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Research Article

Herbal Anti-Hyperglycemic Compound Increases Expression of Glucose Transporter Molecules in Diabetic Rats

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ABSTRACT

Background: In previous studies, Sharma et al. has already isolated an anti-hyperglycemic compound from the fruit pulp of *Eugenia jambolana* using HPLC and other chromatographic techniques. However, the effect of anti-hyperglycemic compound (FIIc) on the expression of Glucose transporters and Kv 1.3 potassium channel in Streptozotocin-Nicotinamide induced diabetic rats has not been studied so far.

Objective: To study the effect of HPLC purified herbal anti-hyperglycemic compound (FIIc) on the expression of GLUT-4, GLUT-8 and Kv 1.3 potassium channel in Streptozotocin-Nicotinamide induced diabetic rats.

Methods: 24 Male Wistar rats were taken and diabetes was induced in group B, C and D rats (n=6 each) by injecting Streptozotocin at a dose of 45 mg/kg of body weight 15 minutes after the administration of Nicotinamide at a dose of 230 mg/kg of body weight, intraperitoneally to overnight fasted rats. Active compound (FIIc) was orally administered to group C and Pioglitazone to group D at a dose of 20 mg/kg of body weight for 6 weeks respectively. Serum was separated for the estimation of Adiponectin and TNF alpha at week 0 and week 6 of the study. Real time mRNA expression of GLUT-4, GLUT-8 and Kv 1.3 potassium channel was measured and compared between healthy and diabetic control rats. Expression of GLUT-4, GLUT-8 and Kv 1.3 potassium channel was also measured at protein level through Immunohistochemistry and compared between healthy and diabetic controls.

Results: After treatment with FIIc for 6 weeks there was a 1.28 folds increase in GLUT-4 mRNA expression in skeletal muscles and 2.67 folds increase in GLUT-8 mRNA expression in liver tissues of group C rats as compared to group B rats. However, Kv 1.3 potassium channel mRNA expression was found to be at par among the four study groups. TNF alpha levels were found to be significantly decreased in group C rats as compared to group B. A slight increase in serum Adiponectin level was observed in group C as compared to group B, which was found to be statistically insignificant.

Conclusion: FIIc treatment for 6 weeks significantly increases the expression of GLUT-4, GLUT-8 mRNA expression in liver and skeletal muscles leading to increased peripheral insulin sensitivity.

Keywords: *Eugenia Jambolana*; FIIc; Diabetes; GLUT 4; GLUT 8; Kv 1.3 Potassium Channel; Pioglitazone; Wistar rats; HPLC

INTRODUCTION

Mammalian cells utilize glucose for the generation of energy in the form of ATP. With the help of glucose transporter proteins, the blood glucose is taken up by the mammalian cells which are encoded by the SLC2 genes [1,2].

GLUT-4 is the primary glucose transporter responsible for insulin stimulated glucose uptake into muscle and adipose tissues. Studies have reported that during diabetes, the insulin-stimulated glucose uptake by GLUT-4 is largely hampered in muscle and adipose tissue leading to the development of insulin resistance. Studies have also reported that over expression of GLUT-4 in the adipose tissue of GLUT-4 knockout mice ameliorate insulin resistance, diabetes and enhance insulin sensitivity [3-6].

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On the other hand GLUT-8 is an intracellular transporter which is responsible for the transport of various hexoses such as glucose, fructose and galactose inside the cell. It is found to be present in high quantities in testis, brain, spleen, liver, heart, skeletal muscle, and adipose tissue. GLUT-8 has been found to be associated with various intracellular compartments such as rough endoplasmic reticulum, Trans Golgi, late endosomes and lysosomes, potentially catalyzing transport of sugars and sugar derivatives into or out of intracellular organelles [7].

