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EFFECT OF *TERMINALIA BELERICA* FRUITS ON STREPTOZOTOCIN-INDUCED DIABETES MELLITUS AND ITS ASSOCIATED COMPLICATIONS

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ABSTRACT

The present study investigated the effect of extracts and fractions of Terminalia belerica fruit extracts on hyperglycemia and associated complications on streptozotocin-induced diabetic rats. The aqueous extract and 95% and 50% ethanol extracts were administered to the normal rats with oral sucrose load and streptozotocin-induced diabetic rats. The extracts significantly decreased hyperglycemia in streptozotocin-induced diabetic rats and also showed lowering of postprandial hyperglycemia in normal rats. Based on antihyperglycemic activity profile the 95% ethanol extract was further fractionated to obtain hexane, chloroform, butanol and aqueous fractions which were administered to STZ-induced diabetic rats. All except aqueous fraction showed significant antihyperglycemic activity and the maximum activity was registered in butanol fraction treated group. The butanol fraction also exhibited significant effect on various parameters in diabetic animals such as lipid profile, serum insulin level, glycated hemoglobin, hepatic and renal function markers. It also enhanced glucose uptake in a concentration-dependent manner in L6 myotubes and stimulated the tyrosine phosphorylation of IRS-1, AKT and GLUT-4 at both mRNA and protein level. The findings suggest that the butanol fraction of 95% ethanol extract of Terminalia belerica fruits is competent in combating various symptoms and complications of diabetes mellitus.

KEYWORDS: Hyperglycemia, diabetes, *T.belerica*, streptozotocin, high-fructose diet.

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in either insulin secretion or insulin action or both.[1] It is one of the most common endocrine disorder affecting nearly 285 million hyperglycemic people in 2010 which is expected to increase by 439 million people in 2030.[2] Hyperglycemia is a crucial factor that plays a central role in the development of diabetic complications and the adverse effects.[3] The secondary complications of diabetes include hyperlipidemia, coronary artery disease, renal failure, stroke, neuropathy, retinopathy and blindness.[4] Due to the multifactorial and the multisymptomatic nature of diabetes mellitus, the treatment of this disease is still considered as the main global problem and successful treatment has yet to be discovered.

Present approaches towards the treatment of diabetes mellitus include insulin treatment and oral drugs belonging to classes like sulphonyl ureas, biguanides, thizaolidinediones, alpha-glucosidase inhibitors like acarbose, mitiglinides like repaglimide and nateglamide and dipeptidylpeptidase-IV inhibitors. The available synthetic drugs do help in effective control of diabetes, but at the same time they also bring serious side effects in long-term use[3]. Therefore, drugs of plant origin are getting great attention in recent years due to their less or negligible side effects and other health benefits as well.

Our present study aims to evaluate the effect of *Terminalia belerica* Roxb (Combretaceae) fruit on diabetes and its related complications. *Terminalia belerica* Roxb (Combretaceae) occurs widely in moist valleys of India and it is commonly used in the Indian traditional system of medicines. Fruits of *T. belerica* is known to have antimicrobial, anti-inflammatory,[5] hepatoprotective, hypotensive, antispasmodic, anti-asthmatic and anti-tussive effects.[4] Although health benefits of *T. belerica* have been known for thousands of years, but a detail study related to the effects and the mechanisms of action with respect to the specific disorders is still required. Fruits of *T. belerica* are the major constituent of a popular ayurvedic formulation Triphala which have been used for the treatment of diabetes.[5, 6]

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ), metformin, 2-DOG, cytochalasin B, IBMX, dexamethasone and insulin were obtained from Sigma Chemical Company, St. Louis, USA, where as gum acacia and sucrose were obtained from Sisco Research Laboratory (India). HG-DMEM, FBS, and horse

serum were purchased from GIBCO. The antibodies, anti-Phospho-IRS-1 (Tyr-612), anti-β-actin were from Santa Cruz Biotechnology. Anti-Phospho-Akt (Ser-437), and anti-GLUT4 were obtained from Cell Signaling Technology, USA. The glucose strips for measuring blood glucose level were obtained from Roche (India).

Preparation of plant extracts and fractions

The fruits of *T. belerica* were purchased from local markets and its identity was authenticated in the laboratory. The dried fruits of *T. belerica* were dried in the shade, powdered in electric blender and the crude powders were stored in airtight plastic containers until used. One part of the crude powder was extracted with 10 volumes of 95% ethanol in the percolator. This process was repeated five times and extract obtained each time were pooled, filtered and concentrated under high vacuum in a rotavapor and the dried substance was termed as 95% ethanol extract. Similarly the 50% ethanol extract and aqueous extract were prepared by extraction with 50% ethanol and water, respectively. In the fractionation process 95% ethanol extract was fractioned using various solvents in the increasing order of polarity viz, hexane, chloroform, butanol and water. All the extracts and fractions were concentrated and dried under vacuum and stored in airtight plastic containers until used.

Procurement of Animals

Male albino rats of Sprague Dawley strain of body weight 160 ± 20 g were procured from the animal colony of Central Drug Research Institute, Lucknow, India. The rats were always housed in animal housing facility where standard conditions of temperature, relative humidity and a 12 h light/dark cycle were always maintained. The animals had always free access to pellet diet and tap water unless stated otherwise. The study was approved by the Institutional Animal Ethical Committee (IAEC) and all research work on animals was conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Streptozotocin-induced diabetes

Albino rats of Sprague Dawley strain of body weight 160 ± 20 g were injected streptozotocin intraperitoneally at the dose of 60 mg/kg body weight to make the animals diabetic. Rats showing fasting blood glucose level between 300-450 mg/dl after 48 h of injection were divided into desired groups depending on the number of rats. Rats of the experimental groups were orally administered the fine suspension of the desired test samples (made in 1.0% gum acacia) at 250 mg/kg (in case of extracts) and 100 mg/kg (in case of fractions) body weight.

Animals of the control group will be given an equal amount of 1.0% gum acacia. The dose of standard antidiabetic drugs in this protocol was 100 mg/kg body weight of metformin. The blood glucose level of each animal was measured by glucostrips at 0, 30, 60, 90, 120, 180, 240, 300 min and at 1440 min post test sample, /standard drug or vehicle treatment.[7] The percentage blood glucose lowering by test substance or standard drug was determined by plotting blood glucose vs time and calculating the area under the curve (AUC) between 0-300 min and 0-1440 min and comparing the AUC of test substance treated/standard drug treated groups to that of sham treated control group.

