6.1. To explore the efficacy of PT on insulin resistance, metabolic syndrome and hepatic oxidative stress in high fructose (65%) diet-induced type 2 diabetic rats (Objective-I).

Several studies have pointed out that high fructose consumption over long periods has deleterious effects on insulin sensitivity and glucose metabolism in human beings as well as in preclinical animal models (Macdonald, 2016; ter Horst et al., 2016). In the current investigation, eight weeks of supplementation with high fructose (65%) diet significantly impaired glucose tolerance associated with hyperinsulinemia, dyslipidemia, insulin resistance and hypertension (evidenced by higher systolic, diastolic and mean arterial pressure) in rats. Moreover, metabolic syndrome parameters such as HbA1c, uric acid, peroxynitrite and hydrogen sulfide were altered in fructose-fed rats. Hence, from these results, it can be inferred that this experimental animal model represents all the pathological features mimicking clinical T2DM associated with insulin resistance and metabolic syndrome. Our findings reiterate earlier reports on the induction of T2DM in animal models (mice and rats) by chronic supplementation with a fructose-rich diet (Lozano et al., 2016; Padiya et al., 2011).

Although there is substantial debate as to the relative contributions of impaired insulin secretion and increased insulin resistance to the pathogenesis of T2DM, it is accepted that both entities unequivocally play essential roles (Tahrani et al., 2016). Substantial evidence documents the presence of insulin resistance in the majority of non-insulin dependent diabetes mellitus (NIDDM) patients and is a powerful predictor of future development of NIDDM in the offspring (Ferrannini, 1998). Not only is insulin resistance the best predictor of the onset of T2DM, but it is also an important therapeutic target once T2DM is present. Since insulin resistance is a key

pathophysiological feature of T2DM, our current investigation focused on reversing insulin resistance. The use of traditional medicines has been promising for diabetes therapy; however, the appearance of adverse effects has puzzled patients. Recent studies have validated the hypoglycemic potential of some dietary polyphenolic compounds that represent a valuable source of anti-diabetic therapies (Kim et al., 2016b). Pterostilbene (PT) is one such naturally occurring compound and is well-known for its diverse health benefits, including in diabetes (Kosuru et al., 2016). Although the antidiabetic activity of PT has been demonstrated in streptozotocin-induced type 2 diabetic models (Bhakkiyalakshmi et al., 2016; Pari & Satheesh, 2006), very few experimental studies have been carried out until recently concerning the effects of PT on insulin resistance in animal models. Recently, Gomez-Zorita et al. (2015) observed a positive effect of PT on improving glycemic control and insulin sensitivity in the obesogenic diet (high in sucrose and fat) induced insulin resistant rats (Gomez-Zorita et al., 2015). Pari and Satheesh (2006) demonstrated that PT significantly ameliorated glucose homeostasis by modulating the enzyme levels of hepatic carbohydrate metabolism in streptozotocin-diabetic rats (Pari & Satheesh, 2006). Similar findings were observed by Bhakkiyalakshmi et al. (2016), in that PT ameliorated hyperglycemia by increasing enzyme levels of hexokinase, glucose-6-phosphate dehydrogenase and by decreasing glucose-6-phosphatase and fructose-1,6-bisphosphatase in liver tissues of streptozotocin-diabetic mice (Bhakkiyalakshmi et al., 2016). Also, PT reduced pancreatic oxidative damage by stimulating Nrf2 dependent antioxidant gene expression (Bhakkiyalakshmi et al., 2016). However, insulin resistance was measured neither in diabetic rats (Pari & Satheesh, 2006) nor diabetic mice (Bhakkiyalakshmi et al., 2016). Furthermore, metabolic syndrome parameters and cardiovascular risk indices were not depicted explicitly in any of the three models described above (Bhakkiyalakshmi et al.,

2016; Gomez-Zorita et al., 2015; Pari & Satheesh, 2006). This inadequacy prompted us to investigate whether oral supplementation of PT would have beneficial effects on reducing insulin resistance and accompanying metabolic syndrome in high fructose diet-induced T2DM rats. The doses of PT used, i.e. 20 and 40 mg/kg (approximately equivalent to one and two times human therapeutic dose), were selected based on previous efficacy studies in rats (Gomez-Zorita et al., 2015; Pari & Satheesh, 2006).

