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Antioxidant, anti-diabetic and renal protective properties of *Stevia rebaudiana*

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ABSTRACT

Background: Stevia rebaudiana Bertoni has been used for the treatment of diabetes in, for example, Brazil, although a positive effect on antidiabetic and its complications has not been unequivocally demonstrated. This herb also has numerous therapeutic properties which have been proven safe and effective over hundreds of years. Streptozotocin is a potential source of oxidative stress that induces genotoxicity.
Objective: We studied the effects of stevia leaves and its extracted polyphenols and fiber on streptozotocin induced diabetic rats. We hypothesize that supplementation of polyphenols extract from stevia to the diet causes a reduction in diabetes and its complications.
Design/Methods: Eighty Wistar rats were randomly divided into 8 groups; a standard control diet was supplemented with either stevia whole leaves powder (4.0%) or polyphenols or fiber extracted from stevia separately and fed for one month. Streptozotocin (60 mg/kg body weight, i.p) was injected to the diabetic groups on the 31st day. Several indices were analyzed to assess the modulation of the streptozotocin induced oxidative stress, toxicity and blood glucose levels by stevia.
Results: The results showed a reduction of blood glucose, ALT and AST, and increment of insulin level in the stevia whole leaves powder and extracted polyphenols fed rats compared to control diabetic group. Its feeding also reduced the MDA concentration in liver and improved its antioxidant status through antioxidant enzymes. Glucose tolerance and insulin sensitivity were improved by their feeding. Streptozotocin was also found to induce kidney damage as evidenced by decreased glomerular filtration rate; this change was however alleviated in the stevia leaves and extracted polyphenol fed groups.
Conclusion: The results suggested that stevia leaves do have a significant role in alleviating liver and kidney damage in the STZ-diabetic rats besides its hypoglycemic effect. It might be adequate to conclude that stevia leaves could protect rats against streptozotocin induced diabetes, reduce the risk of oxidative stress and ameliorate liver and kidney damage.

1. Introduction

Stevia rebaudiana Bertoni is a small perennial shrub that belongs to the family aster or chrysanthemum family. It grows primarily in the Amambay mountain range of Paraguay (Mizutani & Tanaka, 2002). However over 200 species of stevia have been found around the world. Stevia rebaudiana is the only species at present, which possesses the ability to sweeten. The leaves of the stevia shrub contain specific substances (glycosides), which produce a sweet taste but have no caloric value, apart from protein, fibers, carbohydrates, phosphorus, iron, calcium, potassium, sodium, magnesium, rutin (flavonoid), zinc, vitamin C and vitamin A (Kim, Choi, & Choi, 2002). Stevia extracts were used by South Americans for the treatment of diabetes for many years (Curi et al., 1986; Soejarto, Kinghorn, & Farnsworth, 1982). Rebaudioside A and stevioside are the two main steviol glycosides found in the *S. Rebaudiana* herb and are the two predominant derivatives used in high potency sweeteners. Stevioside differs from rebaudioside A by having one less glucose moiety (Wheeler et al., 2008). However, little experimental work has been performed either to prove the clinical efficacy of these extracts in diabetes, to identify the potential active substance(s) in the extracts, or to reveal the mode of action. A number of animal studies have been conducted to investigate the effects of stevioside, having antihyperglycemic, insulinotropic, and glucagonostatic actions in diabetic rat (Jeppesen et al., 2002, 2003, 2000). This herb also has numerous therapeutic properties and has been proven in safety and effectiveness for hundreds of years. Stevia offers an ideal alternative to other sugars or sugar substitutes (Munro, Lirette, Anderson, & Ju, 2000). Chronic diabetic is set with complication such as diabetic nephropathy that causes widespread damage to small and large

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blood vessels, which is a marker for cardiovascular disease, a common cause of death in these patients. Unfortunately, besides having a number of side effects, none of the oral hypoglycemic agents have been successful in maintaining euglycemia and controlling long-term micro- and macro-vascular complications. During the last part of the 1980s, the FDA began seizing stevia from health food manufacturers without adequate explanation (Alvares, Bazzone, Godoy, Cury, & Botion, 1981). Approximately 200 manufacturers including all of the major food manufacturers showed interest in using stevia in the manufacturing of their products. Available toxicological information on stevia is inadequate to demonstrate its safety as a food additive or to affirm its status as GRAS. Stevia leaves used in the studies met all current specifications set by the Joint FAO/WHO Expert Committee on Food Additives (Carakostas, Curry, Boileau, & Brusick, 2008). It is considered by the most of investigators as not toxic, not mutagenic and not carcinogenic (Smirnova, 2001; Takahasshi et al., 2001). In the present study, the *in-vivo* antioxidant properties of stevia leaves were studied with respect to diabetic complications such as antioxidant, hyperlipidemia and nephropathy in streptozotocin (STZ) induced diabetic rats.

2. Material and Methods

2.1. Proximate composition and antioxidant activity of stevia

The stevia leaf powder was obtained from the Institute of Himalayan Bioresource Technology, Palampur, India. The proximate analyses (moisture, ash, fibers, crude fats, proteins and carbohydrates) of all the samples were determined. The moisture and ash were determined using weight difference method. The nitrogen value, which is the precursor for protein of a substance, was determined by micro Kjeldahl method described by Pearson (1976), involving digestions, distillation and finally titration of the sample. The nitrogen value was converted to protein by multiplying a factor of 6.25. Carbohydrate was determined by difference method. All the proximate values are reported in percentage i.e., g/100 g (Anonymous, 1990a,b; Hussain et al., 2009a,b). The total phenols of all extracts were measured at 765 nm by Folin Ciocalteu reagent (McDonald, Prenzler, Autolovich, & Robards, 2001). Aluminium chloride colorimetric technique was used for flavonoids estimation (Chang, Ming-Hua, Hwei-Mei, & Jiing-Chuan, 2002). The amount of chlorophyll in stevia leaves was estimated according to the procedure of extraction in 80% acetone, and then measured the absorbance at 663 nm and 645 nm. The chlorophyll concentration was then calculated using the specific absorption coefficients for chlorophyll a and b (Chaoyang, Zheng, Quan, & Wenjiang, 2008). Stevia powder was extracted with ethanol and flash evaporated. Polyphenols and fiber were extracted according to Mohamed and Chang (2008) to feed the animals. The antioxidant potential of the extract was estimated for Lipid peroxidation inhibition property and Free radical scavenging property (Braca et al., 2001). In brief, DPPH solution (0.004% w/v) and the extract were added followed by serial dilutions (1 μg to 500 μg), the absorbance was read at 515 nm using a spectrophotometer (Shimadzu UV 2001-visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (5 mg/ml). Control sample was prepared containing the same volume without any extract or reference ascorbic acid. 95 % methanol served as blank. % scavenging of the DPPH free radical was measured as per Braca et al. (2001). The inhibition curve was plotted for duplicate experiments and represented as percentage of mean inhibition ± standard deviation. EC_{50} values were obtained by probit analysis (Viturro et al., 1999).

2.2. HPLC analysis of phenolic compounds

Phenolic acid separation was performed on a reverse phase C18 column (150 × 4.5 mm) and the compounds were monitored with Diode array detector (JASCO HPLC system). The solvent system used was 0.1% Formic acid (Solvent A) and methanol (Solvent B). The solvent gradient elution program was used as per the modified method described by Ross, Beta, & Arntfield, 2009 with minor modification. 20 μl of the sample and standards was injected into the column and the phenolic acids were detected at 270 nm. Retention times and UV-Vis spectra of the peaks were compared with those of the standards.