Single dose effect

Oral glucose tolerance test of normal rats

Albino rats of Sprague Dawley strain of body weight 160 ± 20 g showing fasting blood glucose between 60 to 80 mg/dl were selected and divided into groups consisted of five to six animals in each depending on the availability of the animals. Rats of the experimental groups were orally administered the fine suspension of the test samples (made in 1.0% gum acacia) at the dose of 250 mg/kg body weight in the case of extracts, 100 mg/kg in the case of fractions, 100 mg/kg metformin (Sigma). An oral sucrose load (10.0 g/kg body weight) was given to each animal exactly 30 min post administration of the test sample/ vehicle/standard drug. Blood glucose levels of each rat were observed at 30, 60, 90 and 120 min post sucrose load by glucostrips only. Food but not water was withheld from the cages during the course of experimentation.[8] The percentage improvement in glucose tolerance post sucrose load was determined by plotting blood glucose versus time and calculating the area under the curve (AUC) of each group and comparing the AUC of test substance treated group with that of sham treated control group.

Effect on blood glucose of STZ induced diabetic rats

Diabetes was induced by intraperitoneal injection of STZ and fasting blood glucose was measured after 48 h to determine the diabetes status in rats. Animals having fasting blood glucose between 300 to 450 mg/dl were selected for the study. Selected animals were divided into groups having six animals in each group. Plant extracts or fractions and standard drug metformin prepared as suspension in 1% gum acacia were administered to the treatment groups while the control group receives only vehicle i.e., 1% gum acacia suspension. Blood glucose were observed 30, 60, 90, 120, 180, 240, 300 and 1440 min post treatment.

Multiple dose effect

Effect on high fructose diet fed- low dosed streptozotocin-induced diabetic rat model

Male rats of Sprague Dawley Strain having a body weight around 140 g were kept on the high fructose diet (60 % fructose, 13 % saturated fat, Casein 22 %, vital minerals, vitamins) for 12 consecutive weeks. The blood was withdrawn from the retro-orbital plexus of eye for the estimation of their plasma, cholesterol and triglyceride levels. The rats showing their plasma cholesterol and triglyceride level over 150 and 200 mg/dl respectively were separated. STZ at a dose of 30 mg/kg was injected into these rats intraperitoneally. The rats showing their fasting blood glucose profile over 300 mg/dl after 48 hours of STZ injection were taken out and grouped[9]. Each group consisted of 6 animals. The groups were treated with test samples at the desired dose for 30 days. The OGTT of each animal was carried out on day 14^{th} and 28^{th} and the animals were bled on 10^{th} and 30^{th} day when their lipid profiles, i.e. total triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol were measured and liver and kidney function tests were performed on Cobas Integra 400 autoanalyzer using assay kits and instructions of the manufacturers.

Effect on streptozotocin-induced diabetic rat model

STZ-induced diabetic rats showing fasting blood glucose between 250-400 mg/dl after a week of diabetes induction through STZ are selected for the study. STZ induced diabetic rats when left undisturbed for approx a month, develop various complications related to abnormal functioning of liver, kidney and other organs. These rats are grouped on the basis of glycated hemoglobin (HbA1c) level. Animals having HbA1c 10% and above are selected for the study and divided in three groups viz diabetic control, butanol fraction treated and metformin treated group. The test sample and standard drug were administered at the dose of 100 mg/kg bw for 30 consecutive days. OGTT was performed at 14^{th} and 28^{th} day and lipid profile, renal and hepatic function tests were done on 10^{th} and 30^{th} day in plasma samples obtained by collecting blood from the retro-orbital plexus of animals in EDTA coated vials. HbA1c level was measured at the end of the experiment.

Oral glucose tolerance test

Overnight fasted rats were administered with glucose by oral route at the dose of 3g/kg bw and blood glucose was measured at 30, 60, 90 and 120 min from the tail vein. Effect on oral glucose tolerance was obtained by calculating the area under the curve for the values of blood glucose between 0-120 min.

Measurement of plasma lipid profile, insulin, hepatic and renal function markers and HbA1c

Plasma insulin was measured using Mercodia insulin Elisa kit and triglycerides, cholesterol, LDL, HDL, AST, ALT, urea, uric acid, creatinine and HbA1c were measured by Cobas Integra-400 autoanalyser using assay kits provided by manufacturers.

Cell culture of L6 myotubes

L6 myoblasts (originally obtained from ATCC) were cultured in DMEM with 10 % fetal bovine serum (FBS) supplemented with penicillin (120 units/ml), streptomycin (75 µg/ml) in a 5 % CO2 environment. For differentiation, L6 cells were transferred to DMEM with 2 % FBS for 4-6 days post-confluence.

Measurement of 2-deoxy-D-[1-^{3}H] glucose

Measurements of 2-deoxy-D-[^{3}H] -glucose uptake in L6 myotubes were performed as described previously[10]. In brief, after treatment L6 myotubes were incubated for 5 min in HEPES-buffered saline [140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO4, 1 mM CaCl2 (pH 7.4)] containing 10 µM 2-DG (0.5 µCi/ml 2-[^{3}H] DG) at room temperature. For measurement of radioactivity cells were lysed with 0.05 N NaOH, followed by scintillation counting (Beckman Coulter, USA).

RNA extraction/quantitative Real Time PCR

Total RNA was extracted from the cells using TRIZOL reagent (Invitrogen, Life Technologies, USA). An aliquot of 2 µg total RNA from each sample was reverse transcribed to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit, Applied Biosystems (ABI-4368814) according to the manufacturer's instructions. Gene expression was analyzed by relative quantization with the $2^{-\Delta\Delta CT}$ method using real-time PCR Light Cycler 480 System (Roche, Indianapolis, IN).

Western blot analysis

Cells were lysed with PBS containing 1% NP40, 5 mM EDTA, phosphatase inhibitors and protease inhibitors cocktail (RIPA lysis buffer). Electrophoresis was carried out with 10 % SDS-polyacrylamide gels, transferred to PVDF membranes and probed with primary antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by Enhanced Chemiluminescence according to manufacturer's instructions (GE Healthcare, UK).

Statistical analysis

All results are expressed as means ± SEM. The statistical value of $p < 0.05$ was considered as statistical significance. Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA with Dunnet's post hoc test (Graph Pad Prism version 3). Quantitative glucose tolerance of each animal was calculated by the area under the curve (AUC) method.

RESULTS

Effect of crude powder and aqueous as well as ethanolic extracts of *T. belerica* fruits on postprandial hyperglycemia in normal rats

Table 1 shows the effect of crude powder, its 95 and 50 % ethanolic extracts and aqueous extract of *T. belerica* fruits and standard drug metformin on postprandial hyperglycemia in normal rats. On comparing the 0-120 min blood glucose AUC profile of the treated groups to that sham treated control group (Table 1) revealed that only 95% ethanol extract significantly declined the postprandial hyperglycemia to the tune of 16.9 ($p<0.05$). The standard drug metformin showed improvement of around 24.4 % ($p<0.01$).

