World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Effect of *Azadirachta indica* leaves on streptozotocin-induced diabetes mellitus and its associated complications

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Received: 30-01-2015 / Revised: 12-02-2015 / Accepted: 20-02-2015

ABSTRACT

The present study focused on the effect of *Azadirachta indica* leaves on diabetes and its associated complications. Crude powder, aqueous, 95% and 50% ethanolic extracts of the leaves were administered to normal rats with sucrose load and streptozotocin-induced (STZ-induced) diabetic rats in a single dose study. 95% ethanolic extract was found most active and showed significant lowering of blood glucose in STZ-induced diabetic rats and also lowering of postprandial hyperglycemia in normal rats. Therefore, its various fractions were also tested in STZ-induced diabetic rats in single-dose experiment and on the basis of antihyperglycemic activity profile, the chloroform fraction was subjected to detailed study using in vitro and in vivo models. In multiple-dose studies, the chloroform fraction was found effective against various complications of diabetes, such as dyslipidemia and disturbed hepatic and renal functions in high fructose diet fed rats and STZ-induced diabetic rats. It also significantly increased the glucose uptake in treated L6 cells and increased the expression of IRS, AKT and GLUT 4 at both mRNA and protein level. The findings suggest that the chloroform fraction of 95% ethanolic extract of *A.indica* leaves is competent in combating various symptoms and complications of diabetes mellitus.

KEYWORDS: Hyperglycemia, diabetes, *Azadirachta indica*, streptozotocin, high-fructose diet

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by persistent hyperglycemia caused due to insufficient insulin secretion or insulin action or both [1]. Due to the rapidly increasing incidences of diabetes, it has become probably the world's fastest growing metabolic disorder [2, 3]. The complications of diabetes affect almost every organ of the body. Dyslipidemia, impairment of liver function, retinopathy, nephropathy, neuropathy, cardiovascular dysfunction are some of the common complications which are seen in diabetic patients [4]. Chronic hyperglycemia is in the center of all the diabetic complications and therefore proper glycemic control may prevent or delay these complications [5]. Beside insulin present therapeutic approaches to control diabetic hyperglycemia includes various oral antidiabetic agents such as sulfonylureas, biguanides, glinides, α-glucosidase inhibitors, thiazolidinediones, dipeptidyl-peptidase-4 inhibitors [6]. These drugs are effective in their mode of action but exert serious side effects in long term use. Therefore great interest had been developed worldwide for the search of plant based new therapeutic agents which may promise safety along with efficacy in the treatment of diabetes.

Azadirachta indica, has attracted much attention within the worldwide medical community in recent years, due to its wide range of medicinal properties. It has been used extensively in Ayurvedic, homeopathic, and folk medical traditions over thousands of years [7-9]. A vast array of biologically active compounds has been isolated from this plant, many of which have been studied in laboratory conditions for their pharmacological properties. Modern scientific research has validated the traditional uses of *A. indica* for the maintenance of general health and especially for skin disorders including acne. It is also known to be effective in treating arthritis, blood disorders, bronchitis, cough, diabetes, drowsiness, eczema, fever,

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jaundice, malaria, nausea, obesity, parasites, rheumatism, skin diseases, syphilis, and tumors [7, 9, 10-14]. Although, a number of studies are available related to the antihyperglycemic effect of *A. indica* but its effect on complications which develop at the late-stage of diabetes has been scarcely investigated. So the present study focused to investigate the effect of *A. indica* leaves on late-stage complications of diabetes mellitus such as diabetic dyslipidemia, hepatic and renal function disorders in high fructose diet fed and streptozotocin (STZ)-induced diabetic rats. From the present study we identified that the leaves of *A. indica* are competent 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, 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 leaves of *A. indica* were purchased from the local market and its identity was authenticated in the laboratory. The shade dried leaves of *A. indica* were finely powdered in electric blender. This crude powder was extracted separately with 95% ethanolic, 50% ethanolic and water, taking 10 volumes of each and repeated five times. The extract obtained each time were pooled, filtered and concentrated under high vacuum in a rotavapor. Fractionation of 95% ethanolic extract was performed 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: Animals were procured from the animal colony of Central Drug Research Institute, Lucknow, India. Male albino rats of body weight 160 ± 20 g were procured and housed in the animal housing facility by maintaining the standard conditions of temperature, relative humidity and a 12 h light/dark cycle were always maintained. The animals had free access to 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: Streptozotocin was dissolved in Citrate buffer (0.1M) and injected 80 albino rats of Sprague Dawley strain of body weight 160 ± 20 g were injected intraperitoneally at the dose of 60 mg/kg body weight to Sprague-Dawley rats to make them diabetic. Blood glucose was checked after 48 h of injection and animals showing fasting blood glucose level above 270 mg/dl were selected and divided into desired groups. Experimental groups received oral doses of fine suspension of desired test samples prepared in 1% gum acacia at the dose of 250 mg/kg and 100 mg/kg body weight respectively for extracts and fractions. 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 [15].