Studies have reported that Potassium channels also play an important role in glucose-stimulated insulin secretion from the pancreatic beta cells [8,9]. Previous studies showed that gene inactivation or pharmacological inhibition of Kv 1.3 channel activity increased peripheral insulin sensitivity by augmenting the amount of GLUT-4 at the plasma membrane [10,11]. Studies have also reported that potassium channel gene knockout mice demonstrated lower body weight, higher insulin sensitivity, and improved glycemic profile [10,11]. Thus, the modulation of K^+ channel is also a therapeutic approach in the treatment of T2DM.

Studies have reported that adiponectin plays a key role in the suppression of metabolic derangements which results in insulin resistance and T2DM [12]. It also demonstrates a negative correlation with body fat mass and has been found in lower levels in obese subjects as compared to non-obese subjects [13]. Adiponectin has also been reported to be inversely correlated with obesity and low adiponectin level predicts the future development of T2DM [14]. Studies have also reported that overexpression or administering recombinant adiponectin reduces blood glucose levels and ameliorates insulin resistance in obese mice, which are independent of plasma insulin levels [15,16]. Conversely, ablation of the adiponectin gene exacerbates insulin resistance and hyperglycemia in mice fed on a high-fat diet [17-19].

On the other hand, Tumour Necrosis Factor-α (TNF-α) is an inflammatory cytokine secreted by adipocytes. Its overproduction has been reported to be associated with insulin resistance by disrupting insulin signaling pathways [20,21]. PPAR-γ agonists down regulate the expression of TNF-α in adipose tissue and improve TNF-α induced desensitization to insulin action [22,23]. Hence these two cytokines affect the glycemic control by affecting insulin signaling by up regulating/down regulating the PPAR-γ activity. In previous studies, Sharma et al, has already isolated the active anti-hyperglycemic compound known as alpha hydroxy succinamic acid (FIIc) (US Patent no. 6,426,826 dated 6th August 2002; Indian Product Patent no. 2,30,753 February 2009) from the fruit pulp of Eugenia Jambolana [24]. Hence this study was designed to estimate the effect of active herbal compound (FIIc) on peripheral insulin sensitivity by measuring the expression levels of GLUT-4, GLUT-8 and Kv 1.3 Potassium channel at mRNA and at protein level in streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Isolation and purification of active anti-hyperglycemic compound (FIIc)

Active anti-hyperglycemic compound was isolated from the fruit pulp of Eugenia Jambolana by applying Ion exchange chromatography technique. Diethyl Amino Ethyl cellulose-52 (DEAE-52) was used as the stationary phase. This technique was described in detail in the previous studies reported by Sharma et al. [24,25]. From 1 kg of E. jambolana fruit, the pulp obtained was about 650 g. From this pulp the yield of lyophilized water extract obtained was about 10 g.

Experimental animals

Male Wistar albino rats (weighing 220-250 g) were procured from Central Animal House of University College of Medical Sciences (UCMS), University of Delhi, India. The animals were housed in standard conditions of temperature (22 ± 2°C) and at 12 hour light-dark cycle. The rats were fed with commercial diet (Hindustan liver Ltd., Mumbai) and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC), UCMS, Delhi, India (UCMS/IAEC/26 granted on 30th December 2009).

Induction of diabetes

To induce diabetes, a freshly prepared solution of streptozotocin (45 mg/kg of b.w. in 0.1 M citrate buffer, pH 4.5) was injected intraperitoneally to overnight fasted rats. Nicotinamide at a dose of 230 mg/kg b.w. was given 15 minutes prior to STZ injection for the development of stable type 2 diabetes mellitus [26]. After 48 hours of STZ administration, fasting blood glucose (FBG) levels were measured for the confirmation of diabetes.

Study design

Male Wistar rats were taken for the present study and divided into following groups (6 rats each group).

- Group A: Healthy control rats administered with normal saline
- Group B: Diabetic control rats administered with normal saline
- Group C: Diabetic treated rats administered with FIIc (20 mg/kg of b.w.)
- Group D: Diabetic treated rats administered with Pioglitazone (20 mg/kg of b.w.)

