J. of Food and Dairy Sciences, Vol. 1 (4): 209 - 224, 2010

EVALUATION OF BIOACTIVE COMPOUNDS OF *Stevia rebaudiana* LEAVES AND CALLUS

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ABSTRACT

Stevia rebaudiana is considered natural antioxidants, which contained phenolic compounds and flavonoides at levels 24.01 & 18.93 mg/g dry weight basis of leaves and 33.99 & 30.03 mg/g dry weight basis of callus, these substances have been suggested to play a preventive role for human health. Antioxidant activity of water and methanolic extracts of stevia leaves and callus equivalent to gallic acid (GA) and butylated hydroxyl anisole (BHA) were determined. GA was the stronger antioxidant in both water and methanol extracts than BHA. Antibacterial and antifungal of stevia leaves and callus extracted by six types of solvent (acetone, chloroform, hexane, methanol, ethyl acetate and water) were studied. Methanol and acetone extracts had greater antibacterial potential for the strains of *Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* However, acetone, chloroform, methanol and ethyl acetate had antifungal potential for *Asperigillus ochraceus, Asperigillus parasiticus, Asperigillus flavus* and *Fusarium*. The results indicate that stevia leaves and callus extracts may be an ideal candidate for further research into their uses for food preservation and pharmaceutical due to their antioxidants and antimicrobial activities.
Keywords: Antioxidants, antimicrobial, *stevia rebaudiana*, leaves, callus.

INTRODUCTION

Antioxidants are considered important nutraceuticals on account of many health benefits (Valko *et al.,* 2007). They are used to preserve food quality mainly prevention of oxidative deterioration of constituent of lipids. The most commonly used antioxidants at the present time are butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tetrt-butylhydroquinone (TBHQ) (Sherwin, 1990). However, BHA and BHT have suspected of being responsible for liver damage and carcinogenesis (Grice, 1988). Therefore, the development and utilization of more effective antioxidants of natural origin are desired (Oktay *et al.,* 2003). Natural antioxidants can protect the human body from free radicals and retard the progress rancidity in foods (Gülcin *et al*., 2003).

Consumption of antioxidant constituents reported to have protection against oxidative damage induced degenerative and pathological processes including ageing and cancer (Tadhani *et al*., 2007). Antioxidant properties have been studied in several plant species for the development of natural antioxidant formulations in the areas of food, medicine and cosmetics (Miliauskas *et al.,* 2004). However, chemical constituents of medicinal plants depending on several factors such as cultivation area, climatic conditions and genetic modification (Miliauskas *et al*., 2004).

Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have played a remarkable role in the traditional medicine of various countries. In recent years, the prevention of cancer and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants (Virgili *et al*., 2001). The protective effects of plant products are due to the presence of several components which have distinct mechanisms of action; some are enzymes and proteins and others are low molecular weight compounds such as vitamins (Head, 1998), carotenoids (Edge *et al.,* 1997), flavonoids (Zhang and Wang, 2002), anthocyanins and other phenolic compounds (Sanchez-Moreno *et al*., 1998).

In the past few years, there has been increasing interest in research into antioxidants such as phenolic compounds and antioxidant peptides, since they can protect the human body from free radicals and retard the progress of many chronic diseases (Gőktűrk Baydar *et al*., 2007).

Phenolic compounds are commonly found in both edible and non-edible plants. They are important in the plant for normal growth development and defense against infection and injury. The presence of phenolic compounds in injured plants may have an important effect on the oxidative stability and microbial safety. Although phenolic compounds do not have any known nutritional function, they may be important to human health because of their antioxidant potency (Hollman *et al.,* 1996). The importance of the antioxidant constituents in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers as the trend of the future is moving toward functional food with specific health effects (Kahkonen *et al.,* 1999). The beneficial health-related effects of certain phenols or their potential antioxidant properties, especially when these compounds are present in large quantities in foods, are of importance to consumers.

Antimicrobial are compounds protect the living organisms from microbial infection. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and reemerging infectious diseases and development of resistance to the antibiotics in current clinical use (Bauer *et al*., 2003). The screening of plant extracts has been of great interest to scientists in the search for new drugs for greater effective treatment of several diseases (Dimayuga and Garcia, 1991). Therefore, plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments (Erdogrul, 2002).