2.3. Qualitative LC-MS Analysis.

Stevia leaves extract was analyzed on the LCMS- TOF using the conditions found in Table 1. Agilent G6530 Q-TOF with Agilent Jet Stream source was used for the identification of polyphenols in the extract.

2.3. Animals

Eighty in-bred Wistar rats of 3 months age were used in this study. They weighed 180 to 200 g and were maintained at a room temperature of 22 ± 2 °C with 12-h light/dark cycle and 45%–55% relative humidity. The animals had free access to food; water was given through drinking bottles. For the test group, stevia leaf powder was mixed at 4% level with the stock diet and fed. All the experiments were conducted between 09:00 and 14:00 h.

2.4. Experimental design

Rats were segregated randomly into 8 groups of 10 rats each. Groups 1 and 2 were fed with the control diet; groups 3 and 4 with 4.0% stevia leaves powder incorporated diet (4.0 g leaf powder in 96 g dry diet); groups 5 and 6 with equivalent amount of polyphenols extract (through force feeding); groups 7 and 8 with equivalent amount of fiber extracted from 4 g of stevia leaves powder respectively for 5 weeks, all the rats were fed *ad libitum* with free access to water. Food intake and weight gain were monitored weekly. One week prior to sacrifice, all even groups were given a single dose of STZ (60 mg/kg body weight, i.p. using a 5% solution of freshly

Table 1
Analytical Conditions for LCMS-IT-TOF.

LC parameters	
Column	2.1 mm × 150 mm, Eclipse Plus C18 1.8um
Mobile phase A	H2O 10 mM ammonium acetate
Mobile phase B	MeOH
LC Time program (linear gradient)	10% B (0–1 min); 10%–30% B (1–5 min); 30%–90% B (5–10 min); 90% B (10–18 min); 90%–10% B (18–18.5 min).
Flow rate	0.3 ml/min
Injection volume	2.0 μl
MS parameters:	
ESI Positive:	2 GHz mode
Scan Range:	100–1500 m/z
Scan Rate:	2 Hz
Reference ions:	112.9855 m/z, 1033.9881 m/z
Source parameters :	
Drying Gas Temp:	350 °C
Drying Gas Flow:	7 l/min
Nebuliser:	50 p.s.i.
Sheath Gas Temp:	400 °C
Sheath Gas Flow:	11 l/min
VCcap:	4500 V
Fragmentor:	80 V
Nozzle voltage:	0 V

Table 2
Proximate composition and other phytochemical constituents of stevia leaves (n = 3).

Constituents	Qty
Water content (g/100 g)	13.0
Fat (g/100 g)	2.3
Protein (g/100 g)	16.0
Total ash (g/100 g)	6.6
Crude fibre (g/100 g)	15.9
Total sugar by difference (g/100 g)	46.1
Total phenols (mg/g)	91.0
Total flavonoids (mg/g)	23.0
Total chlorophyll (mg/g)	2.6
Chlorophyll a (mg/g)	0.2
Chlorophyll b (mg/g)	0.3
Carotene (mg/g)	Nil
Vitamin C (mg/g)	0.1
Vitamin E (mg/g)	Nil

prepared streptozotocin in 0.1 M citrate buffer pH 4.5). Rats were sacrificed under mild anesthesia using anesthetic ether, after which organs / tissues were quickly excised and stored in liquid nitrogen until analyses (completed immediately within a week).

2.5. Biochemical parameters

Estimation was carried out for creatinine (Henry, 1974); blood sugar estimation was done by using GOD/POD enzymatic method (Tietz, 1976). Measurement of blood glucose concentration for intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) was done using the Accu-Chek Instant Plus blood glucose monitor (Roche Diagnostics Corp.). Insulin was measured at the end of five week treatment in the experimental animals using the enzyme-linked immunosorbent assay (ELISA) Rat/Mouse insulin kit (Millipore, USA). For the measurement of insulin sensitivity, IPGTT and IPITT were performed at the end of five week treatment on the experimental groups. IPGTT was done at the end of four week treatment with minor modifications to the method described by Goren, Kulkarni, and Kahn (2004). Briefly, after an over-night fasting period, 2 g/kg glucose was intraperitoneally injected into all the groups at the end of four week treatment. The blood glucose was measured at 15, 45 and 90 min after the glucose injection. IPITT test was performed with minor modifications following the method described by Wendel, Purushotham, Liu, and Belury (2008). After an over-night fasting period, 1.5 IU/kg insulin was intraperitoneally injected into all the groups at the end of four week treatment. The blood glucose was measured at 15, 45 and 90 min after insulin injection (Wendel et al., 2008). Lipid peroxidation in liver homogenate was assessed by analyzing the formation of thiobarbituric acid reactive substances (TBARS) spectrophotometrically at 535 nm according to the modified method of Nichans and Samuelson (1968). Liver catalase activity was assayed according to the method of Cohen, Dembiec, and Marens (1970); in brief, liver (0.5 g) was homogenized in phosphate buffer (5 M, pH 7.4) and the homogenates were centrifuged at 700×g. The supernatant was used for the assay