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: Overnight fasted 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 consisting six animals in each group. Experimental groups were administered with fine suspension of test samples made in 1% gum acacia at the dose of 250 mg/kg in the case of crude powder and extracts while the control group received the same amount of vehicle that is 1% gum acacia. The standard drug metformin was given at the dose of 100 mg/kg body weight. An oral sucrose load of 10 g/kg body weight was administered to each animal's exactly 30 min post administration of the test sample / vehicle / standard drug. Blood glucose was measured at the intervals of 30, 60, 90 and 120 min post sucrose load with the help of glucostrips. Food but not water was withheld from the cages during the course of the experiment [16]. 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: Rats were made diabetic by i.p. injection of STZ and the animals showing fasting blood glucose between 300-450 mg/dl after 48 h of injection were selected for the study and grouped consisting six animals in each group. Test samples (plant extracts and fractions) and standard drug metformin were prepared as fine suspension in 1% gum acacia were administered to the treatment groups while the control group received the same amount of vehicle i.e., gum acacia suspension. Blood glucose was followed at 30, 60, 90, 120, 180, 240, 300 and 1440 minute 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 [17]. 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 above 270 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, chloroform 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 glycated hemoglobin (HbA1c): Plasma insulin was measured using Mercodia insulin Elisa kit and triglycerides, cholesterol, LDL, HDL, AST, ALT, urea, uric acid, creatinine and glycated hemoglobin (HbA1c) was 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. 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 *A. indica* leaves on postprandial hyperglycemia in normal rats: It is evident from the results in Table 1 that except 95% ethanolic extract neither crude powder nor any extracts showed significant effect on postprandial hyperglycemia of normal rats. Significant activity to the tune of 13.5% ($p<0.05$) and 32.8% ($p<0.01$) were observed in 95% ethanolic extract and metformin treated groups respectively during two hours of study. It implies that leaves of *A. indica* aid in the control of the postprandial rise in blood glucose without causing abnormal hypoglycemia. This increases its significance in the treatment of diabetes as unusual hypoglycemia is a major side effect of many oral antidiabetic agents which often lower down the blood glucose below normal in diabetic patients which may create a medical emergency if did not receive immediate attention.

Effect of crude powder and aqueous as well as ethanolic extracts of *A. indica* leaves on blood glucose profile of STZ-induced diabetic rats: Mass level of beta cell destruction in STZ-induced diabetic rats causes severe insulin deficiency resulting in elevated fasting blood glucose far above the normal. Table 2 shows that when such diabetic rats were treated with crude powder, aqueous and ethanolic extracts of *A. indica* leaves, some extent of lowering of blood glucose was observed in all treated groups. Although a significant decrease in hyperglycemia was found only in 95% ethanolic and aqueous extract to the tune of 24.6% ($p<0.01$) and 23.6% ($p<0.01$) respectively during the 5 h study. The standard drug metformin caused 33.0% ($p<0.01$) lowering of blood glucose in the same duration.

Effect of hexane, chloroform, butanol and aqueous fractions of 95% ethanolic extract of *A. indica* leaves on blood glucose profile of STZ-induced diabetic rats: Since the highest activity was observed in 95% ethanolic extract therefore it was further fractionated with various solvents namely hexane, chloroform, butanol and water to obtain their respective fraction. All the fractions when subjected to study in STZ-induced diabetic rats and it is clear from Table 3 that only chloroform fraction showed significant lowering of 23.3% ($p<0.01$) during 5 h of the study. The standard drug metformin showed significant activity of 28.2% ($p<0.01$) and 31.8% ($p<0.01$) respectively.

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on fasting blood glucose, oral glucose tolerance and plasma insulin level of high fructose diet fed low dosed STZ-induced diabetic rats: Figure 1 shows the effect of the chloroform fraction of *A. indica* leaves on HFD-STZ rats when administered for one month. It is evident from the figure that the chloroform fraction significantly improved fasting blood glucose (Figure 1 A and B), oral glucose tolerance (Figure 1 C and D) and declined plasma insulin level (Figure 1 E) in the treated group. The improvement was registered to the tune of 19.8% ($p<0.05$) and 38.8% ($p<0.01$) for fasting blood glucose and 24.4% ($p<0.01$) and 34.2% ($p<0.01$) of oral glucose tolerance on days 14^{th} and 28^{th} post treatment respectively. The plasma insulin level of the treated group was also found declined by 26.2% ($p<0.01$) on day 30^{th} post treatment.

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on the lipid profile of the high fructose diet fed low dosed STZ-induced diabetic rats: Plasma lipid profile was observed on days 10^{th} and 30^{th} post treatment and Table 4 suggests the positive impact of chloroform fraction on the disturbed lipid profile of HFD-STZ rats. Plasma level of triglyceride was found significantly declined by 19.3% ($p<0.05$) and 37.1% ($p<0.01$) respectively on the mentioned days. The total cholesterol level was also reduced by 22.4% ($p<0.01$) and 40.9% ($p<0.01$) respectively. Marked lowering of LDL-cholesterol to the tune of 28.0% ($p<0.01$) and 48.9% ($p<0.01$) and elevation in HDL-cholesterol by the extent of 30.5% ($p<0.01$) and 54.0% ($p<0.01$) respectively on the same time intervals. There was no

significant improvement in metformin treated group.

