Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 3, 2015

Original Article

EFFECT OF *MOMORDICA CHARANTIA* FRUITS ON STREPTOZOTOCIN-INDUCED DIABETES MELLITUS AND ITS ASSOCIATED COMPLICATIONS

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Received: 16 Dec 2014 Revised and Accepted: 04 Jan 2015

ABSTRACT

Objective: The present study was undertaken to assess the effect of *Momordica charantia* fruits on diabetes mellitus and its related complications.

Methods: Crude powder, aqueous, 95% and 50% ethanol extract of *Momordica charantia* fruits were administered to normal rats post sucrose load and streptozotocin-induced (STZ-induced) diabetic rats in single-dose study. The aqueous extract was further fractionated into butanol and aqueous fractions which were evaluated for the antihyperglycemic activity at a single dose in STZ-induced diabetic rats. The multiple dose effect of aqueous fraction was studied in high-fructose diet fed (HFD) rats and STZ-induced diabetic rats. The effect of the same fraction on cellular glucose uptake and insulin-signaling was studied in rat skeletal muscle cells (L6).

Results: The aqueous extract of *Momordica charantia* fruits showed significant lowering of postprandial hyperglycemia in normal rats and also lowered blood glucose level in STZ-induced diabetic rats. The butanol and aqueous fractions also significantly declined the blood glucose level of STZ-induced diabetic rats in single-dose administration with comparatively higher activity in aqueous fraction. In a multiple - dose study the aqueous fraction significantly improved fasting blood glucose, oral glucose tolerance, plasma insulin level, lipid profile, hepatic and renal parameters in both high-fructose diet fed and STZ-induced diabetic rats. *In vitro* study of L6 cells revealed that the aqueous fraction significantly increased glucose uptake by treated cells by upregulating the expression of IRS, AKT and GLUT4 at both mRNA and protein level.

Conclusion: It can be concluded that the aqueous fraction of aqueous extract of *Momordica charantia* fruits is competent in the control of diabetes and its related complications.

Keywords: Hyperglycemia, Diabetes, *Momordica charantia*, Streptozotocin, High-fructose diet.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by persisting hyperglycemia caused due defect in insulin secretion, action or both [1]. Environmental and genetic factors along with a sedentary lifestyle and dietary habits play major in the development of diabetes [2]. Despite the efforts to control the growing number of diabetes patients, yet, the number of adults with diabetes worldwide is rising continuously every year. Evidences suggest that diabetes is the world's fastest growing metabolic disorder and it is becoming a global public health burden [3]. Globally, as of 2012, an estimated 346 million people have type 2 DM making up about 90 % of DM. Its incidence is increasing rapidly and by 2030, this number is estimated to almost double. Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries [4, 5].

It is predicted that by 2030 India, China and USA will have the largest number of affected individuals [3]. Chronic hyperglycemia is the characteristics of diabetes, which may further lead to several complications by various pathways therefore the effective control of blood glucose level is essential in diabetes [6]. At present the treatment of diabetes includes various hypoglycemic agents such as sulfonylurea, biguanides, meglitinides and α-glucosidase inhibitors [7]. The currently available drugs to combat the impaired insulin secretion, insulin resistance and hyperglycemia may respond good at initial but have various negative side effects in long-term [8].

The management of diabetes without any side effects is still a challenge and current antidiabetic research focus on the development of safe and effective antihyperglycemic without any side effects. Since ancient times plants are used in the treatment of diabetes and other diseases as well, therefore the plant based medicines are considered safe. According to available information more than 800 plants are known for the treatment of diabetes in traditional system of medicines and about 400 traditional plant treatment for diabetes have been reported, although a very small number received scientific and medical evaluation regarding their efficacy [9, 10].

Momordica charantia also known as bitter melon belongs to the family Cucurbitaceae and it is a native to tropics and used as a vegetable in India, China and other countries [11]. In Ayurveda the fruit is considered as tonic, stomachic, stimulant, emetic, antibilous, laxative and antihyperglycemic [12]. It also forms the part of many polyherbal antidiabetic formulations and also known for anticancer, antibacterial, antiulcer, antifertility, anthelmintic, antimalarial, antipsoriasis and immunomodulatory activities [13, 14].