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, essential oils and other aromatic compounds (Kumar and Singh, 1984). In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavonoids (Scalbert, 1991 and Chung *et al*., 1998). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives

(Geissman, 1963). These compounds protect the plant from microbial infection and deterioration (Cowan, 1999). Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects. Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers (Birt *et al*., 2001).

The use of natural products with therapeutic properties has a long history, plant, animal, and mineral products were the main source of medicines (Kao, 1980). There are growing interests in using natural antioxidant and antimicrobial compounds, especially extracted from plants, for the preservation of foods. The plant used in the present study was *Stevia rebaudiana*, which is used traditionally for the source of natural sweetener. The dry extract from the leaves of *Stevia rebaudiana Bretoni* contains sweet diterpene glycosides, flavonoids, water-soluble chlorophylls and xanthophylls, hydroxynnamic acid (caffeic, chlorogenic, etc.), neutral water- soluble oligosaccharides, free sugars, amino acids, lipids, essential oils, and trace elements (Komissarenko *et al*., 1994; Esmat and Ferial 2009).

Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including antihypertensive (Lee *et al*., 2001), antihyperglycemic (Jeppesen *et al*., 2002) and anti-human rotavirus activities (Das *et al.,* 1992).

The best of our knowledge, there is no previous reported work on the antimicrobial activity of *Stevia rebaudiana* for comparison, except that of Tadhani and Subhash (2006).

Therefore, it is of great interest to evaluate the total phenolic compounds, flavonoids, total antioxidant as well as antimicrobial and antifungal activity of *Stevia rebaudiana* due to their beneficial effects on human health and their important in different industrial use.

MATERIALS AND METHODS

Materials:

1- Stevia plant leaves:

-*Stevia* plant leaves *(stevia rebaudiana Bertoni*) were obtained from Stevia International Company from Agro-industry Product (SICAP), Cairo, Egypt.

-Stevia plant leaves were dried in either direct sunlight at temperature ranged 25 to 30 ºC for 24 - 48 hrs and packed in polyethylene bags and stored at -18 °C in refrigerator until used.

2- Reagents:

Antioxidant standards of gallic acid (GA), butylated hydroxyl anisole (BHA), 1-naphtaleneacetic acid (NAA), 6-benzyladenine (BA), sucrose, agar, sodium carbonate, ethyl alcohol, mercuric chloride and formaldehyde as well as organic solvents (acetone, chloroform, hexane, methanol and ethyl acetate) were obtained from Sigma Chemical Co. (St. Louis, Mo). Dimethyl sulphoxide (DMSO) was purchased from Merck (Darmstadt, Germany). For bacterial and fungal assay, nutrient broth and agar, Mueller Hinton agar and potato dextrose agar oxide were used.

3- Bacterial strains:

Bacterial strains of *Listeria monocytogenes* V7 strain syrotype 1 milk isolated was obtained from the government of Food Science, Univ., Madison, USA. *Escherichia coli* 0157: H7 ETCC 6933, *Bacillus cereus* ETCC 33018, *Staphylococcus aureus* ETCC 20231, *Salmonella typhymerium* ETCC 14028 and *Pseudomonas aeruginosa* ETCC 9027 were obtained from American type culture collection (ATCC), Rockville, Maryland 20852, USA.

4- Fungal strains:

Fungal strains of *Aspergillus ochraceus, Aspergillus parasiticus, Aspergillus flavus, Aspergillus niger and Fusarium* were obtained from the Microbiological Research Center (MIRCEN), Fac. of Agric., Ain Shams Univ., Cairo, Egypt.

5- Induction of callus:

Fresh leaves of *Stevia rebaudiana* were surface sterilized which treated with70% ethyl alcohol for 2 min followed by treatment with 0.1% mercuric chloride for 2 min. Finally, the explants were washed with sterile distilled water successively three times and were inoculated on Murashige and Skoog (1962) medium supplemented with 2.0mg/L NAA (1-napthaleneacetic acid), 0.3mg/L BA (6-benzyladenine), 30g/L sucrose, 8g/L agar at pH 5.8 in glass tubes for the production of the callus according to Murashige and Skoog (1962). The cultures were incubated at 25 °C ± 2 for 16h photoperiod. Callus was subcultured onto fresh medium of the same composition for a period of six weeks before being analyzed. The callus were dried in air oven at temperature 40°C for 8 hrs then grounded in a Braun milled (KMM 30) to a powder and stored in plastic bags until required for further analysis.

