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A New Herbal Combination and *Trigonella foenum graceum* Improve Insulin Resistance, Insulin Signaling Genes, Adipokines Level and Body Weight in Type 2 Diabetic Rat Model

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ABSTRACT

Trigonella foenum graceum, Cinnamomum cassia, Momordica charantia, Eugenia jambolana, Gymnema sylvestre and *Camellia sinensis* are well known anti-diabetic nutritional herbs. However, inadequate data is available regarding their molecular mechanism of action. In this study, we used a new combination of the aforementioned herbs and *Trigonella foenum graceum* as a separate treatment. We investigated their effect on the blood glucose level, body weight, expression of some of the insulin signaling genes in skeletal muscle and adipose tissue including insulin receptor (INSR), insulin receptor substrate-1 (IRS-1), protein tyrosine phosphatase-1B (PTP-1B), phosphoinositide 3-kinase (PI3-K), protein kinase B (PKB), and phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase C-theta (PKC-θ) genes and on different adipokines level. Wister rats were used to develop type 2 diabetes using high fat diet and streptozotocin and treated with the extracts. Glucose level and body weight was checked throughout the study. The expression of insulin signaling genes was observed in skeletal muscle and adipose tissue by performing RT-PCR. Moreover, the level of adipokines was measured by using ELISA. Data was analyzed by t-test and One-way ANOVA using SPSS. Significant improvement was observed in the blood glucose level and body weight of diabetic rats that received the herbal combination and *Trigonella foenum graceum* extract. The expression levels of several genes including insulin receptor (INSR), insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3-K), protein kinase B (PKB), and phosphoinositide-dependent protein kinase-1 (PDK-1) were significantly increased whereas those of protein tyrosine phosphatase-1B (PTP-1B) and protein kinase C-theta (PKC-θ) were significantly decreased in both skeletal muscle and adipose tissue of the test groups as compared to the diseased control. Moreover, both the extracts also restored the level of serum adiponectin and leptin. Our data suggests that the antidiabetic activity of these herbs may be due to the action on insulin signaling genes and adipokines level along with the normalization of blood glucose and body weight. These herbs may offer a safe and economic strategy and has nutritional significance for the cure and prevention of type 2 diabetes.

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SE performed all the experiments and wrote first draft. AS supervised the work and finalized the manuscript. MAW supervised the study and conceived the idea.

Key words
Type 2 diabetic rat model, Anti-diabetic herbs, Adipose tissue, Nutritional significance, Skeletal muscle, Obesity, Insuline receptor

INTRODUCTION

According to the IDF Diabetes Atlas, Tenth Edition: In 2021, approximately 537 million adults (20-79 years) are living with diabetes. The total number of people living with diabetes is projected to rise to 643 million by 2030 and 783 million by 2045. Genetic factors play a significant role in the occurrence and progression of type 2 diabetes (Prasad and Leif, 2015). During the course of the disease, several molecular defects have been observed such as low expression of insulin receptors, modifications in the insulin signal transduction processes, abnormalities in pathways triggered by the docking of insulin to its receptor, glucose transport and glycogen synthesis (Potdar Chaudhari, 2016). Obesity is the most plausible factor causing the onset of insulin resistance and type 2 diabetes.

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Abbreviations used
HFD, high fat diet; STZ, streptozotocin; IRS-1, insulin receptor substrate; INSR, insulin receptor; PKB, protein kinase B; PI3-K, phosphoinositide 3-kinase; PDK-1, phosphoinositide-dependent protein kinase-1; PTP-1B, protein tyrosine phosphatase-1B; PKC-θ, protein kinase C-θ, Pip2, phosphatidylinositol 4,5-bisphosphate; Pip3, phosphatidylinositol 3,4,5-triphosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

In this scenario, the negative association between skeletal muscle and adipose tissue generates some metabolic abnormalities which results in insulin resistance (Ferrannini *et al*., 2018).

Studies have shown that adipose tissue and its secreted hormones substantially affect the insulin sensitivity. Abnormalities in adipose tissue cause insulin resistance. It is therefore, becoming a potential therapeutic target to improve insulin resistance and type 2 diabetes (Luo and Liu, 2016). Skeletal muscle also plays a central role in glucose homeostasis and is responsible for most of the body's glucose uptake. The occurrence of insulin resistance in skeletal muscle is likely due to the defects in insulin signal transduction pathway (Wu and Ballantyne, 2017).

Insulin receptor (INSR) is responsible for insulin binding and subsequent signaling. When insulin binds to its receptor, it causes conformational changes which provoke the receptor tyrosine kinase activity through self-phosphorylation of certain tyrosine residues. It phosphorylates other proteins including insulin receptor substrate (IRS-1). IRS-1 is the first intermediate in the insulin signal transduction pathway initiated by the insulin binding to its receptor. It acts as a bridge between insulin receptor and molecules present downstream in the cascade. Reduced IRS-1 expression significantly disrupts the downstream process of insulin signaling which ultimately leads to insulin resistance. Phosphoinositide 3-kinase (PI3-K) induces downstream phosphorylation and dephosphorylation events. The impaired activation of PI3-K leads to the defective insulin function in type 2 diabetic patients (Gutierrez-Rodelo *et al*., 2017).

Phosphoinositide-dependent protein kinase-1 (PDK-1) proceeds the downstream signaling of PI3-K. PDK-1 can activate various kinases including protein kinase B (PKB) (Escribano *et al*., 2017). The phosphorylation and activation of PKB is a critical step for the normal insulin sensitivity (Schultze *et al*., 2011). Protein kinase C-theta (PKC-θ) induces the phosphorylation of IRS-1 at serine residue and this serine phosphorylation weakens the potential of IRS-1 to activate PI3-K. It has been shown that PKC-θ activity opposes insulin signaling pathway (Hage-Sleiman *et al*., 2015). Protein tyrosine phosphatase-1B (PTP-1B) is one of the negative regulators of insulin signal transduction pathway (Gutierrez-Rodelo *et al*., 2017). Down regulation of PTP-1B gene improves glucose tolerance and insulin sensitivity (Pereira de Moura *et al*., 2013).

Adipose tissues synthesize numerous signaling components called as adipocytokines including leptin, adiponectin, resistin, visfatin etc. (Waqar *et al*., 2010). The elevated concentration of leptin in the plasma creates the critical situation of leptin resistance. As leptin is coupled with obesity, it is also linked with insulin resistance and type 2 diabetes (Shebla *et al*., 2017). Adiponectin minimizes the amount of triglycerides and fatty acids in the plasma of mice models experiencing obesity and hyperlipidemia. It stimulates the insulin-induced tyrosine phosphorylation of insulin receptor subtrate-1 (IRS-1) and protein kinase-B in skeletal muscles (Lee and Shao, 2012).

