5. Results

5.1. To investigate the anti-diabetic potential of TMP, using HFD-STZ-induced T2D rat model, and to examine the role of PI3K/Akt pathway in the anti-diabetic mechanism of TMP (Objective I).

5.1.1. Effect of TMP treatment on body weight and FBG level of diabetic rats

Tables 5.1 and **5.2** represents the body weight and FBG level of experimental rats on 0 (before treatment), 14, 21 and 28 days after the treatment with TMP. 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.

Table 5.1: 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 ± S.E.M. (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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

Table 5.2: 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 ± S.E.M. (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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

5.1.2. Effect of TMP treatment on FSI level, HOMA-IR and HOMA-B (%) of diabetic rats

On the day 0 the levels of FSI and HOMA-IR in the diabetic control rats, diabetic rats treated with TMP (100, 150 and 200 mg/kg) and TMP 200 mg/kg + W were significantly ($P < 0.05$) increased compared to the normal control rats (**Table 5.3; Figure 5.1A**). 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 (**Figure 5.1B**). In contrast, on the 28th day the significant ($P < 0.05$) increment in the level of HOMA-B (%) was observed in the diabetic rats treated with TMP (100, 150 and 200 mg/kg) in a dose-reliant manner compared to the diabetic control rats.

Table 5.3: Effect of TMP on 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 ± S.E.M. (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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 µg/kg).

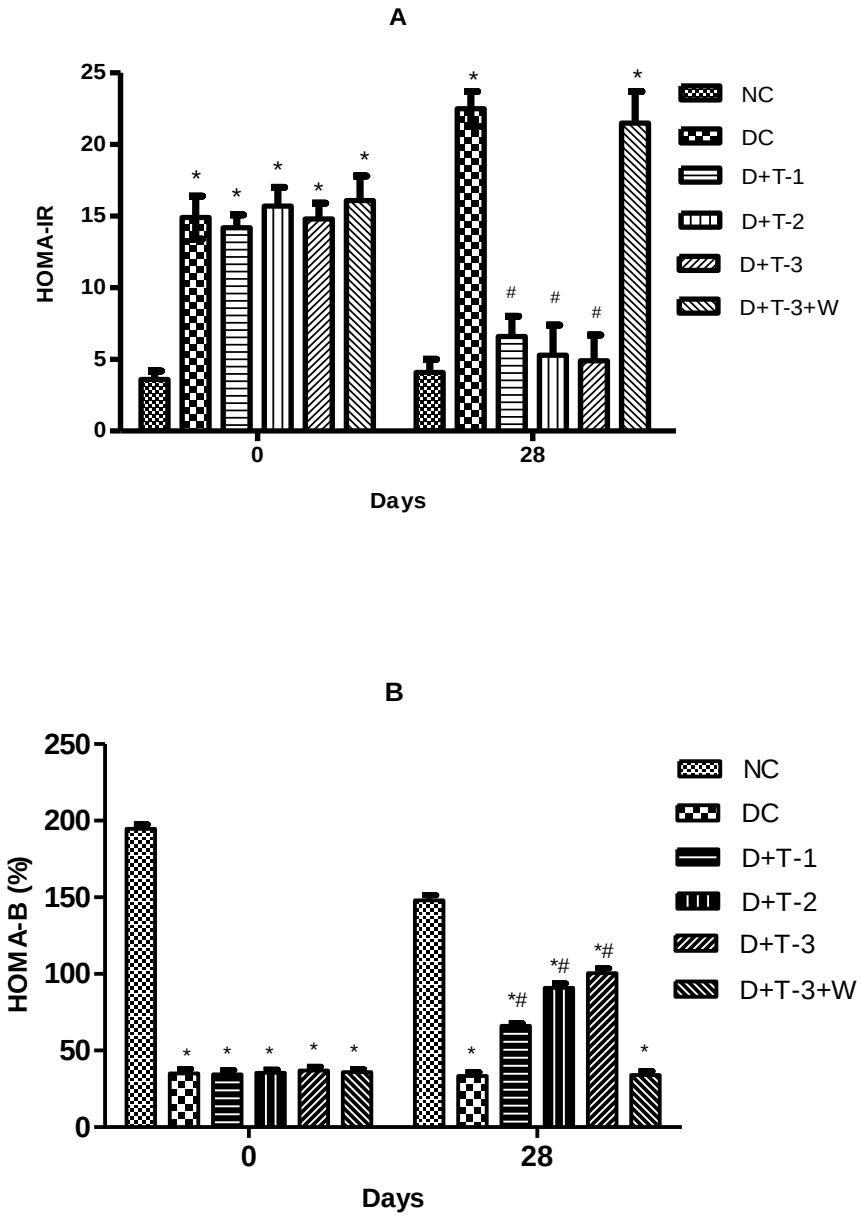


Figure 5.1: Estimated insulin resistance (IR) and β-cell functioning (B%) from the HOMA model. All values are represented as mean ± S.E.M. (n=6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; 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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

5.1.3. Effect of TMP treatment on the level of blood glucose in OGTT and ITT in diabetic rats

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 rats and diabetic rats treated with TMP (200 mg/kg) + W 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 (**Figure 5.2**). Moreover, in ITT, the blood glucose was slightly lowered in diabetic control rats and diabetic rats treated with TMP (200 mg/kg) + W 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 (**Figure 5.3**).

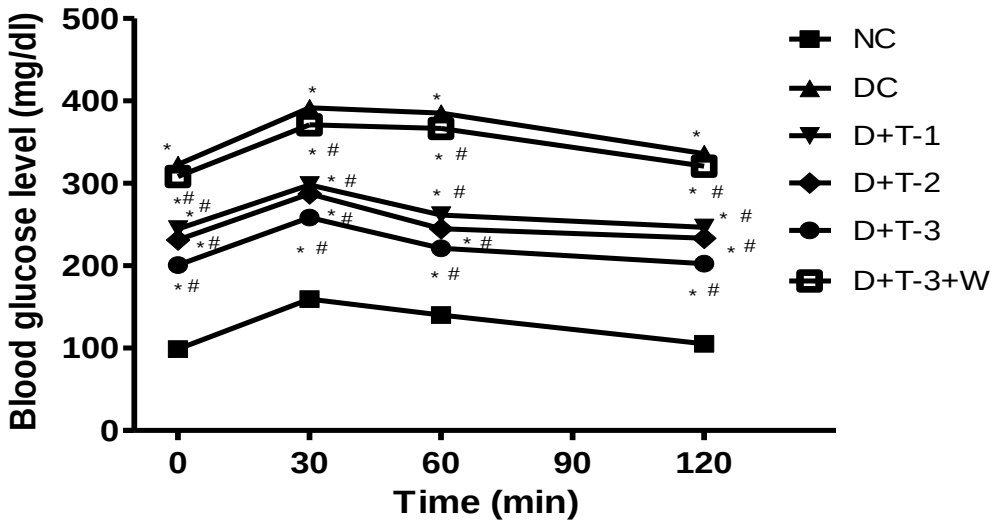


Figure 5.2: Effect of different doses of TMP on blood glucose level in OGTT. All values are represented as mean ± S.E.M. (n=6); Statistical significance was determined by two-way ANOVA followed by Bonferroni post-test, and the significance was set at $P < 0.05$; where * represents significant difference compared to

normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

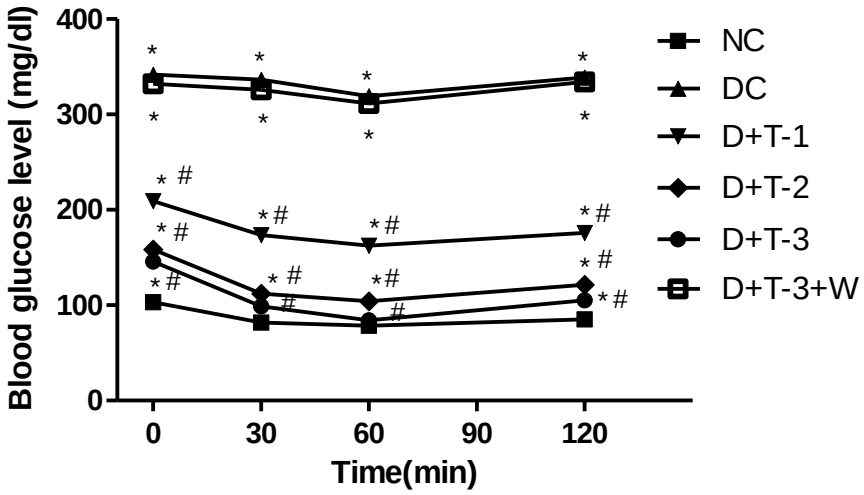


Figure 5.3: Effect of different doses of TMP on blood glucose level in ITT. All values are represented as mean ± S.E.M. (n=6); Statistical significance was determined by two-way ANOVA followed by Bonferroni post-test, and the significance was set at P < 0.05; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

5.1.4. Effect of TMP treatment on the level of glycosylated haemoglobin (HbA1c) in diabetic rats

Figure 5.4 shows the TMP treatment effect on the HbA1c level in HFD-STZ-induced diabetic rats. Diabetic rats, diabetic rats treated with TMP 100 mg/kg and TMP 200 mg/kg + W 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.

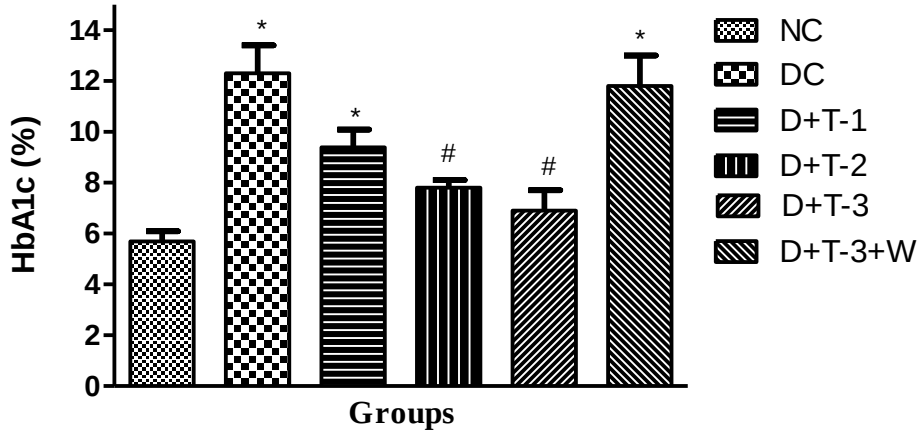
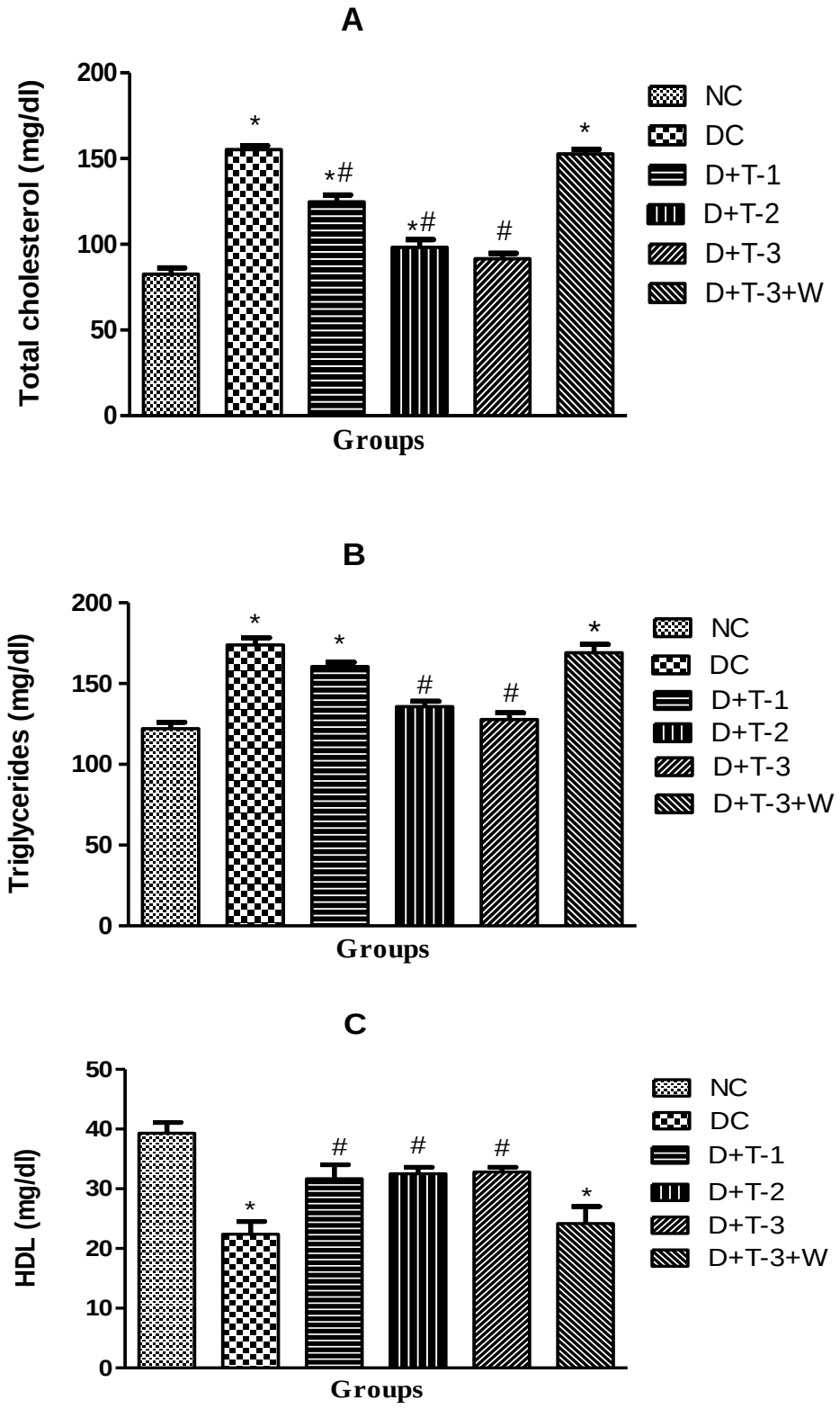


