5.1. 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).

5.1.1. Effect of PT treatment on body weight and weight gain of fructose-fed diabetic rats

During the eight weeks treatment, body weight and weight gain of all experimental rats were noted every week and represented in **Table 5.1**. There was no remarkable difference in body weight gain between C and D groups after four and eight weeks of fructose feeding. However, both doses of PT (20 and 40 mg/kg) significantly decreased the body weight gain, with a reduction potential of 8.1% ± 0.5%, 12.6% ± 0.6% vs. 17.2% ± 0.4% at four weeks and with a reduction potential of 10.1% ± 0.6%, 19.8% ± 0.9% vs. 33.6% ± 0.8% at eight weeks, respectively. However, more reduction in body weight gain was observed in the D+PT20 group and produced an equivalent effect with metformin.

Table 5.1: Effect of PT treatment on body weight and weight gain of rats

Groups	Initial	After 4 weeks		After 8 weeks	
	Body weight (g)	Body weight (g)	Weight gain (%)	Body weight (g)	Weight gain (%)
C	364.5 ± 13.3	426.5 ± 12.7	17.1 ± 0.4	481.5 ± 11.7	32.2 ± 0.8
D	363.3 ± 11.8	427.7 ± 10.5	17.2 ± 0.4	486.7 ± 9.6	33.6 ± 0.8
D+PT20	355.7 ± 28.6	384.2 ± 25.4	8.1 ± 0.5$^{\#\#\#\$\$}$	391.2 ± 21.7$^{\#}$	10.1 ± 0.6$^{\#\#\#\$\$\$}$
D+PT40	351.6 ± 21.7	395.6 ± 14.5	12.6 ± 0.6$^{\#\#}$	420.6 ± 18.7	19.8 ± 0.9$^{\#\#\#}$
D+M	352.2 ± 17.0	379.0 ± 16.9	8.4 ± 1.3$^{\#\#\#\$\$}$	385.1 ± 15.7$^{\#}$	9.4 ± 0.3$^{\#\#\#\$\$\$}$

Data was expressed as mean ± SEM (n=10); C, D, PT20, PT40 and M, stand for control, diabetic, pterostilbene 20 mg/Kg, pterostilbene 40 mg/Kg, and metformin, respectively. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs D; $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ vs D+PT40.

5.1.2. Effect of PT treatment on fasting blood glucose (FBG) levels of fructose-fed rats

After four weeks of fructose feeding, the FBG levels in D group rats were increased but not significantly in comparison to C group rats (**Table 5.2**). However, eight-week fructose diet supplementation pronounced FBG levels considerably in D group rats (P<0.001 vs C). Although four weeks treatment of PT 20 and 40 mg/kg did not alter the basal glucose levels in fructose-fed diabetic rats, eight weeks treatment of PT (both doses) demonstrated significant hypoglycemic activity in fructose-fed diabetic rats. However, more substantial reduction in FBG levels was observed with D+PT40 (reduction rate 19.3%; P<0.001 vs D) than D+PT20 (reduction rate 18.0 %; P<0.01 vs D), but there was no significant difference between the two groups. Pterostilbene (40 mg/kg) produced an equivalent effect with metformin (P>0.05).

Table 5.2: Effect of PT on FBG levels of experimental rats

Groups	Fasting blood glucose (mmol/L) (After weeks)				
	Initial	2 weeks	4 weeks	8 weeks	Reduction rate (%) after 8 weeks
C	5.4 ± 0.3	5.6 ± 0.5	5.7 ± 1.1	6.0 ± 0.3	
D	5.8 ± 0.6	6.3 ± 1.2	10.7 ± 1.3	13.3 ± 0.5***	
D+PT20	5.4 ± 0.4	7.1 ± 0.8	8.6 ± 0.7	11.3 ± 0.3$^{\#\#}$	18.0
D+PT40	5.2 ± 0.7	6.6 ± 0.4	7.1 ± 1.0	11.0 ± 0.2$^{\#\#\#}$	19.3
D+M	5.6 ± 0.3	6.9 ± 0.7	5.5 ± 0.6	9.5 ±0.5$^{\#\#\#\$}$	34.7

Data was expressed as mean ± SEM (n=10); C, D, PT20, PT40 and M stand for control, diabetic, pterostilbene 20 mg/kg, pterostilbene 40 mg/kg, metformin, respectively. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{***}P$<0.001 vs C; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs D; $^{\$}P$<0.05 vs D+PT20.

5.1.3. Effect of PT treatment on OGTT of fructose-fed rats

An oral administration of 2 g/kg load of glucose led to a significant rise in blood glucose levels within 30 min and remained at an elevated level over the next experimental period (until 120 min) in D group in comparison to the C group. However, treatment with PT (20 & 40 mg/kg) significantly decreased the elevated blood glucose levels after oral glucose administration in the fructose-fed diabetic rats (**Figure 5.1A**). The results of OGTT were represented as the area under the curve of blood glucose (AUG; **Figure 5.1B**). The AUG of the PT treated groups (D+PT20 and D+PT40) were markedly less than that of D group (P<0.001 & P<0.01, respectively). However, there was no significant difference between AUG levels of D+PT20 and D+M group (P>0.05).

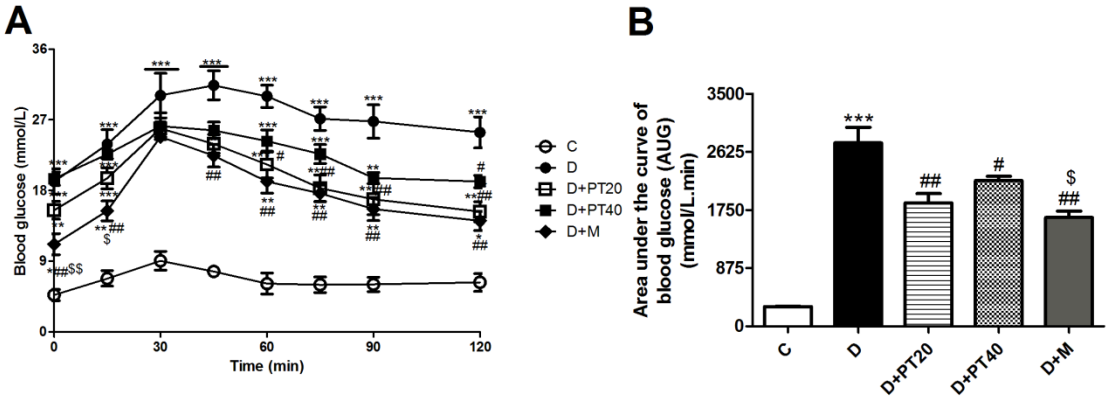

Figure 5.1: Effect of PT administration on OGTT of experimental rats. A) Blood glucose vs time plot graph of OGTT B) Area under the curve of blood glucose (AUG) levels of OGTT. Results are represented as mean ± SD (n=10). C, D, PT20, PT40, and M stand for control, diabetic, pterostilbene 20 mg/kg, pterostilbene 40 mg/kg, and metformin, respectively. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at P < 0.05. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 vs C; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs D; $^{\$}P$<0.05, $^{\$\$}P$<0.01 vs D+PT40. Bar indicates that statistical significance applies to D, D+PT20, D+PT40 and D+M groups.

5.1.4. Effect of PT treatment on insulin sensitivity of fructose-fed diabetic rats

Table 5.3 illustrates the FSI levels, ISI and HOMA-IR values of all the experimental groups. Insulin resistance was observed in D group as evident by increased FSI (P<0.01), HOMA-IR (P<0.001) values and by diminished ISI value (P<0.001), when compared to the C group. After eight weeks treatment, PT 20 mg/kg (P<0.001 vs D) demonstrated a marked reduction in FSI levels, whereas PT 40 mg/kg failed to decrease the FSI levels in diabetic rats. Similarly, PT 20 mg/kg (P<0.001 vs D) helped to increase ISI more significantly than PT 40 mg/kg (P<0.05 vs D) in diabetic rats and produced an equivalent effect with metformin. HOMA-IR values were reduced in both PT-administered groups, but the reduction was more pronounced in the D+PT20 group (−25.3% vs D group) than D+PT40 group (−15.7% vs D group).

Table 5.3: Effect of PT treatment on insulin sensitivity parameters of experimental rats

Groups	Insulin sensitivity		
	FSI (mIU/L)	**ISI**	**HOMA-IR**
C	9.5 ± 0.3	- 4.0 ± 0.1	2.5 ± 0.1
D	$10.7 \pm 0.2^{**}$	$- 5.0 \pm 0.1^{***}$	$6.3 \pm 0.3^{***}$
D+PT20	$9.3 \pm 0.2^{\#\#\#\$\$\$}$	$- 4.7 \pm 0.1^{\#\#\#}$	$4.7 \pm 0.1^{\#\#\#}$
D+PT40	10.7 ± 0.1	$- 4.8 \pm 0.1^{\#}$	$5.3 \pm 0.2^{\#\#}$
D+M	$9.4 \pm 0.2^{\#\#\$\$\$}$	$- 4.5 \pm 0.1^{\#\#\#\$\$\$}$	$4.0 \pm 0.2^{\#\#\#\$\$\$}$

Data was expressed as mean ± SEM (n=10). C, D, PT20, PT40 and M, stand for control, diabetic, pterostilbene 20 mg/Kg, pterostilbene 40 mg/Kg, and metformin, respectively. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{***}P$<0.001 vs C; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs D; $^{\$\$\$}P$<0.001 vs D+PT40

5.1.5. Effect of PT treatment on blood pressure of fructose-fed diabetic rats

The effect of PT on blood pressure measurements of all experimental groups was represented in **Figure 5.2**. After eight weeks of fructose feeding, significant increases in systolic blood pressure (**Figure 5.2A**), diastolic blood pressure (**Figure 5.2B**) and mean arterial blood pressure (**Figure 5.2C**) (all $P<0.001$) were observed in D group rats compared to the C group rats. Although both doses of PT significantly reduced the systolic blood pressure, diastolic blood pressure and mean arterial blood pressure in fructose-fed diabetic rats, maximum reduction was observed in the D+PT20 group (all $P<0.001$ vs D) than D+PT40 group (all $P<0.05$ vs D; **Figure 5.2**).

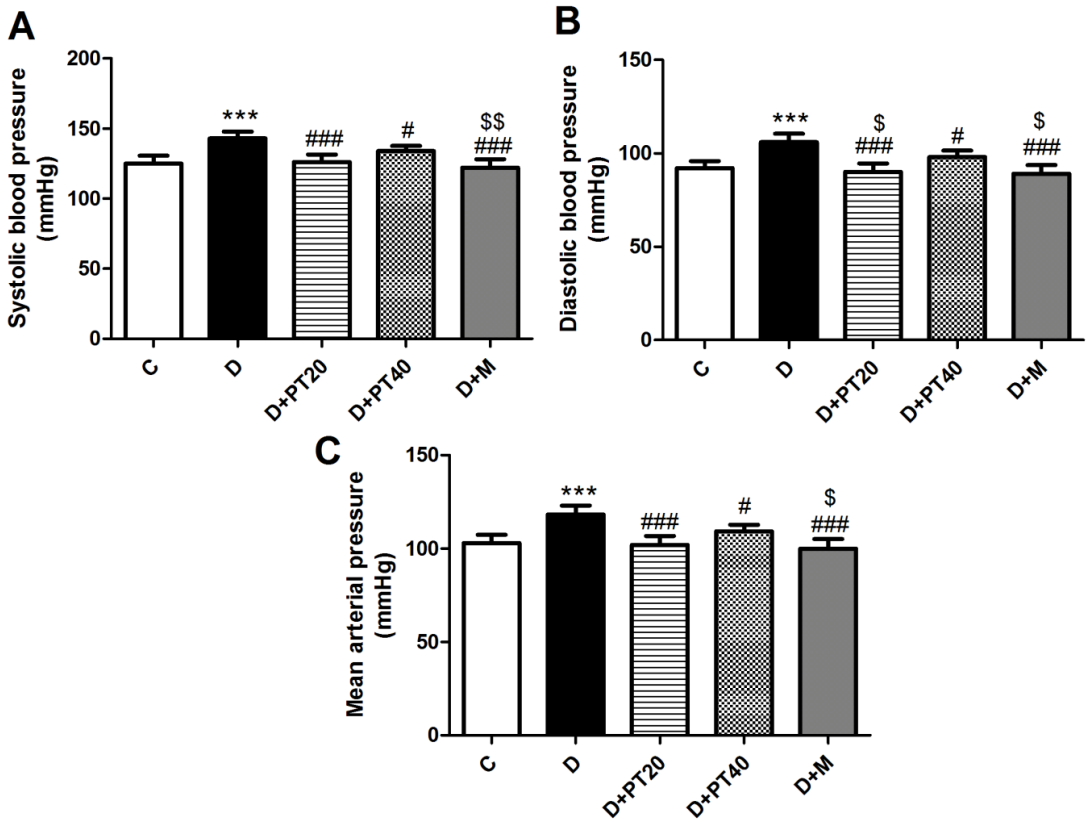

Figure 5.2: Effect of PT on blood pressure of experimental rats. A) Systolic blood pressure B) Diastolic blood pressure C) Mean arterial pressure. Results are represented as mean ± SD (n=6). C, D, PT20, PT40, M stand for control, diabetic, pterostilbene 20 mg/kg, pterostilbene 40 mg/kg, metformin, respectively. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{***}P<0.001$ vs C; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs D; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs D+PT40.

5.1.6. Effect of PT treatment on lipid profile, cardiovascular risk indices and antiatherogenic index (AAI) of fructose-fed diabetic rats

The effect of PT on lipid profile, cardiovascular risk indices and AAI of all experimental groups were represented in **Figure 5.3**. After eight weeks of fructose feeding, there were no changes in TC (**Figure 5.3A**) levels between diabetic and control rats. Conversely, a significant increase in serum TG (P<0.001; **Figure 5.3B**), LDL-C (P<0.01; **Figure 5.3D**) and VLDL-C (P<0.001; **Figure 5.3E**), while a marked (P<0.001) decline in HDL-C levels (**Figure 5.3C**) was observed in the D group in comparison to the C group. Besides, D group rats showed significantly elevated TC/HDL-C ratio (**Figure 5.3F**) and LDL-C/HDL-C ratio (**Figure 5.3G**) (all P<0.001 vs C). Moreover, the AAI was markedly (P<0.001) decreased in D group (**Figure 5.3H**). By comparison, oral administration of PT 20 mg/kg to fructose-fed diabetic rats markedly (P<0.001 vs D) alleviated the altered serum lipid profile, cardiovascular risk indices, and AAI. Although PT 40 mg/kg significantly decreased TG (P<0.01 vs D), VLDL-C (P<0.01 vs D), TC/HDL-C ratio (P<0.001 vs D) and LDL-C/HDL-C ratio (P<0.05 vs D), it did not have any effect on HDL-C, LDL-C, and AAI parameters. However, there was no significant difference observed between D+PT20 and D+M groups (**Figure 5.3**).

