ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Free Calorie Sweetness and Antimicrobial Properties in *Stevia rebaudiana.*

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ABSTRACT

The development of healthy foods with fewer calories with antimicrobial activity is a must. *Stevia rebaudiana* Bertoni produce diterpene glycosides that are low calorie sweeteners, and having therapeutic properties with antimicrobial activity. The present data revealed that among seven extracts, vacuum concentrated methanol-water infusion (1:1) has highest antimicrobial activity against the eleven tested bacterial and fungal species, with *B. subtilis*, *B. cereus* being the most susceptible species. Combination between methanol-water extract of *S. rebaudiana* plus some plant essential oils resulted in 20 out of 55 synergistic cases with higher antimicrobial activity and lower MIC values than single treatment with either *Stevia* extract or essential oils. The combination of *Stevia* extract plus cinnamon oil was the most efficient antimicrobial mixture. *Stevia* extract revealed higher antimicrobial activity than the tested food preservatives when singly added. Combination between *Stevia* extract and food preservatives led to 12 of 55 synergistic cases with lower MIC values the single treatment. Application by adding *Stevia* extract in substitution of 75% of sucrose in commercial food product in Egyption market "Choco Spread" reduced the count of Enterobacteria, Coliform, yeast, molds, *S. aureus* and *Samonella sp*. to the permissible level in foods. The calories in "Choco Spread" decreased by 24.4% in *Stevia* containing "Choco Spread " than sucrose containing product. The taste and sweetener have been not changed in "Choco Spread" containing *Stevia* extract.

Keywords: *Stevia rebaudiana* Bertoin, Antimicrobial Activity, Essential Oils, Food Preservative, Choco Spread.

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ISSN: 0975-8585

INTRODUCTION

Stevia is a genus of about 200 species in the sunflower family Astereaceae (Mishra *et al*. 2010). The plant is perennial herb with extensive root system and brittle stems producing small elliptic leaves (Shock, 1982). Its cultivation has spread to major regions of the world including Canada, Asia, and Europe (Amzad-Hossian *et al*. 2010). Poor seed germination is one of the factors limiting large scale cultivation; consequently, propagation is a special concern for northern growers who must grow *Stevia* as an annual crop. *Stevia* is therefore usually propagated by cuttings which then easily rooted (Nakamura and Tamura, 1985).

The use of *Stevia rebaudiana* leaves extract as an antimicrobial and low calorie sweetener agent could be of immense benefit in restricting microbial contaminants and control calorie intake in the diet foods. Glycosides are compounds mainly found in plants. *Stevia*, the common name for the extract steviode from leaves of *S*. *rebaudiana* Bertoin, is new promising renewable food stuff in the world market as sweetener or sugar substitute (Anton *et al*. 2010). Furthermore the increase in incidence of new and re-emerging infectious disease and the development of resistance to the antibiotics in current use, make it urgent to discover new antibiotic compounds with novel mechanism of action. The screening of plant extracts are of great interest to scientists in the search for new drugs for effective treatment of several diseases.

S. *rebaudiana* leaves are commonly referred to as honey leaves, candy leaves and sweet leaves. This is due to the production of stevoil glycosides sweetening compounds (Brandle and Telmer, 2007). Stevioside is described as a glycoside comprising three glucose molecules attached to an aglycone to steviol moiety. Stevioside has the chemical formula of C_{38} H_{60} O_{18} and is an active compound and show remarkable stability in aqueous solutions over a wide range of pH values and temperature (Virendra and Kalpagam, 2008 and Abou-Arab *et al*. 2010). Other compounds were isolated including rebaudioside A, with a sweetening potency even higher than stevioside (Barrio-Canal *et al*, 2008). Steviosideis about 300 times sweetener than sucrose and can be particularly beneficial to those suffering from obesity, diabetes mellitus, heart disease and dental caries (Ghanta *et al*. 2007). Stevioside was reported to be the most abundant *Stevia* glycosides (4-13%) found in plant leaves. It is followed by rebaudioside A (2-4%), rebaudioside C (1-2%) and dulcoside (0.4-0.7 %) (Makapugay *et al*. 1984 and Geuns, 2003).