Table 1. Effect on postprandial hyperglycemia in *T.belerica* crude powder, extracts and metformin treated normoglycemic rats.

Treatment	Dose (mg/ kg)	Blood Glucose (mg/dl) min post sucrose load					AUC	% improvement
		0'	30'	60'	90'	120'		
Sham	-	71.1±0.94	128.1±2.72	123.5±1.52	128.1±1.57	124.3±1.62	14330±343	-
Crude powder	250	87.8±3.96	143.0±2.07	113.8±2.49	115.8±3.02	120.2±2.33	14300±200	0.20
95% Eth. Ext.	250	69.5±2.29	105.6±4.52	105.5±5.50	101.0±2.48	99.5±3.03	11900±343	16.9*
50% Eth. Ext.	250	69.5±2.07	128.6±4.56	110.6±1.25	104.3±3.50	111.0±2.51	13020±256	9.14
Aqu. Ext.	250	71.0±3.68	126.6±2.43	128.3±3.41	117.3±4.26	118.5±4.04	14020±391	2.16
Metformin	100	70.6±1.81	109.8±2.25	99.3±2.44	81.0±4.04	70.6±1.82	10830±204	24.4**

Results are mean ± SE of six rats; * $p<0.05$, ** $p<0.01$

Effect of crude powder and aqueous as well as ethanolic extracts of T.belerica fruits on blood glucose profile of STZ-induced diabetic rats

Table 2 shows that all the extracts of *T.belerica* fruits showed significant lowering of blood glucose in STZ-induced diabetic rats. Maximum lowering of 26.5% ($p<0.01$) was observed in 95% ethanol extract treated group followed by aqueous and 50% ethanol extract which

showed 24.9% ($p<0.01$) and 22.9% ($p<0.01$) lowering respectively when compared the AUC of blood gluose of 0-5 h. Significant declination of 18.3% ($p<0.05$) was observed only in 95% ethanol extract treated group when AUC of 0-24 h was considered while the lowering was not significant in rest all other extracts. Lowering of blood glucose was not significant in crude powder treated group.

Table 2 Blood gluocose profile of *T. belerica* crude powder, extracts and metformin treated STZ-induced diabetic rats

Treatment	Dose (mg/kg)	Mean AUC		% lowering in blood glucose	
		0-5h	0-24h	0-5h	0-24h
Sham	-	141400±3679	674900±24173	-	-
Crude powder	250	123018±3981	652223±19549	13.0	3.36
95% Eth. Ext.	250	103800±2734	551000±22841	26.5**	18.3*
50% Eth. Ext.	250	108900±3816	578500±19367	22.9**	14.2
Aqu. Ext.	250	106100±1936	575000±13746	24.9**	14.8
Metformin	100	91740±916	431300±11325	35.1**	36.0**

Results are mean ± SE of six rats; * $p<0.05$, ** $p<0.01$

Effect of hexane, chloroform, butanol and aqueous fractions of 95% ethanolic extract of T.belerica fruits on blood glucose profile of STZ-induced diabetic rats

Table 3 indicates the antihyperglycemic potential of various fractions of 95% ethanolic extract of *T. belerica*. All except aqueous fraction significantly declined the blood glucose level of STZ diabetic rats during 5hr and 24 duration of study. The activity of various fractions during 5hr and 24hr were observed as 20.6% ($p<0.01$) and 19.5% ($p<0.01$) in hexane fraction, 16.7% ($p<0.05$) and 17.5% ($p<0.05$) in chloroform fraction and 24.0% ($p<0.01$) and 26.6% ($p<0.01$) in butanol fraction treated groups respectively. Noticeable lowering was also observed in aqueous fraction treated group, but the values were not statistically significant.

Table 3 Blood glucose profile of STZ-induced diabetic rats treated with various fraction of 95% ethanolic extract of *T. belerica* fruits and metformin.

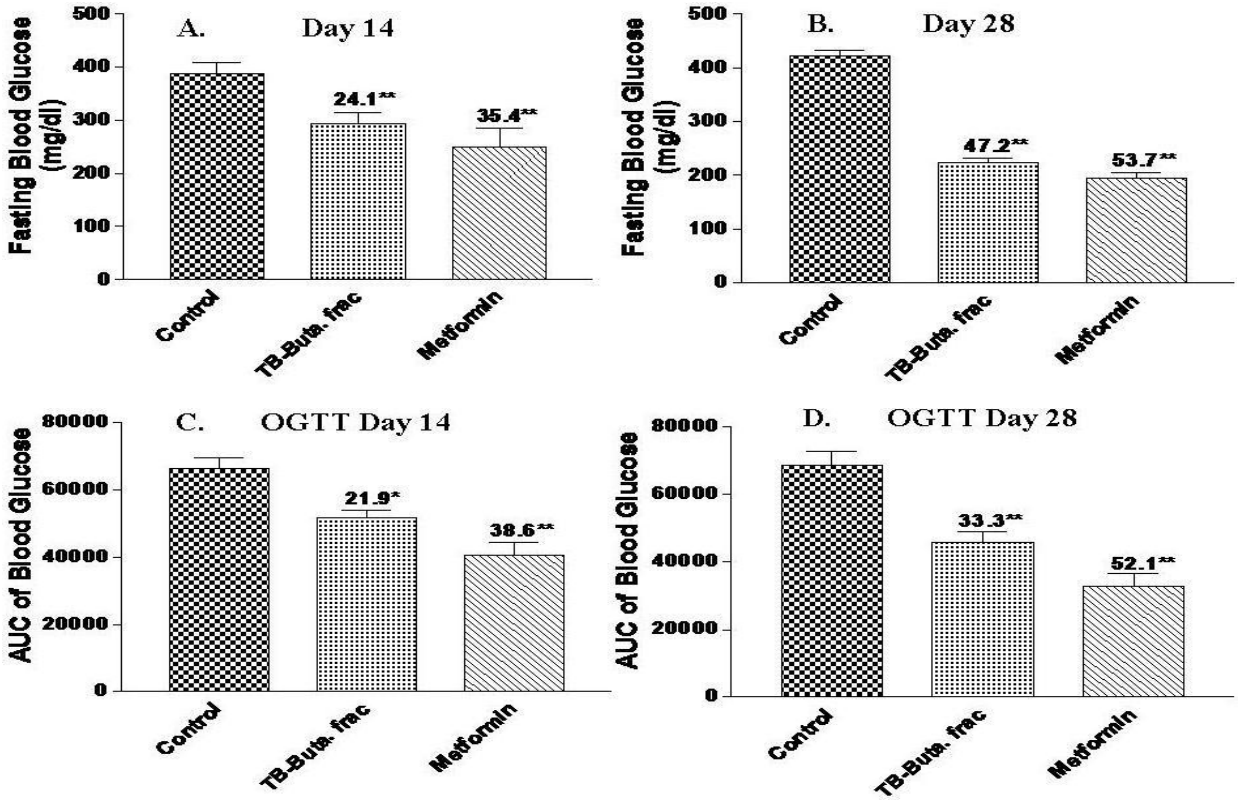
Treatment	Dose (mg/kg)	Mean AUC		% lowering in blood glucose	
		0-5h	0-24h	0-5h	0-24h
Sham	-	129000 ±2549	634600 ± 23850	-	-
Hexane fraction	100	102400 ±4238	510600 ± 11980	20.6**	19.5**
Chloroform fraction	100	107400 ±1422	523100 ± 10240	16.7*	17.5*
Butanol fraction	100	98000 ± 1441	465700 ± 6594	24.0**	26.6**

