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Tetramethylpyrazine prevents diabetes by activating PI3K/Akt/GLUT-4 signalling in animal model of type-2 diabetes

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ABSTRACT

Aims: The present experiment was conceptualised to explore the therapeutic response of tetramethylpyrazine (TMP), a major active constituent of *Ligusticum chuanxiong*, a Chinese traditional medicinal plant, in high-fat diet (HFD)-streptozotocin (STZ)-induced diabetes in rats and to identify the possible mechanism of action.

Main methods: Dose-reliant effect of oral treatment of TMP (100, 150 and 200 mg/kg/day) for 28 days was evaluated by calculating the alteration in body weight, level of fasting blood glucose (FBG), plasma insulin, homeostasis model assessment (HOMA), serum lipids, oral glucose & intraperitoneal insulin tolerance and glycosylated haemoglobin in HFD-STZ-induced type-2 diabetic (T2D) rats and underlying molecular mechanisms of TMP was also studied.

Key findings: TMP treatment prominently reduced the level of FBG, glycosylated haemoglobin and revived body weight gain and level of serum insulin dose-dependently in diabetic rats. TMP treatment considerably improved insulin resistance, as observed in oral glucose tolerance and insulin tolerance tests. Moreover, dose-dependent reduction in the level of pro-inflammatory cytokines, C-reactive protein (CRP) and interleukin-6 (IL-6) was observed and their level was found to be significantly reduced in highest dose TMP (200 mg/kg) treated diabetic rats, pointing towards TMP mediated recovery of insulin signalling and a decrease in insulin resistance. The expressions of p-PI3K-p85/p-Akt/GLUT-4 were also significantly up-regulated by TMP (200 mg/kg), suggesting the connection of the PI3K/Akt signal pathway in the anti-hyperglycemic action of TMP.

Significance: These findings suggest that TMP may be used as a potential agent for type-2 diabetes treatment.

1. Introduction

Diabetes mellitus (DM) is a disorder of metabolic origin which is characterised by postprandial and fasting hyperglycaemia, and hyperlipidaemia, which results from a deformity in protein, fat and carbohydrate metabolism [1,2]. It is recognised as the persistent worldwide disorder arousing effect in all age people. Deterioration of insulin release from the pancreatic β cells leads to type-1 diabetes (T1D) [3], while type-2 diabetes (T2D) is identified by reduced insulin sensitivity and gradual β-cell dysfunction, and it is responsible for > 90% of all diabetic cases. Development of insulin resistance produces a decrease in tissues response to the circulating insulin, in the beginning, there is an increase in the secretion of insulin to maintain normal blood glucose level that ultimately leads to chronic overstimulation and destruction of β cells resulting in an insufficiency of insulin secretion and prominent hyperglycaemia. Therefore, insulin resistance management is very critical in the prevention and treatment of T2D [4,5]. The induction of T2D by a combination of HFD and the low-dose STZ in rats produces the condition similar to the late-stage T2D in humans [6]. The T2D treatment is still a challenging work as the presently accessible conventional drugs like thiazolidinediones, biguanides, sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors and α-glucosidase inhibitors are associated with few unwanted side effects which comprise of weight gain, hypoglycaemia, nausea, gastrointestinal discomfort, hypersensitivity, diarrhoea, heart and liver failure [7]. Beyond conventional treatments, some researcher's reported that products derived from the medicinal plants carry anti-diabetic properties with lesser side-effects and toxicity [8]. Therefore, nowadays in developing countries, naturopathy is

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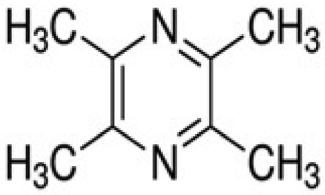

Fig. 1. Chemical structure of tetramethylpyrazine.

widely appreciated as there the resources are in scarcity, and the price of regular medicines is also too high [9,10].

A compound tetramethylpyrazine (TMP) (Fig. 1) is primarily isolated from the rhizomes of the well-recognised traditional medicinal plant *Ligusticum chuanxiong*. Ferulic acid, TMP, alkaloid, chrysophanol, sedanoic acid, and essential oils like butylphthalide and ligustilide are the active ingredients of *Ligusticum chuanxiong* [11]. TMP is the *Ligusticum chuanxiong's* crucial active ingredient [12,13]. In recent times, TMP is well recognised for neurogenesis stimulation in the rat brain after focal ischemia [14] and has also been utilised in the cardiovascular disorder and stroke treatment from the time being in Oriental medicine. It was also reported that TMP might decrease damage to the kidney induced by diabetes by down-regulating the renal vascular endothelial growth factor (VEGF) expression [15]. One of the previous studies has shown that TMP induces neuronal differentiation as a result of TopoIIβ up-regulation through the PI3K/Akt/Sp1 signalling pathway [16]. The literature survey also proves that TMP could protect endothelial cells from the injury induced by upraised blood glucose level, via an increase in NO generation, up-regulation of Akt/eNOS phosphorylation and reduction of ROS production [17].

The binding of insulin with insulin receptor of the surface of the cell stimulates the receptor's tyrosine kinase activity, leading to the tyrosine phosphorylation of insulin receptor substrates (IRS-1 & 2), which in turn activates the downstream effectors of this signalling pathway phosphatidylinositol-3-kinase (PI3K), PI3K-dependent kinase (PDK1) and afterwards protein kinase B (Akt). Akt catalysed phosphorylation of 160 kDa Akt substrate (AS160) leads to the glucose transporter-4 (GLUT-4) translocation from the cytoplasm to the surface of the cell. The GLUT-4 in the plasma membrane promotes extracellular glucose uptake by the cell, which results in the fall of blood glucose level and insulin resistance induced diabetic symptoms [18,19]. In skeletal muscle, glucose uptake is mainly triggered by an insulin signalling pathway through PI3K and Akt activation [20], and in humans, > 70% serum glucose removal occurs through glucose uptake by skeletal muscle [21].

Based on this, we have hypothesised that TMP may produce an anti-diabetic effect by reducing insulin resistance through the up-regulation of PI3Kp85/p-Akt/GLUT4 expression. Thus, the current study was designed with an aim to investigate the anti-diabetic potential of TMP, using HFD – STZ - induced type-2 diabetic rat model, and to illuminate the role of PI3K/Akt pathway in the anti-diabetic mechanism of TMP.

2. Materials and methods

2.1. Drugs and chemicals

Tetramethylpyrazine (cat. no. W323713), Wortmannin (cat. no. W1628) and Streptozotocin (cat. no. S0130) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Huminsulin R 100 IU was procured from Eli Lilly and Company (India). Enzyme-linked immunosorbent assay (ELISA) kits for the determination of rat IL-6 (cat. no. RAB0311), CRP (cat. no. RAB0097) and insulin level (cat. no. RAB0904) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies Phospho-PI3 Kinase-p85 (Tyr458) / p55 (Tyr199) (4228), Phospho-Akt (Ser473) (4060), Glut-4 (1F8) Mouse mAb (2213), PI3 Kinase-p85 (4257), Akt Antibody (9272) and β-actin (4970) were purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary antibodies HRP-linked, IgG anti-rabbit Antibody (7074) and HRP-linked, IgG anti-mouse Antibody (7076) were also procured from Cell Signaling Technology (Danvers, MA, USA). The QIAzol lysis reagent (lot no. 554012939) for RNA isolation was purchased from Qiagen Science (Maryland, USA). RevertAid first strand cDNA synthesis kit (cat.no. K1622) was purchased from Thermo scientific (EU, Lithuania). QuantiFast SYBR Green PCR Kit (cat. no. 204054) was procured from Qiagen Sciences (Hilden, Germany).