All the animals were fed on standard chow diet. Purified active compound (FIIc) was given orally to group C at a dose of 20 mg/kg of body weight/day and Pioglitazone was given as a standard drug orally at a dose of 20 mg/kg of body weight/day for 6 weeks to group D respectively for 6 weeks. This is the effective dose of FIIc standardized in previous studies reported by Sharma et al [24,25]. An equal volume of vehicle was given to healthy control group A and diabetic untreated group B at week 0 and at week 6. Blood was drawn after overnight fasting for the estimation of serum Adiponectin and TNF α at week 0 and at week 6 of the study. At the end of the study, animals were sacrificed using CO_2 inhalation and their liver, skeletal muscles and adipose tissues were dissected out, frozen in liquid nitrogen and stored at -80°C for the estimation of gene expression.

RNA isolation and cDNA preparation

Total RNA was isolated from liver, adipose and skeletal muscles with the help of Trizol reagent, (Thermo Scientific, USA), by using modified Chomczynski and Sacchi method [27]. The RNA yield obtained was of high quality (A 280/260 was 1.90 to 1.95). The concentration of RNA sample obtained was between 4000 ng/µl to 5000 ng/µl. For cDNA preparation we have used First strand cDNA synthesis kit (Thermo Scientific, USA) and only 0.1 µg to 0.5 µg of RNA per sample was used. For cDNA amplification we have used Master cycler nexus gradient thermal cycler (Eppendorf

AG, Germany). Cycling programs were 90°C for 2 minutes followed by 25°C for 5 minutes, 45°C for 60 minutes and a final extension of 70°C for 5 minutes. This cycle was repeated for 40 times.

Real time PCR

For the real time PCR amplification we have used Thermo Scientific Maxima SYBR Green (2X) master mix reagent kit and Qiagen QPCR Rotor Gene 2 Plex with HRM real time PCR machine, Australia. The denaturation step was done at 95°C followed by annealing step at 50°C for all the primers and amplification step at 70°C. This cycle was repeated for 35 times. The following primer sequences were used for the real time PCR amplification.

Glut 4 primer sequence,

Forward Primer-5’CTCATGGGCCTAGCCAATG 3’

Reverse Primer-5’GGGCGATTTCTCCCACATAC 3’

Glut 8 primer sequence,

Forward primer-5’TCATGGACAGAGCAGGGCG 3’

Reverse Primer-5’GCCAGCCAGGCCAGCCCCA 3

Kv 1.3 potassium channel primer sequence,

Forward primer-5’AGTATATGGTGATCGAAGAGG 3’,

Reverse Primer-5’AGTGAATATCTTCTTGATGTT 3’:

Rat tubulin primer sequence,

Forward primer-5’TAGCAGAGATCACCAATGCC 3’,

Reverse Primer-5’ GGCAGCAAGCCATGTATTTA 3’

Immuno histochemical studies

The quantitative expression at protein level of GLUT-4 and Kv 1.3 Potassium Channel was studied in skeletal muscles while the expression at protein level of GLUT-8 was studied in liver tissues in all the study groups. Rats were sacrificed at the end of the study by CO_2 inhalation and their Liver and skeletal muscles were dissected out and fixed in 10% buffered formalin. The tissues were then processed for paraffin embedding, 4 µm thin sections on lysinated slides were prepared for immunohistochemical staining by indirect avidin-biotin peroxidase method. Briefly, slides were deparaffinized using xylene, endogenous peroxidase was blocked by using 3% H_2O_2 in methanol for 30 minutes at room temperature. Heat-mediated antigen retrieval was carried out at 95°C in citrate buffer at pH 6 for 25 minutes. After antigen retrieval the slides were washed 3 times with Tris buffer (3%) for 5 minutes and incubated in milk powder in Tris buffer for 20 minutes at room temperature. Primary antibody (Santa Cruz, USA) was added to the slide sections and were incubated overnight at 4°C. Next day immunoreaction sites were visualized by using appropriate biotinylated secondary antibody and tertiary antibody conjugated with avidin-biotin-peroxidase complex. The dilutions used were as follows-(GLUT 4-1:200, GLUT 8-1:50, Kv 1.3 Potassium Channel-1:150). The slides were counter stained with haematoxylin and the peroxidase activity was revealed with a solution of diaminobenzidine (DAB) followed by microscopical examination.

