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Antidiabetic Activity of Different Fractions of Methanol Extract of *Stevia rebaudiana* Leaves in Streptozotocin Induced Diabetic Rats and its Role in Regulation of Carbohydrate and Lipid Metabolism

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ABSTRACT

The aim of the present study is to exploring benzene, ethyl acetate and ethanol fraction of methanolic leaf extract of Stevia rebaudiana for antidiabetic activities in streptozotocin induced diabetic rats. The acute toxicity studies of different fractions showed that all fractions at the dose of 2000 mg/kg did not show any toxic effects. one day single dose study showed Ethyl Acetate Fraction (EAFSR) at dose of 200 and 400 mg/kg, p.o. decreased blood glucose level significantly ($p<0.05$) in diabetic rats. Three weeks repeated treatment with EAFSR at dose of 200 mg/kg, p.o. lowered blood glucose by 39% on day 21 as compared to control group and GLB Glibenclamide (500 µg/kg) lowered blood glucose by 52% in streptozotocin induced diabetic rats. Treatment with EAFSR exhibited significant changes of lipid profile in diabetic rats ($p<0.05$) TG, TC, Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL) reduced by 19%, 19%, 32% and 18% respectively whereas High Density Lipoprotein (HDL) was increased by 28%. Glyburide at dose of 0.25 mg/kg, p.o. decreased TG, TC, LDL and VLDL by 31%, 29%, 47% and 31% respectively and increased HDL by 40%. EAFSR and GLB treatments enhance insulin level by 68% and 53%, total protein increased by 24% and 25%, albumin decreased by 35% and 43%, urea decreased by 37% and 45%, total hemoglobin increased by 24% and 44%, glycosylated haemoglobin decreased by 41% and 52% and WBCs increased by 23% and 44% respectively. EAFSR seems to have GLB like potential and emerged as a potential candidate for management of diabetes mellitus.

Keywords: *Stevia rebaudiana*, Diabetes, Blood glucose, Antihyperlipidemic, Animal model

INTRODUCTION

Diabetes is a chronic metabolic disorder characterized by impaired carbohydrate metabolism resulting in an increased blood glucose level i.e. hyperglycemia. Over several years diabetes mellitus has become a major health problem worldwide; reaching epidemic proportions [1]. India has earned the dubious distinction of being termed the "Diabetes Capital of the World" by leading the world with largest number of diabetic subjects and the prevalence of diabetes is consistently increasing [2]. Herbal drugs are effective, safer and acceptable to the general society [3]. Currently available oral antidiabetic agents have a number of serious adverse effects. Errors in the choice of drug can also occur; some sulphonylureas like chlorpropamide and glibenclamide are more commonly associated with hypoglycaemia [4]. Thus, the management of diabetes without any side effects is still a challenge. *Stevia rebaudiana is* plant of asteraceae family. Stevia leaves contain a complex mixture of natural diterpene glycosides namely, steviol, steviolbioside, stevioside and rebaudiosides [5]. It is used as antidiabetic, antimicrobial and antifertility agent. It is also used for its immunological potential. The present study was undertaken to investigate the antidiabetic effect of chronic (long term) treatment with different fractions of *S. rebaudiana* leaves on experimental animals.

MATERIAL AND METHODS

Chemicals and reagents

Kits for biochemical parameters such as Lipid profile, Total protein, albumin, creatinin, urea and Enzyme Linked Immunosorbent Assay (ELISA) were purchased from Erba Diagnostics, Mannheim, Germany. Other chemicals used in the study as streptozotocin were purchased from Sigma Aldrich Chemical and Qualigens.

Preparation of fractions of methanol extract of *S. rebaudiana*

The leaves of the *S. rebaudiana* were collected from the nursery at Bhopal, and authenticated by the Botany Department Saifia Science college,

Bhopal (M.P) and voucher specimens were deposited (420/ Bot./saf./14). Shade dried powder of leaves of *S. rebaudiana* were subjected for successive solvent extraction using soxhlation method. On the basis of polarity index, fractionation of methanol extract was done using benzene, ethyl acetate and ethanol by silica gel 60-80 mesh packed chromatographic column. The solvents were removed by distillation and last traces of solvents were removed under vacuum.