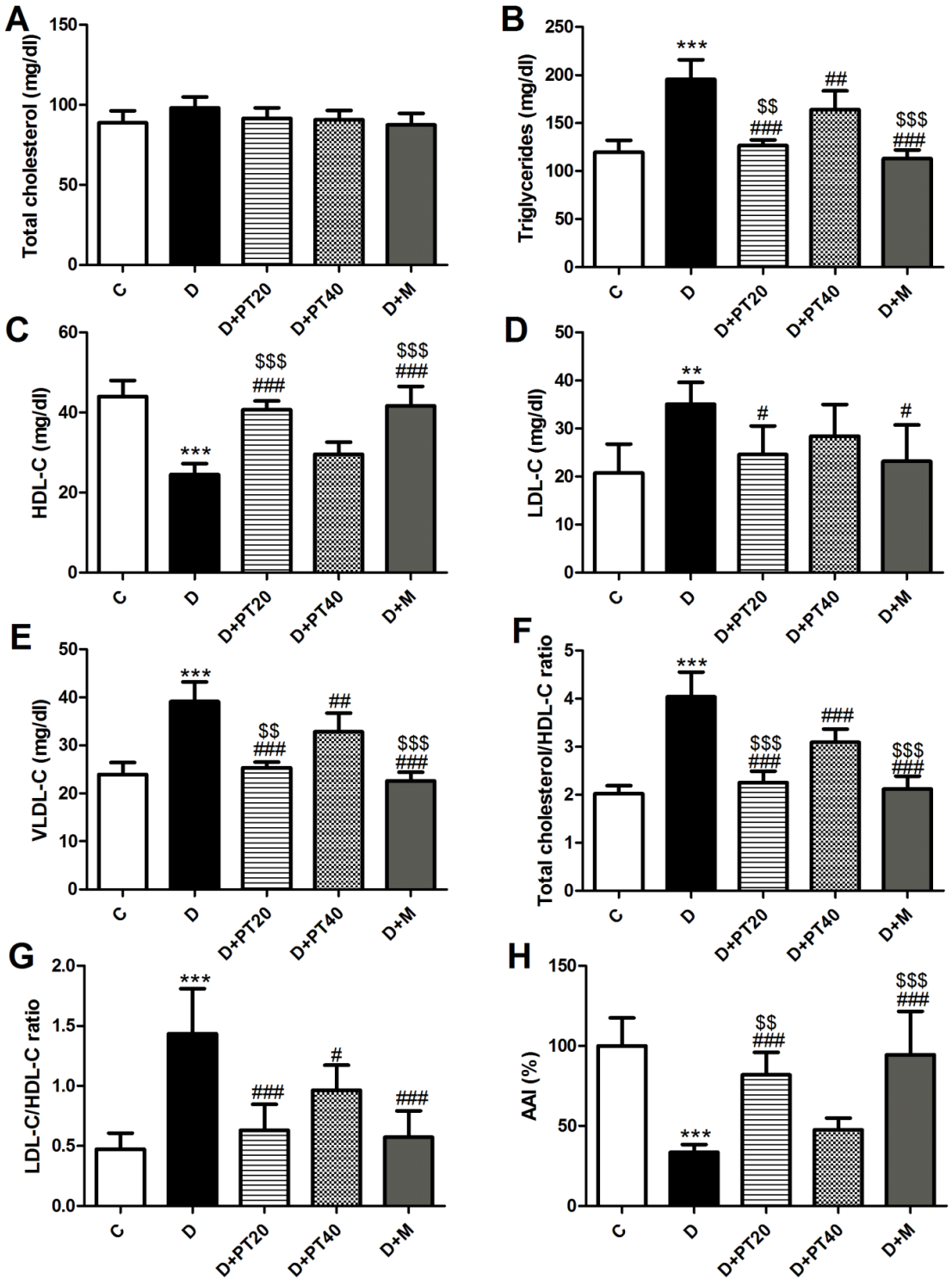

Figure 5.3: Effect of PT on lipid profile, cardiovascular risk indices and antiatherogenic index (AAI) of experimental rats. A) Total cholesterol B) Triglycerides C) HDL-C D)LDL-C E) VLDL-C F) Total cholesterol/HDL-C ratio G) LDL-C/HDL-C ratio H) AAI. C, D, PT20, PT40, M, HDL-C, LDL-C, VLDL-C and AAI stand for control, diabetic, pterostilbene 20 mg/kg, pterostilbene 40 mg/kg, metformin, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol,

very low density lipoprotein-cholesterol, antiatherogenic index respectively. Results are represented as mean ± SD (n=10). Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{**}P<0.01$, $^{***}P<0.001$ vs C; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs D; $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ vs D+PT40.

5.1.7. Effect of PT treatment on glycated haemoglobin, uric acid, peroxynitrite and hydrogen sulfide levels in fructose-fed diabetic rats

After eight weeks fructose feeding, D group rats demonstrated significantly ($P<0.001$) elevated blood levels of HbA1c in comparison to the C group. However, a marked decline in HbA1c levels was seen in D+PT20, D+PT40 and D+M groups as compared to D group (all $P<0.05$; **Figure 5.4A**). There was no significant difference among D+PT20, D+PT40 and D+M groups.

After eight weeks of fructose feeding, D group rats demonstrated significantly ($P<0.001$) enhanced levels of serum uric acid in comparison to the C group. Long-term supplementation of PT (both D+PT20 and D+PT40 groups) markedly (all $P<0.05$) decreased serum uric acid levels in comparison to the D group (**Figure 5.4B**). However, there was no significant difference among D+PT20, D+PT40 and D+M groups.

After eight weeks of fructose feeding, D group rats showed markedly enhanced levels of serum peroxynitrite ($P<0.001$) in comparison to the C group. Long-term supplementation of PT (both D+PT20 and D+PT40 groups) significantly (all $P<0.05$) decreased serum peroxynitrite levels compared to the D group (**Figure 5.4C**). However, there was no significant difference among D+PT20, D+PT40 and D+M groups.

After eight weeks of fructose feeding, serum hydrogen sulfide levels were markedly reduced ($P<0.001$) in the D group in comparison to the C group. Although both doses significantly enhanced serum hydrogen sulfide concentrations in the fructose-fed

diabetic rats, the maximum enhancement was observed in the D+PT40 group (P<0.001 vs D) than D+PT20 group (P<0.05 vs D; **Figure 5.4D**). However, there was no significant difference between D+PT40 and D+M groups.

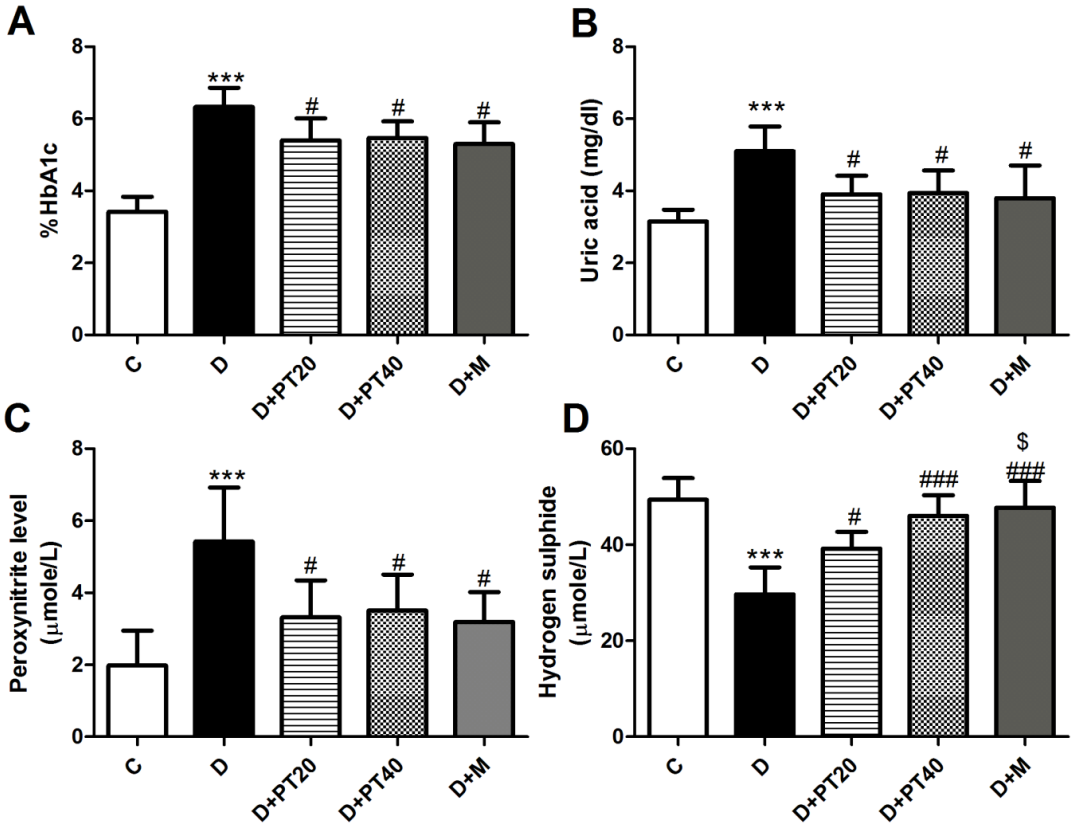

Figure 5.4: Effect of PT treatment on A) serum glycated haemoglobin (HbA1c) levels B) serum uric acid levels C) serum peroxynitrite levels D) serum hydrogen sulphide levels after 8 weeks of fructose feeding. C, D, PT20, PT40, and M stand for control, diabetic, pterostilbene 20 mg/Kg, pterostilbene 40 mg/Kg, and metformin, respectively. Results are represented as mean ± SD (n=10). Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{***}P$<0.001 vs C; $^{\#}P$<0.05, $^{\#\#\#}P$<0.001 vs D; $^{\$}P$<0.05, $^{\$\$\$}P$<0.001 vs D+PT20.

5.1.8. Effect of PT treatment on liver TBARS, SOD and GSH levels of fructose-fed diabetic rats

Table 5.4 illustrates hepatic TBARS, SOD and GSH levels after eight weeks of fructose feeding in all experimental groups. Concerning hepatic lipid peroxidation, D group rats demonstrated significantly enhanced levels of TBARS (P<0.001) in comparison to the

C group rats. Treatment of fructose-fed diabetic rats with PT (20 and 40 mg/kg) significantly alleviated (all $P<0.001$ vs D) hepatic lipid peroxidation levels. However, there was no significant change in lipid peroxidation levels among D+PT20, D+PT40 and D+M groups ($P>0.05$).

On the contrary, after eight weeks of fructose feeding, hepatic SOD ($P<0.001$) and GSH ($P<0.001$) levels were significantly decreased in the D group in comparison to the C group. Although both doses of PT potentially ameliorated hepatic SOD and GSH levels, maximum effect was evident with PT 40 mg/kg (all $P<0.001$ vs D) when compared to D group. However, there was no significant difference observed in liver SOD and GSH levels between D+PT40 and D+M groups ($P>0.05$) (**Table 5.4**).

Table 5.4: Hepatic TBARS, SOD and GSH levels in experimental rats after eight weeks

Groups	Liver		
	TBARS (nmole/gm wet tissue)	**Total SOD (U/gm protein)**	**GSH (nmole/gm wet tissue)**
C	35.0 ± 1.8	59.0 ± 2.5	61.0 ± 2.7
D	55.2 ± 2.0***	26.2 ± 1.6***	40.5 ± 2.6***
D+PT20	43.0 ± 2.1$^{\#\#\#}$	42.2 ± 2.7$^{\#\#}$	57.2 ± 2.7$^{\#\#}$
D+PT40	42.7 ± 1.8$^{\#\#\#}$	57.7 ± 3.8$^{\#\#\#\$\$}$	57.5 ± 2.4$^{\#\#\#}$
D+M	39.2 ± 1.5$^{\#\#\#}$	59.2 ± 3.1$^{\#\#\#\$\$}$	58.6 ± 2.3$^{\#\#\#}$

Data was expressed as mean ± SEM (n=10); C, D, PT20, PT40 and M stand for control, diabetic, pterostilbene 20 mg/Kg, pterostilbene 40 mg/Kg, metformin. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. $^{***}P<0.001$ vs C; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs D; $^{\$\$}P<0.01$ vs D+PT20.

5.2. 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)

5.2.1. Effect of PT treatment on the MAP, heart rate, body weight and hypertrophy index in the fructose-fed diabetic rats

Our earlier study showed that fructose diet for eight-week period induces type 2 diabetes accompanied by metabolic syndrome and insulin resistance in rats (Kosuru & Singh, 2017). PT or vehicle (10% β-cyclodextrin) was administered to fructose-fed rats, and cornstarch fed rats for eight weeks. To reveal whether PT decreases hypertension in the fructose-fed rats, both systolic and diastolic blood pressures were measured using a noninvasive tail-cuff method and estimated the MAP. After eight weeks, the MAP of vehicle-treated fructose-fed rats was increased significantly in comparison to control rats, but in the PT treated fructose-fed rats, it remained at a lower level when compared to D group (**Table 5.5**). The heart rate of PT-administered fructose-fed rats were also markedly lower ($P < 0.05$) than the vehicle-treated fructose-fed rats (**Table 5.5**). However, the body weight of the vehicle-treated fructose-fed rats did not significantly vary from the control rats; PT treatment significantly reduced the body weight in the fructose-fed rats. Furthermore, PT administration decreased the higher ratio of relative heart weight to body weight, a hypertrophy index of heart, in fructose-fed rats, suggesting that PT administration reduces fructose-fed induced hypertension and cardiac hypertrophy (**Table 5.5**). However, PT didn't modulate any of these parameters in nondiabetic rats, i.e. rats fed with cornstarch indicate that PT acts specifically on these physiological parameters only in diabetic condition.