There are a number of studies related to the antidiabetic effect of *M. charantia* but its effect on late-stage complications which mainly develop and become severe in long duration in untreated or loosely treated diabetics has been scarcely investigated. So the present study was designed to investigate the effect of *M. charantia* fruits on late stage complications of diabetes and we identified that it is effective in combating diabetic hyperglycemia as well as the complications found in the late-stage of diabetes.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ), metformin, 2-DOG, cytochalasin B, IBMX, dexamethasone and insulin were obtained from Sigma Chemical Company, St. Louis, USA, whereas 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 *M. charantia* were purchased from the local market and its identity was authenticated in the laboratory. The dried fruits of *M. charantia* were powdered and three separate parts of the crude powder were extracted with 10 volumes of 95% ethanol, 50% ethanol and water 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%, 50% ethanol extract and aqueous extract respectively. In the fractionation process, aqueous extract was fractioned in 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 housed in animal housing facility where all the standard conditions related to temperature, relative humidity and a 12 h light/dark cycle were maintained. The animals had always free access to pellet diet and 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 [15]. 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 [16].

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 [17].

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 was observed 30, 60, 90, 120, 180, 240, 300 and 1440 min post treatment.

Multiple dose effect

Effect on the 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 [18]. 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 14th and 28th and the animals were bled on 10th and 30thday 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, aqueous 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 14th and 28th days and lipid profile, renal and hepatic function tests were done on 10th and 30th 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 was 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. Immuno reactive 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 *M. charantia* fruits on postprandial hyperglycemia in normal rats

It is evident from table 1 that only aqueous extract and standard drug metformin showed significant lowering of postprandial blood glucose to the tune of 13.9% ($p<0.05$) and 36.6% ($p<0.01$) respectively. Lowering was also observed in crude powder and other extracts, but was not statistically significant.

Table 1: Blood glucose profile during an oral glucose tolerance test in *M. charantia* 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	-	70.0±2.33	139.1±1.81	133.8±2.93	123.0±1.82	122.1±1.44	14770±276	-
Crude powder	250	83.8±4.23	142.8±2.85	114.2±3.33	113.6±3.02	113.4±2.44	14080±182	4.67
95% Eth. Ext.	250	70.3±1.99	115.0±2.11	116.5±3.81	114.6±2.74	114.8±3.88	13170±361	10.8
50% Eth. Ext.	250	71.0±2.09	123.1±4.57	109.6±1.66	112.6±1.17	109.6±2.17	13080±287	11.4
Aqu. Ext.	250	70.3±1.80	123.3±5.21	102.5±2.71	108.3±2.91	108.6±2.34	12710±211	**13.9***
Metformin	100	68.5±1.94	99.6±4.66	81.0±2.29	64.5±2.17	64.6±2.49	9353±397	**36.6****

Results are mean ± S. E. of 6 rats; * $p<0.05$, ** $p<0.01$

Effect of crude powder and aqueous as well as ethanolic extracts of *M. charantia* fruits on the blood glucose profile of STZ-induced diabetic rats

STZ causes mass level destruction of beta cells in experimental rats resulting in greatly decrease in plasma insulin level and therefore STZ-induced diabetic rats show high level of fasting blood glucose. After confirmation of diabetes the overnight fasted rats were treated with a single dose of crude powder and various extracts of *M. charantia* fruits. table 2 shows that aqueous extract alone showed significant lowering of blood glucose to the extent of 16.3% ($p<0.05$) during 5 h of treatment.

When calculated for 24 h only aqueous extract was found to be significantly active to the tune of 14.8% ($p<0.05$). Lowering in other extracts was not significant. Activity of standard drug metformin was found to be 28.4% and 26.3% respectively for 5 h and 24 h respectively and both the values were statistically significant.