Statistical analysis

The statistical analysis was carried out by using analysis of variance (ANOVA) followed by tukey’s test.

RESULTS

Table 1 depicts no significant difference in baseline serum Adiponectin and TNF-α level among the four study groups. However, after the induction of diabetes, serum adiponectin levels were found to be significantly decreased in diabetic controls as compared to healthy controls at week 6 of the study. After treatment with FIIc, group C treated rats showed increased serum adiponectin level at week 6 of the study as compared to diabetic controls although it is found to be statistically not significant. Similarly, after the treatment with pioglitazone, group D rats also showed increased serum adiponectin level at week 6 of the study as compared to diabetic controls. It is also found to be statistically not significant (Table 1).

At week 6 of the study serum TNF α levels were found to be significantly increased in diabetic control rats as compared to healthy controls. Among the FIIc treated group, serum TNF-α level were significantly decreased as compared to diabetic controls at week 6 of the study. Similarly, serum TNF-α level were also found to be decreased in group D rats treated with Pioglitazone as compared to diabetic controls, however it is also statistically not significant (Table 1).

Table 1: Serum Adiponectin and serum TNF-α level at week 0 and week 6 after treatment with FIIc and Pioglitazone.

Parameters	Time Points	Group A	Group B	Group C	Group D	p Value
Adiponectin (ng/ml)	0 Weeks	65.65 ± 8.42	59.62 ± 9.12	61.59 ± 8.96	60.078 ± 9.12	a=0.2615
						b=0.7137
						c=0.9324
	6 Weeks	64.21 ± 0.90	45.72 ± 7.34[a]	50.22 ± 8.12[b]	52.91 ± 6.82[c]	a=0.0027
						b=0.3387
						c=0.1096
TNF-α (Pg/ml)	0 Weeks	7.35 ± 0.49	8.86 ± 1.50	8.7 ± 0.98	8.81 ± 1.20	a=0.0411
						b=0.8313
						c=0.9504
	6 Weeks	7.31 ± 0.33	19.43 ± 2.86[a]	16.7 ± 2.84[b]	16.98 ± 2.87[c]	a=0.0001
						b=0.0128
						c=0.1694

Values are mean ± S.D. (n=6). a=group A *vs*. Group B, b=Group B *vs*. Group C, c=Group B *vs*. Group D.

As depicted from Figure 1, a 1.28 and 1.98 folds increase in GLUT-4 mRNA expression levels was observed in group C and D as compared to group B in the skeletal muscles of diabetic rats after treatment with FIIc and Pioglitazone respectively, at week 6 of the study.

As seen from Figure 2, a 2.67 and 1.23 folds increase in GLUT-8 mRNA expression levels was observed in group C and D as compared to group B in the liver tissues of diabetic rats after treatment with FIIc and Pioglitazone respectively, at week 6 of the study.

As depicted from Figure 3, the mRNA expression levels of Kv 1.3 potassium channel in the skeletal muscles of diabetic rats was found to be at par in all the four study groups after treatment with FIIc and Pioglitazone respectively, at week 6 of the study.

In Figure 4, after the induction of diabetes, the GLUT-4 protein expression was found to be decreased in the skeletal muscles of group B rats as compared to healthy controls at week 6 of the study. However, the GLUT-4 protein expression was found to be increased in the skeletal muscles of group C and D rats as compared to group B rats after treatment with FIIc and Pioglitazone respectively, at week 6 of the study.