Acute toxicity studies

Selection of animals

Albino mice of either sex weighing 20-30 g were selected. They were individually housed in polypropylene cages, in well-ventilated rooms, under hygienic condition. Animals were given water (*ad libitum*) and were fed with rat pellet feed. This study was carried out according to guidelines for the care and use of laboratory animals and approved by the Institutional Animal Ethical Committee.

Preparation of drug samples

Benzene Fraction (BFSR), Ethyl Acetate Fraction (EAFSR) and Ethanol Fraction (EFSR) were prepared as 2% gum acacia suspensions. These were administrated through the oral route.

LD_{50} determination

The test substances were administered in a single-dose orally to overnight fasted animals by gavage. Three animals were used in each category and starting dose lied in the range of 2000-5000 mg/kg body weight. $1/10^{th}$ of the lethal dose was used as effective dose for antidiabetic screening where 5, 10 and 20 times of the effective dose of extracts and fractions were optimized. After administration the animals were observed continuously for 1 h for the next 4 h and then upto 24 h.

Table 1: Acute toxicity study of fractions of methanol extracts

S. No.	Fractions	LD_{50} Cut-off (mg/kg body weight)
1	BFSR	2000
2	EAFSR	2000
3	EFSR	2000

Antidiabetic screening models

Experimental animal

Healthy adult male albino rats between 2-3 mon of age and weighing 200-280 g were used for the study. They were housed in group of 6, in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle, 25 ± 10°C, 35-60% humidity). The animals were fed with standard rat pellet diet (Hindustan lever Ltd Mumbai, India) and water *ad libitum*.

Induction of diabetes

Animals were made diabetic by injection of a single dose of streptozotocin (45 mg/kg) in 0.1 M citrate buffer (pH 4.5) intraperitoneally. The rats were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycemia [6]. Fasting blood glucose was estimated at the time of induction of diabetes and postprandial-glucose was checked regularly until stable hyperglycemia was achieved. Control animals were similarly administered with vehicle only. The rats showing fasting blood glucose level more than 250 mg/dl were considered diabetic and selected for the experimentation.

Administration of samples

Samples are administered orally in the form of 2% acacia suspension. Animals are divided in different groups as Diabetic Control (DC), diabetic rats containing standard drug and diabetic rats containing different fractions. Blood glucose was determined using One Touch Glucometer (Accu-Check Sensor, Roche Diagnostics, Germany). After the administration of vehicle, GLB (500 µg/kg) and fractions blood was taken out at 2, 4 and 6 h after the treatment and blood glucose was estimated.

Statistical analysis

All the experimental data for statistical analysis were presented as mean ± SEM. One-way Analysis of Variance (ANOVA) was applied by Dunnett's test. For these calculations a window based package INSTAT was used.

Table 2: Effect of single dose treatment of fractions of methanol extracts of SR on blood glucose level in streptozotocin induced diabetic rats

S. No.	Treatment (mg/kg)	Blood glucose (mg/dl)			
		0 h (FBG)	2 h	4 h	6 h
1.	DC	311.08 ± 6.80	313.10 ± 8.46	316.20 ± 9.40	318.24 ± 10.52
2.	BFSR 100	320.30 ± 7.28	321.12 ± 8.02	320.10 ± 8.02	320.20 ± 6.86
3.	BFSR 200	322.12 ± 4.80	322.80 ± 5.30	320.00 ± 7.24	321.48 ± 9.84
4.	BFSR 400	316.60 ± 10.28	316.30 ± 6.20	314.40 ± 8.08	315.02 ± 10.40
5.	EAFSR 100	318.84 ± 6.48	308.40 ± 9.07	300.38 ± 8.58	294.42 ± 7.42[a,b]
6.	EAFSR 200	312.28 ± 11.64	282.74 ± 8.28[a,b]	260.20 ± 9.42[a,b]	215.52 ± 10.70[a,b]
7.	EAFSR 400	310.68 ± 9.12	284.62 ± 7.90[a,b]	270.46 ± 6.20[a,b]	226.10 ± 8.46[a,b]
8.	EFSR 100	320.46 ± 8.82	321.34 ± 7.64	319.32 ± 8.90	319.00 ± 9.12
9.	EFSR 200	318.68 ± 5.82	320.40 ± 9.48	319.86 ± 10.25	318.28 ± 10.62
10.	EFSR 400	322.00 ± 11.32	323.60 ± 9.46	321.92 ± 10.46	321.50 ± 8.80
11.	GLB	308.00 ± 6.86	266.30 ± 6.48	228.28 ± 7.66	214.20 ± 5.28

n=6 rats in each group, values are mean ± SEM; [a]$p<0.05$ compared to control group (ANOVA applied by Dunnett's test); [b]$p<0.05$ compared to initial (0 h) value followed by paired 't' test; FBG=Fasting Blood Glucose