Table 5.5: Effect of PT treatment on the MAP, heart rate, body weight and hypertrophy index in the control and fructose-fed diabetic rats

Parameters	Groups			
	C	**C+PT20**	**D**	**D+PT20**
MAP (mmHg)	108.50 ± 12.21	106.30 ± 14.45	123.90 ± 8.13$^{*\#}$	109.60 ± 7.31$^{\$}$
Heart rate (beats/minute)	298.60 ± 17.96	290.70 ± 14.77	329.70 ± 27.35$^{*\#}$	304.30 ± 16.06$^{\$}$
Body weight (g)	440.20 ± 12.36	431.60 ± 8.71	437.00 ± 9.77	408.00 ± 7.35$^{\$}$
Hypertrophy index (mg/g)	2.12 ± 0.32	2.12 ± 0.18	2.76 ± 0.16$^{*\#}$	2.30 ± 0.17$^{\$}$

Values are represented as mean ± SD, n = 8. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D. C, control; D, fructose-fed diabetic; PT20, pterostilbene 20 mg/kg/day; MAP, mean arterial pressure.

5.2.2. Effect of PT treatment on glycemic control and cardiac injury markers in fructose-fed diabetic rats

A fructose-fed rat model was used to examine how fructose diet influences glycemic control and cardiac damage and potential benefits of PT. As depicted in **Table 5.6**, the fructose diet markedly increased blood glucose levels, which was efficiently decreased by PT. Serum enzyme activities of LDH, CK-MB and AST are usually regarded as damage markers of myocardial infarction, and also useful for determination of the cardiac structural integrity in diabetic rats (Badole et al., 2015). In the current fructose diet feeding, significant increases were observed on serum LDH, CK-MB and AST, which all were efficiently lessened by PT treatment (**Table 5.6**).

Table 5.6: Effect of PT treatment on blood glucose, serum levels of LDH, CK-MB and AST in control and fructose-fed diabetic rats

Parameters	Groups			
	C	**C+PT20**	**D**	**D+PT20**
Blood glucose (mmol/L)	6.91 ± 2.21	6.02 ± 1.89	13.46 ± 1.27$^{*\#}$	11.05 ± 1.58$^{\$}$
LDH (U/L)	351.45 ± 114.89	336.25 ± 127.51	736.50 ± 153.11$^{*\#}$	515.65 ± 98.732$^{\$}$
CK-MB (U/L)	534.72 ± 149.32	508.46 ± 167.41	812.53 ± 154.73$^{*\#}$	625.70 ± 89.21$^{\$}$
AST (U/L)	172.77 ± 16.70	159.63 ± 27.10	206.25 ± 18.12$^{*\#}$	180.21 ± 14.03$^{\$}$

Values are represented as mean ± SD, n = 8. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D. C, control; D, fructose-fed diabetic; PT20, pterostilbene 20 mg/kg/day; LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB; AST, aspartate aminotransferase.

5.2.3. Effect of PT treatment on the myocardial oxidative stress in fructose-fed diabetic rats

Since oxidative stress is associated with diabetic cardiomyopathy (Golbidi et al., 2014), TBARS (a marker of lipid oxidation), ROS, hydrogen peroxide and peroxynitrite levels were measured to assess the status of oxidative stress in the cardiac homogenate of all experimental rats. Fructose-fed vehicle-treated rats had significantly more oxidative stress in the cardiac tissue based on higher levels of TBARS (**Figure 5.5A**), ROS (**Figure 5.5B**), hydrogen peroxide (**Figure 5.5C**) and peroxynitrite (**Figure 5.5D**) in comparison to control rats, indicating that fructose diet markedly enhanced oxidative stress in the hearts of diabetic rats. PT treatment significantly reversed all of these oxidative stress markers in the myocardium of fructose-fed rats (**Figure 5.5A-D**), but had no influence on oxidative stress in control rats.

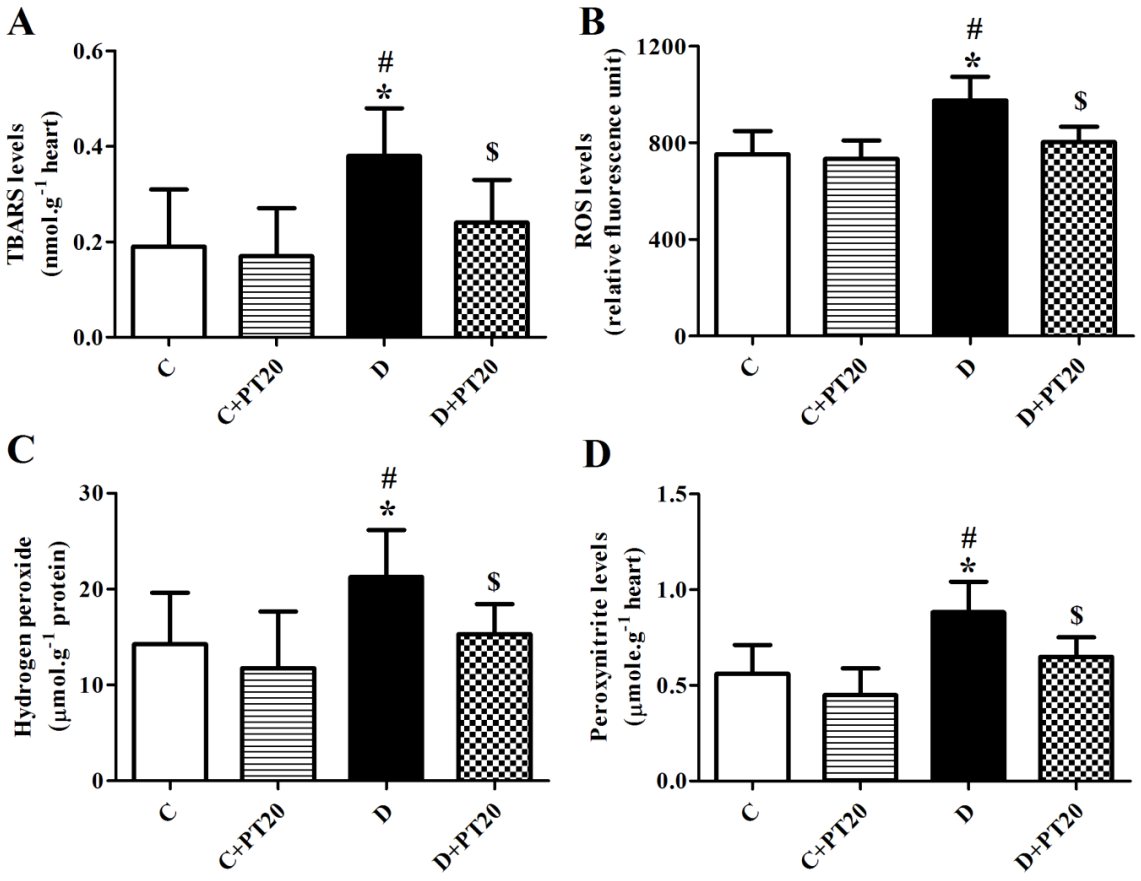

Figure 5.5: Effect of PT on the cardiac oxidative stress in control and fructose-fed diabetic rats. A) Cardiac TBARS levels B) cardiac ROS levels C) cardiac hydrogen peroxide levels D) cardiac peroxynitrite levels. Values are represented as mean ± SD, n = 8. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D.

5.2.4. Effect of PT treatment on the antioxidant defence system in the myocardium of fructose-fed diabetic rats

Both enzymatic (SOD, catalase, GPx) and non-enzymatic (GSH) antioxidant concentrations in the cardiac tissues of all experimental rats were measured to estimated the cardiac antioxidant status. Fructose-fed vehicle-treated rats demonstrated lack of antioxidant defence in the cardiac muscle based on lower levels of total SOD (**Figure 5.6A**), catalase (**Figure 5.6B**), GSH (**Figure 5.6C**), GPx (**Figure 5.6D**) compared to controls, clearly demonstrating fructose diet significantly diminished antioxidant

defence system in the myocardium. PT treatment to fructose-fed rats significantly reversed all of these indicators of antioxidant defence system (**Figure 5.6A-D**), suggesting the PT enhances the antioxidant enzyme levels to guard the myocardium against detrimental oxidative stress.

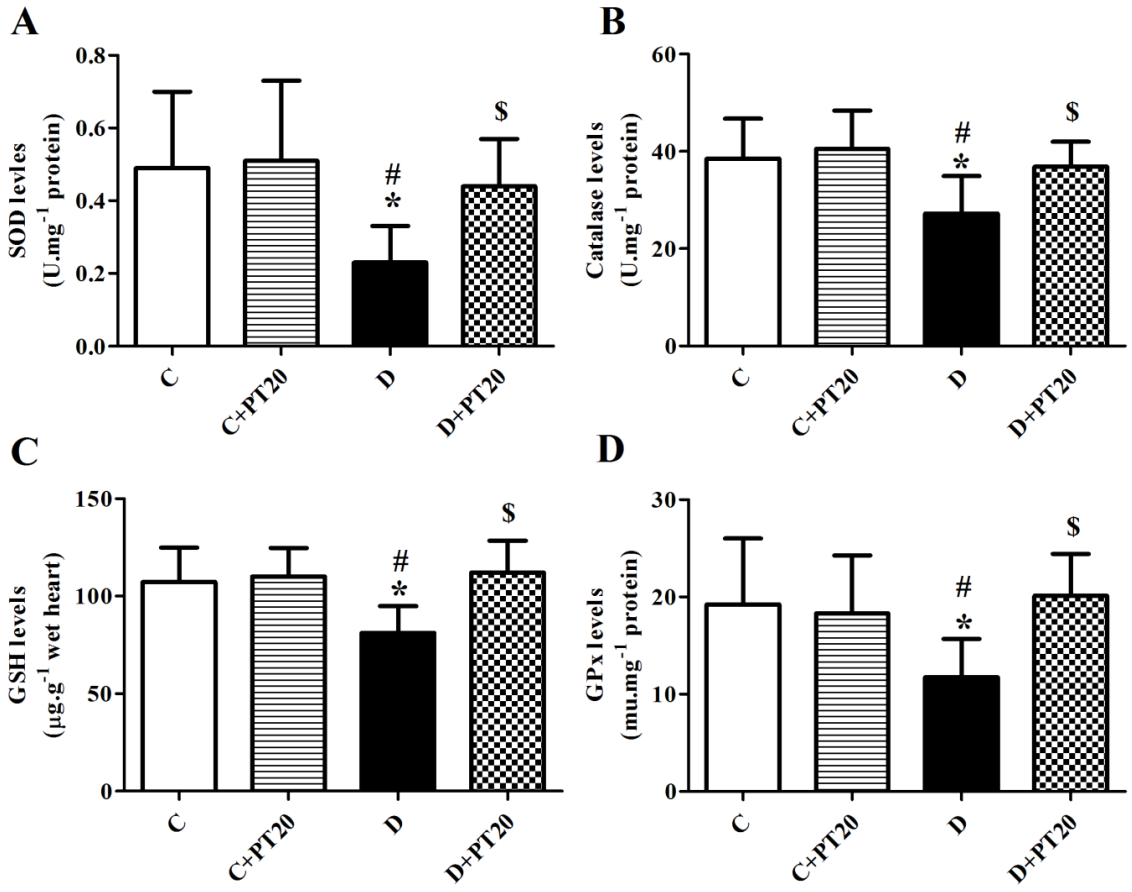

Figure 5.6: Effect of PT on the cardiac antioxidant defence system in control and fructose-fed diabetic rats. A) myocardial SOD levels B) myocardial catalase levels C) myocardial GSH levels and D) myocardial GPx levels. Values are represented as mean ± SD, n = 8. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D. C, control; D, fructose-fed diabetic; PT20, pterostilbene 20 mg/kg/day; SOD, total superoxide dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase.

5.2.5. Effect of PT treatment on the myocardial inflammation in fructose-fed diabetic rats

Since oxidative stress is directly linked to inflammation (Mittal et al., 2014), inflammatory markers were measured in the heart tissues of all experimental groups. It is observed that pro-inflammatory cytokines, such as IL-1β (**Figure 5.7A**), IL-6 (**Figure 5.7B**) and TNF-α (**Figure 5.7C**), were markedly elevated in the myocardium of fructose-fed rats versus controls. However, PT administration significantly normalised the augmented levels of IL-1β, IL-6 and TNF-α in the myocardium of fructose-fed rats (**Figure 5.7A-C**). Furthermore, a similar kind of results was observed in plasma pro-inflammatory cytokines from PT administered fructose-fed diabetic rats and control rats (**Figure 5.7D-F**), suggesting that PT treatment efficiently diminished fructose diet-induced cardiac and systemic inflammation.

