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CUCURBITACIN AUGMENTS INSULIN SENSITIVITY AND GLUCOSE UPTAKE THROUGH TRANSLOCATION AND ACTIVATION OF GLUT4 IN PI3K/AKT SIGNALING PATHWAY

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ABSTRACT

Vegetables that are generally consumed in India as one of the main components of the diet have been proven for their antidiabetic potential. Cucurbitacin is an important member of the Cucurbitaceae family and exhibit potential effects in various metabolic disorders and cancer. The aim of the present study is to investigate the effect and the mechanism of cucurbitacin on glucose uptake in an insulin target skeletal muscle and activation of PI3k/Akt signaling pathways in STZ induced diabetic animal model. STZ induced diabetic rats showed significantly elevated levels of plasma glucose, TBARS, and lipid hydroperoxides, and decreased levels of plasma insulin and enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non -enzymatic antioxidants (vitamin C, E and GSH), and impairment in insulin signaling proteins including IR, IRS-1/2, Akt, PI3K, GLUT4, and PPAR-γ proteins. The administration of cucurbitacin (5 mg/kg b.w/p.o) showed near-normalized levels of plasma glucose, lipid peroxidation products, enzymatic and non-enzymatic antioxidants and improved insulin signaling proteins. Based on the present findings, cucurbitacin improves glucose uptake by enhancing GLUT4 protein activities in skeletal muscle through PI3K/Akt and antioxidant defense in plasma and it also maintains blood glucose homeostasis.

KEYWORDS: cucurbitacin, STZ, insulin signaling protein, GLUT4, diabetes.

Abbreviation

Akt- Protein kinase B/ a serine/threonine-specific protein kinase; ANOVA- One-way analysis of variance; b.w. – body weight; CAT-catalase; EDTA- Ethylene diamine tetra acetic acid; FBG- Fasting blood glucose; GLUT-4- glucose transporter 4; GPx-glutathione peroxidase; GSH- glutathione; IR- insulin receptor; IRS 1, 2- insulin receptor substrate 1,2; PI3K- Phosphatidylinositol-4,5-bisphosphate 3-kinase; PPAR-γ- Peroxisome proliferator-activated receptor gamma; SOD-superoxide dismutase; STZ- streptozotocin; TBARS- Thiobarbituric acid reactive substances.

INTRODUCTION

Diabetes mellitus is a serious metabolic illness worldwide characterized by chronic hyperglycemia resulting from defects in insulin synthesis, distorted insulin action, or both.[1] Insulin resistance also normally designates to insulin deficit, mainly due to β-cell dysfunction, including destruction in the β-cell quantity. The International Diabetes Federation has anticipated that the globally affected individuals with diabetes will elevate from 382 million in 2013 to 592 million by 2035, with 80% of cases taking place in developing and under-developed countries.[2] Insulin has a potent hormone regularly actions in skeletal muscle since it's inducing effect on the metabolism of glucose that relates to diabetes. Upon binding to its insulin receptor, insulin aids glucose uptake via a distinct signaling cascade reaction of proteins, including phosphoinositide 3-kinase (PI3K) and Akt (a serine/threonine-specific protein kinase) signaling system. Akt is normally triggered by phosphorylation at two specific sites by a PI3K dependent protein and induces translocation of the GLUT4, and consequently glucose uptake.[3,4] These GLUT4 translocation and glucose uptakes taking place in the plasma membrane are stimulated by insulin.[5] A complete or relative lacking of this insulin is, as in case, direct to severe dysfunction and distorted insulin signaling pathway leading to a concomitant rises of glucose in the blood and thereby cause diabetes.[6] The study associated with bioactive compounds that induce exocytosis and/or diminish the endocytosis in plasma membrane enhanced GLUT4 expression and augment absorption of glucose.[7] Several naturally occurring triterpenoids have been demonstrated to enhance glucose uptake and stimulate insulin receptor function, which play a vital effect in diabetes.[1] Thus, it has proved that triterpenoids may possibly exert their glucose-lowering effect through insulin cascade signaling.