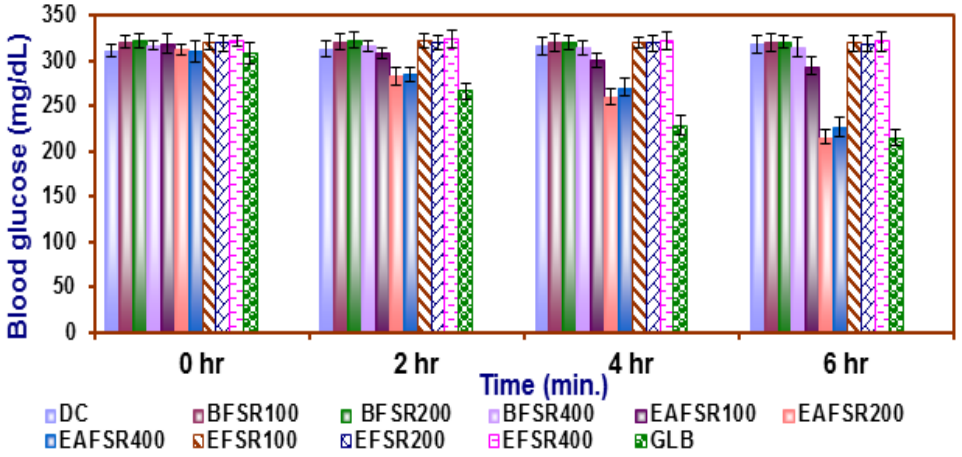

Figure 1: Effect of Single dose treatment of fractions of methanol extracts of SR on blood glucose level in streptozotocin induced diabetic rats

Three weeks treatment of bioactive fractions in streptozotocin-induced diabetic rats

On the basis of one-day single dose treatment of fractions of methanol extract of *S. rebaudiana*, EAFSR showed significant antidiabetic activity and hence it was selected for further study.

Effect of three weeks treatment of EAFSR on blood glucose in streptozotocin induced diabetic rats

EAFSR was administered 21 days at the dose of 200 mg/kg and GLB 500 µg/kg, p.o. as a standard drug orally for three weeks in the streptozotocin-induced diabetic rats. Blood glucose was determined using one touch glucometer and results were expressed in mg/dl (Tables 1-3 and Figures 1-4).

Table 3: Effects of three weeks continued administration of EAFSR on blood glucose level in streptozotocin induced diabetic rats

S. No.	Treatment	Blood glucose (mg/dl)	
		Before Treatment	After Treatment
1	Normal control	82.62 ± 5.60	81.24 ± 4.30
2	Diabetic control	302.26 ± 4.36	304.40 ± 6.20**
5	EAFSR 200	312.82 ± 5.42	191.28 ± 4.20*
7	GLB	310.30 ± 6.14	150.20 ± 4.12*

n=6 rats in each group, values are mean ± SEM; **p<0.05 compared to normal control group (ANOVA applied by Dunnett's test); *p<0.05 compared to diabetic control group (ANOVA applied by Dunnett's)

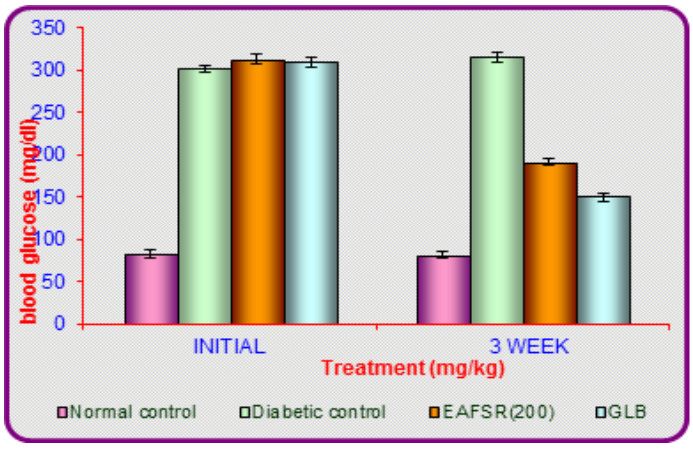

Figure 2: Effects of three weeks continued administration of EAFSR on blood glucose level in streptozotocin induced diabetic rats