2.2. Experimental animals

The present study was carried out in accordance with the standards of the National Institutes of Health (NIH), and all the investigations on animals were performed with the consent of Animal Ethical Committee of the Institution (Reference No. Dean/2016/CAEC/1656) of Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. Adult male Wistar rats about five-weeks-old weighing 180–200 g were procured from the Central Animal House (Registration Number: 542/02/ab/CPCSEA) of IMS, BHU, Varanasi. All the experimental rats were sheltered in polypropylene cages under the regulated environmental conditions (temperature: 25 ± 1 °C; relative humidity: 45–55%; 12-h light/12-h dark cycle). Animals were supported with frequent access to commercial diet available for rats and water ad libitum.

2.3. Development of T2D

After acclimatisation for one week, the rats in the experiment except the normal control rats were fed on standardised HFD containing 70% normal diet, 18% sugar and 12% lard oil for four weeks to build insulin resistance. Followed by, intraperitoneal administration of freshly prepared STZ solution in citrate buffer (0.1 M, pH 4.5) at the 40 mg/kg

Table 1
Effect of TMP on body weight of HFD–STZ-induced diabetic rats.

Groups	Body weight (g)			
	0 day	14th day	21st day	28th day
NC	185.8 ± 2.8	197.5 ± 3.5	204.3 ± 4.1	213.9 ± 3.7
DC	187.4 ± 3.9	190.7 ± 4.6	198.1 ± 2.8	207.8 ± 4.4
D + T-1	183.6 ± 2.9	201.1 ± 4.8	206.3 ± 3.5	215.2 ± 5.4
D + T-2	184.8 ± 4.5	203.4 ± 4.7	216.9 ± 2.3$^{\#}$	227.0 ± 3.8$^{\#}$
D + T-3	189.1 ± 1.9	217.3 ± 2.4$^{*,\#}$	235.6 ± 4.3$^{*,\#}$	254.5 ± 2.8$^{*,\#}$
D + T-3 + W	182.5 ± 4.6	193.8 ± 5.1	199.6 ± 2.2	210.8 ± 3.9

All values are represented as mean ± SEM ($n = 6$); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

Table 2
Effect of TMP on FBG level of HFD–STZ-induced diabetic rats.

Groups	FBG level (mg/dl)			
	0 day	14th day	21st day	28th day
NC	92.4 ± 3.8	96.8 ± 1.9	90.1 ± 2.3	102.4 ± 2.8
DC	283.2 ± 4.1*	319.4 ± 3.7*	337.8 ± 4.9*	346.4 ± 2.6*
D + T-1	278.7 ± 5.4*	241.2 ± 4.6*,#	205.4 ± 3.1*,#	156.6 ± 2.5*,#
D + T-2	287.5 ± 3.3*	235.8 ± 2.7*,#	161.9 ± 1.5*,#	129.2 ± 2.4*,#
D + T-3	275.3 ± 4.9*	202.3 ± 3.5*,#	143.5 ± 3.2*,#	121.7 ± 2.9*,#
D + T-3 + W	289.6 ± 2.7*	310.7 ± 2.4*	329.9 ± 4.6*	337.4 ± 3.1*

All values are represented as mean ± SEM (n = 6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

Table 3
Effect of TMP on fasting serum insulin (FSI) level of HFD–STZ-induced diabetic rats.

Groups	FSI level (μU/ml)	
	0 day	28th day
NC	15.9 ± 0.5	16.2 ± 0.7
DC	21.4 ± 0.8*	26.3 ± 1.4*
D + T-1	20.7 ± 1.0*	17.2 ± 0.6#
D + T-2	22.2 ± 1.3*	16.7 ± 1.0#
D + T-3	21.8 ± 0.9*	16.4 ± 0.3#
D + T-3 + W	22.6 ± 1.1*	25.8 ± 0.9*

All values are represented as mean ± SEM (n = 6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at P < 0.05; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

dose to the rats to create moderate destruction of β-cells [6] excluding normal control group. After 5 days of induction of STZ, blood samples were obtained from the tail vein under mild anaesthesia from rats fasted for overnight, and blood glucose was determined with a One Touch Select Simple glucometer (LifeScan, Scotland, UK). The rats having FBG level over 250 mg/dl were considered as successfully induced with diabetes and included in the experiment. The experiment on the diabetic rats was initiated on the fourth day (day 0) after three days of pacification. HFD was carried on for the overall period of the experiment.

2.4. Design of experiment

In this study total, 36 rats (6 normal and 30 diabetic rats) were used. In five groups (Group II to VI) diabetic rats were randomly allocated with each group of six animals according to the following design of an experiment: Group I (NC): Normal control rats treated with vehicle (1 ml/100 g body weight); Group II (DC): Diabetic control rats treated with vehicle (1 ml/100 g body weight); Group III (D + T-1): Diabetic rats treated with TMP (100 mg/kg body weight); Group IV (D + T-2): Diabetic rats treated with TMP (150 mg/kg body weight); Group V (D + T-3): Diabetic rats treated with TMP (200 mg/kg body weight); Group VI (D + T-3 + W): Diabetic rats treated with TMP (200 mg/kg body weight) + PI3K inhibitor wortmannin (W) (15 μg/kg body weight/day [22], i.v.)- 10 min before TMP administration; The test drugs or vehicle were orally once daily administered in the afternoon, for 28 days using an oral gavage.

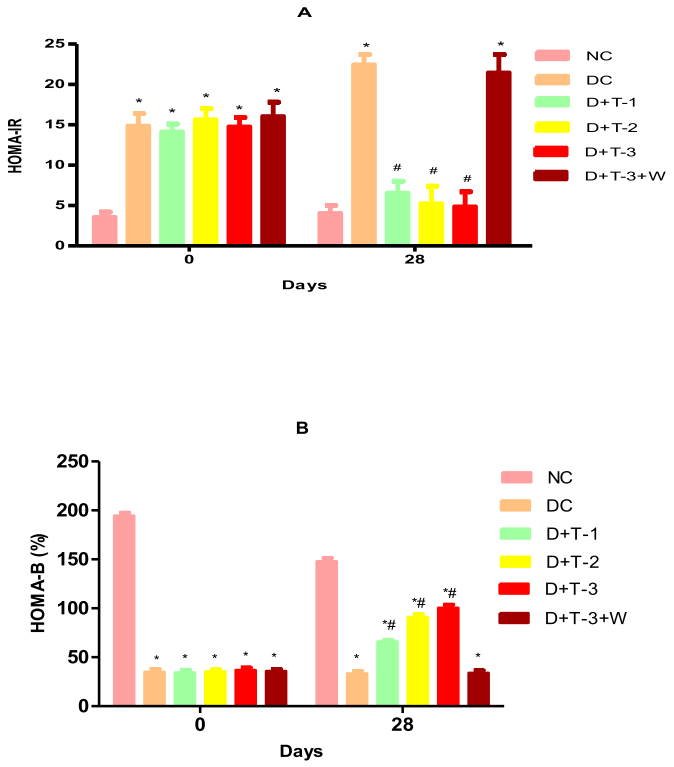

Fig. 2. Estimated insulin resistance (IR) and β-cell functioning (B%) from the HOMA model. All values are represented as mean ± SEM ($n = 6$); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