Methods:

1-Total phenolic compounds:

Total phenolic compounds were estimated according to the method described by Meda *et al*. (2005). Known quantity of stevia leaves or callus powder was taken in100 mL conical flask and 25 mL 0.3N HCl in methanol was added, then kept on shaker at 150rpm for an hour. After shaking, crude extract was filtered through Whatman No.1 filter paper. The filtrate obtained was evaporated to dryness in a boiling water bath. To the residue, hot water was added and final volume was adjusted to 100mL with distilled water. From this, 1mL aliquot was taken in a test tube and 1mL each of Folin–Ciocalteu reagent (diluted 1:2) and 35% sodium carbonate were added and then mixed. After 10min, 2mL of distilled water was added and intensity of the color was recorded at 620nm in the UV spectrophotometer (model T80 x UVNIS Spectrometer PG Instruments Ltd) against the reagent blank. The content of total phenolic compounds was determined using a standard curve prepared with gallic acid.

2-Total flavonoids:

Total flavonoids compounds were determined according to the method described by Meda *et al.* (2005). Known quantity of stevia leaves or

callus powder was taken in100 mL conical flask. To this, 25mL 0.3N HCl in methanol was added and kept on shaker at 150rpm for an hour. After shaking, crude extract was filtered through Whatman No.1 filter paper. The filtrate obtained was evaporated to dryness in a boiling water bath. To the residue, hot water was added and final volume was adjusted to 100mL with distilled water. From this, 1mL aliquot was taken in a test tube and 1mL of 20% HCl and 0.5mL formaldehyde were added and the tubes were allowed to stand for overnight. After 24h the content was centrifuged (Model 2300 Herma) at 3000 xg for 20 min. and 1mL from supernatant was taken and treated as described for phenolic compounds.

3- Total antioxidant activities:

Sample preparation: For the preparation of sample, 150 mg of fine ground powder of stevia leaves or callus was taken in 250mL conical flasks and 50 mL water added then were kept on shaker at 150rpm for an hour. After removing flask, content were filtered using filter paper (Whatman No.1). Similarly, methanol extracts were also prepared. The filtrate was used directly for ferric reducing antioxidant power (FRAP) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay without storage.

Determination of DPPH radical scavenging activity:

The method was adopted from Sharma and Bhat (2009). Methanolic and water extracts of stevia leaves and callus were evaluated in terms of their hydrogen donating or radical scavenging ability using DPPH radical. For assay, 200µL filtrate was taken in test tubes and volume made up to 1mL with methanol. Three milliliters of the freshly prepared solution of DPPH (200mM) in methanol was added to the sample tube and mixed vigorously for 15s. The sample tube was then kept in a water bath at 37°C for 20 min. The absorbance of the sample was measured at 517nm by UV Spectrophotometer (model T80 x UVNIS Spectrometer PG Instruments Ltd)). Gallic acid and BHA were used as standard references. The DPPH radical scavenging effect was calculated as ‘‘inhibition of percentage’’ according to the following formula:

Inhibition of percentage (%) = $[A_{c\ (0)} – A_{a\ (t)}/ A_{c\ (0)}] \times 100$

Where: $A_{c\ (0)}$ is an absorbance of control DPPH solution at 0min and $A_{a\ (t)}$ is an absorbance of test sample after 20min.

FRAP assay:

Ferric reducing antioxidant power (FRAP) was determined according to the procedure described by Benzie and Strain (1996). The principle of this method is based on the reduction of a ferric-tripyridyl-triazine complex to its ferrous, colored form in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5mL of a 10mmol/L TPTZ (2,4,6- tripyridyl-s-triazine) solution in 40mmol/L HCl plus 2.5mL of 20mmol/L FeCl3 6H2O and 25mL of 0.3mol/L acetate buffer, pH 3.6 and was prepared freshly and warmed at 37°C. Aliquots of 40mL sample filtrate were mixed with 0.2mL distilled water and 1.8mL FRAP reagent and the absorbance of reaction mixture at 593nm was measured spectrophotometrically after incubation at 37°C for 10min.