Effect of EAFSR on lipid profile in streptozotocin induced diabetic rats

On completion of the twenty one days of treatment, animals were dissected and whole blood sample was collected and lipid profile estimation was carried out. Total cholesterol, High Density Lipoprotein (HDL) cholesterol and Triglyceride (TG) level in serum were determined according to the instructions of the availed kits protocol (Tran Asia Bio Medical Limited, Mumbai, India). Low density lipoprotein cholesterol was calculated by using Friedwald formula [7]. All the lipid profile estimation data were expressed in mg/dl (Table 4 and Figure 5)

Table 4: Effect of three weeks repeated treatment of EAFSR on lipid profile in streptozotocin-induced diabetic rats

S. No.	Treatment (mg/kg)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	TG (mg/dl)
1.	NC	84.12 ± 4.80	40.28 ± 2.30	26.32 ± 3.10	17.52 ± 1.6*	87.62 ± 7.26
2.	DC	146.82 ± 1.40**	25.0 ± 1.86**	92.14 ± 7.64**	29.68 ± 1.4**	148.42 ± 5.06**
3	EAFSR	118.62 ± 4.22*	32.20 ± 2.12*	62.28 ± 4.28*	24.14 ± 1.4*	120.68 ± 4.76*
4.	GLB	104.46 ± 5.18*	34.98 ± 2.10*	49.06 ± 3.82*	20.42 ± 1.1	102.12 ± 4.38*

n=6 rats in each group, values are mean ± SEM; **p<0.05 compared to normal control group (ANOVA applied by Dunnett's test); *p<0.05 compared to diabetic control group (ANOVA applied by Dunnett's test)

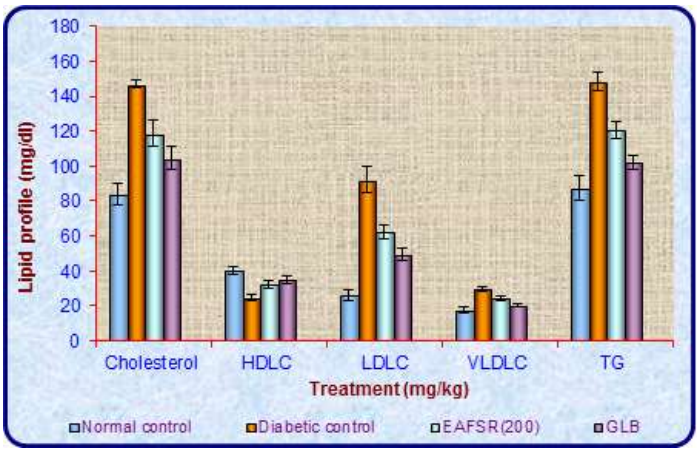

Figure 3: Effect of three weeks repeated treatment of EAFSR on lipid profile in streptozotocin-induced diabetic rats

Effect of EAFSR on biochemical parameter as insulin, total protein, albumin and urea streptozotocin induced diabetic rats

Diabetes Mellitus (DM) is also grossly reflected by profound changes in protein metabolism and by a negative nitrogen (N) balance and loss of nitrogen from most organs.

Table 5: Effects of three weeks continued administration of EAFSR on insulin, total protein, albumin and urea in streptozotocin-induced diabetic rats

S. No.	Treatment (mg/kg)	Insulin	Total protein	Albumin	Urea
1.	NC	13.10 ± 0.54	9.64 ± 0.62	3.74 ± 0.18	32.80 ± 0.52
2.	DC	5.25 ± 0.32**	8.07 ± 0.26**	2.60 ± 0.20**	70.60 ± 0.78**
3.	EAFSR	8.80 ± 0.63*	10.05 ± 0.18*	3.28 ± 0.12*	44.22 ± 0.46*
4.	GLB	11.10 ± 0.42*	10.12 ± 0.68*	3.52 ± 0.10*	38.90 ± 0.48*

n=6 rats in each group, values are mean ± SEM; **p<0.05 compared to normal control group (ANOVA applied by Dunnett's test); *p<0.05 compared to diabetic control group (ANOVA applied by Dunnett's test)