Aqueous fraction	100	109700 ±4312	534100 ± 21720	14.9	15.8
Metformin	100	92580 ± 348.6	432200 ± 8127	28.2**	31.8**

Results are mean ± SE of six rats; * $p<0.05$, **$p<0.01$

Effect of butanol fraction of 95% ethanolic extract of T.belerica fruits on fasting blood glucose, oral glucose tolerance and plasma insulin level of high fructose diet fed low dosed STZ-induced diabetic rats

Dyslipidemia along with the partially damaged beta cells in HFD-STZ rats causes intolerance towards external glucose administration due to slow and less efficient utilization of glucose by the body. Figure 1 C and D shows that multiple dose of butanol fraction of *T.belerica* fruits for 30 days caused significant improvement in glucose tolerance, which was found to be 21.9% ($p<0.01$) and 33.3% ($p<0.01$) at days 14th and 28th post treatment respectively. Improvement in metformin treated group was 38.6% ($p<0.01$) and 52.1% ($p<0.01$) respectively at same day intervals. It is evident from Figure 1 A and B that fasting blood glucose of butanol fraction treated group were found significantly improved and the improvement was 47.2% ($p<0.01$) which was comparable to standard drug metformin 53.7% ($p<0.01$) at 28th day of treatment. Insulin level generally get elevated in HFD-STZ rats and Figure 1 E shows that the treatment with butanol fraction for 30 days lowered the plasma insulin level by 19.0% ($p<0.05$) as measured on final day of treatment.



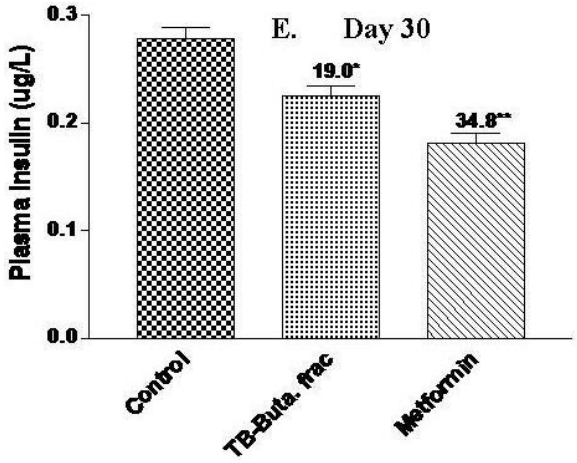


Figure 1: Effect of butanol fraction of 95% ethanol extract of *T.belerica* fruits on fasting blood glucose (A and B), oral glucose tolerance (C and D) and plasma insulin level (E) of high fructose diet fed low dosed STZ-induced diabetic rats. Significance *p<0.05; **p<0.01

Effect of butanol fraction of 95% ethanolic extract of T.belerica fruits on lipid profile of high fructose diet fed low dosed STZ-induced diabetic rats

HFD-STZ rats display disturbed lipid profile with abnormally high plasma triglycerides, total cholesterol and LDL-c level and depletion in HDL-c level. Table 4 shows that the treatment of butanol fraction for 4 weeks turned plasma lipid level towards normal with significant decrease in plasma triglycerides, total cholesterol, LDL-c and increase in HDL-c in treated group. Maximum decline of 45.2% ($p<0.01$), 44.5% ($p<0.01$) and 57.7% ($p<0.01$) of plasma triglycerides, total cholesterol, LDL-c and increase of 45.5% ($p<0.01$) of HDL-c level were observed on day 30th post treatment. Effect of standard drug metformin was not significant for any lipid parameters.

Table 4. Effect of butanol fraction of 95% ethanolic extract of *T.belerica* fruits on lipid profile of high fructose diet fed low dosed STZ-induced diabetic rats

Group	Day	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	LDL-c (mg/dl)	HDL-c (mg/dl)
Control	10th	274.7±10.8	195.5±14.2	118.7±15.4	21.83±1.81
	30th	303.2±6.88	218.2±12.1	137.0±12.7	20.50±1.09
Butanol fraction (100 mg/kg)	10th	218.0±7.43**(20.6**)**	152.7±7.02**(21.8**)**	79.07±7.37**(33.4**)**	30.00±2.18**(37.4**)**
	30th	166.0±5.40 **(45.2**)**	121.0±4.25 **(44.5**)**	57.97±4.99 **(57.7**)**	29.83±2.09**(45.5**)**
Metformin (100 mg/kg)	10th	253.6±4.79 **(7.68)**	184.6±1.39 **(5.54)**	110.7±2.28 **(6.70)**	23.1±1.76 **(6.10)**
	30th	265.6±3.51 **(12.3)**	194.5±8.15 **(10.8)**	118.7±7.96 **(13.3)**	22.6±1.54 **(10.5)**

Results are mean ± SE of six rats; **p<0.01

Effect of butanol fraction of 95% ethanolic extract of T.belerica fruits on hepatic and renal parameters of high fructose diet fed low dose STZ-induced diabetic rats

AST and ALT are the main biochemical markers used in liver function test and elevated level of these, indicate impaired liver function. As it is evident from Table 5 that plasma AST and ALT level were reduced by the significant level in treated group as compared to the control HFD-STZ group. The lowering observed on day 30th were 34.9% ($p<0.01$) and 33.3% ($p<0.01$) of AST and ALT level respectively in butanol fraction treated group and 41.6% ($p<0.01$) and 40.7% ($p<0.01$) in metformin treated group. Similarly Table 5 also shows decline of kidney function parameters which indicates improved kidney functions in treated groups as compared to control. Declination of 43.2% ($p<0.01$), 30.1% ($p<0.01$) and 26.0% ($p<0.01$) of plasma urea, uric acid and creatinine were observed on final day i.e. day 30th post treatment in butanol fraction treated group and 51.1% ($p<0.01$), 53.0% ($p<0.01$) and 35.9% ($p<0.01$) respectively in metformin treated group. Results indicate towards the hepato- and renoprotective action of butanol fraction on treated rats.