Cucurbitaceae is a family of about 110 genera and 640 species found abundantly in the tropics and subtropics. Its members are mostly herbs, climbing by tendrils with abundant sap and a very rapid growth. The main genera include *Cucurbita* (five species), *Cucumis* (25 species), *Citrullus* (three species), *Luffa* (six species), *Bryonia* (four species), and *Momordica* (45 species). The blood glucose-lowering efficacy of a few members of *Cucurbitaceae* family has been assessed by many researchers worldwide.[8-10] Pointed gourd (*Trichosanthes dioica* Roxb (L.)) is known by the name of *parwal, palwal, parmal, patol*, and *potala* in different parts of India and Bangladesh and is one of the important vegetables.[11] The fruits and leaves are the edible parts of the plant which are cooked in various ways, either alone or in combination with other vegetables or meats.[12] The study revealed that the fruit extracts of *T.dioica* produce a significant amount of tetra and pentacyclic triterpenes, the toxic bitter principles cucurbitacins, a group of often highly oxygenated tetracyclic compounds.[12] The aqueous fruit extract of *T.dioica* has documented as a potential of hypoglycemic,[13] antioxidant,[14] and cholesterol lowering activities.[15] Further, the studies indicated that cucurbitacins has cytotoxic effects on various cancer cells, including bladder, pancreas, liver, mammary gland and blood.[16-18] In addition, the study further reported that cucurbitacins inhibit JAK-STAT signaling in various cancer cell lines.[19,20] Furthermore, the recent study showed inhibitory role of cucurbitacin on adipocytes *in vitro*.[21] However, the role of cucurbitacins in diabetes and related metabolic complications in rodent study has not been investigated yet. Therefore, the purposes of this study were to evaluate the effects of cucurbitacin on plasma glucose and insulin signaling cascade proteins and to explore the possible related mechanisms using an STZ-induced diabetic rat model.

MATERIALS AND METHODS

Chemicals and antibodies

Cucurbitacin and STZ were obtained from Sigma-Aldrich. (St. Louis, MO, USA). SOD, CAT, GPx kits were purchased from Millipore (Billerica, MA, U.S.A.). IR, IRS-1-2, PI3K, Akt, GLUT 4, PPAR-γ and β-actin primary antibodies were acquired from Santa Cruz Biotechnology, California, USA. Anti-rabbit secondary antibody was procured from GeNei, Bangalore, India. All other chemicals and solvents were of analytical grade obtained from local suppliers (India).

Animals

Six-week-old adult male Wistar albino rats weighing 180–200g were maintained at animal house of our department. They were maintained in environmentally controlled conditions (temperature of 22 ± 2^{0}C, a relative humidity of 65 ± 5%, and 12/12h light /dark cycle) for 7days. The animal shad free access to commercial standard pellet diet before start the experiment and water was provided *ad libitum* throughout the period of the experiment. The animal facilities and all experimental protocols were approved by the Institutional Animal Ethics Committee following the principles and guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), India.

Induction of experimental diabetes

After acclimatization for 7days, experimental rats excluding the normal control and normal treated groups were given free access to standardized high-fat diet for 2 weeks prior to single intraperitoneal injection with STZ (40mg/kg, dissolved in 0.1 M citrate buffer, pH 4.5). Citrate buffer (carrier) alone was injected to normal control and normal treated rats. STZ-injected animals were administered glucose solution (20%) to block existing drug-induced hypoglycemia. Fasting blood glucose (FBG) level was measured 5 days after the STZ induction.[22] The rats with an FBG level above 240 mg/dl were considered diabetic and grouped further for clinical study. The treatment was started on the subsequent day and this was considered as 1st day of treatment.

Experimental design

The experimental animals were randomly divided into five groups of six animals each. Cucurbitacin were dissolved in 5% dimethyl sulfoxide and glibenclamide was diluted in water and administered orally to experimental groups using intragastric tube daily for a period of 45 days. Group I as normal control rats; Group II as normal with cucurbitacin (5mg/kg b.w.); Group III as diabetic control rats; Group IV as diabetic with cucurbitacin (5mg/kg b.w.); Group V as diabetic with glibenclamide (600µg/kg b.w.).

After 45 days of treatment, the animals were anesthetized using ketamine by sacrificed cervical decapitation. Blood samples were collected in tubes containing EDTA. The plasma was obtained after centrifugation (3000rpm for 10 min at 4^{0}C) and used for various biochemical estimations. After plasma separation, the buffy coat containing leucocyte was discarded and the existing RBC was washed thrice with normal saline. A sufficient volume of red blood cells was lysed with phosphate buffer at pH 7.4. These hemolysate was separated

by centrifugation at 5000rpm for 10 min, and the supernatant was used for the quantification of enzymatic antioxidants. Skeletal muscle tissue was excised immediately after the decapitation of the animals and used to carry out immunoblotting.

Biochemical analysis

Plasma glucose was estimated using a commercial kit by the method followed by Trinder.[22] Plasma insulin was assayed by ELISA kits by the method of Bürgi et al.[23] Plasma lipid peroxidation was estimated spectrophotometrically by measuring TBARS and lipid hydroperoxides by the method of Fraga et al.[24] and Jiang et al.[25] respectively. SOD and CAT were estimated by the methods of Kakkar et al.[26] and Sinha[27] respectively. GSH and GPx were determined by the method of Ellman[28] and Rotruck et al.[29] respectively. Plasma vitamin E and C were assayed by the method of Baker et al.[30] and Omaye et al.[31] respectively.

