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ANTIHYPERGLYCEMIC AND ANTIHYPERLIPIDEMIC EFFECTS OF DIFFERENT FRACTIONS OF *STEVIA REBAUDIANA* LEAVES IN ALLOXAN INDUCED DIABETIC RATS

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ABSTRACT

The present study carried out to investigate the antihyperglycemic, oral glucose tolerance test (OGTT) and antihyperlipidemic (Total cholesterol and triglycerides) effects of the different fractions (Petroleum ether, ethyl acetate and chloroform) of ethanolic extract of *Stevia rebaudiana* leaves. The different fractions of the extract were administered orally as a single dose of 150 mg/kg body weight to alloxan induced hyperglycemic rats and found to reduce blood glucose level significantly ($p<0.05$). The different fractions resulted in the significant reduction of lipid content which was increased in hyperglycemic rats. The plant fractions also improve the glucose tolerance in the glucose induced rats. The effects of plant fractions were compared with standard drug metformin. The phytochemical screening tests indicated that the different constituents such as saponins, tannins, triterpines, alkaloids, flavonoids and glycosides etc. were present in the plant which has antidiabetic and hypolipidemic properties. Thus, this investigation paves the way for plant based antihyperglycemic and antihyperlipidemic treatment and indicates that various fractions (Petroleum ether, ethyl acetate and chloroform) of the ethanolic extract of *Stevia rebaudiana* leaves have favorable effect in bringing down the severity of hyperglycemia, enhancing antihyperlipidemic activity and also improving glucose tolerance activity.

INTRODUCTION: Hyperglycemia or diabetes is one of the most commonly occurring problems around the globe. Technically it is known as Diabetes Mellitus. It is the single most important metabolic disorder. This can affect nearly every organ system in the body [1]. According to World Health Organization projections, the prevalence of diabetes is likely to increase by 35% by the year 2025 [2]. Recently, the treatment of diabetes mainly involves a sustained reduction in hyperglycemia by the use of biguanides, thiazolidinediones, sulfonylureas D-phenylalanine and α-glucosidase inhibitors in addition to insulin. However, due to unwanted side effects the efficacies of these compounds are debatable and there is a demand for new compounds for the treatment of diabetes [3, 4].

Hence, plants have been suggested as a rich, as yet unexplored source of potentially useful antidiabetic drugs. Many traditional plants treatment for diabetes are used throughout the World. Plant drugs and herbal

formulations are frequently considered to be less toxic and free from side effects than synthetic one [5]. The anti-hyperglycemic effect of these plants are for their ability to restore the function of pancreatic tissues by increasing insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependant processes. Medical plants has been recently an increasing interest to treat DM. Ethnobotanical information indicates that more than 500 plants are used as traditional remedies for DM treatment [6]. Hence, treatment with herbal drugs has an effect on protecting *β*-cells and smoothing out fluctuation in glucose levels [7].

Hypercholesterolemia and hypertriglyceridemia are common complications of diabetes mellitus in addition to hyperglycemia. The frequency of hyperlipidemia in diabetes is indeed very high depending on the type of diabetes and its degree of control. Diabetes mellitus is one of the oldest diseases affecting millions of people all over the world [8]. Diabetes is a metabolic disorder featured by hyperglycemia as well as hyperlipidemia. The alterations in carbohydrate, fat and protein metabolism associated with absolute or relative deficiency of insulin secretion and insulin action [9].

Although, numerous oral hypoglycemic and hypolipidemic drugs exist alongside insulin, still there is no promising therapy to cure diabetes[10]. Over the last few decades the reputation of herbal remedies has increased globally due to its therapeutic efficacy and safety. In recent years, numerous traditional medicinal plants were tested for their antidiabetic, hypolipidemic and hepatoprotective potential in the experimental animals [11]. Hyperlipidemia is the current medical as well social problem, specially associated with diabetes mellitus leading to increasing morbidity and mortality. The major risk factors of hyperlipidemia are associated with atherosclerosis which predisposes ischemic heart disease and cerebrovascular disease. The study of the effect of *Stevia rebaudiana* on lipid profile in hyperlipidemic individuals showed that it significantly reduces the lipid level (cholesterol and triglyceride) [12].