In the present study, oral administration of PT for eight weeks showed the expected effects on body weight gain, altered glucose homeostasis and insulin resistance in a rat model of T2DM. Although body weight gain is associated with fructose-induced insulin resistance (Lozano et al., 2016), we did not notice any enhancement of body weight gain between diabetic and control rats. It has been demonstrated that 20 weeks of a high fructose diet does not increase body weight, but does result in increased liver adiposity and lipid deposition in the liver (Abdullah et al., 2009). Interestingly, eight weeks of treatment with PT (20 and 40 mg/kg/day) significantly reduced body weight gain in diabetic rats, suggesting that PT reduces insulin resistance. Similarly, a lower dose of PT (20 mg/kg/d) significantly ameliorated insulin resistance, as evidenced by reduced OGTT, HOMA-IR and increased ISI than a higher dose of PT (40 mg/kg/d). Although PT (40 mg/kg/day) had a weaker effect on insulin resistance, it had a substantial impact on lowering fasting blood glucose (FBG) levels than PT (20 mg/kg/d). These results suggest that the lower dose of PT (20 mg/kg/d) exerted a significant antidiabetic effect by ameliorating both insulin resistance and glucose homeostasis in fructose-fed diabetic rats than the higher dose of PT (40 mg/kg/d). Our results are in agreement with the findings published by Gómez-Zorita et al. (Gomez-Zorita et al., 2015), in that low dose PT (15 mg/kg/d) significantly ameliorated glycemic control in obesogenic fed insulin resistant rats compared to rats given the high dose of

PT (30 mg/kg/d). Besides, the antidiabetic effect of lower dose PT was mediated by both the liver and skeletal muscle, whereas only skeletal tissue was responsible for the higher dose PT antidiabetic effect (Gomez-Zorita et al., 2015). From these results, it can conclude that PT is helpful to improve insulin sensitivity by increasing the body's utilization of insulin to accelerate glucose metabolism.

In the range of doses employed in the current investigation, the lack of change in various metabolic parameters in the D+PT40 group was surprising. This phenomenon is a standard feature observed with polyphenols when assessing beneficial actions. Low dose resveratrol (0.005%) is more potent than its corresponding high dose (0.02%) in preventing adiposity, hepatic steatosis, and dyslipidemia in an obese mouse model (Cho et al., 2012). Similarly, resveratrol at a low dose of 15 mg/kg/d significantly decreased non-HDL-C, liver triacylglycerol infiltration and serum transaminases in obese Zucker rats, but failed to produce any beneficial effects at a higher dose of 30 mg/kg/d (Gomez-Zorita et al., 2012). It seems that PT exhibits a similar kind of response as its parent molecule, resveratrol. Furthermore, it has been reported that higher doses of PT are beneficial in the treatment of cancer through proapoptotic, antiproliferative actions, whereas lower doses of PT are useful in the amelioration of metabolic diseases through antiapoptotic and antioxidant effects (Kosuru et al., 2016).

Stringent glycemic control is a prime concern in diabetic population because it negatively correlates with diabetic microvascular complications (Rodriguez-Gutierrez & Montori, 2016). Glycated haemoglobin (HbA1c) is generated by the non-enzymatic modification of haemoglobin with glucose, and its concentrations reflect average blood glucose levels in the patient during the previous two or three months before blood collection (Hanas & John, 2010). HbA1c is regarded as the gold standard for long-term

follow-up of glycemic control, and each 1% decrease in HbA1c has been linked with a 37% decline in microvascular complications and 21% fewer diabetes-associated deaths (Adler et al., 2000). The findings of our study show an apparent difference between the control (HbA1c 3.4%) and fructose-fed diabetic (HbA1c 6.3%) groups, and PT treatment significantly decreased elevated HbA1c levels in diabetic rats. It has already been proposed that PT exerts antidiabetic effects through insulinotropic effects (Manickam et al., 1997) and stimulation of the phosphoinositol 3-kinase (Manickam et al., 1997), Akt (Gomez-Zorita et al., 2015), cardiotrophin-1 (Gomez-Zorita et al., 2015) and Nrf2 pathways (Bhakkiyalakshmi et al., 2016). Moreover, PT significantly increased serum hydrogen sulfide levels in diabetic rats, indicating that PT has beneficial effects in preventing disease progression in T2DM since lowered hydrogen sulfide levels have been found in diabetic patients (Suzuki et al., 2017).

Fructose induces dyslipidemic state by enhancing the expression of sterol regulatory element binding protein-1c (a critical lipogenic enzyme in the liver) and by increasing liver fatty acid synthesis through boosting the production of hepatic triose-phosphate levels (Kim et al., 2016a). Furthermore, fructose stimulates the expression of fatty acid synthase and acetyl-CoA carboxylase enzymes in the liver through activation of the carbohydrate-responsive element binding protein (Kim et al., 2016a). In the present study, dyslipidemia was observed, as evidenced by decreased HDL-C and augmented serum TG, LDL-C, VLDL-C in fructose-fed diabetic rats, a significant cardiovascular risk factor for insulin resistant and T2DM patients (Schofield et al., 2016). Cardiovascular risk indices (total cholesterol to HDL-C ratio and LDL-C to HDL-C ratio) were significantly augmented, while AAI was markedly reduced in diabetic rats. However, the lower dose of PT (20 mg/kg/day) significantly ameliorated dyslipidemia, AAI and reduced cardiovascular risk indices in diabetic rats than the higher dose (40