Table 2: Blood glucose profile of *M. charantia* 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	-	128400±3959	585600±22679	--	--
Crude powder	250	108241±4116	544139±19432	7.08	7.08
95% Eth. Ext.	250	116100±2164	550000±17645	9.57	6.07
50% Eth. Ext.	250	115700±5184	571500±19864	9.89	2.40
Aqu. Ext.	250	107400±3119	498900±12186	**16.3***	**14.8***
Metformin	100	91830±2648	431400±15695	**28.4****	**26.3****

Results are mean ± S. E. of six rats; * $p<0.05$, ** $p<0.01$

Table 3: Blood glucose profile of STZ-induced diabetic rats treated with various fractions of aqueous extract of *M. charantia* fruits and metformin

Treatment	Dose (mg/kg)	Mean AUC		% lowering in blood glucose	
		0-5h	0-24h	0-5h	0-24h
Sham	-	135400±5150	601600±15380	-	-
Butanol fraction	100	111800±4763	558800±25370	**17.4***	7.11
Aqueous fraction	100	102800±3070	589200±24320	**24.0****	2.06
Metformin	100	93660±1089	456100±9580	**30.8****	**24.2****

Results are mean ± S. E. of six rats; * $p<0.05$, ** $p<0.01$

Effect of butanol and aqueous fractions of aqueous extract of *M. charantia* fruits on the blood glucose profile of Streptozotocin-induced diabetic rats

Since the aqueous extract showed highest activity in normal rats post sucrose load and STZ-induced diabetic rats therefore the same was further subjected to fractionation and butanol and aqueous fractions were obtained which were administered in a single dose to overnight fasted STZ-induced diabetic rats and as it is clear from table 3 both the fractions showed significant activity of 17.4% ($p<0.05$) and 24.0% ($p<0.01$) respectively during 5 h of study. The metformin treated group showed significant activity of 30.8% ($p<0.01$) and 24.2% ($p<0.01$) when AUC compared to 5 h and 24 h respectively.

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on fasting blood glucose, oral glucose tolerance and plasma insulin level of high fructose diet fed low dosed STZ-induced diabetic rats

Fig. 1 shows the remarkable effect of aqueous fraction on fasting blood glucose of treated animals. A highly significant improvement of 31.8% ($p<0.01$) and 56.0% ($p<0.01$) was observed in fraction treated group respectively on days 14th and 28th post treatment. The lowering observed on day 28th was even greater than the metformin treated group i. e., 47.3% ($p<0.01$) (fig. 1A and B). It also had a positive impact on oral glucose tolerance and significant improvement of 17.7% ($p<0.05$) and 29.1% ($p<0.01$) was registered on days 14th and 28th post treatment (fig. 1C and D). Plasma insulin level, which gets elevated in HFD-STZ rats was also brought down from 19.7% ($p<0.05$) as compared to an untreated control group (fig. 1E).

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on the lipid profile of the high fructose diet fed low dosed STZ-induced diabetic rats

High fructose diet generally causes disturbances in lipid profile leading to dyslipidemia, which get further deified by the low dose treatment of STZ causing highly elevated plasma triglycerides, total cholesterol, LDL level and decreased plasma HDL level. table 4 shows that the treatment with aqueous fraction for one month significantly improved plasma lipid profile and the significant decline of triglycerides, total cholesterol and LDL-cholesterol was found in the tune of 35.7% ($p<0.01$), 32.4% ($p<0.01$) and 33.8% ($p<0.01$) respectively on day 30th post treatment. While the plasma HDL level was found increased by the significant extent of 41.5% ($p<0.01$). Thus the aqueous fraction of *M. charantia* was found sufficiently effective against diabetic dyslipidemia caused by high fructose and low dose STZ in animals.