2.5. Experimental procedure

The body weights and FBG were measured on days 0 (before treatment), 14, 21 and 28 during the study. The insulin level in rats was measured in the serum on 0 and 28th day. The insulin resistance (IR), and the function of β-cell (B%) were calculated with the help of fasting glucose and fasting insulin levels through a homeostatic model assessment (HOMA). The following two equations were used for the calculation: HOMA-IR = (Glucose × Insulin) / 405 and HOMA-B = (360 × Insulin) / (Glucose-63) % [23]. On the 14th day, the oral glucose tolerance test (OGTT) was carried out. In brief, 6 h fasting was given to each rat in the study and 2 g/kg body weight glucose solution was orally given to the rats through intra-gastric gavage exactly 30 min

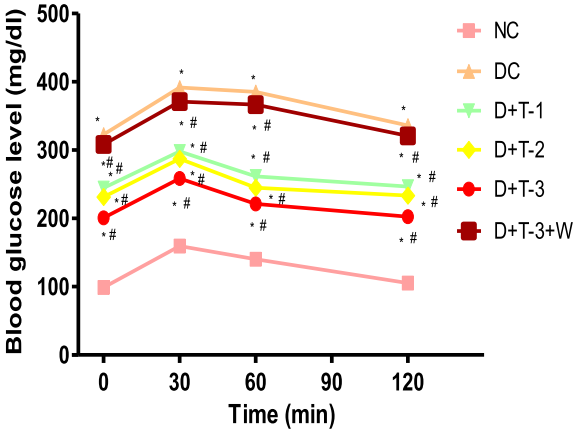

Fig. 3. Effect of different doses of TMP on blood glucose level in oral glucose tolerance test (OGTT). All values are represented as mean ± SEM ($n = 6$); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

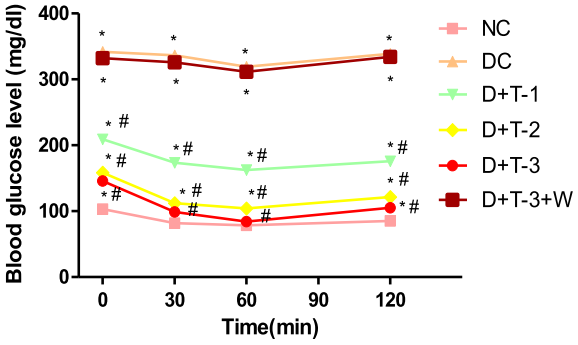

Fig. 4. Effect of different doses of TMP on blood glucose level in insulin tolerance test (ITT). All values are represented as mean ± SEM (n = 6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

after the administration of TMP and vehicle. At time 0 (before glucose infusion) and 30, 60 and 120 min after glucose intake the blood glucose level in each rat was estimated. After treatment of 21 days, the rats were subjected to an insulin tolerance test (ITT). In short, after 6 h of fasting, animals were treated with insulin (1.2 U/kg body weight) in normal saline intraperitoneally, and blood glucose level was evaluated as in OGTT. At the study end, the animals were given fasting for overnight, and blood was withdrawn from the orbital vein for the evaluation of serum biochemical parameters and inflammatory cytokines. Then, the rats were euthanised by cervical dislocation under mild anaesthesia following animal ethical guidelines. The skeletal muscle, heart and adipose tissue were excised and frozen rapidly in liquid nitrogen for successive western blot and gene expression analysis.

2.6. Estimation of biochemical parameters

In the serum, the level of triglyceride (TG), total cholesterol (TC), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol were measured using

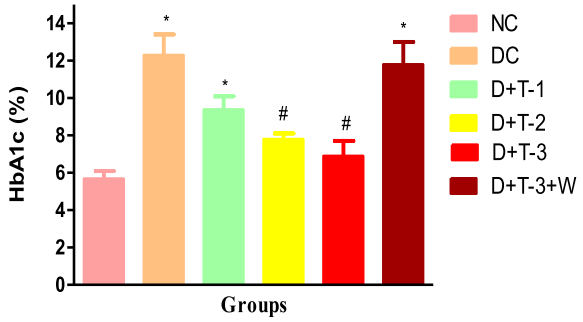

Fig. 5. Effect of TMP on HbA1c (%) in HFD–STZ-induced diabetic rats. All values are represented as mean ± SEM (n = 6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

auto-analyser (Fisher scientific, Biomaster touch screen biochemistry analyser). Nayak and Pattabiraman [24] proposed the method through which the level of glycosylated haemoglobin (HbA1c) was assessed in whole blood.

2.7. Estimation of pro-inflammatory cytokines in serum

Using the commercially available ELISA kits the pro-inflammatory cytokines IL-6 and CRP level were estimated. Here the concentrations of test samples were obtained through the construction of a standard curve.

2.8. Western blot analysis

Western blotting was performed as explained by the method proposed by Martin et al. [25] with few modifications. In brief, the lysis buffer (20 mM Tris–HCl (pH 7.4), 2 mM EGTA, 2 mM EDTA, 30 mM NaF, 1 mM PMSF, 30 mM sodium pyrophosphate, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail) was used to make the 10% *w/v* tissue homogenate and the homogenate was solubilized at 4 °C for 1 h and centrifuged at 8000*g* for 15 min. The supernatants were collected, and the protein content was estimated according to Bradford's method [26] and then stored at −20 °C until further analysis. On the individual lanes of 10% polyacrylamide gel, 40 μg of protein samples were loaded and separated by Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE; Bio-Rad, Mini-PROTEAN® Tetra Cell). Proteins were relocated to polyvinylidene difluoride (PVDF) (Millipore Corporation, MA, USA) membrane using Bio-Rad (Mini Trans-Blot® Electrophoretic Transfer Cell). The membranes were blocked with bovine serum albumin (5%) in TBS-T (0.1%Tween20) for 2 h and overnight incubated at 4 °C with primary antibodies: Phospho-PI3 Kinase-p85, PI3 Kinase-p85, Phospho-Akt, Akt and Glut-4. The β-actin antibody was used as an internal control. The membranes were then washed with TBS-T solution gently and subsequently incubated with either HRP-linked, IgG anti-mouse or anti-rabbit secondary antibody for 1 h. The desired blots were washed with TBS-T three times (each of 10 min), and were visualised using 3, 3′-diaminobenzidine (DAB) (cat. no. 94524; SRL, Mumbai, India) and H_2O_2 (SDFCL, Mumbai, India) as substrates. The densitometric analysis was achieved using NIH Image J analysis software.