Immunoblotting

Immunoblotting was performed to analyze the expression pattern of insulin signaling proteins, GLUT-4, PPAR-γ by Laemmli.[32] The skeletal muscle tissue sample was homogenized with ice-cold RIPA lysis buffer and protein inhibitor cocktail (10μg/ml). The cells were then centrifuged at 20,000 g for 15 min at 4°C. The supernatants were used as total protein extracts. The total and nuclear protein contents were determined by the Bio-Rad protein kit with BSA as the standard. The lysate containing 20μg of protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Separated proteins were transferred electrophoretically to PVDF membrane, blocked with 3% BSA solution for 1 h, and then incubated with specific primary antibodies (diluted 1:1000) overnight at 4°C. The membranes were washed with TBST thrice for 15 min and the blots were incubated with goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase-conjugated (HRP-conjugated) secondary antibody (diluted 1:1000) in blocking solution for 1 h at room temperature. Then, the membranes were washed with TBST thrice for 15 min. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Tokyo, Japan). Band densities were determined by an image analyzer (Multi Gauge V3.1, FUJIFILM Corporation, Valhalla, NY, USA) and normalized to β-actin for total protein and nuclear protein. Bands were scanned using a scanner and quantitative by Image J, a public domain Java image processing software.

Statistical analysis

All the experimental data were expressed as the mean±SD. Statistical analysis was performed by using SPSS 22 software. One-way analysis of variance (ANOVA) was performed. Statistically significant differences between the groups were determined by Dunnett's post-hoc test. The differences were considered significant at $p<0.05$.

RESULTS

As shown in Figure 1 and 2, there was significantly elevated ($p<0.05$) plasma glucose and decrease insulin levels in STZ induced diabetic rats compared with normal control rats. Oral administration of cucurbitacin (5mg/kg b.w) and glibenclamide (600µg/kg b.w) significantly ($p<0.05$) inhibited the plasma glucose and insulin levels when compared with diabetic rats. However, there were no changes in the plasma glucose and insulin levels when treated with cucurbitacin (5mg/kg b.w) alone in normal control groups. Figures 3-10 showed the levels of TBARS, lipid hydroperoxides, enzymatic antioxidants (SOD, CAT, GPx) and non-enzymatic antioxidants (vitamin C, E, and GSH) in normal and experimental rats. Cucurbitacin (5mg/kg b.w) administration significantly ($p<0.05$) reduced the TBARS and hydroperoxide when compared with STZ treated diabetic rats (Figures 3, 4). The enzymatic (Figures 5-7) and non-enzymatic antioxidants (Figures 8-10) were also significantly ($p<0.05$) reduced in STZ treated rats. However, the Oral administration of cucurbitacin (5mg/kg b.w) and glibenclamide (600µg/kg b.w) significantly ($p<0.05$) enhanced those enzymes activities in STZ induced diabetic rats, which was very close to normal rats. Furthermore, there were no changes in the enzymatic and non-enzymatic antioxidant levels when treated with cucurbitacin (5mg/kg b.w) and normal control groups.

Immunoblotting expressions and densitometric analysis of insulin signaling proteins, including IR, IRS-1 and 2, PI3K, Akt, GLUT-4, and PPAR-γ in skeletal muscles of control and experimental rats were illustrated in Figures 11 and 12. STZ treated diabetic rats significantly ($p<0.05$) showed downstream regulation of insulin signaling proteins compared to normal control rats. Oral administration of cucurbitacin (5mg/kg b.w) significantly ($p<0.05$) showed upstream regulation of insulin signaling proteins when compared with STZ treated diabetic rats. These results were compared with the density ratio of β-actin expression.

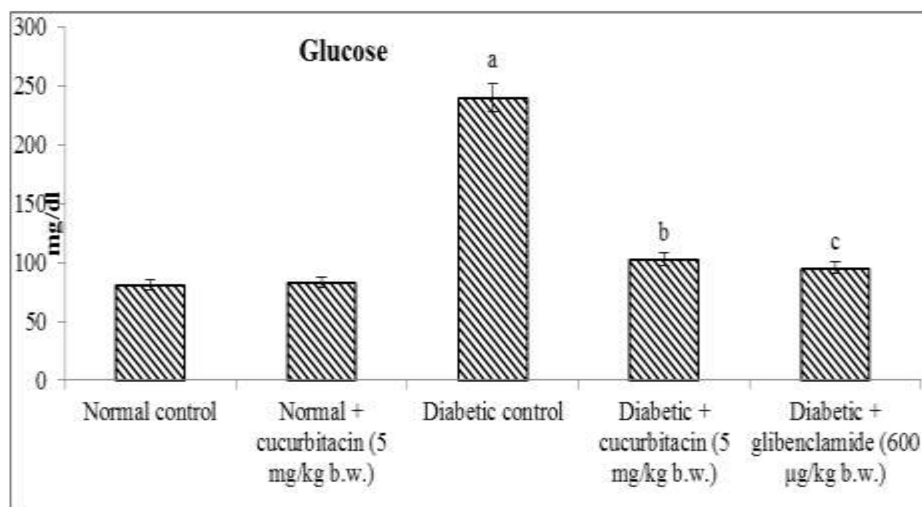


Figure 1: Effect of cucurbitacin on plasma glucose in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

