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Research Article

Antioxidant Activity of Stevia(*Stevia rebaudiana*L.) Leaf Powder and A Commercial Stevioside Powder

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ABSTRACT

Stevia leaf powder (SLP) and a commercial stevioside powder (CSP) were analysed for their polyphenol content, isothermal sorption behavior and their antioxidant activity was evaluated by DPPH radical scavenging activity, ferric reducing power and ABTS assay. Polyphenol contents were higher (5.6%) in SLP than in CSP (2.3%). It was observed that the SLP was non-hygroscopic and CSP was hygroscopic in nature as seen from sorption isotherms. The equilibrium relative humidity of SLP and CSP respectively was 62 and 24%. The antioxidant activity of the methanolic extracts were determined and DPPH radical scavenging activity for SLP showed 30.33% for 20 µg/ml and 52.46% inhibition at 100 µg/ml, which were slightly higher compared to CSP stevioside, which showed 29.5% and 47.64% inhibition for similar concentrations.Higher ferric reducing power was seen in CSP (0.632) than SLP (0.166) for 100 µg.The antioxidant activity measured by ABTS assay also indicated higher activity (37.5% inhibition for10 µg/ml) for SLP than CSP (39.66% for 40 µg/ml).

Keywords: **S**tevia, Stevioside, methanol extract, total polyphenols, antioxidant activity

1. Introduction

The stevia (*Stevia rebaudiana*Bertoni) plant is known as sweet leaf, sugar leaf, honey leaf and candy leaf. The plant is a bushy shrub belonging to *Asteraceae* family and native of Paraguay. Historically the natives of Paraguay and Brazil have been using the leaves of stevia as a sweetening essence for tea and to make medicines more palatable. It is also used as a sweetener in food and beverages in Japan and China (Geuns, 2003). In the 1930's and 1940's stevia was introduced in UK and Russia as a sugar substitute and Japan has been using it extensively since early 1970 (Mindore& Rank, 2002).

The chemical, mineral, and amino acid content; antioxidant activity and stability of active components in stevia were also described. It was suggested that stevioside and rebaudiana A are two major sweetening agents that are thermostable even up to 200 °C and suitable for use in cooked foods (Lemus-Mondaca et al., 2012). The structure of the principal sweetening compounds was established as diterpenoid glycosides with molecular formula $C_{38}H_{60}O_{18}$. The stevioside is 150 to 300 times sweeter than sucrose with a strong residual flavor. Similarly, rebaudioside A has more sweetness about 250 to 400 times than the sucrose with a smaller residual flavor and has a wide range of food applications (Brandle, 1998; Kennelly, 2002; Starrat et al., 2002; Chang & Cook, 1983; Flavia et al., 2007; Kroyer, 1999; FDA, 2007; Kinghorn, 1998). The usage of safe and effective low-calorie intense sweeteners is gaining popularity in processed foods with increased consumer awareness on dietary calorie intakes. The Joint FAO/WHO Expert Committee on Food Additives reviewed the safety of steviol glycosides (steviol equivalents) and recommended an acceptable daily intake (ADI) limit of 4 mg/kg body weight/day (EFSA, 2010).Medicinal properties such as lowering of blood sugar, increased

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urination, and dilation of blood vessels which in turn help lowering blood pressure, anti-inflammatory activity, wound healing were reported for stevia leaf extracts. Antibacterial, antifungal, anti-inflammatory, antimicrobial, antiviral, antiyeast, cardiotonic, diuretic and anti- hyperglycemic effect of stevia leave extract have been reviewed (Curi et al., 1986).

Polyphenols such as flavonoids, catechins and anthocyanins are the major compounds, which provide flavor, colour and taste to fruits and vegetables and they are recognized as health promoters by way of their radical inhibition capacities (Elmastas et al., 2007; Klimczak et al., 2007; Mattila &Hellstrom, 2007; Iacopini et al., 2008). Phenolic components, particularly flavonoids play an important role in prevention of cancer and heart disease (Kahkonen et al., 1999). Stevia leaves possessing a total phenol content of 2.5% (on dry basis) exhibited an IC_{50} (50% inhibition) of DPPH radicals at 11.04 µg(Tadhani et al., 2007). High antioxidant activity was noticed for *Stevia rebaudiana*aqueous extract when compared to α-tocopherol, BHA and green tea extract in sardine oil and linoleic acid systems (Xi, et al., 1998). In vitro and in vivo experiments revealed that stevioside and rebaudioside A are not genotoxic (Benford et al., 2006). The aqueous and solvent extracts of stevia leaf were detected to contain terpenes, carvacrol, caryophyllene, caryophyllene oxide, spathulenol, cardinol, α-pinene, limonene, isopinocarveol along with ibuprofen and showed antioxidant, anti-inflammatory and antimicrobial activities (Muanda et al., 2011).