2.9. RNA extraction, synthesis of cDNA and Real-time PCR

Total RNA was isolated from the 100 mg of skeletal muscle, adipose and cardiac tissue using QIAzol lysis reagent according to the guidelines

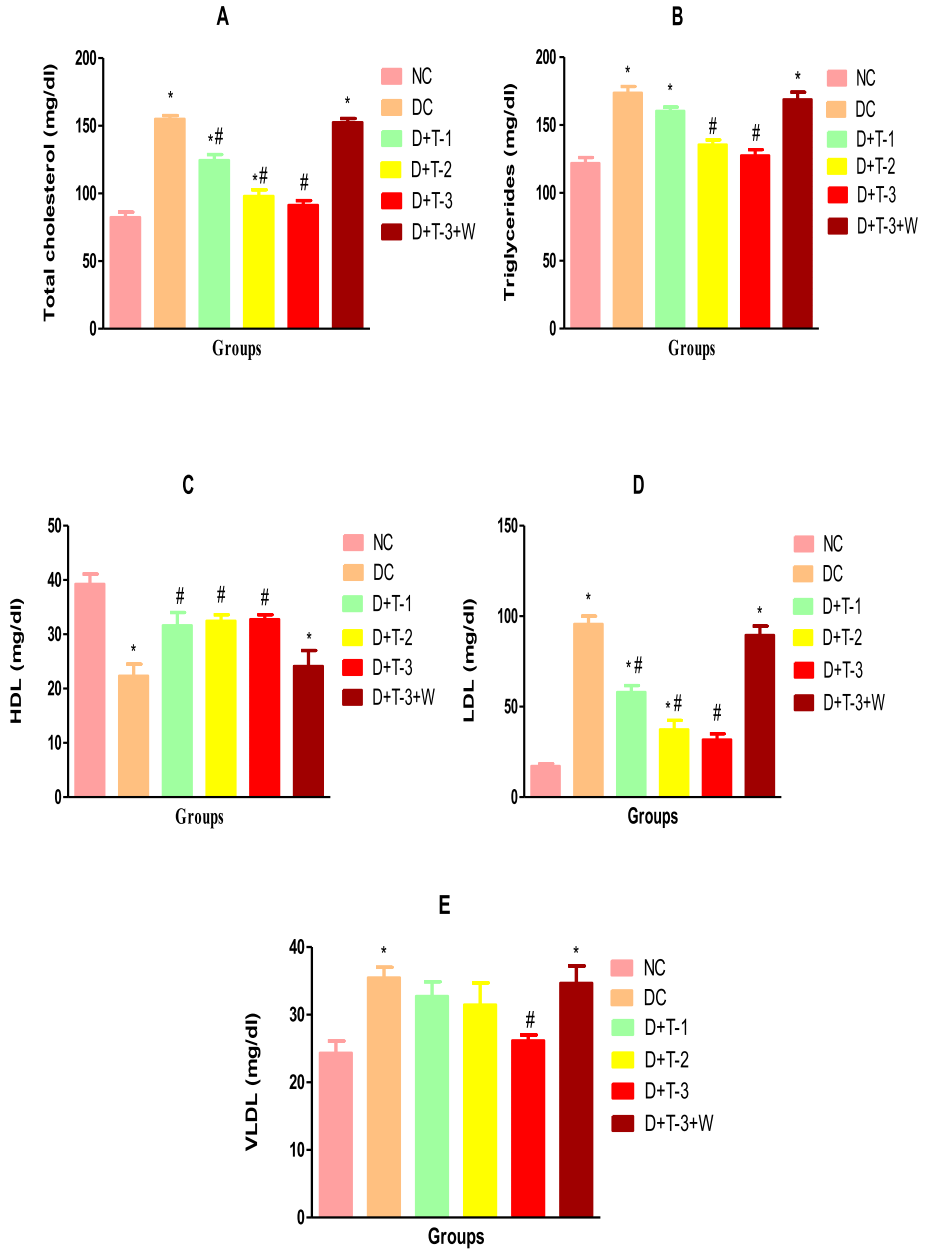

Fig. 6. Effect of TMP on lipid profile levels in the serum of HFD–STZ-induced diabetic rats. All values are represented as mean ± SEM (n = 6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

of the manufacturer, and its quantification was performed through Nanodrop instrument (Thermo Scientific, USA). Before cDNA synthesis, RNA was treated with DNase I, RNase-free (#EN0521; Thermo scientific, EU, Lithuania) to remove trace amounts of DNA. Then, cDNA was synthesised from isolated and purified RNA by using RevertAid first strand cDNA synthesis kit. Real-time PCR was carried out on the Rotor-Gene Q 2plex HRM real-time PCR System (Qiagen, Germany) using the QuantiFast SYBR Green PCR Kit, according to manufacturer's protocol. After initial pre-incubation and denaturation for 10 min at 95 °C, PCR amplification was executed at 95 °C for 10 s (denaturation) and at 60 °C for 45 s (combined annealing/extension) for 40 cycles. The relative gene expression levels were normalised using β-actin gene and quantified by the $2^{-\Delta\Delta Ct}$ method. The primer sequences employed in the study were listed in Table S1 in supplemental materials.

2.10. Statistical analysis

All the results of the study were expressed as mean ± standard error of the mean (SEM) ($n = 6$), and the results were analysed using one-way ANOVA followed by Tukey's multiple comparison post hoc tests. Statistical analysis was accomplished by Graph Pad Prism 5.0 software (San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of TMP on body weight and FBG

Tables 1 and 2 represents the levels of body weight and FBG of study rats on 0 (before treatment), 14, 21 and 28 days after the treatment

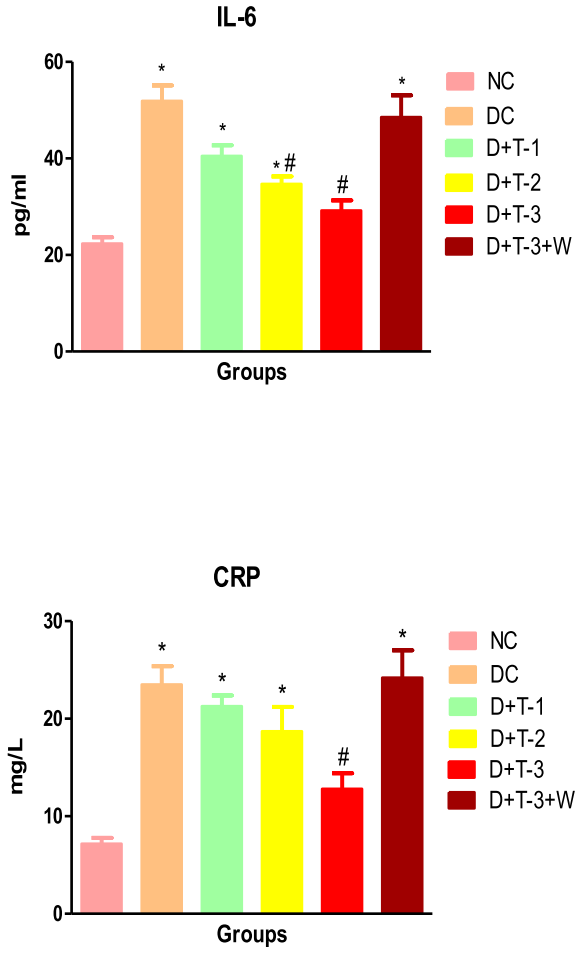

Fig. 7. Effect of TMP on IL-6 and CRP levels in the serum of HFD–STZ-induced diabetic rats. All values are represented as mean ± SEM (n = 6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at P < 0.05; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

with drug. According to the results, the body weight gain in TMP treated groups was higher than the diabetic control group in a dose-dependent manner over the whole period of the experiment. In the TMP 200 mg/kg group, the body weight was significantly ($P < 0.05$) higher compared to diabetic control and normal control groups. While the weight gain in TMP 200 mg/kg + W group was comparable to diabetic control. In the diabetic control and TMP 200 mg/kg + W groups, there was significant ($P < 0.05$) rise in blood glucose level compared to the normal control group for the whole course of study. Whereas, TMP (100, 150 and 200 mg/kg) treated diabetic rats demonstrated significant reduction ($P < 0.05$) in the level of blood glucose after treatment of 14, 21 and 28 days in a dose-dependent way compared to the diabetic control group.