mg/kg/day). PT has been demonstrated to exert beneficial effects on lipid metabolism through PPAR-α induction (Rimando et al., 2005), AMPK stimulation (Gomez-Zorita et al., 2014) and by downregulating leptin, PPAR-γ, CCAAT/enhancer binding protein (C/EBP)-α, resistin and fatty acid synthase (Hsu et al., 2012). Furthermore, PT enhances lipid oxidation in insulin-resistant rats, through the induction of mitochondrial oxidative capacity and mitochondriogenesis by increasing carnitine palmitoyl transferase-1B, mitochondrial transcription factor A (a marker of mitochondriogenesis), citrate synthase and cytochrome c oxidase subunit II activities (Gomez-Zorita et al., 2015). Thus, it can be presumed that PT decreases dyslipidemia and cardiovascular risk indices through antihyperlipidemic actions.

Hyperuricemia during fructose consumption might be attributed to the activation of adenosine monophosphate deaminase activity stimulated by fructose metabolism resulting in enhanced production of uric acid (Hallfrisch, 1990). Hyperuricemia in fructose-fed rats has been considered as a risk factor in the development of metabolic syndrome associated with insulin resistance and hypertension (Tran et al., 2009). Besides, fructose leads to hypertension by diverse mechanisms such as stimulation of the sympathetic nervous system, impaired endothelial relaxation and enhanced activity of vasoconstrictors (Klein & Kiat, 2015). In agreement with this, in the present study, fructose-fed diabetic rats demonstrated a marked elevation in serum uric acid levels and blood pressure, compared to control rats. However chronic supplementation of PT significantly decreased hyperuricemia and hypertension in diabetic rats. These results suggest that PT ameliorates T2DM by reducing metabolic syndrome parameters.

Substantial evidence suggests that consumption of a high fructose diet contributes to oxidative stress and leads to the induction of pathological insulin resistance in rats

(Lozano et al., 2016). In diabetes, enhanced glucose oxidation accelerates the generation of free radical oxidative species, which can damage vital cellular components like DNA, lipids and proteins. Further, the fructose-induced hyperglycemia observed in this study might be a contributing risk factor for enhanced lipid peroxidation, resulting in the simultaneous decline of the hepatic endogenous antioxidant system, indicating the development of oxidative stress in fructose-fed diabetic rats in agreement with other studies (Padiya et al., 2011). Lipid peroxidation was evident from the augmented levels of thiobarbituric acid reactive substances (TBARS) in fructose-fed diabetic rats. The enhanced vulnerability of cells to fructose-induced lipid peroxidation could be related to energy depletion associated with augmented fructose catabolism and also due to a free radical induction potential similar to that of glucose (Rajasekar & Anuradha, 2007).

Peroxynitrite, a product of the reaction of nitric oxide with superoxide anions, triggers nitrosative stress with concomitant protein nitration, mitochondrial dysfunction and impaired stress signalling (Stavniichuk et al., 2014). The possibility that diabetic complications like peripheral neuropathy are linked with augmented nitrosative damage is supported by the detection of enhanced serum peroxynitrite levels in diabetic patients (Edwards et al., 2015). In our study, PT decreased serum peroxynitrite levels in diabetic rats. Paul et al. (2009) demonstrated that PT treatment decreases oxidative/nitrosative stress in colon cancer cell lines through downregulation of inducible nitric oxide synthase and cyclooxygenase-2 (Paul et al., 2009).

Superoxide dismutase (SOD) and glutathione (GSH) are regarded as endogenous enzymatic and non-enzymatic antioxidant defences respectively, protects from ROS-induced cellular damage. Some studies reported that increased SOD activity in diabetic rats (Ramanathan et al., 1999), whereas most of the studies demonstrated decreased

SOD activity in streptozotocin-induced diabetic rats (Sheweita et al., 2016). In the present study, SOD activity was reduced in fructose-fed diabetic rats; this decline could be responsible for the damaging effects of enhanced oxidative free radicals and uninterrupted glycation of enzymatic proteins (Nowotny et al., 2015). The diminished SOD activity may contribute to the rise of superoxide radicals which in turn decrease the function of glutathione peroxidase (Blum & Fridovich, 1985) that catalyzes the reduction of lipid peroxides at the expense of GSH. Additionally, a significant decrease in GSH content has been observed in streptozotocin diabetic rats (Sheweita et al., 2016). Thus, the diminution of GSH impairs the antioxidant enzyme defence, and the resultant oxidative damage can then contribute to the pathogenesis of diabetes.