The objective of the present work was to assess the total polyphenol content and antioxidant activity of stevia leaf powder and a commercial stevioside powder. Further, the samples were assessed for storage behavior by determining their equilibrium relative humidity.

2. Materials and methods

2.1. Materials

Stevia leaf powder (SLP) was purchased from M/s Trishakthi Stevia Farms, Hyderabad, India. The commercial stevioside (90% pure) powder (CSP) was provided by M/s Stanpack Pharma Pvt. Ltd., Mumbai, India. Chemicals and solvents used in the study were of analytical grade and procured form M/s. S. D. Fine-Chem. Ltd., Mumbai, India. Potassium ferricyanide, DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were purchased from M/s Sigma-Aldrich Fine Chemicals, Bangalore, India.

2.2. Estimation of moisture content and bulk density

SLP and CSP were analysed for moisture content by determining the loss of weight on drying 5 g at 103 ± 2°C in an air oven for 16 h (Ranganna, 1986). The bulk density of SLP and CSP was measured by noting the volume occupied by 25 g sample in a 100 ml graduate cylinder.

2.3. Determination of sorption behavior

The sorption isotherms of SLP and CLP were determined by studying the relation between equilibrium moisture content (EMC) and relative humidity (RH). The samples were exposed to different RH conditions such as 10, 30, 50, 70, 90 and 100% maintained by using appropriate concentrations of sulphuric acid in airtight glass desiccators at room temperature (RT) of 28 ± 2°C. The samples were observed critically for changes in colour, lump formation and mold growth during the study. The samples were weighed at daily intervals till they attained constant weight. Based on the data generated, moisture sorption isotherms were plotted using EMC and the corresponding RH as x and y coordinates. (Ranganna, 1986) Nature of hygroscopicity and hygro-emmissivity of the powders were assessed using the initial moisture content (IMC). The critical moisture content (CMC) and the corresponding RH was identified by noting the appearance of lump, mold or discolouration during the study.

2.4. Preparation of methanol extract

SLP (5 g) was extracted using methanol (100 ml) on a magnetic shaker for 2 h at RT and CSP was dispersed in methanol. The aliquots were further diluted to obtain 1 mg/ml concentration. The extracts were centrifuged at 8000 rpm (Remi, Model C-30 BL, Mumbai, India) and the clear supernatant was collected for analyzing total polyphenols and antioxidant activity.

2.5. The total polyphenol content

The total polyphenol content in SLP and CSP was determined using Folin ciocalteu reagent (Sadasivam & Manickam, 1997). The methanol extract (0.5 ml) was added with 0.5 ml Folin–Ciocalteau reagent and 8 ml using distilled water. The contents were vortexed for 2 min and allowed to stand at RT for 5 min and 1 ml saturated sodium carbonate was added. The contents were again vortexed for 2 min and allowed at RT for 1 h. The intensity of colour developed was read at 675 nm and per cent total polyphenol content was calculated and expressed as gallic acid equivalents, g/100g.

2.6. DPPH radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured according to Theodore, Sivakumar and Kristinsson (2008). Varying quantities (20, 40, 60, 80 and100 µg) of SLP and CSP were taken in different test tubes and the volume was made up to 1 ml with methanol. DPPH (0.004%) 4 ml was added to the test tubes and the contents were incubated at RT for 30 min and the absorbance was read at 517 nm. The solution without sample was treated as control. The DPPH radical scavenging activity was compared with the activity of butylated hydroxytolulene (BHT) and the antioxidant activity was measured as percent inhibition.

2.7. Ferric reducing power

The ferric reducing power of the SLP and CSP was measured by taking methanol extract containing 20-100 µg/ml in different test tubes and added with 2.5 ml of phosphate buffer. The contents were mixed with

potassium ferricyanide (2.5 ml) 1% solution and incubated for 20 min at 50 °C. Then, 2.5ml of 10% tri-chloroacetic acid was added to each test tube and centrifuged at 8000 rpm for 10 min. The supernatant of 2.5 ml was mixed with 2.5 ml distilled water and 0.5 ml 0.1% ferric chloride and the absorbance was read at 700 nm. The reducing power activity was compared with those of BHT according to a reported method in the literature (Theodore et al., 2008).