3.2. Effect of TMP on fasting serum insulin (FSI) level, HOMA-IR and HOMA-B (%)

On the day 0 the levels of FSI and HOMA-IR in the diabetic control, diabetics treated with TMP (100, 150 and 200 mg/kg) and TMP 200 mg/kg + W groups were significantly ($P < 0.05$) increased compared to the normal control group (Table 3; Fig. 2A). However on the 28th day, significant ($P < 0.05$) reduction in their levels was observed in TMP (100, 150 and 200 mg/kg) treated groups in a dose-dependent manner compared to the diabetic control group. The significant ($P < 0.05$) reduction in the level of HOMA-B (%) was observed in all the diabetics treated and untreated groups on the day 0 compared to the normal control group (Fig. 2B). In contrast, on the 28th day the significant ($P < 0.05$) increment in the level of HOMA-B (%) was observed in the diabetics treated with TMP (100, 150 and 200 mg/kg) groups in a dose-reliant manner compared to the diabetic control group.

3.3. Effect of TMP on OGTT and ITT

An oral administration of glucose (2 g/kg) in OGTT led to a significant blood glucose level rise within 30 min in all the groups, and it remained elevated in the diabetic control and diabetic treated with TMP (200 mg/kg) + W groups until the last experimental period (120 min). The significant ($P < 0.05$) decline in blood glucose level was recognised in TMP (100, 150 and 200 mg/kg) groups in a dose-dependent way compared to diabetic control at 60 min and it gets lowered back to the initial level at the end of study (Fig. 3). Moreover, in ITT, the blood glucose is slightly lowered in diabetic control and diabetic treated with TMP (200 mg/kg) + W groups after 30 and 60 min of insulin administration and again rise back to the original level at 120 min. In contrast, TMP (100, 150 and 200 mg/kg) treated diabetic rats showed a significant ($P < 0.05$) glucose clearance in a dose-dependent manner compared to diabetic control rats over the complete period of study (Fig. 4).

3.4. Effect of TMP on the level of glycosylated haemoglobin (HbA1c)

Fig. 5 shows the TMP treatment effect on the HbA1c level in HFD-STZ-induced diabetic rats. Diabetic rats, diabetic treated with TMP 100 mg/kg and TMP 200 mg/kg + W rats had shown the significantly ($P < 0.05$) increased level of HbA1c compared to normal control rats. However, TMP (150 and 200 mg/kg) treated diabetic rats had presented a significant ($P < 0.05$) lowering in HbA1c level in a dose-reliant manner compared to diabetic control rats.

3.5. Effect of TMP on serum lipid profile

As shown in Fig. 6A, B and D, there was a significant ($P < 0.05$) increment in the level of TC, TG and LDL in diabetic control, diabetic treated with TMP 100 mg/kg and TMP 200 mg/kg + W groups compared to normal control group. Whereas, significant ($P < 0.05$) decrease in their level was observed in diabetic treated with TMP (150 and 200 mg/kg) groups in a dose-dependent manner compared to the diabetic control group. The VLDL level was significantly (P < 0.05) raised in diabetic control and diabetic treated with TMP 200 mg/kg + W groups compared to normal control. However, its level was reduced significantly ($P < 0.05$) in TMP (200 mg/kg) treated diabetic rats compared to diabetic control rats (Fig. 6E). In the present study (Fig. 6C), the significant ($P < 0.05$) decrease in HDL level was recognised in the diabetic control and diabetic treated with TMP 200 mg/kg + W groups compared to normal control group and the HDL level increased significantly ($P < 0.05$) in the TMP (100, 150 and 200 mg/kg) treated diabetic rats in a dose-dependent way compared to diabetic control rats.

3.6. Effect of TMP on the level of pro-inflammatory cytokines

As presented in Fig. 7, the pro-inflammatory cytokines IL-6 and CRP levels were raised significantly (P < 0.05) in diabetic control, diabetic treated with TMP (100 and 150 mg/kg) and diabetic treated with TMP 200 mg/kg + W groups compared to the normal control group. In contrast, the level of IL-6 was decreased significantly ($P < 0.05$) in diabetic treated with TMP (150 and 200 mg/kg) groups in a dose-dependent manner and the level of CRP was reduced significantly ($P < 0.05$) only in diabetic treated with TMP 200 mg/kg group

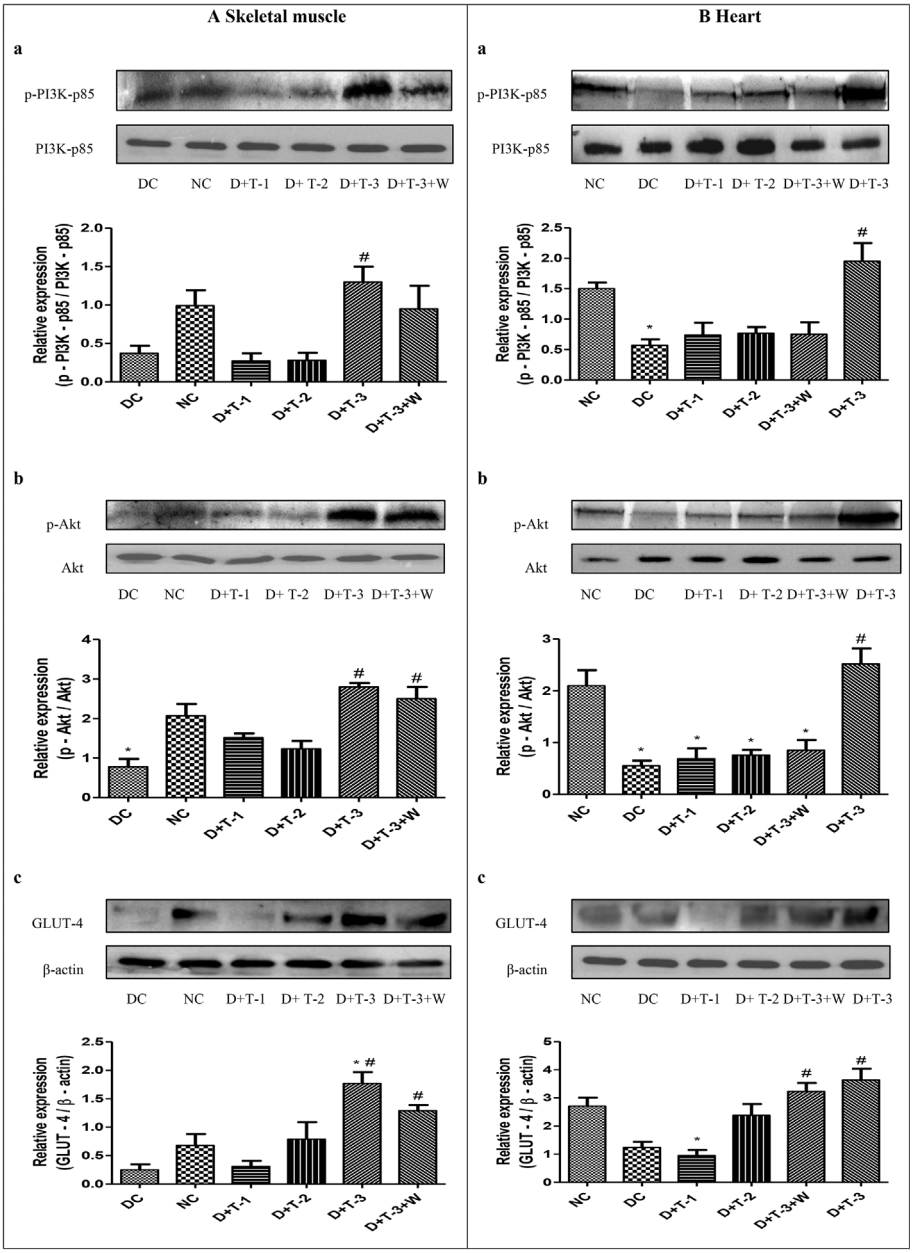

Fig. 8. Analysis of the effect of TMP administration on the expression of the insulin signalling pathway proteins PI3K, Akt and GLUT4 in the skeletal muscle, heart and adipose tissue of HFD–STZ-induced diabetic rats through western blotting; a relative expression of p-PI3K-p85, b Relative expression of p-Akt, c relative expression of GLUT-4. Experiments were 3 times repeated independently. The shown blots were the outcome of one of the experiments. Intensities of band in each blot were determined after normalisation by the corresponding total protein, and the values are represented as mean ± SEM, $n = 3$ independent experiments. Differences were estimated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

compared to diabetic control group.