Figure 5.4: Effect of TMP treatment on HbA1c (%) in HFD–STZ-induced diabetic rats. All values are represented as mean ± S.E.M. (n=6); Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance was set at $P < 0.05$; where * represents significant difference compared to normal control, # represents significant difference compared to diabetic control; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

5.1.5. Effect of TMP treatment on serum lipid profile in diabetic rats

As shown in **Figure 5.5A, B and D**, there was a significant ($P < 0.05$) increment in the levels of TC, TG and LDL in diabetic control rats, diabetic rats treated with TMP 100 mg/kg and TMP 200 mg/kg + W compared to the normal control rats. Whereas, significant ($P < 0.05$) decrease in their level was observed in diabetic rats treated with TMP (150 and 200 mg/kg) in a dose-dependent manner compared to the diabetic control rats. The VLDL level was significantly ($P < 0.05$) raised in diabetic control rats and diabetic rats treated with TMP 200 mg/kg + W compared to normal control rats. However, its level was reduced significantly ($P < 0.05$) in TMP (200 mg/kg) treated diabetic rats compared to diabetic control rats (**Figure 5.5E**). In the present study (**Figure 5.5C**), the significant ($P < 0.05$) decrease in HDL level was recognised in the diabetic control rats and diabetic rats treated with TMP 200 mg/kg + W compared to normal control rats 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.



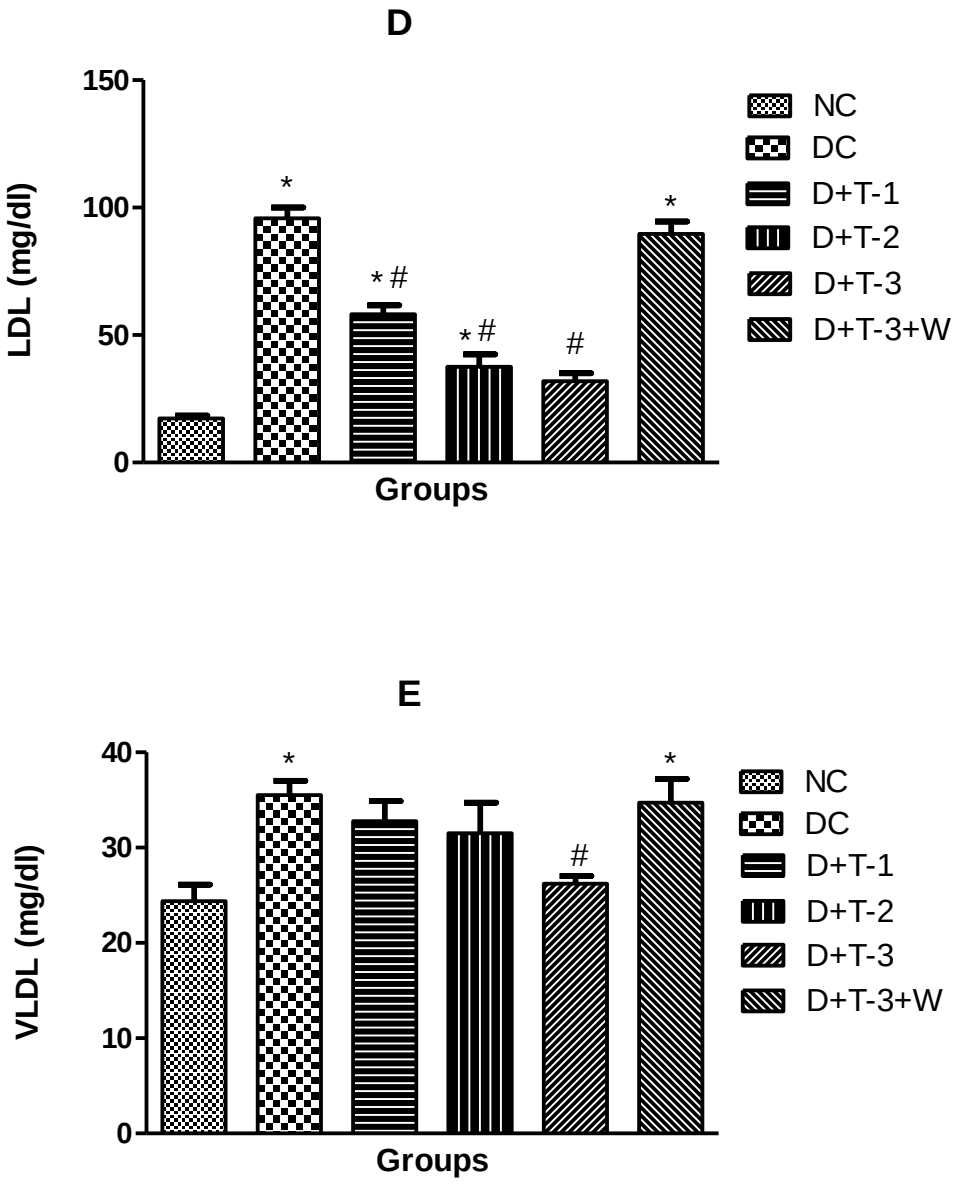


Figure 5.5: Effect of TMP treatment on lipid profile levels in the serum of HFD–STZ-induced diabetic rats; A, total cholesterol (TC); B, triglycerides (TG); C, high-density lipoprotein (HDL); D, low-density lipoprotein (LDL); E, very low-denisty lipoprotein (VLDL). All values are represented as mean ± S.E.M. (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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

5.1.6. Effect of TMP treatment on the level of pro-inflammatory cytokines in diabetic rats

As presented in **Figure 5.6**, the pro-inflammatory cytokines IL-6 and CRP levels were raised significantly ($P < 0.05$) in diabetic control rats, diabetic rats treated with TMP (100 and 150 mg/kg) and diabetic rats treated with TMP 200 mg/kg + W compared to the normal control rats. In contrast, the level of IL-6 was decreased significantly ($P < 0.05$) in diabetic rats treated with TMP (150 and 200 mg/kg) in a dose-dependent manner and the level of CRP was reduced significantly ($P < 0.05$) only in diabetic rats treated with TMP 200 mg/kg compared to diabetic control rats.

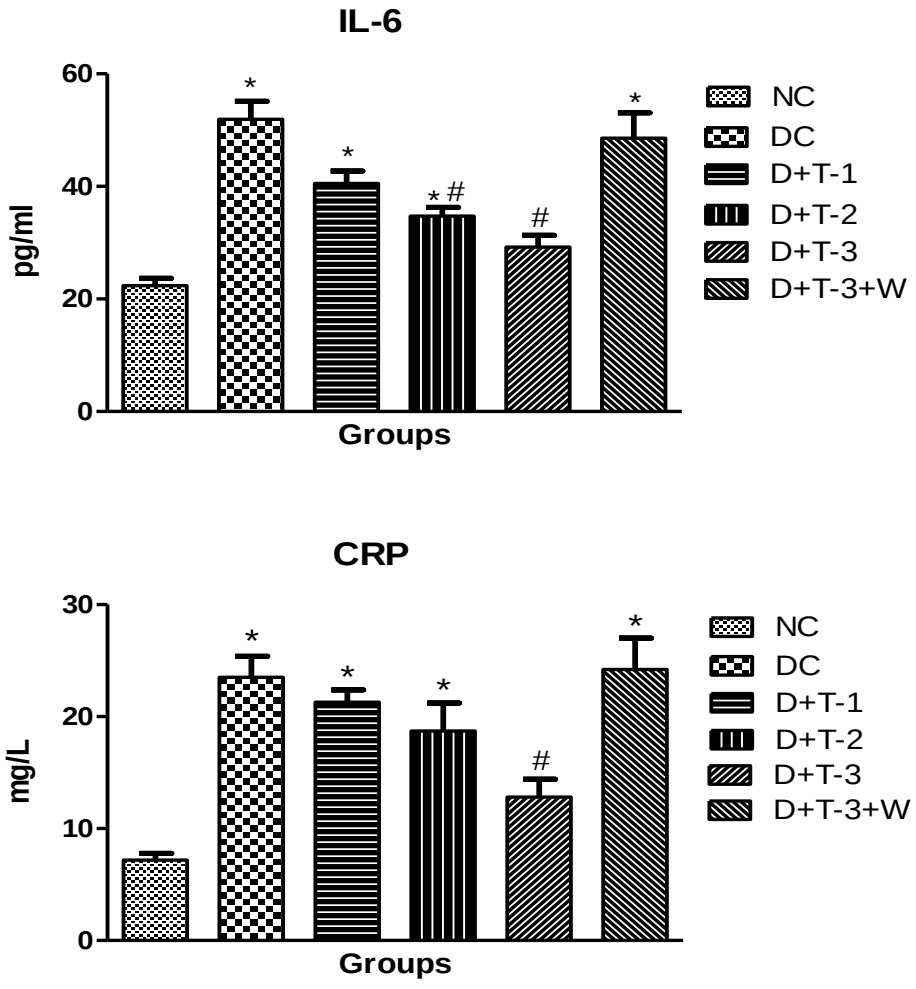


Figure 5.6: Effect of TMP treatment on IL-6 and CRP levels in the serum of HFD–STZ-induced diabetic rats. All values are represented as mean ± S.E.M. (n=6);

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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

5.1.7. Western blotting analysis

5.1.7.1. TMP has increased the expression of the p-PI3K-p85 protein in HFD–STZ-induced diabetic rats

As displayed in **Figure 5.7A-a, B-a** and **C-a**, the expression of p-PI3K-p85 was reasonably decreased in diabetic control rats and diabetic rats treated with TMP 200 mg/kg + W compared to the normal control rats 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 the diabetic group receiving TMP 200 mg/kg + W 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 rats treated with TMP 200 mg/kg compared to the diabetic control rats in all the three types of tissues.

