6. Discussion

6.1. 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).

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 [93]. 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 fed 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 [94]. 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 [95-97].

The reduction in body weight gain observed in diabetic rats might be the result of structural fats and proteins deterioration due to carbohydrate deficiency for energy metabolism [98]. 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 5.1**). 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 [99]. The anti-hyperglycemic action of TMP may be due to the restoration of insulin sensitivity (**Table 5.2**).

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 experimental procedure [100]. In this study TMP administration significantly reduced insulin resistance and improved β-cell activity compared to diabetic control (**Figure 5.1A** 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 rats and diabetic rats treated with TMP (200 mg/kg) + W through the generation of insulin resistance as displayed in the results (**Figure 5.2** and **5.3**), 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 a 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 [101]. In this investigation, TMP treatment significantly lowered (**Figure 5.4**) 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 [102]. 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 [103, 104]. In this experiment, TMP treatment significantly lowered the level of TC, TG, LDL and VLDL and raised the HDL level in diabetic rats (**Figure 5.5A-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 [105, 106]. The results of this study demonstrated that TMP has a dose-dependent anti-inflammatory activity through the reduction of IL-6 and CRP cytokine levels (**Figure 5.6**) 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 IRS-1 stimulates the phosphorylation and activation of the p85 subunit of PI3K, which provokes insulin-induced transport of glucose [107]. The results in our experiment indicated that TMP treatment (administration) had significantly ($P<0.05$)

increased the expression of p-PI3K-p85 (**Figure 5.7A-a, B-a** and **C-a**) at the highest dose in all tissue samples suggesting the activation of downstream 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) [108]. In this study, the expression of p-Akt was found to be significantly elevated (**Figure 5.7A-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 [109]. GLUT-4 is the primary insulin-regulated glucose transporter and is expressed mainly in adipose tissues, heart and skeletal muscle [110]. 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 (**Figure 5.7A-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 [107]. 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 [111, 112]. 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 [113]. 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 (**Figure 6.1**). 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 (**Figure 5.8A, 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. Although, The PI3K/Akt signalling pathway prominently controls the normal cellular processes involved in cell growth and proliferation but, aberrant activation of this pathway promotes the survival and proliferation of tumour cells [108]. Therefore, this may be the

potential adverse effect in TMP induced PI3K/Akt activation and further studies are required for the proper examination of this adversity.

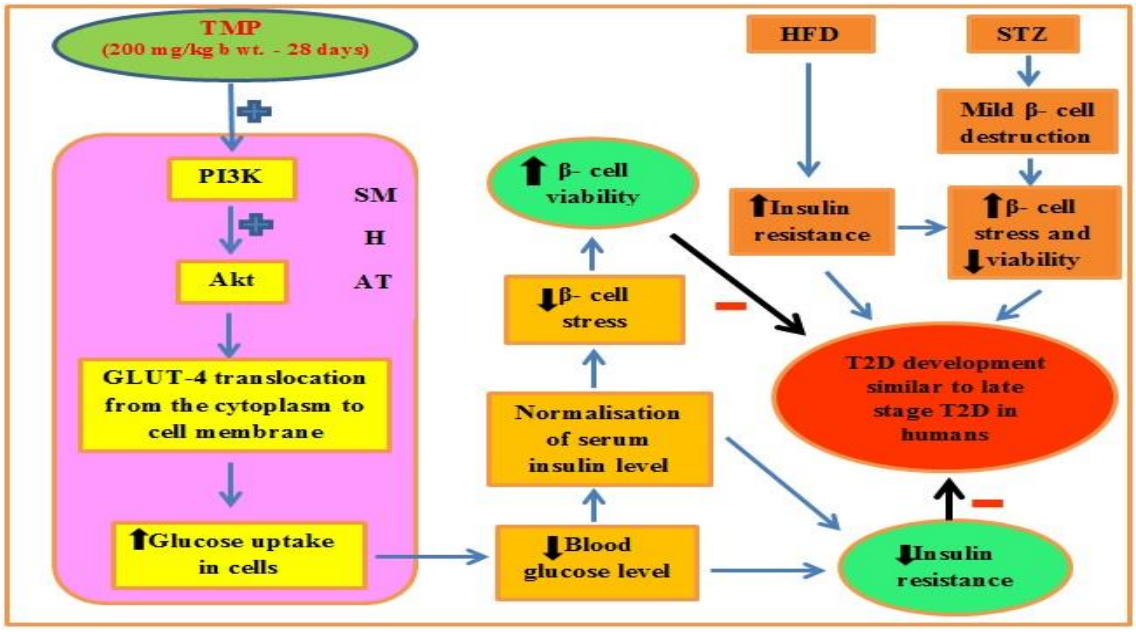

Figure 6.1: Illustration of the mechanism of action of TMP in amelioration of HFD-STZ-induced T2D in rats. Abbreviations: TMP, tetramethylpyrazine; PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; GLUT-4, glucose transporter-4; HFD, high-fat diet; STZ, streptozotocin; T2D, type-2 diabetes; SM, skeletal muscle; H, heart; AT, adipose tissue; ↑, increase; ↓, decrease; +, activation; -, inhibition.