3.7. Western blotting analysis

3.7.1. TMP increases the expression of the p-PI3K-p85 protein

As displayed in Fig. 8 A-a, B-a and C-a, the expression of p-PI3K-p85 was reasonably decreased in diabetic control and diabetic treated with TMP 200 mg/kg + W groups compared to the normal control group in the context of skeletal muscle and adipose tissue samples. However, its expression was decreased significantly ($P < 0.05$) in the diabetic control group and remarkably in diabetic receiving TMP 200 mg/kg + W group compared to normal control in the case of the heart tissue sample. In contrast, the expression of p-PI3K-p85 was significantly ($P < 0.05$) increased in diabetic treated with TMP 200 mg/kg group compared to the diabetic control group in all the three types of tissues.

3.7.2. TMP increases the expression of the protein p-Akt

As exhibited in Fig. 8 A-b, B-b and C-b, the expression of p-Akt in skeletal muscle was significantly ($P < 0.05$) decreased in the diabetic control group, and its expression in heart and adipose tissue samples was significantly ($P < 0.05$) lowered in diabetic control and diabetic treated with TMP 200 mg/kg + W groups compared to normal control group. However, the expression of p-Akt in all the tissue types was significantly ($P < 0.05$) elevated in diabetic treated with TMP 200 mg/kg group in comparison of the diabetic control group.

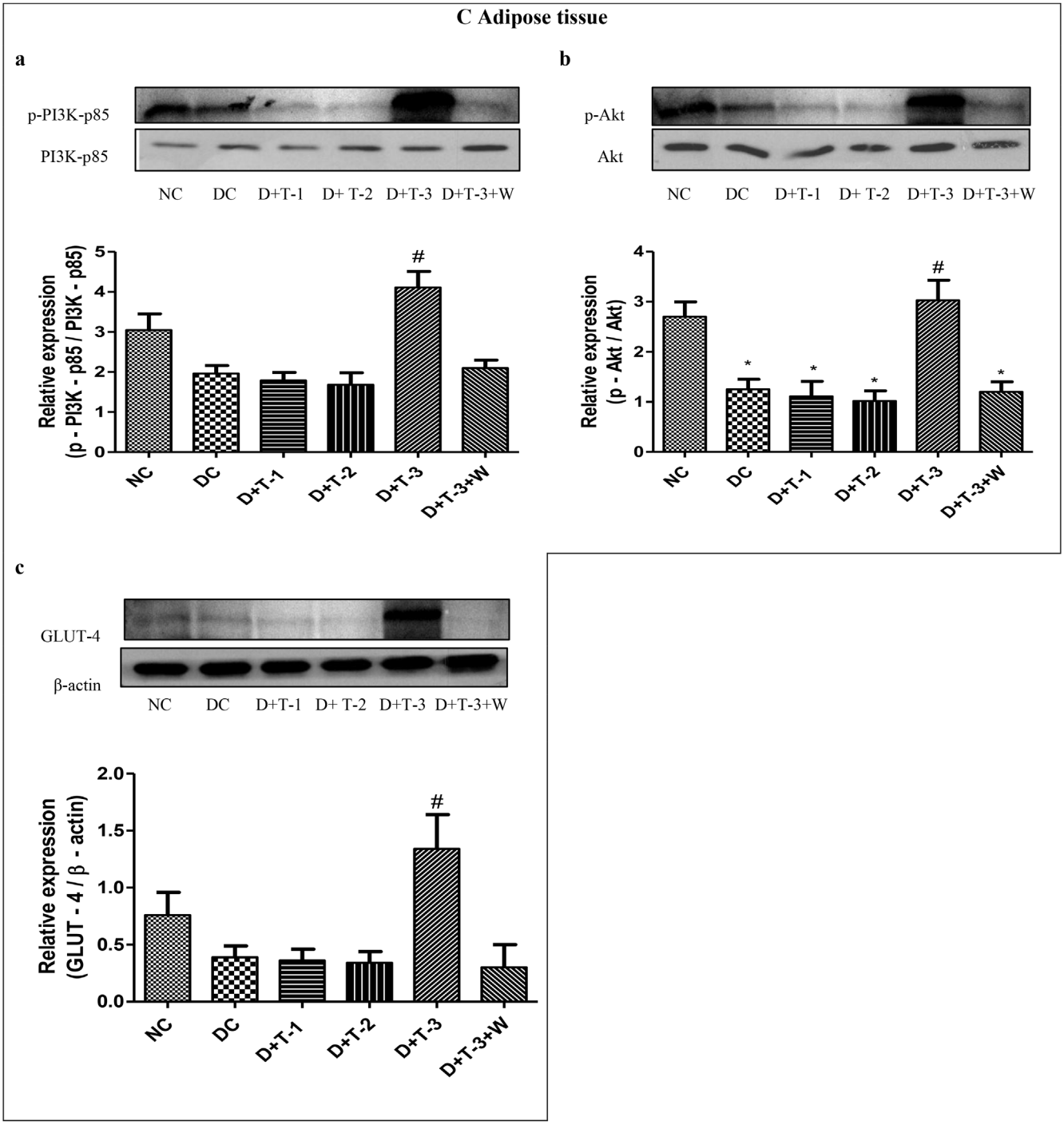

Fig. 8. (*continued*)

3.7.3. TMP boosts the expression of the GLUT-4 protein

As represented in Fig. 8 A-c, B-c and C-c, the expression of GLUT-4 in the skeletal muscle and heart was notably reduced in diabetic control group and in the adipose tissues remarkable decrease was observed in the diabetic control and diabetic control treated with TMP 200 mg/kg + W groups compared to normal control group. Whereas, the expression of GLUT-4 in the diabetic treated with TMP 200 mg/kg group was significantly ($P < 0.05$) elevated compared to the diabetic control group in all the tissue forms.