Stevia rebaudiana (family- asteraceae) is one of them which are herbaceous perennial plant native to subtropical and tropical rainforest areas of South America (Brazil, Venezuela, Colombia and Paraguay) [13]. The leaves are used traditionally in various regions of the world including China, Japan, Korea, Taiwan, Thailand, Malaysia and Paraguay, India and Bangladesh etc. The leaves have been known to contain 100 useful alkaloids among other pharmacologically active compounds. It has been used for the treatment of diabetes and its anti-diabetic effect has been valuated in diabetic animals in many countries and significant hypoglycemic activities of powdered form of Stevia (*Stevia rebaudiana*) leaves have been reported [14]. *Stevia rebaudiana* is the single sweetener which has antidiabetic property [15].

Thus, the leaves of *Stevia rebaudiana* are advocated in the management of diabetes mellitus. Here we investigated the antihyperglycemic, antihyperlipidemic (Total cholesterol and triglycerides) and oral glucose tolerance effects of petroleum ether, ethyl acetate and chloroform fractions of ethanolic extract of *Stevia rebaudiana leaves* in alloxan and glucose induced diabetic rats.

MATERIALS AND METHODS:

Plant Materials: Fresh leaves of *Stevia rebaudiana* (Local name- stevia, sweet leave) were collected from medicinal plant garden, Department of Pharmacy, Atish Dipankar University of Science & Technology, Dhaka, Bangladesh. After thorough washing the leaves were dried completely under mild sun and ground in electric grinder into a coarse powder. The plants were authenticated by Mr. A.H.M. Mahbubur Rahman, Department of Botany, University of Rajshahi, Bangladesh.

Preparation and Fractionation of Crude Extracts: The crude extract was obtained through cold extraction process. The coarse powder was submerged in ethanol and allowed to stand for 10 days with occasional shaking and stirring. When the solvent became concentrated the alcohol content was filtered through cotton and then through filter paper (What man filter paper no. 1). Then the solvent was allowed to evaporate using rotary evaporator at temperature 40-45^{o}C. Thus, the highly concentrated crude extract was obtained. That was then fractionated using petroleum ether, ethyl acetate and $CHCl_3$. The solvents of these

fractions were evaporated by rotary evaporator and then dried under mild sun. The dried fractions of extract were then preserved in the freeze for the experimental uses [16].

Drugs and Chemicals used: The standard drug metformin was the generous gift samples from Square Pharmaceuticals Ltd., Pabna Bangladesh. Alloxan was purchased from Sisco Research Laboratories Pvt. Ltd. Mumbai, India. Total cholesterol (TC) and triglyceride (TG) wet reagent diagnostic kits were the products of Crescent diagnostic kits. Dimethyl sulfoxide (DMSO) was purchased from Loba Chemie, Bombay, India and used to dissolve metformin and the different fractions of extract of *Stevia rebaudiana* [17].

Phytochemical Screening Methods: The following Phytochemical screening methods were used for the tests: [18]

Test for saponins: 300 mg of extract in 5 ml water was boiled for two minutes. Mixture was cooled, mixed vigorously and left for three minutes. The formation of frothing indicated the presence of saponins.

Test for tannins: To an aliquot of the extract added sodium chloride to make to 2% strength. This was filtered and mixed with 1% gelatin solution. Precipitation indicated the presence of tannins.

Test for triterpenes: 300 mg extract mixed with 5 ml chloroform and warmed for 30 minutes. To the chloroform solution small volume of concentrated sulfuric acid was added and mixed properly. The appearance of red color indicated the presence of triterpenes.

Test for alkaloids: 300 mg extract was digested with 2 molar HCl. The acidic filtrate was mixed with amyl alcohol at room temperature and the alcoholic layer was examined. Pink color indicated the presence of alkaloids.

Test for flavonoids: The presence of flavonoids was determined using 1% aluminium chloride solution in methanol, concentrated HCl magnesium turnins and potassium hydroxide solution. Red color indicated the presence of flavonoids.

Test for glycosides: A small amount of an alcoholic extract of the plant material was taken in water and alcohol and boiled with Fehling's solution. Brick-red precipitate was considered as an indication for the presence of glycosides.

Preparation of dosage of active drug and plant extract:

Metformin: Metformin was in microcrystalline form and freely soluble in water. The dosage was prepared in solution form using sterilized water in such a concentration that, each 0.1 ml of solution contained metformin according to the dose of 150 mg/kg body weight since metformin is effective in such dose.

Stevia rebaudiana: The fractionated extracts of *Stevia rebaudiana* leaves were dissolved in 99% DMSO to prepare the solution where each 0.1 ml contained *Stevia rebaudiana* according to the dose of 150 mg/kg body weight [19]. 0.1 ml of each solution was administered orally to every 100 gm body weight of the rats during treatment to achieve required dose of fractions of plant extract.