6.2. 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).

An increasing percentage of the world population is affected by diabetes, and one of the major target organs of diabetic complication is kidney [114]. In DN numerous functional and morphological changes occur such as glomerular mesangial matrix expansion, nodular glomerulosclerosis and basement membrane thickening. During the initial phase of DN, hypertrophy of the tubules can be recognised which later develops into tubular atrophy with interstitial fibrosis along with hyalinosis of the arterioles with T- lymphocytes and macrophage infiltration in advanced cases [7].

The TMP treatment considerably improved the metabolic parameters of type-2 diabetes such as body weight, FBG, OGTT, HbA1c and lipid profile (**Figure 5.9-5.12**). It can be assumed that this TMP mediated response is mainly due to its insulin sensitising action and reduction of insulin resistance as it is already known that TMP stimulates PI3K/Akt signalling [22]. Activation of this pathway can promote GLUT-4 translocation to the surface of the plasma membrane of the cell [115]. It then provokes the uptake of glucose and utilisation in the major organs of glucose uptake such as skeletal muscle, cardiac and adipose tissue [110]. Thus, the normalisation of diabetic metabolic markers is an indication of the reduced hyperglycemic condition, and it promotes the improvement of oxidative stress-induced renal inflammation, which is one of the major determining factors of nephropathic complication [29]. It has also been reported that TMP produces a protective effect on pancreatic islets and also increases its microcirculation which promotes insulin secretion leading to normalisation of the hyperglycemic condition [116, 117]. In this study also the TMP treatment significantly increased the FSI level in diabetic rats indicating its insulin secretory potential (**Figure 5.9C**). Along with the insulin-sensitising action, the induction of insulin release from the β-cells of pancreatic islets by TMP too contributes to the normalisation of the hyperglycemic condition and to the reduction of oxidative stress-induced renal injury.

Various experiments have been performed to demonstrate the role of oxidative stress in the DN pathogenesis, and the reduction of oxidative stress has proven to exert beneficial therapeutic effects in the prevention and treatment of diabetic nephropathic complications [118, 119]. In our study, we investigated the levels of SOD, MDA and GSH-Px, 3 critical markers of oxidative stress, in kidney and serum samples [120]. From the data represented in **Figure 5.13A, B** and **C**, it can be concluded that TMP significantly reduced the oxidative stress in both the kidney and serum through

increments in the SOD and GSH-Px activity and reduction of MDA level. Therefore, TMP alleviated nephropathic symptoms by oxidative stress reduction.

Furthermore, the functional parameters of kidney injury such as BUN, SCR and 24 h urinary protein level were considerably improved by TMP treatment (**Figure 5.14**). In addition, the deterioration of renal morphological markers, GMEI and TDI were estimated by histological analysis and found to be effectively alleviated by the therapeutic action of TMP (**Figure 5.15**). Renal dysfunctions objectivated by, raised BUN, SCR and urinary protein level, as well as the morphological changes like expansion of glomerular mesangial matrix and tubulointerstitial injury, are the major pathological changes of DN and these changes are related to hyperglycemia and oxidative stress [121, 122]. As investigated in this experiment, hyperglycemia and oxidative stress were significantly alleviated by TMP treatment. Therefore, it can be considered that TMP could improve renal functional and morphological parameters of DN by reducing hyperglycemia-induced reactive oxygen species production.

The Akt signalling pathway induces various intracellular actions in response to extracellular signals. The activation of Akt occurs through phosphorylation and, it mediates various down-stream functional responses like cell migration, proliferation, survival and growth [123]. The linkage between DN and Akt signalling has already been established, and for nephropathic occurrence and development, inhibition of the Akt pathway is needed. It has also been reported that Akt signalling inhibition is responsible for the apoptosis of kidney proximal tubule cells in DN complication [29] whereas nephropathic complications in rats can be alleviated through Akt signalling activation. Thus, increasing the expression of Akt pathway proteins proves to be a beneficial therapeutic strategy in DN treatment. When the phosphorylated Akt expression decreases, downstream effectors of this pathway such as GSK-3β gets