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on Hepatic and Renal parameters of the high fructose diet fed low dosed STZ-induced diabetic rats: AST and ALT serve as biochemical markers for the liver function test and its elevated level in plasma indicate liver dysfunction. It is clear from Table 5 that plasma AST and ALT level were found significantly reduced in treated groups as compared to control and the lowering observed on day 30th were 37.8% ($p<0.01$) and 36.2% ($p<0.01$) respectively and the results were comparable to the standard drug metformin. Table 5 also shows the effect of treatment on some renal function markers. It is evident from the table that there was a significant lowering of urea, uric acid and creatinine level to the tune of 41.4% ($p<0.01$), 39.2% ($p<0.01$) and 23.5% ($p<0.05$) was observed on the final day of treatment that is on day 30th. The results suggest the hepatoprotective and the renoprotective activity of the chloroform fraction of *A. indica* leaves.

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on fasting blood glucose, oral glucose tolerance, plasma insulin and glycated hemoglobin (HbA1c) of STZ-induced diabetic rats: STZ-induced diabetic rats show abnormally high levels of fasting blood glucose, glycated hemoglobin, low level of plasma insulin and decreased tolerance towards external glucose administration. Such rats were treated with a chloroform fraction of *A. indica* for 30 days and it is evident from Figure 2 A and B that lowering of 13.8% and 31.3% ($p<0.01$) was observed in fasting blood glucose of fraction treated group on days 14th and 28th post treatment. The improvement in oral glucose tolerance was 13.4% on day 14th and 23.2% ($p<0.01$) on 28th day of treatment (Figure 2 C and D). Plasma insulin and HbA1c were measured on day 30th post treatment and it was found that the insulin level was increased by 35.4% ($p<0.01$) (Figure 2 E) and the level of HbA1c was decreased by 33.7% ($p<0.01$) (Figure 2 F).

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on lipid profile of STZ-induced diabetic rats: Table 6 shows the effect of chloroform fraction on plasma lipid profile of treated animals. Plasma lipid level was determined on days 10th and 30th post treatment and lowering of triglycerides, total cholesterol, LDL and increase in HDL were observed at both intervals although the changes in all parameters were not significant on day 10. At the final day of study, i.e., on day 30th, significant decline of 36.1% ($p<0.01$), 21.7% ($p<0.01$) and 40.8% ($p<0.01$) was observed in the plasma level of triglycerides, total cholesterol and LDL respectively.HDL-cholesterol level was found raised by the significant level of 15.0% ($p<0.05$) 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 17.8% ($p<0.05$) on the final day of treatment.

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on hepatic and renal parameters of STZ-induced diabetic rats: Hepatic and renal dysfunctions are more prominent in STZ-induced diabetic rats left untreated for a few weeks and which shows the high level of HbA1c as compared to the diet induced model. Table 7 shows the effect of chloroform fraction on hepatic parameters and renal parameters on day 30th post treatment. Lowering of plasma AST and ALT level was observed to the tune of 43.0% ($p<0.01$) and 24.4% ($p<0.01$) respectively. Similar activity was observed in metformin treated group. It is also evident from the table that the treatment with chloroform fraction lowered the plasma level of renal function markers in the treated group and the lowering were found to be 41.1% ($p<0.01$), 22.7% ($p<0.01$) and 27.0% ($p<0.01$) of urea, uric acid and creatinine respectively on day 30th post treatment. Results shown in Table 7 confirm the hepatoprotective and renoprotective activity of chloroform fraction.

Concentration dependent effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on glucose uptake in L6 cells: It is evident from Figure 3 that the chloroform fraction of *A.indica* increases basal and insulin-stimulated glucose uptake in a concentration dependent manner in L6 cells. An increase of 1.37 fold ($p<0.05$) in basal glucose uptake was registered at the minimum concentration of 10 ug/ml. The maximum increase of 1.66-fold ($p<0.01$) was observed at 20 ug/ml. Effect of chloroform fraction on insulin-induced increase in glucose uptake was also observed. Insulin alone enhanced a significant increase of 1.69 fold ($p<0.01$) in glucose uptake. Myotubes pre-incubated with different concentration chloroform fraction for 16 h with insulin (100 NM) added for final 20 min, resulted in a dose-dependent increase of insulin response in an additive manner that is 1.84-fold, $p<0.01$, 1.91-fold, $p<0.01$ and 2.02-fold, $p<0.01$ at 5 , 10 and 20 μg/ml concentration, respectively vs. control.

Effect of chloroform fraction of of 95% ethanolic extract *A. indica* on mRNA expression of insulin signaling gene in L6 cells: Gene

expression profile of chloroform fraction treated cells is shown in Figure 4. The expressions of IRS-1 (Insulin receptor substrate-1), PIK3CG (phosphatidylinositol 3-kinase, catalytic, alpha), AKT2 (Protein Kinase-B) and GLUT4 gene are upregulated in fraction treated cells. These results suggest that chloroform fraction of *A. indica* stimulates insulin signaling pathway genes which may account for the antihyperglycemic effects of this plant fraction.

Effect of chloroform fraction of 95% ethanolic extract of *A. indica* on IRS-1, AKT and GLUT4 proteins in L6 cells: One of the mechanism which can stimulate or increase glucose uptake in cells is the increased translocation of glucose transporter 4 (GLUT 4) from the intracellular site to the plasma membrane. This could be mediated by insulin signaling pathway as suggested by gene expression profile. Therefore, western blot analysis was performed to study the expression of the protein molecules involved in insulin signaling pathway. Figure 5 shows that the protein expression of p-IRS-1, p-AKT and GLUT-4 were upregulated in chloroform fraction treated cells, which further confirms that the chloroform fraction of *A.indica* increases glucose uptake in treated cells occur through the insulin signaling pathway.