In Figure 5, the GLUT-8 protein expression was also found to be decreased in the liver tissues of group B rats as compared to healthy

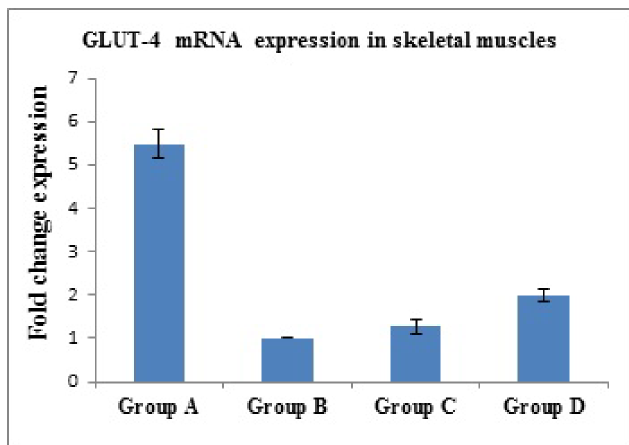

Figure 1: Showing GLUT-4 mRNA expression level in skeletal muscle at week 6 after treatment with FIIc and Pioglitazone in Streptozotocin-Nicotinamide induced diabetic animals.

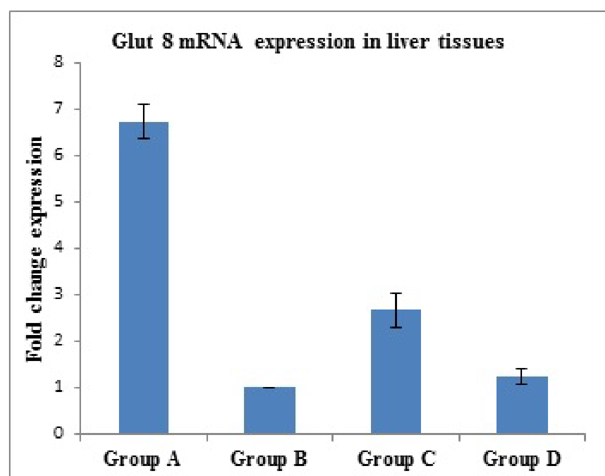

Figure 2: Showing GLUT-8 mRNA expression level in liver tissue at week 6 after treatment with FIIc and Pioglitazone in Streptozotocin-Nicotinamide induced diabetic animals.

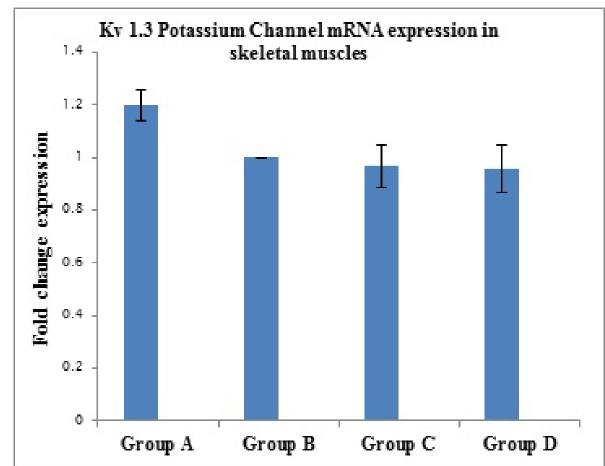

Figure 3: Showing Kv 1.3 Potassium channel mRNA expression level in skeletal muscle at week 6 after treatment with FIIc and Pioglitazone in Streptozotocin-Nicotinamide induced diabetic animals.