Several anti-diabetic herbs have been used since long as part of traditional medicine but basic mechanisms are still undefined. The traditional medicine comprises several herbal and non-herbal constituents and it is hypothesized that they have potential to act on a number of targets through various mechanisms (Choudhury *et al*., 2017). The cocktail comprising of various nutritional components and herbs may have synergistic effects (Dey *et al*., 2002). *Trigonella foenum graceum* is one of the ancient medicinal herbs. It is long been used in Ayurvedic and Chinese medicine. Polyphenols and quercetin are present in *Trigonella* seeds in high concentration (Kiss *et al*., 2018). This herb also contains bioactive saponins, coumarins, alkaloids, and 4-hydroxyisoleucine (Evans, 2010). *Trigonella* seeds are also anti-hyperlipidemic in nature. Although, the hypoglycemic activity of the aqueous extract of *Trigonella* seeds and leaves is well documented, there is no comprehensive study to clarify the cellular and molecular mechanism of actions of these extracts (Kassaee *et al*., 2018).

The use of *Momordica charantia* (bitter melon or bitter gourd) is very common in Asian countries. Its fruit pulp, seeds and leaves have anti-diabetic potential (Habicht *et al*., 2014). *Camellia sinensis* (green tea) is used in many regions of the world and is known to have strong anti-diabetic potential. *Gymnema sylvestre* (Gurmar; meaning sugar destroyer) has long been used in Ayurvedic medicine. It lowers fasting blood glucose level, stimulates insulin secretion and regeneration of pancreas (Tiwari *et al*., 2014). *Cinnamomum cassia* (cinnamon) has been used for a long period of time for number of medical conditions including diabetes. It regulates blood glucose level and its polyphenols show insulin like activity in type 2 diabetic subjects (Aijaz *et al*., 2014). *Eugenia jambolana* (jamun) is also known as *Syzygium cumini* or *Syzygium jambolanum*. It contains phenolic acids, flavonoids with anti-hyperglycemic activity as well as improves insulin secretion (Raza *et al*., 2017).

In the present study, we explored the possible molecular targets of anti-diabetic herbs in the rat model of type 2 diabetes through analysis of the gene expression pattern in adipose tissue and skeletal muscle. Abnormality

in the expression and function of any of these insulin signaling genes triggers the onset of insulin resistance and ultimately type 2 diabetes. The nutritional herbs included in the combination extract are *Trigonella foenum graceum* (TFG), *Cinnamomum cassia, Momordica charantia, Eugenia jambolana, Gymnema sylvestre* and *Camellia sinensis*. It is interesting to elucidate the effect of this new combination of herbs on the expression of some of the critical diabetogenes involved in the insulin signaling pathway. To the best of our knowledge, this is the first such report for the use of this herbal combination and its effect on the insulin signaling genes, adipokines, blood glucose and body weight.

MATERIALS AND METHODS

Administration of high fat diet and determination of insulin resistance

Wistar male rats, weighing 180-200g were housed in the Animal House Facility of the International Center for Chemical and Biological Sciences (ICCBS). Temperature was maintained at 21±1°C and humidity around 57% at 12:12 h standard light and dark cycle. Animals were grouped into normal, test, and diabetic control. The number of animals was six in each group. The diabetic control group was given high fat diet and streptozotocin only and no extract was administrated. The test group 1 was given high fat diet, streptozotocin (STZ) and combination extract. The test group 2 was given high fat diet (HFD), STZ and *Trigonella foenum graceum* (TFG) extract. The normal group did not receive HFD, STZ or any of the used extracts. To prepare HFD, butter was mixed with normal diet ingredients in the ratio of 4:6, respectively (Eijaz *et al*., 2014). HFD was given to two groups for six months. Rats had free access to HFD and water. One group was fed normal diet throughout the experiment and was considered as the normal group (Warden and Fisler, 2008).

These rats were non-diabetic as compared to the rats in the test and diabetic control groups. During the administration of HFD, weights of all the rats were recorded twice a month to observe the effect of HFD on body mass. Before the analysis of insulin resistance, animals were fasted overnight. Weights of all the rats in each group were recorded. To determine the effect of HFD, oral glucose tolerance test (OGTT) was performed by the oral administration of glucose (1g/mL/kg) of both the test and diabetic control groups. Blood samples were collected by venipuncture from the tail. Glucose levels at fasting (0 min) and after the oral glucose administration at 30, 60 and 120 min were recorded with glucometer (Roche).

Administration of STZ and induction of type 2 diabetes

STZ (MP Bio Medicals Inc., Illkirch Cedex, France) was prepared in 0.1 M citrate buffer pH 4.3 (containing citric acid and sodium citrate) and administered intravenously (35mg/kg/mL) to the test and diabetic control groups while rats were in 12 h fasting condition. Normal group was administered citrate buffer only. OGTT was performed to monitor the onset of type 2 diabetes as described in case of insulin resistance.

Treatment of diabetic rats with herbal combination and TFG extracts

TFG (seeds), *Cinnamomum cassia, Momordica charantia* (fruit pulp), *Eugenia jambolana* (fruit pulp), *Gymnema sylvestre* (leaves) and *Camellia sinensis* (leaves) were purchased in dry form from a local herbal market, Karachi, Pakistan. They were washed and dried under sunlight. Ethanolic extracts were prepared by soaking in 80% ethanol (Fisher Scientific, Waltham, USA) for 72 hr. The extracts were filtered and mixed with the normal diet ingredient. In the herbal combination diet, extract of each part was added as 1g. Both, the herbal combination and TFG extracts were administered as 1g/kg/day and continued for one month. Weights of all rats were recorded twice a month. The diabetic control group was not given the herbal combination and TFG extract.

Measurement of blood glucose level and body weight after extracts treatment

The effect of herbal combination and TFG extracts were analyzed on blood glucose level by performing OGTT. Body weights were also recorded before and after treatment.

Analysis of gene expression after extracts treatment in skeletal muscle and adipose tissue by RT-PCR

After the treatment with herbal combination and TFG extracts, rats of all groups were sacrificed and skeletal muscle and adipose tissues were dissected out. After isolation, muscle and adipose tissues were preserved immediately in RNA stabilization reagent (Qiagen, Hilden, Germany) and stored at -20°C until later use. RNA isolation was done with SV Total RNA Isolation System (Promega, Madison, USA) and quantified at 260 nm using spectrophotometer. 0.5µg total RNA was subjected to cDNA synthesis by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, Leon-Rot, Germany). cDNA was amplified by RT-PCR using GoTaq green master mix (Promega, Madison, USA). cDNA samples corresponding to PTP-1B, IRS-1, INSR, PI3-K, PKB, PKC-θ and PDK-1 genes were subjected to denaturation for 5 min at 94°C, followed by 30 cycles

of amplification (denaturation at 94°C, annealing at 50-61°C and extension at 72°C for 1 min each) and a final extension at 72°C for 10 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primers were designed using primer 3 online software (http://frodo.wi.mit.edu/). The primer sequences used as:

