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Antioxidant and anti-diabetic potential of rebaudioside A and a mixture of steviol glycosides in alloxan-induced diabetic rats

Ena Gupta[1], Abubakar Mohammed[2], Neha Mishra[3*], Shalini Purwar[1], Syed Ibrahim Rizvi[2] and Shanthy Sundaram[1]

[1]Centre of Biotechnology, [2]Department of Biochemistry, University of Allahabad, Allahabad 211002, Uttar Pradesh, India

[3]Ethelind College of Home Science, Sam Higginbottom University of Agriculture, Allahabad 211007, Uttar Pradesh, India

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Diabetes mellitus, a metabolic disorder rapidly spreading worldwide affects multiple organs and body functions. *Stevia rebaudiana* (Bertoni), belonging to the Asteraceae family is a herb with medicinal and commercial importance to cure chronic diseases like diabetes mellitus. The present study was aimed to explore the antidiabetic effect of two reference standards like Steviol Glycosides System Suitability (SGSS, a mixture of nine steviol glycosides) and Rebaudioside A in alloxan-induced diabetic rats. In this regard, diabetes was induced in rats by administration of a single intraperitoneal (i.p.) injection of alloxan monohydrate (150 mg/kg body weight). After exposure to alloxan monohydrate, the overall intracellular antioxidant functions get disturbed which significantly increase the blood glucose levels, plasma malondialdehyde and sialic acid content. The oral administration of SGSS and Reb A at a dose of (20 and 30 mg/kg b.wt.) for a period of 21 days to diabetic rats significantly (P <0.05) reduced the blood glucose levels when compared with alloxan-induced diabetic control (DC) rats of group II and restore the antioxidant potential by decreasing the oxidative damage and also normalizes hyperlipidemic indices. The study demonstrated that bioactive components (steviol glycosides) of *S. rebaudiana* have potential therapeutic prospects to attenuate diabetes and its related complications.

Keywords: Alloxan, Diabetes mellitus, Insulin, Rebaudioside A, Steviol glycosides.

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Introduction

Diabetes mellitus is a group of metabolic disorder mainly identified as long term hyperglycemia due to insufficient action or secretion of insulin in the body[1]. Long term increase in levels of blood glucose is associated with major complications leading to neuropathy, retinopathy, cardiomyopathy, and nephropathy. Simultaneously, many other factors play a major role in the pathogenesis of diabetes by damaging beta cells and decreasing insulin secretion[2]. In diabetes, there is an increased generation of free radicals or reactive oxygen species (ROS) due to glucose autoxidation, increased polyol (sorbitol) pathway, non-enzymatic glycation of proteins, hyperglycemia, decreased antioxidant defences and lipid peroxidation leading to damage of enzymes, cellular mechanisms and modulation in insulin signalling due to oxidative stress[3].

Plant-derived bioactive compounds or natural antioxidants are efficient to reduce the free radicals formation and thus prevent oxidative damage effectively[4]. Traditional practices are the best option for the treatment of diabetes when compared with synthetic ones as many plants with their natural bioactive components initiate less toxicity more efficiently and cost-effectively with a low incidence of side effects. The natural bioactive compounds show their efficacy through different mechanisms which help in the reduction of high blood glucose levels. The few promising mechanisms are an increase in regeneration of β-cell mass, enhancing insulin's activity and inhibiting insulinase activity[5]. Therefore, in recent years there is an urgent need to investigate naturally originated plant-derived anti-diabetic and antioxidative agents with the vast reserve of phytochemicals.

Stevia rebaudiana (Bertoni), belonging to the family Asteraceae is a worldwide recognized herb with medicinal and commercial importance mainly approved as a low-calorie natural sweetener. Natural bioactive compounds like phenols, flavonoids, alkaloids, sugars, glycosides, and essential oils are present in the crude extracts of *S. rebaudiana* leaf

*Correspondent author
Email: neha.alladuniv@gmail.com
Mob.: 9807060312

which repairs the body systems and enhances the endogenous antioxidant levels. Approximately thirty *ent*-kaurene diterpene glycosides have been isolated from leaves of different species of *Stevia* plants generally recognized as steviol glycosides - stevioside, rebaudiosides A – E, dulcoside A, steviolbioside etc. The aglycone part of such glycosides is steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid) which is involved in constructing a C19-ester linkage between the glucose unit and a C19-carboxylic function, together with the formation of ether linkages using combinations of glucose, xylose and rhamnose moieties with C13-hydroxy group[6]. The glycoside of major interest because of its sweetening property is Rebaudioside A (4α)-13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-Dglucopyranosyl-β-D-glucopyranosyl)-oxy] kaur-6-en-8 oic acid β-D-glucopyranosyl ester). It is a glucosylated steviol glycoside found in large quantities and an active noncaloric, non-toxic and non-glycemic natural sweetener, extracted and purified from *S. rebaudiana*, approximately 1% in dried leaves. It has been reported that these different steviol glycosides and majorly Rebaudioside A possess additional functionalities by maintaining and regulating different metabolic processes in the body with possible beneficial effects on health including antioxidant, antimicrobial, anti-diabetic, antihypertension and cardio-protective activities[7]. Earlier studies established the protective effect of Rebaudioside A by stimulating the secretion of insulin from pancreatic β-cells and thus decreasing the condition of hyperglycemia due to its antioxidant property through scavenging ROS[8].