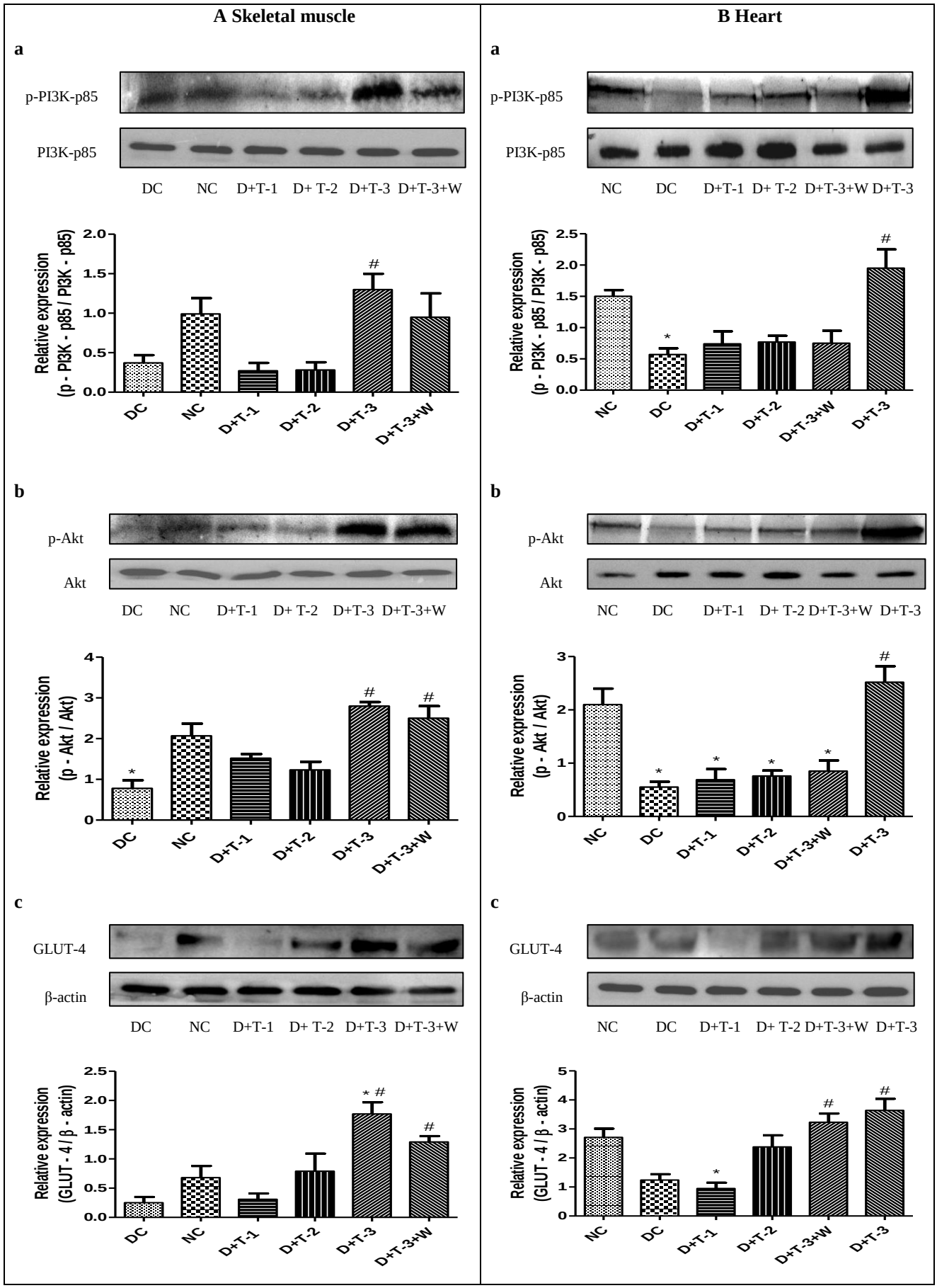
5.1.7.2. TMP has increased the expression of the protein p-Akt in HFD–STZ-induced diabetic rats

As exhibited in **Figure 5.7A-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 rats and diabetic rats treated with TMP 200 mg/kg + W compared to normal control rats. However, the expression of p-Akt in all the tissue types was significantly

($P < 0.05$) elevated in diabetic rats treated with TMP 200 mg/kg in comparison to the diabetic control rats.

5.1.7.3. TMP has elevated the expression of the GLUT-4 protein in HFD–STZ-induced diabetic rats

As represented in **Figure 5.7A-c, B-c** and **C-c**, the expression of GLUT-4 in the skeletal muscle and heart was notably reduced in the diabetic control group and in the adipose tissues a remarkable decrease was observed in the diabetic control rats and diabetic rats treated with TMP 200 mg/kg + W compared to the normal control rats. Whereas, the expression of GLUT-4 in the diabetic rats treated with TMP 200 mg/kg was significantly ($P < 0.05$) elevated compared to the diabetic control rats in all the tissue forms.


A Skeletal muscle
B Heart
a
p-PI3K-p85
PI3K-p85
DC NC D+T-1 D+ T-2 D+T-3 D+T-3+W
NC DC D+T-1 D+ T-2 D+T-3+W D+T-3
Relative expression (p - PI3K - p85 / PI3K - p85)
b
p-Akt
Akt
Relative expression (p - Akt / Akt)
c
GLUT-4
β-actin
Relative expression (GLUT - 4 / β - actin)

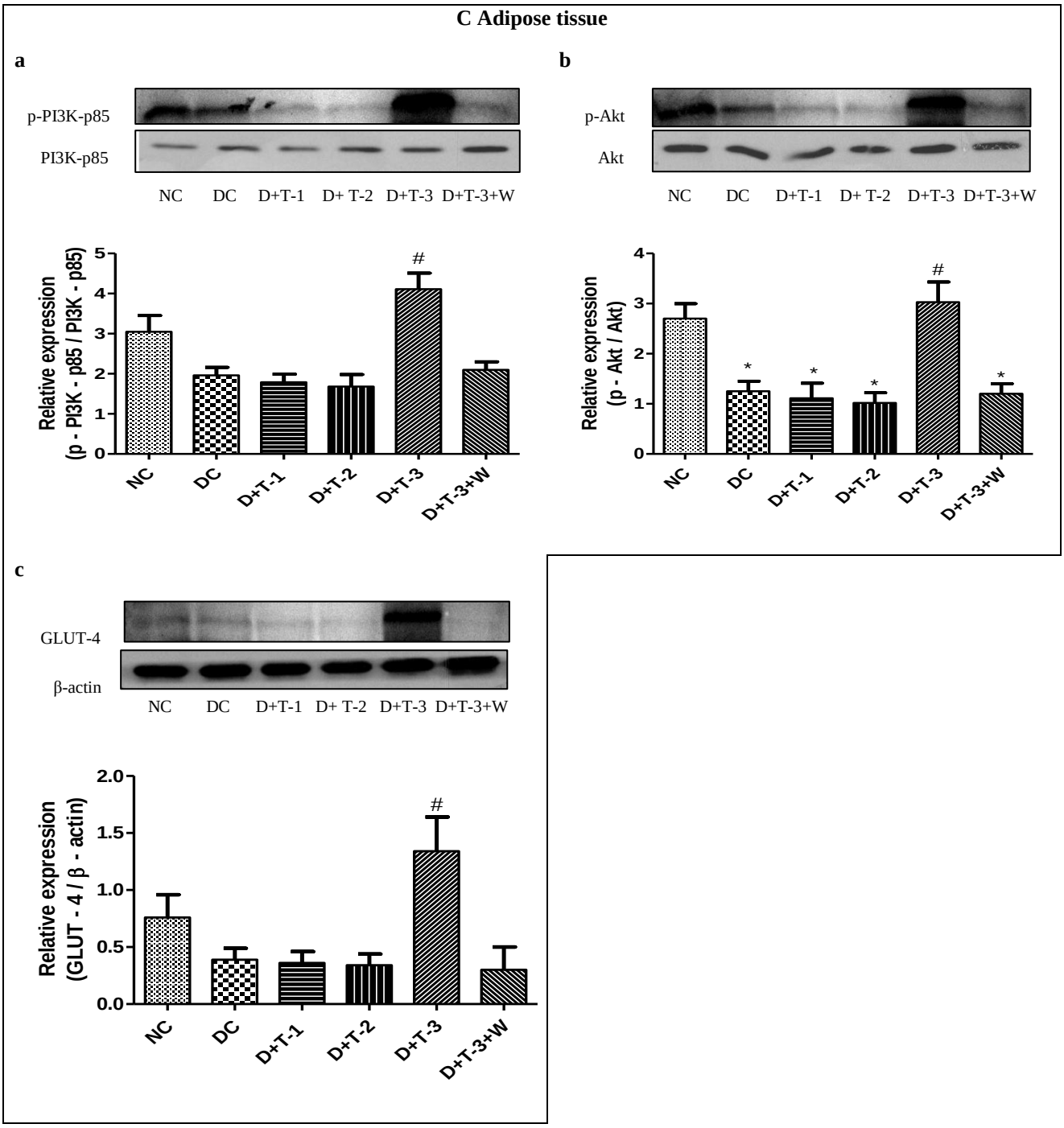
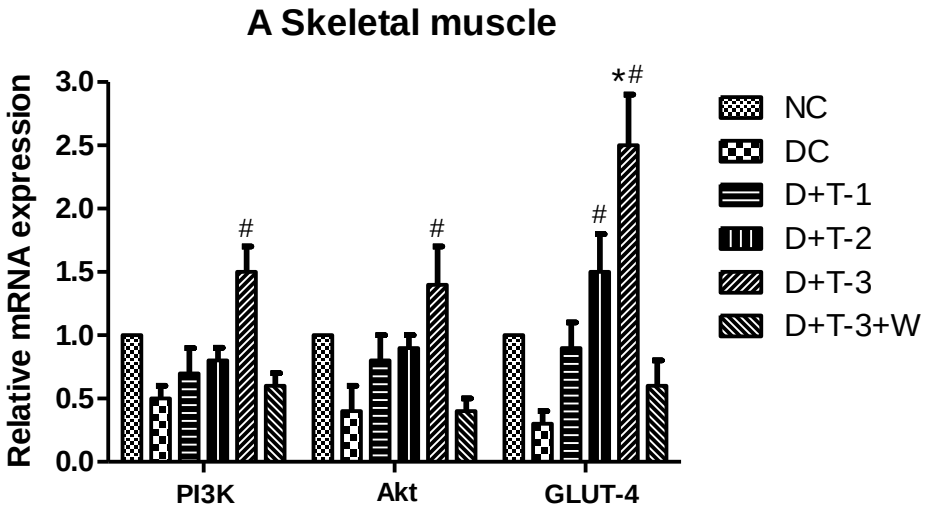


Figure 5.7: Analysis of the effect of TMP administration on the expression of the insulin signalling pathway proteins PI3K, Akt and GLUT4 in the skeletal muscle, heart and adipose tissue of HFD–STZ-induced diabetic rats through western blotting; a relative expression of p-PI3K-p85, b Relative expression 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 ± S.E.M., 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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

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

From the results presented in **Figure 5.8A, B** and **C**, the relative mRNA expression of PI3K in skeletal muscle, heart and adipose tissue was significantly ($P < 0.05$) increased in diabetic rats treated with TMP 200 mg/kg compared to the diabetic control rats. Whereas, the relative mRNA expression of Akt was significantly ($P < 0.05$) elevated in diabetic rats treated with TMP 200 mg/kg compared to the diabetic control rats 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 rats treated with TMP (150 and 200 mg /kg) and in contrary, its expression in heart tissue was significantly ($P < 0.05$) marked up only in TMP 200 mg/kg treated group compared to diabetic control group.