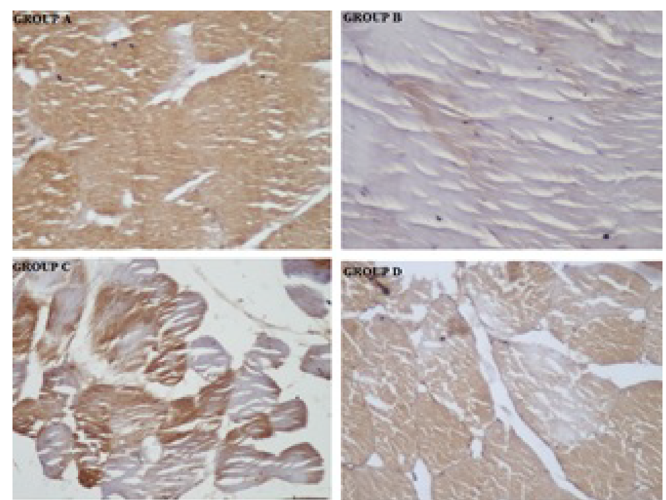

Figure 4: Showing GLUT-4 protein expression in skeletal muscle at week 6 after treatment with FIIc and Pioglitazone in Streptozotocin-Nicotinamide induced diabetic animals. (Manufacturer-Santa Cruz, USA, Dilution -1:200, Magnification-200X).

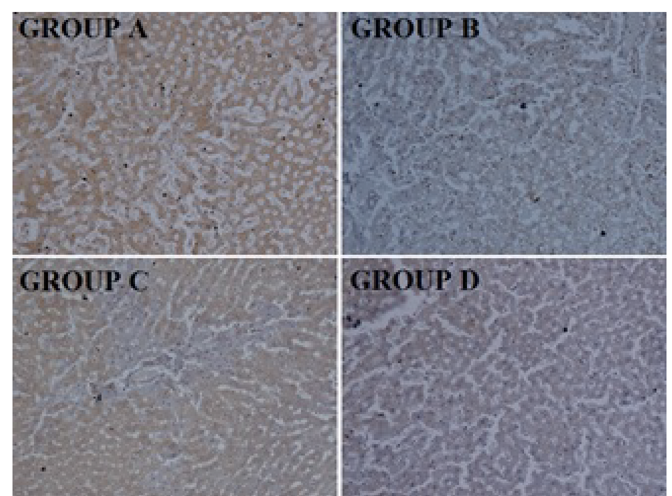

Figure 5: Showing GLUT-8 protein expression in liver tissue at week 6 after treatment with FIIc and Pioglitazone in Streptozotocin-Nicotinamide induced diabetic animals. (Manufacturer-Santa Cruz, USA, Dilution -1:50, Magnification-200X).

controls at week 6 of the study. However, the GLUT-8 protein expression was found to be increased in the liver tissues of group

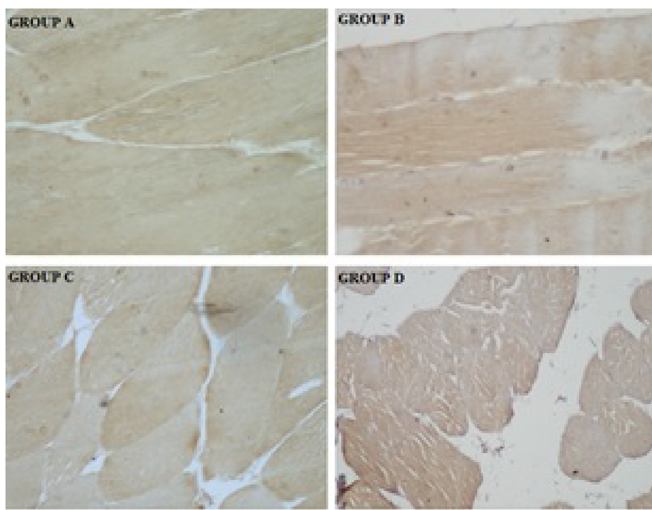

Figure 6: Showing Kv 1.3 Potassium channel protein expression in skeletal muscle of Streptozotocin-Nicotinamide induced diabetic rats after treatment with FIIc and Pioglitazone at week 6 of the study (Manufacturer-Santa Cruz, USA, Dilution -1:150, Magnification-200X).

C and D rats as compared to group B rats after treatment with FIIc and Pioglitazone respectively, at week 6 of the study.