DISCUSSION

A. indica is known for its medicinal properties since ancient time. Every part of this plant found its place in traditional system of medicines [18]. The present study aimed to investigate the effect of *A. indica* leaves on diabetes and its associated complications. In the present study it was seen that among crude powder and various extracts of *A.indica* leaves, only 95% ethanolic extract showed significant improvement of the postprandial blood glucose profile in normal rats and lowering of blood glucose in STZ-induced diabetic rats. Therefore the same extract was subjected to fractionation using various solvents viz., hexane, chloroform, butanol and water in the increasing order of polarity to obtain their respective fractions. From all the fractions only chloroform fraction was found to be capable of lowering the blood glucose level of STZ-induced diabetic rats to the significant extent during 5 hours. On the basis of the blood glucose profile of STZ-diabetic rats post treatment with various fractions, it was justified to conduct the detail study of chloroform fraction on various animal models to investigate the overall effect of the fraction on diabetes associated complications. Therefore, the effect of the chloroform fraction was studied in high-fructose diet fed low dose STZ-induced diabetic rats and only low dose STZ-induced diabetic rats administered with chloroform fraction for one month.

Diet intervention models are frequently used in the study of diabetes as it resembles the characteristics of human type 2 diabetes [19-21]. Therefore the chloroform fraction of *A. indica* was subjected to multiple dose study in HFD-STZ rats for one month. These animals display some common features of untreated diabetes like high level of plasma triglycerides and cholesterol level, glucose intolerance and disturbed liver and kidney functions [22]. Multiple dose treatment on HFD-STZ rats showed marked improvement in oral glucose tolerance lowered fasting blood glucose and revert lipid profile towards normal and also improved liver and kidney functions. The results clearly suggest the potential of the chloroform fraction in the treatment of diabetes and its associated complications.

The complications of diabetes can be more prominently seen in STZ-induced diabetic rats as compared to diet models, and therefore more suitable for the study of diabetic complications related to liver and kidney functions. STZ diabetic rats with high level of HbA1c were administered with chloroform fraction for one month and the fraction treated rats showed increased tolerance towards external glucose administration which was well reflected from the significant decline in HbA1c level. Plasma triglycerides, total cholesterol and LDL-cholesterol were declined and HDL-cholesterol was raised to the significant level, which confirms the outcome observed in HFD-STZ model and thus the antidyslipidemic activity of the chloroform fraction of *A. indica.* It is evident from the decreased level of plasma AST, ALT, urea, uric acid and creatinine level in the present as well as in diet induced model that the chloroform fraction also possesses hepatoprotective and renoprotective activity.

Skeletal muscles serve as the large disposal site for the body glucose. GLUT 4 transporter regulates the glucose transport in skeletal muscle cells [23]. Hence the translocation and redistribution of GLUT 4 protein play vital role in glucose uptake by skeletal muscles. In the present study, the effect of the chloroform fraction was studied in L6 cells and it was evident from the study that it increases glucose uptake in a concentration dependent manner in skeletal muscle cells. Importantly, effect of chloroform fraction was additive to insulin response to stimulate glucose uptake, indicating that involvement of distinct but convergent signalling pathway. To find the effect of the fraction on expression level of GLUT 4, the L6 myotubes were treated with the chloroform fraction

of *A,indica* and as the resultant expression of GLUT 4 significantly increased at both mRNA and protein level. Hence it suggests that the chloroform fraction of *A. indica* increases glucose uptake in L6 myotubes by upregulating the expression of GLUT 4.

Binding of insulin to its receptor, increases the tyrosine phosphorylation of insulin receptor substrate (IRS-1) and subsequent activation of PI-3-kinase and AKT protein, which regulates the process of glucose uptake and GLUT4 translocation in skeletal muscle cells in insulin dependent pathway. In the present study, we found that the chloroform fraction enhanced tyrosine phosphorylation of IRS-1 in L6 myotubes and also increased the mRNA level of the same. Beside this it also increased the expression of PI3K. Apart from this our 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 *A.indica* on glucose uptake is mediated via PI-3-K/AKT pathway.

CONCLUSION

It may be concluded that chloroform fraction of 95% ethanolic extract of *A. indica* leaves is highly effective in the control of diabetes mellitus and its associated complications.

ACKNOWLEDGEMENTS

The authors would like to thanks 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 thank to Director CSIR-CDRI, for show his keen interest and providing necessary facilities for the study. This manuscript bears CSIR-CDRI communication number-8918.