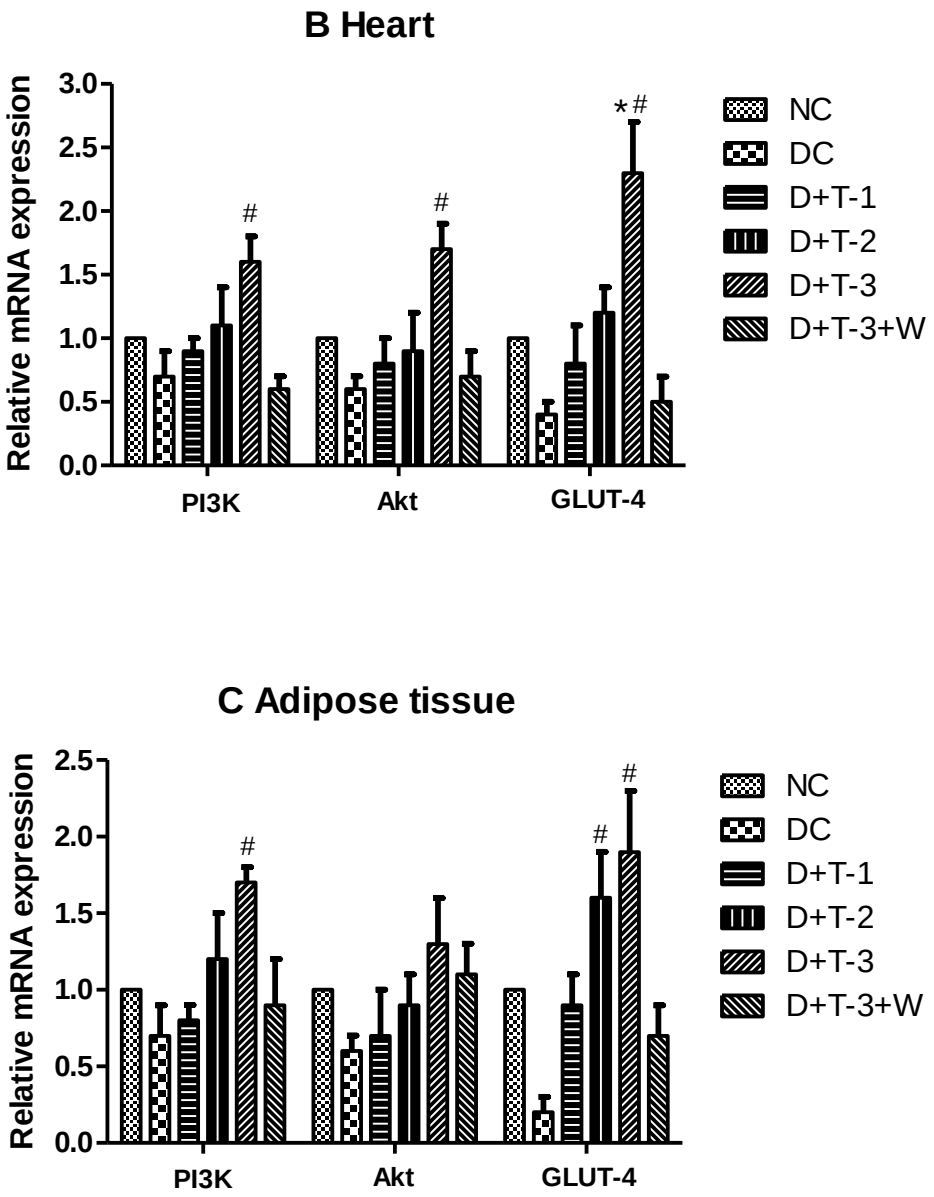


Figure 5.8: Confirmation of the effect of TMP administration on the expression of genes corresponding to the proteins of insulin signalling pathway PI3K, Akt and GLUT4 in the skeletal muscle, heart and adipose tissue of HFD–STZ-induced diabetic rats through qRT-PCR. The relative level of expression for 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 S.E.M. 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 (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; W, wortmannin (15 μg/kg).

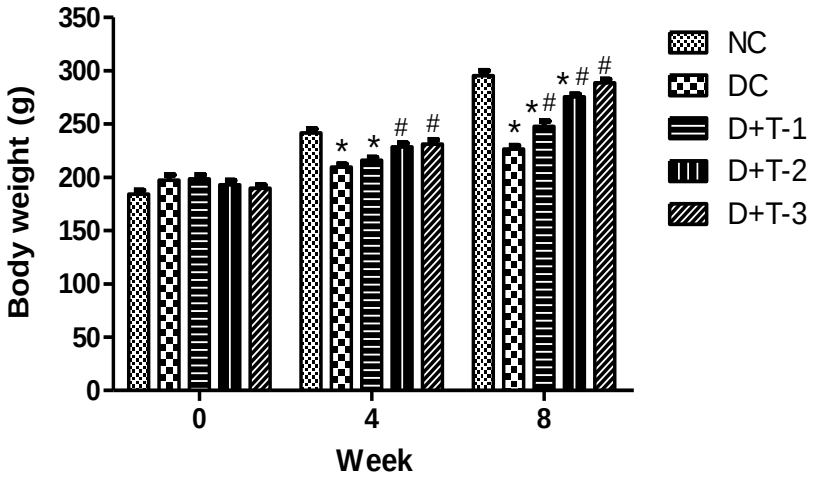
5.2. To explore the protective effect of TMP on DN, using STZ-NCT-induced T2D rat model, and to identify the role of Akt signalling pathway and oxidative stress in providing good therapeutic resolution for DN treatment (Objective II).

5.2.1. The effect of TMP treatment on body weight, FBG, FSI, OGTT, HbA1c and lipid profile in diabetic rats

As shown in **Figure 5.9A**, the body weight of diabetic control rats was observed to be significantly ($P < 0.05$) lower compared to normal control rats at the end of week 4 and 8 during the study. However, there was a significant ($P < 0.05$) rise in body weight of diabetic rats treated with TMP (150 and 200 mg/kg) at week 4 and in diabetic rats treated with TMP (100, 150 and 200 mg/kg) at the 8^{th} week of study in a dose-dependent manner compared to diabetic control rats. FBG level was significantly ($P < 0.05$) elevated in all diabetic treated and untreated groups after diabetes induction just before the initiation of the study (at day 0) compared to normal control rats (**Figure 5.9B**). However, at the end of the 4^{th} week and the 8^{th} week of study, the FBG level was found to be significantly elevated only in diabetic control rats compared to the normal control group. Moreover, the significant ($P < 0.05$) decline in FBG level was observed in diabetic rats treated with TMP (100, 150 and 200 mg/kg) in a dose-dependent manner compared to diabetic control at the end of both 4^{th} and 8^{th} week of the experiment. The FSI level was found to be significantly ($P < 0.05$) reduced in the diabetic control group compared to the normal control group (**Figure 5.9C**). Whereas, its level was significantly ($P < 0.05$) elevated in diabetic rats treated with TMP (150 and 200 mg/kg) in a dose-dependent manner compared to diabetic control rats. In OGTT, after the oral administration of 2g/kg glucose load, the blood glucose level was found to be remarkably increased in all groups after 30 min of glucose administration (**Figure 5.10**), and it was significantly elevated until the end of study (120 min) in the diabetic control

group. In diabetic rats treated with TMP (100, 150 and 200 mg/kg) significant ($P < 0.05$) fall in blood glucose level was observed in a dose-dependent way compared to diabetic control rats after 60 min, and it finally fell to the initial level at the end of investigation period. At the end of the study, the HbA1c level was observed (**Figure 5.11**), and it was significantly ($P < 0.05$) elevated in diabetic control rats compared to the normal control group. However, in diabetic rats treated with TMP (100, 150 and 200 mg/kg) significant ($P < 0.05$) decline in HbA1c level was observed in a dose-dependent manner compared to diabetic control rats. As represented in **Figure 5.12A, B, C** and **D**, the levels of TC, TG, LDL and VLDL were significantly ($P < 0.05$) elevated in the diabetic control group compared to the normal control group. However, significant ($P < 0.05$) decline in TC, TG and LDL levels were observed in diabetic rats treated with TMP (100, 150 and 200 mg/kg) and prominent ($P < 0.05$) decrease in VLDL level was observed only in TMP 150 and 200 mg/kg groups in a dose-dependent way compared to the diabetic control group. The HDL level was significantly ($P < 0.05$) decreased in the diabetic control group compared to the normal control group, and its level was elevated significantly ($P < 0.05$) only in highest dose TMP (200 mg/kg) treated group compared to the diabetic control group (**Figure 5.12E**).

A



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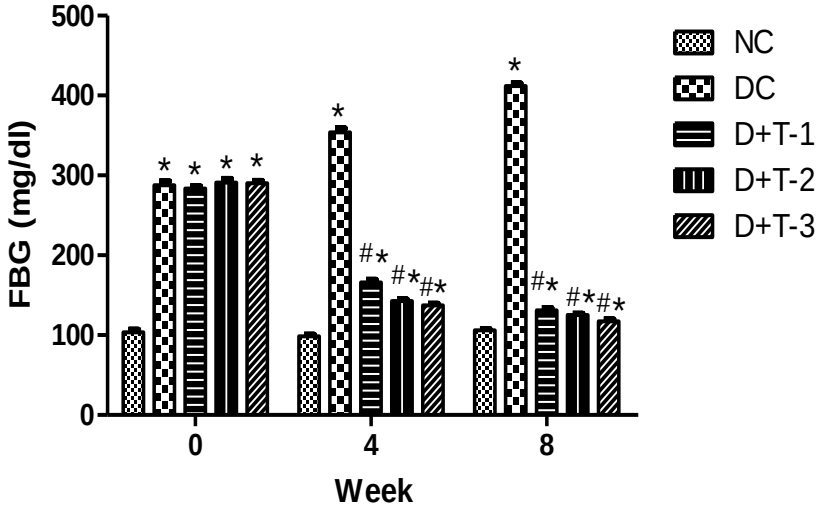

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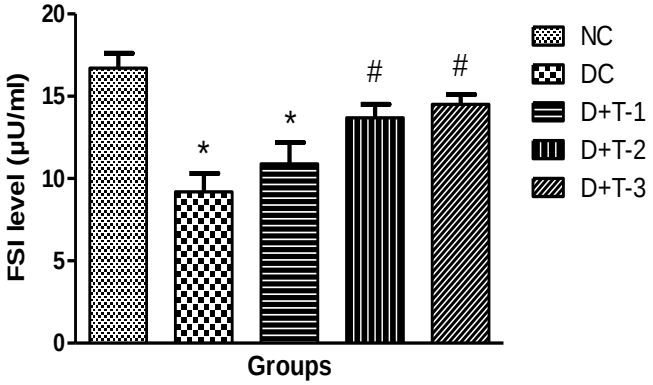


Figure 5.9: Effect of TMP administration on (A) body weight, (B) FBG level and (C) FSI level of experimental rats. All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.

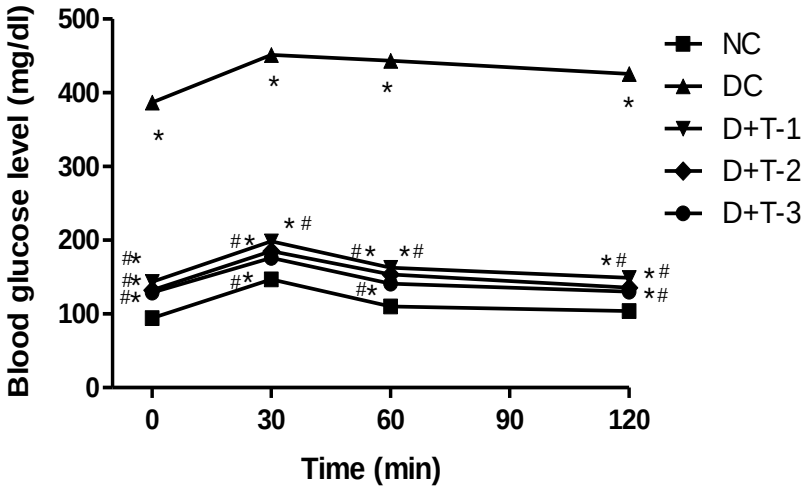


Figure 5.10: Effect of TMP administration on blood glucose level in OGTT of experimental rats. All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by two-way ANOVA followed by Bonferroni post-test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.