In Figure 6, Kv 1.3 potassium channel protein expression measured in liver tissue was found to be at par among all the study groups after treatment with FIIc and Pioglitazone respectively, at week 6 of the study.

DISCUSSION

This study was designed to estimate the effect of active herbal compound FIIc and pioglitazone on the parameters of peripheral insulin sensitivity such as GLUT-4, GLUT-8 and Kv 1.3 Potassium channel at mRNA and at protein levels in streptozotocin induced diabetic rats.

In this study we observed that after the induction of diabetes, GLUT-4 mRNA expression was found to be decreased in the skeletal muscles of group B, C and D rats as compared to healthy controls at week 6 of the study. However, the GLUT-4 mRNA expression was found to be increased in skeletal muscles by 1.28 and 1.98 fold in group C and group D rats, as compared to diabetic controls after treatment with FIIc and Pioglitazone respectively, at week 6 of the study (Figure 1). Similarly, after the induction of diabetes, the GLUT-8 mRNA expression was also found to be decreased in the liver tissues of group B, C and D rats as compared to healthy controls at week 6 of the study (Figure 2). However GLUT-8 mRNA expression was found to be increased in liver tissues by 2.67 and 1.23 fold in group C and group D rats as compared to diabetic controls after treatment with FIIc and Pioglitazone respectively, at week 6 of the study (Figure 2). No change was observed in the Kv 1.3 Potassium channel mRNA expression level and it was found to be at par in all the study groups after treatment at week 6 of the study (Figure 3).

After conducting immunohistochemistry, the GLUT-4 protein expression was found to be increased in skeletal muscles in group C and group D as compared to group B after treatment with FIIc and Pioglitazone respectively, at week 6 of the study (Figure 4). Similarly, GLUT-8 protein expression in liver tissue was also found to be increased in group C and group D as compared to group B after treatment with FIIc and Pioglitazone respectively, at week 6 of the study (Figure 5). However, the Kv 1.3 potassium channel protein expression was found to be similar in all the study groups at week 6 of the study (Figure 6).

The initial base line serum adiponectin and TNF-α level were found to be in normal range in all the study groups. However, after the induction of diabetes, a significant decrease in serum adiponectin and increase in serum TNF-α level in group B rats was observed, as compared to healthy controls at week 6 of the study. After treatment with active compound FIIc and Pioglitazone in group C and D respectively, an increase in serum adiponectin levels was observed at week 6 of the study as compared to group B. However, the difference is found to be statistically insignificant. Similarly, serum TNF-α level were also found to be significantly decreased in group C and D compared to group B at week 6 of the study (Table 1).

Adipose tissue and skeletal muscle play an important role in the regulation of insulin-mediated glucose homeostasis. It has been reported that glucose transport into skeletal muscles has been impaired in insulin resistance [28,29] through inhibition of GLUT-4 translocation to the cell membrane [30]. Studies have also reported adipose specific GLUT-4 gene knockout results in insulin resistance in muscle and liver [31]. Development of insulin resistance and glucose intolerance has also been observed in muscle specific GLUT-4 gene knockout animal models [32]. Studies have also reported that muscle-specific over expression of GLUT-4 ameliorated insulin action in diabetic mice [33,34], which may be an outcome of increased accumulation of GLUT-4 at the cell membrane [34]. There are various studies which have reported that treatment with herbal drugs resulted in increased glucose transporter levels in diabetic animal models [35-39].