Japan was the first country in Asia to market stevioside as a sweetener in the food and drug industry. It applied as a substitute of sucrose in many industries (Pol *et al*. 2007). Stevioside, along with rebaudioside, steviol and isosteviol may also offer therapeutic benefit, as they have antihyperglycemic, antimicrobial, anti-hypersensitivities, anti-inflammatory, antitumor, diuretic and immunomodulatory effects (Chatsudlhipong and Muanprasat, 2009). Debanth, (2008) even claim that using *Stevia* help to prevent the onset of cold and flu. It used also traditionally in treating wounds, sores and gum diseases and also to treat yeast infections or reoccurring streptococcal infections.

In some studies, the antimicrobial activity of various extracts of *S*. *rebaudiana* (with water, acetone, chloroform, methanol, ethyl acetate or hexane) as solvent have been investigated and its effect on some selected organisms such as *Salmonella typhinorium*, *Aeromonas hydrophila*, *Vibrio cholerae*, *B. subtilis*, *Streptococcus aureus* and other have been studied (Debanth, 2008; Ghosh *et al*. 2008; Jagarman *et al*. 2008 and Seema, 2010).

S. *rebaudiana* contain non-caloric sweeteners, whose accumulation could exert beneficial effects on human health (Gardana *et al*. 2010). Regular consumption of these compounds decreases the content of sugar, radionuclides and cholesterol in the blood (Atteh *et al*. 2008). *S*. *rebaudiana* pure extract from leaves are commercially available in Japan, Korea, China, South East Asia and South America to sweet a variety of foods and beverages (Koyama *et al*. 2003). In USA *Stevia* powders are used as dietary supplement and skin care products.

- The aim of present investigation was to assess the antimicrobial activity of *S*. *rebaudiana* using different solvents and determination of MIC against some pathogenic bacteria and yeast.
- The present work exclusively deal with addition of *S*. *rebaudiana* extract to one of the commercial food product in Egyptian market "Choco Spread" as antimicrobial agent and as sucrose substitute which not being undertaken by earliest workers.

ISSN: 0975-8585

MATERIALS AND METHODS

Plant material:

Fresh leaves of *Stevia rebaudiana* Bertoni were collected from young plants grown in agriculture research center, Cairo, Egypt. Plant leaves were washed and dried at temperatures below 30°C to avoid decomposition of thermolabile compounds. It kept in dark to be protected from sunlight because of the potential for chemical to be transformed when exposed to ultraviolet radiation (Sarker *et al.* 2008).

Preparation of extracts:

Dry powdered *Stevia* leaves (100g) were extracted separately with methanol, ethanol, water and methanol (1:1). Soxhelet, boiling and infusion for 3 days at 4 °C were used as different methods for extraction. Extracts of different solvent were filtrated and concentrated under vacuum on a rotary evaporator. The water bath temperature used was below 40°C to prevent decomposition of thermolabile components (Sarker *et al.* 2008).

Determination of Antimicrobial activity of *Stevia* extracts by disc diffusion technique:

Nine bacterial isolates (*Staphlococcus aureus* ATCC 6538, *Enterococcus hirae* ATCC 10541, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 33495, *Kocuria rhizophila* ATCC 9341, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 13076, *Lactobacillus delbrueckii* NCTC 12712 & *Bacillus cereus* NCTC 7464) and two yeasts isolates (*Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763) were used for testing antimicrobial activity in *Stevia* extracts. These microorganisms were selected for microbial assay as they are common pathogens cause food spoilage. All the bacterial and yeast strains used for the experimental purpose were grown and maintained on nutrient agar medium. The bacterial and yeast, 1ml of 24h old culture suspension (10^{-6} CFU/ml) were seeded onto 100 ml of nutrient agar and mixed gently. Nutrient agar plates were prepared by pouring 20 ml of seeded agar in sterile Petri dishes and allow solidifying. Sterilized filter paper discs of 5 mm diameter supplemented with 5 µl of *Stevia* extracts were placed on the surface of inoculated plates and the diffusion occur by placing the plates for 1h in refrigerator at 5°C. The plates were incubated at 37°C for 24 hours. At the end of the period, inhibition zones formed on the medium were measured in mm (Harly Prescott, 2002).