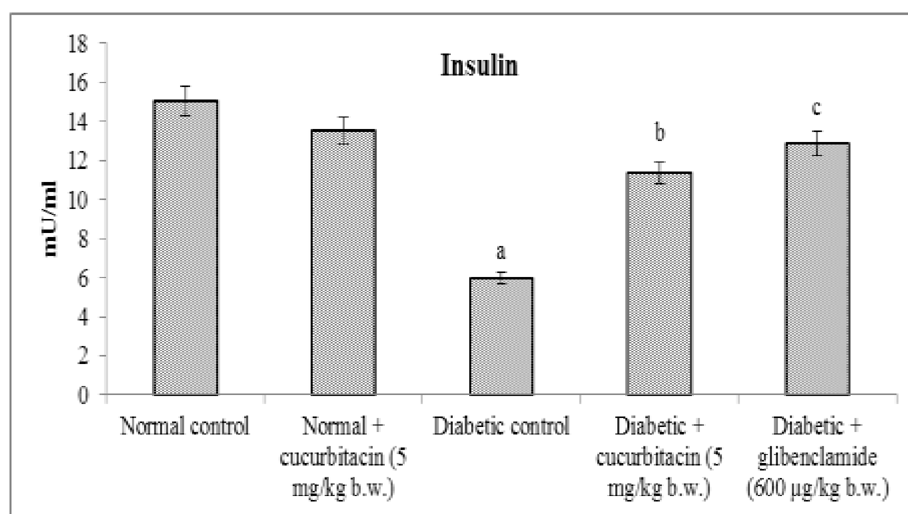


Figure 2: Effect of cucurbitacin on plasma insulin in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

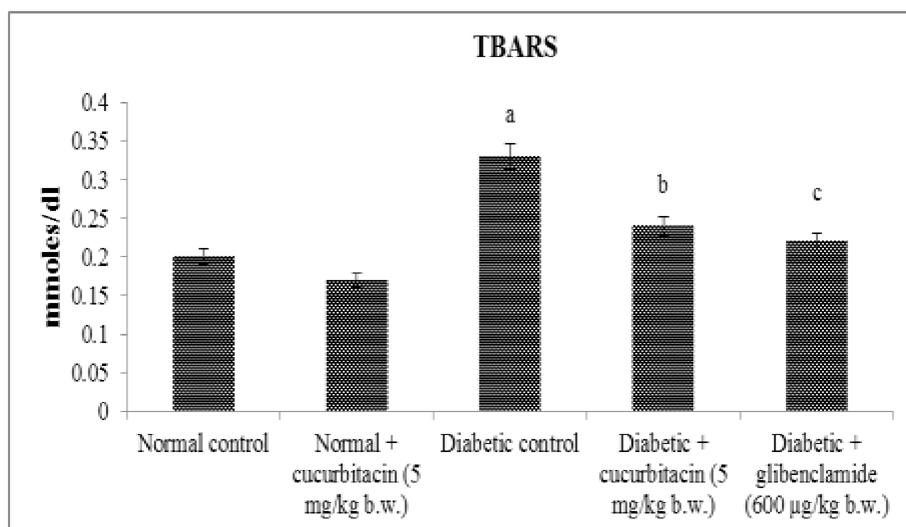


Figure 3: Effect of cucurbitacin on plasma TBARS in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

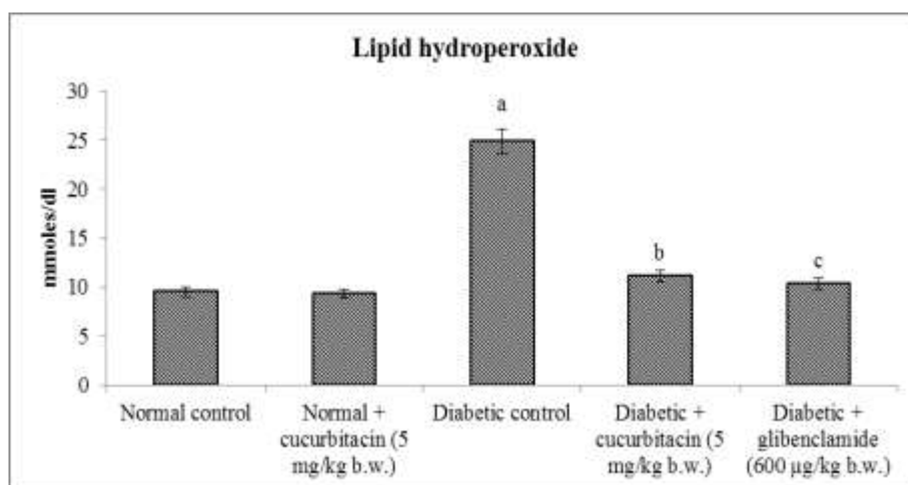


Figure 4: Effect of cucurbitacin on plasma lipid hydroperoxide in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