Table 1. Effect on postprandial hyperglycemia in *A. indica* 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	-	62.0± 0.81	124.5± 4.62	121.3± 3.28	121.5± 2.83	123.8± 3.15	13810± 204	-
Crude powder	250	74.8± 3.77	121.0± 2.02	114.6± 1.56	119.0± 2.60	113.4± 3.29	13460± 129	2.53
95% Eth. Ext.	250	62.6± 0.80	106.1± 4.34	105.8± 3.46	102.6± 2.23	104.0± 3.61	11940± 339	**13.5***
50% Eth. Ext.	250	63.6± 0.76	110.5± .99	112.3± 1.92	116.3± 2.33	110.8± 2.00	12800± 254	7.31
Aqu. Ext.	250	62.3± 0.95	106.0± 3.08	115.3± 0.66	114.5± 1.78	111.6± 2.13	12690± 98.0	8.11
Metformin	100	63.5± 1.56	99.6± 4.66	81.0± 2.29	64.5± 1.40	64.6± 2.49	9278± 168	**32.8****

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

Table 2. Blood gluocose profile of *A. indica* 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	-	138900±2861	647300±17635	--	--
Crude powder	250	119592±3194	592926±11674	13.9	8.40
95% Eth. Ext.	250	104700±1364	585700±21849	**24.6****	9.51
50% Eth. Ext.	250	122300±3263	604900±15387	11.9	6.55
Aqu. Ext.	250	106100±2418	584700±21819	**23.6****	9.67
Metformin	100	92930±1945	432500±15345	**33.0****	**33.1****

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

Table 3. Blood glucose profile of STZ-induced diabetic rats treated with various fractions of 95% ethanolic extract of *A. indica* leaves 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	113300±4795	610600±15470	12.1	3.78
Chloroform fraction	100	98880±5483	548600±26830	**23.3****	13.5
Butanol fraction	100	115900±7262	562900±33460	10.1	11.3
Aqueous fraction	100	112600±1938	588100±15290	12.7	7.32
Metformin	100	92580 ± 348.6	432200 ± 8127	**28.2****	**31.8****

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

Table 4. Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves and standard drug metformin on lipid profile of high fructose diet fed low dosed STZ-induced diabetic rats

Group	Day	Triglycerides (mg/dl)	Cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Control	10^{th}	269.1±10.8	195.5±14.2	106.6±15.4	20.3±1.81
	30^{th}	299.6±6.88	217.2±12.1	124.2±12.7	19.8±1.09
Chloroform fraction	10^{th}	217.0±11.5 **(19.3*)**	151.7±8.29 **(22.4**)**	76.7±9.69 **(28.0**)**	26.5±1.23 **(30.5**)**
	30^{th}	188.3±4.29 **(37.1**)**	128.2±5.82 **(40.9**)**	63.4±7.02 **(48.9**)**	30.5±1.08 **(54.0**)**
Metformin (100 mg/kg)	10^{th}	241.8±8.84 (10.1)	189.4±8.63 (3.12)	98.4±1.63 **(**7.69)	22.4±1.14 (10.3)
	30^{th}	256.3±4.86 (14.2)	203.0±8.36 (6.53)	105.4±5.64 (15.1)	21.8±1.29 **(**10.1)

Values are mean ± SE; Significance *p<0.05, **p<0.01

Table 5. Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on hepatic and renal parameters of high fructose diet fed low dosed STZ-induced diabetic rats at 30^{th} 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	8.16±0.34	36.1±1.65	91.4±3.46	9.53±0.41	0.659±0.064
Chloroform fraction (100 mg/kg)	5.07±0.66 **(37.8**)**	23.0±1.07 **(36.2**)**	53.5±3.04 **(41.4**)**	5.79±0.28 **(39.2**)**	0.504±0.044 **(23.5**)**
Metformin (100 mg/kg)	5.29±0.59 **(35.1**)**	19.9±1.36 **(44.8**)**	42.6±2.06 **(53.3**)**	4.91±0.36 **(48.4**)**	0.446±0.049 **(32.3**)**

Values are mean ± SE; Significance **p<0.01

6. Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves and standard drug metformin on lipid profile of STZ-induced diabetic rats

Group	Day	Triglycerides (mg/dl)	Cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Control	10^{th}	177.2±3.46	132.7±2.96	49.8±3.24	47.8±1.18
	30^{th}	182.6±6.37	137.4±2.68	58.2±3.11	54.3±2.11
Chloroform fraction	10^{th}	137.0±2.88 **(22.7**)**	121.7±3.99 (8.29)	44.2±2.19 (11.4)	44.6±2.51 (6.86)
	30^{th}	116.7±1.97 **(36.1**)**	107.6±3.59 **(21.7**)**	34.5±3.72 **(40.8**)**	46.2±2.63 **(15.0*)**
Metformin (100 mg/kg)	10^{th}	169.0±2.36 (4.63)	130.0±3.18 (1.98)	45.8±3.98 (7.98)	45.5±3.11 (4.81)
	30^{th}	163.6±2.64 (10.4)	128.6±2.84 (6.34)	47.8±4.41 **(17.8*)**	49.4±3.26 (8.96)

Values are mean ± SE; Significance *p<0.05, **p<0.01

Table 7. Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves 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)	UricAcid (mg/dl)	Creatinine(mg/dl)
STZ Control	125.6±3.42	95.1±1.88	115.3±3.991	7.473±0.219	1.055±0.0154
Chloroform fraction (100 mg/kg)	71.6±2.60 **(43.0**)**	71.9±2.62 **(24.4**)**	67.9±2.966 **(41.1**)**	5.777±0.370 **(22.7**)**	0.770±0.0319 **(27.0**)**
Metformin (100 mg/kg)	73.5±2.19 **(41.5**)**	60.9±2.63 **(35.9**)**	64.7±1.68 **(43.8**)**	5.35±0.196 **(28.4**)**	0.719±0.020 **(31.8**)**