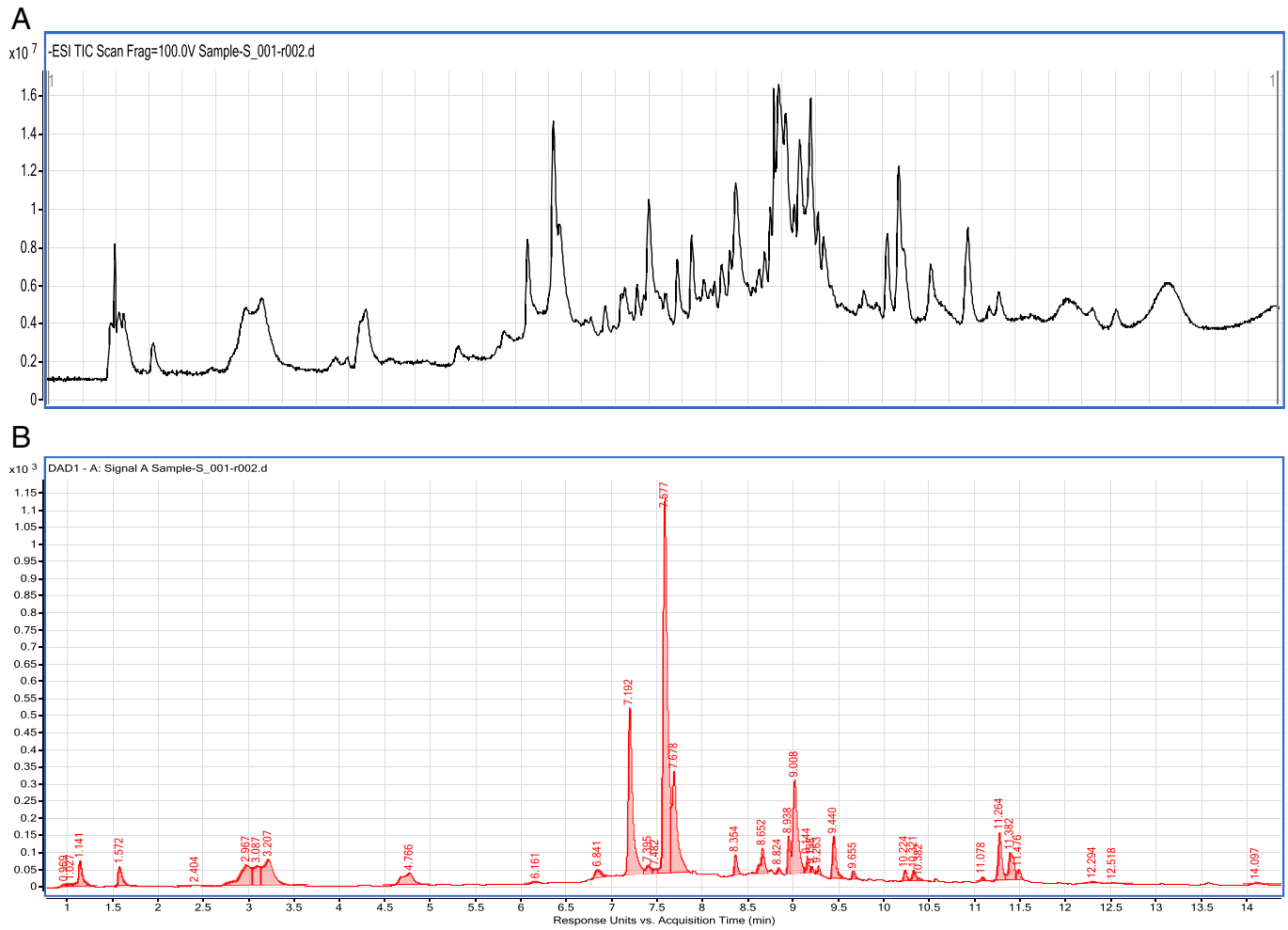

Fig. 1. (**A**) Total ion current (TIC) chromatogram from the HPLC separation of polyphenols present in the extract. (**B**) HPLC–UV—vis chromatogram at 280 nm of the extract.

with hydrogen peroxide as a substrate. Glutathione peroxidase (GSHPx) activity was determined by the method of Weiss, Maker, and Lehrer (1980) in the supernatant of liver homogenate prepared in phosphate buffer (0.5 M pH 7.0) using H_2O_2 and NADPH as substrates. L-γ-glutamyl transpeptidase in kidney was estimated by the method of Meister, Tate, & Griffith, 1981 using L-γ-glutamyl-*p*-nitroanilide as substrate in the homogenate prepared in 0.1 M Tris–HCl buffer, pH 7.4. Superoxide dismutase (SOD) was measured by monitoring the inhibition of cytochrome C reduction mediated via superoxide anions generated by xanthine–xanthine oxidase (Samanta & Chainy, 1995). One unit of SOD is defined, as the amount of enzyme required to inhibit the reduction of cytochrome C by 50 %. Protein in tissues was measured according to Lowry, Rosebrough, Faro, and Randall (1951). Plasma glutathione (GSH) and glutathione disulfide (GSSG) were estimated by the methods of Ellman (1958). Plasma α-tocopherol and Vitamin C were determined according to Keplowitz (1980). Creatinine (Folin & Wu, 1919) was estimated by Folin's method in blood and urine (24 h collection). Glomerular filtration rate (GFR) was determined (Yokozawa, Chung, He, & Qura, 1996) using the formula,

$$\mathrm{GFR(ml/min)} = \frac{\text{Urinary Creatinine(mg/dl)} \times \text{Urine volume (ml)} \times 100\text{(g)}}{\text{Plasma creatinine(mg/dl)} \times \text{Body weight (g)} \times 1440\text{(min)}}$$

Table 3
Characterization and Quantitation of Polyphenolic Compounds in polyphenol extract of stevia leaves Using LC-MS Detection.