Determination of Minimum Inhibitory Concentration (MIC):

The minimum inhibitory concentration (MIC) was determined by macrobroth dilution method. Crude *Stevia* extract or essential oils or preservatives were serially diluted two-fold in nutrient broth medium (2, 4, 8, 16, 32, 64, 128, 256 and 512 µg ml^{-1}). Duplicate tubes of each dilution were inoculated with $5x10^5$ cells (CFU) of the test organisms and the cultures incubated at 37°C for 18 hours. The recommended wavelength for measuring the absorbance of plate is 530 nm. MIC was taken as the highest dilution (least concentration) of extract showing no detectable growth in the broth culture (EUCAST, 2003 and Preethi *et al*. 2011).

Choco Spread product:

"Choco Spread" is one of the commercial food products in Egyption market and manufactured by El-Rashidi El Mizan Confectionery for food products. Ingredients of "Choco Spread": pure sesame paste, sugar, cocoa, hazelnut, vegetable oil, emulsifies, soya lecithin (E322), flavors.

Microbial analysis for Choco spread product:

The following microbial analysis was carried out in absence and presence of *Stevia* extract substituting 75% sucrose.

Total plate count:

10g from product added to 90 ml of sterilized maximum recovery diluent (0.1% peptone and 0.85% sodium chloride in distilled water) and mixed to make initial suspension (10^{-1} dilution). Other dilutions 10^{-2} and

10^{-3} were then prepared. Place 1ml of each dilution (10^{-1}, 10^{-2} & 10^{-3} dilutions) into each of two sterile Petri dishes. Add about 15 ml of molted plate count agar (PCA) tempered to 44-47°C to each plate. Each plate mixed well and after complete solidification incubated at 30°C for 72h **(ISO 4833:2003)**. After incubation the colonies counted on plates and the number of CFU/g of test sample is calculated

Yeasts and moulds count:

Place 1ml of the dilution (10^{-1}, 10^{-2} & 10^{-3}) into each of two sterile Petri dishes. Add about 15 ml of molted Dichloran 18% glycerol agar (DG18) tempered to 44-47°C to each plate. After complete solidification the plates incubated at 25°C for 5-7 days. After incubation count the colonies on plates **(ISO21527-2: 2008).**

***Enterobacteriaceae* count:**

Place 1ml of the dilution (10^{-1}, 10^{-2} & 10^{-3}) into each of two sterile Petri dishes. Pour into each Petri dish approximately 10 ml of violet red bile glucose media which has been prepared then cooled to 44- 47°C in water bath. Allow to solidify and incubate at 37°C for 24h. After incubation characteristic colonies which are pink to red or purple counted **(ISO 21528-2:2004)**.

Coliform count:

Place 1ml of the dilution (10^{-1}, 10^{-2} & 10^{-3}) into each of two sterile Petri dishes. Pour into each Petri dish approximately 15 ml of Crystal violet neutral red bile lactose agar (VRBL) which has been prepared then cooled to 44- 47°C in water bath. Allow solidification and incubate at 37°C for 24h. After incubation characteristic colonies are purplish red with diameter of least 0.5 mm counted **(ISO 832:2006).**

***Staphylococcus aureus* count:**

After preparing and sterilization of Baird-Parker agar base medium which used in detection and count of *S. aureus,* medium cooled to 47°C and 50 ml of egg-yolk tellurite emulsion added to 1L of base medium and mixed well. Appropriate quantity of the complete medium placed into sterile Petri dishes in order to obtain an agar thickness of about 4 mm, and allow solidifying. Transfer 1ml of the dilution (10^{-1}, 10^{-2} & 10^{-3}) into each of Baird-Parker agar plates and spread the inoculums over the surface of the agar plat and incubate plates for 24h at 37°C. After incubation count typical colonies (black or grey, shining and convex and surrounded by a clear zone) **(ISO 6888-1:1999)**.