Table 5. Effect of butanol fraction of 95% ethanolic extract of *T.belerica* fruits on hepatic and renal parameters of high fructose diet fed low dosed STZ-induced diabetic rats 30th day of treatment.

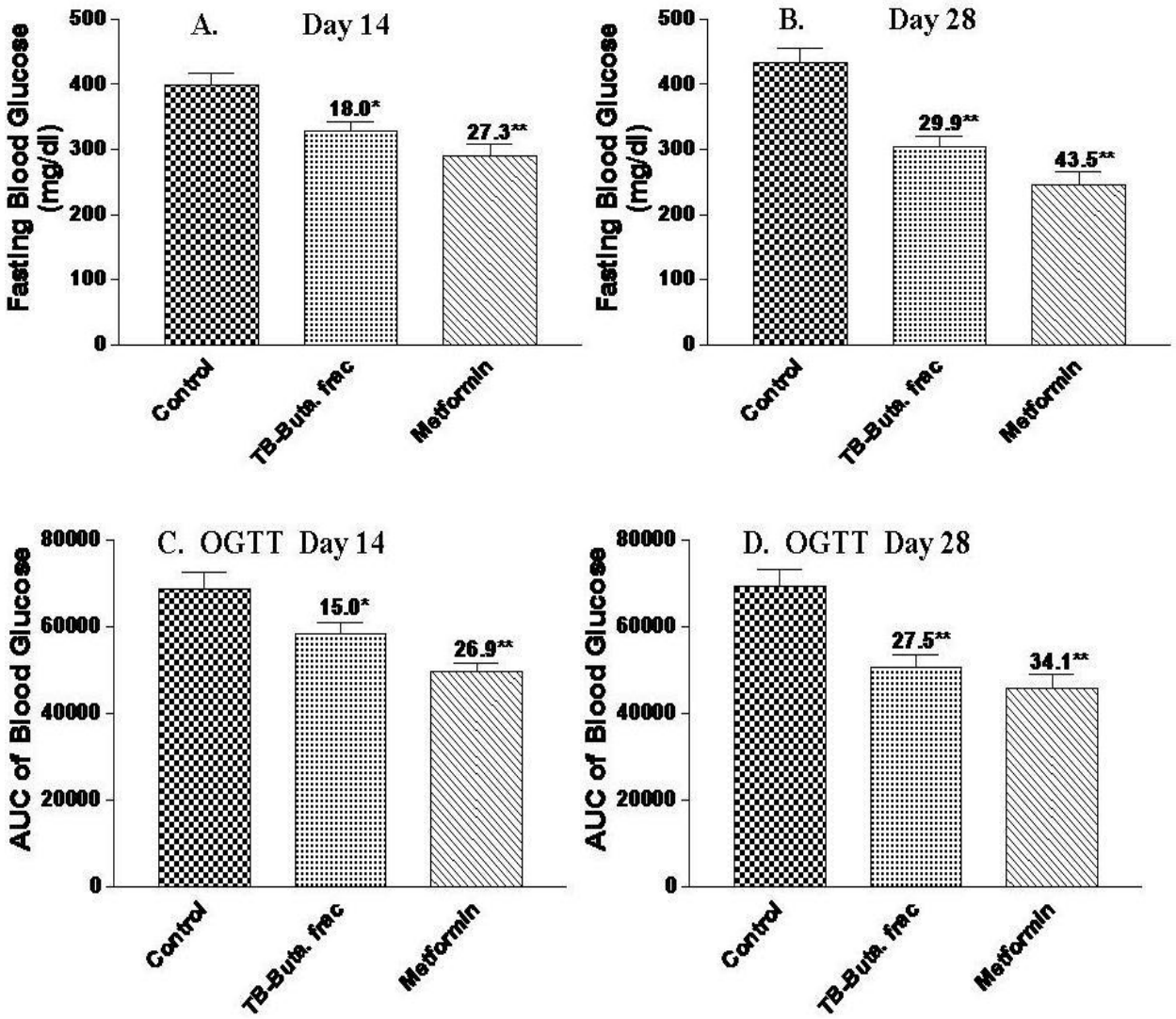
Group	Hepatic parameters		Renal parameters		
	AST (U/I)	ALT (U/I)	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine (mg/dl)
HFD-STZ Control	7.645±1.85	34.20±1.70	80.50±5.43	8.250±0.76	0.7417±0.075
Butanol fraction (100 mg/kg)	4.975±0.42 **(34.9**)**	22.80±2.19 **(33.3**)**	45.67±3.04 **(43.2**)**	5.760±0.28 **(30.1**)**	0.5483±0.027 **(26.0**)**
Metformin (100 mg/kg)	4.462±0.41 **(41.6**)**	20.25±1.08 **(40.7**)**	39.33±1.78 **(51.1**)**	3.877±0.27 **(53.0**)**	0.4750±0.033 **(35.9**)**

Results are mean ± SE of six rats; **p<0.01

Effect of butanol fraction of 95% ethanolic extract of T.belerica fruits on fasting blood glucose, oral glucose tolerance, plasma insulin and glycated harmoglobin (HbA1c) level of STZ-induced diabetic rats

Elevated fasting blood glucose and reduced glucose tolerance are characteristic of STZ-induced diabetic rats due to extreme declination of insulin level resulting from massive beta cells destruction. Figure 2 shows that the treatment with butanol fraction for 30 consective days improved glucose tolerance of the treated animals to the tune of 15.0% ($p<0.05$) and 27.5% ($p<0.05$) on days 14th and 28th post treatment respectively (Fig. 2C and D). Fasting

blood glucose was declined to 18.0% ($p<0.05$) and 29.9% ($p<0.01$) as compared to STZ control group on the same mentioned days respectively (Fig. 2A and B). Plasma insulin level in treated group was found elevated to 42.3% ($p<0.01$) as observed on final day i.e on day 30th post treatment (Fig. 2E). Standard drug metformin also showed marked improvement in fasting blood glucose and oral glucose tolerance but the rise in plasma insulin level was not found significant after the completion of 4 weeks of treatment. According to WHO guidelines 2011 glycated haemoglobin or HbA1c now forms the major diagnostic criteria of diabetes mellitus. Animals showing HbA1c level 10 and above were selected for study. Figure 2F shows that the oral administration of butanol fraction of *T.belerica* declined the HbA1c level to the tune of 36.2% ($p<0.01$) which was close to the 41.2% ($p<0.01$) reduction showed by metformin treated group on 30th day.