No.	Label	Formula	score	Mass	Avg mass	Mass (Data base)	m/z	RT
1	Cpd1:threo-isocitric acid	$C_6\ H_8\ O_7$	86.86	192.0271	192.0871	192.027	191.0198	0.894
2	Cpd2:$C_7H_{12}O_6$	$C_7H_{12}O_6$	83.58	192.0626	192.1462		191.0549	0.986
3	Cpd3:Dulcitol	$C_6H_{14}O_6$	99.65	182.0793	182.1647	182.079	181.072	1.045
5	Cpd5:Caffeic acid	$C_9H_8O_4$	99.82	180.0424	180.1639	180.0423	179.0351	1.576
6	Cpd6:Chlorogenic acid	$C_{16}\ H_{18}\ O_9$	98.95	354.0945	354.1436	354.0951	353.0872	2.931
7	Cpd7: Chlorogenic acid	$C_{16}\ H_{18}\ O_9$	97.03	354.0943	354.2999	354.0951	707.1822	3.175
8	Cpd8: Picrotoxin	$C_{15}\ H_{16}\ O_6$	99.58	292.0945	338.3085	292.0947	337.0927	40.318
9	Cpd9: chlorogenic acid	$C_{15}\ H_{18}\ O_9$	99.17	354.0947	354.3003	354.0951	354.0873	4.716
10	Cpd10: $C_{17}\ H_{20}\ O_9$	$C_{17}\ H_{20}\ O_9$	99.87	368.1103	368.3177		367.1033	5.102
11	Cpd11:m-Salicylic acid	$C_7\ H_{20}\ O_3$	99.87	138.0318	138.1192	138.0317	137.0245	5.392
12	Cpd12: $C_{19}\ H_{28}\ O_{11}$	$C_{19}\ H_{28}\ O_{11}$	98.43	432.1626	432.4928		431.1556	6.13
13	Cpd13: dicaffeoylquinic acid	$C_{25}\ H_{24}\ O_{12}$	99.01	516.1262	516.4829		515.1191	6.827
14	Cpd14: dicaffeoylquinic acid	$C_{25}\ H_{24}\ O_{12}$	97.98	516.1259	516.4466		515.1184	7.172
15	Cpd15: dicaffeoylquinic acid	$C_{25}\ H_{24}\ O_{12}$	97.16	516.1259	516.4517		1031.2445	7.568
16	Cpd16: dicaffeoylquinic acid	$C_{25}\ H_{24}\ O_{12}$	97.47	516.1273	516.4499		1031.2447	7.66
17	Cpd17: $C_{34}\ H_{30}\ O_{15}$	$C_{25}\ H_{30}\ O_{15}$	98.37	678.1575	678.5898		677.15	8.331
18	Cpd18:luteolin-7-*o*-glucoside	$C_{21}\ H_{20}\ O_{11}$	98.64	448.0998	448.3878	448.1006	447.0925	8.575
19	Cpd19:Rutin:	$C_{27}\ H_{30}\ O_{16}$	98.43	610.1525	610.5096	610.1534	609.1451	8.632
20	Cpd20: $C_{21}\ H_{26}\ O_{10}$	$C_{21}\ H_{26}\ O_{10}$	99.15	438.1521	438.4118		437.1449	8.666
21	Cpd21: Quercetin-3-*o*-xyloside	$C_{20}\ H_{18}\ O_{11}$	99.42	434.0847	434.3469		433.0772	8.799
22	Cpd22:$C_{20}\ H_{40}\ O_{10}$	$C_{20}\ H_{40}\ O_{10}$	97.87	440.2614	440.4922		439.2544	8.82
23	Cpd23: $C_{18}\ H_{36}\ O_8$	: $C_{18}\ H_{36}\ O_8$	98.82	380.2404	380.7034		415.2099	8.822
24	Cpd24: luteolin-7-*o*-rutinoside	$C_{27}\ H_{30}\ O_{15}$	97.26	594.1573	594.5157		593.15	8.913
25	Cpd25:Emodin 8-glucose	$C_{21}\ H_{20}\ O_{15}$	81.58	432.1052	432.2891	432.1056	431.098	8.962
26	Cpd26:QUERCITIN	$C_{21}\ H_{20}\ O_{11}$	98.05	448.0998	448.3969	448.1006	447.0924	8.995
27	Cpd27: $C_{21}\ H_{21}\ N\ O_{14}$	$C_{21}\ H_{21}\ N\ O_{14}$	95.86	511.0957	511.3732		510.0883	9.006
28	Cpd28:$C_{36}\ H_{36}\ O_9$	$C_{36}\ H_{36}\ O_9$	98.06	772.184	772.6459		771.177	9.034
29	Cpd29:PSOROMIC ACID	$C_{18}\ H_{14}\ O_8$	99.15	358.0684	418.3241	358.0689	417.0823	9.118
30	Cpd30:PSOROMIC ACID	$C_{18}\ H_{14}\ O_8$	99.19	358.0684	418.3409	358.0689	417.0823	9.172
31	Cpd31: $C_{36}\ H_{36}\ O_{18}$	$C_{36}\ H_{36}\ O_{18}$	97.93	756.1889	756.6464		577.1814	9.244
32	Cpd32: $C_{42}\ H_{40}\ O_{20}$	$C_{42}\ H_{40}\ O_{20}$	98.57	864.2103	864.7279		863.2033	9.415
33	Cpd33: Apigenin-7-*O*-glucoside	$C_{21}\ H_{20}\ O_{10}$	97.71	432.1049	432.3982	432.1056	431.0975	9.42
34	Cpd34:2,5 Dimethoxy cinnamic	$C_{11}\ H_{12}\ O_4$	99.45	208.0735	208.2071	208.0736	207.0662	9.635
36	Cpd36:$C_{44}\ H_{70}\ O_{23}$	$C_{44}\ H_{70}\ O_{23}$	98.3	966.4295	967.0338		965.4227	10.063
37	Cpd37: $C_{26}\ H_{42}\ O_{10}$	$C_{26}\ H_{42}\ O_{10}$	99.69	514.2767	514.6625		513.2698	10.079
38	Cpd38:Genistein	$C_{15}\ H_{10}O_5$	99.07	270.0527	270.2324	270.0528	269.0454	10.199
39	Cpd39: $C_{50}\ H_{80}O_{28}$	$C_{50}\ H_{80}\ O_{28}$	97.63	1128.4818	1129.15		1127.4746	10.244
40	Cpd40:Rosmaric acid	$C_{18}\ H_{16}\ O_8$	9.74	360.084	360.3075	360.0845	359.0767	10.305
42	Cpd42:$C_{23}\ H_{33}\ Cl\ N\ O_2$	$C_{23}\ H_{33}\ Cl\ N\ O_2$	82.7	390.2194	391.1744		389.2092	210.734
49	Cpd49: $C_{39}\ H_{62}\ O_{20}$	$C_{39}\ H_{62}\ O_{20}$	97.07	850.3823	850.8797		489.375	10.934
53	Cpd53: $C_{43}\ H_{68}\ O_{22}$	$C_{43}\ H_{68}\ O_{22}$	97.64	936.4188	936.9702		935.4124	10.996
56	Cpd56: $C_{38}\ H_{60}\ O_{17}$	$C_{38}\ H_{60}\ O_{17}$	95.89	788.3814	788.8595		787.3742	11.058
57	Cpd57: $C_{32}\ H_{50}\ O_{12}$	$C_{32}\ H_{50}\ O_{12}$	98.336	626.3294	626.7086		625.3224	11.061
63	Cpd63: $C_{21}\ H_{34}\ O_6$	$C_{21}\ H_{34}\ O_6$	41.61	382.2351	382.4207		381.2277	11.242
64	Cpd64: $C_{34}\ H_{54}\ O_{15}$	$C_{34}\ H_{54}\ O_{15}$	97.85	702.3454	702.7223		701.3383	11.248
65	Cpd9: $C_{32}\ H_{50}\ O_{13}$	$C_{32}\ H_{50}\ O_{13}$	98.51	642.3242	642.7128		641.3165	11.26
66	Cpd66: $C_{32}\ H_{51}\ N\ O_{16}$	$C_{32}\ H_{51}\ N\ O_{16}$	98.87	705.3204	705.6879		704.3131	11.263
67	Cpd67:$C_{32}\ H_{51}\ Cl\ O_{13}$	$C_{32}\ H_{51}\ Cl\ O_{13}$	98.68	678.3012	679.1902		677.2938	11.267
68	Cpd68: $C_{59}\ H_{100}\ N2\ O_{28}$	$C_{59}\ H_{100}\ N2\ O_{28}$	96.04	1284.6487	1285.434		1283.6417	11.277
77	Cpd77:$C_{26}\ H_{38}\ O\ S$	$C_{26}\ H_{38}\ O\ S$	85.36	398.2655	398.5101		397.2588	11.416
78	Cpd78: $C_{21}\ H_{36}\ O_6$	$C_{21}\ H_{36}\ O_6$	98.4	384.2505	384.4998		383.2431	11.416
79	Cpd79: $C_{20}\ H_{34}\ O_4$	$C_{20}\ H_{34}\ O_4$	76.06	338.2453	338.9114		373.2135	11.419
83	Cpd 83: $C_{26}\ H_{40}$ O8	$C_{26}\ H_{40}$ O8	99.38	480.272	480.7756			
86	Cpd 86: $C_{25}\ H_{48}\ O_{11}\ S$	$C_{25}\ H_{48}\ O_{11}\ S$	98.78	556.291	556.6852		555.2835	12.212
88	Cpd 88: $C_{21}\ H_{36}\ O_5$	$C_{21}\ H_{36}\ O_5$	99.03	368.2557	368.5069		367.2482	12.735
90	Cpd 90: $C_{22}\ H_{38}\ O_5$	$C_{22}\ H_{38}\ O_5$	95.26	382.2712	382.5168		381.2638	12.737
98	Cpd98: $C_{34}\ H_{40}\ O_9$	$C_{34}\ H_{40}\ O_9$	99.67	592.2675	592.7051		591.2602	14.072

Table 4
Antioxidant activity (*in-vitro*) of Stevia leaves (n = 3).

Antioxidant properties of Stevia	EC_{50} (μg)			
	Stevia	BHA[a]	BHT[a]	TBHQ[a]
1. Lipid per-oxidation inhibition property	2.6 ± 0.05	2.9 ± 0.04	2.2 ± 0.02	3.8 ± 0.02
2. Free radical scavenging property (DPPH scavenging)	10.6 ± 1.91	13.0 ± 1.72	43.2 ± 2.84	8.6 ± 0.11

[a] Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and Tertiary butyl hydroquinone (TBHQ).

3. Results and Discussion

Diabetes has been one of the major killer diseases of the present day life. This disease is not only genetic but is linked to lifestyle and stress, both physical and mental. Nearly 12% of population worldwide is suffering from diabetes. In this study, we examined oxidative stress markers in rats with STZ induced diabetes and its modulation on feeding of stevia. STZ is a generally employed compound for the induction of type-I diabetes in rats (Cameron & Cotter, 1999; Tomlinson, Gardiner, Hebden, & Bennett, 1992).