Detection of *Salmonella spp*.:

25g from product sample added to 225 ml of sterilized Buffered peptone water broth medium and incubated at 37°C for 18 h. after incubation 0.1 ml of this culture transferred to 10 ml of Rappaport-Vassiliadis medium with soya (RVS broth) and incubated for 24h at 41°C. After incubation, the culture obtained in RVS broth were inoculated by means of a loop to the surface of one Petri dish containing XLD agar medium and incubated for 24h at 37°C, after incubation examine the plates for the presence of typical colonies of *Salmonella* (have a black center and lightly transparent zone of reddish) **(ISO6579:2002)**.

Chemical analysis for Choco spread product:

Determination of carbohydrates:

Carbohydrates determination by HPLC model Hp Hewlett Packared series 1100 and column used Hyper REZ XP carbohydrate Ca^{++} counter-ion, particle size 8µm. Column 300 x 7.7 mm, mobile phase water, flow rate 0.6 ml/min, detection RI and temperature 85°C were condition used in determination.

Determination of protein:

The sample is digested in H_2SO_4 to convert the protein N to $(NH_4)_2SO_4$ at a boiling point elevated by the addition of K_2SO_4 with a CU catalyst to enhanced the reaction rate. Ammonia is liberated by alkaline steam distillation and quantified titirimetrically with standardized acid **(AOAC: 2003)**.

Determination of fat by Soxhlet extractor apparatus:

This method involves the extraction of fat from food product by an exhaustive extraction with solvent using a Soxhlet extractor apparatus. Fat content in food product is reported as the difference between the weight of the flask before addition of the sample and after concentration of the sample, multiplied by 100% and divided by the original weight of the sample. It gives the percentage of fat from the sample. This calculation assumes 100% extraction of fat from the sample (**AOAC: 1990)**.

Taste evaluation for "Choco Spread" product:

Taste evaluation is the process of using the human five senses to analyze and evaluate a food product (ISO 6658:2005). Color, odor, texture, taste, and after taste for two samples ("Choco Spread" with stevia & "Choco Spread" with sugar) were assessed by random sample of Choco spread consumers and reported any significant difference between two Choco Spread samples.

RESULTS AND DISCUSSION

In the present study, among eight extractions to *S*. *rebaudiana* leaves, methanol- water infusion (1:1 v/v) had the highest potential antimicrobial activity. It affect 5 of 11 tested microorganisms (Table: 1). However when this extract subjected to concentration under vacuum, the spectrum of antimicrobial activity was broaden to affect the 11 tested microorganisms. *B. cereus*, *L. delbrue*ckii, *S. cerevisiae* and *B. subtilis* showed high susceptibilities to the vacuum concentrated methanol-*water extract. K. rhizophila, S. aureus* and *E. coli* achieved median sensitivity while, *K. pneumonia, C. albicans* and *S. typhemorum* achieved weak susceptibility. *Stevia* leaves extraction by water infusion or water decoction failed to exert any antimicrobial activity. The other extractions (ethanol/infusion, ethanol/soxhelet, methanol / infusion and methanol/soxhelet) selectively affect *B. subtilis, B. cereus and S. aureus*. We suggest that the dependence of the antimicrobial activity on the solvent and the extraction process was due to their potentiality to dissolve lipids in the cell wall of plant which results in increasing its permeability to the active metabolites. In relation to our results, the antimicrobial activity of the clonal propagated *S. rebaudiana* Bertoin against pathogenic bacterial and fungal strains, with chloroform and methanol extracts was the most potential (Debnath, 2008). Water extract of *Stevia* leaves showed activity against *B. subtilis*, *S. aureus*; methanol extract against *P. aeruginosa*, ethyl acetate and hexane against yeast (Manish *et al*. 2006). Petroleum ether extract from flower and leaves of *S*. *rebaudiana* gave very low MIC values against all tested pathogenic strains (Preeth *et al*. 2011). 250 ug/ml of *S*. *rebaudiana* leaves extracted by Petroleum ether completely inhibited the growth of *S. aureus*, *E. coli* and *Penicillium chrysogenum* (Ghosh *et al*. 2008). They concluded that *S*. *rebaudiana* Bertoni leaves may have a role to be used as pharmaceutical and/or preservative. The acetone extract of *S*. *rebaudiana* showed greater activity against *Epidermophyton* species and *C. albicans* (Jayaraman *et al*. 2008). Recently, Barba *et al*. (2014) found that the treatment of *Listeria monocytogenes* for 5 min with 2.5% (w/v) of *S*. *rebaudiana* extract succeeded in activating over 5 log cycles of the bacteria.