3.8. Confirmation of PI3K/Akt/GLUT-4 signalling through gene expression analysis by RT-PCR

From the results presented in Fig. 9 A, B and C, the relative mRNA expression of PI3K in skeletal muscle, heart and adipose tissue was significantly ($P < 0.05$) increased in diabetic treated with TMP 200 mg/kg group compared to the diabetic control group. Whereas, the relative mRNA expression of Akt was significantly ($P < 0.05$) elevated in diabetic treated with TMP 200 mg/kg group compared to the diabetic control group only in the course of skeletal muscle and heart tissue samples. The genetic expression of GLUT-4 in skeletal muscle and adipose tissues was significantly ($P < 0.05$) heightened in diabetic treated with TMP (150 and 200 mg /kg) groups and in contrary, its

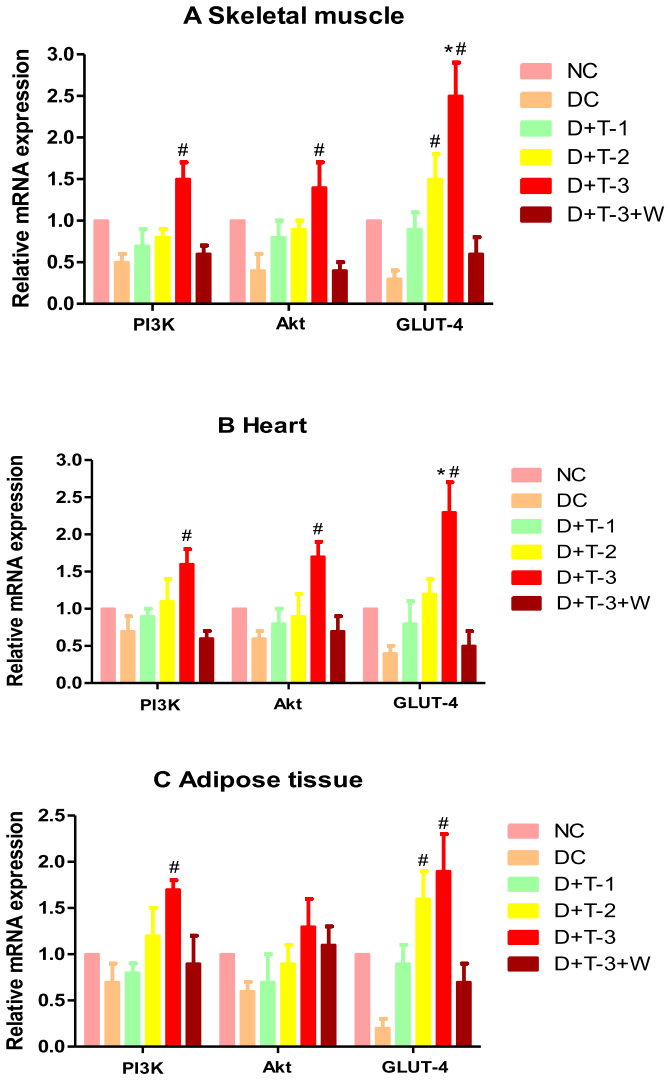

Fig. 9. Confirmation of the effect of TMP administration on the expression of genes corresponding to the proteins of insulin signalling pathway PI3K, Akt and GLUT4 in the skeletal muscle, heart and adipose tissue of HFD–STZ-induced diabetic rats through qRT-PCR. The relative level of expression for the indicated genes compared to normal control was determined by the $2^{-\Delta\Delta Ct}$ method using β-actin gene as a normaliser. The average relative expression determined in three independent experiments (n = 3) is plotted on the histogram with error bars representing the SEM. Differences were estimated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100 mg/kg); T-2, TMP (150 mg/kg); T-3, TMP (200 mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

expression in heart tissue was significantly ($P < 0.05$) marked up only in TMP 200 mg/kg treated group compared to diabetic control group.

4. Discussion

T2D is identified as the most prevalent disorder of health in the whole world, and gradually it is growing to be the prominent cause of morbidity and mortality in humans [27]. The T2D which is induced by a standardised HFD and STZ at low-dose (40 mg/kg body weight) combination in rats mimics the situation identical to the insulin-resistant condition in human beings [6]. The HFD feeding to rats produces insulin-resistant state via inhibition of insulin receptor substrate's (IRS) tyrosine phosphorylation as IRS is a critical target protein of insulin signalling pathway [28]. Moreover, STZ at a low dose produces moderate damage to the β-cells of the pancreas which is similar to the typical metabolic feature of the T2D at the critical phase. This animal model was also used earlier to induce T2D by various researchers [29–31].

The body weight gain reduction observed in diabetic rats might be the result of structural fats and proteins deterioration due to carbohydrate deficiency for energy metabolism [32]. A significant increase in body weight of diabetic rats treated with TMP (200 mg/kg) showed the blood glucose stabilisation effect which results in prevention of the body weight loss (Table 1). HFD induced insulin resistance, and STZ caused β-cell anomaly leads to an imbalance between internal glucose load and insulin sensitivity causing immediate hyperglycemia in rats with diabetes [33]. The anti-hyperglycemic action of TMP may be due to the restoration of insulin sensitivity (Table 2).

HOMA-IR and HOMA-B (%) models were employed to calculate the insulin resistance and β-cell functions respectively by employing FBG and FSI. That is based on the presumption that the relationship between insulin and glucose in the elementary phase shows the harmony between the secretion of insulin and output of glucose from the liver, and it has been shown to correspond accordingly with the experiential procedure [34]. In this study TMP administration significantly reduced insulin resistance and improved β-cell activity compared to diabetic control (Fig. 2A and B). These results indicated that TMP behaved as a good insulin sensitiser reasonably due to increased uptake of glucose in the major organ sites. Additionally, these results also show that the TMP administration assisted in the insulin aided uptake of glucose into peripheral tissues. There was a significant reduction of increased insulin level, HOMA-IR and a significant increase in HOMA-B (%) in TMP treated diabetic rats in comparison to the diabetic control rats indicating that TMP possesses significant insulin sensitisation activity along with recovery in the glucose homeostasis apparently due to the normalised function of the β-cell.

T2D was successfully developed in diabetic control and diabetic treated with TMP (200 mg/kg) + W groups through the generation of insulin resistance as displayed in the results (Figs. 3 and 4), and the therapeutic potential of TMP was indicated by its relieving effects on T2D. In both OGTT and ITT, in rats treated with the highest dose of TMP more prominent insulin sensitising effect was observed in comparison to the rats treated with the lower dose.

HFD causes insulin resistance, and STZ leads to the selective pancreatic β cells destruction that ultimately induces the free radicals generation through excess glucose oxidation, the non-enzymatic glycation of proteins and subsequential glycated proteins oxidative degradation, consequently, the HbA1c level increases [35]. In this investigation, TMP treatment significantly lowered (Fig. 5) the level of HbA1c in diabetic rats, and it may be due to the reduction of insulin resistance at the major sites of insulin action.

There is always a high risk of dyslipidemia and cardiovascular disorder in patients with pre-diabetes and diabetes [36]. It is well recognised that patients with T2D are more procumbent than the rest of people to be dyslipidemic. The normalisation of lipid profile including the increase in HDL level plays a crucial role in reducing the risk of cardiovascular disease in T2D [37,38]. In this experiment, TMP treatment significantly lowered the level of TC, TG, LDL and VLDL and raised the HDL level in diabetic rats (Fig. 6A-E) indicating that TMP is a prominent lipid-normalising agent.