Selection of animals: A total number of 55 long-Evans male rats weighing about 150-180 gm, age 2 months were purchased from animal house of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Prior to commencement of the experiment all the rats were acclimatized to the new environmental condition for a period of one week.

During the experimental period the rats were kept in a well ventilated animal house at room temperature of 25^{o}C and were supplied with standard pellets supplied from ICDDR, B and fresh drinking water. All the rats were kept in cages with wide square mesh at bottom to avoid coprophagy and maintained with natural 12 hour light and dark cycle [20].

Grouping of experimental rats: 55 long-Evans male rats were randomly assigned into 11 groups, 5 rats in each group.

Group 1	Normal Control
Group 2	Diabetic Control
Group 3	Diabetic+ Metformin (150mg/kg body wt.)
Group 4	Diabetic+ Petroleum ether fraction SR (150mg/kg

	body wt.)
Group 5	Diabetic+ Ethyl acetate fraction SR (150mg/kg body wt.)
Group 6	Diabetic+ Chloroform fraction SR (150mg/kg body wt.)
Group 7	Normal control (Glucose)
Group 8	Glucose+ Metformin (150mg/kg body wt.)
Group 9	Glucose+ Petroleum ether fraction SR (150mg/kg body wt.)
Group 10	Glucose+ Ethyl acetate fraction SR (150mg/kg body wt.)
Group 11	Glucose+ Chloroform fraction SR (150mg/kg body wt.)

Experimental induction of diabetes: Group 1 animals were used for normal control receives only vehicle (DMSO). Groups 2-6 animals were allowed to fast for 12 hrs and were induced diabetic by injection intraperitoneally a freshly prepared solution of alloxan (110 mg/kg body wt.) in normal saline after base line glucose estimation was done. The alloxan treated animals were allowed to feed over night to overcome drug induced hypoglycemia.

After 48 hours blood glucose content was measured by using Bioland G- 423 test meter (Bioland, Germany) using blood sample from the tail vein of the rats. When the condition of diabetes was established animals with blood glucose levels above 11.1 m mol/L was selected for the study. Group 7 animal were used for Normal control receives only glucose and groups 8-11 animals were allowed to fast for 12 hrs were rendered diabetic by oral administration of glucose for the test of OGTT[21].

Antihyperglycemic effect of plant extracts: The groups 2-6 were prepared for resting antihyperglycemic effect after alloxan induction. All the rats were starved at water for 16 hours and then were tested for baseline glucose level. The group 2 was selected for diabetic control group which does not receive extract or metformin. The group 3 stands for metformin control group which was administered metformin orally at a dose of 150 mg/kg body weight. The groups 4-6 received the different fractions of extract of the plants. The blood glucose level was then tested by using glucometer (Bioland Glucometer, Germany). In this case, the blood was collected by picking the tail vein in 0, 2, 6, 16 and 24 hours after drug and plant extract administration [22].

Test of antihyperlipidemic effects of the plant extracts (TC and TG test): The blood serum collected from hearts after sacrificing the rats (Group 1- Group 6) was used for testing the serum total cholesterol (TC) and triglyceride (TG) levels. The concentrations were analyzed by taking absorbance by UV spectrophotometer (Shimidzu UV-1200, Tokyo, Japan) using commercial wet reagent diagnostic kits (Crescent diagnostic kits) according to manufacturer's protocol [23].

Oral Glucose Tolerance Test: Groups 7-11 were selected for OGTT test after starving at water for 16 hours. The base line glucose level was measured by glucometer [24]. Group 7 stands for normal control group received glucose. Group 8 was treated with metformin (150 mg/kg body weight). Group 9 - Group 11 was used for testing the different fractions of extract. These fractions were administered orally at the dose of 150 mg/kg body weight. 2 ml glucose (2 gm/kg body weight) solution was administered orally by intra gastric tube. The blood glucose level was then tested by using glucometer (Bioland Glucometer, Germany). In this case, the blood was collected by picking the tail vein in 0, 30, 60, 90, 150 and 270 minutes[25].

Statistical Analysis: The results were expressed as mean±SEM using Graph Pad Prism (version 4.0) computer program (Graph pad Software San Diego, CA, USA). We used a one-way analysis of variance (ANOVA), followed by Scheffe's post-hoc test or students paired or unpaired t-test where appropriate. The statistical method applied in each analysis was described in each figure. Results were considered to be significant when p values were less than 0.05 ($p<0.05$).