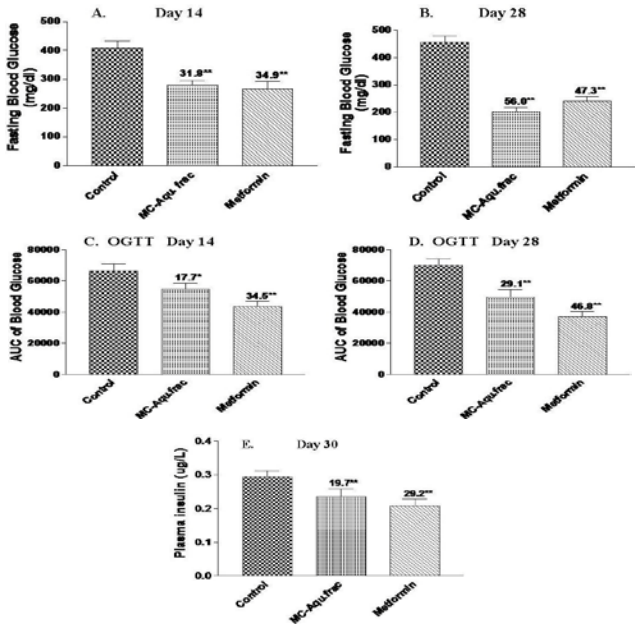

Fig. 1: Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits and standard drug metformin on fasting blood glucose (A and B), oral glucose tolerance (C and D) and plasma insulin level (E) of high fructose diet fed low dosed STZ-induced diabetic rats during 30 days of treatment. Significance *p<0.05; **p<0.01

Table 4: Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on the lipid profile of the high fructose diet fed low dosed STZ-induced diabetic rats

Group	Day	Triglycerides (mg/dl)	Cholesterol (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)
Control	10th	286.5±12.1	206.3±9.85	115.8±11.9	22.6±1.09
	30th	321.9±14.6	231.6±11.9	129.8±12.8	21.4±1.45
Aqueous fraction	10th	227.5±9.78 (20.5*)	168.3±6.30 (18.4*)	94.6±7.22 (18.3*)	28.1±1.72 (24.3**)
(100 mg/kg)	30th	206.7±5.67 (35.7**)	156.5±4.87 (32.4**)	85.8±5.22 (33.8**)	30.3±2.34 (41.5**)
Metformin	10th	261.3±9.86 (8.79)	198.4±4.63 (3.82)	106.6±3.63 (7.94)	23.8±1.13 (5.30)
(100 mg/kg)	30th	279.1±8.96 (13.2)	213.9±6.36 (7.64))	115.7±7.96 (10.8)	22.9±1.26 (7.00)

Results are mean ± S. E. of six rats; * p<0.05, **p<0.01

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on hepatic and renal parameters of the high fructose diet fed low dosed STZ-induced diabetic rats

High fructose diet may cause dyslipidemia, hyperinsulinemia and insulin resistance which are the characteristics of diabetes type 2 and the extent of severity may get increased by the low dose of STZ which may further add the symptoms like hepatic and renal dysfunction in the experimental animals. table 5 shows that in our present study treatment of aqueous fraction was found to improve the plasma level of hepatic and renal function markers. There was a significant decline of plasma AST and ALT level and the lowering of 31.2% ($p<0.01$) and 23.5% ($p<0.01$)

respectively was observed on the final day of treatment. Accordingly, significant decline of plasma level of urea, uric acid and creatinine to the tune of 38.4% (p<0.01), 27.5% (p<0.01) and 25.0% respectively was observed on final day i. e. 30th day of treatment.

Table 5: Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on hepatic and renal parameters of the high fructose diet fed low dosed STZ-induced diabetic rats

Group	Hepatic parameters		*Renal parameters*		
	AST (U/l)	ALT (U/l)	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine (mg/dl)
HFD-STZ Control	6.91±0.59	31.9±1.83	78.9±3.86	7.65±0.49	0.764±0.051
Aqueous fraction	4.75±0.35	24.1±1.37	48.6±2.78	5.54±0.37	0.573±0.027
(100 mg/kg)	**(31.2**)**	**(23.5**)**	**(38.4**)**	**(27.5**)**	**(25.0**)**
Metformin	3.86±0.26	18.6±1.44	35.6±1.19	4.36±0.16	0.519±0.048
(100 mg/kg)	**(44.1**)**	**(41.6**)**	**(54.8**)**	**(43.0**)**	**(32.0**)**