The type 2 diabetes is considered as an auto-inflammatory disease as the rise in blood glucose level leads to an increase in the level of pro-inflammatory cytokines such as IL-6 and CRP. The elevated levels of IL-6 and CRP are fundamentally associated with the increased risk of T2D as these cytokines have a potentiating effect on insulin resistance. These cytokines through their insulin signalling pathways interaction contribute to the physiology and pathology of T2D [39,40]. The results of this study demonstrated that TMP has a dose-dependent anti-inflammatory activity through the reduction of IL-6 and CRP cytokine

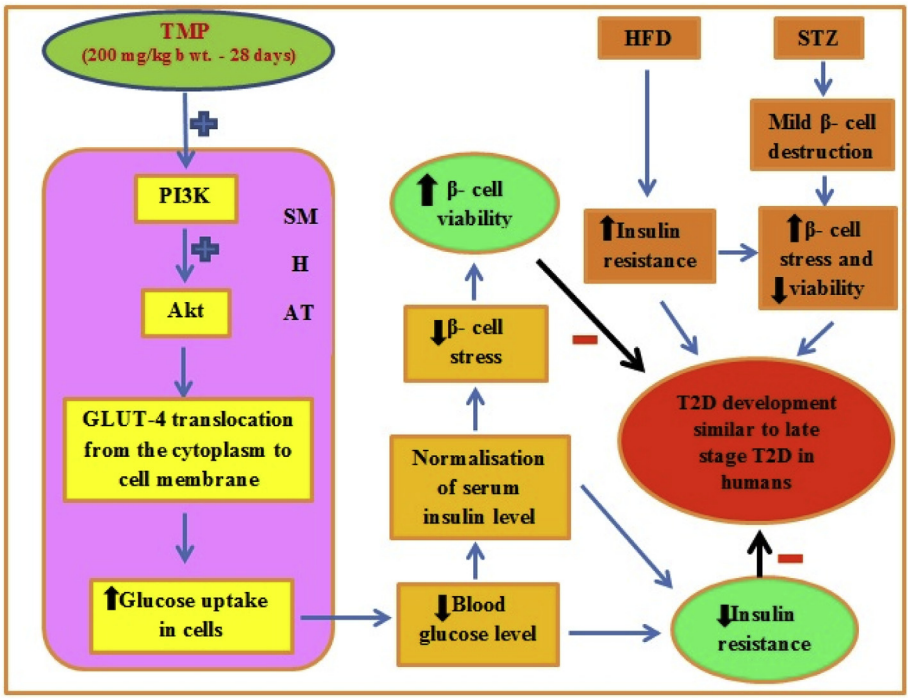

Fig. 10. Illustration of the mechanism of action of TMP in amelioration of HFD-STZ-induced T2D in rats. Abbreviations: TMP, tetramethylpyrazine; PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; GLUT-4, glucose transporter-4; HFD, high-fat diet; STZ, streptozotocin; T2D, type-2 diabetes; SM, skeletal muscle; H, heart; AT, adipose tissue; ↑, increase; ↓, decrease; +, activation; −, inhibition.

levels (Fig. 7) which may be the result of the normalised glycemic condition due to insulin sensitisation.

Amid the insulin signalling pathways that are essential for the amelioration of resistance associated with insulin, the PI3K pathway is a primary glucose transport systems and glucose uptake pathway. Naturally, the phosphorylation of insulin receptor substrate-1 (IRS-1) stimulates the phosphorylation and activation of the p85 subunit of phosphatidylinositol-3-kinase (PI3K), which provokes insulin-induced transport of glucose [41]. The results in our experiment indicated that TMP treatment significantly increased the expression of p-PI3K-p85 (Fig. 8 A-a, B-a and C-a) at the highest dose in all tissue samples suggesting the activation of afterwards proteins in this pathway such as Akt and GLUT-4 and improvement in insulin sensitisation.

Akt is usually known as one of the primary effectors of PI3K-induced cell signalling and, extracellular stressors like oxidative stress, Ca^{2+} influx and growth factors are the major determinants of PI3K guided activation of Akt. Numerous downstream signalling pathways such as those that stimulates glucose metabolism, protein synthesis and cellular proliferation and, inhibits the apoptosis are mainly regulated by Phosphorylated Akt (p-Akt) [42]. In this study, the expression of p-Akt was found to be significantly elevated (Fig. 8 A-b, B-b and C-b) in all three types of tissue samples in TMP 200 mg/kg treated diabetic rats compared to diabetic control. Thus, this experiment indicated the confirmatory results on p-PI3K-p85 and p-Akt, which showed that TMP may be a promising candidate with therapeutic efficiency in this signalling pathway activation.

The relative levels of expression of GLUT-4 protein were portrayed to illustrate the glucose metabolic mechanism in diabetic rats. The facilitative GLUTs forms the association of deeply connected membrane proteins, and there are numerous isoforms with similar homology of the sequence. Amid these, GLUT-4 performs a crucial act in preserving glucose homeostasis through PI3K/Akt signal pathway [43]. GLUT-4 is the primary insulin-regulated glucose transporter and is expressed mainly in adipose tissues, heart and skeletal muscle [44]. In this experiment, the expression of GLUT-4 was increased significantly in the highest dose TMP treated diabetic rats in comparison to diabetic control in all types of tissues (Fig. 8 A-c, B-c and C-c). These results indicated that TMP could improve the level of GLUT-4 at the highest dose. Therefore, it would be a plausible contender to restore glucose homeostasis.

The PI3K signal pathway is the principal glucose uptake and glucose transport systems pathway [41]. Activation of PI3K and Akt are the crucial steps in insulin-mediated action, where Akt acts as a mediator in the signal pathway of glucose uptake, which is regulated in fat, muscle and heart tissues by insulin [45,46]. From the results, it was also proved that Akt activation eventualise after the activation of PI3K as in the rats treated with wortmannin (putative PI3K inhibitor) the expressions of p-Akt and GLUT-4 were also diminished along with p-PI3K-p85, showing that PI3K was the very first target protein of this pathway whose activation leads to the activation of Akt and lastly GLUT-4. Glucose transporter activation is a major episode in the insulin signalling cascade that promotes efficient disposal of glucose into peripheral tissues [47]. In this experiment, the expressions of p-PI3K-p85/p-Akt/GLUT-4 were increased after the administration of the highest dose of TMP (200 mg/kg). Accordingly, the results displayed that the PI3K/Akt signalling pathway activation leads to the intracellular GLUT-4 translocation to the surface of the cell membrane, resulting in increased uptake of glucose and reduction in insulin resistance (Fig. 10). The potential hypoglycemic mechanism of TMP on HFD-STZ-induced diabetic rats was through the up-regulation of PI3K/Akt/GLUT-4 signalling pathway. The activation of the PI3K/Akt signalling pathway by TMP as detected through protein expression analysis was also confirmed through relative mRNA expression study of the corresponding genes (Fig. 9 A, B and C). Accordingly, it can be stated that TMP at the highest dose (200 mg/kg) produced potent stimulation of PI3K/Akt/GLUT-4 signalling pathway as the genetic expression was analogous to corresponding protein expression.

5. Conclusion

The antidiabetic activity of TMP on the high-fat diet and streptozotocin-induced type-2 diabetes in rats and its plausible mechanism via PI3K/Akt/GLUT-4 signalling pathway were first time explored in this experiment. Our results demonstrated that the TMP has dose-dependent hypoglycemic activity as determined through various biochemical parameters. Also, TMP (200 mg/kg) could up-regulate the expression of the crucial PI3K/Akt signal pathway proteins such as p-PI3K-p85, p-Akt and GLUT-4 and it was also confirmed through gene expression analysis

of corresponding genes, indicating that TMP could produce compelling stimulation of this insulin signalling pathway. In conjunction, TMP also reduced the level of pro-inflammatory cytokines such as IL-6 and CRP through insulin sensitisation and glycemic control that lead to the inhibition of inflammation-induced potentiation of insulin resistance. Therefore, it can be stated that TMP produces antidiabetic activity in T2D through PI3K/Akt/GLUT-4 signalling and suppression of inflammation-induced facilitation of insulin resistance. To demonstrate, the prominent clinical applications of TMP further clinical experiments are recommended.

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Declaration of competing interest

The authors declare that there is no conflict of interests.

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