In this study, MIC determination of *S. rebaudiana* extract and 5 plant essential oils (cinnamon oil, basil, peppermint, ginger and clove) revealed that *Stevia* extract achieved lower MIC values than all essential oils (Table: 2). *Stevia* extract achieved lower MIC against *K. pneumonia*, *L. delbrueckii*, *B. subtilis*, *B*. *cereus*, *S*. *aureus*, *S. cerevisiae* and *C. albicans*. Cinnamon essential oil was efficient against *E. haira*, *E. coli*, *S. typhemorum* and *K. rhizophila* with low MIC. The essential oils of basil, peppermint, ginger and clove achieved high MIC values against all tested microorganisms (Table: 2).

Combination between *S*. *rebaudiana* extract plus essential oil (one by one) resulted in 20 of 55 synergistic cases with lower MIC and higher antimicrobial activities than the single treatments (Table: 3). Combination between *Stevia* extract and cinnamon oil gave 8 of 11 cases of synergy, followed by *Stevia* plus basil oil (6 of 11), *Stevia* plus clove (4 of 11) and *Stevia* plus peppermint (3 of 11) cases of synergy against microorganisms. Oppositely *Stevia* plus ginger were antagonistic to almost all microorganisms and elevate the MIC values than the single treatments.

ISSN: 0975-8585

Table (1): Antimicrobial activity of *Stevia rebaudiana*leaves extracted by different solvents and different methods.

Solvent used and extraction method	Antimicrobial activity Mean inhibition zone diameter (mm)										
	S. typhemorum	*E. coli*	*K. pneumonia*	*E. haira*	*K. rhizophila*	*B. subtilis*	*B. cereus*	*S. aureus*	*L. delbrueckii*	*S. cerevisiae*	*C. albicans*
Ethanol / Infusion(3 days)	– ve	– ve	– ve	– ve	– ve	8±0.58	12±0.58	13±0.58	– ve	– ve	– ve
Ethanol/ Soxhelet	– ve	– ve	– ve	– ve	– ve	8±0.58	11±0.58	11±1.0	– ve	– ve	– ve
Methanol / infusion (3days)	– ve	– ve	– ve	– ve	– ve	8±0.58	12±0.58	13±0.58	– ve	– ve	– ve
Methanol / Soxhelet	– ve	– ve	– ve	– ve	– ve	10±1.0	9±0.29	8±0.76	– ve	– ve	– ve
Methanol + water (1:1) / infusion (3days) (MWE)	– ve	8±0.58	– ve	12±0.58	– ve	12±0.76	14±0.58	15±0.0	– ve	– ve	– ve
Vaccum conc. (MWE)	9±1	8±0.58	10±1.15	12.5±1.3	13±0.50	16±0.89	18±0.76	12±1.04	17±1.04	16±0.58	8.5±0.5
Water / infusion (3 days)	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve
Water / decoction	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve	– ve

* -ve: no antimicrobial activity.

*Diffusion plate method was applied and the results were expressed as the inhibition zone diameter (mm) .

*Triplicates were assayed for each microorganism.

Table (2): MIC (ug/ mL) of *Stevia rebaudiania* extract and some essential oils tested singly against some microorganisms:

Treatment	MIC (ug/ ml)					
	Stevia rebaudiania CMWE	Cinnamon oil	Basil oil	Peppermint oil	Ginger oil	Clove oil
S. typhemorum	64	32	256	256	512	256
E. coli	128	32	256	512	512	128
K. pneumonia	128	256	512	512	512	256
E. haira	32	16	32	256	512	256
K. rhizophila	64	32	512	512	512	256
L. delbrueckii	16	32	512	512	512	256
B. subtilis	16	32	512	512	512	512
B. cereus	8	32	512	512	512	256
S. aureus	32	64	256	512	512	256
S. cerevisiae	32	128	512	512	512	256
C. albicans	32	128	512	512	512	256

*CMWE: Conc. methanol/ water extract.