phosphorylated and activated, leading to reduction of anti-apoptotic factor Bcl-2 expression and increases Bax and cleaved caspase-3 pro-apoptotic factors level [124]. In our study, we investigated the role of Akt signalling in TMP induced amelioration of DN. In diabetic untreated rats, we found a decrease in Akt signalling as evidenced by reduced p-Akt and anti-apoptotic factor Bcl-2 levels together with increases in the expression of the pro-apoptotic determinants p-GSK-3β, Bax and cleaved caspase-3. In diabetic rats treated with TMP, TMP appeared to be able to counteract and dose-dependently reverse all these abnormalities. The expression of p-Akt and Bcl-2 were restored to normal levels, whereas p-GSK-3β, Bax and cleaved caspase-3 were reduced to the level found in normal control rats (**Figure 5.16**). Taken together, our results suggest that TMP protects against diabetes-induced functional and morphological renal defects at least in great part via an effect on Akt signalling pathway (**Figure 6.2**). However, the role of Akt signalling pathway (p-Akt, p-GSK-3β, Bax, Bcl-2 and cleaved CASP-3) in reducing the renal apoptosis and diabetic nephropathy complication needs more extensive investigation and it worth testing in future to effectively create a link between Akt signalling, renal apoptosis and diabetic nephropathy.

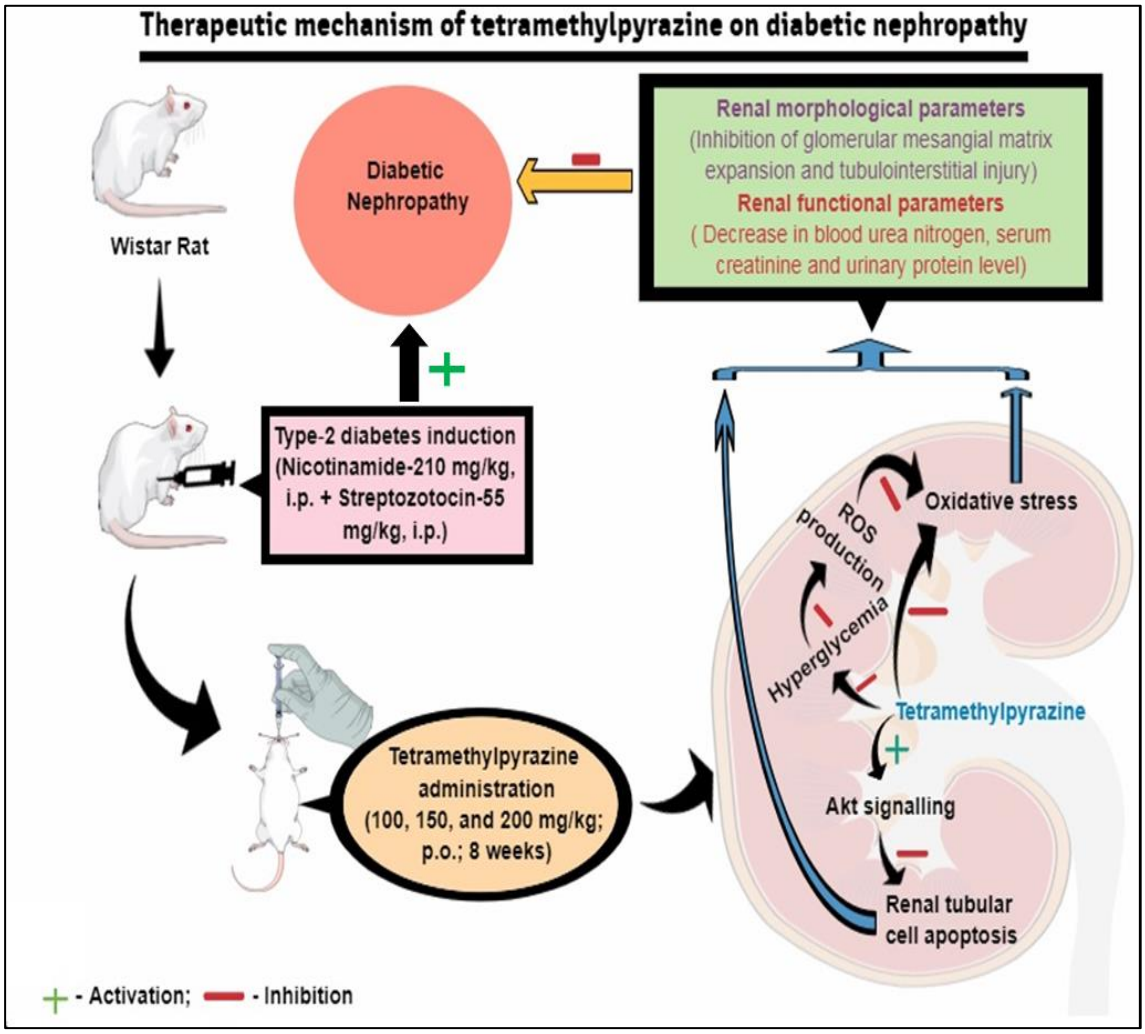

Figure 6.2: Elucidation of the mechanism of action of TMP in the amelioration of diabetic nephropathy in STZ-NCT-induced T2D rats.