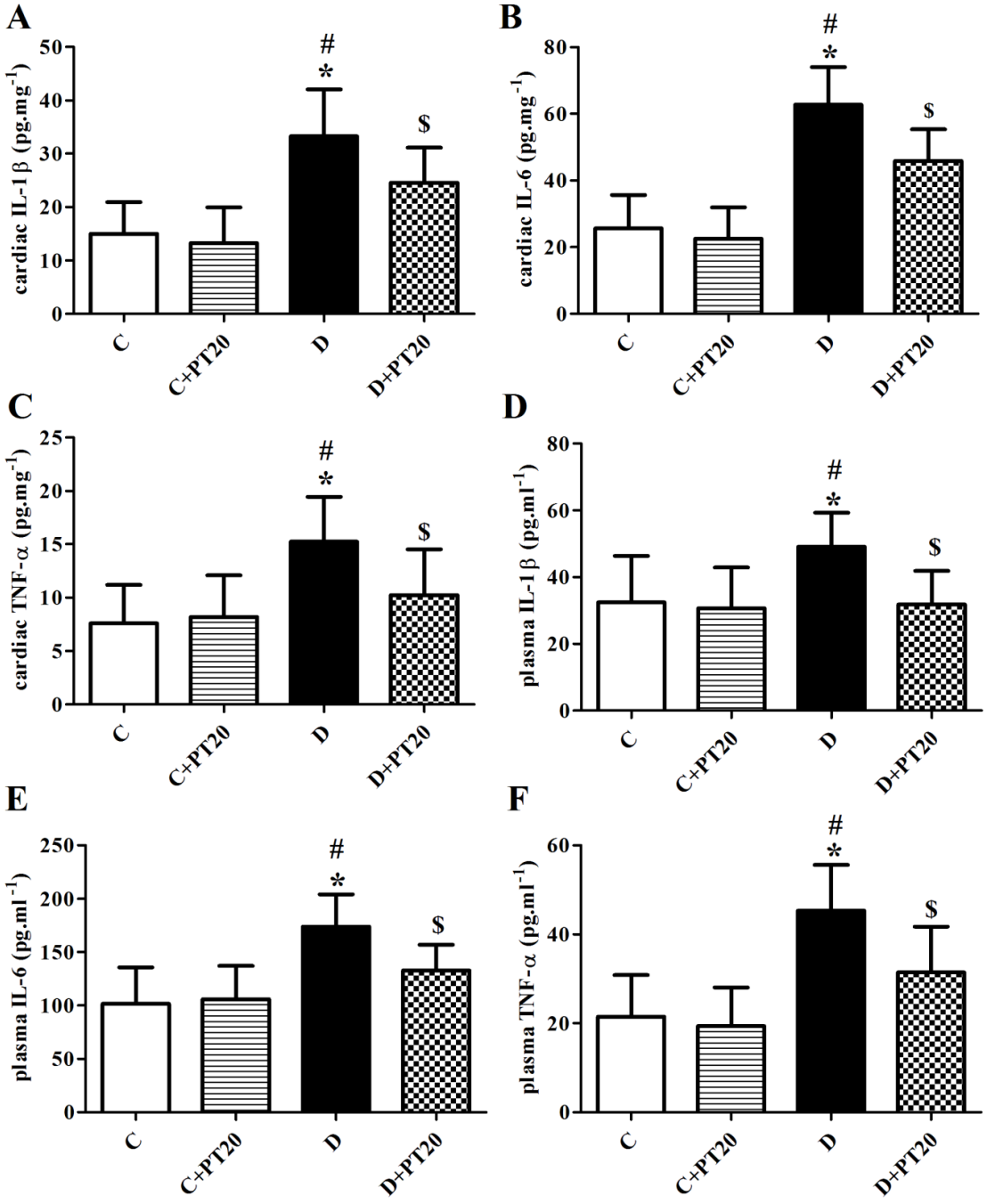

Figure 5.7: Effect of PT supplementation on cardiac and plasma inflammatory cytokines of control and fructose-fed diabetic rats. A) cardiac IL-1β levels B) cardiac IL-6 levels C) cardiac TNF-α levels D) plasma IL-1β levels E) plasma IL-6 levels F) plasma TNF-α levels. Values are represented as mean ± SD, n = 6. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D. C, control; D, fructose-fed diabetic; PT20, pterostilbene 20 mg/kg/day; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

5.2.6. Effect of PT treatment on the myocardial NF-κB expression and inflammasome in fructose-fed diabetic rats

Fructose-fed diabetic rats demonstrated marked increase in the myocardial mRNA and protein expression of NF-κB when compared to control rats. A significant decrease in mRNA (**Figure 5.8A**) and protein (**Figure 5.8B**) expression of cardiac NF-κB were observed with PT administration to fructose-fed diabetic rats (**Figure 5.8A-B**) whereas CC treatment enhanced the same. However, co-administration with CC markedly prevented the inhibitory effect of PT on NF- κB, suggesting that PT administration might decrease NF-κB-mediated inflammation in fructose-fed diabetic rats through AMPK dependent manner.

Furthermore, fructose-fed diabetic rats demonstrated elevated mRNA expressions of myocardial TLR4 (**Figure 5.8C**), NLRP3 (**Figure 5.8D**) and ASC (**Figure 5.8E**) in comparison to control rats. PT effectively reduced the mRNA levels of the same, whereas CC treatment markedly enhanced them (**Figure 5.8C-E**). However, co-administration with CC prevented the suppressive effect of PT on mRNA expressions of inflammasome components, indicating that PT effectively attenuated cardiac inflammation via inhibition of NLRP3 inflammasome through activation of AMPK signalling.

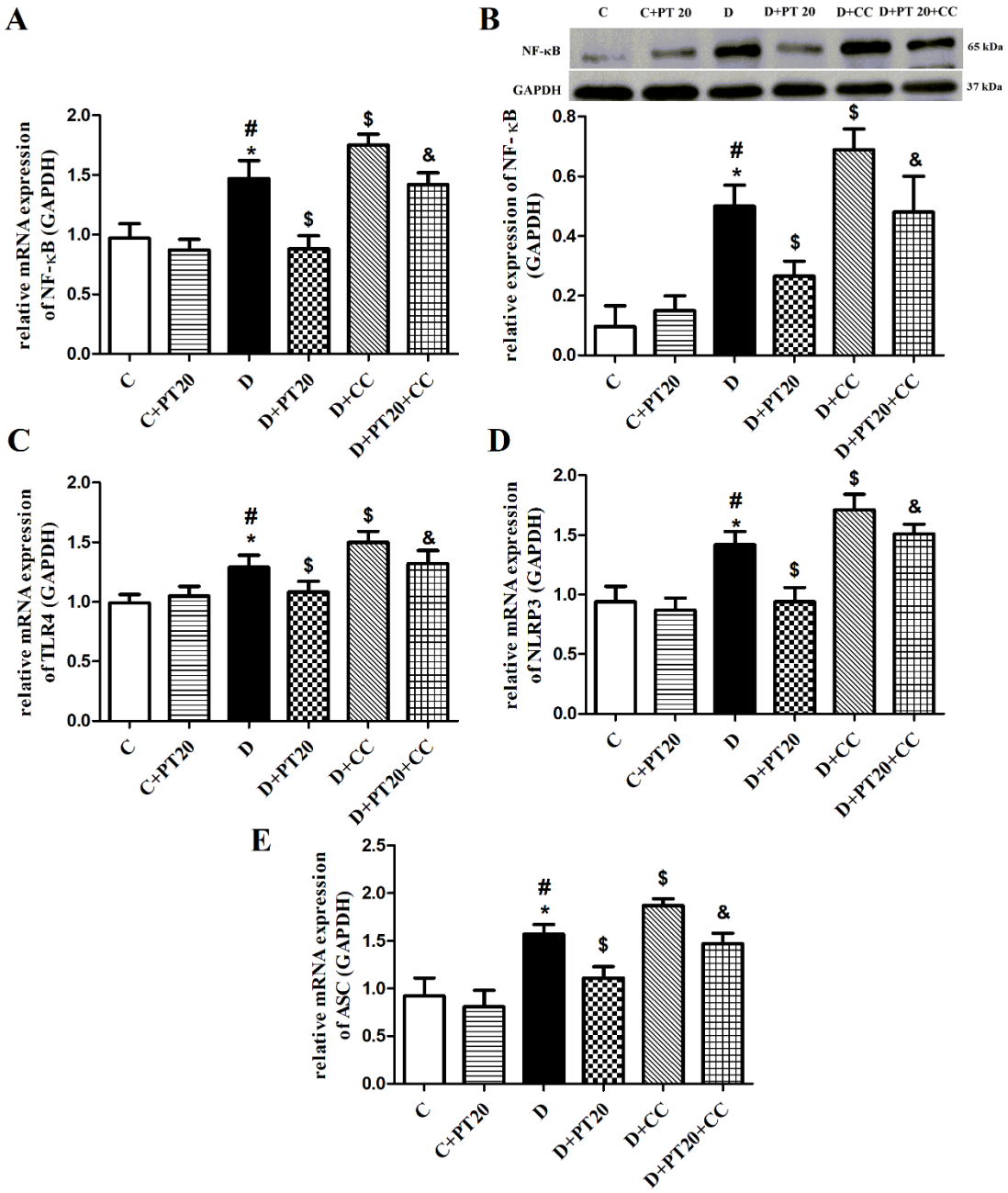

Figure 5.8: Effect of PT treatment on inflammatory response in cardiac tissues of control and fructose-fed diabetic rats. A) NF-κB mRNA expression B) NF-κB protein expression C) TLR4 mRNA expression D) NLRP3 mRNA expression E) ASC mRNA expression. Values are represented as mean ± SD, n = 4 independent experiments. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D; (&) significantly different compared to D+PT20.

5.2.7. Effect of PT treatment on mitochondrial biogenesis in the hearts of fructose-fed diabetic rats

Mitochondria are crucial elements involved in the regulation of cellular energetic, including ATP generation and fatty acid beta-oxidation. Previous reports demonstrated that oxidative stress is known to cause mitochondrial dysfunction (Okonko & Shah, 2015), determination of mitochondrial biogenesis, a direct index of mitochondrial activity, in the myocardium of diabetic rats indicates the status of mitochondrial health. Fructose-fed diabetic rats demonstrated markedly decreased levels of PGC-1α, Complex III and Complex V, in comparison to control rats (**Figure 5.9A-D**). PT treatment significantly enhanced the mRNA expression of PGC-1α (**Figure 5.9A**) and protein levels of myocardial PGC-1α (**Figure 5.9B**), Complex III (**Figure 5.9C**) and Complex V (**Figure 5.9D**) in fructose-fed diabetic rats, whereas CC treatment markedly decreased the mRNA and protein levels (**Figure 5.9A-D**). However, co-administration with CC abolished the stimulant effect of PT on mitochondrial biogenesis indicating that PT administration alleviates fructose diet-induced diminution of cardiac mitochondrial biogenesis through AMPK stimulation.

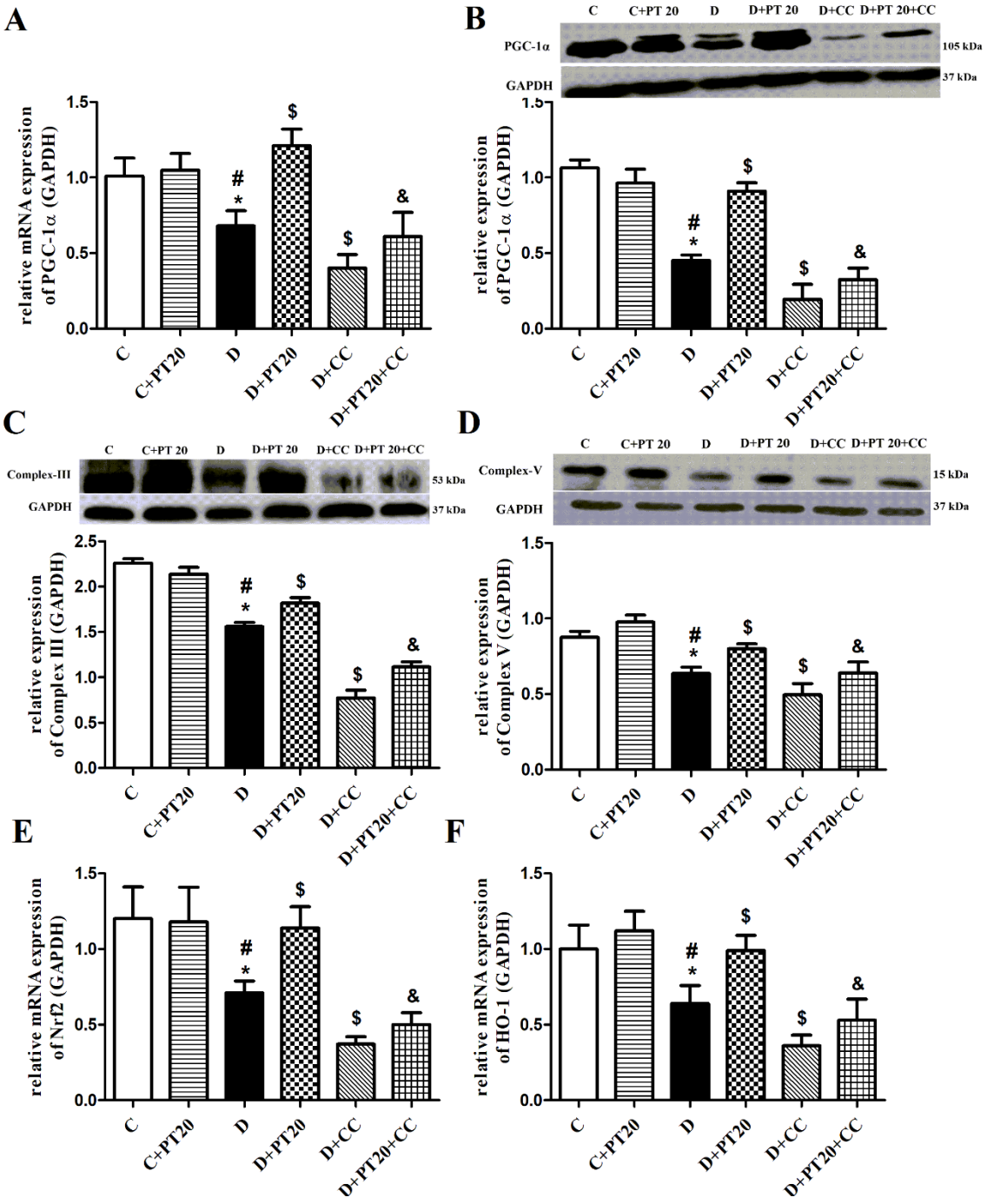

Figure 5.9: Effects of PT administration on the expression of the mitochondrial biogenesis proteins, Nrf2, HO-1 in the hearts of control and fructose-fed diabetic rats. Cardiac A) mRNA expression of PGC-1α B) protein expression of PGC-1α C) protein expression of Complex III D) protein expression of Complex V E) mRNA expression of Nrf2 F) mRNA expression of HO-1. Values are represented as mean ± SD, n = 4 independent experiments. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D; (&) significantly different

compared to D+PT20. C, control; D, fructose-fed diabetic; PT20, pterostilbene 20 $mg.kg^{-1}.day^{-1}$; CC, compound C; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator; Nrf2, nuclear factor erythroid 2–related factor 2; HO-1, heme-oxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

5.2.8. Effect of PT treatment on the mRNA expression of Nrf2, HO-1 in cardiac tissues of fructose-fed diabetic rats

It is known that Nrf2/HO-1 axis acts as stress-protective signalling by regulating cellular redox balance and protective antioxidant enzymes (Loboda et al., 2016). Thus mRNA expression of Nrf2, HO-1 in cardiac tissues of control and fructose-fed diabetic rats were estimated to validate the Nrf2/HO-1 axis. A marked decrement ($P < 0.05$) in mRNA expressions of Nrf2 (**Figure 5.9E**) and HO-1 (**Figure 5.9F**) was observed in the cardiac tissue of fructose-fed diabetic rats in comparison to control rats. Oral administration of PT significantly enhanced the mRNA expressions of Nrf2 and HO-1 in heart tissues of fructose-fed diabetic rats, whereas CC treatment significantly reduced the same (**Figure 5.9E, F**). However, co-administration with CC abrogated PT's stimulant effect indicating that PT augments the mRNA expression of Nrf2 and HO-1 through AMPK dependent manner.