3.1. Proximate composition

The proximate composition of stevia leaves is given in Table 2. The leaves contain more of carbohydrates (cellulose / soluble fiber) and ash, but no significant amount of fat or proteins. Among other phytoconstituents (Table 2) analyzed phenols were 91 mg/g. A good amount of total phenols is a positive indication for antioxidant and antidiabetic properties of stevia.

3.2. Phenolic compounds identification

The identification of major polyphenols (Fig. 1) was based on comparison of their retention times with those of reference compounds and their elution order on reversed-phase C18 columns. Peak assignment was confirmed by mass spectrometry. Major compounds identified are dicaffeoylquinic acid, chlorogenic acid, Quercetin 3-*O*-xyloside, Apigenin-7-*O*-glucoside, 3,4-Dimethoxycinnamic acid, Luteolin 7-*O*-rutinoside, Caffeic acid etc (Table 3).

Stevia was found to oppose the hyperglycemic actions in the diabetic treated rats (Curi et al., 1986; Soejarto et al., 1982). Previous studies had also noted the importance of hypoglycemic components of Stevia is because of Rebaudioside A and stevioside that are concentrated in leaves (Wheeler et al., 2008). In our study none of the compounds identified in the polyphenol extract was Rebaudioside A or stevioside, since the mass was not matching with compounds detected (Table 3). This explains the involvement of polyphenolic compounds in preventing diabetic and its complications caused by streptozotocin (Baynes and Thorpe, 1999; Bennett and Pegg, 2004; Imaeda et al., 2002; Jain et al., 1990; Kakar et al., 1995; Kinalski et al., 2000; Nrc, 1994; Petzold and Swenberg, 1978; Reaven, 1988; Traverso et al., 2004; Wiseman and Halliwell, 1996; Wolff and Dean, 1987).

3.2. Antioxidant property (in-vitro)

Antioxidant property of stevia leaves was estimated and compared with other commercial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and Tertiary butyl hydroquinone (TBHQ). Stevia leaves were having better antioxidant properties such as free radical scavenging and inhibition of lipid peroxidation w.r.t. their EC_{50} values (Table 4).

3.3. Antioxidant property (in-vivo)

Thiobarbituric acid reaction substances, conjugated dienes and hydroperoxides; products of lipid peroxidation is a measure of oxidative stress in liver. The levels of malondialdehyde, conjugated dienes and hydroperoxides increased upon injection of STZ significantly (Table 5). Peroxidation was reduced significantly in rats prefed with stevia leaves powder and polyphenols extracted to 25%&30% in liver compared to diabetic group.

Since high blood glucose is susceptible to oxidation, hyperglycemia causes high ROS production and, in turn, leads to high Malondialdehyde (MDA) levels in tissues (Das, Vasisht, Snehalta Das, & Shrivastava, 2000). However, the increment of lipid peroxidation has been found to be involved in observed tissue damages in diabetes (Ahmed, Naqvi, & Shafiq, 2006; Can et al., 2004;Mahboob, Rahman, & Grover, 2005; Packer, Rossen, Tritschter, King, & Azzi, 2000; Sathishsekar & Subramanian, 2005; Sitasawad, Shewade, & Bhonde, 2000).

STZ administration resulted in significant decrease in vitamin C content by 27%, increased in vitamin E levels by 130% and GSH or GSH/GSSG ratio by 45% compared to controls. Pre-feeding stevia however did not alter the levels of antioxidants in plasma (Table 6).

Vitamin E and vitamin C have been demonstrated to be an antioxidant that scavenges the free radicals generated in cell membranes (Bartov & Frigg, 1992; Halliwell & Gutteridge, 1989; Luadicina & Marnett, 1990; Zuprizal, Larbier, Channeau, & Geraert,

Table 5
Effect of Stevia on lipid peroxidation in liver in STZ treated rats (n = 8).

	TBARS ($\times 10^{-10}$ mol / g)	Hydroperoxides ($\times 10^{-4}$ mol / g)	Conjugated dienes ($\times 10^{-4}$ mol / g)
Control	$0.06 \pm 0.01^{**}$	$0.07 \pm 0.02^{**}$	$0.12 \pm 0.02^{**}$
Streptozotocin	$0.16 \pm 0.03^{*}$	$0.17 \pm 0.03^{*}$	$0.24 \pm 0.06^{*}$
Stevia leaves Powder	$0.08 \pm 0.02^{**}$	0.11 ± 0.03	$0.09 \pm 0.02^{**}$
STZ + Stevia leaves Powder	$0.12 \pm 0.03^{*}$	$0.10 \pm 0.02^{**}$	$0.17 \pm 0.04^{**}$
Stevia leaves polyphenols	$0.07 \pm 0.01^{**}$	0.09 ± 0.02	$0.10 \pm 0.02^{**}$
STZ + Stevia leaves polyphenols	$0.11 \pm 0.02^{*}$	$0.08 \pm 0.01^{**}$	$0.14 \pm 0.03^{**}$
Stevia leaves fiber	$0.05 \pm 0.01^{**}$	$0.08 \pm 0.02^{**}$	$0.15 \pm 0.03^{**}$
STZ + Stevia leaves fiber	$0.17 \pm 0.02^{*}$	$0.15 \pm 0.03^{*}$	$0.25 \pm 0.05^{*}$

Note: Significance is defined as $p<0.05$. Superscript '*' indicates the value is significantly different in comparison with Control ($P<0.05$) and superscript '**' indicates the value is significantly different in comparison with the treated group (STZ) at $P<0.05$.

Table 6
Effect of Stevia on plasma antioxidant levels in STZ treated rats (n = 8).

Plasma (mg/dl)	Vitamin E	Vitamin C	GSH	GSSG	GSH/GSSG
Control	1.47 ± 0.05**	1.43 ± 0.29**	24.58 ± 0.51**	0.10 ± 0.02	246**
STZ	3.55 ± 0.13*	1.05 ± 0.1*	13.58 ± 0.40*	0.10 ± 0.03	136*
Stevia leaves Powder	2.06 ± 0.42**	1.54 ± 0.07**	22.00 ± 0.62**	0.13 ± 0.02	169 **
STZ + Stevia leaves Powder	2.81 ± 0.09*	1.30 ± 0.18*	14.71 ± 0.86*	0.11 ± 0.01	134*
Stevia leaves polyphenols	2.13 ± 0.22**	1.70 ± 0.09**	21.11 ± 0.51**	0.15 ± 0.02	141**
STZ + Stevia leaves polyphenols	2.78 ± 0.10*	1.26 ± 0.15*	13.60 ± 0.56*	0.12 ± 0.03	113*
Stevia leaves fiber	1.65 ± 0.11**	1.29 ± 0.12**	23.77 ± 0.41**	0.14 ± 0.06	170 **
STZ + Stevia leaves fiber	3.61 ± 0.11*	1.10 ± 0.09*	13.81 ± 0.34*	0.11 ± 0.05	126*