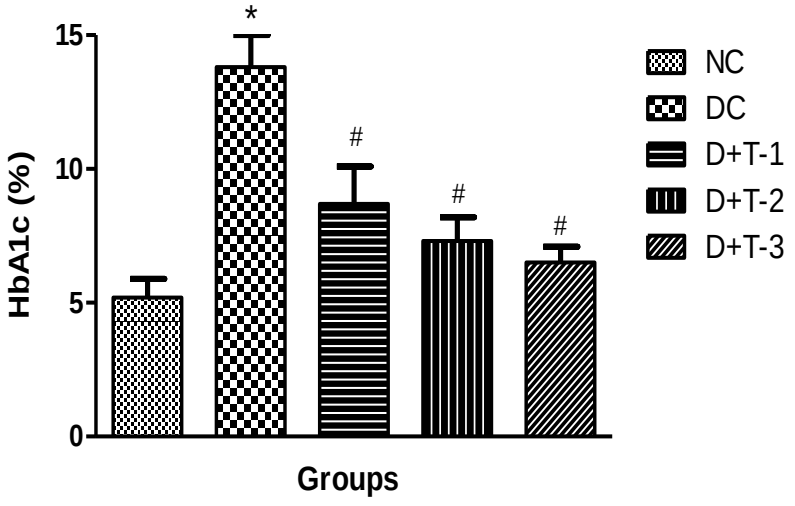
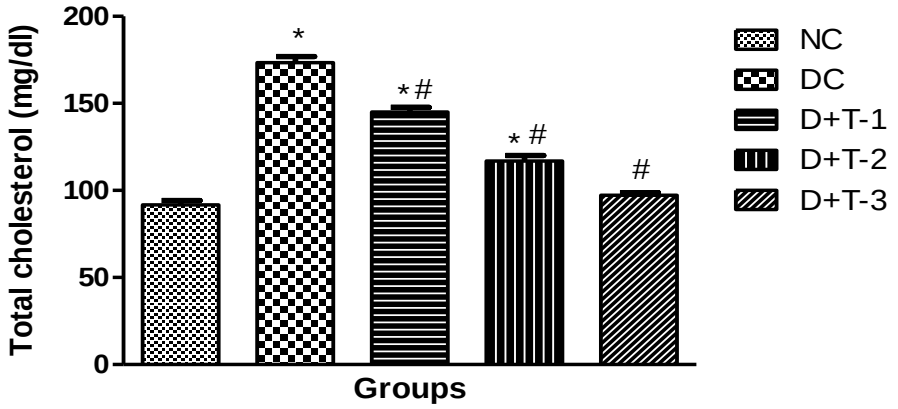


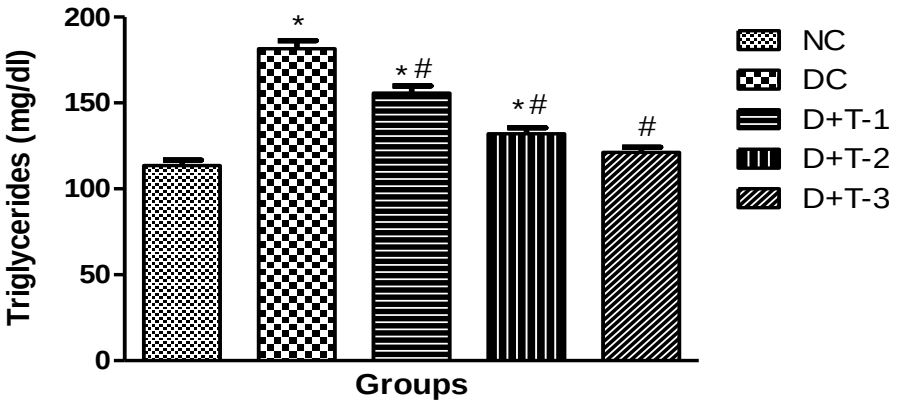
Figure 5.11: Effect of TMP administration on glycosylated haemoglobin (HbA1c-%) level of experimental rats. All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance

compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.

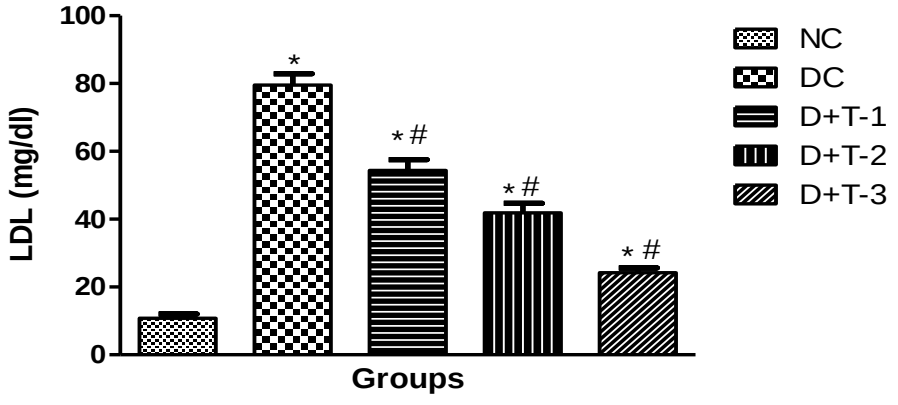
A



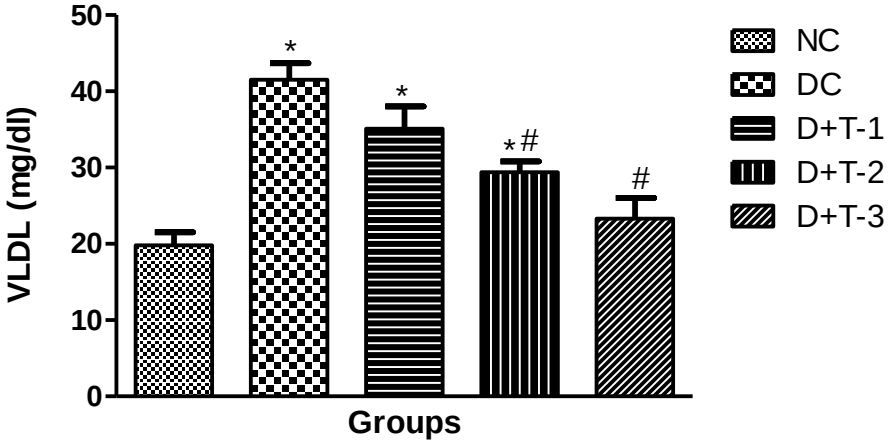
B



C



D



E

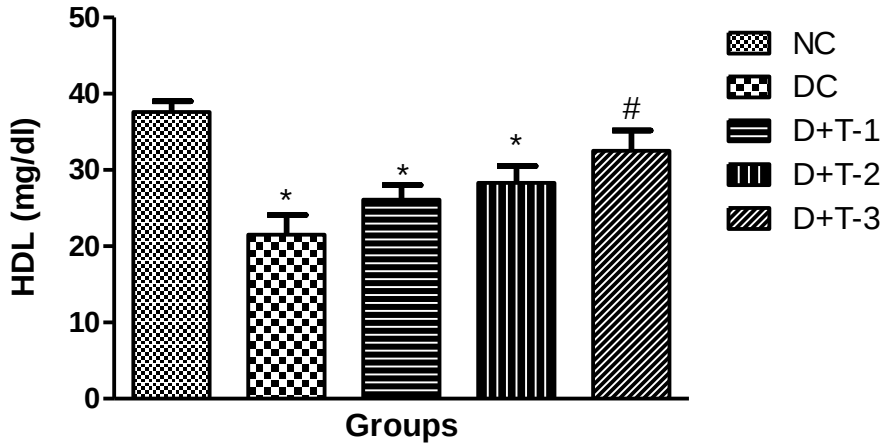
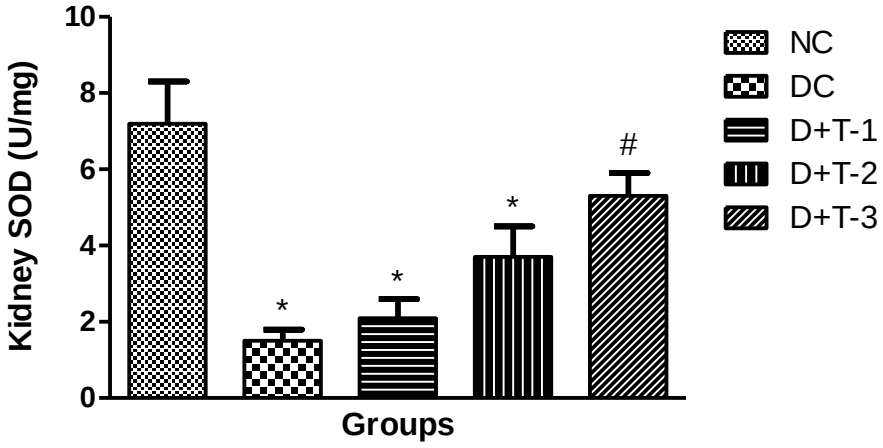


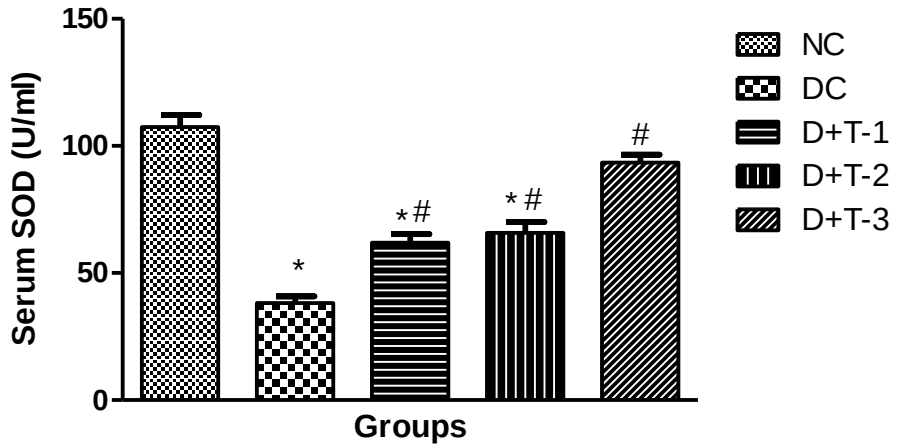
Figure 5.12: Effect of TMP administration on serum lipid profile levels of experimental rats; A, total cholesterol (TC); B, triglycerides (TG); C, low-density lipoprotein (LDL); D, very low-density lipoprotein (VLDL); E, high-density lipoprotein (HDL). All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.

5.2.2. Effect of TMP treatment on the oxidative stress parameters in kidney and serum of diabetic rats

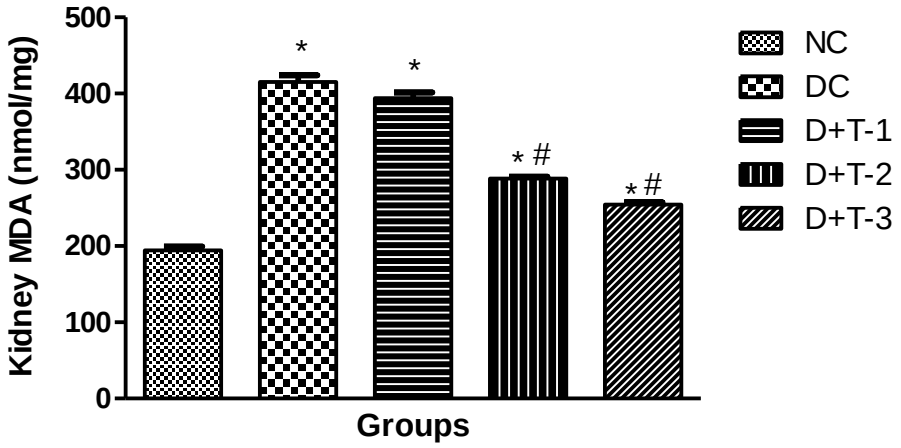
As presented in **Figure 5.13A**, the level of SOD was significantly ($P < 0.05$) reduced in both kidney and serum of diabetic control rats compared to normal control rats. Moreover, the SOD level was significantly ($P < 0.05$) elevated only in the kidney of highest dose TMP (200 mg/kg) treated diabetic rats and in the serum of TMP (100, 150 and 200 mg/kg) treated diabetic rats in a dose-dependent way compared to diabetic control rats. The MDA level was found to be significantly ($P < 0.05$) raised in the kidney and serum of diabetic control rats compared to normal rats (**Figure 5.13B**), and its level was reduced significantly ($P < 0.05$) only in the kidney of TMP (150 and 200 mg/kg) treated diabetic rats dose-dependently compared to diabetic control group. **Figure 5.13C** shows that the level of GSH-Px was significantly ($P < 0.05$) reduced in the kidney and serum of diabetic rats compared to normal rats. However, the level of GSH-Px was significantly ($P < 0.05$) increased in the kidney and serum of diabetic rats treated with TMP (100, 150 and 200 mg/kg) in a dose-dependent way compared to diabetic control rats.