There is only a little information available regarding the biological actions of Rebaudioside A in reducing the complications of diabetes in rat model[7]. Additionally, more evidence is required on the antidiabetic activity of a mixture of steviol glycosides and individual glycoside Rebaudioside A for their various biological actions that reduce diabetic complications directly or indirectly in alloxan-induced diabetic rats. The Joint Food and Agriculture Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) purposely requested for additional studies to be performed involving repeated exposure to dietary and therapeutic doses of steviol glycosides in people with diabetes to help describe an acceptable dietary intake (ADI) of steviol glycosides[9]. In the United States and Europe, the first *Stevia*-based sweeteners were widely used. In a variety of food products, mixtures of different glycosides including Rebaudioside A are now being regularly used.

To date, there have been no research articles reported on the antidiabetic and protective effect of standard compounds Steviol Glycosides System Suitability (SGSS, a mixture of nine steviol glycosides) and individual glycoside components like Rebaudioside A (Reb A) in alloxan-induced diabetic rats. It was aimed to study whether the mixture of steviol glycosides/SGSS or individual glycoside Reb A possesses a similar property to reduce tissue complications by increasing insulin secretion from beta cells which helps in maintaining blood glucose homeostasis[7]. To better understand the complexity of the disease, the blood glucose, body weight and other biochemical parameters are integrated to find the overall effect of the mixture of nine steviol glycosides and individual Reb A, which was compared with the effect of standard drug insulin.

Materials and Methods

Chemicals

Analytical grade reagents were used to perform the experiments. Alloxan monohydrate (2,4,4,6- tetra-oxohexahydropyrimidine) was procured from Loba Chemie, Mumbai, India. Insulin was purchased from SRL chemicals. N-Acetylneuraminic acid (NANA) and resorcinol were purchased from Sigma Aldrich, India. ERBA Mannheim (Transasia Bio Medicals Ltd., Daman, India) kits were purchased for the estimation of biochemical parameters.

Test Supplements

Two reference standards SGSS, (a mixture of nine steviol glycosides i.e., stevioside, rebaudioside A-F, dulcoside A, rubusoside and steviolbioside and RebaudiosideA (Reb A) were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Animals and Diets

Healthy Wistar albino rats of both sexes (4-5 months old, weighing 150-200 g) were used for the study. Before the initiation of the experiment, all the rats were acclimatized for one week to the new standard laboratory conditions. They were housed under a controlled facility (25±5 °C) with a 12 h light/ 12 h dark cycle, fed with nutrient-rich diet pellets and water ad libitum. Safe handling and treatment of all the rats were done according to the principles of the institutional animal ethics committee constituted

under the directions of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), India. All the experimental protocols were approved by the Animal Care and Ethical Committee (849/GO/Re/04/CPCSEA) of the University of Allahabad.

Diabetes induction

The overnight fasted Wistar albino rats were injected intraperitoneally (i.p.) with alloxan monohydrate (2, 4, 5, 6-tetraoxyprimidine) dissolved in 0.9% normal saline at a dose of 150 mg/kg b.wt.[10]. Alloxan administration massively destroys pancreatic beta cells which induce toxicity, hyperglycemia and insulin-dependent diabetes mellitus (IDDM). The alloxan-injected rats were allowed to access 20% glucose solution for the next 24 hours to prevent initial drug-induced hypoglycemic shock. The diabetes was confirmed after one week of alloxan injection by measuring the levels of elevated blood glucose with the help of a glucometer (Accu-Chek, Roche Diagnostics, USA). The rats showing fasting blood glucose above (160 mg/dL) were considered diabetic and selected for further study.

Acute toxicity study for determining the dosage

After one week of diabetes induction, the overnight fasted rats were included in the study and body weight was recorded just before use. They were randomly divided into control and treatment groups, with four rats in each group. The control group received vehicle only, whereas the treatment groups were orally administered with pure compounds (SGSS) and individual glycoside (Reb A) ranging from 10 to 50 mg/kg b.wt. Animals were closely observed for any behavioural changes and other physical activities and after 5 hrs the blood glucose was determined[11]. The maximum antihyperglycemic effect brought by the lowest dose of each compound was given through oral intubations for repeated administration. The doses selected for the pure compounds (20 and 30 mg/kg b.w.) were administered every day till the completion of the experimental period (i.e., 21 days).

Experimental protocol

After inducing diabetes successfully, the experimental rats were divided into 7 groups containing 6 rats in each group (n=6). Pure compounds like (SGSS) and Reb-A were dissolved in distilled water which was administered orally to the experimental groups using the intragastric tube for 21 days. Group I: Normal control (NC) receiving no treatment (without alloxan or supplementation); Group II: Diabetic control (DC) injected with a single dose of alloxan monohydrate intraperitoneally[10]; Group III: Diabetic insulin group (DI) injected subcutaneously with three units of NPH insulin (NPH huminsulin, Lilly Egypt)[12] dissolved in distilled water were given twice a day; Group IV: Diabetic + SGSS (20 mg/kg b.w.); Group V: Diabetic + Reb A (20 mg/kg b.w.); Group VI: Diabetic + SGSS (30 mg/kg b.w.); and Group VII: Diabetic + Reb A (30 mg/kg b.w.).