*Microdilution broth method was applied.

*Triplicates were assayed for each microorganism.

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Table (3): MIC (ug/mL) and FICI values of *Stevia rebaudiania* in combination with some essential oils

Microorganism	S/C		S/B		S/P		S/G		S/Cl	
	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
S. typhemorum	4	S	32	I	32	I	512	A	512	I
E. coli	4	S	4	S	512	I	512	A	256	I
K. pneumonia	2	S	32	S	256	I	512	I	32	S
E. haira	4	S	16	I	4	A	256	I	32	I
K. rhizophila	16	I	32	I	256	S	512	A	32	I
L. delbrueckii	16	S	32	I	256	I	512	A	256	I
B. subtilis	2	I	2	S	2	I	32	I	2	S
B. cereus	2	S	2	S	2	S	32	A	2	S
S. aureus	4	S	32	I	256	S	512	A	32	I
S. cerevisiae	2	S	32	S	256	A	512	A	32	I
C. albicans	2	S	32	S	256	I	512	A	32	S

- S/C: *Stevia* + Cinnamon oil.
- S/B:*Stevia* +Basil oil.
- S/P:*Stevia* + Peppermint oil.
- S/G:*Stevia* + Ginger oil.
- S/Cd: *Stevia* + Clove oil.
- FICI: Functioal Inhibitory Concentration Index.
- FICI =

$$\frac{\text{MIC in combination}}{MIC_A} + \frac{\text{MICin combination}}{MIC_B}$$

- FICI of < 0.5 considered synergy (S), FICI of > 4 considered antagonism (A), and FICI of 0.5 to 4 considered no interaction or indifference (I).

* Microdilution broth method was applied.
* Triplicates were assayed for each microorganism.

Muanda *et al*. (2011) found that combination between essential oils plus extract of *S. rebaudiana* possess high antioxidants, anti-inflammatory and antimicrobial properties against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *A. niger* and *C. albicans*. It has been reported that antimicrobial activity of the extracts of *S. rebaudiana* is due to the flavonoids, aromatic acids, terpenoids, phenolic acids and ester content. HPLC indicated that ethanol extract contain carvacrol, caryophyllene, caryophyllene oxide, spathulenal, candinol, α-pinene, limonene, isopinocarveol and ibuprofen as a major components. Furthermore in water extract and methanol water extract the major components were quercetin dehydrate, protocatechnic acid, and quercetin glycosyl. All of these components are biologically active compounds (Ho *et al*. 2006). Plant essential oils especially ethanol extracts have recently gained popularity and scientific interest (Balunas and kinghora, 2005; Shibamato, 2007). The protective effects of plant extracts are due to the presence of several components which have distinct mechanisms of action such as phenolic compounds, vitamins, carotenoid (Tadhani *et al*. 2007 and Zhang and Wang, 2002). The essential oils of the plant varied to several factors; geographical condition, culture conditions, collection time, altitude, climate. All these factors can influence their composition and biological activities (Ozkan *et al*. 2010).

In this study, measurement of MIC of some food preservatives were done, the data in table (4) indicated selective activity against microorganisms achieved by different food preservative. Nisin efficiently inhibit the growth of *K. pneumonia*, *E. haira*, *K. rhizophila*, *L. delbrueckii*, *B. cereus* and *S. aureus* with low MIC ranged between 16-32ug/ml nisin. K-sorbate and Na-benzoate were highly active against *K. pneumonia*, *E. haira*, and *L. delbrueckii* with low MIC values. Conversely, EDTA and Na-metabisulfate showed low antimicrobial activity against the tested fungi.