Values are mean ± SE; Significance **p<0.01

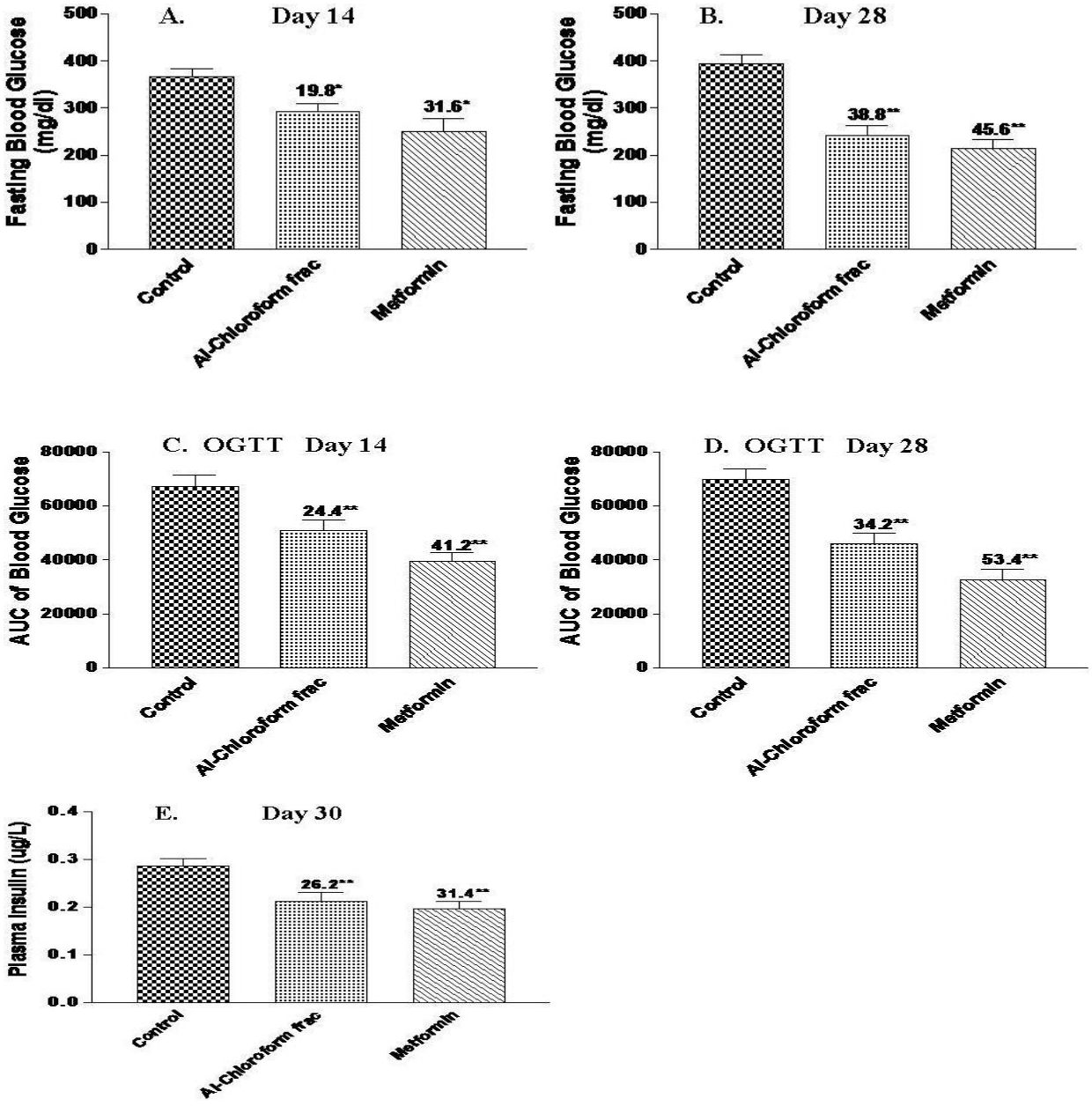

Figure **1. Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on fasting blood glucose (A and B), oral glucose tolerance (C and D), Plasma insulin level (E) of high fructose diet fed low dosed STZ-induced diabetic rats during 30 days of treatment. Significance *p<0.05, **p<0.01**