INSR (F) 5'GGATGGTCAGTGTGTGGAGA 3'
(R) 5' TCGTGAGGTTGTGCTTGTTC 3',
IRS-1 (F) 5' CACCCAGTTTTTCGACAC 3'
(R) 5' GAGTTGAGCTTCACAAAG 3'
PTP-1B (F) 5' CCACACCATCTCCCAGAAGT 3'
(R) 5' CGGAACAGGTACCGAGATGT 3'
PKB (F) 5'ACCTCATGCTGGACAAGGAC 3'
5' TGAGCTCGAACAGCTTCTCA 3'
PDK-1 (F) 5'CTCACAGAAGGGCCACATTT3'
(R) 5' AGCATCTGGACTGCTCTGGT 3'
PI3-K (F) 5'AGCCACAGGTGAAAATACGG 3'
(R) 5' TTTTCTTTCCGCAACAGCTT 3'
PKC-θ (F) 5'CCGAGAAGGACCAATTGAAA3'
(R) 5' AAACTTCCTTTCCCCAGCAT3'
GAPDH (F) 5'GGAAAGCTGTGGCGTGATGG3'
(R) 5' GTAGGCCATGAGGTCCACCA 3'

The annealing temperatures used were 55°C, 50°C, 55°C, 60°C, 61°C, 58°C, 58°C and 55°C for INSR (563 bp), IRS-1 (600 bp), PTP-1B (174 bp), PKB (246 bp), PDK-1 (228 bp), PI3-K (199 bp), PKC-θ (240 bp), GAPDH (414 bp) genes, respectively (F; Forward, R; Reverse; bp; base pairs).

Each PCR product was electrophoretically resolved on 3% agarose gel. Bands were visualized under UV light in the FluorChem Imaging System (Alpha Innotech, San Leandro, USA). The relative expression ratio of each gene was calibrated with GAPDH and comparison was done between normal, test, and diabetic control groups.

Measurement of serum adiponectin hormone after extracts treatment

The effect of both the extracts on adiponectin hormone was measured by using ELISA technique. The assay was performed by following the steps provided with the rat adiponectin ELISA kit (Abcam, Cambridge, UK, ab108784, UK). The standard stock solution (100ng/mL) was used to make working concentrations as 100ng/mL, 50ng/mL, 25ng/mL, 12.50ng/mL, 6.250ng/mL, 3.125ng/mL, 0ng/mL. 50µL of standard or sample was added per well. Wells were covered with a sealing tape and incubated for one hour at room temperature. Wells were washed five times with 200µL of wash buffer manually. The plate was inverted each time and the contents were decanted by tapping the plate 4-5 times on absorbent paper to completely remove the liquid. 50µL of biotinylated adiponectin antibody was added to each well and incubated for one hour. Microplate was washed as described above. 50µL of streptavidin-peroxidase conjugate was added to each well and incubated for 30 min at room temperature. Microplate was again washed as described above. 50µL of chromogen substrate was added per well in the dark and incubated for about 10 min, till the development of optimal blue color. The plate was tapped gently to ensure thorough mixing. Finally, 50µL of stop solution was added to each well and the color was changed from blue to yellow. The absorbance was read at a wavelength of 450 nm immediately by using Elisa reader (Sunrise absorbance reader, Tecan, Mannedorf, Switzerland). The mean value of optical density (OD) for each standard (triplicate) was calculated and the standard graph was plotted by taking the standard protein concentrations on the x-axis and the corresponding means at 450 nm absorbance on the y-axis. The mean value of optical density (OD) of each sample was plotted by using the standard curve.

Measurement of serum leptin hormone after extracts treatment

The effect of both the extracts on leptin hormone was measured by using ELISA technique. The assay was performed at room temperature (20-30°C) by following the steps provided with the rat leptin ELISA kit (Abcam, Cambridge, UK, ab100773, UK). The standard stock solution (50ng/mL) was used to make working concentrations as 50,000ng/mL, 8,000ng/mL, 2,667ng/mL, 888.9ng/mL, 296.3ng/mL, 98.77ng/mL, 32.92ng/mL, 0ng/mL. 100uL of each standard and sample were added into appropriate wells. The plate was covered and incubated for 2.5 h at room temperature with gentle shaking. The solution was discarded and wells were washed with 300uL of 1x wash solution. Washing was done 4 times. After the last wash, any remaining wash buffer was removed by decanting the plate and tapping on clean adsorbent paper. 100µL of 1x prepared biotinylated antibody (supplied with the kit) was added to each well. The plate was incubated for one hour at room temperature with gentle shaking. The solution was discarded and washing was done as described above. 100uL of prepared Streptavidin solution (supplied with the kit) was added to each well and the plate was incubated for 45 min at room temperature with gentle shaking. The solution was discarded and washing was done as described above. 100uL of TMB One-Step Substrate Reagent (supplied with the kit) was added into each well and the plate was incubated for 30 min at room temperature in the dark with

gentle shaking. Finally, 50uL of Stop solution (supplied with the kit) was added to each well and the plate was read at 450nm immediately by using ELISA reader (Sunrise absorbance reader, Tecan, Mannedorf, Switzerland). The mean value of optical density (OD) for each standard triplicate (supplied with the kit) was calculated and the standard graph was plotted by taking the standard protein concentrations on the x-axis and the corresponding means at 450nm absorbance on the y-axis. The mean value of OD of each sample was plotted by using the Standard curve.

Statistical analysis

Statistical analysis was performed by using the Sigma plot 11.2.0. For statistical analysis, Student's t-test and One- way ANOVA followed by Bonferroni post hoc test were performed to compare the groups with level of significance $p \leq 0.05$; (where*= $p<0.05$, **=$p<0.01$, ***= $p<0.001$). Data are presented as mean ± SEM.

RESULTS

Effect of HFD on blood glucose level

After the administration of HFD for six months, OGTT showed a significant increase in the blood glucose levels of the overnight fasted rats at 0 min and during all time periods after glucose administration. The readings of normal group were compared with diabetic control group as 0 min (p <0.001***), 30 min (p <0.01**), 60 min (p 0.01*), 120 min (p <0.001***); test group 1 (herbal combination) 0 min (p <0.01**), 30 min (p <0.05*), 60 min and 120 min (p <0.05*), test group 2 (TFG) 0 min (p <0.001***), 30 min (p <0.001***), 60 min (p 0.01**) and 120 min (p <0.01**) min (Fig. 1A, B).

Effect of STZ on blood glucose level in insulin resistant rats

After one month of STZ administration, OGTT showed persistent high blood glucose level in all test and diabetic control groups during all time periods as compared to the normal group showing that these groups developed type 2 diabetes. The readings of normal group were compared with diabetic control group as 0 min (p <0.001***), 30 min (p <0.001***), 60 min (p <0.001***), 120 min (p <0.001***); test group 1 (herbal combination), 0 min (p <0.001***), 30 min (p <0.001***), 60 min (p <0.001***), and 120 min (p <0.001***); test group 2 TFG, 0 min (p <0.001***), 30 min (p <0.001***), 60 min (p <0.001***), and 120 min (p <0.001***) (Fig. 1C, D).

Effect of herbal combination extract on blood glucose level in treated rats

After one month, OGTT showed significant difference in the blood glucose levels of the overnight fasted rats (0 min) and after glucose administration during all time periods when test group (herbal combination extract treated) was compared with the diabetic control group at 0 min (p <0.001***), 30 min (p <0.001***), 60 min (p <0.001***), and 120 min (p <0.001***) and with the normal group; 0 min (p <0.05*), 30 and 60 min (p <0.001***), and 120 min (p 0.01**) (Fig. 1E).