1993). The synergic effects between these two vitamins are particularly efficient for reducing production of reactive oxygen species. Tissue antioxidant concentrations such as vitamin E decrease due to its mobilization to blood in control diabetic rats (Craven, Derubertis, Kagan, Melhem, & Studer, 1997; Douilleta, Bostb, Accominottic, Borson-Chazotd, & Ciavattia, 1998). Levels of vitamin E were found to be higher in patients of acute myocardial infarction (AMI) as compared to controls due to oxidative stress (Reena et al., 2007). Increased ascorbic acid plasma levels during oxidative stress along with cortisol (Gleeson, Robertson, & Maughan, 1987; Umegaki, Daohua, Sugisawa, Kimura, & Higuchi, 2000) or to the ascorbate recycling and efflux from neutrophils induced by exercise (Mastaloudis, Leonard, & Traber, 2001; Peake, 2003; Tauler, Aguilo, Fuentespina, Tur, & Pons, 2002). Increase in the vitamin E and C level observed in diabetic rats could be a protective mechanism against increased peroxidation in diabetes due to stored vitamin E mobilization and vitamin C synthesis (Stanely Mainzen Prince & Menon, 1998). GSH another efficient antioxidant molecule in the body protects the tissue from free radical damage by inhibiting free radical mediated lipid peroxidation (Meistor & Anderson, 1983). Decreased levels of plasma GSH/GSSH ratio in diabetic rats could be due to the utilization of GSH as a result of increased ROS generation by increased levels of glucose (Park et al., 2001; Stanely Mainzen Prince & Menon, 1998; Vallabhji et al., 2001) in STZ treated animals.

STZ administration resulted in significant reduction of hepatic antioxidant enzymes SOD and catalase by 50% compared to control group, when rats were prefed with stevia leaves powder and polyphenols extracted the enzyme activity was restored to normal and was significant when compared to diabetic group (Table 7). Hepatic phase I catalase activity was reduced by 51% on STZ treatment (Table 7), which on pre-feeding with stevia leaves powder and polyphenols extracted the activity increased to 55%&50% compared to diabetic group. No significant change was seen in extracted fiber fed group.

Stevia also increased the level of antioxidant enzymes and could be effective through scavenging these free radicals (Wu & Ng, 2008). SOD and CAT are considered primary antioxidant enzymes, since these are involved in direct elimination of ROS (Bhor, Raghuram, & Sivakami, 2004; Halliwell & Cutteridge, 1985; Kakkar et al., 1997). Pre-treatment of stevia stimulated SOD and CAT to reverse oxidative damage. The results indicate that stevia showed significant protection against the oxidative damage induced by STZ in liver.

3.4. Anti-diabetic property

Treatment of animals with STZ, a known diabetogen (Marianna, Chaonan, QingPing, Yanling, & Ping-A, 2006) led to polydypsia, polyphagia and polyurea. Food consumption increased from 65 g to 91 g/week, while water intake almost doubled from 27 ml/day to 59 ml/day. Urine output increased from 39 ml to 98 ml/day. The body weight which was 306 g on the day of injection of STZ was 240 g at the weekend i.e, a loss of 22% in one week. Pre-feeding the rats with stevia leaves powder and polyphenols extracted before injecting STZ prevented these changes significantly; food consumption was brought down by 15%&8%, water consumption by 28%&12%, urine output by 42%&54% and loss in body weight was limited to 5&10 g/week by stevia leaves powder and polyphenols extracted. The diabetic rats showed a significant increase in the blood glucose level when compared to the normal rats by 226% ($P<0.05$). Stevia leaves powder and its polyphenol extract reduced the elevated levels of blood glucose by 36&64% compared to the untreated diabetic control ($P<0.05$). But there was no reduction in blood glucose level in stevia fiber fed rats (Table 8).

STZ treatment of rats led to hyperglycemia, a diabetic condition and also its associated symptoms like increased thirst, increased hunger, increased urine output etc,. These findings correlate with those reported by others (Cameron & Cotter, 1999], [Golden, Wals, & Okajima, 1979] and [Bishop, 1970). STZ injection also led to increased oxidative stress apart from inducing liver damage. Stevia leaves powder prefed to rats for 4 weeks before and one week after injection with STZ was found to provide significant protection from all the serious effects of STZ. Increased food consumption and decreased body weight observed in diabetic rats indicate polyphagic condition and weight loss due to excessive breakdown of tissue proteins (Raju, Gupta, & Rao, 2001). Pre-feeding of stevia failed to achieve euglycemia but caused a significant reduction in glucose levels. The mechanism involved in the lowering of blood glucose levels by stevia could be due to modulation of glucose transport (Curi et al., 1986; Yamasaki et al., 1993) or glucose disposal (Yokozawa, Kobayashi,

Table 7
Effect of Stevia on liver antioxidant enzymes levels in STZ treated rats (n = 8).

	Glu-S-transferase	GSH-Px	Catalase	SOD	GSSG reductase
Control	2.1 ± 0.6	1.1 ± 0.52**	1.02 ± 0.05**	2.41 ± 0.91**	2.5 ± 0.17
STZ	2.2 ± 0.91	2.15 ± 0.42*	0.52 ± 0.07*	1.21 ± 0.05*	2.6 ± 0.27
Stevia leaves Powder	2.85 ± 0.72	2.72 ± 0.31*	1.52 ± 0.11**	2.69 ± 0.61**	3.02 ± 0.13*,**
STZ + Stevia leaves Powder	2.4 ± 0.66	2.5 ± 0.32*	0.81 ± 0.2**	2.08 ± 0.43**	2.9 ± 0.08*
Stevia leaves polyphenols	2.68 ± 0.51	2.71 ± 0.10*	1.49 ± 0.09**	2.72 ± 0.52**	3.00 ± 0.09*,**
STZ + Stevia leaves polyphenols	2.37 ± 0.54	2.47 ± 0.22*	0.78 ± 0.17**	2.01 ± 0.32**	2.81 ± 0.10*
Stevia leaves fiber	2.05 ± 0.55	1.21 ± 0.37**	1.11 ± 0.10**	2.37 ± 0.53**	2.46 ± 0.25
STZ + Stevia leaves fiber	2.33 ± 0.82	2.30 ± 0.39*	0.48 ± 0.11*	1.18 ± 0.07*	2.50 ± 0.15

Table 8
Effect of stevia on Anti-diabetic and kidney functions in STZ treated rats (n = 8).

	Control	Stevia	STZ	STZ + Stevia leaves powder	Stevia polyphenol	STZ + Stevia polyphenol	Stevia fiber	STZ + Stevia fiber
Weight gain (g/week)	25.8 ± 9.70	25.7 ± 14.3	6.8 ± 1.8*,**	11.7 ± 1.60*,**	21.5 ± 11.4	10.3 ± 0.95*,**	22.5 ± 9.12	6.0 ± 2.1*,**
Food intake (g/day)	9.4 ± 3.7	10.2 ± 3.00	13 ± 1.9	11 ± 3.3	9.8 ± 2.50	10.1 ± 2.3	9.1 ± 2.6	11 ± 1.1
Water intake (ml/day)	27.9 ± 16.1	20.8 ± 13.3	59.5 ± 28.4*,**	42.6 ± 23.3*,**	22.1 ± 16.1	52.6 ± 25.2*,**	25.1 ± 14.3	55.2 ± 22.1*,**
Urine volume (ml/24 h)	39.0 ± 17.7	31.1 ± 14.7	98.4 ± 27.9*,**	57.2 ± 16.8*,**	28.3 ± 11.6	53.1 ± 11.5*,**	42.2 ± 15.1	89.9 ± 17.8*,**
Blood glucose (mg/dl)	109.2 ± 29.60	99.8 ± 22.0	356.3 ± 65.40*,**	228.2 ± 22.10*,**	101.4 ± 17.2	129.2 ± 12.50*,**	99.3 ± 10.46	342.0 ± 42.50*,**
Plasma Creatine	0.47 ± 0.11	0.40 ± 0.14	0.57 ± 0.02	0.53 ± 0.12	0.50 ± 0.22	0.45 ± 0.09	0.48 ± 0.08	0.60 ± 0.10
Urinary Creatine	0.071 ± 0.003	0.082 ± 0.014	0.058 ± 0.002*,**	0.062 ± 0.002*,**	0.080 ± 0.01	0.059 ± 0.008*,**	0.069 ± 0.01	0.059 ± 0.004*,**
GFR (ml/min/kg body wt)	5.55 ± 0.24^{a}	6.42 ± 0.32*	4.56 ± 0.17^{a}	4.89 ± 0.18^{a}	6.01 ± 0.12*	4.95 ± 0.06^{a}	5.49 ± 0.11^{a}	4.44 ± 0.20^{a}