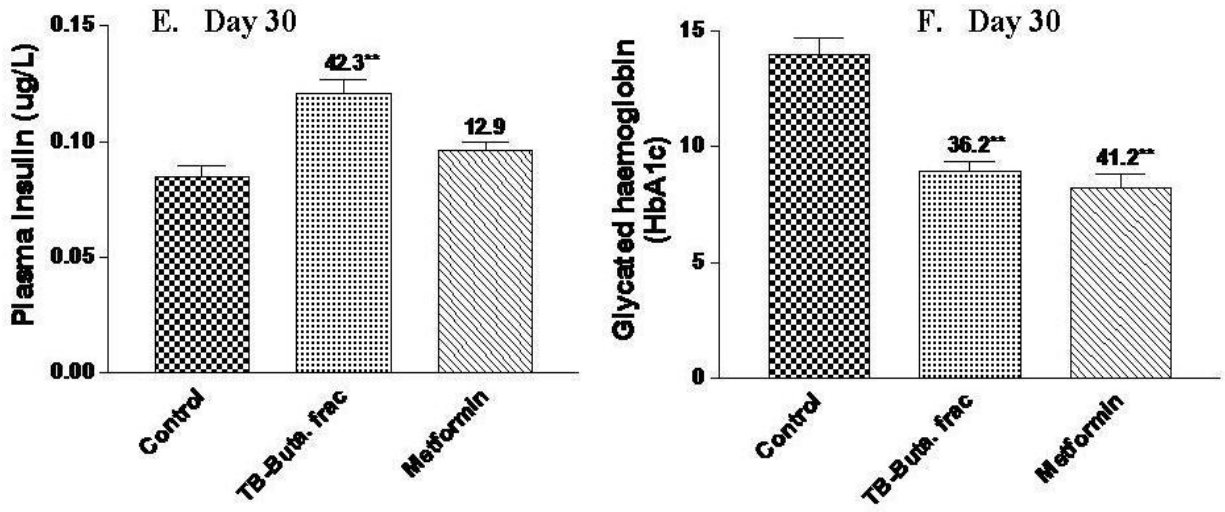


Figure 2: Effect of butanol fraction of 95% ethanolic extract of *T.belerica* fruits on fasting blood glucose (A and B), oral glucose tolerance (C and D), plasma insulin (E) and HbA1c level (F) of STZ-induced diabetic rats.

Effect of butanol fraction of 95% ethanolic extract of T.belerica fruits on lipid profile of STZ-induced diabetic rats.

Table 6 shows that the plasma triglyceride level was found reduced by 39.9% ($p<0.01$) while total cholesterol and LDL-c were dropped down by 25.4% ($p<0.01$) and 52.3% ($p<0.01$) on day 30th post treatment in butanol fraction treated group. HDL-c level was found raised by the significant level of 21.2% ($p<0.01$). There was no considerable improvement in metformin treated group except the plasma LDL-c which was found reduced by the significant level of 21.4% ($p<0.01$) on final day of treatment. The results confirm the antidyslipidemic activity of the butanol extract as seen in HFD-STZ model.

Table 6. Effect of butanol fraction of 95% ethanolic extract of *T.belerica* fruits on lipid profile of STZ-induced diabetic rats

Group	Day	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	LDL-c (mg/dl)	HDL-c (mg/dl)
Control	10th	164.5±8.38	121.8±4.25	48.10±5.82	40.83±2.55
	30th	167.0±6.37	124.7±4.68	51.27±4.25	40.00±2.53
Butanol fraction (100 mg/kg)	10th	130.3±5.37**(20.7**)**	113.0±3.64 **(7.22)**	42.43±4.94 **(11.8)**	44.50±2.57 **(9.06)**
	30th	100.3±4.42**(39.9**)**	93.00±2.21**(25.4**)**	24.43±2.68**(52.3**)**	48.50±1.52**(21.2**)**
Metformin (100 mg/kg)	10th	159.0±3.46 **(3.34)**	118±2.95 **(2.95)**	44.2±3.28 **(8.10)**	42.1±2.84 **(3.18)**
	30th	146.5±4.51 **(12.2)**	112±3.99 **(10.1)**	40.2±5.38 **(21.4**)**	42.5±2.23 **(6.25)**

Results are mean ± SE of six rats; **p<0.01

Effect of butanol fraction 95% ethanolic extract of T.belerica fruits on hepatic and renal parameters of STZ-induced diabetic rats

It is evident from Table 7 that butanol fraction treated group showed marked decline in plasma AST and ALT as well as urea, uric acid and creatinine level which clearly reflects improvement in hepatic and renal performance as compared to the untreated control group. Table 7 shows that maximum reduction in AST and ALT level was observed to be 53.8% ($p<0.01$) and 35.5% ($p<0.01$) in butanol fraction treated group on day 30th post treatment and in metformin treated group it was 47.1% ($p<0.01$) and 40.6% ($p<0.01$). Results in Table 7 also shows that the treatment of butanol fraction significantly depleted urea, uric acid and creatinine level and maximum depletion was observed on day 30th post treatment by the tune of 46.6% ($p<0.01$), 28.3% ($p<0.01$) and 32.0% ($p<0.01$) respectively and in metformin treated group it was 51.5% ($p<0.01$), 32.5% ($p<0.01$) and 34.3% ($p<0.01$) respectively. Results proved hepato- and renoprotective potential of butanol fraction of 95% ethanol extract of *T.belerica.*

Table 7. Effect of butanol fraction of 95% ethanolic extract of *T.belerica* fruits on hepatic and renal parameters of STZ-induced diabetic rats on 30th day of treatment

Group	Hepatic parameters		Renal parameters		
	AST (U/I)	ALT (U/I)	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine(mg/dl)
STZ Control	120.5±2.08	92.1±2.00	119.1±4.21	7.417±0.192	1.081±0.021
Butanol fraction (100 mg/kg)	55.6±3.36 **(53.8**)**	59.8±2.54 **(35.5**)**	63.5±2.28 **(46.6**)**	5.317±0.410 **(28.3**)**	0.735±0.027 **(32.0**)**
Metformin (100 mg/kg)	63.6±1.92 **(47.1**)**	54.6±2.98 **(40.6**)**	57.7±1.99 **(51.5**)**	5.00±0.33 **(32.5**)**	0.709±0.025 **(34.3**)**