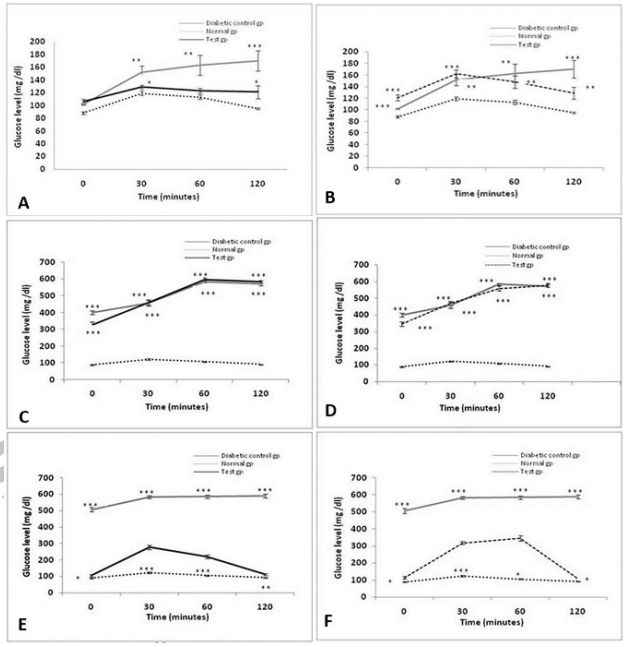

Fig. 1. Effect of HFD (A and B); STZ (C and D); herbal combination and *Trigonella foenum graceum* extracts (E and F) on blood glucose levels of diabetic control, test and normal groups. HFD was given for six months, while STZ, the herbal combination and *Trigonella foenum graceum* extract for one month. Analysis was done by Student's t-test using Sigma plot software. Data are presented as means ± SEM and level of significance is $p < 0.05$ (where * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$; n = 6).

Effect of TFG *on blood glucose level in treated rats*

After one month of treatment, OGTT showed significant difference in the blood glucose levels of the overnight fasted rats (0 min) and after glucose administration during all time periods when test group (TFG extract treated) was compared with the diabetic control group at 0 min (p <0.001***), 30 min (p <0.001***), 60 min (p <0.001***), and 120 min (p <0.001***) and with the normal group at 0 min (p <0.05*), 30 min (p <0.001***), 60 min (p <0.001***), 120 min (p <0.05*) (Fig. 1F).

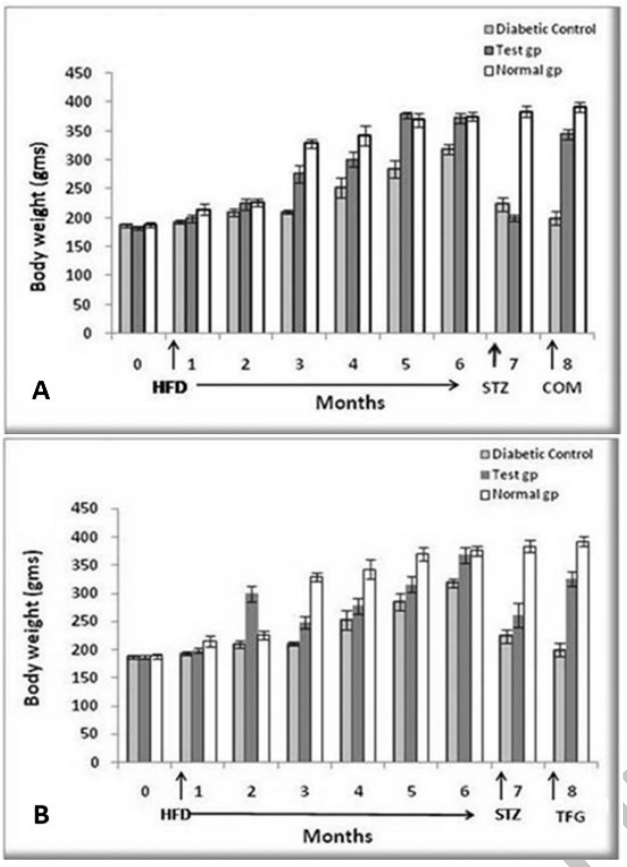

Fig. 2. Effect of herbal combination (A) and *Trigonella foenum graceum* extract (B) on body weight of diabetic control, test and normal groups. The weights were recorded after every one month during the administration of HFD, STZ and herbal combination extract. HFD was given for six months, while STZ and the herbal combination extract for one month. Analysis was done by Student's t-test using Sigma plot software. Data are presented as means ± SD and level of significance is $p < 0.05$ (where * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$; n = 6).

Effect of HFD, STZ, herbal combination and TFG *extracts on body weight*

After the administration of HFD, all rats gained weight gradually. At the end of the sixth month, weight gain was gradual in the test group as shown by monthly recordings. As the STZ was administered to rats it showed marked decrease in the body weight of diabetic control, herbal combination treated (p <0.001***) and TFG treated group (p <0.01**) as compared to the normal group. After treatment of one month, significant increase in the body weight of herbal combination treated (p <0.001***) and TFG treated group (p <0.01**) was observed as compared to the diabetic control group (Fig. 2A, B).

Effect of herbal combination extract on insulin signaling genes in skeletal muscle

The expression levels of IRS-1 (p 0.01**), PDK-1 (p 0.01**) and PKB (p <0.05*) genes were significantly increased and PTP-1B (p <0.001***) and PKC-θ (p 0.01**) were significantly decreased by the herbal combination test group as compared to the diabetic control group. When test group compared with normal group, the gene expression level of IRS-1 (p <0.05*), PDK1 (p 0.01**) and PKB (p <0.05*) significantly increased in test group.

The gene expression was also compared between diabetic and normal groups. The levels of IRS-1 (p <0.05*) and PDK-1 (p 0.01**) were significantly decreased in diabetic control group while PTP-1B (p <0.001***) and PKC-θ (p <0.01**) were significantly increased (Fig. 3).

Effect of TFG *extract on insulin signaling genes in skeletal muscle*

Statistically significant difference was observed between diabetic control and the TFG treated group as IRS-1 (p <0.001***), PKB (p <0.01**) and PI3-K (p <0.01**), PDK1 (p <0.05*) were significantly increased and PTP-1B (p <0.001***) was significantly decreased. The expression level of PDK1 (p <0.05*) gene significantly increased in the test group as compared to the normal group.

The gene expression was also observed in the normal group and compared with diabetic control group. PKB (p <0.05*) was significantly decreased while PTP-1B (p <0.001***) and PKC-θ (p <0.001***) were significantly increased in diabetic control group (Fig. 4).

Effect of herbal combination extract on insulin signaling genes in adipose tissue

The combination of herbal and nutritional showed its potential to significantly restore the normal expression of genes which are involved in the onset of type 2 diabetes. After treatment, the genes' expression profile was analyzed in the adipose tissue as IRS-1 (p <0.001***) was significantly increased and PTP-1B (p <0.001***) was significantly decreased as compared to the diabetic control group.