Kawai, Oura, & Kawashima, 1984) or a better insulin secretion (Dao & Le, 1995; Waki, Kyo, Yasuda, & Kimura, 1982).

In the STZ group, the serum insulin level was lower than that of the normal group ($P<0.05$). The serum insulin level in the stevia leaves powder and its polyphenol extract fed groups was higher than that of the STZ group ($P<0.05$). There was no change observed by feeding extracted stevia fiber (Fig. 2). Impaired glucose tolerance was seen in the STZ group. The blood glucose increased in the STZ group (455 ± 21) peaking at 45 min after glucose injection and was higher during 90 min after glucose was administered, compared to the control (144 ± 8) ($P<0.05$). Significantly, lower blood glucose level was observed in the stevia leaves powder and its polyphenol extract groups during 90 min after glucose injection when compared to the diabetic rats ($P<0.05$) (Fig. 2). The fasting serum insulin level was significantly lower in the STZ group compared to the control ($P<0.05$). However, the serum insulin level was significantly higher in stevia leaves powder and its polyphenol extract groups ($P<0.05$) than that of the STZ group (Fig. 2). The blood glucose started to diminish after insulin was administered. The results of IPITT showed increased insulin sensitivity in the treated diabetic groups after four weeks of treatment (Fig. 3). The blood glucose level slowly decreased within 90 min after insulin injection in the STZ group compared to the normal control ($P<0.05$). However, the glucose concentrations rapidly diminished after insulin injection in the stevia leaves powder and its polyphenol extract groups compared to the untreated STZ group ($P<0.05$) and were close to the normal control.

Glycogen is the primary intracellular storable form of glucose and its levels in various tissues especially liver are a direct reflection of insulin activity as insulin promotes intracellular glycogen deposition by stimulating activities of glycogen synthase and inhibiting glycogen phosphorylase. Since STZ causes selective destruction of β-cells of islets of Langerhans resulting in marked decrease in insulin levels, it is rational that glycogen levels in tissue decrease as they depend on insulin for influx of glucose (Bishop, 1970; Cameron & Cotter, 1999; Golden et al., 1979; Weber, Lea, & Fisher, 1966). Streptozotocin is a generally employed compound for the induction of type-I diabetes in rats (Tomlinson et al., 1992). Streptozotocin causes diabetes by the rapid depletion of β-cells, which leads to a reduction in the insulin release. An insufficient release of insulin causes high blood glucose (hyperglycemia), which results in oxidative damage by the generation of reactive oxygen species (Kangralkar, Shivraj, Patil, & Bandivadekar, 2010) and the development of diabetic complications (Donnini, Zambito, Perella, Ambesi-Impiombat, & Curcio, 1996). The results of this study showed increased serum insulin level in the stevia leaves powder and its polyphenol extract groups. This means, stevia would enhance the number of β-cells of pancreatic islets in diabetic treated rats. The increase in insulin levels suggested that stevia would enhance the secretion of insulin from β-cells of islets of Langerhans.

Cell resistant to insulin is one of the most important factors in the initiation and development of type II diabetes (Zhang, Lv, Li, Xu, & Chen, 2008). The diabetic rats showed significant hyperglycemia in IPGTT and IPITT compared to the control (Figs. 2 and 3). However, it was evident that the stevia leaves powder and its polyphenol extract treatment restored the plasma glucose clearance rate, in contrast to that of the normal animal (Figs. 2 and 3). This clearly shows that stevia has the ability to improve glucose tolerance and increase cellular insulin sensitivity.

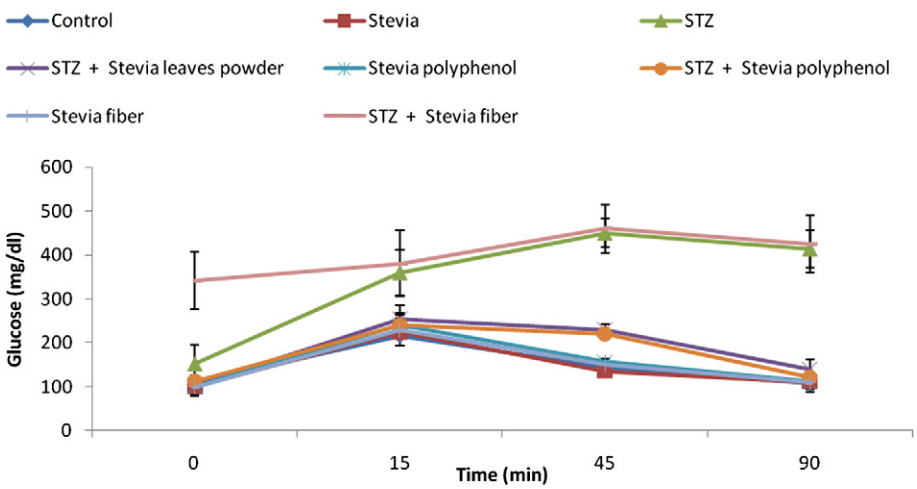

Fig. 2. Blood glucose level changed during GTT after one week treatment. Error bar = ±SD (n = 10).

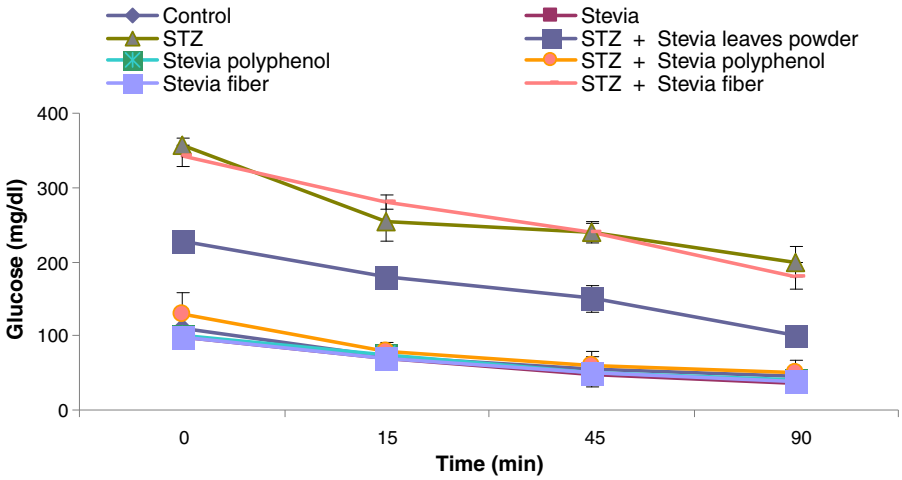

Fig. 3. Blood glucose level changed during ITT after one week treatment. Error bar = ±SD (n = 10).