Administering PT to fructose-fed diabetic rats restored the SOD activity and GSH levels in comparison with levels in diabetic rats and further reduced oxidative stress. Sateesh and Pari (2006) reported that PT significantly increased SOD, GSH, glutathione peroxidase and catalase in liver and kidney tissues of streptozotocin-induced diabetic rats (Amarnath Satheesh & Pari, 2006). Furthermore, Bhakkiyalakshmi et al. (2016) highlighted the antioxidant potential of PT and reported that PT diminished pancreatic cell oxidative damage by activating NF-E2-related factor 2 (Nrf2) mediated antioxidant genes expression including SOD, catalase, heme oxygenase-1 and glutathione peroxidase in diabetic mice (Bhakkiyalakshmi et al., 2016). In aged (SAMP8) mice, PT was also shown to improve cognitive performance by limiting oxidative stress through the upregulation of MnSOD via induction of its upstream molecule, PPARα (Chang et al., 2012). Thus the ability of PT to increase antioxidant activity by augmenting SOD activity and GSH levels contribute to its antidiabetic activity and may be able to salvage the diabetic liver against the deleterious effects of oxidative stress.

6.2. To investigate the therapeutic potency and signalling mechanism of pterostilbene against diabetes induced-cardiac oxidative stress, inflammation and mitochondrial impairment in fructose-fed diabetic rats (Objective-II).

It has been reported that type II diabetes is strongly coupled with the onset and development of myocardial hypertrophy and cardiac dysfunction (Lehrke & Marx, 2017), warrants further scientific investigations to comprehend the precise mechanisms. To facilitate studies, chronic supplementation of fructose diet has been employed to produce apparent cardiac impairment in animal models. In mice, high fructose intake induced myocardial hypertrophy and diastolic dysfunction after 20 weeks feeding (Zhang et al., 2016b) and profound perturbations of myocardial calcium handling and myofilament responsiveness after 12 weeks feeding (Mellor et al., 2012). While in rats, four weeks fructose feeding induced blood pressure, left ventricular hypertrophy by activating renin-angiotensin and sympathetic nervous system (Kamide et al., 2002). Three weeks of fructose feeding developed cardiac oxidative stress, myocardial hypertrophy and hypertension in Sprague-Dawley rats (Delbosc et al., 2005). 66% fructose diet for four weeks was reported to reduce cardiac tolerance to ischemic insults in Wistar rats (Morel et al., 2003). While in the present investigation, eight-week high-fructose diet feeding was employed to examine the myocardial impairment with an emphasis on mitochondrial biogenesis, thereby suggesting potential targets that credit for the protective functions of PT. Pterostilbene significantly decreased the body weight of fructose-fed diabetic rats compared to normal rats. These results indicate that treatment of diabetic rats by PT has a positive influence on body weight reduction in diabetic rats. Our results were in agreement with others findings (Manickam et al., 1997; Riche et al., 2014c). Manickam and colleagues reported that PT from Pterocarpus marsupium (20 $mg.kg^{-1}$, p.o.) significantly decreased body weight by 20% in a

streptozotocin-induced rat model (Manickam et al., 1997). Furthermore, weight loss effects of PT were also observed in hypercholesterolemic patients (Riche et al., 2014c). Weight loss during PT treatment may be attributed to its ability to reduce adipose tissue mass (Gomez-Zorita et al., 2014). Since weight loss decreased blood pressure and left ventricular hypertrophy in a cohort of overweight patients (Hinderliter et al., 2002), PT's ability to normalise hypertension and myocardial hypertrophy in diabetic rats could be related partly to weight loss effect.

The physiological concentrations of reactive oxygen species (ROS) are essential to preserve basal cellular activities, but an excess production of ROS could surpass the antioxidant enzyme capacity and results in the induction of oxidative stress. Accumulating evidence indicates that surplus level of superoxide radicals induced by diabetic hyperglycemia lead to oxidative stress and cardiac structural alterations, eventually contributing to diabetic myocardial damage (Golbidi et al., 2014). Further, excess superoxide radicals combine with nitric oxide and generate secondary reactive free radical like peroxynitrite, leading to damage to proteins, lipids, DNA and triggering cardiac injury (Szabó et al., 2012). It has already been demonstrated that high fructose intake has a causative role in the induction of human metabolic diseases including type II diabetes, insulin resistance, dyslipidemia and hypertension through the resultant overload of ROS levels (Rebollo et al., 2012). Thus cytotoxic free radicals are implicated in the progression of cardiac cell injury, and the data of the current study hypothesised that these ROS could be essential in the pathogenesis of myocardial damage at the later stages of type II diabetes.

Hyperglycemia increased the susceptibility of cardiac tissues to oxidative stress because of the lack of antioxidant defence evidenced by lowered functional activities of SOD,