Results are mean ± S. E. of six rats; * p<0.05, **p<0.01

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on fasting blood glucose, oral glucose tolerance, plasma insulin and HbA1c of STZ-induced diabetic rats

Most of the beta cells get destroyed in STZ-induced diabetic rats and therefore animals display high level of fasting blood glucose and intolerance towards external glucose administration. Fig. 2A and B shows that treatment of aqueous fraction for one month significantly improved the fasting blood glucose level to the tune of 15.4% (p<0.05) and 28.1% (p<0.01) as monitored on days 14th and 28th post treatment. Improvement in oral glucose tolerance was not significant on day 14th while the significant improvement of 21.3% (p<0.05) was registered on day 28th (fig. 2C and D). Plasma insulin level in the treated group was found elevated to 16.2% (p<0.05) (fig. 2E). Glycated hemoglobin or HbA1c reflects the average concentration of glucose in the blood for a prolonged period of time and HbA1c level is generally found elevated in the untreated or late diagnosed diabetes. Hence the reduction in HbA1c level reflects the effective control of blood glucose level. In the present study animals showing HbA1c level 10 and above were selected for study. Fig. 2F shows that the oral administration of the aqueous fraction of *M. charantia* declined the HbA1c level to the tune of 24.1% (p<0.01) in the treated group on day 30th post treatment.

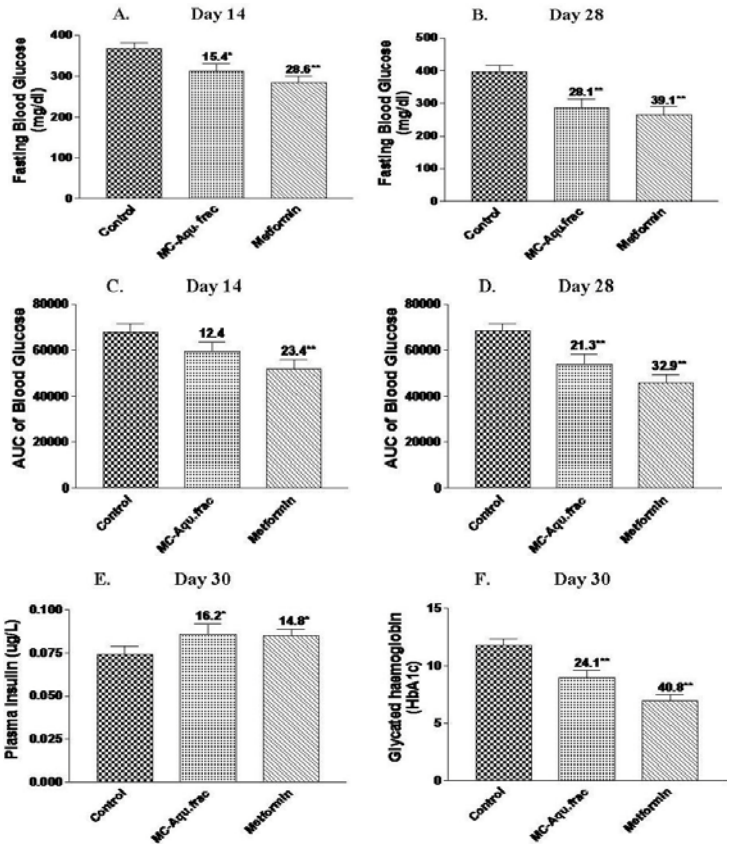

Fig. 2: Effect of the aqueous fraction of aqueous extract on fasting blood glucose (A and B), oral glucose tolerance (C and D), Plasma insulin (E) and HbA1c level (F) of STZ-induced diabetic rats during 30 days of treatment. Significance *p<0.05; **p<0.01

Effect of aqueous fraction of aqueous extract of *M. charantia* fruits on the lipid profile of STZ-induced diabetic rats