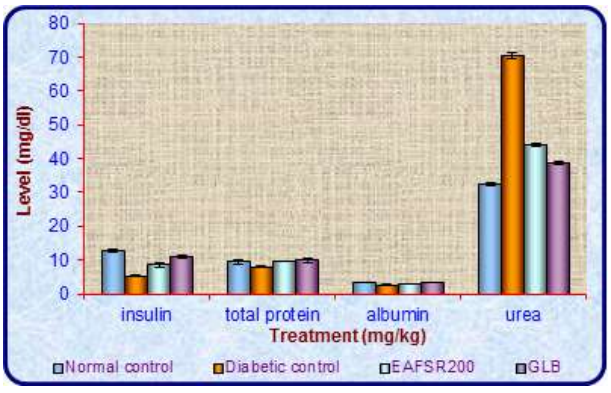

Figure 4: Effect of three week treatment of EAFSR on biochemical parameter in diabetic rats

After three weeks treatment with bioactive fractions in streptozotocin-induced diabetic rats were examined for activity. Moreover associated enzymatic anomalies exhibited glucose metabolic disorder and other complications eventually.

Effect on glucose-6-phosphatase and hexokinase

Liver is the candidate organ involved in glucose homeostasis. It is the main site for glycolysis, a process where glucose is degraded and gluconeogenesis, where glucose is synthesized from lactate, amino acids and glycerol. These are the two important complementary events that balance the glucose load in our body [8]. Hexokinase is the prime enzyme catalyzing glucose phosphorylation. Impairment of hexokinase activity suggest the impaired oxidation of glucose via glycolysis leading to its accumulation resulting in hyperglycemia. Glucose-6-phosphatase and fructose-1,6-bisphosphatase, are the regulatory enzymes in gluconeogenic pathway.

Table 6: Effects of three weeks continued administration of EAFSR on enzymes in streptozotocin-induced diabetic rats

S. No.	Treatment (mg/kg)	Enzyme level	
		Glucose-6phosphatase	Hexokinase
1.	Normal control	0.182±0.004	0.144±0.002
2.	Diabetic control	0.324±0.008**	0.106±0.1**
6.	EAFSR	0.180±0.005*	0.124±0.008*
7.	GLB	0.176±0.003*	0.148±0.004*

n=6 rats in each group, values are mean ± SEM

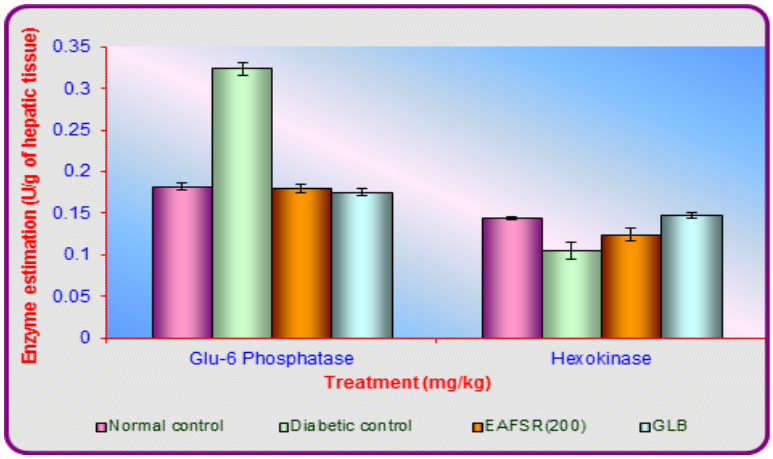

Figure 5: Effect of three week treatment of EAFSR on glucose-6-phosphatase and hexokinase level in diabetic rats

Effect of repeated dose treatment of EAFSR on Hematological parameter in streptozotocin induced diabetic rats

Assessment of hematological parameters can be used to determine the extent of deleterious effect of foreign compounds including plant extracts on the blood constituents of an animal. It can also be used to explain blood relating functions of chemical compounds/plant extract. Haemoglobin was determined using Cynmeth-hemoglobin solution [9]. The RBC and WBC counting methods were based on the dilution of obtained blood with diluting fluids in RBC and WBC counting pipettes [10].