catalase, GSH and GPX in fructose-fed diabetic rats. SOD, catalase and GPx are considered as enzymatic antioxidants that catalyse the detoxification of superoxide anion, hydrogen peroxide, lipid/hydroperoxides respectively, eventually, decreases the oxidative stress (Kurutas, 2016). The non-enzymatic antioxidant GSH acts as a cosubstrate for the function of GPx that oxidises the GSH into oxidised glutathione, which can be recovered back to GSH by glutathione reductase (Kurutas, 2016). Hence, the functions of these antioxidants are reduced during oxidative stress. In this context, multiple reports have indicated the decreased functions of these antioxidant enzymes in the diabetic heart and various natural products with antioxidant activity have been shown to restore the functions of these antioxidant enzymes in diabetic rats (Cao et al., 2015; Cheng et al., 2014). PT diminished pancreatic oxidative injury by its potential to stimulate Nrf2-mediated antioxidant enzyme expression in diabetic mice (Elango et al., 2016). Furthermore, in our previous study, PT reduced hepatic oxidative stress by decreasing lipid peroxidation and by upregulating SOD and GSH levels in fructose-fed diabetic rats (Kosuru & Singh, 2017). In the current study, PT treatment to fructose-fed diabetic rats notably declined the levels of cardiac TBARS, ROS, hydrogen peroxide, peroxynitrite (**Figure 5.5**) and restored the levels of cardiac antioxidant enzymes SOD, catalase, GPx and GSH (**Figure 5.6**). Thus the free-radical scavenging nature and antioxidant effects of PT could contribute to attenuate the fructose-induced cardiac oxidative stress and may be able to salvage the diabetic heart against detrimental effects of oxidative stress.

Hyperglycemia-induced oxidative stress in the cardiac tissue is reported to be linked with the inflammation through the excess generation of pro-inflammatory cytokines, and polyphenolic antioxidants reduce plasma pro-inflammatory cytokines by decreasing oxidative stress (Hussain et al., 2016). Besides, stimulation of NF-κB, a pro-

inflammatory signalling molecule, in the cardiac tissue enhances oxidative stress and inflammation, while blockade of NF-κB decreases both oxidative stress and pro-inflammatory response, and alleviates cardiac dysfunction in type II diabetic mice (Mariappan et al., 2010). In the heart, activation of NF-κB signalling has been linked to different pathophysiological contexts, including myocardial infarction, heart failure, cardiac hypertrophy, and diabetic cardiomyopathy (Kumar et al., 2013; Maier et al., 2012). Paradoxically, a prosurvival function of NF-κB has also been highlighted in the heart under certain circumstances including hypoxic or ischemic preconditioning (Jancso et al., 2004). An accepted view is that acute stimulation of NF-κB is requisite for cardioprotection (such as in preconditioning), whereas chronic stimulation contributes to heart failure. The lack of agreement among different experiments is likely due to complexity of multiple components of NF- κB signalling and the cell type and disease conditions studied (Kumar et al., 2013). Thus, the protective or detrimental role of NF-κB to cardiac injury may merely depend on the cellular context and nature of the stimulus. In the current study, both mRNA and protein expression of NF-κB activity was augmented in the hearts of fructose-fed diabetic rats and PT treatment reduced them; suggesting that PT might inhibit NF-κB-mediated cardiac inflammation. Interestingly, NF-κB transactivation and toll-like receptor 4 (TLR4)-mediated ROS production account for priming and upregulation of inflammasomes (Boaru et al., 2015; Gurung et al., 2015). These are multiprotein cytosolic complex made up of nucleotide-binding oligomerization domain-like receptor (NLR) protein NLRP3, the adapter protein apoptosis-associated speck-like protein containing caspase recruitment domain (ASC) and procaspase-1 (Davis et al., 2011). NLRP3 pathway affects insulin sensitivity and concurrently increases cardiac cytokine levels and macrophage infiltration (Kawaguchi et al., 2011; Wen et al., 2012). In fact, NLRP3 inflammasomes regulate

downstream inflammatory events of lipotoxicity and glucotoxicity during the development of T2DM (Vandanmagsar et al., 2011). Moreover, NLRP3 enhances the pool of proinflammatory cytokines such as interferon-γ, IL-1β and IL-18 and promotes insulin resistance in M1 macrophages (Vandanmagsar et al., 2011). Thus NLRP3 inflammasome may also responsible for cardiac inflammation in T2DM. In the present study, it is observed that cardiac expression of TLR4, NLRP3 and ASC were substantially upregulated in fructose-fed diabetic rats and PT decreased the NLRP3 inflammasome-mediated cardiac inflammation. Recent evidence suggests that PT and allopurinol decreased fructose-induced glomerular podocyte injury through inhibition of microRNA-377-mediated O_2^-/p38 mitogen-activated protein kinase/thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome pathway activation (Wang et al., 2015b). Furthermore, PT attenuates early brain injury following subarachnoid haemorrhage through suppression of NLRP3 inflammasome and Nox2-induced oxidative stress (Liu et al., 2017). Surprisingly, coadministration with CC prevented the inhibitory potential of PT on NF-κB- and NLRP3-inflammasome-mediated cardiac inflammation in fructose-fed diabetic rats through AMPK dependent manner.