Estimation of body weight

The body weight of the control group and all the treated groups were recorded on day 0 (pre-treatment) and days 3, 5, 7, 14 and 21 post-treatment. A calibrated and standardized electronic balance was used for taking the body weight of rats (expressed in g).

Blood samples collection and estimation of blood glucose

On day 0 (pre-treatment) and during the treatment (day 3, 5, 7, 14 and 21), the blood samples were collected from the rats tail vein for estimating blood glucose parameters (expressed as mg/dL) using glucose test strips of an electronic glucometer (Accu-Chek, Roche Diagnostics, USA).

Isolation of plasma and serum

The rats were sacrificed under the influence of light anaesthesia at the end of the experimental period. The cardiac was punctured and blood samples were collected into heparin rinsed 10 unit/mL of anticoagulant syringes (for serum collection, anticoagulant was not used) which were transferred into the test tubes and then red blood cells (RBCs) were pelleted by centrifugation at 800 g for 10-15 minutes at 4 °C. The separated plasma was stored immediately at -80 °C until used for biochemical estimations[13]. The RBCs and 15% of packed red blood cells (PRBCs) were washed twice with cold phosphate-buffered saline (PBS) (0.9% NaCl and 10 mmol L^{-1} Na_2HPO_4; pH 7.4).

Determination of erythrocyte malondialdehyde (MDA) content

Erythrocyte MDA was evaluated according to the method by Esterbauer and Cheeseman[14] with slight modification. Packed erythrocytes of 0.2 mL were suspended in 3 mL PBS containing 0.5 mM glucose (pH 7.4). The suspension of 0.2 mL was added to 1 mL of 10% trichloroacetic acid and 2 mL of 0.67% thiobarbituric acid. The obtained solution was boiled

for 20 minutes at 90–100 °C and then cooled. Consequently, the mixture was centrifuged at 1000 g for 5 minutes and the absorbance of the supernatant was read at 532 nm. The MDA concentration in erythrocytes was calculated using extinction coefficient (ε = 31,500) and expressed as nmol·mL^{-1} of packed erythrocytes.

Determination of membrane and plasma sialic acid (NANA) levels

The sialic acid level was estimated in membrane/plasma according to the method given by Spyridaki and Siskos[15]. Periodic acid-schiff (0.041 M) of 0.10 mL was added in a test tube containing 500 µL diluted (20 times) sample solution. The solution was thoroughly mixed and allowed to stand in an ice bath for 30 min. Afterwards, 1.25 mL of resorcinol working solution (5 mL of 6.0% resorcinol solution, 0.125 mL of 0.1 M copper sulphate solution and 19.875 mL of distilled water along with 50 mL of 10 M HCl and final volume was raised) was added, mixed and heated at 98 °C for 5 minutes. In an ice bath, the tubes were allowed to cool for 2 minutes and 3.25 mL of n-butanol was finally added. The solutions were vigorously mixed and the tubes were placed in a water bath at 37 °C for 3 minutes (for colour stabilization). The absorbance was immediately measured after removing the solutions from the water bath at 625 nm against a reagent blank set at zero. For the preparation of the calibration graph, standard solutions of NANA in the range of 20–200 µM and the unknown concentrations of total sialic acid in samples were calculated. A level of sialic acid in the membrane is calculated in terms of µg/mg membrane protein whereas in plasma sialic acid is measured as µM.

Total antioxidant activity (FRAP)

Ferric reducing ability of plasma (FRAP) assay was performed to study the total antioxidant potential of plasma according to the method reported by Benzie and Strain[16] with slight modification. Working FRAP reagent was prepared from 300 mmol/L acetate buffer (pH 3.6), 20 mmol/L ferric chloride and 10 mmol/L 2,4,6-tripyridyl-s-triazine made up in 40 mmol/L hydrochloric acid. These solutions were mixed in the ratio of 10:1:1 (vol/vol/vol). In 100 µL of plasma, the prepared FRAP reagent (3 mL) was mixed thoroughly and the absorbance was recorded at 593 nm at 30s intervals for 4 min. Fe (II) concentration (100–1000 µmol/L) in an aqueous solution was used for calibration. The regression equation was used for calculating the FRAP values (µmol Fe (II)/L) in plasma.

Biochemical estimations

Standard diagnostic kits were used for estimating biochemical parameters (total cholesterol, bilirubin, alkaline phosphatase, urea, creatinine, and uric acid) from previously isolated heparinised plasma.

Statistical analysis

The data obtained were presented as mean±standard deviation. One way analysis of variance (ANOVA) test was performed by using statistical analysis, followed by Bonferroni test using graph pad prism software package, version 5.0. The values of P <0.05 were considered statistically significant.