Table 7: Effect of repeated dose treatment of EAFSR on hematological parameter in streptozotocin induced diabetic rat

S. No.	Treatment (mg/kg)	Hb	Glycosylated Hb	RBC	WBC
1.	NC	13.20 ± 5.84	2.86 ± 1.60	5.72 ± 1.25	5.04 ± 2.6
2.	DC	9.02 ± 4.82**	8.34 ± 2.50**	4.20 ± 2.10**	3.24 ± 1.2**
3.	EAFSR	11.20 ± 4.10*	4.92 ± 2.48*	4.22 ± 2.14	4.00 ± 2.8*
4.	GLB	13.02 ± 6.30*	4.00 ± 2.06*	5.26 ± 3.26*	4.68 ± 2.20*

n = 6 rats in each group, values are mean ± SEM; **p<0.05 compared to normal control group (ANOVA applied by Dunnett's test); *p<0.05 compared to diabetic control group (ANOVA applied by Dunnett's)

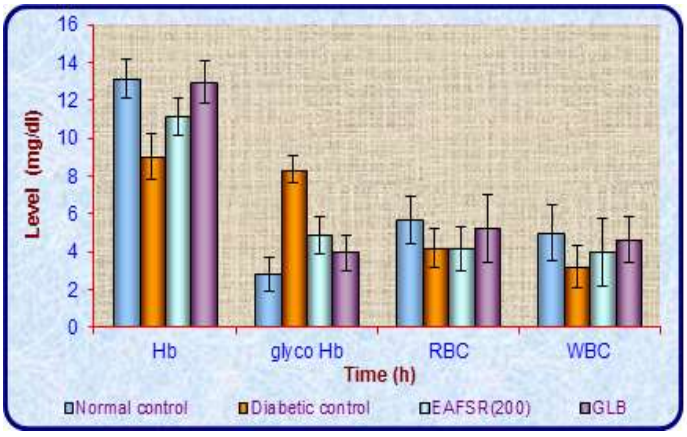

Figure 6: Effect of repeated dose treatment of EAFSR on hematological parameter in streptozotocin induced diabetic rats

RESULTS AND DISCUSSION

The acute toxicity studies of different fractions showed that administration of single dose of benzene, ethyl acetate and ethanol fraction at the dose of 2000 mg/kg did not show any toxic effects. These animals remained, healthy and active during the observation period. Thus these fractions were considered safe for oral administration (Table 1).

Effect of one day single dose treatment of fractions of extract on blood glucose in streptozotocin-induced diabetic rats at dose of 100 mg/kg, p.o. seems to have marginal anti hyperglycemic action in diabetic rats and decreased blood glucose level significantly ($p<0.05$) at 6 h of their

treatment by 7%. EAFSR at dose of 200 and 400 mg/kg, p.o. decreased blood glucose level significantly ($p<0.05$) in diabetic rats at 2 h which persisted reducing blood glucose level up to 4 and 6 h by 9%, 17%, 31% and 8%, 13%, 27% respectively. GLB treated rats produced significant ($p<0.05$) reduction of glucose level at 2 h, 4h and 6 h by 14%, 26%, 31% respectively compared to diabetic control rats. Other fractions i.e., BFSR and EFSR at optimized dose failed to exhibit an antihyperglycemic action (Table 2 and Figure 1).

Effect of three weeks repeated treatment of bioactive fractions and Shilajit on blood glucose level in Streptozotocin-induced diabetic rats

The effect of three weeks repeated treatment of EAFSR on blood glucose level in streptozotocin-induced diabetic rats showed that on continued administration at the dose level of 200 mg/kg significantly ($p<0.05$) lowered blood glucose by 39% on day 21 as compared to control group. GLB showed significant ($p<0.05$) antidiabetic activity in streptozotocin- induced diabetic rats 52% after its three weeks repeated administration (Table 3 and Figure 2).

Estimation of lipid profile in streptozotocin-induced diabetic rats

Liver and kidney participates in the uptake, oxidation and metabolic conversion of free fatty acids, synthesis of cholesterol, phospholipids, and triglycerides. During diabetes, a profound alteration in the concentration and composition of lipids occurs. Although cholesterol and lipids are important for the various metabolic processes of the body cells, they are also important features in diabetes. When their level exceeds than normal values, they are deposited in the blood vessels leading to atherosclerosis and other associated complications. The emerging evidences confirm the pivotal role of hyperlipidemia, mainly elevated blood cholesterol, particularly LDL cholesterol and VLDL cholesterol in the development of atherosclerosis related disease. Chronic diabetes usually results in disturbance of the plasma lipid profile including increased plasma cholesterol and triglyceride [11]. This study confirms the previous finding that the plasma lipids were increased in the diabetic rats.