RESULTS: The effect of the different fractions of ethanolic extract of *Stevia rebaudiana* leaves on the fasting blood glucose (FBG) level, antihyperlipidemic (total cholesterol and triglyceride) activity and oral glucose tolerance test (OGTT) were investigated in the alloxan-induced diabetic rats using metformin as standard antidiabetic agents.

Effect of different fractions of *Stevia rebaudiana* leaves on fasting blood glucose (FBG) level in diabetic rats: The mean blood glucose concentrations of normal, drugs and plant fractions treated animals were

estimated on the 2, 6, 16, and 24 hours, respectively as shown in **Fig. 1**.Their baseline glucose concentrations were also measured. In case of alloxan induced diabetic rats metformin reduced blood glucose level to 72%, 52%, 34% and 24% in 2, 6, 16, and 24 hours, respectively. In case of petroleum ether fraction reduced blood glucose level to 80%, 55%, 44% and 31% in 2, 6, 16, and 24 hours, respectively. Ethyl acetate fraction reduced blood glucose level to 76%, 58%, 44% and 31% in 2, 6, 16, and 24 hours, respectively. $CHCl_3$ fraction reduced blood glucose level to 83%, 62%, 45% and 33% in 2, 6, 16, and 24 hours, respectively. The results were compared with diabetic control group.

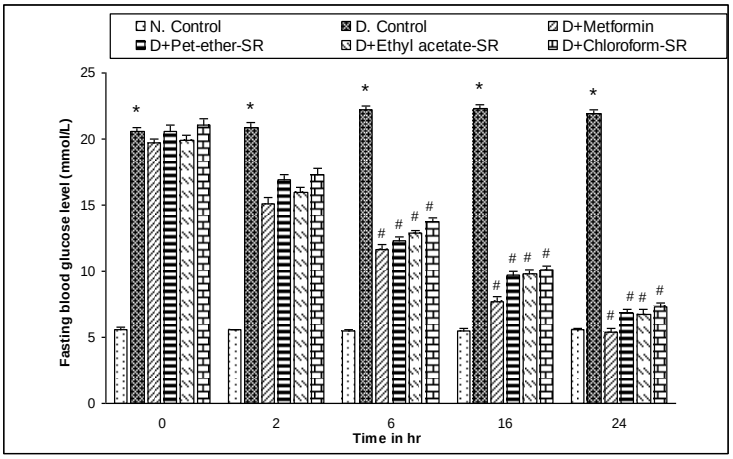

FIG. 1 EFFECT OF DIFFERENT FRACTIONS OF *STEVIA REBAUDIANA* LEAVES ON THE FBG LEVEL ON DIABETIC RATS COMPARED TO NORMAL RATS.

* indicates significant change in blood glucose level compared with normal control group (p<0.05). # indicates significant changes in FBG level in diabetic rats after treatment (p< 0.05). The results are expressed as means ± SEM.

Effect of different fractions of *Stevia rebaudiana* leaves on total cholesterol and triglyceride levels in diabetic rats: In case of the effect of different fractions of *Stevia rebaudiana* it was observed that the metformin, petroleum ether, ethyl acetate and $CHCl_3$ fractions reduced cholesterol level to 41%, 44%, 50% and 51%, respectively than the diabetic control group (**Fig. 2**). On the other hand, it was observed that the metformin, petroleum ether, ethyl acetate and $CHCl_3$ fractions of *Stevia rebaudiana* reduced triglyceride level to 39%, 45%, 47% and 48% respectively, than the diabetic control group (**Fig. 3**).

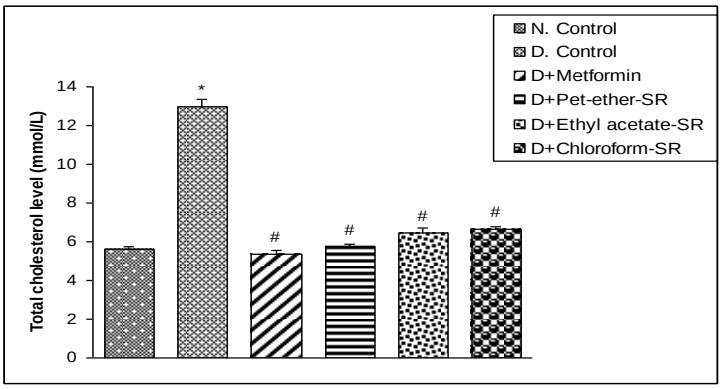

FIG. 2 EFFECT OF DIFFERENT FRACTIONS OF *STEVIA REBAUDIANA* LEAVES ON THE TOTAL CHOLESTEROL LEVEL IN DIABETIC RATS.