Results

Effects on body weight

In diabetes, one of the major symptoms is weight loss which was also noticed in this study. The change in body weight was observed in all the groups noted on days 0, 3, 5, 7, 14, and 21. The administration of the drug alloxan damages the pancreatic beta cells and provokes a condition of insulin deficiency which accelerates the body weight reduction leading to a condition of wasting. Significant (P <0.05) difference was observed for the change in body weight in alloxan-induced diabetic control (DC) rats when compared with (SGSS and Reb A) treated diabetic rats (Fig. 1). Stable body weight was observed in the normal control (NC) group whereas a significant decrease in body weight (155.26, 128.01, 115.67, 109.12, 98.34, and 85.89 g) on days 0, 3, 5, 7, 14, and 21

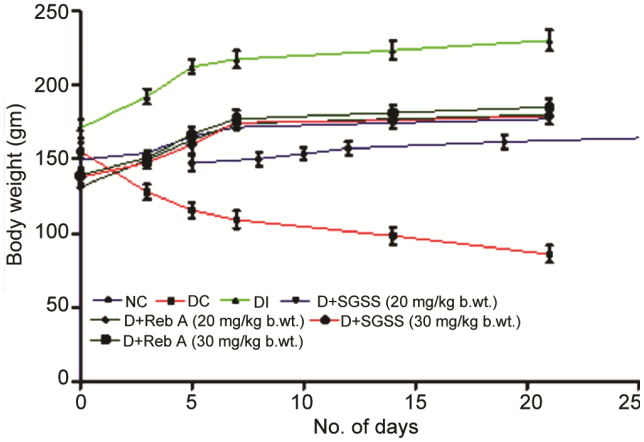

Fig. 1 — Graphical representation of changes in the body weight (gm) of normal and diabetic rats.

days were seen in group II (alloxan-induced negative control) rats. Rats of group III (positive control treated with standard drug Insulin) showed a significant increase in the body weights from 171.01 to 230.22 g. Oral administration of SGSS and Reb A (20 mg/kg b.w.) to diabetic induced rats in groups IV and V significantly increase the body weight (177.23 and 180.01 g) whereas groups VI and VII (30 mg/kg) also showed a significant increase in body weight (179.31 and 185.12 g) on day 21 as compared to day 0 value. Post-treatment with SGSS and Red A (20 and 30 mg/kg b.w.) significantly (P <0.05) increase the body weight in alloxan-induced diabetic rats compared with diabetic control rats (DC).

Effect on blood glucose

Oral administration of SGSS and Reb A (20 and 30 mg/kg b.w.) daily for a period of 21 days to diabetic rats significantly (P <0.05) decreased the level of blood glucose when compared with alloxan-induced diabetic control (DC) rats of group II (Table 1). During the study, period the diabetic rats of group III treated with antidiabetic reference drug insulin also showed a significant decrease in blood glucose levels from day 5 to 21 and maintained it to normal standard levels. SGSS and Reb A treated (20 mg/kg b.w.) diabetic rats of groups IV and V showed normal blood glucose levels (95.11 and 92.13 mg/dL) on day 21, while diabetic rats of groups VI and VII were treated with SGSS and Reb A(30 mg/kg b.w.) also exhibit a remarkable decrease in blood glucose (96.23 and 88.53 mg/dL), close to the value of day 0. The produced results by the standard compounds were found to be dose-dependent.

Erythrocyte malondialdehyde (MDA) content

Plasma malondialdehyde content was significantly (P <0.05) lower in a normal control group, insulin-treated group, SGSS and Reb-A treated diabetic group compared with the diabetic untreated control group. Administration of these pure compounds (20 and 30 mg/kg/day) significantly reduces the level of plasma malondialdehyde (MDA) content in diabetic rats of groups IV to VII, similar to insulin. It protects the erythrocytes from alloxan-induced hyperglycemia, as evidenced by the decreased MDA levels (Fig. 2).

Plasma sialic acid (NANA) levels

A significant (P <0.05) increase in plasma sialic acid content was observed in alloxan treated rats in group II as compared to normal control rats in group I. Administration with pure compounds SGSS and Reb-A at a dose of (20 and 30 mg/ kg b.w.) in alloxan-induced diabetic rats in groups IV to VII, retains more sialic acid content in plasma when compared with normal rats in group I. Furthermore, treatment with insulin also reduces the sialic acid level in diabetic rats of group III as shown in Fig. 3.

Effect on biochemical parameters

The results of SGSS and Reb-A at a dose of (20 and 30 mg/kg/day) on plasma total cholesterol, bilirubin (total and direct), urea, creatinine, uric acid and alkaline phosphatase of normal and alloxan-

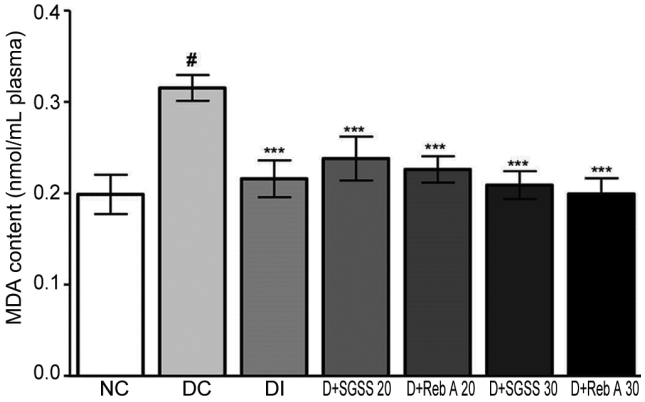

Fig. 2 — Levels of malondialdehyde (MDA) content in normal control and experimental groups. The value represents the mean±SE for six rats per group.