2.8. ABTS assay

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)assay was carried out according to Mandana et al. (2012) with minor modifications. The ABTS solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulphate and then incubated in the dark at RT for 16 h. The mixture was diluted with 80% (v/v) methanol to obtain an absorbance of 0.700 at 734 nm. ABTS solution (3.9 ml) was mixed with 0.1 ml sample containing of 20-100 µg SLP or CSP and shaken vigorously. The blank was prepared using methanol instead of samples and its absorbance was recorded at 734 nm after 10 min and the ABTS (%) was calculated. The ABTS antioxidant activity was compared with BHT.

2.9. Statistical analysis

Experiments were carried out in triplicate and the results are expressed as mean values with standard deviation were computed using MS Excel 2007.

3. Result and discussion

3.1. Experimental sorption isotherms

Photographs of stevia leaf powder (SLP) and commercial stevioside powder (CSP) used in the study are presented Figure 1. Data on moisture content and bulk density of SLP and CSP are presented in Table 1. Moisture content was found to be 8.19 and 4.09% in SLP and CSP respectively. The bulk density of SLP was higher (0.46 g/cc) than CSP (0.32 g/cc). Earlier, the bulk density of stevia leaf was estimated to be 0.44 g/ml (Mishra et al., 2010). Experimental sorption isotherms of SLP and CSP are presented in Figure 2. SLP gained moisture (42.5%) rapidly above 90% relative humidity (RH), whereas CSP gained moisture (15%) gradually up to 100% RH. Initial moisture contents (IMC) of 8.19 and 4.09% of SLP and CSP, were equilibrated at 68 and 24% RH at room temperature respectively. Similarly, the CMC of SLP and CSP were noted as 10.02 and 12.5%, which were equilibrated at 84 and 91% RH respectively. It was observed that SLP was more shelf-stable than CSP at RT. The low CMC of SLP may be due to the presence of proteins and carbohydrates, which are more susceptible to microorganisms, where these are absent in CSP. The results indicated that CSP is hygroscopic in nature and should be packed with high moisture barrier properties for storage at RT. The IMC of 7.2%, which equilibrated at 51% RH at room temperature for wood apple seed powder, was reported by Narsing Rao et al. (2011). The CMC was found to be 9.9%, which equilibrated at 69% RH. They concluded that the moisture content increased sharply at higher RHs beyond 70% indicating that the non-hygroscopic in nature of seed powder and can be stable at room temperature in polyethylene (PE) pouch, which is good congruent with SLP.

Fig1. (a) *Stevia rebaudiana* leaf powder (b) commercial stevioside powder

Table 1. Physico-chemical analysis of stevia leaf powder (SLP) and commercial stevioside powder (CSP)

Parameter	SLP	CSP
Moisture (%)	8.19 ± 0.24	4.09 ± 0.20
Bulk density (g/cc)	0.46 ± 0.02	0.32 ± 0.01
Total polyphenols (%)	5.60 ± 0.09	2.26 ± 0.11

[a]Values are average of triplicate analysis with ± SD

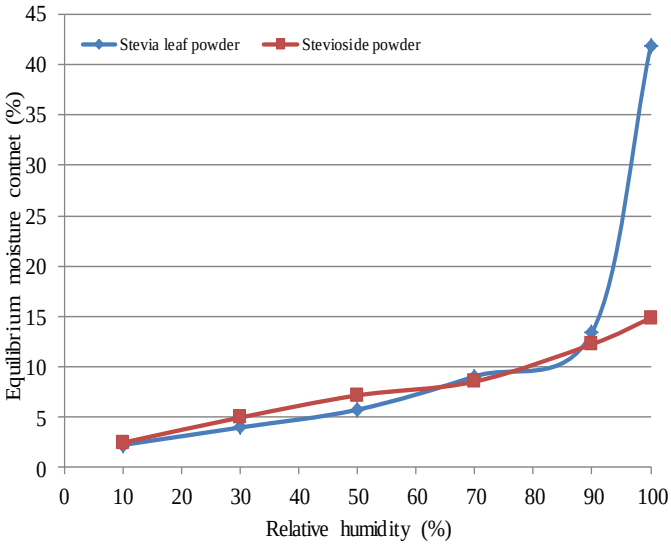

Fig2. Sorption isotherm of stevia leaf powder and commercial stevioside powder

3.2. Polyphenols

Polyphenol contents were 5.6 and 2.26% in SLP and CSP respectively (Table 1). Total phenolic content ranged between 2.53 – 6.52% gallic acid equivalents in ethanol extracts of stevia leaf (Jahan et al., 2010). The lower polyphenol content of 4.2% (weight on dry basis) was reported in stevia leaf by Kaushik et al. (2010). Very high polyphenol content (14%) was reported in dry leaves of *Celtis africana* (Adedapo et al., 2009). Lower polyphenol contents were observed in bael (*Aegle marmelos*) seed