STZ-induced diabetic animals which develop a high HbA1c level and other complications in long duration are generally lean animals with disturbed lipid profile also but the level of triglycerides and cholesterol are not as much elevated as in the diet induced model. In the present model medium elevation of triglycerides, total cholesterol and LDL was noticed and slight declined of HDL level. table 6 shows that the plasma triglyceride level was found to reduce by 25.4% (p<0.01) while total cholesterol and LDL-cholesterol were dropped down by 18.2% (p<0.05) and 38.8% (p<0.01) on day 30th

post treatment. HDL-cholesterol level was found raised by the level of 13.7%, which is not significant on the above said day of treatment. There was no considerable improvement in metformin treated group except the plasma LDL-cholesterol, which was found reduced by the significant level of 18.3% ($p<0.05$) on final day i. e. day 30th post treatment.

Table 6: Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on the lipid profile of STZ-induced diabetic rats

Group	Day	Triglycerides (mg/dl)	Cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Control	10th	159.8±4.95	130.3±3.89	50.3±2.98	45.1±1.68
	30th	161.5± 6.37	135.4±3.91	55.0±2.64	52.1±2.89
Aqueous fraction	10th	141.0±8.15 (11.8)	123.3±2.53 (5.37)	45.1±2.96 (10.3)	42.6±1.62 (5.63)
	30th	120.5±5.02 (25.4**)	110.0±3.50 (18.2*)	33.6±3.76 (38.8**)	45.0±1.87 (13.7)
Metformin	10th	151.5±4.16 (5.19)	126.2±2.86 (3.18)	46.5±3.15 (7.43)	43.4±2.19 (3.72)
(100 mg/kg)	30th	143.1±3.76 (11.4)	121.2±2.11 (10.5)	44.9±2.96 (18.3*)	47.9±3.15 (8.11)

Results are mean ± S. E. of six rats; * $p<0.05$, ** $p<0.01$

Table 7: Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on hepatic and renal parameters of STZ-induced diabetic rats

Group	Hepatic parameters		Renal parameters		
	AST (U/l)	ALT (U/l)	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine (mg/dl)
STZ Control	112.5±2.95	98.0±2.66	121.4±3.926	7.689±0.348	1.093±0.0347
Aqueous fraction	64.7±6.39	69.3±3.21	80.5±4.956	5.890±0.308	0.830±0.0318
(100 mg/kg)	(42.5**)	(29.3**)	(33.7**)	(23.4**)	(24.1**)
Metformin	63.1±2.01	61.3±2.52	65.3±2.99	5.082±0.348	0.742±0.0311
(100 mg/kg)	(43.9**)	(37.4**)	(46.2**)	(33.9**)	(32.1**)

Results are mean ± S. E. of six rats; ** $p<0.01$

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on hepatic and renal parameters of STZ-induced diabetic rats

Diabetes effect almost all the organs of the body and liver and kidney functions also get highly disturbed in diabetic animals which is well reflected in liver and kidney function markers such as AST, ALT, urea, uric acid and creatinine. It is evident from table 7 that multiple dosing of aqueous fraction in diabetic animals for one month 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. On the final day of treatment lowering in above mentioned parameters was found to be 42.5% ($p<0.01$), 29.3% ($p<0.01$), 33.7% ($p<0.01$), 23.4% ($p<0.01$) and 24.1% ($p<0.01$) respectively.

Concentration dependent effect of aqueous fraction of aqueous extract of *M. charantia* fruits on glucose uptake in L6 cells

Treatment of aqueous fraction led to the increase of basal as well as insulin-stimulated glucose uptake in a concentration dependent manner in L6 cells. Fig. 3 shows a significant increase of 1.41-fold ($p<0.05$) was observed in treated L6 myotubes at the minimum concentration of 5 ug/ml. The maximum increase of 1.66 fold ($p<0.01$) was observed at 10 ug/ml. Effect of aqueous fraction on insulin-induced increase in glucose uptake was also studied. When pre-incubated myotubes with various concentrations of aqueous extract were provided with insulin for final 20 min, a dose-dependent increase of 1.88-fold ($p<0.01$), 1.91-fold ($p<0.01$) and 2.12-fold ($p<0.01$) respectively at 2.5, 5.0 and 10.0 µg/ml concentration were observed.