Gallic acid, and BHA were used as the standard. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of mg of standard used per gram stevia leaves and callus on dry weight basis.

4. Antimicrobial and antifungal activity:

Test organisms:

Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhymerium, Bacillus cereus and Escherichia coli were used to test antibacterial activity while *Asperigillus ochraceus, Asperigillus parasiticus, Asperigillus flavus, Asperigillus niger and Fusarium* were used to assess antifungal activity.

Preparation of plant extract:

25g of air-dried powder of *Stevia rebaudiana* leaves and callus was immersed in 100 mL organic solvent (acetone, chloroform, hexane, methanol, ethyl acetate) and water separately in conical flask. It was incubated at room temperature (25 ± 2°C) for 48 hour at 150 rpm in orbital shaker. The suspension was filtered and concentrated to dryness at 40 °C in hot air oven. The extract was dissolved in 0.25 % Dimethyl Sulphoxide (DMSO, Merck) to concentration of 100 mg/mL (Jayaraman *et al*., 2008).

Assay for antibacterial activity:

Preparation of inoculum

Stock cultures were maintained at 4 °C on nutrient agar slants. Active cultures for experiments were prepared by transferring a loopful of culture to 10 mL of nutrient broth and incubated at 37 °C for 24 hours for bacterial proliferation.

Agar –well diffusion method:

Agar –well bioassay was employed for testing antibacterial activity of *Stevia rebaudiana* leaves (Lindat, 1962). Each extracts were made to a final concentration of 50 mg/mL 24 hour old cultures of test organisms (0.05 mL) were seeded onto Mueller Hinton agar plate and uniformly spread with a spreader. Wells (5 mm) were made in the agar plate with a sterile cork borer. The plant extract was introduced into the well and the plates were incubated at 37 °C for 24 hours. The antibacterial activity of the plant extract was determined by measuring the diameter of the inhibition zone. Controls contained only DiMethyl Sulfoxide (DMSO). The antibacterial assay for each of the extracts against all microorganisms tested was performed in triplicates.

Assay for antifungal activity:

Potato dextrose agar was prepared and 1 mL (50 mg/mL) of plant extract was added to the medium. After solidification a loopful of culture was placed in the centre of the plates and incubated at 25 °C for 4 days (Lindat, 1962). The growth of the fungal cultures was measured and compared with the respective control plates. The antifungal assay for each of the extracts against all microorganisms tested was performed in triplicates.

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 DISCUSSIONS

Antioxidant activity of *stevia rebaudiana*

The total phenolic compounds and flavonoids content of stevia leaves and callus were determined and data shown in Table (1). It could be noticed that the total phenolic compounds were 24.01 and 33.99 mg/g in stevia leaves and callus on dry weight basis, respectively. Flavonoid contents were 19.93 mg/g for stevia leaves and it was 30.03 mg/g for stevia callus on dry weight basis. These results indicate that the content of total phenolic compounds and flavonoids showed high significant differences (P< 0.05) in callus

Table 1: Total phenolic compounds and flavonoid contents of leaves and callus *Stevia rebaudiana*

Item	Contents (mg/g dry weight basis)		LSD at 5 %
	Stevia leaves	Stevia callus	
Total phenolic	$24.01^{b} \pm 0.29$	$33.99^{a} \pm 0.29$	0.66
Flavonoids	$19.93^{b} \pm 0.25$	$30.03^{a} \pm 0.44$	0.80

- All values are means of triplicate determinations ± standard deviation (SD).
- Means within columns with different letters are significantly different (P <0.05).

Compared to the leaves.

The ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996) are commonly used and are the representative method frequently used in various investigations. We selected the FRAP and DPPH assay to evaluate the antioxidant activities of the leaves and callus stevia.

Table 2. show the absorbance at 593nm against various concentrations of gallic acid and butylated hydroxyl anisole (BHA) as standards in water and methanol leaves and callus extracts, respectively. Gallic acid was the strongest antioxidant in both water and methanol extracts whereas BHA was proved to be a weak antioxidant in water and methanol extracts.

To the best of our knowledge and from the cited literature, this is first time that the antioxidant activity of stevia leaves and callus has been reported.

Methanolic extract of stevia leaves showed significantly (P<0.05) high antioxidant activity equivalent to gallic acid (10.77 mg equivalent) and BHA (34.75 mg equivalent).

