والتنقية كان نسبة استرجاع الأستيوسيد والنقاوة ٩٢,٣٤ و ٧٩,٨١٪ على التوالي. تم تقدير الخواص الطبيعية والكيميائية للأستيوسيد المستخلص بواسطة الطرق الثلاثة التى اشتملت عليها الدراسة. كما تم تحليل مستخلصات الأستيوسيد الثلاثة بواسطة جهاز الـ HPLC. فوجد أن أعلى كمية من الأستيوسيد كانت ٢٣,٢٠٪ التى تم الحصول عليها من الإستخلاص بالميثانول. أيضاً تم تقييم الخواص الحسية والتى تشير إلى أن هذا المحلى يمكن استخدامه كبديل لسكر القصب (السكروز) في المشروبات المختلفة ومنتجات المخابز.

### قام بتحكيم البحث

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