Results are mean ± SE of six rats; **p<0.01

Concentration dependent effect of butanol fraction of 95% ethanolic extract of T. belerica on glucose uptake in L6 cells

Effect of butanol fraction of *T. belerica* on glucose uptake was evaluated using 2-deoxyglucose (2-DOG) in L6 cells. *T. belerica* increases basal and insulin-stimulated glucose uptake in concentration dependent manner in L6 cells. Figure 3 shows butanol fraction of *T. belerica* increases basal glucose uptake in L6 myotubes to a significant level of 1.55 fold ($p<0.05$) at a minimum concentration of 10 µg/ml. Maximum stimulation of 2.20 fold ($p<0.01$) was observed at 20 µg/ml concentration. Effect of *T. belerica* on insulin-induced increase in glucose uptake was also observed. Insulin alone enhanced a significant increase of 1.61-fold ($p<0.01$) in glucose uptake. Pre-incubation of myotubes with different

concentration of *T. belerica* for 16 h with insulin (100 nM) added for final 20 min, resulted in dose-dependent increase of insulin response in an additive manner which was found to be 1.88-fold (p<0. 01), 2.19-fold (p<0.01) and 2.94-fold (p<0.01) at 5, 10 and 20 µg/ml concentration, respectively vs. control.

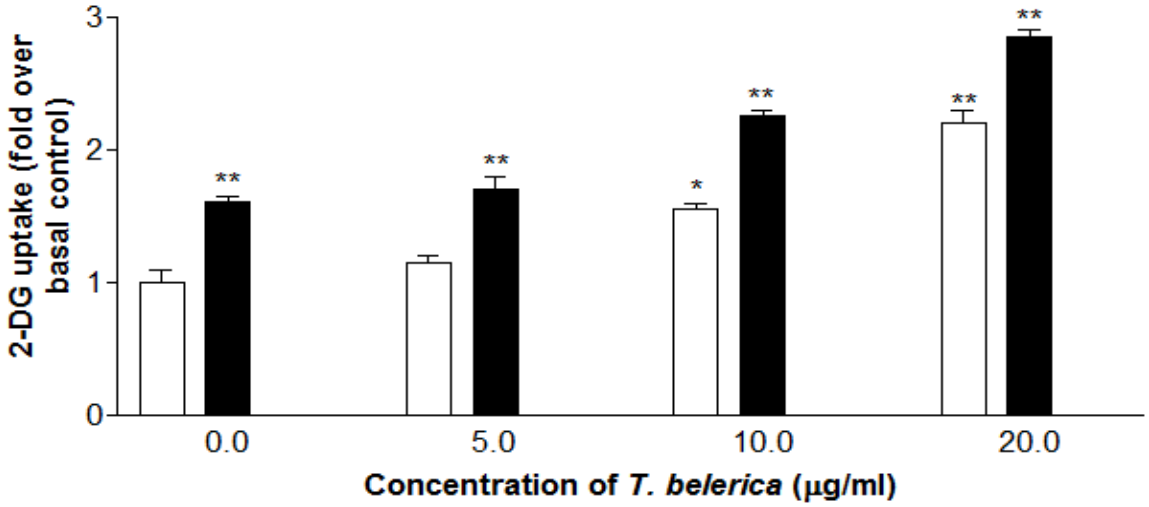


Figure 3. Concentration-dependent effect of butanol fraction of 95% ethanol extract of *T.belerica* on 2-deoxyglucose uptake in L6 myotubes. Cells were incubated for 16 h with different concentrations of *T.belerica*. After incubation myotubes were left untreated (white bars) or stimulated with 100 nM insulin (black bars) for 20 min, followed by the determination of 2-DG uptake. Results are expressed as fold stimulation over control basal. Significance *p<0.05; **p<0.01

Effect of butanol fraction of 95% ethanolic extract of T. belerica on mRNA expression of insulin signaling gene in L6 cells

Our gene expression profile in Figure 4 showed that butanol fraction of *T.belerica* could upregulate the expression of IRS-1(Insulin receptor substrate-1), PIK3CG (phosphatidylinositol 3-kinase, catalytic, alpha), AKT2 (Protein Kinase-B), and GLUT4 gene. These results suggest that butanol fraction of *T.belerica* stimulates insulin signaling pathways genes which may account for the antihyperglycemic effects of this drug.

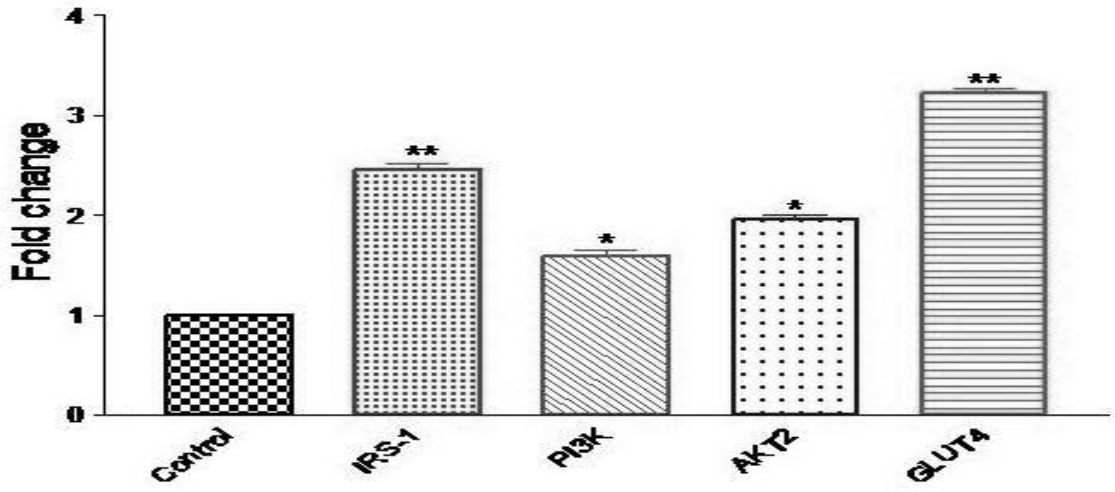


Figure 4. Effect of the butanol fraction of 95% ethanol extract of *T. belerica* on the expression of IRS-1, PI-3Kinase, AKT2 and GLUT4 genes in L6 myotubes. L6 myotubes were treated with 20 µg/ml concentrations of *T. belerica* for 16 h and then subjected to Real Time PCR analysis. Experiments are performed in triplicate. Results shown are mean ± SE of three independent experiments. *p<0.05, **p<0.01, relative to control.