5.2.9. Effect of PT on AMPK/Nrf2/HO-1 signalling pathway in fructose-fed diabetic rats

The mechanism through which PT treatment reduces oxidative stress, inflammation and augments mitochondrial biogenesis in the hearts of fructose-fed rats was also investigated. Phosphorylated AMPK stimulates Nrf2, which increases the expression of antioxidant defence proteins, including HO-1, which safeguard against oxidative stress insult accelerated by injury and inflammation (Mo et al., 2014; Zimmermann et al., 2015). It is observed that p-AMPK/AMPK ratio was significantly reduced in the myocardium of fructose-fed vehicle-treated rats, and PT administration stimulated it

(**Figure 5.10A**). Administration of CC diminished p-AMPK/AMPK ratio considerably, whereas coadministration of CC significantly blocked PT-mediated phosphorylation of AMPK (**Figure 5.10A**). Similarly, downstream proteins including Nrf2 and HO-1 levels were enhanced in response to PT administration to fructose-fed rats (**Figure 5.10B, C**). However, coadministration of CC markedly abolished the PT-mediated augmentation of Nrf2 and HO-1 levels (**Figure 5.10B, C**). These data indicate that PT treatment decreases oxidative stress, inflammation and ameliorates mitochondrial biogenesis in the myocardium of fructose-fed rats mediated by activation of the AMPK/Nrf2/HO-1 signalling pathway.

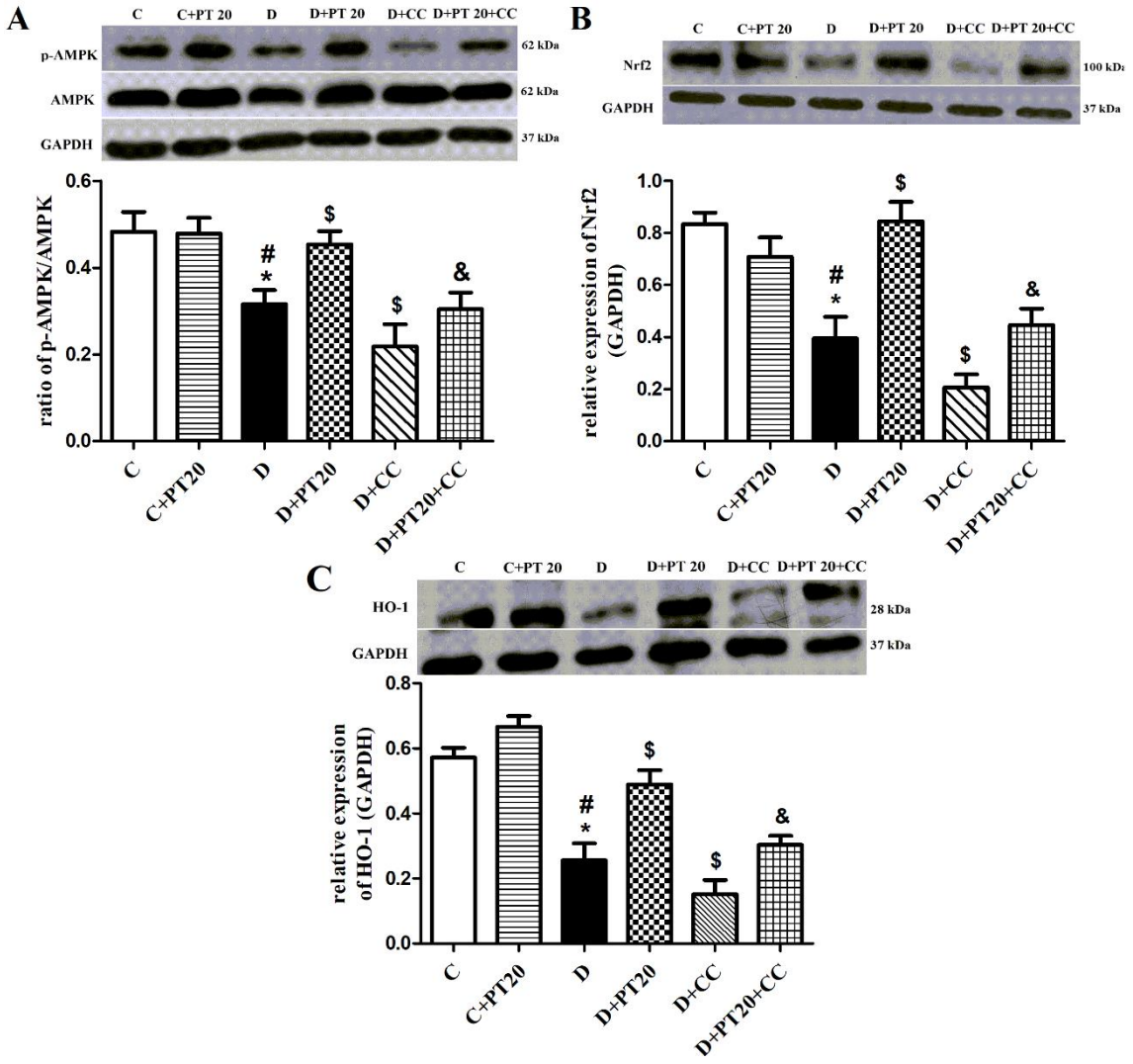

Figure 5.10: Effects of PT administration on the protein expression levels of AMPK/Nrf2/HO-1 signalling components in the hearts of control and fructose-fed diabetic rats. Myocardial expression of A) AMPK, p-AMPK B) Nrf2 C) HO-1. Values are represented as mean ± SD, n = 4 independent experiments. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison post hoc tests, and the significance was set at $P < 0.05$. (*) Significantly different compared to C; (#) significantly different compared to C+PT20; ($) significantly different compared to D; (&) significantly different compared to D+PT20. C, control; D, fructose-fed diabetic; PT20, pterostilbene 20 $mg.kg^{-1}.day^{-1}$; CC, compound C; AMPK, AMP-activated protein kinase; Nrf2, nuclear factor erythroid 2–related factor 2; HO-1, heme-oxygenase. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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

5.3.1. Effect of PT treatment on general characteristics of streptozotocin diabetic rats

As shown in **Table 5.7**, four weeks of PT treatment at both doses (20 and 40 mg/kg) significantly decreased plasma glucose levels (all $P<0.001$ vs D+IR), increased heart weight (all $P<0.001$ vs D+IR) and body weight (all $P<0.05$ vs D+IR) when compared to diabetic rats without treatment. Intriguingly, PT (at either 20 or 40 mg/kg) significantly reduced the ratio of heart weight to body weight (an indirect indicator of myocardial hypertrophy) in diabetic rats (all $P<0.001$ vs D+IR). In line with this, PT (20 and 40 mg/kg) significantly (all $P<0.001$) decreased the diabetes-induced alteration of cardiomyocyte cross-sectional area in diabetic rats (**Figure 5.11A**), indicating that PT can attenuate cardiac hypertrophy in diabetes.

Table 5.7: Effect of PT treatment on general characteristics of experimental rats.

General characteristics	D	D+IR	D+IR+PT20	D+IR+PT40
Plasma glucose (mM)	23.24±0.06	22.60±0.24	15.68±0.08***	17.98±0.21$^{***\#\#\#}$
Heart weight (g)	1.18±0.01	1.20±0.01	1.38±0.02***	1.35±0.02***
Body weight (g)	238.75±10.14	250.25±12.44	347.50±24.39*	336.00±14.30*
Heart weight/body weight (mg/g)	4.90±0.01	4.85±0.01	3.98±0.02***	4.01±0.03***

All values are represented as mean ± standard error mean (SEM). N=8/group. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. $^{*}P<0.05$, $^{***}P<0.001$ vs D+IR; $^{\#\#\#}P<0.001$ vs D+IR+PT20 group.

5.3.2. Effect of PT treatment on post-ischemic myocardial injury in streptozotocin-induced diabetic rats

To examine whether PT decreased IR-induced myocardial apoptotic cell death, myocardial infarct size, area at risk, plasma CK-MB, LDH release, free 8-isoprostane and myocardial apoptotic index after 2-hour of reperfusion in different groups were further measured. TTC staining was employed to demonstrate infarct size (**Figure 5.11B**). Myocardial infarct size was markedly increased in D+IR group when compared with the D group (48.60±2.75% vs. 0.9±0.02%; n=8; P<0.001). In contrast, PT treatment significantly reduced myocardial infarct size, at both doses (20 and 40 mg/kg) (29.08±1.84%, 20.63±1.37% vs 53.76±2.17%, respectively; n=8; all P<0.001). However, the infarct-limiting effect of PT 40 was better than that of PT 20 in diabetic rats since the post-ischemic myocardial infarct size in PT 40 group was significantly smaller than that in the PT 20 group (P<0.05). No significant difference in the ratio of the area at risk to left ventricle was observed among D+IR, D+IR+PT 20 and D+IR+PT 40 groups (P>0.05, **Figure 5.11C**).

As shown in **Figure 5.11D, 5.11E,** plasma levels of LDH and CK-MB were remarkably increased in D+IR group when compared with D group. After two hours (2h) of reperfusion, treatment with PT at dosages 20 and 40 mg/kg significantly attenuated the post-ischemic levels of LDH (P<0.05 and P<0.01) and CK-MB (P<0.05 and P<0.01) in diabetic rats, in a dose-dependent manner when compared with the D+IR group.

After 2h of post-ischemic reperfusion, the plasma free 8-isoprostane level was markedly higher in the D+IR than that in the D group (**Figure 5.11F**, 367.20±9.45 vs 270.5±9.76; n=8; P<0.001). PT (20 mg/kg and 40 mg/kg) significantly reduced the IR-induced

elevations in plasma free 8-isoprostane levels when compared to D+IR group (331.70±9.45; n=8; $P<0.05$ and 321.60±6.56; n=8; $P<0.01$, respectively).

The apoptotic index was significantly higher in the D+IR group when compared to the D group (**Figure 5.12A,** 11.20±0.67% vs 2.77±0.38; n=6; $P<0.001$), which was markedly attenuated by treatments with employed doses of PT (20 mg/kg, 7.53±.39%; n=6; $P<0.01$ vs D+IR; PT 40 mg/kg, 4.93±0.44%; n=6; $P<0.001$ vs D+IR). Taken together, these data suggested that both doses of PT (20 and 40 mg/kg/d) could protect diabetic hearts against IR-induced cell necrosis, oxidative stress, and apoptosis.

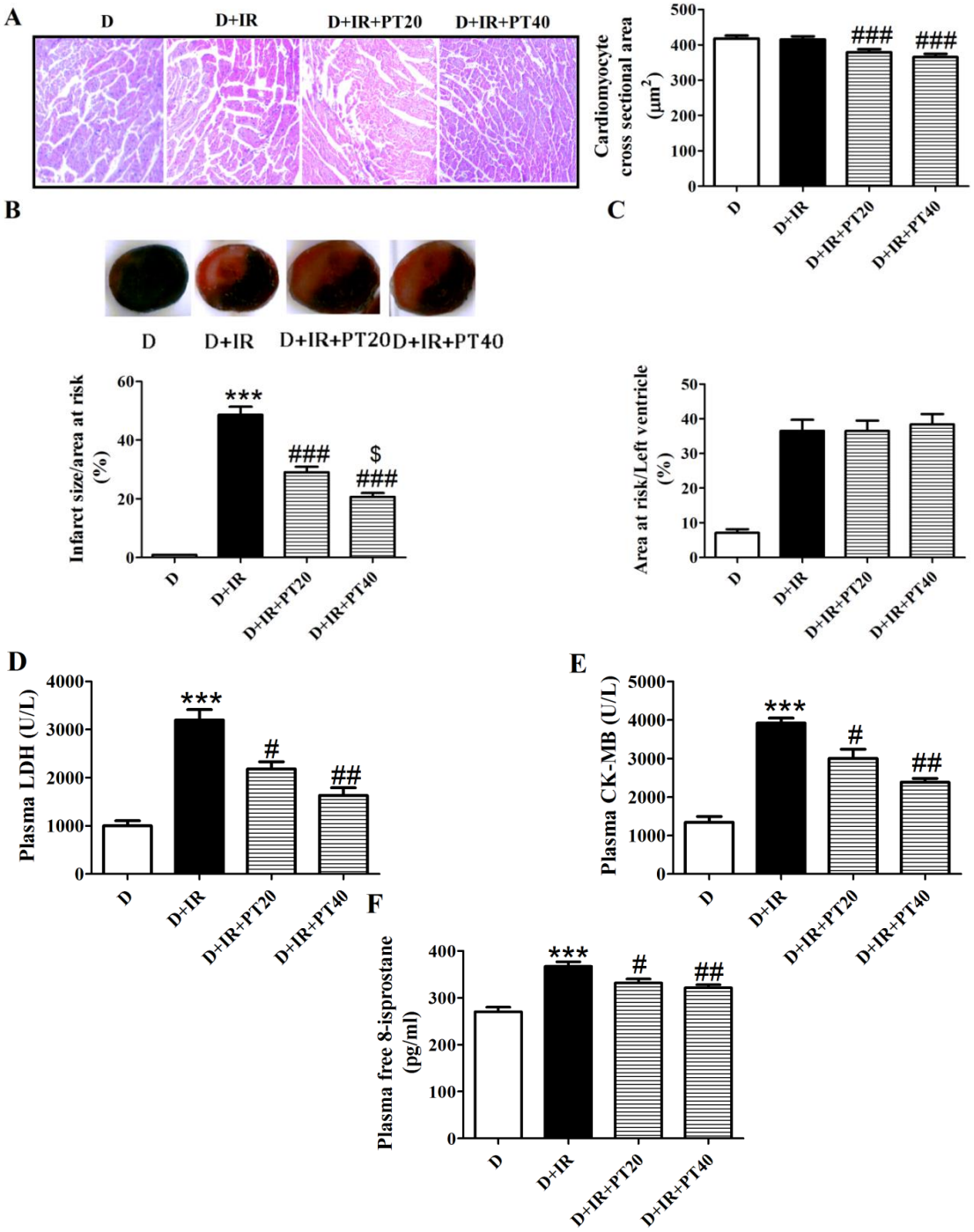

Figure 5.11: Effect of PT on myocardial IR injury in diabetic rats (infarct size, necrosis, and oxidative stress). (A) Cardiomyocytes cross-sectional area 8 weeks post-treatment by H-E staining. (B) Myocardial infarct size in diabetic rats subjected to 30 minutes ischemia, followed by 2 hours reperfusion. (C) The area at risk is expressed as percentage of left ventricle (D) Plasma lactate dehydrogenase (LDH) levels. (E) Plasma creatine kinase-MB (CK-MB) levels. (F) Plasma free 8-isoprostane levels. Blue-staining demonstrates live& non-ischemic area, red-staining demonstrates the area at risk, and pale area demonstrates infracted area. Infarct size expressed as a percentage of area at risk. All values are presented as mean ± SEM. N=8/group. $^{***}P$<0.001 vs D, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs D+IR, $^{\$}P$<0.05 vs

D+IR+PT 20. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests.