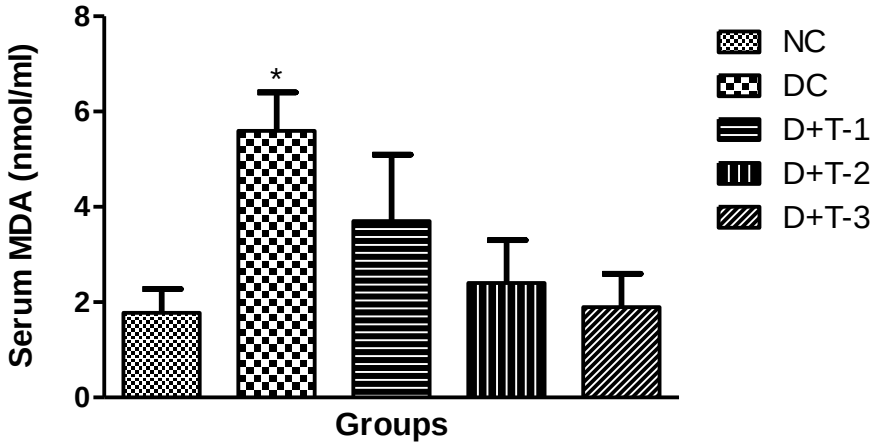
A




150
100
50
0
Serum SOD (U/ml)
*
*#
*#
#
Groups
NC
DC
D+T-1
D+T-2
D+T-3

B


500
400
300
200
100
0
Kidney MDA (nmol/mg)
*
*
*#
*#
Groups
NC
DC
D+T-1
D+T-2
D+T-3


8
6
4
2
0
Serum MDA (nmol/ml)
*
Groups
NC
DC
D+T-1
D+T-2
D+T-3

C

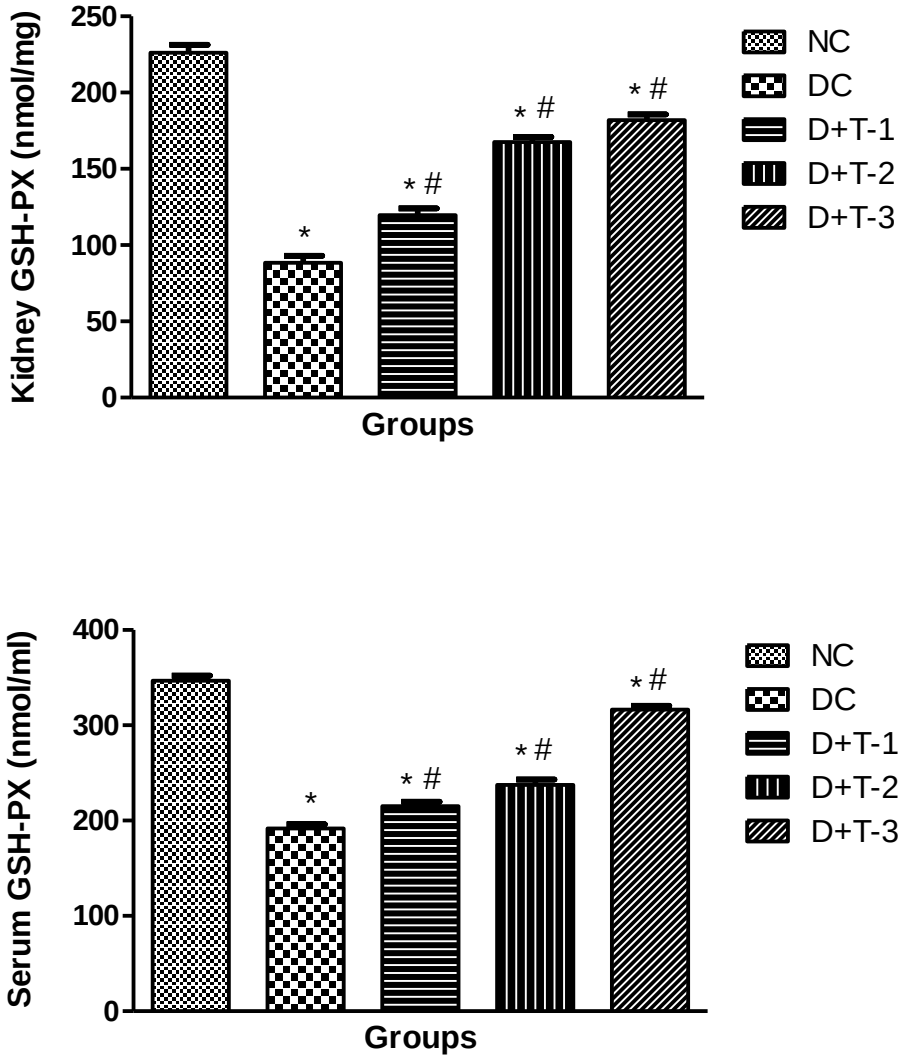
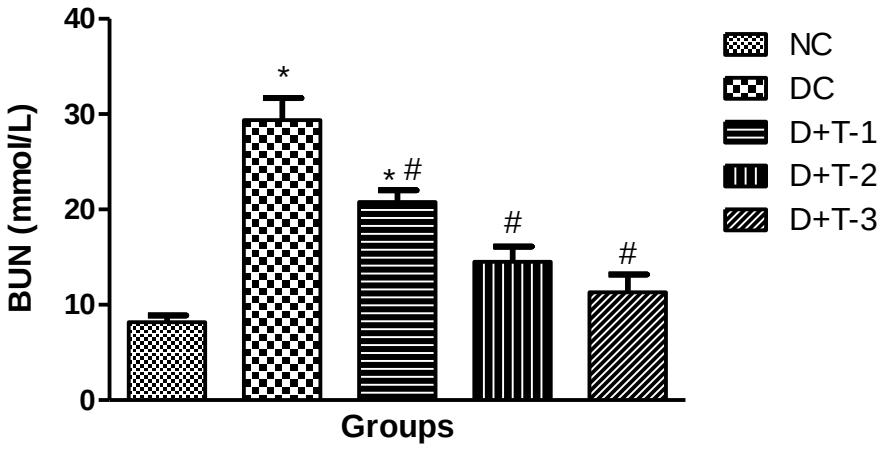


Figure 5.13: Effect of TMP on kidney and serum oxidative stress parameters of experimental rats. A, SOD activity in kidney and serum; B, MDA activity in kidney and serum; C, GSH-Px activity in kidney and serum. All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.

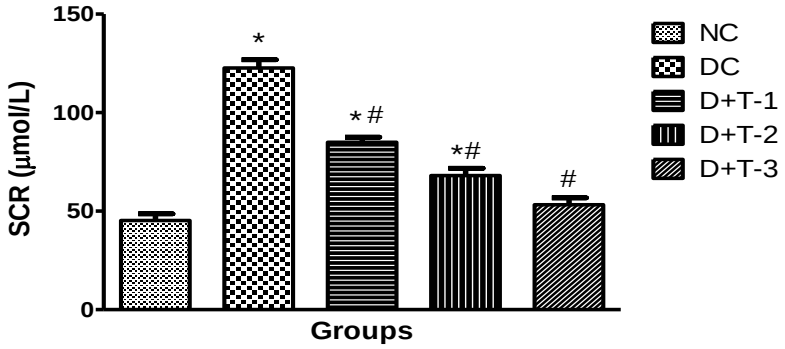
5.2.3. Effect of TMP treatment on renal biochemical markers in diabetic rats

In **Figure 5.14A, B** and **C** it is shown that the levels of BUN, SCR and 24 h urinary protein were significantly ($P < 0.05$) raised in the diabetic control group compared to normal rats. However, the levels of these biochemical indicators were significantly ($P < 0.05$) reduced in TMP 100, 150 and 200 mg/kg treated diabetic rats in a dose-dependent manner compared to diabetic control rats.

A



B



C

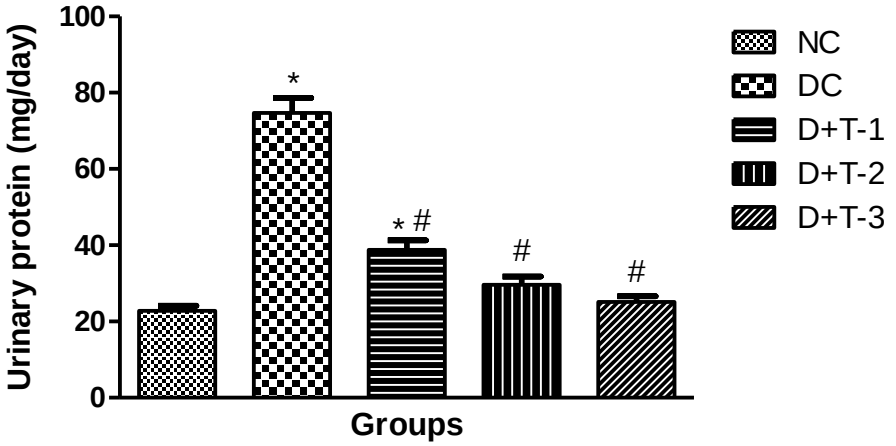


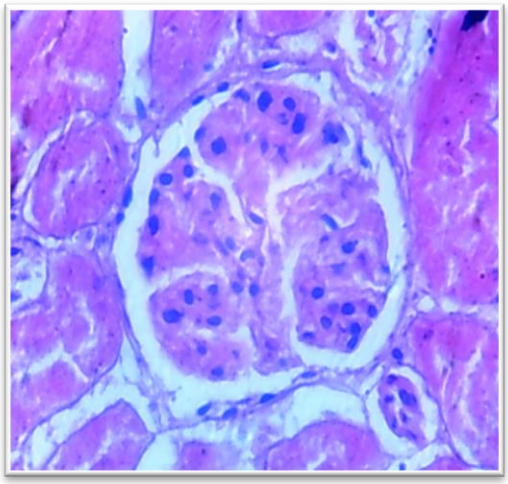
Figure 5.14: Effect of TMP treatment on kidney function parameters of diabetic rats. BUN level (A), SCR level (B) and 24-hour urinary protein content (C) were measured. All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.

5.2.4. Effect of TMP treatment on renal morphology of diabetic rats

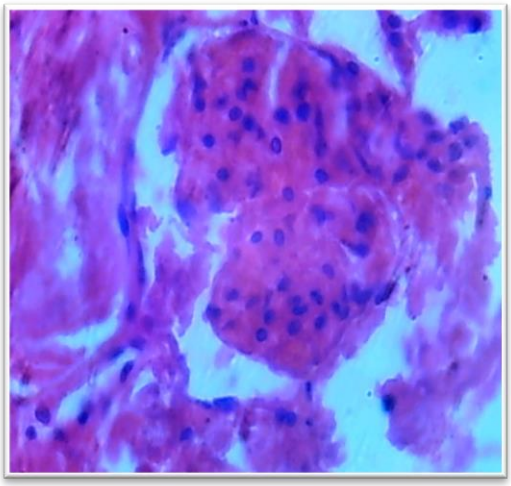
As represented in **Figure 5.15A** HE renal photomicrographs, the mesangial matrix expansion was evident in the glomerulus of diabetic control rats, and TMP treatment clearly reduced the glomerular mesangial matrix expansion. The micrograph of the glomerulus of the highest dose TMP (200 mg/kg) treated diabetic rats was found to be comparable to that of the normal rats. The GMEI score was significantly ($P < 0.05$) elevated in diabetic control rats in comparison to normal rats (**Figure 5.15B**). However, it was reduced significantly ($P < 0.05$) only in the diabetic rats treated with the highest dose of TMP (200 mg/kg) compared to diabetic control rats. From the renal micrographs of **Figure 5.15C**, it can be observed that the tubulointerstitial injury was prominent in diabetic rats and reasonably reduced after treatment with TMP. The TDI

score was significantly ($P < 0.05$) increased in diabetic control rats compared to normal rats (**Figure 5.15D**), and it was reduced significantly ($P < 0.05$) in TMP 150 and 200 mg/kg treated diabetic rats in a dose-dependent way compared to diabetic control rats.