When the test group compared to the normal group, expression level of IRS-1 (p <0.001***) and PDK1 (p <0.01**) genes was significantly increased while expression level of PKC-θ (p <0.05*) and PTP-1B (p 0.01**) was significantly decreased. The gene expression of diabetic control group was also compared with the normal group. IRS-1 (p <0.001***), PDK-1 (p <0.05*) were significantly decreased while PTP-1B (p <0.01**) and PKC-θ (p <0.01**) were significantly increased (Fig. 5).

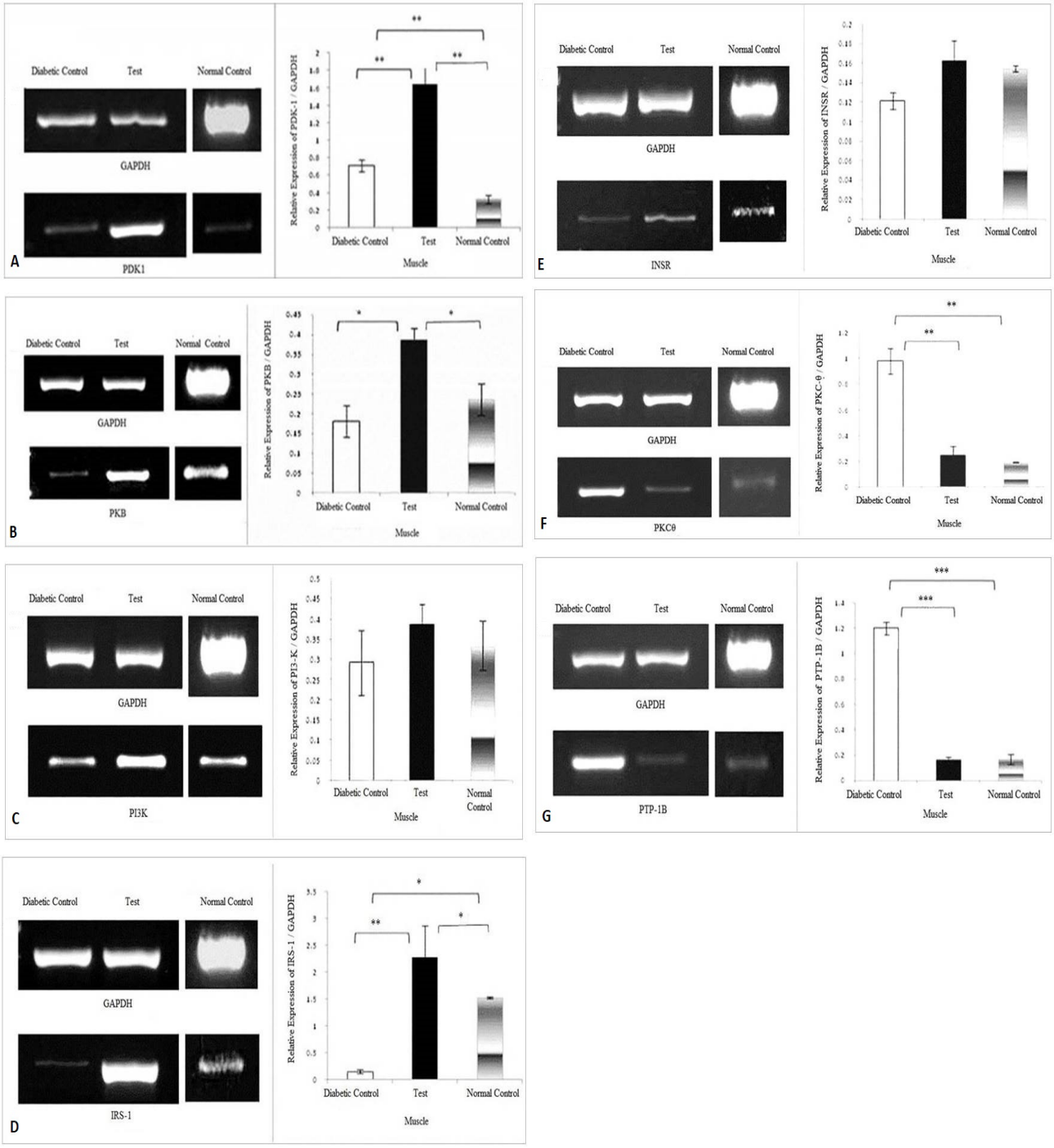

Fig. 3. Effect of herbal combination extract on the expression levels of (A) PDK-1, (B) PKB, (C) PI3-K, (D) IRS-1, (E) INSR, (F) PKC-θ, (G) PTP-1B, genes in the skeletal muscle. The density of each band was measured as Integrated Density Values (IDVs). The graphs are showing expression of genes relative to the expression of GAPDH. Analysis was done by one way ANOVA followed by Bonferroni's post hoc test for comparison between groups using Sigma plot software. Data are presented as means ± SD and level of significance is $p < 0.05$ (where * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$; n = 6).