In the light of above finding our research reveals that streptozotocin-induced diabetes in rats significantly ($p<0.05$) changed biochemical parameters such as increase in serum total cholesterol and triglyceride and decrease in serum HDL-cholesterol compared to normal control healthy group, bioactive fractions and glibenclamide treated diabetic group of rats. Repeated dose treatment for consecutive three weeks treatment of EAFSR at 200 mg/kg, p.o. exhibited significant changes of lipid profile in diabetic rats ($p<0.05$) while reducing harmful cholesterol and increasing favorable cholesterol as compared to diabetic rats. Conclusively EAFSR at dose of 200 mg/kg, p.o. decreased harmful lipid cholesterol i.e., TG, TC, LDL and VLDL by 19%, 19%, 32% and 18% respectively whereas HDL was increased by 28%. Glyburide at dose of 0.25 mg/kg, p.o. decreased harmful cholesterol i.e., TG, TC, LDL and VLDL by 31%, 29%, 47% and 31% respectively and increased favorable cholesterol HDL by 40% (Table 4 and Figure 3). Finally it may be hypothesized that bioactive fractions with respect to promoting favorable lipid profile and reducing to harmful lipid profile, seems to have GLB like potential and emerged as a potential candidate for management of diabetes mellitus.

Estimation of insulin, total protein, albumin, creatinine and urea

EAFSR and glibenclamide treatments have shown remarkable enhancement ($p<0.05$) in insulin level by 68% and 53%. Among the parameters of protein metabolism the present study showed an overall reduction in serum total protein in diabetic rats and the tested fractions showed an elevation of total protein by 24% and 25% by EAFSR and GLB respectively. Three weeks repeated dose Treatment with EAFSR and GLB produce significant effect on albumin decreased by 35% and 43%. Increased urea nitrogen production in diabetes may be accounted for by enhanced catabolism of both liver and plasma proteins. In the present study EAFSR and GLB significantly decreased level of urea by 37%, 45% respectively (Table 5 and Figure 4).

Estimation of glucose-6-phosphatase and hexokinase enzymes activity

Biochemical estimation of various enzyme activities and tissue glycogen content serve as a marker of excess blood glucose level in diabetic condition as it aggravates various complications and induces a 2-3 fold increase in Glc-6-pase activity and decreases hexokinase and glycogen content in liver[12]. Therefore the effect of fractions in consecutive three weeks repeated dose treated streptozotocin-induced diabetic group of rats was observed. The study revealed that EAFSR at dose of 200 mg/kg, p.o, exhibited significant ($p<0.05$) increase in hexokinase level in liver, was 29% and 54% respectively. Overall it could be concluding that regulation of glucose homeostasis might be not due to counter-balancing through one defined process only but in any factors may be responsible to regulate glucose metabolism (Table 6 and Figure 5).

Estimation of hematological parameters

It has been suggested that anaemia occurrence in DM is due to the increased non-enzymatic glycosylation of RBC membrane proteins, which correlates with hyperglycaemia [13]. Oxidation of these glycosylated membrane proteins and hyperglycaemia in DM cause an increase in the production of lipid peroxides causing a hemolysis of RBC. During diabetes the excess glucose present in blood reacts with haemoglobin. Therefore the total haemoglobin levels decreased in alloxan diabetic rats [14].

Induction of diabetes changed hematological parameter such as Hb, RBCs and WBCs, significantly ($p<0.05$) decreases and glycosylated Hb as compared to normal control group. Administration of EAFSR and GLB to diabetic rats significantly ($p<0.05$) restored the changes in the level of total hemoglobin increased by 24%and 44%, glycosylated haemoglobin decreased by 41% and 52%, RBCs increased 25% by GLB and WBCs increased by 23% and 44% respectively, to near normal levels, (Table 7 and Figure 6).

CONCLUSION

It was observed that the administration of ethyl acetate fractions of methanolic extract of *S. rebaudiana* leaves showed antidiabetic activity, by producing significant restoration of blood glucose level as well as illustrated some beneficial effects such as reduced hypercholesterol and hypertriglyceride level in streptozotocin-induced diabetic rats. This study will pave the way for plant based specific treatment of diabetes avoiding the complications of artificial drug substances.

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