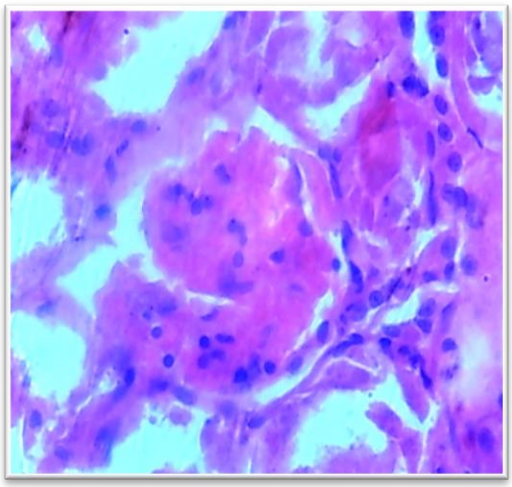
A



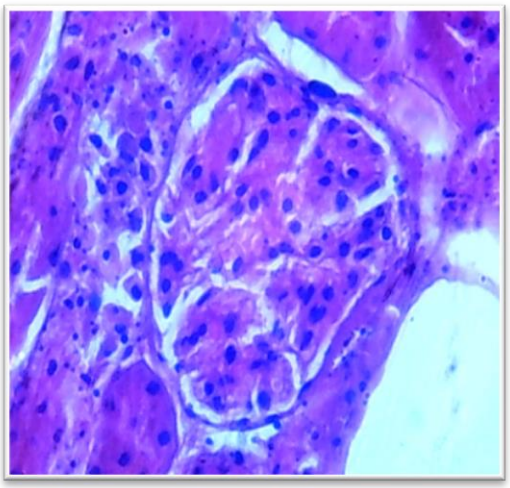
NC



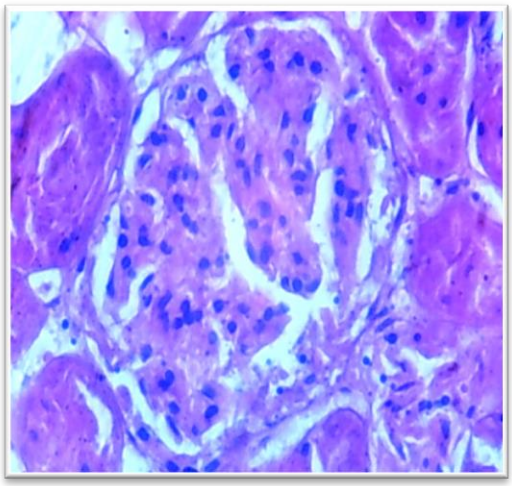
DC



D+T-1

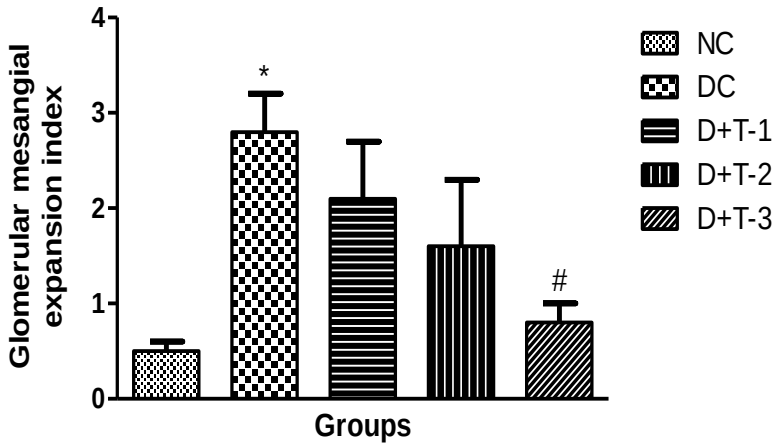


D+T-2

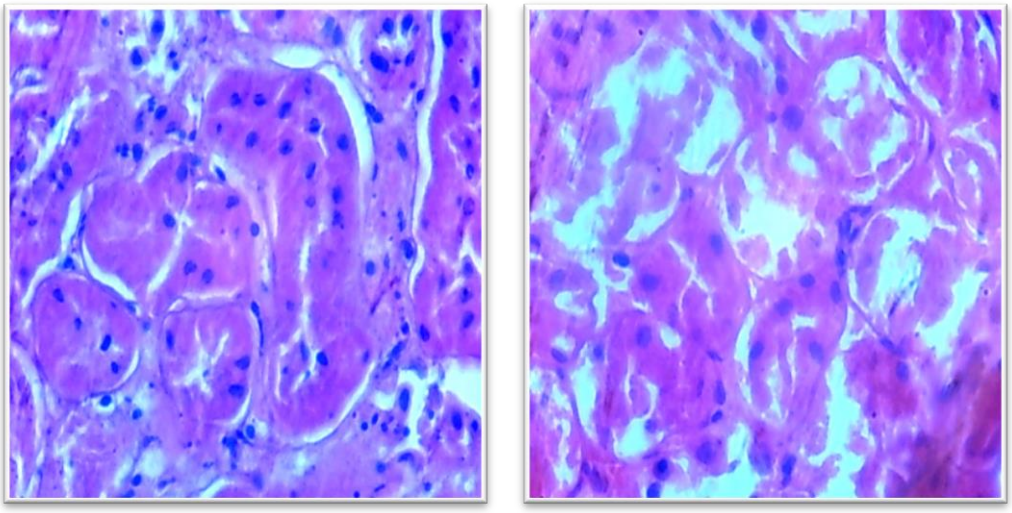


D+T-3

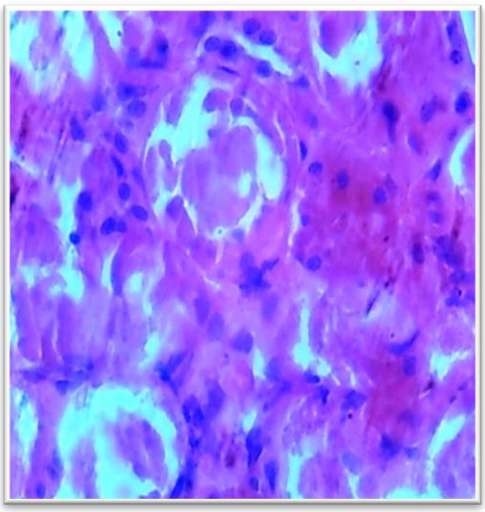
B


Glomerular mesangial expansion index
4
3
2
1
0
*
#
Groups
NC
DC
D+T-1
D+T-2
D+T-3

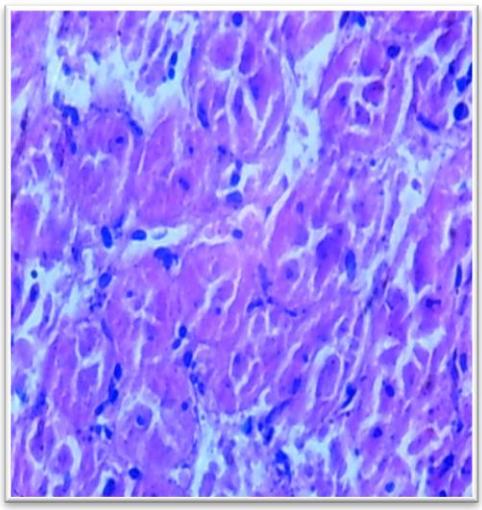
C



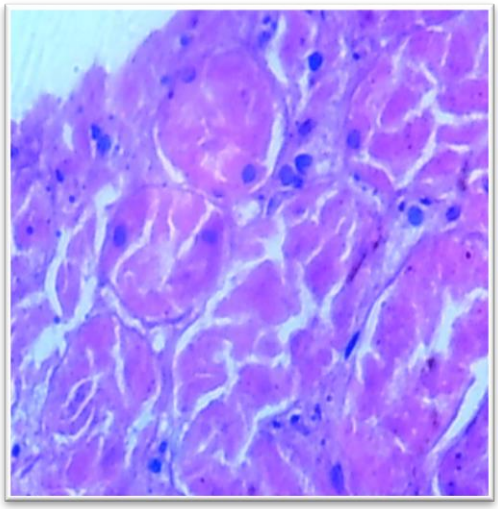
NC **DC**



D+T-1



D+T-2



D+T-3

D

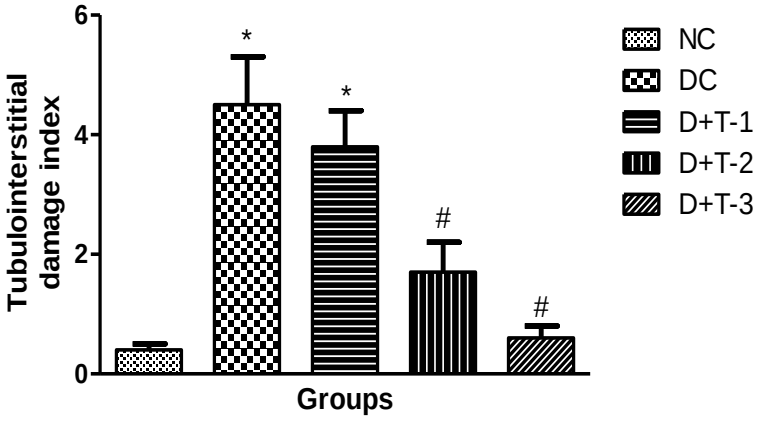
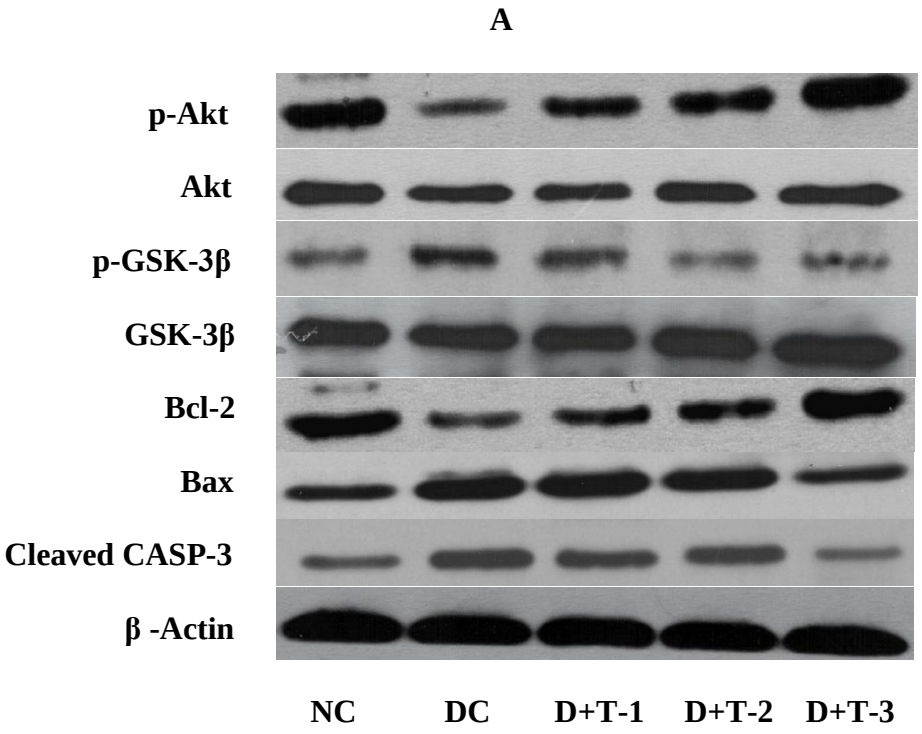