Effect of butanol fraction of 95% ethanolic extract of T. belerica on IRS-1, AKT and GLUT4 proteins in L6 cells

Glucose uptake can be mediated by the insulin signaling pathway, which can stimulate the translocation of GLUT4 protein from cytoplasm to plasma membrane. To investigate the mechanistic aspects of the antidiabetic action of *T. belerica* expression of genes involved in insulin signaling pathway were studied by western blot analysis. As shown in the Figure 5 butanol fraction of *T. belerica* increases the proteins expression profile of p-IRS-1, p-AKT and GLUT-4.

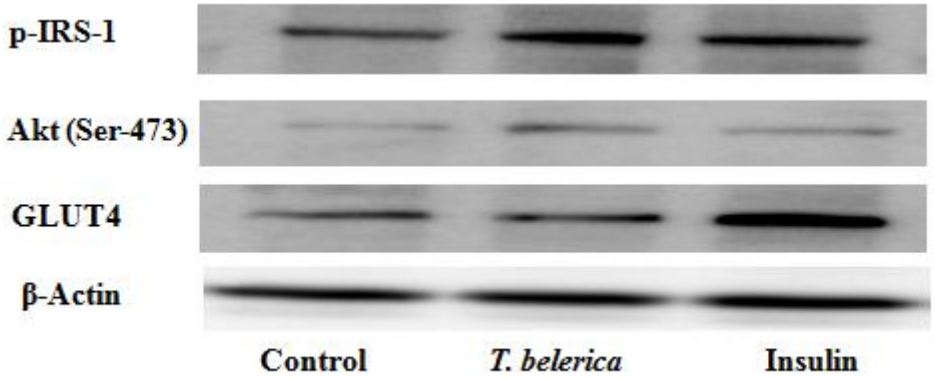


Figure 5. Effect of the butanol fraction of *T. belerica* on the expression of IRS-1, AKT2 and GLUT4 protein in L6 myotubes. L6 myotubes were treated with 10 µg/ml concentrations of *T. belerica* for 16 h and then subjected to western blot analysis. Experiments are performed in triplicate. Results shown are mean ± SE of three independent experiments.

DISCUSSION

Terminalia belerica has been used in Ayurvedic system of medicine for centuries in India[11]. A large number of pharmacological activites such as analgesic, anti diarrhoeal, antihypertensive, antispasmodic, bronchodilatory and antimicrobial has been reported in various studies[12,4,13,14,15]. The present study was undertaken to investigate the effect of *T. belerica* fruits on diabetes and its associated complications in validated animal models of diabetes.

Terminalia belerica fruit extracts significantly reduced blood glucose level in STZ-induced diabetic rats in the single dose experiment. 95% ethanolic extract being the most active, was subjected to further fractionation using various solvents viz., hexane, chloroform, butanol and water in the order of their increasing polarity. Antihyperglycemic effect was observed in all the fractions when administered to STZ-induced diabetic rats with maximum activity at the given dose in butanol fraction comparable to the metformin treated group which demands for the multiple dose study in validated animal models to investigate its overall effect on various symptoms and complications of diabetes. Therefore the effect of butanol fraction was studied in high fructose diet fed low dose STZ (HFD-STZ) induced diabetic rat model showing elevated triglycerides, cholesterol and fasting blood glucose level and low dose STZ-induced diabetic rats showing abnormally high HbA1c above 10% and display other complications as well in more pronounced manner.

The HFD-STZ rats share some resemblance with human type 2 diabetes mellitus having disturbed lipid profile with elevated triglycerides and cholesterol level, abnormally high fasting blood glucose and decreased tolerance towards external glucose administration[16,17,18,19]. These animals when treated with butanol fraction, showed marked improvement in oral glucose tolerance, lowered fasting blood glucose and revert lipid profile towards normal. There was marked improvement in liver and kidney functions parameters. The results clearly suggest the antidyslipidemic and antidiabetic activity of the butanol fraction.

Although the HFD-STZ rat model resembles human type 2 diabetes, but the complications of long term diabetes related to hepatic and renal functions are more pronounced in STZ-induced diabetic rat model with a high level of HbA1c. Therefore the STZ-induced diabetic rats serve as a model which express the symptoms of full blown diabetes with various complications more severe than the diet-induced model. Severe and persistent hyperglycemia in STZ-rats were reflected in their glycated hemoglobin status (HbA1c) which were far above from the normal. The diabetic complications related to hepatic and renal functions are generally more pronounced in these animals evident from the highly elevated level of their corresponding plasma markers such as AST, ALT, urea, uric acid and creatinine. butanol fraction significantly improved oral glucose tolerance and also lowered plasma triglycerides and cholesterol level in the treated group. The remarkable decline in the plasma concentration of AST, ALT, urea, uric acid and creatinine indicates restoration of liver and kidney functions of treated animals. HbA1c level measured on the 30th day of treatment showed significant lowering which indicates the efficient control of blood glucose by butanol fraction.

The study of butanol fraction in L6 cells showed its potential to stimulate glucose uptake by skeletal muscles. Enhancement of glucose transport in skeletal muscle cells is regulated by translocation and redistribution of GLUT4 protein in the plasma membrane and defect in this process causes insulin resistance[20]. Thus, it is necessary to stimulate glucose uptake in skeletal muscle cells to reduce insulin resistance. In the present study, we have found that like insulin, butanol fraction *T. belerica* fruits significantly stimulate glucose uptake in a concentration dependent manner, indicating that it enhances glucose metabolism in skeletal muscle cells. Since GLUT4 protein mediate glucose uptake in skeletal muscle cells[21], hence we examined the effect of butanol fraction of *T. belerica* on GLUT4 protein in L6 myotubes and found that treatment with butanol fraction significantly enhanced the GLUT4 expression at both mRNA and protein level in L6 myotubes, suggesting the potential activity of butanol fraction of *T. belerica* to stimulate glucose uptake in L6 myotubes was due to increased in the total amount of GLUT4 protein. The process of GLUT4 mediated glucose uptake in skeletal muscle cells can be regulated by insulin dependent pathway[20,22]. When insulin binds to its receptor it increases the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), and subsequently activation of PI-3-kinase and AKT protein that commonly regulates the both process glucose uptake and GLUT4 translocation in skeletal muscle cells[23,24]. In our study, we found an increase in the expression of IRS-1, AKT at gene as well as protein level

suggesting that stimulatory effect of *T. belerica* on glucose uptake is mediated via PI-3-K/AKT pathway.

CONCLUSION

From the present study, it may be concluded that butanol fraction of 95% ethanolic extract of *T. belerica* fruits are highly effective in the control of hyperglycemia and the associated complications in streptozotocin-induced diabetes mellitus in rats. Further study is needed to identify and elucidate the mechanism(s) of action of antidiabetic components in the butanol fraction of *T. belerica.*

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