* indicates significant change compared with normal control group (p<0.05). # indicates significant changes in diabetic rats after treatment (p< 0.05). The results are expressed as means ± SEM.

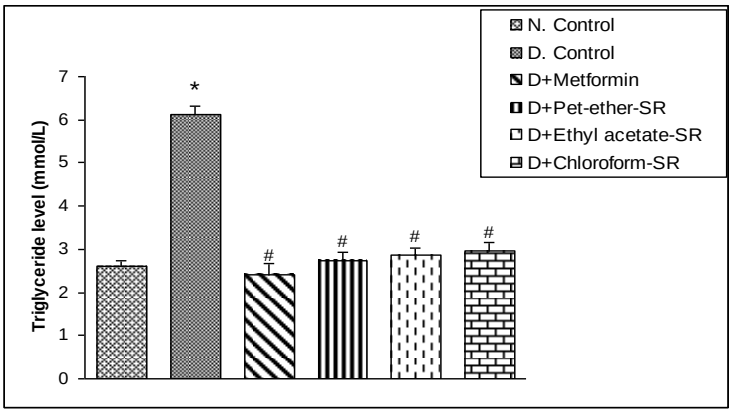

FIG. 3 EFFECT OF DIFFERENT FRACTIONS OF *STEVIA REBAUDIANA* LEAVES ON THE TRIGLYCERIDE LEVEL IN DIABETIC RATS

* indicates significant change compared with normal control group (p<0.05). # indicates significant changes in diabetic rats after treatment (p< 0.05). The results are expressed as means ± SEM.

Effect of different fractions of *Stevia rebaudiana* leaves on fasting blood glucose (FBG) level in the glucose-induced hyperglycemic rats: In case of the effect of different fractions of *Stevia rebaudiana* metformin reduced blood glucose level to 62%, 59% and 67% in 30, 60 and 90 minutes, respectively. Petroleum ether fraction reduced blood glucose level to 68%, 71% and 72% in 30, 60 and 90 minutes, respectively. Ethyl acetate fraction reduced blood glucose level to 72%, 71% and 75% in 30, 60 and 90 minutes, respectively. $CHCl_3$ fraction reduced blood glucose level to 75%, 73% and 73% in 30, 60 and 90 minutes, respectively as shown in **Fig. 4**. The results were compared with glucose induced (normal control) group.

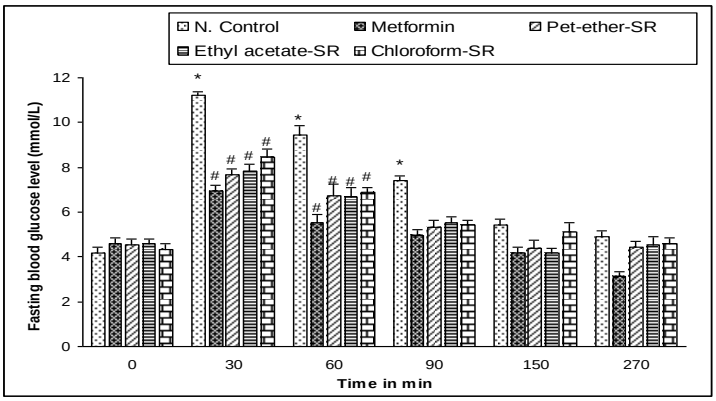

FIG. 4 EFFECT OF DIFFERENT FRACTIONS OF *STEVIA REBAUDIANA* ON THE GLUCOSE-INDUCED HYPERGLYCEMIA IN NORMAL RATS.

* indicates significant changes in FBG level compared to normal rats after treatment (p<0.05). # indicates significant changes in diabetic rats after treatment (p< 0.05). The results are expressed as means ± SEM.

Phytochemical Screening: The phytochemical screening tests indicated the different constituents such as saponins, tanins, triterpines, alkaloids flavonoids and glycosides were present in the plant *Stevia rebaudiana* leaves which have the antihyperglycemic and antihyperlipidemic properties. The results are summarized in **Table 1**.