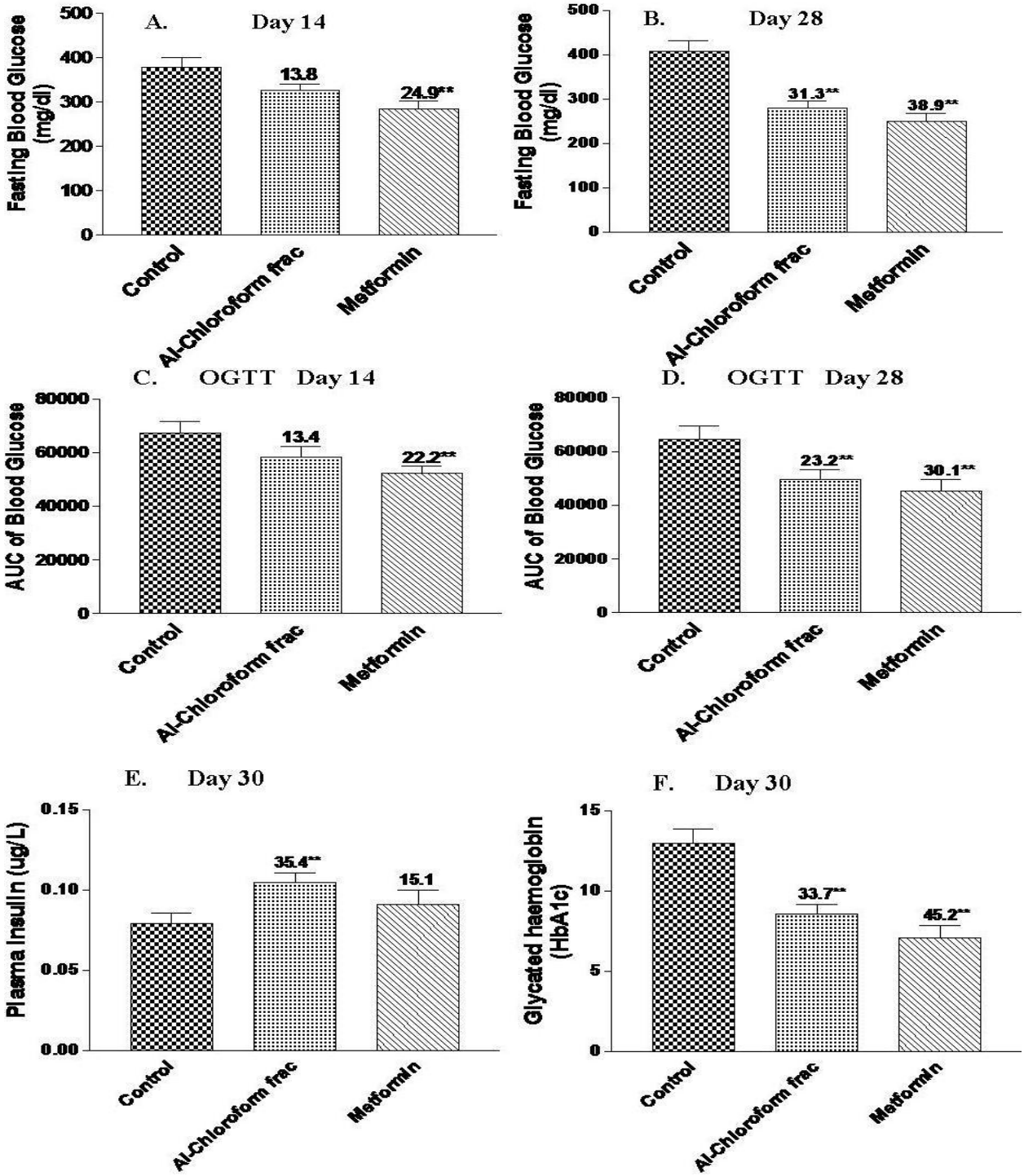

***Figure* 2. Effect of chloroform fraction of 95% ethanolic extract of *A. indica* leaves on fasting blood glucose (A and B), oral glucose tolerance (C and D), Plasma insulin (E) and HbA1c level (F) of STZ-induced diabetic rats during 30 days of treatment. Significance **p<0.01**

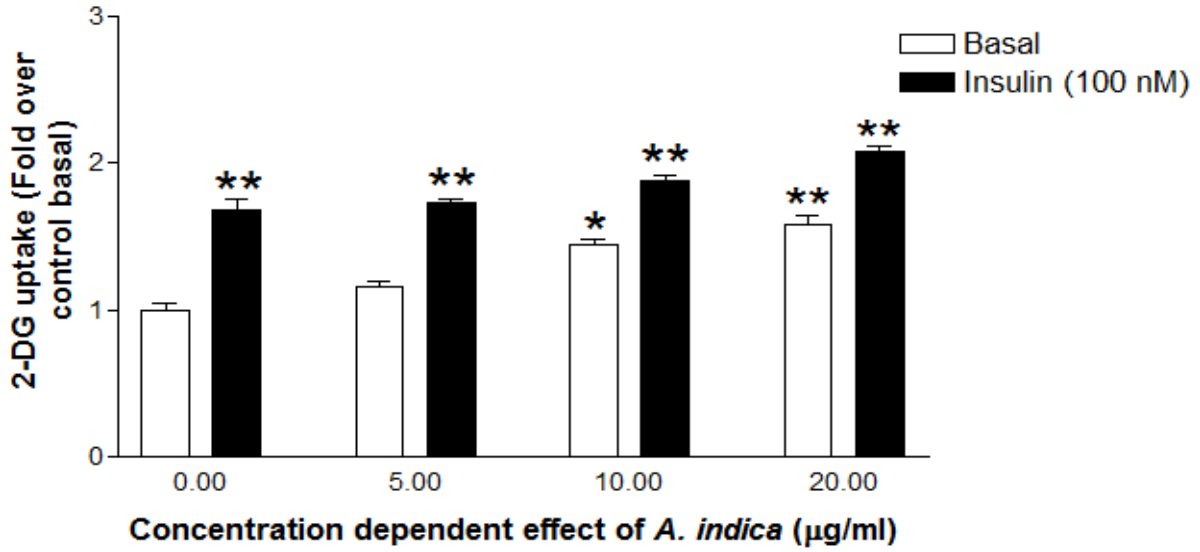

***Figure* 3: Concentration-dependent effect of the chloroform fraction of 95% ethanolic extract of *A. indica* leaves on 2-deoxyglucose uptake in L6 myotubes. Cells were incubated for 16 h with different concentrations of *A. indica* . After incubation myotubes were left untreated (white bars) or stimulated 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**