powder (0.75%), dry quamachil aril powder (0.83%) and tomato powder (1.15%) by Narsing Rao et al. (2011); Narsing Rao et al. (2011); Narsing Rao et al. (2011). The study indicated that the high polyphenols (5.6%) present in SLP can be of importance for food and medicinal uses.

Interest on natural food additives has been increasing and plant extracts possessing natural food antioxidants were shown to influence human health when consumed daily (Martinez-Tome et al., 2001). The phenolic compounds present in the herbs and spices have been reported to show natural antioxidant activity and applied as food preservatives (Rice-Evans et a., 1996; Zheng & Wang, 2001). Hydrodistillation yielded very high total phenols 14.7% in the extract, which is equivalent to 3.62% polyphenols in herbs (Hinneburg et al., 2006). The hydrodistilled extracts are reported to be used in the functionalization of foods and beverages as phenolic compounds have been ascribed with health-promoting properties (Harborne & Williams, 2000). Medicinal herbssuch as *O. basilicam, O. vulgare* and *T. vulgaris* were reported to be rich in polyphenols which are in the range1.38 – 4.18% (Daniel & Balcerek, 2009; Kosar et al., 2005; Surveswaran et al., 2007). Polyphenols are known to protect lipids, carbohydrates and proteins from degradation (Halliwell, 1997).

3.2. Antioxidant activity of BHT

Antioxidant activity of BHT was measured by DPPH radical scavenging, iron reducing power and ABTS assay was shown in Table 2.

Table 2. Antioxidant activity of BHT

Concentration, µg	DPPH inhibition (%)	Ferric reducing power (OD)	Concentration, µg	ABTS assay (%)
4	22.61 ± 0.78	0.031 ± 0.007	10	36.04 ± 1.151
8	34.75 ± 1.29	0.04 ± 0.003	20	45.01 ± 0.445
16	54.15 ± 0.72	0.079 ± 0.004	30	54.57 ± 0.908
24	68.26 ± 1.66	0.106 ± 0.003	40	66.01 ± 0.811
32	78.53 ± 1.45	0.126 ± 0.010	50	79.00 ± 0.568

[a] Values expressed on samples basis and average of triplicate analysis with ± SD

3.3. DPPH radical scavenging activity

DPPH radical scavenging activityof SLP and CSP was presented in Figure 3. The substances cause change in colour of DPPH from violet to yellow upon reduction by electron-donation can be considered as antioxidants or radical scavengers (Brand-Williams et al., 1995). BHT showed percent inhibition of 22.6% at 4 µg and 78% at 32 µg/ml. In the present study, DPPH radical scavenging activity was found to be higher in SLP (52.46%) than CSP (47.64%) for 100 µg. The activity at lower concentration (20 µg) was almost similar for both the powders (~ 30%). The variation in polyphenol content might be responsible for changes in % inhibition. The DPPH activity of ethanolic extract and aqueous extract of stevia (20 - 200 µg/ml) was increased from 36.93 to 68.76% and 40-72.37% in a dose dependent manner and the total phenolic content were measured as 6.15 and 5.67%. (Shukla et al., 2009; Shukla et al., 2011). The higher total phenol (131 µg) content in stevia leaf extract showed greater antioxidant activity than that of stevia callus extract containing 44 µg (Kim et al., 2011).The higherDPPH radical scavenging activity (77.7%) was reported when 250 µg/ml methanolic extract of stevia leaf was used (Ahmad et al., 2010).