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Table (4): MIC (ug/mL) values of food preservatives against some microorganisms:

Microorganism	MIC (ug/ml)				
	EDTA	Na - metabisulfate	k- sorbate	Na- benzoate	Nisin
S. typhemorum	256	256	128	128	128
E. coli	256	256	256	256	128
K. pneumonia	128	128	64	64	32
E. haira	512	128	64	32	16
K. rhizophila	128	128	256	128	64
L. delbrueckii	256	128	64	64	64
B. subtilis	512	512	256	512	128
B. cereus	512	512	512	64	32
S. aureus	256	128	128	256	64
S. cerevisiae	256	256	256	256	128
C. albicans	512	512	512	512	512

* Microdilution broth method was applied.
* Triplicates were assayed for each microorganism.

Combination between *S. rebaudiana* extract and food preservative (table: 5) resulted in 13 of 55 cases of synergy, while the remaining cases were of antagonistic or indifferent interactions. Interaction between *Stevia* extract plus nisin resulted in four synergistic cases against *S. typhi, E. coli, K. pneumonia* and *S. aureus*. The pathogenic bacteria *K. pneumonia* was highly affected by the synergistic interaction between *Stevia* extract plus all tested food preservatives. Dry *S. rebaudiana* leaves contain phenolic compounds, flavonoids and other component with beneficial effects on human health (Belda-Galbis *et al*. 2014). Synergistic effect was detected between *S. rebaudiana* and *Equisetum arvense* extract against *Aspergillus flavus* and *Fusarium verticilloides* growth and their aflatoxin production. Toxicological studies have shown that *Stevia* doesn't have mutagenic or carcinogenic effects. It has no allergic reaction when it used as food sweetener or preservative (Pol-Hohnova *et al*. 2007). Steviosides promoted a slight increase in the MIC of potassium sorbate preservative against *Zygosaccharomyses bailii* and *Z. roaxi.* However the main action of steviosides was the protected action on sorbate destruction. This action was essential to ensure that the "Preservatives Residual level" was higher than MIC of the preservative to prevent the growth of *Z. bacilli* and *Z. raaxii* during storage. The results revealed that steviosides represent low calorie sweet products and protect potassium sorbate from destruction and decrease browning food products (Hracek *et al*. 2010).

Table (5): MIC (ug/mL) and FICI values of *Stevia rebaudiania* in combination with some food preservatives

Microorganism	S/E		S/SM		S/SB		S/PS		S/N	
	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
S. typhemorum	32	I	32	I	16	S	16	S	8	S
E. coli	64	I	64	I	32	S	32	S	16	S
K. pneumonia	16	S	16	S	16	S	32	S	16	S
E. haira	128	A	128	A	256	A	128	A	64	A
K. rhizophila	64	A	64	A	64	I	32	A	16	I
L. delbrueckii	64	I	64	A	32	I	64	A	16	I
B. subtilis	128	A	128	A	128	A	64	A	32	A
B. cereus	16	A	16	I	16	I	16	I	16	I
S. aureus	32	I	32	I	16	I	16	S	16	S
S. cerevisiae	128	I	64	I	64	I	64	I	16	I
C. albicans	256	A	256	A	256	A	256	A	256	A

- S/E: *Stevia* + EDTA, S/SM: *Stevia* + Na-metabisulfate, S/SB: *Stevia* + Sod. Benzoate, S/PS: *Stevia* + Pot. sorbate, S/N: *Stevia* + Nisin. FICI: Functioal Inhibitory Concentration Index.
- FICI =

$$\frac{\text{MIC in combination}}{\text{MIC}_A} + \frac{\text{MIC in combination}}{\text{MIC}_B}$$

  - FICI of < 0.5 considered synergy (S), a FICI of > 4 considered antagonism (A), and FICI of 0.5 to 4 considered no interaction or indifference (I).

* Microdilution broth method was applied.
* Triplicates were assayed for each microorganism.