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on mRNA expression of insulin signaling gene in L6 cells:

Gene expression profiles in fig. 4 suggest that the expression of IRS-1 (Insulin receptor substrate, PI3K (Phosphatidylinositol 3-kinase), AKT2 (Protein kinase-B) and GLUT4 genes were upregulated by the treatment of aqueous fraction. Thus, it is clear that the aqueous fraction of *M. charantia* stimulates the genes of insulin signaling pathway which may lead to the antihyperglycemic effect of the fraction.

Effect of the aqueous fraction of aqueous extract of *M. charantia* fruits on IRS-1, AKT and GLUT4 proteins in L6 cells

Insulin signaling pathway can influence glucose uptake by the translocation of GLUT4 containing vesicles to the plasma membrane and thus facilitates the transportation of glucose across the plasma membrane. Drugs affecting the insulin signaling may modulate the glucose uptake in this manner.

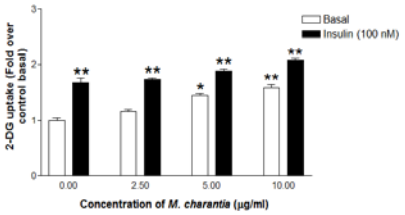

Fig. 3: Concentration-dependent effect of aqueous fraction of aqueous extract of *M. charantia* fruits on 2-deoxyglucose uptake in L6 myotubes. Cells were incubated for 16 h with different concentrations of aqueous fraction. After incubation myotubes were left untreated (white bars) or stimulated with 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

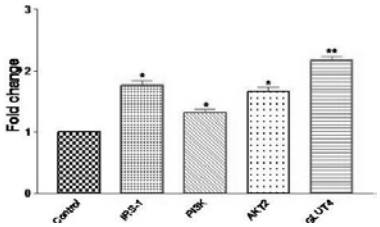

Fig. 4: Effect of aqueous fraction of aqueous extract of *M. charantia* fruits on the expression of IRS-1, PI-3Kinase, AKT2 and GLUT4 genes in L6 myotubes. L6 myotubes were treated with 20 µg/ml concentrations of *M. charantia* for 16 h and then subjected to Real Time PCR analysis. Experiments are performed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

Fig. 5 shows that similar to the gene expression profile, treatment of aqueous fraction increases the protein expression of of p-IRS-1, p-AKT and GLUT4. Thus, it is clear that the aqueous fraction of *M. charantia* does effect insulin signaling pathway in *in vitro* and as a consequence increase glucose uptake by cells.

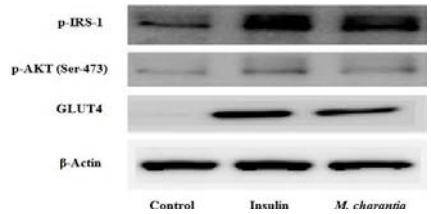

Fig. 5: Effect of aqueous fraction of aqueous extract of *M. charantia* fruits on the expression of IRS-1, AKT2 and GLUT4 protein in L6 myotubes. L6 myotubes were treated with 10 µg/ml concentrations of *M. charantia* for 16 h and then subjected to Western blot analysis. Experiments are performed in triplicate. Results shown are mean ± SE of three independent experiments