Table 1 — Effect of Steviol Glycosides System Suitability (SGSS) and Rebaudioside (Reb A) on blood glucose concentration

		Blood glucose concentration (mg/dL)					
Groups	Treatments	Day 0	Day 3	Day 5	Day 7	Day14	Day 21
I	NC	82.34±4.98	88.12±5.19	86.23±5.14	88.98±4.98	89.01±3.15	85.05±5.15
II	DC	97.56±5.14	372.91±6.14	398.99±5.88	409.01±4.72	458.91±4.25	465.32±5.65
III	D+I	86.19±4.99	132.18±5.72	108.51±5.63	101.27±3.98	97.86±5.02	89.01±5.72
IV	D+SG (20 mg)	98.07±5.57	295.28±4.28	221.76±5.89	125.15±3.98	107.15±4.78	95.11±5.61
V	D+Reb-A (20 mg)	85.56±4.28	312.21±5.93	207.43±4.21	109.27±5.62	103.01±3.32	92.13±4.80
VI	D+SG (30 mg)	105.14±4.52	286.67±5.21	216.35±4.86	120.08±5.78	105.21±5.65	93.23±4.91
VII	D+Reb-A (30 mg)	82.14±5.28	323.21±3.93	201.01±5.21	107.36±5.62	101.72±5.32	88.53±5.21

Each value represents mean±SD, n=6, P <0.05 significant

induced diabetic rats after 21 days of treatment are presented in Table 2.

The total cholesterol was found to be significantly (P <0.05) lower in normal rats (80.92 mg/dL) when compared to alloxan-induced diabetic rats (99.42 mg/dL). Rats of group III (Insulin treated) restore cholesterol level (84.98 mg/dL) close to normal. Administration with SGSS and Reb-A at a dose of (20 mg/kg) in alloxan-induced rats of IV and V resulted in a significant decline in levels of total cholesterol (82.40 and 83.85 mg/dL) while rats in groups VI and VII (30 mg/kg) also showed a significant decrease in total cholesterol (80.15 and 81.32 mg/dL) as shown in Table 2.

The direct and total bilirubin levels were shown in Table 2. Significantly (P <0.05) higher levels of bilirubin (direct and total) were observed in alloxan-induced diabetic rats (0.36 and 3.05 mg/dL) when compared with normal control rats of Group I (0.25 and 0.76 mg/dL). The rats in group III (insulin-treated) shows normal parameters for direct and total bilirubin (0.26 and 1.71 mg/dL). The oral Administration of SGSS and Reb-A at a dose of 20 mg/kg in alloxan-induced rats of groups IV and V resulted in a significant decline in the direct (0.31 and 0.34 mg/dL) and total bilirubin (1.55 and 1.47 mg/dL) whereas rats in groups VI and VII (30 mg/kg) also showed a decreased direct (0.28 and 0.31 mg/dL) and total bilirubin (1.43 and 1.44 mg/dL).

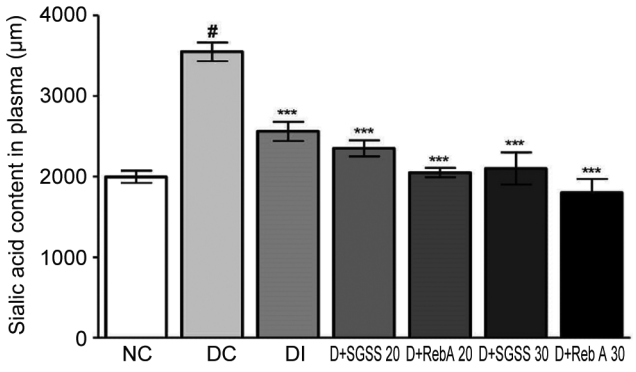

Fig. 3 — Levels of plasm sialic acid content in normal, control and experimental groups. The values represent the mean±SE for six rats per group.

Significant (P <0.05) increase in the concentration of plasma alkaline phosphatase was observed in alloxan treated rats in group II (422.19 mg/dL) when compared to normal control rats in group I (197.65 mg/dL). Decreased level of alkaline phosphate was also observed in insulin-treated rats (230.51 IU/L). Administration of SGSS and Reb A at a dose of 20 mg/kg in alloxan-induced rats of groups IV and V offered a significant decline in the level of alkaline phosphatase (211.14 and 213.18 IU/L), while the dose 30 mg/kg administered to rats in groups VI and VII (207.01 and 210.21 IU/L) restored the level of alkaline phosphatase close to a normal level as shown in Table 2.