Tubulointerstitial damage index
6
4
2
0
*
*
#
#
Groups
NC
DC
D+T-1
D+T-2
D+T-3

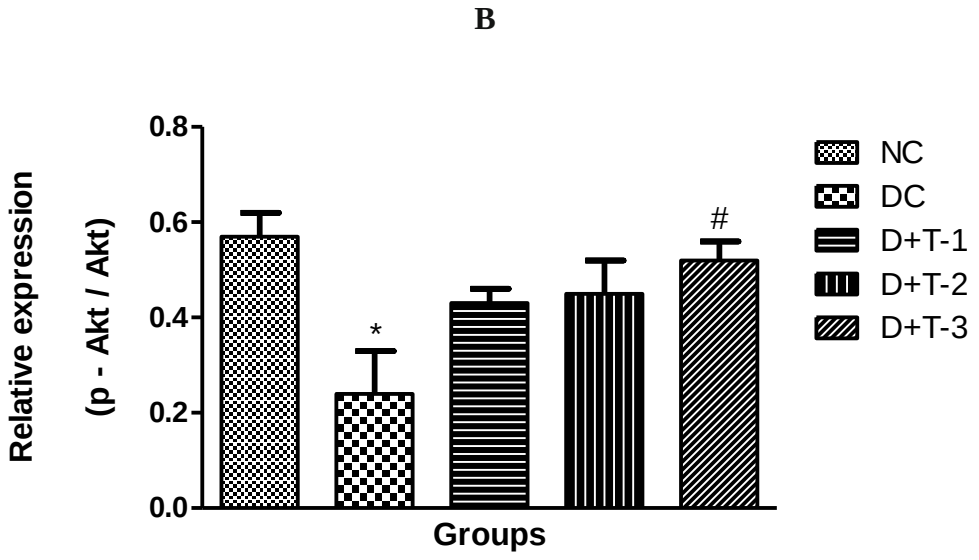
Figure 5.15: Histopathological analysis of TMP treatment effect on renal morphology of diabetic rats. (A) Typical HE staining photomicrograph indicating effect of TMP treatment on glomerular mesangial matrix expansion, scale bar: 50 µm, magnification: X40; (B) Values of GMEI; (C) HE staining photomicrograph showing TMP treatment effect on the tubulointerstitial injury, scale bar: 50 µm, magnification: X40; (D) Values of TDI. All values are expressed as mean ± S.E.M., n=6; Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc test, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC; NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine; HE, hematoxylin and eosin.

5.2.5. Effect of TMP treatment on the expression of Akt signalling pathway proteins in the kidney tissue of diabetic rats

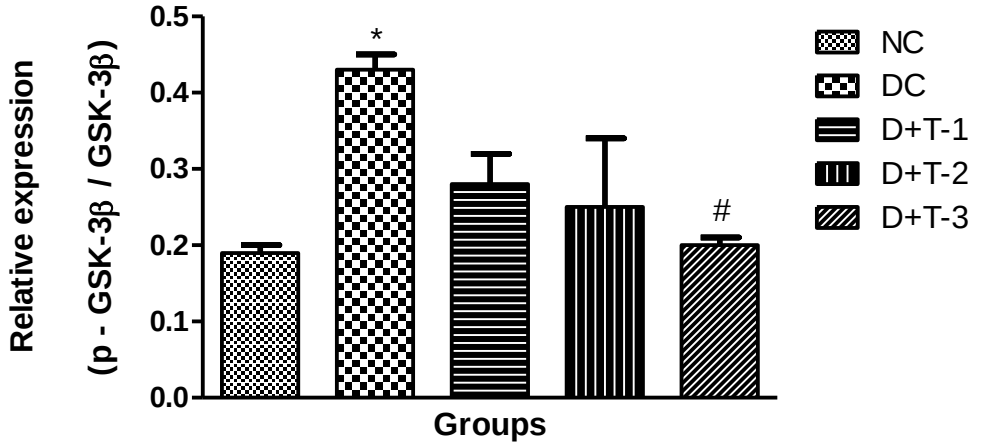
To explore the role of Akt signalling pathway in the TMP mediated DN treatment the expressions of p-Akt, Akt, p-GSK-3β, GSK-3β, Bcl-2, Bax, cleaved caspase-3 and β-actin proteins were observed in the kidney tissues of rats of all groups through western blotting (**Figure 5.16A**). As shown in **Figure 5.16B**, the expression of p-Akt was significantly ($P < 0.05$) reduced in the diabetic control group in comparison to the normal control group. Moreover, the reasonable dose-dependent increase in the expression of p-Akt was observed in all TMP treated groups but the expression of p-Akt was significantly ($P < 0.05$) increased only in TMP 200 mg/kg group compared to diabetic control. The expression of p-GSK-3β was found to be significantly ($P < 0.05$) elevated in diabetic control in comparison to the normal control group (**Figure 5.16C**), and its expression was reduced significantly ($P < 0.05$) in TMP (200 mg/kg) treated diabetic rats compared to diabetic control rats. As presented in **Figure 5.16D**, the expression of Bcl-2 was decreased significantly ($P < 0.05$) in diabetic control rats in comparison to normal rats and significantly ($P < 0.05$) raised in highest dose TMP (200 mg/kg) treated diabetic rats compared to diabetic control rats. The Bax and cleaved caspase-3 expressions were significantly ($P < 0.05$) increased in diabetic rats in

comparison to normal rats. However, their expressions were reduced in the TMP treated diabetic rats in a dose-dependent manner but their expression was significantly ($P < 0.05$) reduced only in TMP (200 mg/kg) treated diabetic rats compared to diabetic control rats (**Figure 5.16E and F**).

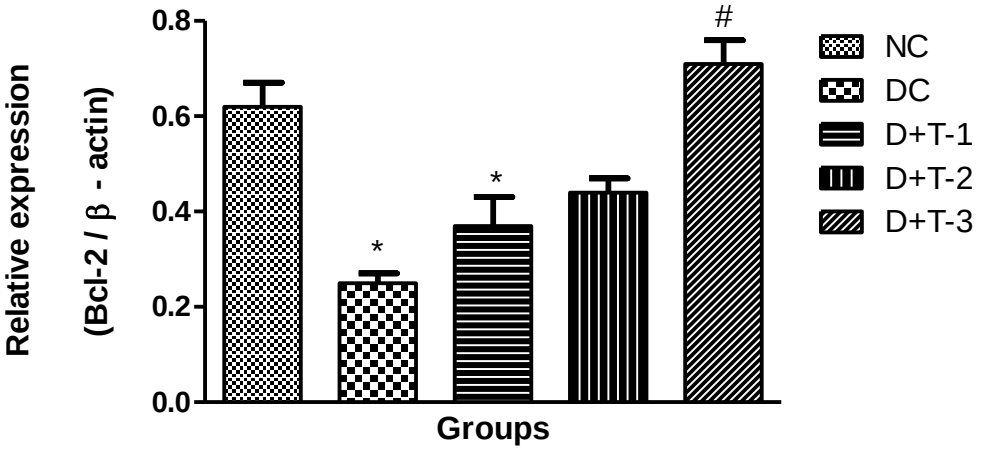




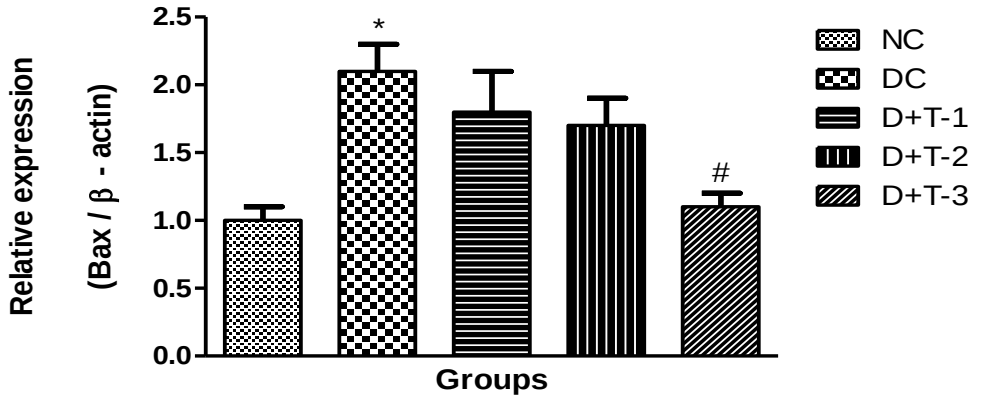
C


Relative expression
(p - GSK-3β / GSK-3β)
0.5
0.4
0.3
0.2
0.1
0.0
*
#
Groups
NC
DC
D+T-1
D+T-2
D+T-3

D


Relative expression
(Bcl-2 / β - actin)
0.8
0.6
0.4
0.2
0.0
*
*
#
Groups
NC
DC
D+T-1
D+T-2
D+T-3

E


Relative expression
(Bax / β - actin)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Groups
NC
DC
D+T-1
D+T-2
D+T-3

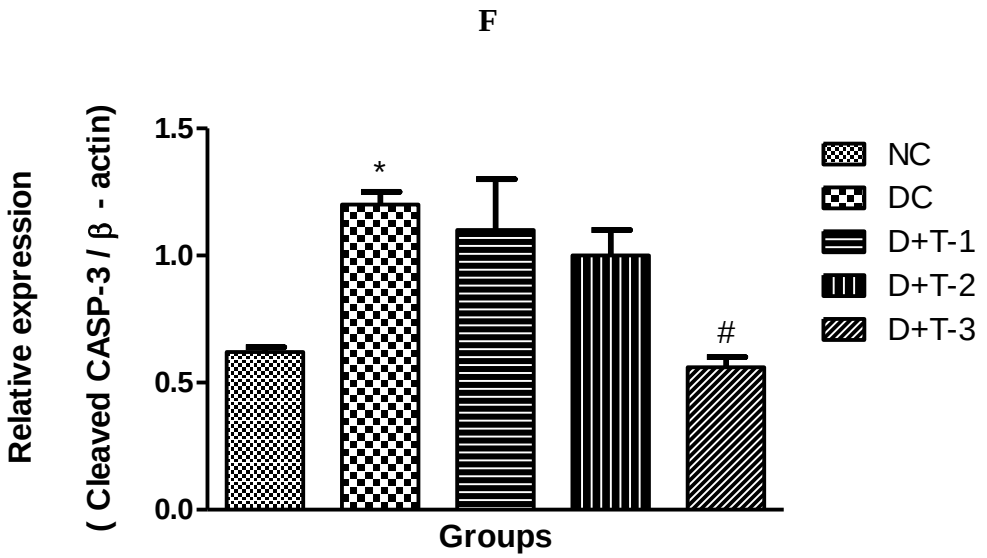


Figure 5.16: Analysis of the TMP treatment effect on the expression of the Akt signalling pathway proteins in the kidney tissue of STZ-NCT-induced diabetic rats through western blotting (A); (B) relative expression of p-Akt, (C) relative expression of p-GSK-3β, (D) relative expression of Bcl-2, (E) relative expression of Bax, (F) relative expression of cleaved caspase-3. Experiments were independently repeated 3 times. The shown blots were the result of one of the investigation. Intensities of the band in each blot were evaluated after normalisation by the corresponding total protein, and the values are presented as mean ± S.E.M., where n = 3 independent experiments. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance level was set at $P < 0.05$; where * denotes statistical significance compared to NC, and # denotes statistical significance compared to DC. NC, normal control; DC, diabetic control; D, diabetic; T-1, TMP (100mg/kg); T-2, TMP (150mg/kg); T-3, TMP (200mg/kg); TMP, tetramethylpyrazine.