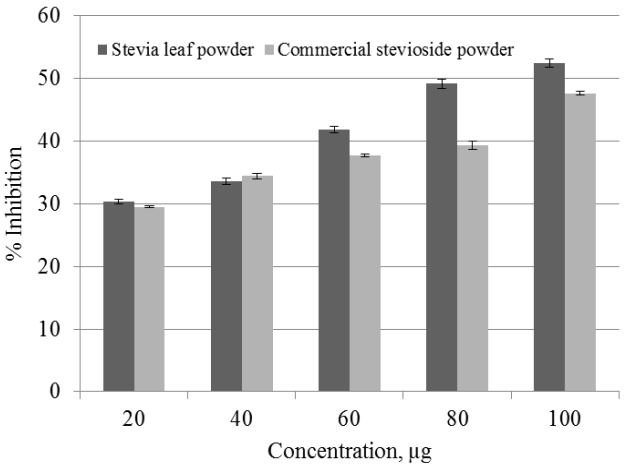

Fig 3. DPPH radical scavenging activity of SLP and CSP

3.4. Ferric reducing power

Ferric reducing power of SLP and CSP was shown in Figure 4. Fe(III) to Fe(II) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim et al., 2001). Ferric reducing power of BHT

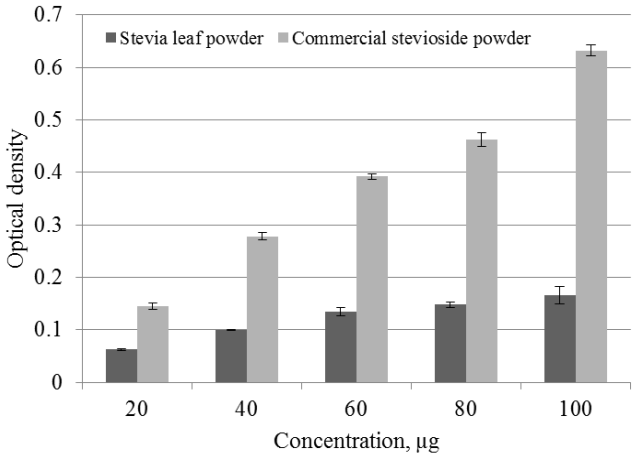

Fig. 4. Ferric reducing power of SLP and CSP

showed an optical density of 0.031 at 4 µg and 0.126 at 32 µg. It was observed that SLP exhibited a lesser reduction (0.06 OD) at 20 µg as compared to 0.145 OD for CSP. The optical densities were measured as 0.166 and 0.632 at higher concentration of 100 µg for them. The ferric reducing properties are generally associated with the presence of reductions by breaking the free radical chain by donating a hydrogen atom (Duh, Tu, & Yen, 1999). The ferric reducing power is widely used in the evaluation of the antioxidant component in dietary polyphenols (Luximon-Ramma et al. 2005). A highly positive relationship between total phenols and antioxidant activity was observed in many plant species by Oktay et al. (2003).

3.5. ABTS antioxidant assay

ABTS antioxidant activity of SLP and CSP was presented in Table 3. In this assay, SLP showed very high activity when compared to the CSP as observed in DPPH activity. It can be observed 10 times higher for SLP from the assay results. The percent ABTS assay was measured as 36% (4 µg) and 79% (32 µg) for BHT. CSP exhibited lower ABTS assay percent of 32.76 and 58.62% for 20 and 100 µg as compared to values of 23.21 and 37.50% with 2 and 10 µg for SLP.Lower value of 27.0% (100 µg) was reported for ethanol extract of winter melon seeds by Mandana et al. (2012). The methanol extract of *Celtis africana* leaves exhibited higher activity (98.8%) at 20 µg level (Adedapo et al., 2009).

Table 3.Antioxidant activity of methanolic extract of SLP and CSP by ABTS assay

SLP (µg)	ABTS % assay	CSP (µg)	ABTS % assay
2	23.21 ± 0.08	20	32.76 ± 0.13
4	28.57 ± 0.10	40	39.66 ± 0.38
6	30.36 ± 0.20	60	44.82 ± 0.62
8	32.14 ± 0.46	80	55.17 ± 0.50
10	37.50 ± 0.35	100	58.62 ± 0.45

[a] Values expressed on samples basis and average of triplicate analysis with ± SD

4. Conclusions

The study revealed the polyphenol content and antioxidant activity of both stevia leaf powder and commercial stevioside. The higher polyphenols (5.6%) in the leaf powder were observed compared to the commercial stevioside powder (2.3%). Stevia leaf powder was non-hygroscopic and stevioside was hygroscopic in nature as seen from sorption isotherms. The methanol extracts from SLP and CSP were found to have the highest antioxidant activity in DPPH inhibition and ABTS assays. The CSP showed the best performance in the ferric reducing power. The DPPH inhibition and ABTS assay correlated well with the content of total phenols. There is ample scope for both SLP and CSP for use not only as sweetener, but also source of polyphenols with potent antioxidant activity in the preparation of low calorie functional foods.

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