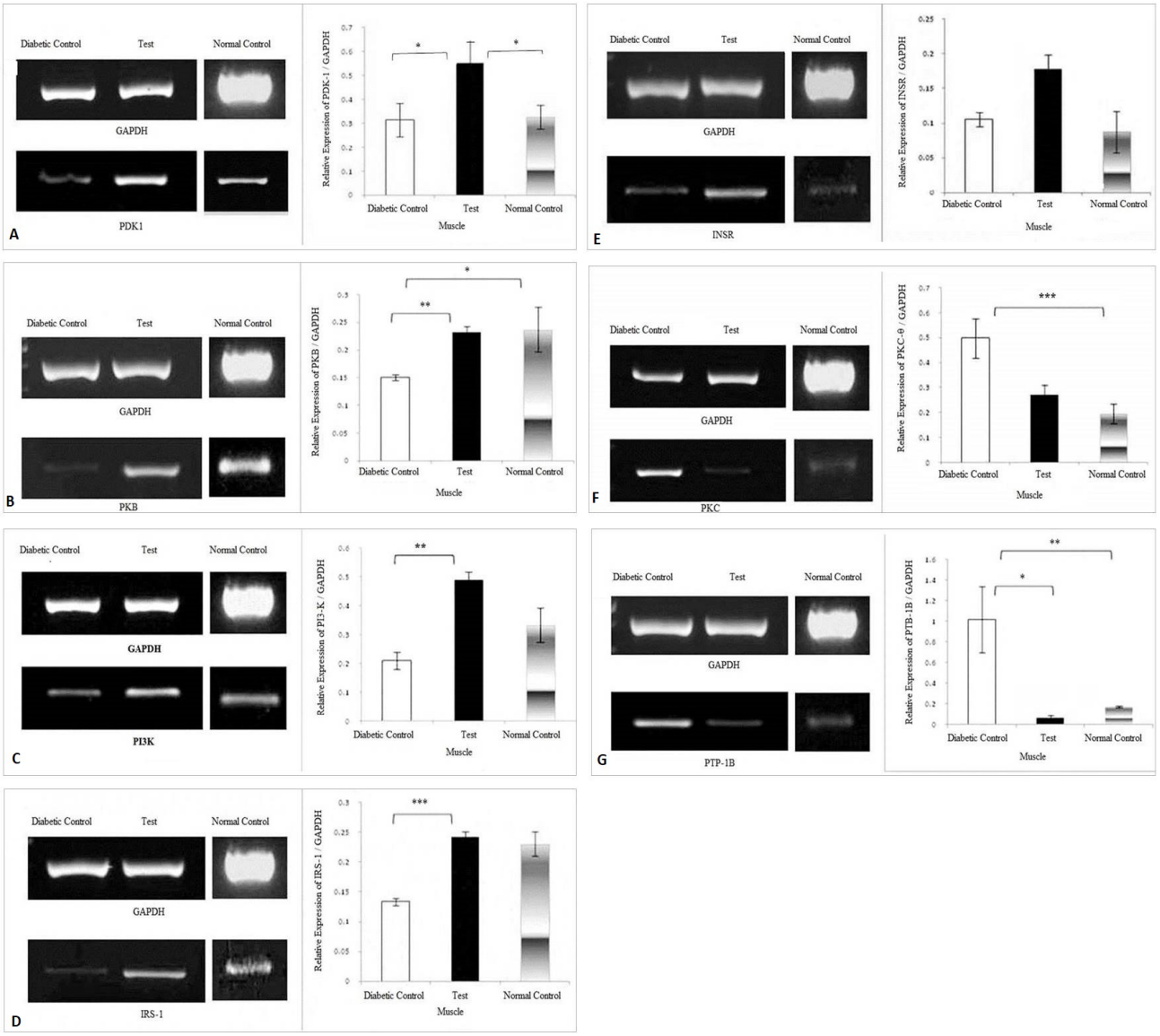

Fig. 4. Effect of *Trigonella foenum graceum* extract on the expression levels of (A) PDK-1, (B) PKB, (C) PI3-K, (D) IRS-1, (E) INSR, (F) PKC-θ, (G) PTP-1B, genes in the skeletal muscle. The density of each band was measured as Integrated Density Values (IDVs). The graphs are showing expression of genes relative to the expression of GAPDH. Analysis was done by one way ANOVA followed by Bonferroni's post hoc test for comparison between groups using Sigma plot software. Data are presented as means ± SD and level of significance is $p < 0.05$ (where * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$; n = 6).

Effect of TFG *extract on insulin signaling genes in adipose tissue*

It was observed that the expression level of INSR (p 0.01**) and PKB (p <0.05*) was significantly increased and PTP-1B (p <0.01**) was significantly decreased in the TFG treated group as compared to diabetic control group. After treatment with TFG, test group showed significant increase in the expression level of IRS-1 (p <0.001***), PDK1 (p <0.05*), PKB (p <0.05*), PI3-K (p 0.01**) and INSR (p <0.05*) genes while decrease in PKC-θ (p 0.01**) gene's expression.

Statistically significant difference was also observed between diabetic control and normal groups as the expression level of IRS-1 (p <0.001***), PDK-1 (p <0.05*) and PI3-K (p <0.05*) were significantly decreased while PTP-1B (p <0.01**) and PKC-θ (p <0.01**) were significantly increased in diabetic control group (Fig. 6).

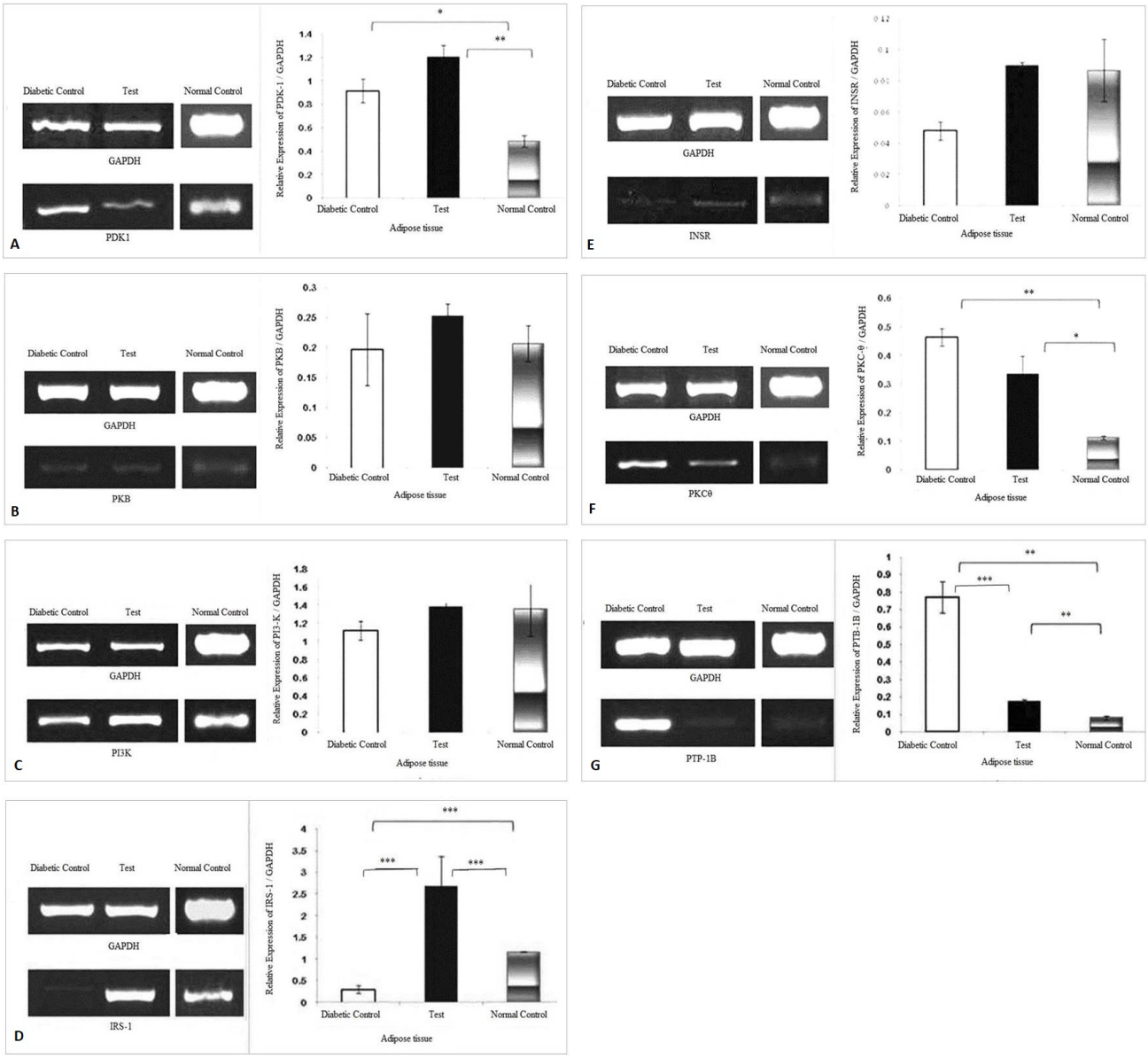

Fig. 5. Effect of herbal combination extract on the expression levels of (A) PDK-1, (B) PKB, (C) PI3-K, (D) IRS-1, (E) INSR, (F) PKC-θ, (G) PTP-1B, genes in the adipose tissue. The density of each band was measured as Integrated Density Values (IDVs). The graphs are showing expression of genes relative to the expression of GAPDH. Analysis was done by one way ANOVA followed by Bonferroni's post hoc test for comparison between groups using Sigma plot software. Data are presented as means ± SD and level of significance is $p < 0.05$ (where * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$; n = 6).