Next, the effect of PT on IR-induced cardiac apoptosis in diabetic rats was further assessed by measuring the changes of pro- and anti-apoptotic proteins. Myocardial IR injury decreased Bcl-2/Bax ratio (P<0.001, **Figure 5.12B**) and increased cleaved caspase3/caspase3 ratio (P<0.05, **Figure 5.12C**) when compared to sham-operated diabetic rats. PT at two doses significantly reversed the altered ratios of Bcl-2/Bax (all P<0.001) and cleaved caspase-3/caspase-3 (all P<0.05) when compared to D+IR group. These results demonstrated that PT decreased IR-induced myocardial apoptosis in diabetic rats.

5.3.3. Effect of PT treatment on the phosphorylation of AMPK in diabetic rats

As shown in **Figure 5.13**, despite no change in total protein levels of AMPK in all treatment groups, the p-AMPK level was significantly increased in the D+IR group when compared to the D group (P<0.05). As anticipated, PT treatment at both doses significantly further increased p-AMPK levels when compared to D+IR group (P<0.01, PT dose 20 mg/kg vs D+IR; P<0.05, PT dose 20 mg/kg vs D+IR), respectively. A slight decrease in p-AMPK levels was observed in PT 40 group when compared to PT 20 group; however, this difference did not reach statistical significance (P>0.05).

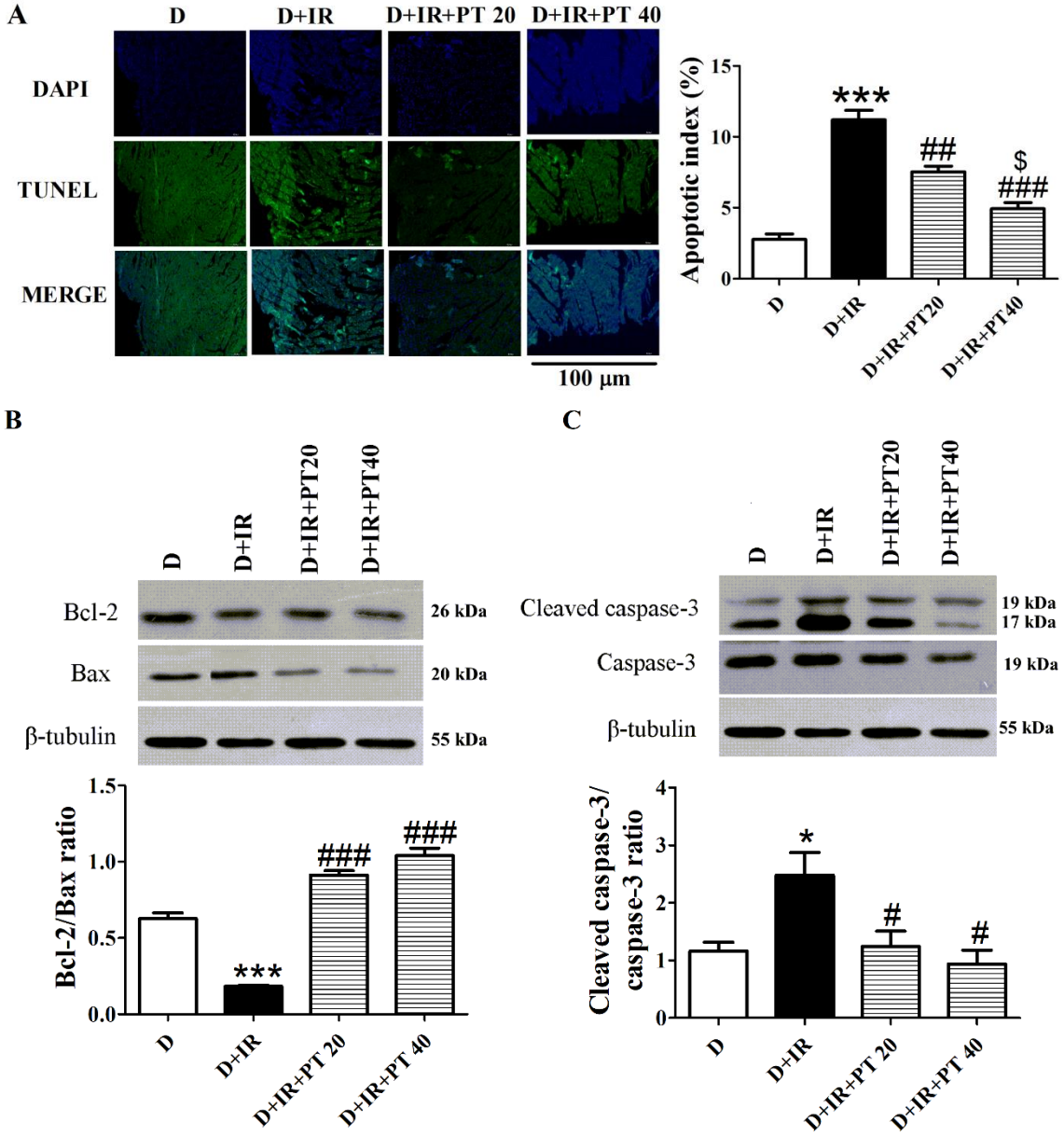

Figure 5.12: Effect of PT on cardiac apoptosis in diabetic rats. (A) Terminal deoxynucleotidyl nick-end labelling (TUNEL) staining in myocardial IR heart tissue from the experimental groups. (B) Bcl-2/Bax ratio (C) cleaved caspase-3/caspase-3 ratio. Green fluorescence indicates TUNEL-positive cardiomyocytes; blue fluorescence indicates nuclei of total primary cardiomyocytes. Apoptotic index was depicted as a histogram. All values are presented as mean ± SEM. N=6/group. $^{*}P<0.05$, $^{***}P<0.001$ vs D, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs D+IR, $^{\$}P<0.05$ vs D+IR+PT 20. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests.

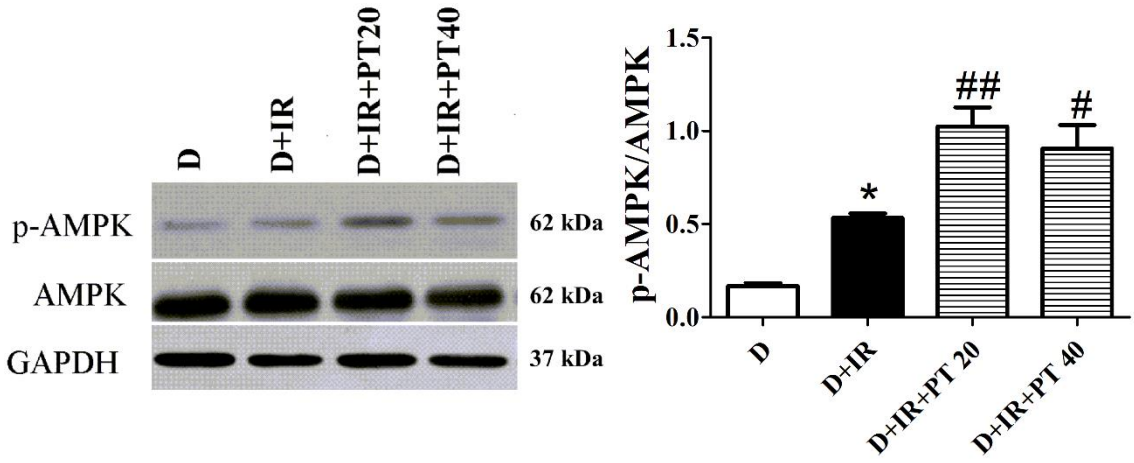

Figure 5.13: Effect of PT treatment on AMPK phosphorylation in heart tissues of diabetic rats. All values are presented as mean ± SEM. N=6/group. $^{*}P<0.05$ vs D, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs D+IR. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests.

5.3.4. Effect of PT treatment on cardiomyocytes viability exposed to hypoxia-reoxygenation under high glucose condition

To investigate the underlying mechanisms of cardioprotective effects of PT on myocardial IR injury in diabetic rats, *in-vitro* studies in adult rat primary cardiomyocytes challenged with 45 minutes hypoxia and 2 hours reoxygenation under high glucose (HG) condition were performed.

Primary cardiomyocytes were exposed to low glucose (LG) and varying concentrations of PT (0.1, 0.5, 1 μM) at the onset of re-oxygenation to determine the effective concentration of PT. Cell viability and LDH release, indices of primary cardiomyocytes injury, were measured by MTT and LDH assay, respectively. As shown in **Figure 5.14A and 5.14B,** after being challenged with HR, cell viability in LG+HR group was significantly reduced to 66.00%±3.00%, and LDH increased to 157.60%±6.80% when compared to LG (n=6; all $P<0.001$). PT (0.1 and 0.5 μM) markedly reduced HR-induced cell death, increasing viability rate to 76.40%±1.21% (n=6; $P<0.05$) and

84.78%±3.11% (n=6; P<0.01) and decreasing LDH release to 139.20%±1.93% (n=6; P<0.05) and 125.60%±3.78% (n=6; P<0.01), respectively. By contrast, PT at 1 μM failed to show protection against HR induced cell death. Also, DMSO did not affect HR induced cell injury. Taken together, these results indicated that PT markedly preserved post-hypoxic cell viability and attenuated injury at the concentrations of 0.1 and 0.5 μM, and the highest cellular viability was observed at 0.5 μM concentration of PT.

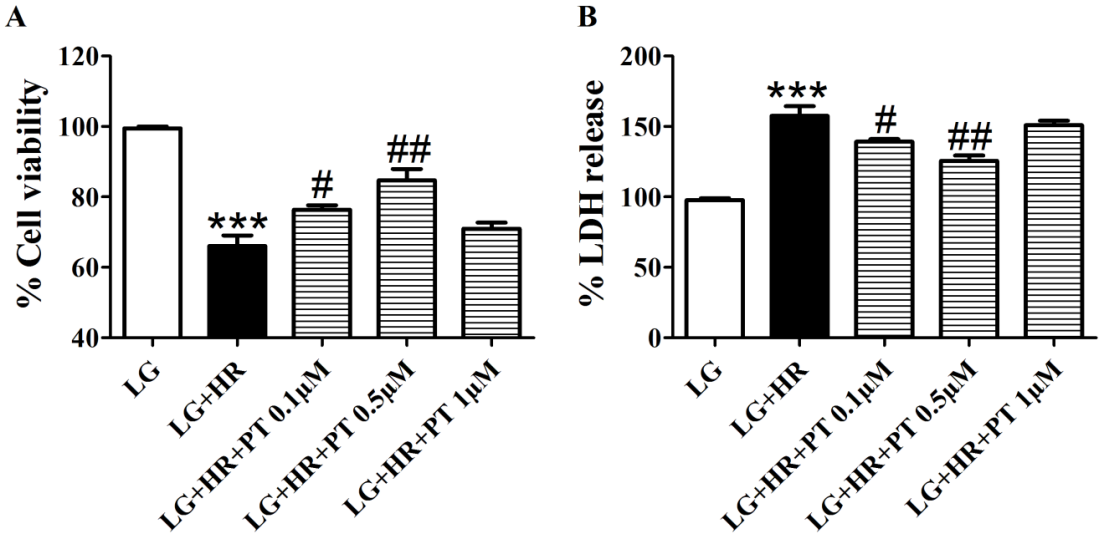

Figure 5.14: Dose selection of PT for in-vitro cell line studies based on MTT and LDH assays. Primary cardiomyocytes exposed to low glucose (LG) or PT were challenged with hypoxia-reoxygenation (HR). Cell viability and LDH release were measured by MTT and LDH assay, respectively. (A) Cell viability in primary cells administered PT (0.1, 0.5, 1 μM) during HR. (B) LDH assay in primary cells administered PT (0.1, 0.5, 1 μM) during HR. $^{***}P$<0.001 vs LG, $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs LG+HR. Results demonstrated as mean ± SEM, N=6/group. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests.

The effect of PT (0.5 μM) on primary rat cardiomyocytes exposed to LG+HR and HG+HR in the presence or absence of the AMPK inhibitor, Compound C (CC, 5 μM, administered one hour before HR stimulation) was determined. The rat primary cardiomyocytes were randomly divided into the following groups: (1) LG (5 mM glucose, Control); (2) LG+HR; (3) LG+HR+PT; (4) LG+HR+CC (5) LG+HR+PT +CC; (6) HG (30mM glucose)(hyperglycemic control); (7) HG+HR; (8) HG+HR+PT; (9)

HG+HR+CC (10) HG+HR+ PT +CC. As shown in **Figure 5.15A, 5.15B,** HR treatment significantly decreased cell viability (69.82%±3.20% vs 100.4%±4.13%, n=8, *P*<0.001 vs LG; and 49.26%±3.08% vs 78.82%, n=8, *P*<0.001 vs HG, respectively) and increased LDH levels (35.10%±3.74% vs 14.80%±2.70%, n=8, *P*<0.001 vs LG; and 52.17%±3.25% vs 26.97%±2.20%, n=8, *P*<0.001 vs HG, respectively). PT treatment significantly reversed the LG+HR-induced and HG+HR-induced damage to cardiomyocytes, as evidenced by significantly increased cell viability (88.63%±3.38%, n=8, *P*<0.01 vs LG+HR; and 69.33%±5.03%, n=8, *P*<0.01 vs HG+HR) and decreased LDH levels (35.10%±3.74%, n=8, *P*<0.01 vs LG+HR; and 52.17%±3.25%, n=8, *P*<0.001 vs HG+HR). Whereas CC treatment significantly decreased the cell viability (55.06%±2.21%, n=8, *P*<0.05 vs LG+HR; and 34.87%±2.16%, n=8, *P*<0.05 vs HG+HR) and markedly increased LDH levels (46.45%±2.1%, n=8, *P*<0.05 vs LG+HR; and 64.23%±2.2%, n=8, *P*<0.05 vs HG+HR). In addition, the effect of PT on HR-induced cardiac damage in both LG and HG conditions was abrogated in the presence of the AMPK inhibitor CC.