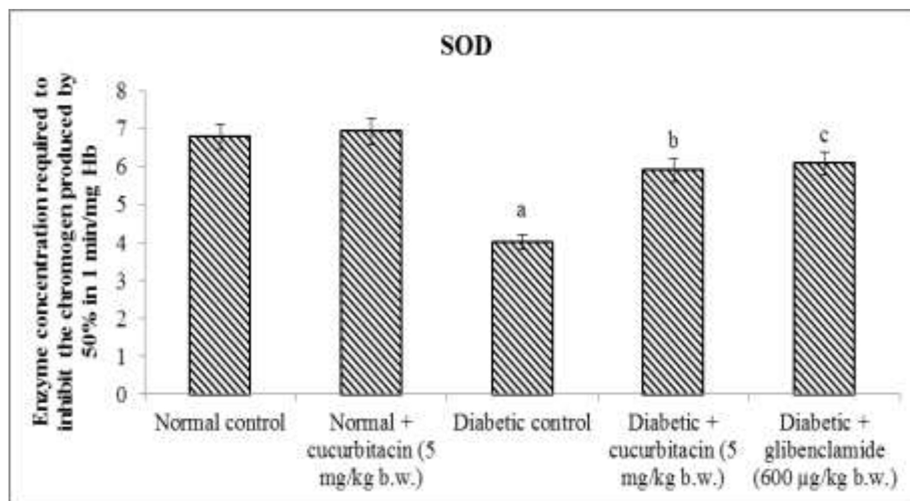


Figure 5: Effect of cucurbitacin on SOD in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

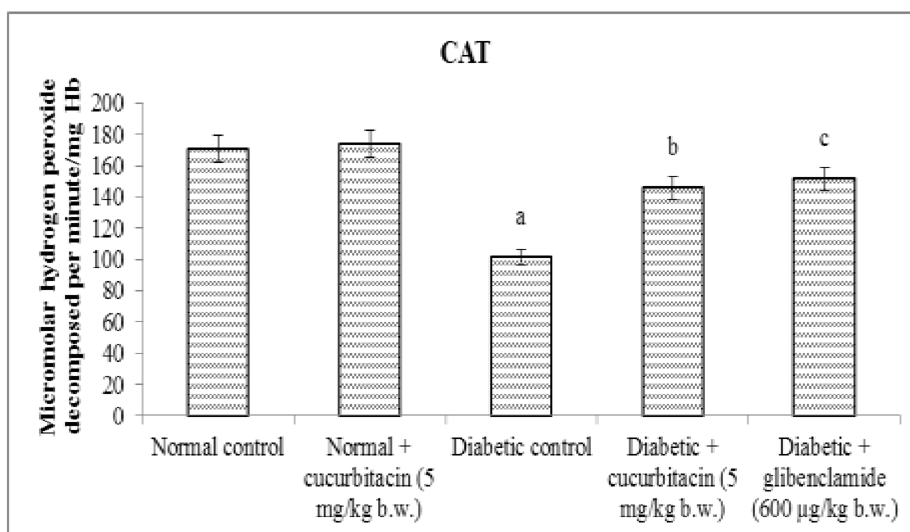


Figure 6: Effect of cucurbitacin on CAT in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

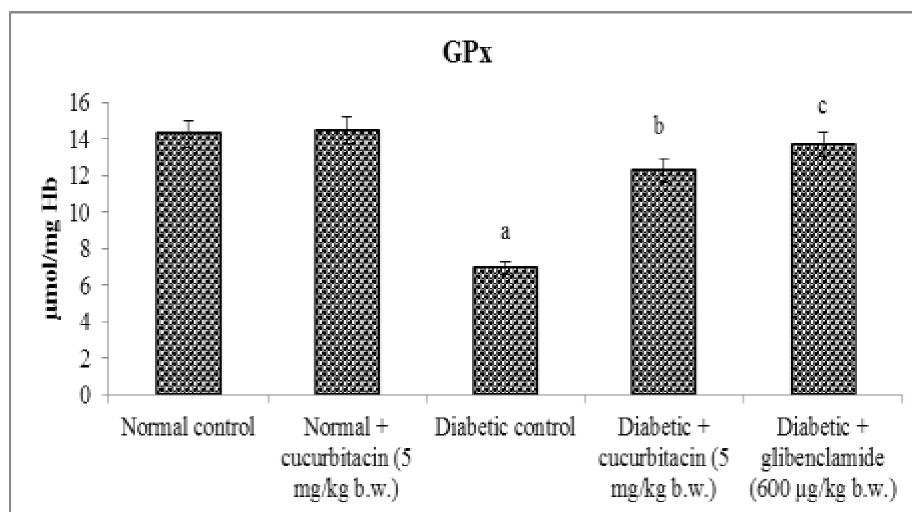


Figure 7: Effect of cucurbitacin on GPx in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