Effect of herbal combination extract on serum adiponectin and leptin hormones

The treatment with herbal combination extract significantly increased the production of adiponectin hormone in the serum of treated group (p <0.05*). In case of leptin hormone, the herbal combination extract significantly reduced the production of leptin (p <0.001***) (Fig. 7A, B).

Effect of TFG *extract on adiponectin and leptin hormones*

The treatment with TFG extract significantly increased the production of adiponectin hormone in the serum of treated group (p <0.05*). In case of leptin hormone, the herbal combination extract significantly reduced the production of leptin (p <0.001***) (Fig. 7C, D).

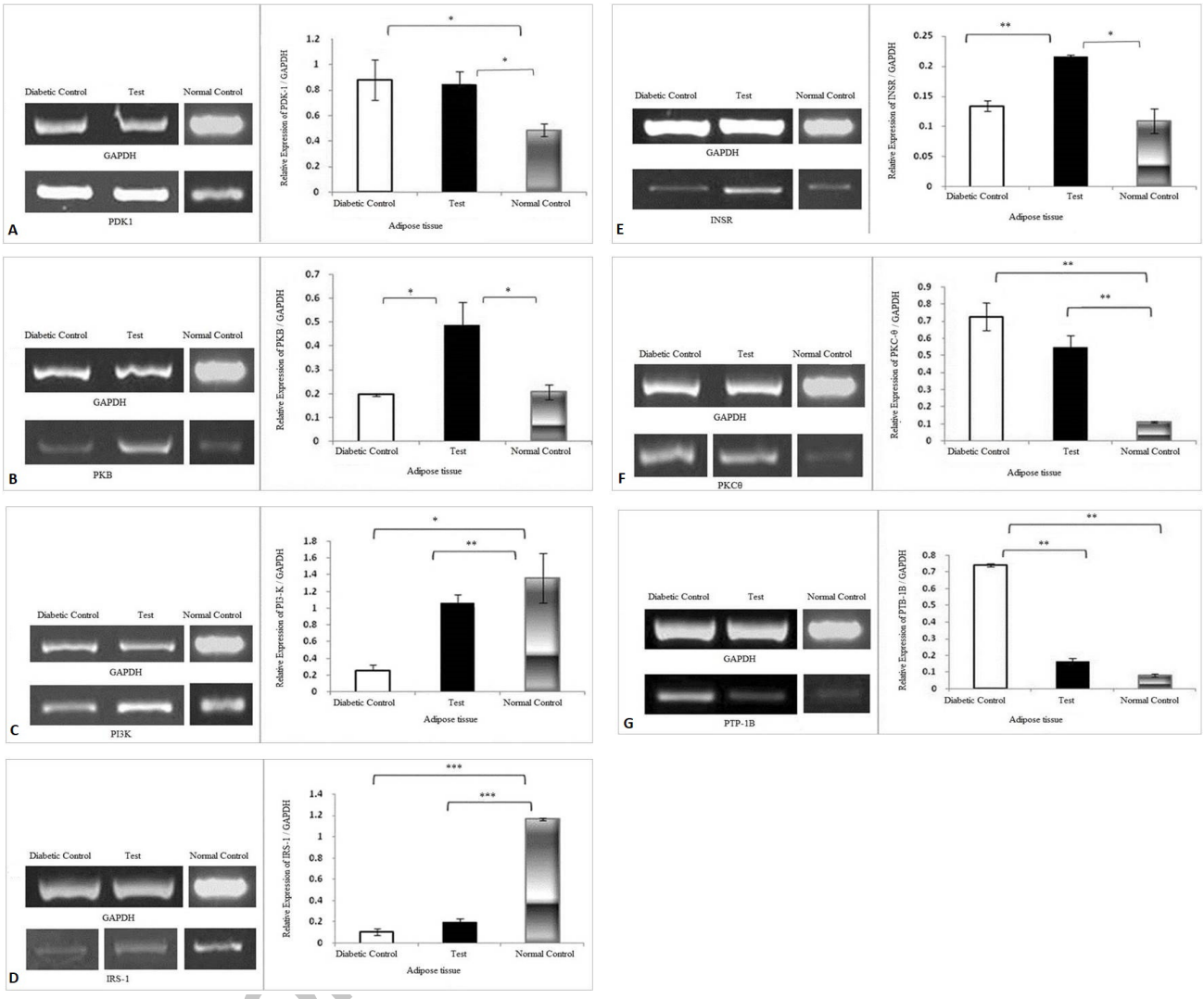

Fig. 6. Effect of *Trigonella foenum graceum* extract on the expression levels of (A) PDK-1, (B) PKB, (C) PI3-K, (D) IRS-1, (E) INSR, (F) PKC-θ, (G) PTP-1B, genes in the adipose tissue. The density of each band was measured as Integrated Density Values (IDVs). The graphs are showing expression of genes relative to the expression of GAPDH. Analysis was done by one way ANOVA followed by Bonferroni's post hoc test for comparison between groups using Sigma plot software. Data are presented as means ± SD and level of significance is $p < 0.05$ (where * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.001$; n = 6).

DISCUSSION

In this study, we have elucidated the possible gene targets of combination of some nutritional herbs as well as of TFG which was given separately. We analyzed the effect of a novel combination of the aforementioned antidiabetic herbs and TFG on the expression of different diabetogenes including PTP-1B, INSR, IRS-1, PI3-K, PKB, PKC-θ and PDK-1. The abnormal expression of these genes triggers the onset of insulin resistance and ultimately type 2 diabetes. Insulin resistance was developed in rats using high fat diet (HFD). HFD negatively affects the insulin function and disrupts the process of insulin signaling. The rats fed with HFD experienced impaired glucose tolerance and insulin sensitivity as evident by their glucose level measured through glucose tolerance test. The increase was persistent which proved the onset of impaired insulin action and sensitivity. Increase in the body weights was also observed owing to the accumulation of spare fat. We used streptozotocin STZ to develop type-2 diabetes in insulin resistant rats. After one month of STZ administration, OGTT results showed marked increase in blood glucose level that remained elevated even after 2 h of oral glucose administration, while that of the normal group returned

back to normal after 2 h. The rats developed type 2 diabetes and certain metabolic disturbances occurred. Consequently, the body tissues fail to metabolize glucose and start utilizing the surplus amount of fats as energy source which were accumulated inside the body. As the excess amount of fats was consumed, the body weight of obese diabetic rats declined (Pataky *et al*., 2017).