Mitochondrial dysfunction enhances the production of oxidative free radicals, which causes cardiac oxidative stress in diabetic rats (Boudina et al., 2007). Eight weeks of fructose feeding leads to mitochondrial impairment, mitochondrial DNA damage, reduced mitochondrial DNA repairing capacity and decreased mitochondrial biogenesis in the rats (Cioffi et al., 2017). In the heart, overexpression of PGC-1α, a critical regulator of mitochondrial biogenesis, robustly enhances mitochondrial DNA content (Russell et al., 2004a), and knockout of PGC-1α resulted in reduced expression of the citric acid cycle and oxidative phosphorylation genes (Arany et al., 2005). AMPK

stimulation regulates cellular energy content by increasing ATP generation through increasing mitochondrial pool via upregulation of mitochondrial biogenesis, which is requisite to restrain diabetes-triggered oxidative stress (Kukidome et al., 2006) and prevents fructose diet-induced lipid accumulation in hepatic tissue (Woods et al., 2017a). AMPK inhibition was reported to worsen pressure overload-induced eccentric left ventricular hypertrophy in fructose-fed rats (Bouchard-Thomassin et al., 2011). In the cardiac muscle, chronic PT treatment demonstrated a beneficial effect on the fructose diet-induced reduction in mitochondrial biogenesis by enhancing the expression of PGC-1α, Complex III and V subunits. Interestingly, coadministration with CC substantially suppressed the PT enhancement of mitochondrial biogenesis in hearts of diabetic rats, indicating that PT improves mitochondrial biogenesis in diabetic myocardium through AMPK pathway activation.

Oxidative stress triggers AMPK phosphorylation, which stimulates Nrf2 and its downstream antioxidant enzymes, including HO-1 (Zimmermann et al., 2015). Furthermore, AMPK stimulation conferred neuroprotective effect against prenatal stress-induced cognitive deficit through regulation of mitochondrial stores and Nrf2 pathway (Cao et al., 2014). Impaired Nrf2 signalling contributes to hepatic oxidative stress, inflammation in fructose-fed mice (Nigro et al., 2017), and activation of Nrf2 protects cardiomyocytes against doxorubicin-induced cardiomyopathy by inducing mitochondrial biogenesis (Piantadosi et al., 2008). Nrf2 is also discussed to be responsible for stimulation of inflammasomes. It is suggested that Nrf2 expression is required to activate NLRP3 inflammasome in murine cells in-vitro and in-vivo (Zhao et al., 2014). Paradoxically, Nrf2 is a negative regulator of NLRP3 inflammasome activation by regulating thioredoxin 1/TXNIP complex (Hou et al., 2018). Furthermore, specific Nrf2 activators seem to restrain NLRP3 inflammasome activation at high

concentrations (Greaney et al., 2016; Liu et al., 2016a; Maier et al., 2015). The seemingly conflicting findings suggest both effects are mediated independently of Nrf2 target gene expression, but rather by physical interaction of Nrf2 with caspase-1 through Keap1/Cul3/Rbx1 components (Garstkiewicz et al., 2017). Surprisingly, Nrf2 also blocks inflammatory response by inhibiting proinflammatory cytokine transcription (Kobayashi et al., 2016). PT is a potent stimulator of Nrf2 and ameliorates pancreatic beta cell apoptosis and oxidative damage by augmenting antioxidant signalling pathways in streptozotocin-treated rats (Bhakkiyalakshmi et al., 2014; Elango et al., 2016). Furthermore, PT decreased cerebral ischemia-reperfusion-induced mitochondrial oxidative damage by preserving complex I, complex IV activity and mitochondrial membrane potential through activation of HO-1 signalling (Yang et al., 2016b). In the present study, Nrf2/HO-1 axis was considered as a marker of antioxidant enzymes and found that PT could efficiently enhance the expressions of Nrf2, HO-1 through activation of AMPK in the myocardium of fructose-fed rats. To further reveal the participation of AMPK signalling pathway in PT-induced cardioprotective effects, AMPK inhibitor was given to fructose-fed diabetic rats along with PT. CC co-administration not only prevented PT-induced AMPK phosphorylation but also decreased Nrf2 and HO-1 expression in diabetic myocardium. These results suggest that PT stimulates AMPK/Nrf2/HO-1 signalling pathway to ameliorate high fructose diet-mediated cardiac oxidative stress and inflammation.

Till now, no straight-forward studies are indicating the PT modulation of AMPK and NF-κB in myocardial systems and its application in the prevention of diabetic cardiomyopathy. The novelty of present study is that we have provided the direct evidence of the cardioprotective potential of PT against diabetic cardiomyopathy. Using

fructose-fed type II diabetic rats, we have demonstrated that PT treatment stimulates AMPK/Nrf2/HO-1 signalling in myocardial tissue, causing the diminution of oxidative stress, and improves mitochondrial biogenesis.

The primary drawback of the current study is the lack of positive control group for comparison of results. Since resveratrol is a well established cardioprotective molecule (Gu et al., 2014) and also a structural analogue of pterostilbene, the inclusion of resveratrol group as a positive control group would have strengthened the data. Furthermore, the present study is limited by specific gene knockout animal species to validate the current PT's signalling mechanism.