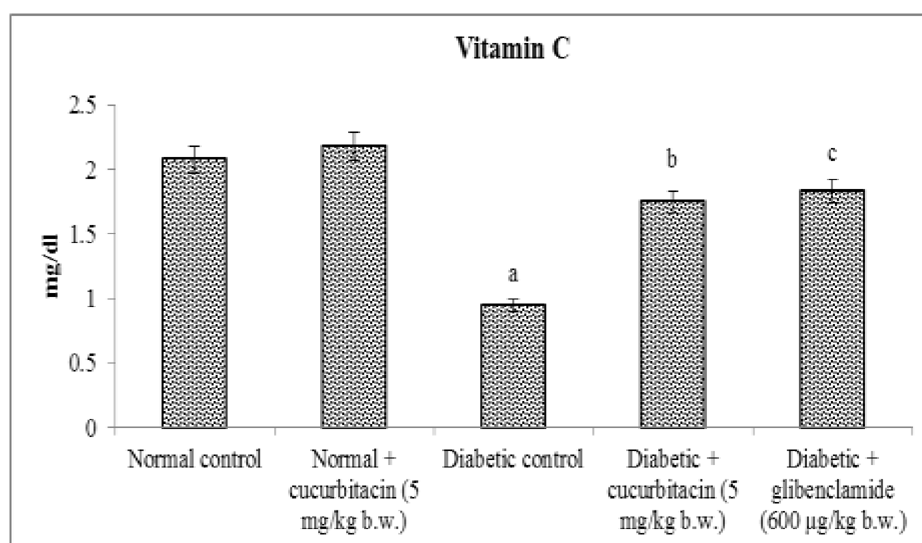


Figure 8: Effect of cucurbitacin on Vitamin C in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

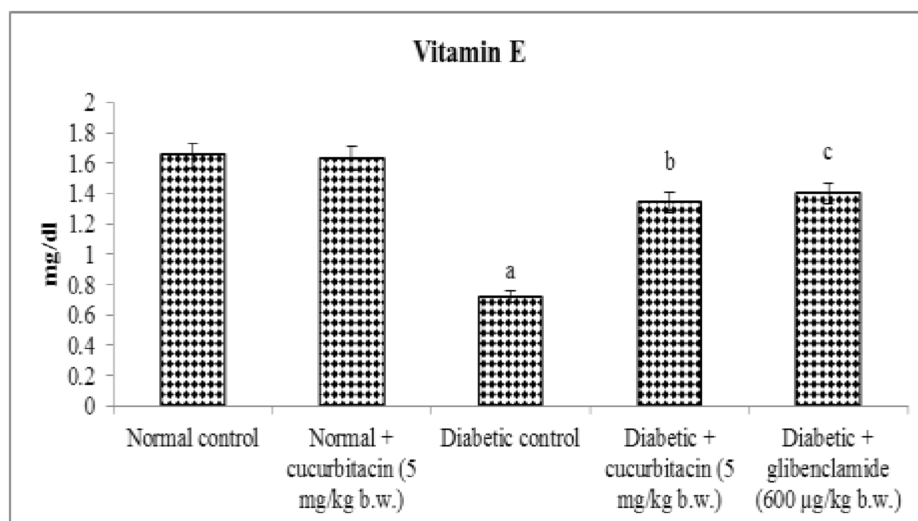


Figure 9: Effect of cucurbitacin on Vitamin E in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

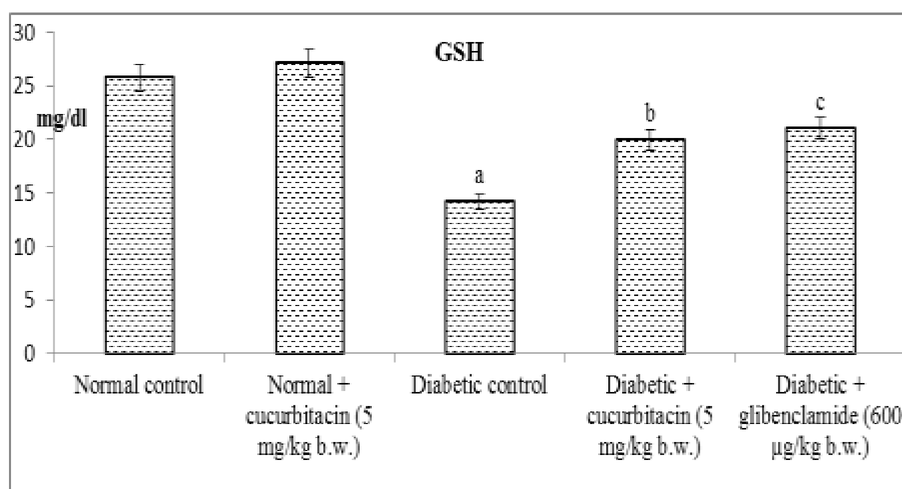
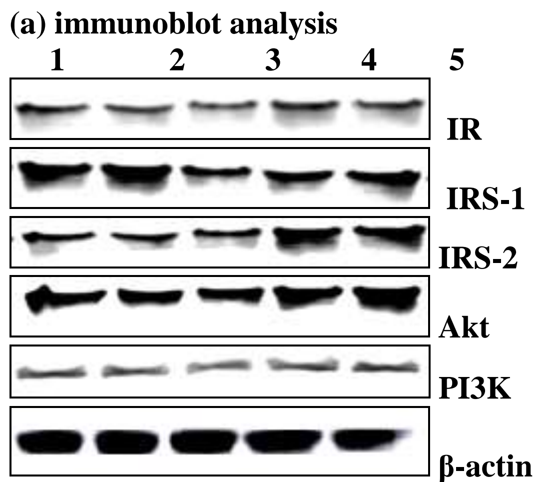