Table 2: **Antioxidant activity of water and methanolic extracts of *Stevia rebaudiana* leaves and callus equivalent to gallic acid and butylated hydroxyl anisole (BHA)**

Item	Mg equivalent per gram on dry weight basis				LSD at 5 %
	Leave extract		Callus extract		
	Water	Methanolic	Water	Methanolic	
Gallic acid	$8.72^{c} \pm 0.13$	$10.77^{a} \pm 0.17$	$8.52^{c} \pm 0.25$	$9.65^{b} \pm 0.26$	0.32
BHA	$19.52^{b} \pm 0.49$	$34.75^{a} \pm 0.18$	$18.77^{c} \pm 0.13$	$17.60^{d} \pm 0.21$	0.54

- All values are means of triplicate determinations ± standard deviation (SD).
- Means within columns with different letters are significantly different (P < 0.05).

Water extract of stevia leaves and callus did not show significant difference in antioxidant activity when expressed in terms of BHA but it was significantly (P<0.05) high compared to the methanolic extract of leaves and callus. On expressing the antioxidant activity in terms of gallic acid as standards the methanolic extracts of leaf and callus showed slightly higher values whereas on expressing in terms of BHA the methanolic leaf extract showed higher value compared to the water extract.

DPPH radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. When DPPH radical is scavenged, the color of the reaction mixture changed from purple to yellow with decreasing of absorbance at wave length 517nm.

The inhibition percent of DPPH radical with different extracts of stevia leaves and callus were recorded (Table 3). This inhibition with water extracted of stevia leaves and callus were found to be 37.36% and 53.76%, respectively, whereas it was found to be 31.61% and 54.80% for methanolic extract of stevia leaves and callus, respectively (Fig. 1). The same table proved that IC_{50} was found to be 626.37 and 462.38 µg of leaves and callus samples for water extract whereas it was 683.90 and 451.38 µg of sample for methanolic extract of stevia leaves and callus, respectively. Inhibition percent of DPPH radical by water extract of stevia leaf was significantly (P<0.05) differ from methanol extract of leaf. Water and methanolic extract of callus showed a significantly (P<0.05) higher inhibition percent of DPPH radical compared to water and methanolic extract of stevia leaves In the DPPH assay, callus showed higher antioxidant activity compared to the stevia leaves.

Table 3: **The percent inhibition of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical with different extracts of Stevia leaves and callus**

Item	Leave extract		Callus extract		LSD at 5 %
	Water	Methanolic	Water	Methanolic	
Inhibition (%)	$37.36^{b} \pm 1.47$	$31.61^{c} \pm 0.90$	$53.76^{a} \pm 0.88$	$54.8^{a} \pm 1.12$	2.10
IC_{50} µg of sample	$626.37^{b} \pm 14.67$	$683.90^{a} \pm 8.79$	$462.38^{c} \pm 8.79$	$451.38^{c} \pm 11.23$	2.03

- All values are means of triplicate determinations ± standard deviation (SD)
- Means within columns with different letters are significantly different (P < 0.05).
- IC_{50}: Concentration required for 50 % inhibition

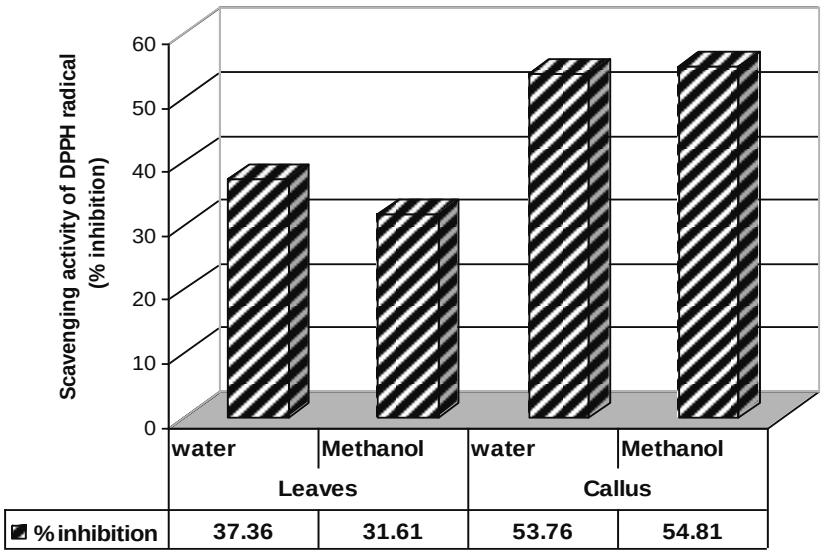

Fig. 1: Scavenging activity of water and methanolic extracts of *Stevia rebaudiana* leaves and callus against DPPH radical