6.3. To investigate the cardioprotective potential and mechanistic pathway of pterostilbene against myocardial ischemia-reperfusion injury in streptozotocin-induced diabetic rats (Objective-III).

In this study, the novel findings were: 1) four weeks treatment with PT attenuated myocardial IR injury in rats with streptozotocin-induced diabetes (evidenced by reduced infarct size, LDH, CK-MB, free 8-isoprostane and cardiac apoptosis after myocardial IR), and decreased *in vitro* HR injury in primarily cultured rat cardiomyocytes incubated with HG (evidenced by preserved cardiomyocytes viability and decreased LDH, oxidative stress and apoptotic index). 2) AMPK activation is essential for the anti-oxidative, anti-apoptotic action of PT as evidenced by the finding that, compound C, an inhibitor of AMPK, blunted the protective effects of PT against myocardial IR injury in diabetes.

Oxidative stress occurs as consequence of an enhanced generation of reactive oxygen and nitrogen species and poor antioxidant enzyme defence and is responsible for cardiac remodelling in diabetic hearts after myocardial IR injury (Eguchi et al., 2012). Prostaglandin isoprostane isomers are identified as a new class of oxidative stress markers and produced mostly from oxidative alterations of phospholipids *via* a free radical catalyzed mechanism (Morrow et al., 1990). Of these, 8-isoprostane is regarded as the sensitive quantitative measurement of the myocardial oxidative stress *in vivo* (Delanty et al., 1997). Smith et al. reported that enhanced level of myocardial 8-isoprostane, oxidized glutathione in the diabetic heart after myocardial infarction is coupled with the increased functional severity of heart failure (Smith et al., 2005). Similarly, an elevated level of myocardial 8-isoprostane is correlated with depressed indices of left ventricular hemodynamic function and decreased cardiac proteome levels of antioxidant defence and apoptotic resistance in rats with diabetic cardiomyopathy

(Hamblin et al., 2007). These findings provide a potential link between oxidative stress and myocardial dysfunction in diabetes after myocardial IR injury. In our study, enhanced oxidative stress in the diabetic myocardium was evidenced by elevated plasma free 8-isoprostane levels and DHE staining. However, PT treatment significantly alleviated myocardial IR-induced oxidative stress in diabetic rats. Interestingly, four weeks treatment with PT significantly decreased the plasma glucose levels in streptozotocin-induced diabetic rats, and therefore, PT-induced attenuation of hyperglycemia can save the diabetic myocardium from excessive oxidative stress. Thus, the protective effects of PT against diabetic myocardial IR injury may be ascribed to its potent antioxidant nature and its suppressive activity against hyperglycemia-induced oxidative damage.

It is reported that enhanced oxidative stress in the ischemic myocardium leads to the alteration of the membrane integrity which results in the release of cardiac damage markers like LDH and creatine kinase into the serum (Rossoni et al., 2008). The CK-MB is a sensitive marker of post-ischemic myocardial infarction in acute myocardial ischemic patients (Christenson et al., 2000), while infarct size is widely accepted as the gold standard for measuring the index of IR-induced cardiac injury (Christenson et al., 2000; Turer et al., 2005). In acute myocardial infarction patients undergoing thrombolytic therapy, serum concentrations of CK-MB attain peak level after ten hours of ischemia and correlate well with maximal indices of cardiac infarct size after five to seven days following reperfusion (Christenson et al., 2000). Similar findings were observed in a rat model of myocardial IR injury, in which post-ischemic CK-MB level reached maximum minutes after reperfusion while significant myocardial infarct size became apparent only after a lag period of one hour following reperfusion (Xia et al., 2005). In our study, IR-induced enhanced levels of LDH, CK-MB in diabetic rats that

were correlated with increased indices of cardiac infarct size and apoptosis. However, PT treatment significantly restricted the diabetic IR-induced elevation of cardiac damage markers in serum, indicating that PT ameliorates the post-ischemic cardiac injury in diabetic rats.

Substantial evidence suggests that enhanced oxidative stress during myocardial IR can trigger cardiac apoptosis *via* the mitochondrial (intrinsic)-mediated apoptosis, and represents a significant contributor to cardiomyocyte death in diabetes (Crow et al., 2004; Eefting et al., 2004). The Bcl-2 family proteins, consists of both pro- and anti-apoptotic members, are essential regulators of mitochondrial apoptosis in the myocardium (Gustafsson & Gottlieb, 2007). Bcl-2, an anti-apoptotic protein that decreases cell apoptosis by opposing Bax (a pro-apoptotic protein) mediated release of cytochrome c from mitochondria to the cytoplasm and eventually inhibits caspase cascade (van Empel et al., 2005). It has been reported that down-regulation of apoptosis could diminish IR-induced cardiomyocyte damage, and rescue the contractile function and therefore delay or even inhibit the incidence of heart failure (Chen et al., 2014). Thus, it would be interesting to investigate the direct effect of PT in diabetic myocardial IR injury related to or beyond its anti-apoptotic capacity. Western blot analysis revealed that PT attenuated cardiac apoptosis after myocardial IR injury in diabetic rats. It is noteworthy that PT (10 mg/kg, administered intraperitoneally, once a day for five consecutive days) significantly attenuated myocardial IR-induced-inflammation, oxidative stress, and myocardial apoptosis *via* up-regulating Gas6/Axl pathway in non-diabetic rats (Wu et al., 2017). Furthermore, PT administration (10 minutes before reperfusion) markedly reduced myocardial caspase-3 activity, *via* lowering nitrative/oxidative stress by attenuating peroxynitrite production, ROS generation and inflammatory response following myocardial IR injury (Yu et al., 2016). Previous