ISSN: 0975-8585

Today there is a trend to replace synthetic sweeteners (saccharin and aspartame) with natural ones, in this field, *Stevia* gains a great interest. *Stevia* extract from leaves of plant is 300 time sweetener than sucrose and also has low "Glyceamic Index" making it attractive for diabetic people (Geuns, 2003). The use of *Stevia* as food additive has permitted in USA since 2008 and in Europe in 2010 (Parischa, 2010). In the present work, *Stevia* powder was added to Choco spread (a product of El-Rashidy factory for food industry) substituting 75% of sucrose. It added as low calorie sweetener and as preservative. It was found that the count of enterobacteria, coliform, yeast, molds, *S. aureus* and *Samonella sp*. were reduced to the permissible level in food products (Table: 6). Studying the sucrose containing "Choco Spread" in comparison with *Stevia* containing one revealed a reduction in calories from 213 to 161 calorie, respectively, representing 32.29%. The soluble sugar content also reduced by 55.5% in *Stevia* containing "Choco spread". The sweetness had been affected with no bitterness in taste. Furthermore, the content of fats, protein, fibers and sodium remain unchanged in *Stevia* containing "Choco Spread" compared with sucrose containing product (Table:7).

Table (6): Microbiological study on "Choco spread" product after replacing 75% of sucrose by 1.5 g *Stevia* powder

Storage period	Total plate count (CFU/g)	Enterobacteria count (CFU/g)	Coliform count (CFU/g)	Yeast & mold count (CFU/g)	*S. aureus* count (CFU/g)	*Salmonella* count (CFU/g)
24h	8000	30	< 10	< 10	< 10	– ve
One week	5000	< 10	< 10	< 10	< 10	– ve
One month	2000	< 10	< 10	< 10	< 10	– ve
3 month	1000	< 10	< 10	< 10	< 10	– ve
6 month	900	< 10	< 10	< 10	< 10	– ve

* Stevia powder added as sweetener and preservative.
* Choco Spread product is manufactured by El-Rashidi El Mizan Confectionery for food products, Egypt.
* -ve : not present

Table (7): Nutritive value for "Choco Spread" after replacing 75% of sucrose by 1.5 g *Stevia* powder

Content	Nutritive value / 40g	
	Choco Spread (with *Stevia* powder)	Choco Spread (with sucrose)
Fats	10.4±0.06 g	10.4±0.1 g
Sodium	34±0.58 mg	34±0.29 mg
Carbohydrates	10.4±0.15 g	23.4±0.58 g
Total sugars	8.92±0.08 g	21.6±0.57 g
Fibers	0.4±0.04 g	0.4±0.04 g
Protein	6.5±0.26 g	6.5±0.21 g
Energy	161 calories	213 calories
Energy from fats	94 calories	94 calories

*Energy calculation: Energy = (calories from fat) + (calories from protein) + (calories from sugars).
*1gm fat = 9 calories, 1gm protein = 5.3 calories & 1gm sugar = 3.95 calories.

FDA (2008) stated that rebaudioside A from *S. rebaudiana* can be considered as "Generally Recognized As Safe" (GRAS). The steviod glycosides are currently in use as sweetener in soft drinks or fruit drinks. The natural sweeteners of *S. rebaudiana* leaves called steviod glycosides, are diterpenes, isolated and identified as steviosides A, B, C, D, E & F (Goyal *et al*. 2010). Sheela (2004) calculated an energy value in dry leaves of *S. rebaudiana* to be 2.7 kcal/g. so it considered a low caloric sweetener as compared with chemical sweeteners such as aspartame (4 kcal/g), saccharine (calorie free) and sucralose (5 Kcal/g).

Stevia powder is used safely in deserts, sauces, bread, biscuits, cereals, yoghurt, soy sauce and sea food (Wallin, 2007; Koyama, 2003and Tadhani and Subush, 2006). The bactericidal activity of fermented hot water extract from *S. rebaudiana* Bertoni towards enterohaemorrhagic *E. coli* and other food borne pathogenic bacteria such as *Salmonella typhinorium, B. subtilis and S. aureus* have been investigated by (Debnath, 2008 and Ghosh *et al*. 2008). Hot water appeared to be the preferred extraction medium in food industry, since the bitter tasting of rebaudioside A was less soluble than stevioside in water (Rank and Midmore, 2006).

CONCLUSION

The leaves of *S. rebaudiana* are likely to become the major source of high-potency sweetener for the growing natural food and pharmaceutical markets in the future, due to its safety, low calorie sweetening and other biological activities including antimicrobial activity.

ACKNOWLEDGEMENTS

I would like to acknowledge Cairo University – Faculty of Science for funding and facilitating the accomplishment of this research work.

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