Antibacterial activity of *Stevia rebaudiana*

The antibacterial activities of the solvents (acetone, chloroform, hexane, ethyl acetate and water) of *Stevia rebaudiana* leaves and callus extracts were showed significant variations ($P<0.05$) as shown in Table 4 and 5. Among the sixth extracts tested, methanol extract had greater antibacterial potential for the strains of *Listeria monocytogenes, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus* followed by acetone extract with the same strains. The inhibitory activity was measured by zone diameter (mm) of inhibition. Data in Table 4 showed that in leaves extracts, the largest zones of inhibition were observed against *Bacillus cereus* (25 and 20 mm) and *Listera monocytogenes* (20 and 11mm). However, *Staphylococcus aureus and Pseudomonas aeruginosa* were inhibition zone (16 and14 mm) and (15 and15 mm), respectively. On the other hand, leaves extracted by chloroform and hexane affected only on *Pseudomonas aeruginosa* which recorded 11 mm of inhibition zone. In contrast the other extracted leaves (by ethyl acetate and water) didn't affect on the studied strains. These results confirmed by statistical analysis which data showed highly significant differences ($P<0.05$) with methanol extract than others.

The antibacterial activities (the same strains) of the *Stevia rebaudiana* callus extracts showed significant variations as shown in Table 5. Methanol extract had greater antibacterial potential followed by acetone extract than other extracts. The inhibitory activity measured by zone of inhibition showed that the largest zone of inhibition was observed with methanol extract against *Staphylococcus aureus* (9 mm) followed by *Pseudomonas aeruginosa* (8 mm), *Listera monocytogenes* (6 mm) and *Bacillus cereus* (5 mm). Regarding to acetone extract, it was very effective against *Escherichia coli* (10 mm) followed by *Listera monocytogenes* (3 mm), *Staphylococcus aureus* (2 mm) and *Bacillus cereus* (2 mm). However, *Pseudomonas aeruginosa* didn't affect with callus extracted by acetone. In contrast as methanol extract. Regarding to chloroform, hexane and ethyl acetate, they affected only on *Escherichia coli* (2 mm), *Bacillus cereus* (10 mm) and *Staphylococcus aureus* (3 mm), respectively.

The higher antibacterial activities of the methanol and acetone extracts *may be due to the greater solubility of the extract in these organic* solvents (De Boer *et al*., 2005). On the other hand, Tadhani and Subhash (2006) showed that methanol extracts was effective against *Staphylococcus aureus* and *E. coli* which zones of inhibition were 8.33 and 13.0 mm, respectively. These variations in diameter zone as well as effects on *E. coli* with our results may be due the type of strains and their activities.

The water extract of *Stevia rebaudiana* leaves or callus were practically ineffective against the test organisms. This finding in similar to that of Tadhani and Subhash (2006) who also recorded very low antibacterial activity for water extracts of *Stevia rebaudiana* leaves. Several workers (Martin, 1995 and Vlietinck *et al*., 1995) have reported that water extracts do not have much activity against bacteria. However, the growth media also seems to play an important role in the determination of antibacterial activity (Lin *et al*., 1999).

Antifungal activity of *stevia rebaudiana*

The antifungal activities of the solvent extracts by (acetone, chlorophorm, hexane, ethyl acetate and water) of *Stevia rebaudiana* leaves and callus were varied significantly among the tested organisms (*Aspergillus ochraceus*, *Aspergillus parasiticus*, *Asprgillus flavus, Aspergillus niger* and *Fusarium* as shown in Table 6 and 7. The inhibitory activity measured by zone of inhibition. In leaves extract, data in Table 6 showed that the largest zones of inhibition were observed with methanol extract against *Fusarium* (15 mm) and *Aspergillus niger* (14 mm). However, *Aspergillus ochraceus, Aspergillus parasiticus* and *Asprgillus flavus* were the same inhibition zones (13 mm). This may be due to the greater solubility of the active principles in the solvent (Jayaraman *et al*., 2008).