studies also lend the support for an anti-apoptotic role of PT in non-cardiac tissues such as brain (Zhou et al., 2015) and skeletal muscle (Cheng et al., 2016) following IR injury. In the present study, it is observed that PT decreased cardiac apoptosis in primary rat cardiomyocytes by up-regulating Bcl-2/Bax ratio, down-regulating cleaved caspase-3/caspase-3 ratio. Guo et al. demonstrated that PT attenuated HR-induced apoptosis *via* restoration of sirt1 activity in H9C2 cardiomyocytes (Guo et al., 2016). Thus, our results suggest that PT confers cardioprotection against diabetic myocardial IR injury by suppressing cardiac apoptosis.

AMPK is an "endogenous survival mechanism" (Qi & Young, 2015), and its activation plays a vital role in protecting against diabetes mellitus (Coughlan et al., 2014) and myocardial IR injury (Russell et al., 2004c). AMPK has been reported to exert an infarct-sparing effect by increasing glucose uptake and glucose transporter-4 translocation (Russell et al., 2004c) and precluding cardiomyocyte apoptosis (Ma et al., 2010; Russell et al., 2004c). Furthermore, AMPK activation is known to increase myocardial resistance to IR and oxidative stress of different magnitudes by up-regulating sulfonylurea receptor 2A (SUR2A), a major cardioprotective protein in the heart (Mohammed Abdul et al., 2017). Mohammed Abdul et al. (2017) demonstrated that AMPK-mediated activation of SUR2A enhanced the trafficking and activity of sarcolemmal ATP-sensitive K+ channels in mice, which in turn offers cardioprotection against prolonged hypoxic injury (Mohammed Abdul et al., 2017). Also, AICAR (an AMPK activator) treatment reduced the myocardial sensitivity to hypoxic insult by increasing the SUR2A expression in H9C2 cardiomyocytes (Mohammed Abdul et al., 2017). Therefore, manipulation with AMPK has been suggested to be a potential therapeutic approach to cure cardiac ischemic diseases where enhanced cardiac resistance to stress is indispensable. Also, PT can stimulate AMPK activity to induce

suppressive effects on fat accumulation and apoptosis in both adipocytes and vascular endothelial cells (Gomez-Zorita et al., 2014; Zhang et al., 2013a). In our study, myocardial IR significantly increased p-AMPK levels in diabetic rats, and the rise of p-AMPK was not sufficient to combat apoptosis, and this led to the enhanced cardiac oxidative stress and apoptosis in diabetic hearts. However, treatment with PT markedly potentiated IR-induced increase in p-AMPK and significantly reduced the oxidative stress and apoptosis in the myocardium of diabetic rats.

To further reveal the participation of AMPK signalling pathway in PT-induced cardioprotective effects against myocardial IR injury in diabetes, AMPK inhibitor compound C was applied to primary rat cardiomyocytes incubated with HG condition. Compound C co-administration not only prevented PT-induced AMPK phosphorylation but also decreased PT-mediated antioxidative and anti-apoptotic effects in diabetic cardiomyocytes exposed to HR challenge. Thus, phosphorylation of AMPK by PT and its suppression by CC bestow robust support for the contribution of AMPK pathway in PT-induced safeguard in diabetic myocardial IR injury.

Recent studies showed that restoration of AMPK activation reduced oxidative stress (Liu et al., 2016b; Qi & Young, 2015), while other studies have linked AMPK stimulation to enhanced oxidative stress (Jiang et al., 2014; Jung et al., 2004). Although the exact relationship between AMPK and oxidative stress is a matter of debate, our results support the notion that restoration of AMPK activation contributes to the amelioration of oxidative stress injury. Such an effect may be related to the attenuated fatty acid synthesis caused by phosphorylation of acetyl-CoA carboxylase enzyme (Ford et al., 2015), although further studies are essential to validate this. Furthermore,

additional experiments are required to determine the mechanisms of PT induced cardioprotection from IR-induced oxidative stress and apoptosis in diabetes.

The findings suggest the PT can be considered to have a potential therapeutic value in the prevention and rescue for diabetes-associated cardiovascular complications. Thus, our studies may facilitate the development of novel and effective therapies for the treatment of cardiovascular complications which are common in patients with diabetes.