Figure 10: Effect of cucurbitacin on GSH in normal and experimental rats

The values indicate mean ± SD for six animals in each group. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.



(b) Band intensity scanned by densitometer

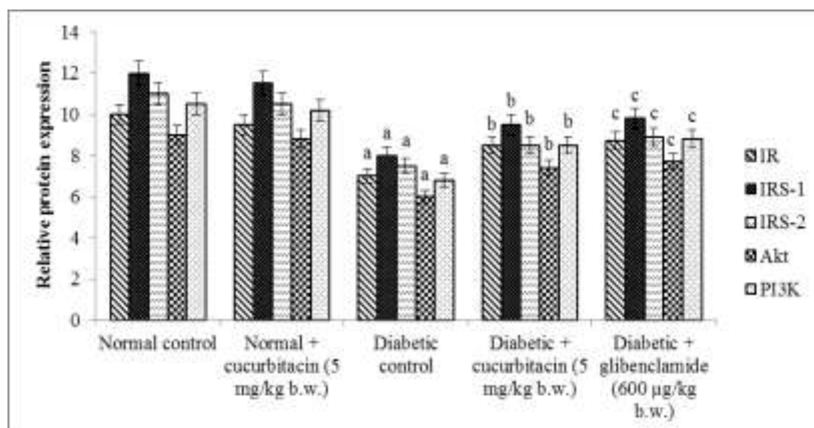
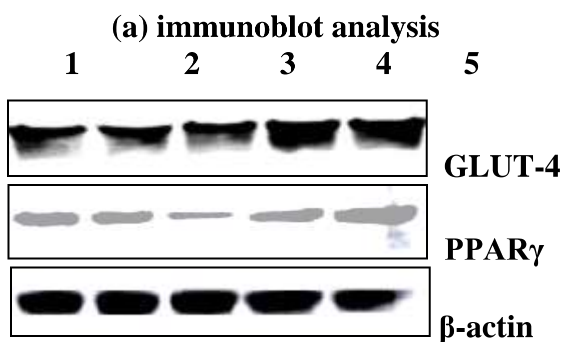


Figure 11: Effect of cucurbitacin on insulin signaling protein (IR, IRS-1, IRS-2 Akt and PI3K) expression in normal and experimental rats

The histogram quantification values indicate mean ± SD of six independent experiments. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.



(b) Band intensity scanned by densitometer

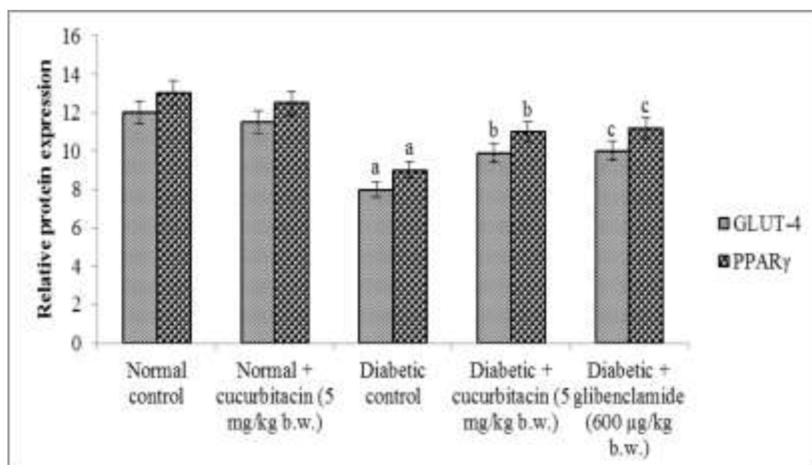


Figure 12: Effect of cucurbitacin on GLUT-4 and PPARγ expression in normal and experimental rats

The histogram quantification values indicate mean ± SD of six independent experiments. One-way ANOVA is followed and statistically significant differences ($p < 0.05$) between the groups were determined by Dunnett's post-hoc test. [a]Diabetic control rats were compared with normal rats; [b]Cucurbitacin-treated diabetic rats were compared with diabetic control rats; [c]Glibenclamide-treated diabetic rats were compared with diabetic control rats.