Oxidative stress plays a crucial role in the pathogenesis of ischemic heart diseases in the context of diabetes. In the present study, DHE staining was employed to determine HR- and HG (diabetes)-induced myocardial O2− production in primary cardiomyocytes (**Figure 5.15C**). HR significantly increased the number of positive DHE-stained cardiomyocytes under normal and diabetic conditions (all *P*<0.05 vs LG and HG). PT (0.5 μM) treatment significantly attenuated the LG+HR- and the HG+HR-induced increase of positive DHE-stained cells (*P*<0.01 LG+HR+PT 0.5μM vs LG+HR; and *P*<0.001 HG+HR+PT 0.5μM vs HG+HR), while CC treatment significantly (*P*<0.05 LG+HR+CC vs LG+HR; and *P*<0.05 HG+HR+CC vs HG+HR) enhanced DHE-stained cardiomyocytes. Furthermore, co-administration of CC reversed the PT's suppressive

effect on myocardial O2− production (P<0.01 LG+HR+PT 0.5μM+CC vs LG+HR+PT 0.5μM; and P<0.001 HG+HR+PT 0.5μM+CC vs HG+HR+PT 0.5μM).

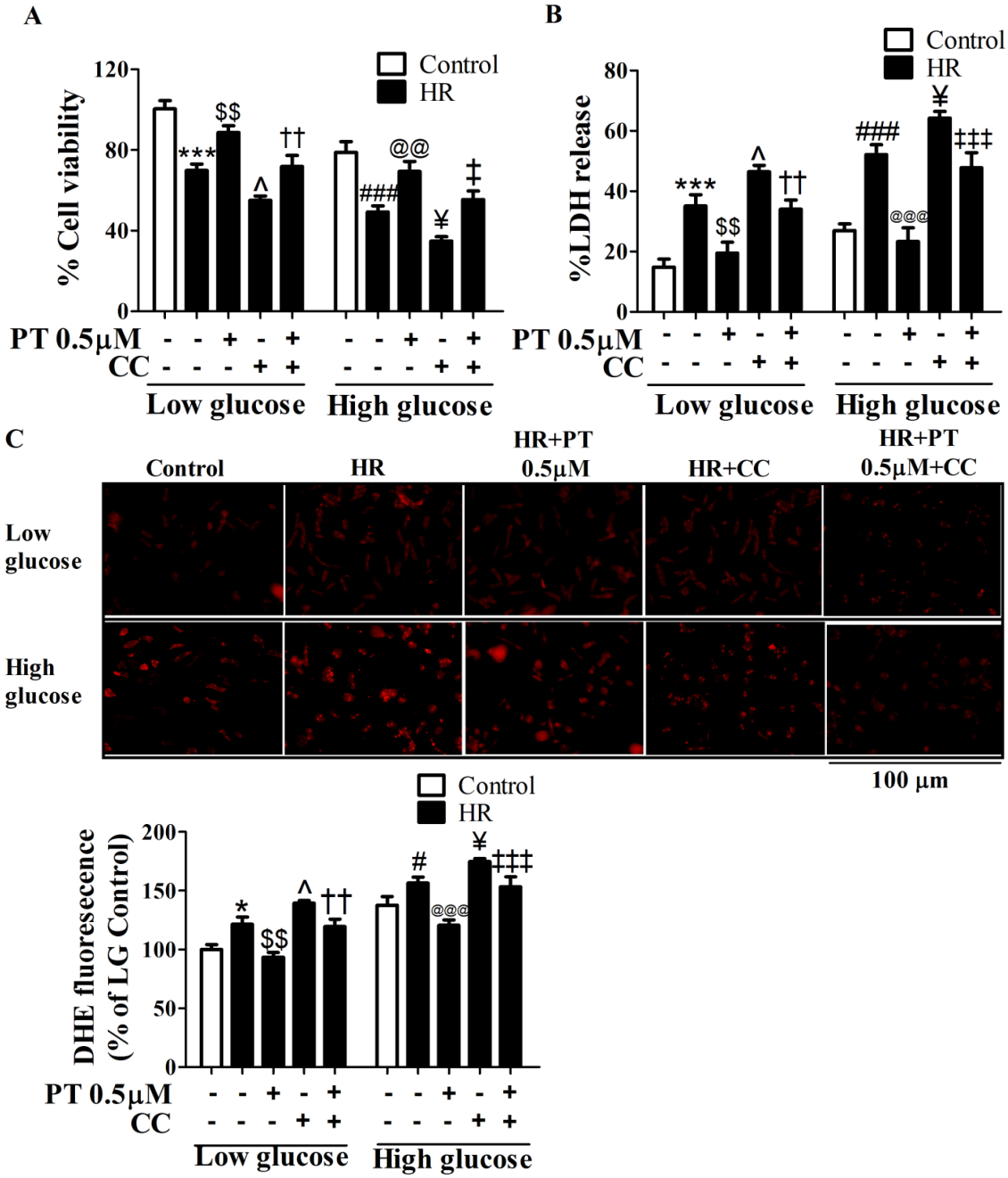

Figure 5.15: Effect of PT on HR-induced injury in primary rat cardiomyocytes (cell viability, LDH release and oxidative stress) under normal and diabetic conditions. (A) Cell viability in different treatment groups. (B) LDH release in different treatment groups. (C) Oxidative stress determined by DHE staining. Results were expressed as fold of LG (Control) and demonstrated as mean ± SEM. N=8/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{*}P$<0.05, $^{***}P$<0.001 vs LG, $^{\$\$}P$<0.01 vs LG+HR, $^{\wedge}P$<0.05 vs LG+HR, $^{\dagger\dagger}P$<0.01 vs LG+HR+PT 0.5μM, $^{\#}P$<0.05, $^{\#\#\#}P$<0.001 vs HG;

$^{@@}P<0.01$, $^{@@@}P<0.001$ vs HG+HR, $^{¥}P<0.05$ vs HG+HR, $^{‡}P<0.05$, $^{‡‡‡}P<0.001$ vs HG+HR+PT 0.5μM.

5.3.5. Effect of PT treatment on the phosphorylation of AMPK in primary cardiomyocytes subjected to HG+HR

To further explore the molecular mechanism underlying PT-mediated cardioprotection, p-AMPK/AMPK expression in primary cardiomyocytes was measured. There was no significant difference in total protein levels of AMPK between treatment groups at baseline (**Figure 5.16**). HR treatment significantly ($P<0.05$ vs LG and HG) enhanced the p-AMPK levels under normal and diabetic conditions. However, PT (0.5 μM) treatment further enhanced the post-hypoxic p-AMPK and significantly increased p-AMPK/AMPK ratio (all $P<0.001$ vs LG+HR or HG+HR), whereas CC treatment significantly diminished it (all $P<0.001$ vs LG+HR or HG+HR). Pretreatment with CC significantly blocked PT-mediated phosphorylation of AMPK (all $P<0.001$ vs LG+HR+PT 0.5 μM or HG+HR+PT 0.5 μM).

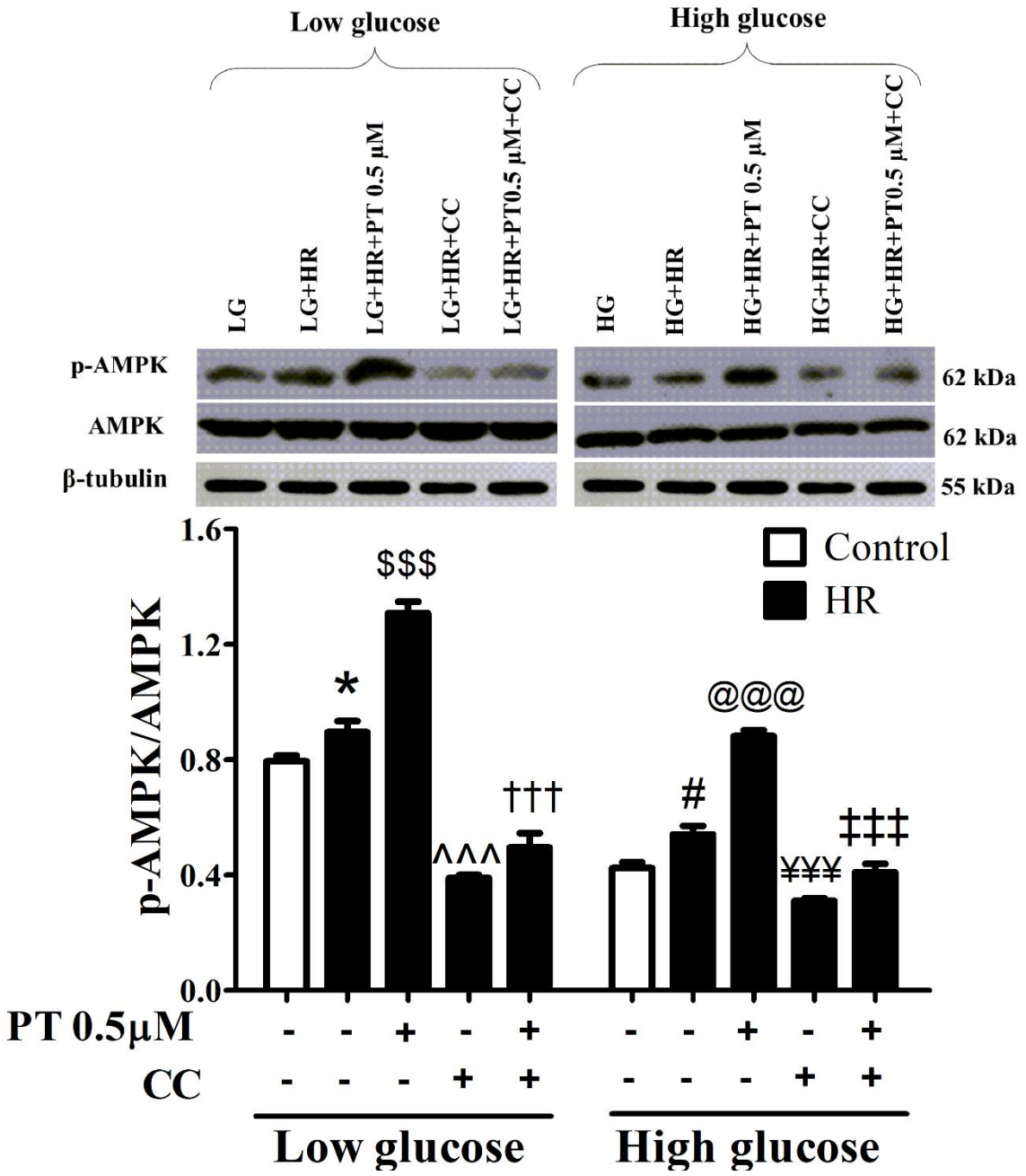

Figure 5.16: Effect of PT on AMPK phosphorylation in rat primary cardiomyocytes. Results were expressed as mean ± SEM. N=6/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{*}P<0.05$ vs LG, $^{\$\$\$}P<0.001$ vs LG+HR, $^{\wedge\wedge\wedge}P<0.001$ vs LG+HR, $^{\dagger\dagger\dagger}P<0.001$ vs LG+HR+PT 0.5µM, $^{\#}P<0.05$ vs HG; $^{@@@}P<0.001$ vs HG+HR, $^{¥¥¥}P<0.001$ vs HG+HR, $^{\ddagger\ddagger\ddagger}P<0.001$ vs HG+HR+PT 0.5µM.

5.3.6. Effect of PT treatment on apoptosis in primary cardiomyocytes subjected to HG+HR

Cellular apoptosis was determined by TUNEL staining (**Figure 5.17A**). HR significantly increased the TUNEL positive cells and apoptotic index under normal glucose and high glucose conditions (all $P<0.001$ vs LG and HG). PT (0.5 μM) treatment significantly attenuated HR-induced apoptotic index in both control and diabetic/high glucose conditions ($P<0.001$ LG+HR+PT 0.5μM vs LG+HR; and $P<0.001$ HG+HR+PT 0.5μM vs HG+HR), whereas CC treatment further exacerbated posthypoxic apoptosis ($P<0.001$ LG+HR+CC vs LG+HR; and $P<0.05$ HG+HR+CC vs HG+HR). Furthermore, co-administration with CC reversed the suppressive effect of PT on cardiomyocyte apoptosis (all $P<0.001$). These results suggested that PT had a direct cardioprotective impact on HR-induced cardiac apoptosis via AMPK activation in both normal and diabetic condition.

To determine whether PT confers protection against HR-induced apoptosis, Bcl-2 and Bax expressions were measured in primary cardiomyocytes. HR significantly downregulated Bcl-2 (an antiapoptotic protein) expression, upregulated Bax (a pro-apoptotic protein) expression, and eventually, decreased the Bcl-2/Bax ratio under normal and diabetic condition (all $P<0.001$ vs LG and HG) (**Figure 5.17B**). Pretreatment with PT (0.5 μM) significantly increased Bcl-2/Bax ratio ($P<0.001$ LG+HR+PT 0.5μM vs LG+HR; and $P<0.001$ HG+HR+PT 0.5μM vs HG+HR), while treatment with CC decreased Bcl-2/Bax ratio ($P<0.001$ LG+HR+CC vs LG+HR; and $P<0.05$ HG+HR+CC vs HG+HR). Furthermore, co-administration of CC prevented PT-induced increase of Bcl-2/Bax ratio in cardiomyocytes under normal ($P<0.001$ LG+HR+PT 0.5 μM+CC vs LG+HR+PT 0.5 μM) and diabetic ($P<0.001$ HG+HR+PT 0.5 μM+CC vs HG+HR+PT 0.5 μM) condition.