In our study, the possible explanation of increased GLUT-4 expression after treatment with FIIc is attributed through phosphorylation and activation of AMPK which leads to increased expression and translocation of GLUT-4 to the cell membrane in skeletal muscle. As reported by Bao et. al. [40] while studying the effect of Catalpol, an iridoid glycoside isolated from the root of Rehmanniae glutinosa on diabetic mice. He observed that Catalpol ameliorated diabetes, insulin resistance and increased the expression of GLUT-4 in diabetic mice. Studies have reported the importance of AMPK a serine/threonine protein kinase, as a key drug target in diabetes due to its role in modulation of glucose metabolism [41]. In animal model of diabetes the levels of p-AMPK has found to be decreased in the peripheral tissue [42]. After treatment with Catalpol the expression of p-AMPK and GLUT-4 were found to be increased as compared to controls. It is proposed to be mediated via phosphorylation and activation of AMPK which in turn increases the translocation of GLUT-4 from intracellular storage vesicles to cell membrane in skeletal muscle and adipose tissues [43,44]. Similar results have been reported by Choi et. al. [45] and Kumar et. al. [46]. In our study we also observed increased expression of GLUT-4 in the skeletal muscles of FIIc treated rats. Hence we hypothesize that the same mechanism might be operating in FIIc treated rats leading to increased expression of GLUT-4 in skeletal muscles. Pioglitazone treated rats also showed similar results rats at week 6 of the study.

We have also observed increased GLUT-8 expression in the liver tissues of FIIc treated rats as compared to diabetic controls at week 6 of the study. Previous studies have reported increased GLUT-8 expression in the mid piece region and its translocation to acrosomal region after insulin stimulation in spermatozoa

[47]. Studies have also reported reduced GLUT-8 motility and mitochondrial potential of spermatozoa after targeted disruption of GLUT-8 [7]. Studies have also reported that translocation of GLUT-8 from intracellular compartments to the cell membrane takes place in insulin-treated blastocysts [47,48]. It has also been reported that insulin through the activation of PI3K, results in phosphorylation of PKB/Akt, which in turn leads to increased GLUT-8 translocation to the plasma membrane [48]. This results in increased glucose uptake, and increased glycemic control. In our study we have observed increased beta cell mass and insulin levels after treatment with FIIc. Hence, we hypothesize that the increased serum insulin levels might be responsible for increased GLUT-8 expression and translocation in the liver tissues.

Studies have also reported that an edible Chinese medicinal herb known as Radix Astragali were frequently used as a traditional medicine to treat various diseases including diabetes from past many centuries [49-51]. The authors reported that upon treatment with herbal drug a significant elevation of serum adiponectin was observed which was associated with a significant improvement in hyperglycemia, insulin resistance, and glucose intolerance in both dietary and genetic obese mice. Increased serum adiponectin also attenuates TNF-α secretion from macrophages and adipocytes which suggest that adiponectin may work as anti atherogenic agent. Unlike the PPAR-γ agonists, long-term treatment with Chinese herbal drug for 6 weeks does not cause undesirable weight gain [49-51]. Hence in consonance with the previous studies, we have observed increased serum adiponectin and significantly decreased TNF-α levels in group C and D rats treated with FIIc and Pioglitazone respectively, compared to group B at week 6 of the study.

However we have not observed any difference in the expression of Kv 1.3 at mRNA & at protein level in skeletal muscles in the four study groups at week 6 of the study. Hence, we hypothesize that the possible mechanism of action of FIIc is mediated through the up regulation of serum insulin and glucose transporters activity.

CONCLUSIONS

1. After the induction of diabetes, the expression of GLUT-4 and GLUT-8 at mRNA and at protein level was found to be reduced in group B rats as compared to healthy controls at week 6 of the study.
2. However, the expression of GLUT-4 and GLUT-8 was found to be increased at mRNA level in FIIc and Pioglitazone treated rats as compared to diabetic controls at week 6 of the study.
3. Immuno-histochemical studies showed increased GLUT-4 and GLUT-8 expression at protein level in FIIc and Pioglitazone treated rats as compared to diabetic controls at week 6 of the study.
4. No significant difference was observed in the expression of Kv 1.3 potassium channel at mRNA and at protein level after treatment with FIIc and Pioglitazone among the four study groups at week 6 of the study.

A significant decrease in TNF-α level in FIIc treated rats as compared to diabetic controls was observed at week 6 of the study.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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