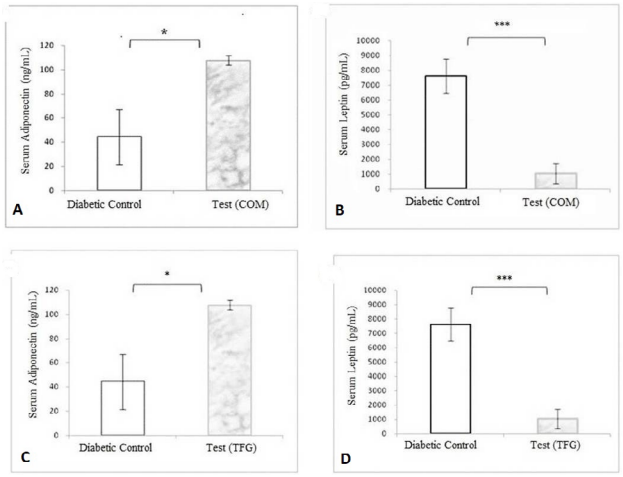

Fig. 7. Quantification of adipokines after herbal combination extract treatment using ELISA: (A) Adiponectin, (B) Leptin and Quantification of Adipokines after *Trigonella foenum graceum* treatment, (C) Adiponectin, (D) Leptin. Data is presented as means ± SEM where n = 3. For statistical analysis, a Shapiro-Wilk Normality test was performed and all comparisons were made with Student's *t*- tests. Level of confidence was $p \leq 0.05$; (*= $p<0.05$, **=p<0.01, ***= p<0.001).

Following the development of type 2 diabetes, diabetic groups were treated with the herbal combination and TFG extracts. After one month of treatment, blood glucose level started to decrease gradually and finally reached normal values after 2 h of glucose administration as compared to the diabetic control group. The body weight of diabetic rats which was drastically reduced after the development of type 2 diabetes, also showed statistically significant increase after the treatment with both the extracts.

We selected adipose tissue and skeletal muscle for the analysis of genes that play significant role in insulin signaling. Being the two major targets of high fat diet, these tissues are drastically affected and show increased insulin resistance. A number of enzymes and protein molecules are involved in insulin signaling. Some of these genes are upregulated for the normal insulin signaling to occur while the others are down regulated. Insulin receptor (INSR) is responsible for insulin binding and subsequent signaling. Upon insulin binding, tyrosine kinase activity of the receptor causes self-phosphorylation of certain tyrosine residues. It phosphorylates other proteins including insulin receptor substrate (IRS-1) that mediates the binding and activation of phosphoinositide 3-kinase (PI3-K) and formation of lipid products *Pip2* and *Pip3* on the plasma membrane (Gutierrez-Rodelo *et al*., 2017), a crucial event in the process of insulin signaling. *Pip3* triggers the phosphorylation of PKB by PDK-1 protein and PKB then further phosphorylates other downstream proteins (Escribano *et al*., 2017).

There are a number of protein tyrosine phosphatases which cause dephosphorylation of insulin receptor. One of the examples is protein tyrosine phosphatase-1B (PTP-1B) which catalyzes the dephosphorylation of insulin receptor and IRS-1 (Gutierrez-Rodelo *et al*., 2017; Pereira de Moura *et al*., 2013). PKC-θ is a serine-threonine kinase and induces the phosphorylation of IRS-1 at the serine residue. This serine phosphorylation weakens the potential of IRS-1 to activate PI3-K. It has been shown that the activity of PKC-θ opposes the insulin signaling pathway (Hage-Sleiman *et al*., 2015).

Our results show that the expression of most of the genes was significantly affected by these extracts; however, the pattern of expression depends on the tissue type. In case of combination extract treatment, most prominent result was obtained in case of skeletal muscle where the expression levels of IRS-1, PKB, PI3-K and PDK-1 genes were significantly increased while that of PTP-1B and PKC-θ were significantly decreased as compared to the diabetic control group. In the adipose tissue, the expression level of only IRS-1 was significantly increased while that of PTP-1B was decreased. INSR and PKB expressions were also increased but not significantly. After TFG treatment, the expression level of IRS-1, PKB and PI3-K was significantly increased and of PTP-1B was significantly decreased in skeletal muscle. In adipose tissue, the expression of only INSR was significantly increased and that of PTP-1B was significantly decreased. The expression level of other genes was either increased or decreased after both the treatments; however, it was not statistically significant.

The studied genes IRS-1, PKB, PI3-K, PDK-1, PI3-K and INSR are the crucial part of insulin signaling pathway and their abnormal and low expression cause the onset of insulin resistance and type 2 diabetes. On the other hand, the increased expression of PTP-1B and PKC-θ deteriorates the normal insulin signaling pathway leading to insulin resistance and type 2 diabetes. The novel herbal combination and TFG extracts normalized the expression of most of these genes and so restored the normal insulin signaling. Our experimental treatments ultimately improved the insulin resistant and type 2 diabetic states

by normalizing the glucose uptake process in both skeletal muscle and adipose tissue.

The hypoglycemic activity of all herbal components of the combination is well documented; however, there is no comprehensive study to clarify the cellular and molecular mechanism of actions of these extracts. Only a few studies are available demonstrating the anti-diabetic action of these herbs when administered individually. We earlier reported the effect of *Cinnamomum cassia* on insulin signaling genes (Aijaz *et al*., 2014). It has been demonstrated that anti-diabetic action of *Camellia sinensis* is due to its effect on the expression of genes engaged in glucose uptake and insulin signaling process (Qiu-Y *et al*., 2017). However, the exact molecular mechanism is still not very well understood (Cao *et al*., 2007). Also, various mechanisms of action of *Gymnema sylvestre* are reported such as, improved glucose uptake and metabolism, enhanced insulin secretion and beta cell mass. However, the underlying mode of action is still to be defined.

We also analyzed the effect of both the extracts on adipokines including adiponectin and leptin. The increased amount of leptin and decreased concentration of adiponectin is imperatively associated with obesity, insulin resistance and the development of type 2 diabetes (Shebla *et al*., 2017; Lee and Shao, 2012). Our results show that both the extracts significantly increase the level of adiponectin and decrease the level of leptin in the blood which ultimately play role in the improvement of insulin resistance and type 2 diabetic states.

CONCLUSION

Our findings demonstrate that the anti-diabetic nutritional herbs present in the combination extract regulate the expression of genes of insulin signaling pathway. The presence of these anti-diabetic herbs in a single combination may result in an improved action, however further studies are needed to confirm this. These herbs act in synergistic fashion and hit multiple molecular targets more in case of skeletal muscle and therefore have the potential to reduce the blood glucose level and decrease insulin resistance. Our findings also suggest the possible molecular mechanism of action of well-known anti-diabetic herb TFG in both skeletal muscle and adipose tissue of type 2 diabetic rats.

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IRB approval

Animal study was endorsed by the Institutional Animal Care and Use Committee (International Center for Chemical and Biological Sciences, University of Karachi, Pakistan).

Ethical statement

International guidelines were followed for the care and use of laboratory animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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