Acetone, chloroform and ethyl acetate extracts showed variable inhibitory activity against different organisms. The highest inhibition zones were 15, 12 and 11 mm with *Aspergillus ochraceus,* in stevia leaves extracted by acetone, chloroform and ethyl acetate, respectively. The other strains revealed inhibition zone ranged from 9 to 11 mm.

Similar results obtained by Tadhani and Subhash (2006) who reported that the methanol and ethyl acetate extracts showed inhibitory activity against *Asprgillus niger*. Zones of inhibition were found to be 10 mm and 8.33 mm, respectively.

The antifungal activities of the solvents *Stevia rebaudiana* callus extracts also varied significantly (P<0.05) among the test organisms (Table 7). The largest zone of inhibition was observed with acetone extract against *Aspergillus parasiticus* (35 mm).

The chloroform extract showed higher inhibitory activity against *Aspergillus ochraceus* which zone of inhibition was 19 mm followed by *Aspergillus parasiticus* (15 mm). This may be due to the greater solubility of the active principles in the solvent (Jayaraman *et al*., 2008).

The methanol extract showed higher inhibitory activity against *Aspergillus ochraceus* which zone of inhibition was 16 mm followed by *Fusarium* (7 mm), and *Aspergillus flavus* (4 mm).

The hexane extract showed higher inhibitory activity against *Aspergillus flavus*, inhibition zone was (10 mm), followed by *Aspergillus ochraceus* (4 mm).

Similar results reported by Tadhani and Subhash (2006) who showed that the methanol extract caused inhibitory activity against *Asprgillus niger*. Zones of inhibition were found to be 10 mm and 8.33 mm, respectively.

Table 6: Antifungal activity of the *Stevia rebaudiana* leaves extracts

Test organism	Zone of Inhibition (mm)						
	Acetone	Chloroform	Hexane	Methanol	Ethyl acetate	Water	LSD at 5%
Aspergillus ochraceus	15.0^{a}±0.01	12.0 ^{bc}±0.01	-	13.0^{b}±0.01	11.0^{c}±0.01	-	0.15
Aspergillus parasiticus	10.0^{b}±0.01	11.0^{b} ±0.01	-	13.0^{a}±0.01	10.0^{b}±0.01	-	0.15
Aspergillus flavus	-	10.0^{b} ±0.01	-	13.0^{a}±0.01	10.0^{b}±0.01	-	0.13
Aspergillus niger	10.0^{b}±0.01	10.0^{b} ±0.01	-	14.0^{a}±0.01	10.0^{b}±0.01	-	0.15
Fusarium	9.0^{b}±0.01	10.0^{b} ±0.01	-	15.0^{a}±0.01	-	-	0.13

- All values are means of triplicate determinations ± standard deviation (SD).
- Means within columns with different letters are significantly different (P < 0.05).

Table 7: Antifungal activity of the *Stevia rebaudiana* callus extracts

Test organism	Zone of Inhibition (mm)						
	Acetone	Chloro-form	Hexane	Methanol	Ethyl acetate	Water	LSD at 5%
Aspergillus ochraceus	-	19.0 ^{a} ±0.01	4.0 ^{c} ±0.01	16.0 ^{b} ±0.01	-	-	1.26
Aspergillus parasiticus	3.05 ^{a} ±0.01	15.0 ^{b} ±0.01	3.0 ^{c} ±0.01	-	-	-	1.26
Aspergillus flavus		-	10.0 ^{a} ±0.01	4.0 ^{b} ±0.01	-	-	1.03
Aspergillus niger	-	-	-	-	-	-	-
Fusarium	-	-	-	7.0 ±0.01	-	-	0.73

- All values are means of triplicate determinations ± standard deviation (SD).
- Means within columns with different letters are significantly different (P < 0.05).

Conclusion

It could be concluded that *Stevia rebaudiana* is considered natural antioxidants, which contained phenolic compounds, flavonoids as well as gallic acid. These substances have been suggested to play a preventive role for human health. Also, The results of the present work indicate that stevia leave and callus extracts may be an ideal candidate for further research into their uses for food preservation as well as pharmaceutical and natural plant-based products due to their antimicrobial and antioxidant activities.