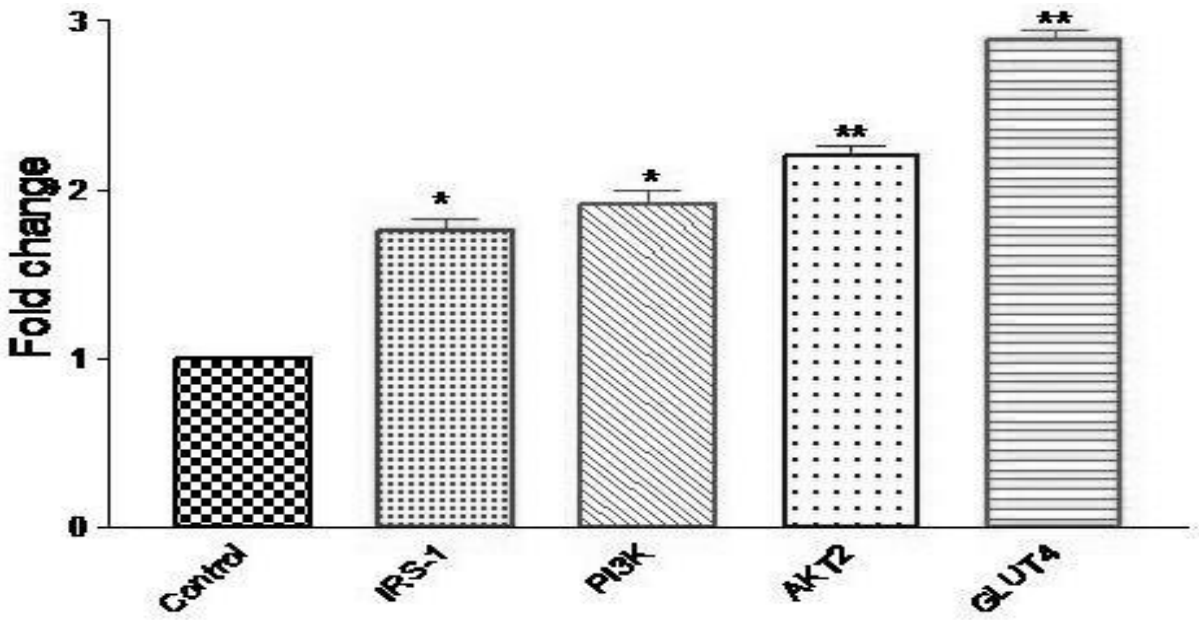

***Figure* 4. Effect of the chloroform fraction of 95% ethanolic extract of *A. indica* leaves on the expression of IRS-1, PI-3Kinase, AKT2 and GLUT4 genes in L6 myotubes. L6 myotubes were treated with 20 µg/ml concentrations of *A. indica* for 16 h and then subjected to Real Time PCR analysis. Experiments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

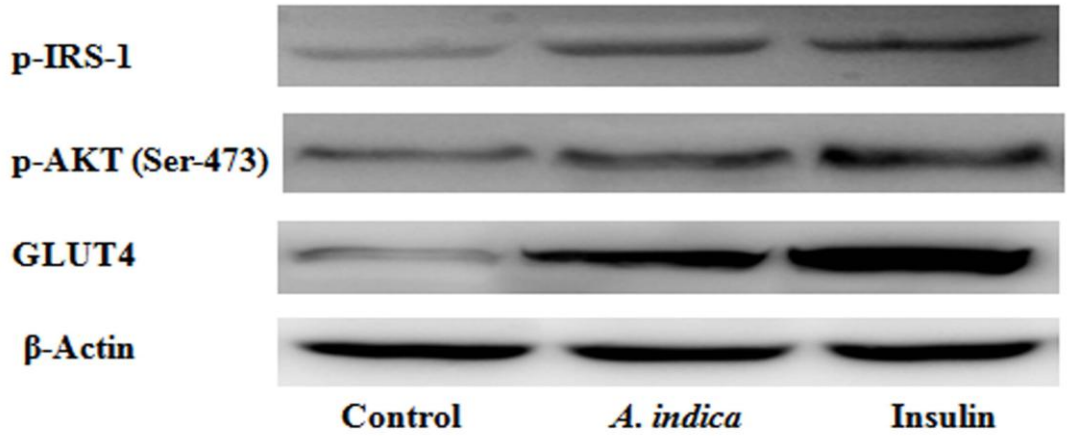

***Figure* 5. Effect of the chloroform fraction of *A. indica* leaves on the expression of IRS-1, AKT2 and GLUT4 protein in L6 myotubes. L6 myotubes were treated with 20 µg/ml concentrations of *A. indica* for 16 h and then subjected to western blot analysis. Experiments are performed in triplicate. Results shown are mean ± SE of three independent experiments.**

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