DISCUSSION

M. charantia is a common vegetable in Indian cuisine and also known for its various medicinal properties [11, 12]. It is also used in many herbal antidiabetic formulations. A study of polyherbal formulation containing *M. charantia* showed significant lowering of blood glucose, HbA1c and also elevated plasma insulin level in lab animals [13]. Our present study was focused to evaluate the effect of *M. charantia* fruits on diabetes mellitus and its associated complications. Initially from single dose administration of crude powder and various extracts of *M. charantia* fruits in normal rats post sucrose load and STZ-induced diabetic rats we found that among all extracts only aqueous extract showed significant lowering of postprandial blood glucose in normal rats post sucrose load and also significant lowering of blood glucose in STZ-induced diabetic rats. Therefore the aqueous extract was subjected to fractionation with butanol and water to obtain their respective fraction. Single dose administration of butanol and aqueous fractions in STZ-induced diabetic rats revealed that both the fractions showed significant lowering of blood glucose but the activity was higher in the aqueous fraction. Further the aqueous fraction was subjected to the multiple dosing in HFD-STZ rat model which shares some characteristics with human type 2 diabetes [19]. Fasting blood glucose level and oral glucose tolerance were markedly improved in the aqueous fraction treated group which supports the outcome of in vitro study showing increased glucose uptake by treating cells. Dyslipidemia is the characteristic feature of HFD-STZ rats [20] and aqueous fraction effectively restored the lipid profile of treated rats by the significant lowering of triglycerides, total cholesterol, LDL and enhancing the plasma HDL level. Declined level of hepatic transaminases and plasma level of urea, uric acid and creatinine indicates towards hepato and reno protective action of aqueous fraction.

Since the diabetic complications related to liver, kidney and other organs are more severe in STZ-induced diabetic rats left for a few weeks with untreated hyperglycemia and hence it is commonly used in the study of diabetic complications. Therefore the aqueous fraction was also studied in STZ-induced diabetic rats showing an abnormally high level of HbA1c. Such animals were treated with aqueous fraction for one month and there was significant improvement in fasting blood glucose level and oral glucose tolerance of treated animals.

The improvement in fasting blood glucose was well reflected in the declination of HbA1c level of the treated animals by the significant extent. Plasma triglycerides, total cholesterol and LDL were significantly reduced and HDL-level was raised significantly, which confirms the antidyslipidemic effect of aqueous fraction in the diet induced model. There was also marked lowering of hepatic transaminases and plasma level of urea, uric acid and creatinine indicating towards the hepato and reno protective activity of the aqueous fraction of *M. charantia.*

Aqueous fraction treatment in L6 cells enhanced basal as well as insulin-stimulated glucose uptake in concentration dependent manner. GLUT4 translocation and distribution is vital in the glucose uptake by cells [21]. Effect of aqueous fraction on GLUT4 expression was studied by treating L6 myotubes with aqueous fraction and as a result expression of GLUT4 significantly increased at both mRNA and protein level. Hence the increase in glucose uptake was due to the upregulation of the GLUT4 expression by L6 myotubes. The present study also suggests that the aqueous fraction increased tyrosine phosphorylation of IRS-1 in L6 myotubes and also increased the mRNA level of the same. PI3K expression was also found increased in treated L6 myotubes. Beside this the aqueous fraction also increased mRNA level of AKT in L6 myotubes and also stimulated the phosphorylation of AKT at Ser-473 suggesting that the stimulatory effect of the aqueous fraction of *M. charantia* on glucose uptake is mediated via PI-3-K/AKT pathway.

CONCLUSION

Therefore, it may be concluded that aqueous fraction of aqueous extract of *M. charantia* fruits is effective in controlling of diabetic hyperglycemia and dyslipidemia and other complications related to hepatic and renal functions.

ACKNOWLEDGEMENT

The authors would like to thank Council of Scientific and Industrial Research (CSIR), New Delhi for providing financial support in the form of Senior Research Fellowship to Arvind Mishra, Sudeep Gautam, Akansha Mishra, Savita Pal and Arun K. Rawat. The authors also thanks to Director CSIR-CDRI, for show his keen interest and providing necessary facilities for the study. This manuscript bears CSIR-CDRI Communication Number 8911.

ABBREVIATION

Area under the curve, AUC; Diabetes mellitus, DM; High fructose diet, HFD; Oral glucose tolerance test, OGTT; Ethanolic extract, Eth. Ext.; Aqueous extract, Aqu. Ext.; Aqueous fraction, Aqu. frac; Streptozotocin, STZ.

CONFLICT OF INTERESTS

Declared None

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