Significantly (P <0.05) higher concentration of urea (61.50 mg/dL), creatinine (2.50 mg/dL), and uric acid (0.99 mg/dL) was observed in alloxan treated rats of group II when compared with normal rats in Group I for urea (38.52 mg/dL), creatinine (1.33 mg/dL) and uric acid (0.58 mg/dL). Insulin treated diabetic rats in group III also showed decreased levels of urea (41.02 mg/dL), creatinine (0.84 mg/dL) and uric acid (0.38 mg/dL). However, administration with SGSS and Reb-A at a dose of (20 mg/kg) in alloxan treated

Table 2 — Effect of ethanolic leaf extracts of *S. rebaudiana* on biochemical parameters in normal and alloxan-induced diabetic rats after 21 days of treatment

	Biochemical assays						
Treatment	Total Chloesterol (mg/dL)	Bilirubin (Direct) (mg/dL)	Bilirubin (Total) (mg/dL)	Alkaline Phosphatase (IU/L)	Urea (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
Normal Control	80.92±0.79	0.25±0.15	0.76±0.09	197.65±5.97	38.52±2.26	1.33±0.32	0.58±0.09
Diabetic Control	99.42±3.67	0.36±0.12	3.05±0.62	422.19±7.78	61.50±7.82	2.50±0.19	0.99±0.32
Diabetic+Insulin	84.98±0.46	0.26±0.05	1.71±0.02	230.51±14.9	41.02±5.11	0.84±0.89	0.38±0.05
Diabetic+SGSS (20 mg/kgb.wt)	82.40±0.44	0.31±0.34	1.55±0.34	211.14±8.12	36.81±3.10	0.59±0.155	0.64±0.08
Diabetic +Reb A (20 mg/kg b.wt)	83.85±0.73	0.34±0.06	1.47±0.527	213.18±15.0	35.90±6.34	0.48±0.14	0.61±0.24
Diabetic+SGSS (30 mg/kgb.wt)	80.15±3.77	0.28±0.13	1.43±1.26	207.01±12.5	36.19±2.10	0.54±0.10	0.59±0.13
Diabetic+Reb A (30 mg/kgb.wt)	81.32±1.33	0.31±0.29	1.44±0.09	210.21±14.2	34.81±4.63	0.45±0.28	0.57±0.88

Each value represents mean±SD, n=6, P <0.05 significant

rats of groups IV and V significantly reduce the blood urea (36.81 and 35.90 mg/dL), creatinine (0.59 and 0.48) and uric acid (0.64 and 0.61) concentration, while administration of (30 mg/kg) to diabetic rats in groups VI and VII also showed decreased urea (36.19 and 34.81 mg/dL), creatinine (0.54 and 0.45 mg/dL) and uric acid levels (0.59 and 0.57 mg/dL) as shown in Table 2.

Discussion

Diabetes mellitus is a metabolic disorder majorly characterized by hyperglycemia, insulin resistance, and several other long term complications. Increased oxidative stress, free radicals production, and impaired antioxidant defences lead to the development and progression of diabetes. For the management of diabetes, many drugs are available which pose to have drawbacks like restricted pharmacokinetic properties, secondary failure rates, high costs and serious adverse effects. Therefore, it is required to investigate more effective and safer therapeutic and natural medicines from traditional medicinal plants which offer great potential to work as hypoglycaemic agents[17]. The presence of broad arrays of active principles in plants represents diverse biological activities that demonstrate insulinomimetic activity and anti-diabetic properties. This study focused on investigating the potential effect of pure compounds like SGSS and Reb-A on alloxan treated diabetic rats.

The higher doses (20 and 30 mg/kg b.wt.) of SGSS and Reb-A could produce a significant difference in initial and final body weight and the blood glucose levels of normal and diabetic rats. After 21 days of treatment, the blood glucose levels were found to be significantly elevated with reduced body weight in diabetic control in comparison to normal control, insulin-treated and pure compounds treated experimental groups. Oral administration of pure compounds to alloxan treated rats produce potent *in-vivo* anti-diabetic or hypoglycaemic effect by maintaining blood glucose homeostasis and improving body weight without causing any toxic effect, unlike other artificial drugs[18]. This anti-hyperglycemic effect might be due to the presence of different steviol glycosides in the leaves of *S. rebaudiana* which thereby enhance glucose metabolism by stimulating the efficiency of β-cells of islets of Langerhans of the pancreas to produce more insulin. These bioactive compounds possess antioxidant properties as they scavenge free radicals and inhibit cellular oxidative damage and lipid peroxidation. According to Saravanan *et al.*[7] oral treatment with Reb-A to experimental diabetic rats significantly (P <0.05) lower the blood glucose levels by decreasing the oxidative stress and protecting the integrity of pancreatic β-cells, thus decreasing the condition of hyperglycemia. Gregersen *et al.*[19] studied the short term supplementation effects of diterpene glycoside (stevioside) in a test meal of type 2 diabetic patients. It was found that in type 2 diabetic patients, stevioside reduces postprandial blood glucose levels and exert beneficial effects in metabolizing blood glucose by increasing insulin secretion and improving diabetes regulation.