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تقييم المركبات النشطة حيوياً للأوراق والكالس من نبات الاستيفيا

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يعتبر نبات الاستيفيا مصدر من مضادات الأكسدة الطبيعية، والتى تحتوى على مركبات فينولية وفلافونويدات عند مستويات ٢٤,٠١و ١٨,٩٣مللجم/جم على أساس الوزن الجاف للأوراق و ٣٣,٩٩ و٣٠,٠٣ مللجم/جم على أساس الوزن الجاف للكالس، حيث تلعب هذه المواد دورا وقائيا لصحة الانسان٠ تم تقدير نشاط مضادات الأكسدة لكل من المستخلص المائى ومستخلص الميثانول للأوراق و الكالس من نبات الاستيفيا والتى تعادل حمض الجليك والبيوتيليتدهيدروكسى أنيسول (BHA)٠ وجد أن حمض الجليك كان أقوى مضاد أكسدة فى كل من المستخلص المائى ومستخلص الميثانول عن البيوتيليتدهيدروكسى أنيسول (BHA)٠كما تم تقدير النشاط كمضاد للبكتيريا والفطريات لكل من الأوراق و الكالس من نبات الاستيفيا والمستخلص بواسطة ستة أنواع من المذيبات (الأسيتون والكلوروفورم والهكسان والميثانول وخلات الإيثيل والماء)٠ لوحظ أن مستخلص الميثانول و الأسيتون كانت لها قدرة أكبر كمضاد للبكتيريا وخاصة سلالات *Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* بينما وجد أن مستخلص الأسيتون والكلوروفورم و الميثانول وخلات الإيثيل كانت لها قدرة أكبر كمضادة للفطريات وخاصة سلالات *Asperigillus ochraceus, Asperigillus parasiticus, Asperigillus flavus* and *Fusarium*.

وتشير النتائج الى أن مستخلص الأوراق و الكالس من نبات الاستيفيا قد يكون مثاليا للمزيد من البحث فى إستخداماتها فى حفظ الأغذية فضلا عن الأدوية بسبب نشاطهم كمضاد للأكسدة وكمضاد للميكروبات٠

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Table 4: Antibacterial activity of the *Stevia rebaudiana* leaves extracts

Test organism	Zone of Inhibition (mm)						
	Acetone	Chloroform	Hexane	Methanol	Ethyl acetate	Water	LSD at 5%
Listeria monocytogenes	17.0^{b}±0.01	-	-	20.0^{a}±0.01	-	-	0.10
Staphylococcus aureus	14.0^{b}±0.01	-	-	16.0^{a}±0.01	-	-	0.10
Pseudomonas aeruginosa	15.0^{a}±0.01	11.0^{b}±0.01	11.0^{b}±0.01	15.0^{a}±0.01	-	-	0.15
Salmonella typhymerium	-	-	-	-	-	-	-
Bacillus cereus	20.0^{b}±0.01	-	-	25.0^{a}±0.01	-	-	0.10
Escherichia coli	-	-	-	-	-	-	-

- All values are means of triplicate determinations ± standard deviation (SD).
- Means within columns with different letters are significantly different (P < 0.05).

Table 5: Antibacterial activity of the *Stevia rebaudiana* callus extracts

Test organism	Zone of Inhibition (mm)						
	Acetone	Chloroform	Hexane	Methanol	Ethyl acetate	Water	LSD at 5%
Listeria monocytogenes	3.0^{b}±0.01	-	-	6.0 ^{a} ±0.01	-	-	1.03
Staphylococcus aureus	2.0^{b} ±0.01	-	-	9.0 ^{a} ±0.01	3.0^{b}±0.01	-	1.26
Pseudomonas aeruginosa	-	-	-	8.0 ±0.01	-	-	0.73
Salmonella typhymerium	-	-	-	-	-	-	-
Bacillus cereus	2.0^{b} ±0.01	-	10.0^{bc}± 0.01	5.0 ^{a} ±0.01	-	-	1.03
Escherichia coli	10.0^{a}±0.01	2.0^{b} ±0.01	-	-	-	-	1.03

- All values are means of triplicate determinations ± standard deviation (SD).
- Means within columns with different letters are significantly different (P < 0.05).