DISCUSSION

Generally, pancreas β-cells do not articulate the glucose transporters, which are resistant to streptozotocin. Amongst insulin target tissues, particularly skeletal muscle is accounted for greater than 75% of glucose clearance in response to insulin during postprandial condition.[33] The maintenance of glucose in skeletal muscle is thus quantitatively significant in terms of energy balance, glucose uptake, storage, and clearance.[34] The plant-derived bioactive compounds have been shown to prove the favorable effect on glucose transport.[35] In the current study, administration of cucurbitacin showed significant control in postprandial glucose levels along with rapid elimination of blood glucose compared to the STZ induced diabetic rats. These present findings confirmed that cucurbitacin played a crucial responsibility in regulating insulin-dependent glucose clearance and maintaining glucose homeostasis compared with previous studies.[1,10]

It has also been well known that oxidative stress associated lipid peroxidation improved under the diabetic conditions. Diabetes is noticeable by enhanced these reactive oxygen species (ROS) or impairment of endogenous antioxidant defense. During glucose oxidization,

greater quantities of SOD anion radicals produced and convert to high reactive hydroxyl radicals.[36] However, these ROS are managed by endogenous enzymatic and non-enzymatic antioxidants.[37] Oxidative stress associated lipid peroxidation and ROS generations are strongly connected with the diabetic rodent study and clinical models.[38] Cucurbitacin, tri, tetra or Penta-terpenoids have been proven as an exogenous antioxidant and ROS scavenger and thereby inhibit the elevation of lipid peroxidation in diabetic rats.[39] Due to their ROS scavenging potential, antioxidant and non-enzymatic antioxidants are known to be decreased in diabetic conditions.[40] However, administration of cucurbitacin restores all these vitamins to normal range and upregulate the activities of all those antioxidant enzymes.[41,42]

Oxidative stress associated with diabetic complications has been shown to impair insulin sensitivity.[43] Insulin-dependent pathway is a major signaling pathway in the stimulation of glucose uptake in skeletal muscle. It occupies insulin receptor substrate (IRS), PI3K, and protein kinase B (Akt)-mediated signaling activated by the interaction of insulin with its receptor. IRS mediates diverse metabolic and mitogenic responses of insulin.[44] Among the six IRS proteins identified, IRS-1 and IRS-2 are major substrates in the regulation of glucose metabolism.[45] It is identified that, on insulin-mediated signal transduction, IRS-1 is more imperative for Akt activation than IRS-2. Moreover, IRS-1 principally regulates the phosphorylation of Akt, mostly at Ser473 and partly at Thr308, and regulates AS160. Phosphorylation of AS160 of upstream proteins results in GLUT4 storage vesicle fusion with the plasma membrane, resulting in increased glucose uptake. In the present study, administration of cucurbitacin (5mg/kg b.w) has increased the phosphorylation of IRS-1, PI3K, and Akt, the study which is positively correlates with the previous findings.[46]

Based on the phosphorylation of AKT is recognized to arbitrate glucose uptake by facilitating GLUT4 translocation from intracellular storages to the plasma membrane.[47] Among 13 transporter proteins in the human body, glucose transporter 4 (GLUT4) is potentially expressed in skeletal muscle and is actually translocated to the plasma membrane by insulin and other stimuli.[48] GLUT4 is primarily required for basal glucose uptake.[49] Defects in GLUT4 trafficking or protein expression are known to cause insulin resistance.[50] We show that treatment of cucurbitacin increased protein expression of GLUT4 at normal and STZ induced diabetic conditions. Similar positive stimulatory effects on GLUT4 protein expression have also been reported in triterpenoids[35] and pentacyclic triterpenoids.[46] PPARγ plays key roles in the regulation of glucose and lipid metabolism, which is

downregulated in tissues during insulin resistant conditions.[51] An increase of PPARγ expression is detected in the skeletal muscle of cucurbitacin treated diabetic rats, suggesting that it might play critical role in pathophysiology of diabetic skeletal muscle. Our study was positively compared with the previous study associated with cucurbitacin and expression of PPARγ in skeletal muscle and adipose tissue.[18,21]

In conclusion, our present findings suggest that administration of cucurbitacin shows authoritative antioxidant potential and elevated insulin secretion through signal transduction in rats. In addition, cucurbitacin also exerts antihyperglycemic effect by enhancing glucose uptake into skeletal muscle via PI3K-Akt signaling pathway. Thus, these results provide an origin for the use of cucurbitacin since they have vital for the anticipation of diabetes and associated complications.

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