In diabetes, insulin resistance is developed due to the production of high levels of reactive oxygen species (ROS), which is accompanied by impaired antioxidant defence mechanisms causing damage to cellular proteins and enzymes along with the increase in lipid peroxidation, nitrate concentration, hydroxyl radicals, and hyperglycemia[20]. Malondialdehyde content is one of the most common indicators for estimating lipid peroxidation, with its increased level in erythrocytes alters membrane permeability, integrity and functional loss in lipid bilayer membrane fluidity. The observation of this study suggests the protective effect of SGSS and Reb-A at a dose of (20 and 30 mg/kg b.wt.) significantly (P <0.05) decrease the MDA concentration in plasma of alloxan treated diabetic rats when compared with untreated diabetic control rats. According to Shivanna *et al.*[21], administration of *Stevia* leaves and their extracted constituents (polyphenols and fibre) to streptozotocin-induced diabetic rats reduce the concentration of MDA in the liver and improve the status of antioxidant enzymes. The main source of oxidative stress and free radical generation is glucose oxidation which increases the plasma sialic acid content in diabetes. This study shows a significant (P <0.05) increase in plasma sialic acid levels in diabetic rats, administration of SGSS and Reb-A (20 and 30 mg/kg b.wt.) to diabetic treated rats restore the plasma sialic acid to normal.

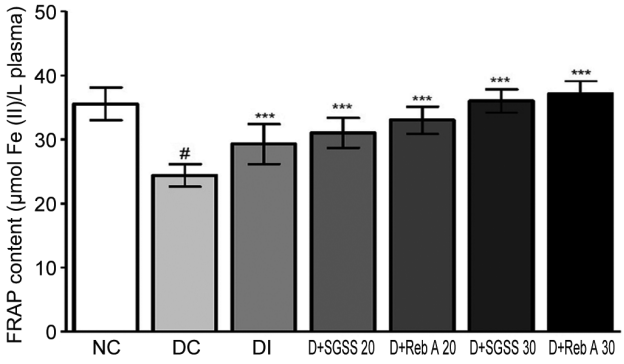

Fig. 4 — The total antioxidant activity (FRAP) in normal control and experimental groups. The values represent the mean±SE for six rats per group.

Cakatay and Kayali[22] reported that in diabetic animals the total antioxidant capacity (FRAP) levels is significantly decreased when compared to non-diabetic control groups. Similar observations were found in this study, where a significant increase in plasma (FRAP) of normal control and pure compounds treated diabetic rats when compared with untreated diabetic rats (Fig. 4). Treatment with SGSS and Reb-A (20 and 30 mg/kg) significantly (P <0.05) enhance the antioxidant potential in treated diabetic rats when compared with the untreated diabetic control group.

The biochemical parameters (total cholesterol, bilirubin, alkaline phosphatase, urea, creatinine and uric acid) indicate that administration with SGSS and Reb-A (20 and 30 mg/kg) provide protective effect by improving the condition of diabetes mellitus in alloxan treated rats and uphold the stable condition in normal rats.

Chronic hyperglycaemia promotes lipid peroxidation of low-density lipoprotein (LDL) resulting in free radical generation through a superoxide-dependent pathway. In diabetes, there is a decrease in endogenous insulin release which inactivates the production of lipoprotein lipase enzymes resulting in hypertriglyceridemia.

In diabetic dyslipidemia, there are an increased concentration in plasma triglyceride and LDL cholesterol particles which enhance the free fatty-acid release from insulin-resistant fat cells[23]. However, administration of methanolic root extract of *S. rebaudiana* significantly reduces the level of total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) by 53.5, 37.4, 82.3, and 45.6% respectively when compared with diabetic control group[24].

The liver is the largest organ that helps in maintaining the concentration of normal blood glucose in fasting and postprandial states. Liver dysfunction due to insulin resistance leads to glycogenolysis (increased production of hepatic glucose). Lack of proper storage of triglycerides and lipolysis in the liver (insulin-sensitive tissue) elevates the hepatic enzyme alkaline phosphatase (ALP) and bilirubin in the bloodstream.

In diabetes, the blood vessels (glomeruli) in the kidneys get damaged which affects the glomerular filtration rate (GFR) with the decreased synthesis of protein and increased tissue proteolysis with raised blood urea, creatinine and uric acid levels (Table 2). Administration with effective and safe hypoglycemic compounds (SGSSand Reb-A) at a dose of (20 and 30 mg/kg b.wt.) restores all the disturbed parameters in alloxan treated rats to the normal level.

Conclusion

In conclusion, the results suggest that two reference standards steviol glycosides system suitability (containing a mixture of nine steviol glycosides) and rebaudioside A has significant antioxidative, antihyperglycemic and antihyperlipidemic properties. These pure compounds in small doses (20 and 30 mg/kg b.w.) are potentially effective in decreasing oxidative damage by enhancing antioxidant levels and maintaining glucose homeostasis in diabetic rats. Thus, these compounds could be used as a potential drug or as a food additive (natural sweetener) in regulating the complications of diabetes and improving stress-related pathological conditions. Toxicological evaluation including clinical trials is required to better understand the mechanism of action at the molecular level and to settle issues associated with safety concerns. Therefore, further studies are necessary to cover a wide spectrum of applications of the bioactive compounds (steviol glycosides) of the herb *S. rebaudiana* before recommending the patients with type 2 diabetes.

Conflict of interest

The authors have declared no conflict of interest.

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References

1 Sundus S, Hira K, Sohail N, Tariq A, Ara J, *et al.*, Protective role of Pandanus tectorius Parkinson ex Du Roi in diabetes, hyperlipidemia, liver and kidney dysfunction in alloxan diabetic rats, *Clin Phytoscience*, 2021, **7**(1), 1-3.