Caspases regulate myocardial apoptosis, and caspase-3 is regarded as the final executioner of the apoptotic process. HR significantly enhanced cleaved caspase-3/caspase-3 ratio (all $P<0.001$ vs LG and HG, **Figure 5.17C**). PT (0.5 μM) treatment significantly attenuated the cleaved caspase-3/caspase-3 ratio ($P<0.001$ vs LG+HR; and $P<0.001$ vs HG+HR), while CC treatment markedly enhanced the cleaved caspase-3/caspase-3 ratio ($P<0.05$ vs LG+HR; and $P<0.01$ vs HG+HR). However, co-treatment with CC inhibited PT-mediated decrease of the cleaved caspase-3/caspase-3 ratio (all $P<0.001$ vs LG+HR+PT 0.5 μM and HG+HR+PT 0.5 μM).

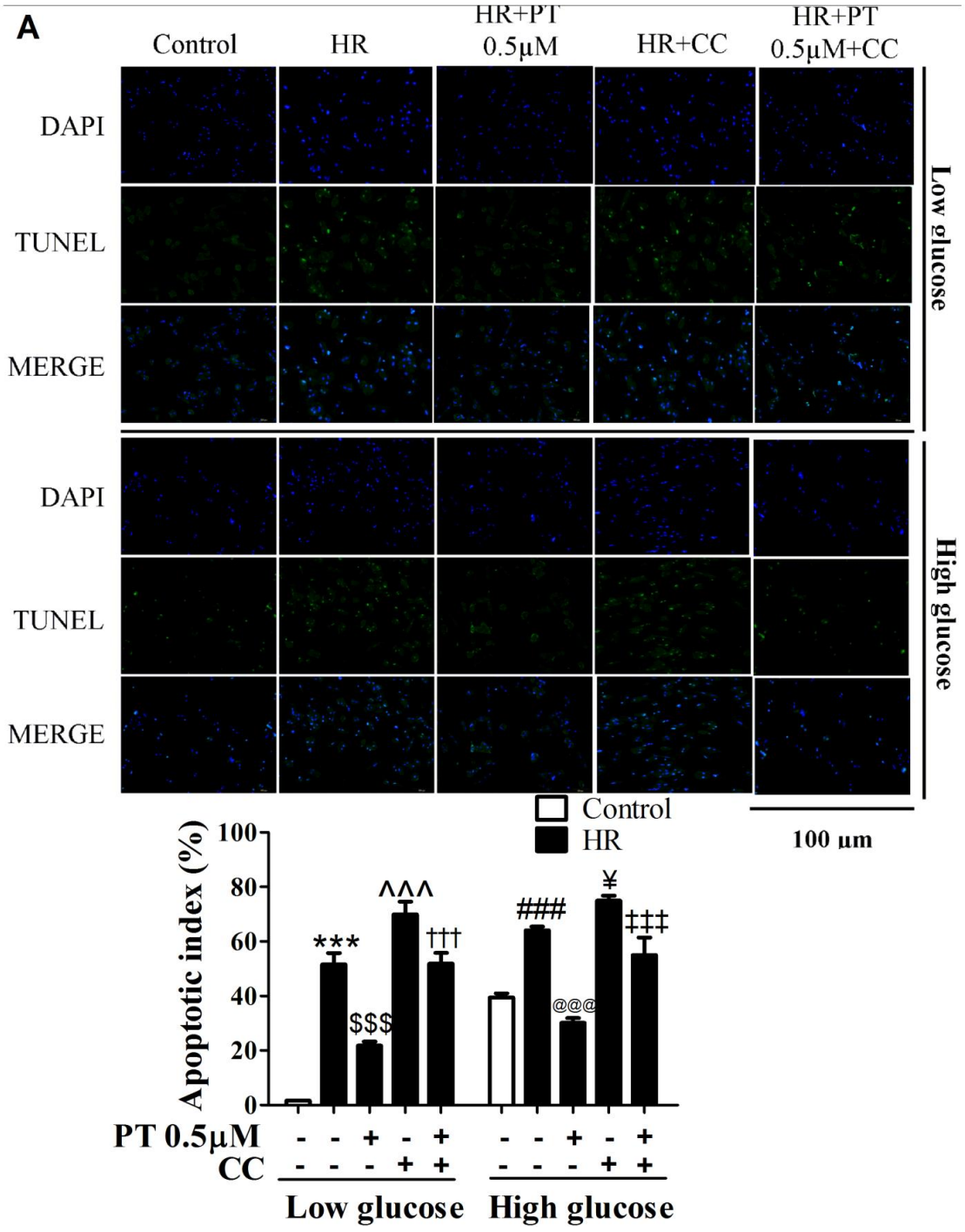

Figure 5.17: Effect of PT on cardiac apoptosis in primary cardiomyocytes under normal and diabetic conditions. (A) HR-induced apoptosis as determined by TUNEL staining. Results were expressed as fold of LG (Control) and demonstrated as mean ± SEM. N=6/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{***}P$<0.001 vs LG, $^{\$\$\$}P$<0.001 vs LG+HR, $^{\wedge\wedge\wedge}P$<0.001 vs LG+HR, $^{\dagger\dagger\dagger}P$<0.001 vs LG+HR+PT 0.5μM, $^{\#\#\#}P$<0.001 vs HG; $^{@@@}P$<0.001 vs HG+HR, $^{¥}P$<0.05 vs HG+HR, $^{\ddagger\ddagger\ddagger}P$<0.001 vs HG+HR+PT 0.5μM.

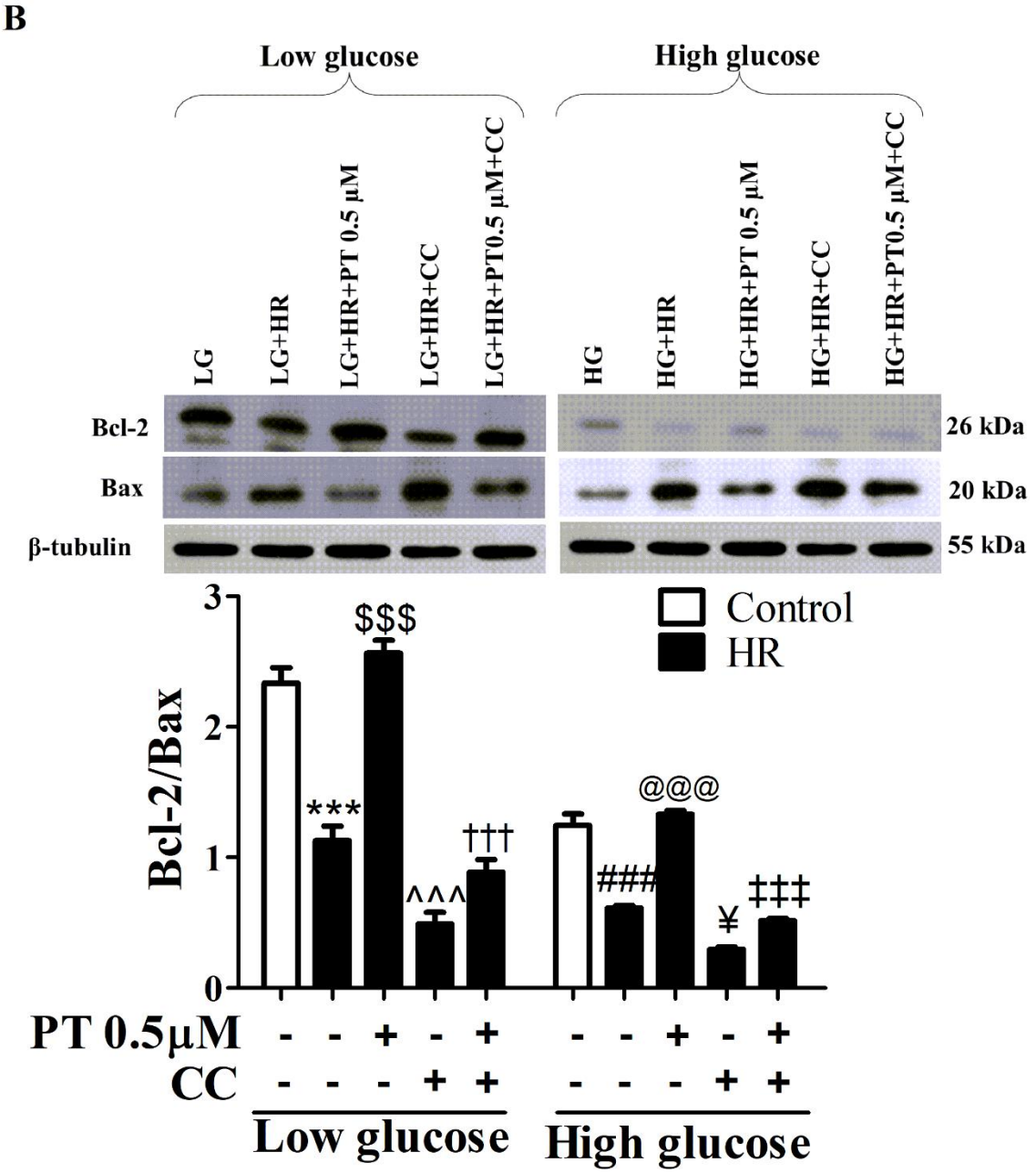

Figure 5.17: Effect of PT on cardiac apoptosis in primary cardiomyocytes under normal and diabetic conditions. (B) Bcl-2/Bax ratio. Results were expressed as fold of LG (Control) and demonstrated as mean ± SEM. N=6/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{***}P<0.001$ vs LG, $^{\$\$\$}P<0.001$ vs LG+HR, $^{\wedge\wedge\wedge}P<0.001$ vs LG+HR, $^{\dagger\dagger\dagger}P<0.001$ vs LG+HR+PT 0.5µM, $^{\#\#\#}P<0.001$ vs HG; $^{@@@}P<0.001$ vs HG+HR, $^{¥}P<0.05$ vs HG+HR, $^{\ddagger\ddagger\ddagger}P<0.001$ vs HG+HR+PT 0.5µM.

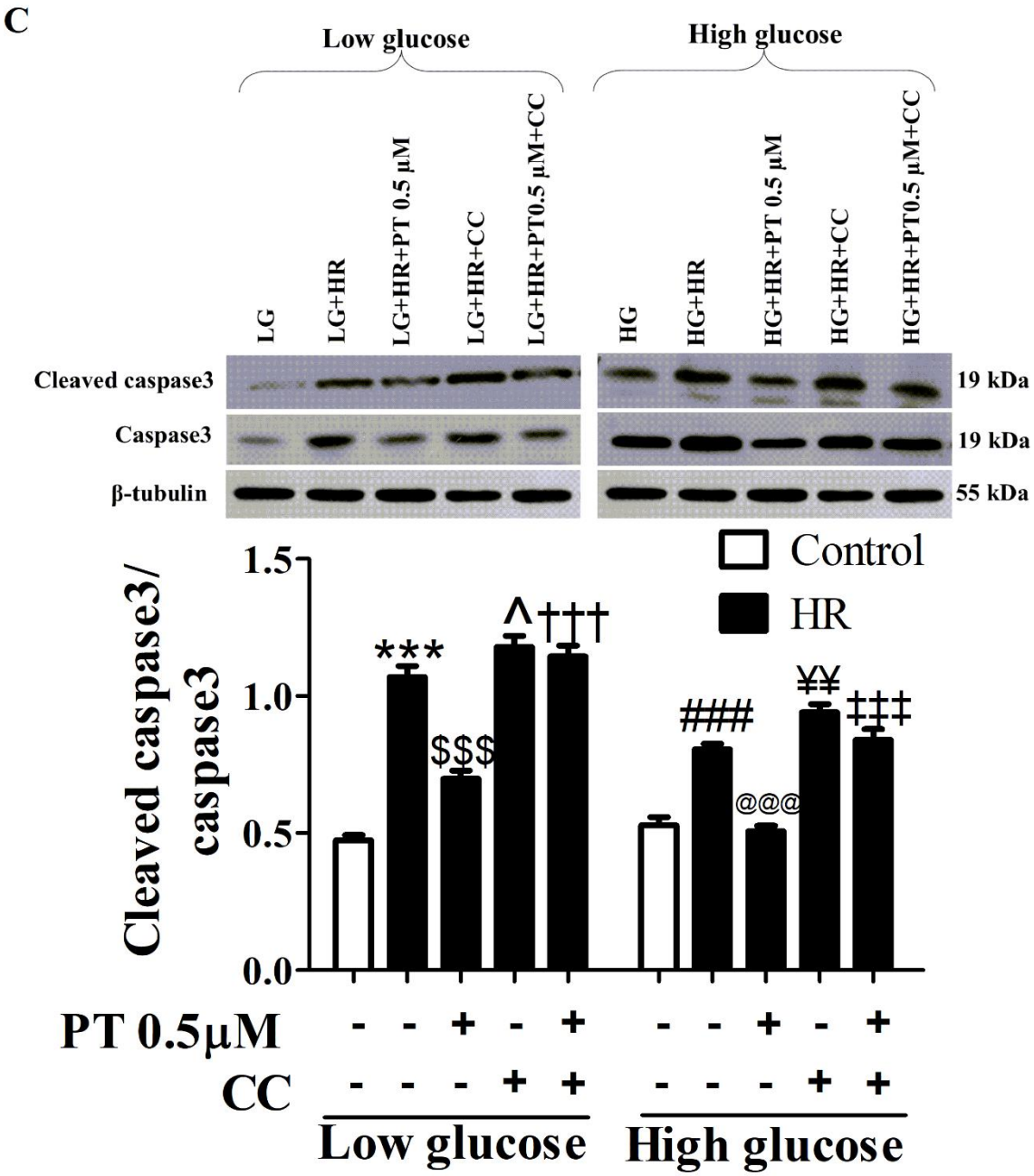

Figure 5.17: Effect of PT on cardiac apoptosis in primary cardiomyocytes under normal and diabetic conditions. (C) Cleaved caspase-3/caspase-3 ratio. Results were expressed as fold of LG (Control) and demonstrated as mean ± SEM. N=6/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{***}P<0.001$ vs LG, $^{\$\$\$}P<0.001$ vs LG+HR, $^{\wedge}P<0.05$ vs LG+HR, $^{\dagger\dagger\dagger}P<0.001$ vs LG+HR+PT 0.5µM, $^{\#\#\#}P<0.001$ vs HG; $^{@@@}P<0.001$ vs HG+HR, $^{¥¥}P<0.01$ vs HG+HR, $^{\ddagger\ddagger\ddagger}P<0.001$ vs HG+HR+PT 0.5µM.