TABLE 1: THE PHYTOCHEMICAL CONSTITUENTS OF THE EXPERIMENTAL PLANT FRACTIONS OBTAINED BY PHYTOCHEMICAL SCREENING TESTS

Partitionates	Saponin	Tannins	Triterpines	Alkaloids	Flavonoids	Glycosides
Pet-ether	+	+	+	+	+	+
Ethyl acetate	+	+	+	-	+	+
Chloroform	+	+	-	+	+	+

(+)=Present ;(-) =Absent

DISCUSSION: The pathogenesis of diabetes mellitus and the possibility of its management by existing therapeutic agents without any side effects have stimulated great interest in recent years[26].For the treatment of diabetes Plant medicines have a long history. With a disturbing rise in the prevalence of this metabolic disease and associated healthcare costs, interest in alternative or complementary therapies has grown [27].

In this study the different fractions of ethanolic extract of *Stevia rebaudiana* leaves reduced blood glucose level significantly in the hyperglycemic rats. The significant antihyperglycemic activity of petroleum ether, ethyl acetate and chloroform fractions of *Stevia rebaudiana* as shown in Fig.1 may be due to the presence of hypoglycemic saponins, tanins, triterpines, alkaloids and flavonoids etc. (Table 1).

Stevia rebaudiana lowered hyperglycemia [28] and it may be useful for the treatment of diabetes and associated complications. It could be envisaged that the plant extracts may also contain some biomolecules that may sensitize the insulin receptor to insulin or stimulates the *β*-cells of islets of langerhans to release insulin which may finally lead to improvement of carbohydrate metabolizing enzymes towards the re-establishment of normal blood glucose level. The extract might be promoting glucose uptake and metabolism or inhibiting hepatic gluconeogenesis [29].

In hyperglycemic rats there was a significant increase in lipids (total cholesterol and triglycerides). The most common lipid abnormalities in diabetes are hypercholesterolemia and hypertriglyceridemia [27]. Oral administration of different fractions of ethanolic extract of *Stevia rebaudiana* leaves resulted in a significant reduction of serum lipid levels [30] in rats viz. total cholesterol and triglyceride levels (Fig. 2 and Fig. 3). The antihyperlipidemic activity of *Stevia rebaudiana* may be attributed due to the presence of flavonoids, ascorbic acid etc [1].

Flavonoids are known for their diverse biological activities including hypolipidemic activity resulting from their antioxidant activity [31]. *Stevia rebaudiana* plant fractions demonstrated the presence of flavonoids and other different constituents such as saponins, tanins, triterpines, glycosides and alkaloids (Table 1). With respect to the lipid lowering capacity of these plant fractions, it could be suggested that the constituents of these plant fractions may acted as inhibitors for enzymes such as hydroxyl-methyl-glutaryl-CoA reductase, which participates in *de novo*

cholesterol biosynthesis as has been suggested for some plants earlier [32, 33].

Oral glucose tolerance test (OGTT) measures the body's ability to use glucose, the body's main source of energy [24]. *Stevia rebaudiana* leaves are used as antidiabetic plant in traditional medicine [34]. The extracts of *Stevia rebaudiana* leaves have been reported to produce fall in fasting blood glucose (FBG) level and improve glucose tolerance [35]. In our study, it was observed that various fractions have also hypoglycemic effect in glucose induced hyperglycemic rats. The fractions of plant extract enhanced glucose utilization [36]. So the blood glucose level was significantly reduced in the glucose loaded rats (Fig. 4). This may be due to the presence of hypoglycemic Glycosides, saponins, tanins, triterpines, alkaloids and flavonoids etc. (Table 1).

CONCLUSION: Our preliminary phytochemical analysis has indicated that flavonoids and alkaloids have been reported to exert potent hypoglycemic and hypolipidemic effects. Thus, in the light of our pharmacological studies it was observed that the administration of plant fractions of *Stevia rebaudiana* leaves extract demonstrated antihyperglycemic activity [37] by producing significant restoration of blood glucose level as well as illustrated some beneficial effects such as reduced hyper cholesterol and hyper triglyceride level in alloxan-induced diabetic rats and also improvement of oral glucose tolerance [38] in glucose induced diabetic rats.

Further, comprehensive pharmacological investigations are needed to elucidate the exact chemical compounds responsible for antihyperglycemic, antihyperlipidemic activity as well as improvement of oral glucose tolerance and also their exact mechanism of actions. However this study will pave the way for plant based specific treatment of diabetes avoiding the complications of artificial drug substances.

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