3.5. Renal protective activity/capacity

A slight reduction, though not significantly; in the kidney weight was observed (Fig. 4) in stevia leaves powder and polyphenols extracted groups, which increased on STZ treatment. Also there was a significant decrease in GFR on STZ treatment which was enhanced by 7% and 9% on pre-feeding of stevia leaves powder and polyphenols extracted respectively (Table 8). The data on the effect of stevia on kidney antioxidant enzymes viz. G-6-PDH, and detoxifying enzyme γ-glutamyl transpeptidase (Table 9) showed administration of STZ resulted in decreased G-6-PDH and increased γ-glutamyl transpeptidase activity. The STZ-induced elevation in renal G-6-PDH was brought down by stevia leaves powder but both the enzyme activities were bought down by the polyphenols extracted. No significant change was seen in extracted fiber fed group.

Kidney enlargement is an early feature of diabetes due to an increase in the capillary length and diameter and was correlated with the degree of glycemic control (Melis, 1995; Melis, 1996; Melis, 1999; Seyer-Hansen, 1977). In our experiment there is a significant reduction in the kidney weight with stevia supplementation. Increase in γ-glutamyl transpeptidase levels were similar to numerous drugs that can raise γ-glutamyl transpeptidase levels, by inducing hepatic microsomal production, or it may cause the leakage of γ-glutamyl transpeptidase from hepatocytes (Barouki et al., 1983). Increase in GFR, shows the characteristic features of hyperfunctional kidney, which is common during early stages of diabetes (Christiansen, Gammelgaard, Frandsen, & Parring, 1981). Long term metabolic control is known to reduce kidney filtration in diabetic subjects (Feldt-Rammussen, Hegedus, Mathiesen, & Deckert, 1991). In our study, GFR increased considerably in diabetic group. Stevia feeding to diabetic rats showed significant reduction in GFR. Therefore, it was likely that the stevia alleviated lipid peroxidation and tissue liver injuries through antioxidant enzyme activity.

3.6. Hepatoprotective activity

Normal values of ALT and AST in serum for rats is 46–80 and 18–30 IU/dl respectively (Kalyanasundarm, 1985). Serum ALT and AST levels were significantly increased in STZ treated animals by 89% and 42% compared to control group (Fig. 5). Pre-feeding of stevia leaves powder and extracted polyphenols to diabetic animals reduced the activity to 45%&38% and 13%&6% compared to diabetic group respectively. No significant change was seen in extracted fiber fed group.

ALT and AST are common intracellular enzymes that increase the liver damage induced by diabetes (Can et al., 2004). The results of this

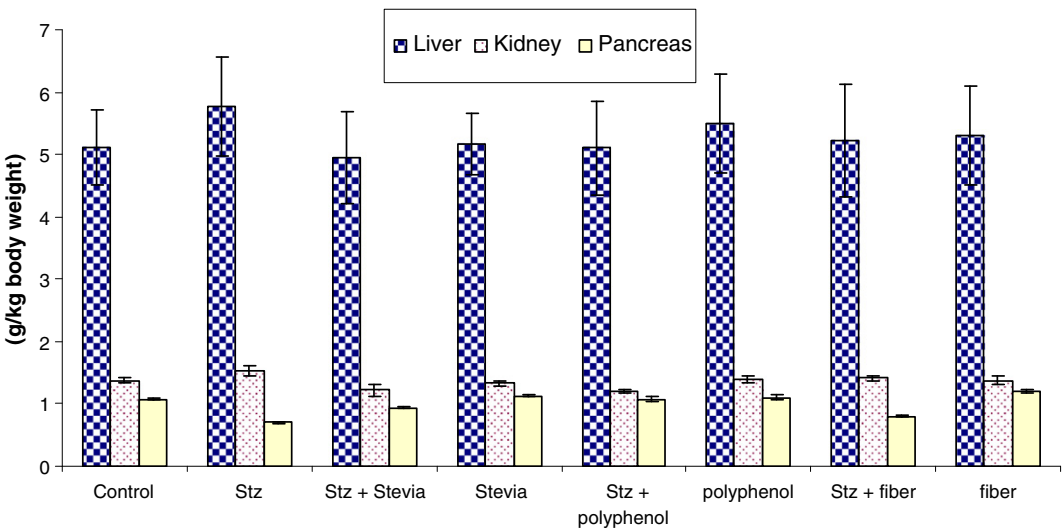

Fig. 4. Effect of STZ on organ weights.

Table 9
Effect of Stevia on kidney detoxifying enzymes levels in STZ treated rats (n = 8).

	γ-glutamyl transpeptidase	Glu-6-phosphate dehydrogenase
Control	5.42 ± 1.12**	60.4 ± 5.21**
STZ	23.4 ± 3.42*	45.2 ± 3.0*
Stevia leaves Powder	12 ± 2.02**	71.41 ± 6.12**
STZ + Stevia leaves Powder	25.614 ± 3.61*	57 ± 4.08**
Stevia leaves polyphenols	9 ± 2.2**	73.31 ± 5.82**
STZ + Stevia leaves polyphenols	25.614 ± 3.61*	55 ± 5.0**
Stevia leaves fiber	6.41 ± 2.02**	55.4 ± 4.21**
STZ + Stevia leaves fiber	20.14 ± 3.51*	42.12 ± 3.1*

study clearly showed the high level of ALT and AST serums in the STZ group. In contrast, they were decreased in the stevia leaves powder and its polyphenol extract groups. Stevia leaves powder and its polyphenol extract caused a significant decrease in the levels of ALT and AST serums and had a protective effect on the liver damage of diabetic treated rats (Abdelsattarelbatran, Elgengaihi, & Elshabrawy, 2006). It was likely that the reduced levels of ALT and AST serums by the stevia were an indication of alleviation of plasma membrane damage produced by diabetes (Fig. 5).

3.7. Statistical Analysis

In all the tables of this chapter the values are represented as mean ± SD from 8 experiments. Differences in mean values were analyzed using Student t-test and ANOVA. Significance is defined as $p<0.05$. Superscript '*' indicates the value is significantly different in comparison with Control ($P<0.05$) and superscript '**' indicates the value is significantly different in comparison with the treated group (STZ) at $P<0.05$.

4. Conclusion

All these results are quite interesting and very encouraging. One single plant could have multifaceted benefits. However, the study brings forth many questions which need further thorough investigation, like the mechanism of action of stevia leaves powder inactivating the STZ in the blood itself, or does it prevent its transport across into pancreatic and hepatic cells? Whether the extract simply mops off the reactive oxygen species or is it a chain breaking antioxidant? Whether the prevention of the liver damage seen with stevia leaves is the result of its antioxidant property or does it induce the activity of any other liver enzymes? The plant seems to be worth investigating further and could be an excellent source for nutraceuticals. In conclusion, polyphenols present in stevia leaves are mainly responsible for its multi-beneficial properties.

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Fig. 5. Effect of STZ on serum AST and ALT activities (IU/dl).

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