2 Kangralkar VA, Patil S D and Bandivadekar R M, Oxidative stress and diabetes: A review, *Int J Pharm Appl*, 2010, **1**(1), 38–45.

3 Erejuwa O O, *Oxidative stress in diabetes mellitus: is there a role for hypoglycemic drugs and/or antioxidants, Oxid Stress Dis*, (IntechOpen), 2012, 217–46.

4 Ashour M N, Megahed A H, Morsy S M, Eltoukhy S I, Youness E R, *et al.*, Antioxidant and radical scavenging properties of garlic oil in streptozotocin induced diabetic rats, *Aust J Basic Appl Sci, 2011,* **5**(10), 280–86.

5 Janani C, Sundararajan B, Moola A K and Kumari B R, Antidiabetic activity of methanolic leaves extract of transformed soybean plantlets in streptozotocin (STZ) induced diabetic rats, *J Stress Physiol Biochem*, 2021, **17**(2), 66-78.

6 Gupta E, Purwar S, Sundaram S, Tripathi P and Rai G, Stevioside and rebaudioside A – predominant e*nt*-kaurene diterpene glycosides of therapeutic potential: A review, *Czech J Food Sci*, 2016, **34**(4), 281–99.

7 Saravanan R, Vengatashbabu K and Ramachandran V, Effect of rebaudioside A, a diterpenoid on glucose homeostasis in STZ-induced diabetic rats, *J Physiol Biochem*, 2012, **68**(3), 421-31.

8 Abudula R, Matchkov V V, Jeppesen P B, Nilsson H, Aalkjaer C, *et al.*, Rebaudioside A directly stimulates insulin secretion from pancreatic beta cells: A glucose dependent action via inhibition of ATP-sensitive K+ channals, *Diabetes Obes Metab*, 2008, **10**(11), 1074–85.

9 JECFA, Steviol glycosides, In: 63rd Meeting of the joint FAO/WHO expert committee on food additives, Geneva, Switzerland, World Health Organization (WHO), Geneva, Switzerland, WHO Technical Report Series 928, 2005, 34–39, 138.

10 Burade K B and Kuchekar B S, Antidiabetic activity of madhunashini (MD-19) in alloxan induced diabetes mellitus, *J Cell Tissue Res*, 2011, **11**(I), 2515–20.

11 Karunanayake E H, Welihinda J, Sirimanne S R and Sinnadoria H, Oral hypoglycaemic activity of some medicinal plants of Sri Lanka, *J Ethnopharmacol*, 1984, **1**(2), 223-231.

12 Leite A C R, Ara´ujo T G, Carvalho B M, Silva N H, Lima V L, *et al.*, Parkinsoniaaculeata aqueous extract fraction: Biochemical studies in alloxan-induced diabetic rats, *J Ethnopharmacol*, 2007, **111**(3), 547–552.

13 Takahashi M, Makino S, Kikkawa T and Osumi N, Preparation of rat serum suitable for mammalian whole embryo culture, *J Vis Exp*, 2014, **3**(90), 51969.

14 Esterbauer H and Cheeseman K H, Determination of aldehydic lipid peroxidation products: Malondialdehyde and 4-hydroxynonenal, *Methods Enzymol*, 1990, **186**, 407–421.

15 Spyridaki M H E and Siskos P A, "An improved spectrophotometric method for the determination of free, bound and total N-acetylneuraminic acid in biological fluids," *Anal Chim Acta*,1996, **327**(3), 277–285.

16 Benzie I F F and Strain J J, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, *Anal Biochem*, 1996, **239**, 70-76.

17 Kumar R and Hemalatha S, An overview on antidiabetic medicinal plants having insulin mimetic property, *Asian Pac J Trop Biomed*, 2012, **2**(4), 320–330.

18 Losso J N, Holliday D L, Finley J W, Martin R J, Rood J C, *et al.*, Fenugreek bread: A treatment for diabetes mellitus, *J Med Food*, 2009, **12**(5), 1046-1049.

19 Gregersen S, Jeppesen P B, Holst J J and Hermansen K, Antihyperglycemic effects of stevioside in type 2 diabetic subjects, *Metabolism*, 2004, **53**(1), 73–76.

20 Maritim A C, Sanders R A and Watkins J B, Diabetes, oxidative stress, and antioxidants: A review, *J Biochem Mol Toxicol*, 2003, **17**(1), 24–38.

21 Shivanna N, Naika M, Khanum F and Kaul V K, Antioxidant, anti-diabetic and renal protective properties of *Stevia rebaudiana*, ***J Diabetes Complications*****,** 2013, **27**(2), 103-113.

22 Cakatay U and Kayali R, The evaluation of altered redox status in plasma and mitochondria of acute and chronic diabetic rats, *Clin Biochem*, 2006, **39**(9), 907-912.

23 Williamson J R, Chang K, Frangos M, Hasan K S, Ido Y, Kawamura T, *et al.*, Hyperglycemic pseudohypoxia and diabetic complications, *Diabetes*, 1993, **42**(6), 801–813.

24 Singh S, Garg V and Yadav D, Antihyperglycemic and antioxidative ability of *Stevia rebaudiana* (Bertoni) leaves in diabetes induced mice, *Int J Pharm Pharm Sci*, 2013, **5**(2), 297-302.

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