CHAPTER V
THE EFFECTS OF NOVEL HERBAL RECIPE (PWCG) ON DIABETIC CATARACT AND RETINOPATHY MODELS

1. Introduction

Diabetic cataract and diabetic retinopathy are chronic progressive, potentially sight-threatening diseases which are associated with hyperglycemia (Engerman and Kern, 1986; Pollreisz and Schmidt-Erfurth, 2010). The epidemiological study has shown that the prevalence of both cataract and retinopathy in diabetic patients are increased (Ding and Wong, 2012; Obrosova *et al.*, 2010) in accompany with the increased diabetic patients worldwide. The effective treatments of diabetic cataract and diabetic retinopathy require the skillful physician. Therefore, these conditions still induce the important heath problems in the developing countries especially in a rural area so the prevention strategy is very much important.

Recent findings have demonstrated that the increased aldose reductase activity and the elevated oxidative stress contribute the important roles on the development of cataract and retinopathy in diabetes mellitus (Drel *et al.*, 2008; Lee and Chung, 1999; Madsen-Bouterse and Kowluru, 2008; Obrosova and Kador, 2011). Anthocyanins rich diet such as purple waxy corn and colored rice could prevent diabetic cataract (Morimitsu *et al.*, 2002).. In addition, ginger extract also exerts anti-cataractogenesis (Li *et al.*, 2012; Saraswat *et al.*, 2010). Our pilot data show that the antioxidant effect of the combination of purple waxy corn and ginger (PWCG) is more potent than the antioxidant effect of either purple waxy corn or ginger alone. However, all of the evidence, mentioned earlier is the in vitro study. Therefore, this study aimed to determine the in vivo effect of purple waxy corn seeds extract (PWC) and the combination extract of purple waxy corn and ginger (PWCG) on cataract, retinopathy, oxidative stress and aldose reductase in lens of diabetic rats.

2. Materials and Methods

2.1 Experimental design in glucose-induced cataract genesis

Male Wistar rats, weighing 280-300 g, were used in this study (n=6 per group). The animals were maintained and treated in accordance with the

guideline and approval of the Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU 98/2555). When the rats were sacrificed, the eyes were enucleated via posterior approach to avoid the damage. The isolated transparent lens were incubated artificial aqueous humor (NaCl 140 mM, KCl 5 mM, $MgCl_2$ 2 mM, $NaHCO_3$ 0.5 mM, Na_2HPO_4 0.5 mM, $CaCl_2$ 0.4 mM, Glucose 5.5 mM) for 72 hours at room temperature. The pH is maintained at 7.8 throughout the incubation period. To prevent microbial contamination, strict aseptic techniques were performed. Antibiotic drugs including penicillin 32 mg and streptomycin 250 mg were added to the culture media. In addition, glucose at concentration of 55 mM was added to the media in order to develop the model of diabetic cataract. All lens were divided into various groups as following

Experiment I: Anti-cataract effect of purple waxy corn grains extract (PWC)

Group I Normal lens (Glucose 5 mM)

Group II Diabetic cataract (Glucose 55 mM)

Group III Diabetic cataract+Quercetin (2.4 μg/ml); a phenolic compound which previously demonstrated anti-cataract effect: this group was served as positive control

Group IV-VI Diabetic cataract+the combination extract of purple waxy corn (PWC) at doses of 2, 10 and 50 $mg.kg^{-1}$ BW, respectively

Experiment II: Anti-cataract effect of the combination extract of purple waxy corn and ginger (PWCG)

Group I Normal lens (Glucose 5 mM)

Group II Diabetic cataract (Glucose 55 mM)

Group III Diabetic cataract+Quercetin (2.4 μg/ml)

Group IV-VI Diabetic cataract+the combination extract of purple waxy corn and ginger (PWCG) at doses of 50, 100 and 200 $mg.kg^{-1}$ BW, respectively

Glucose at concentration of 55 mM was added to all lens in group II-VI in order to mimic the diabetic cataract. After 72 hours of incubation, Lens opacity was determined and photographs were taken by placing the lens on the paper with posterior surface touching the paper, and the number of visible clear squares was observed through the lens during the evaluation of lens opacity (Singh, 2011).

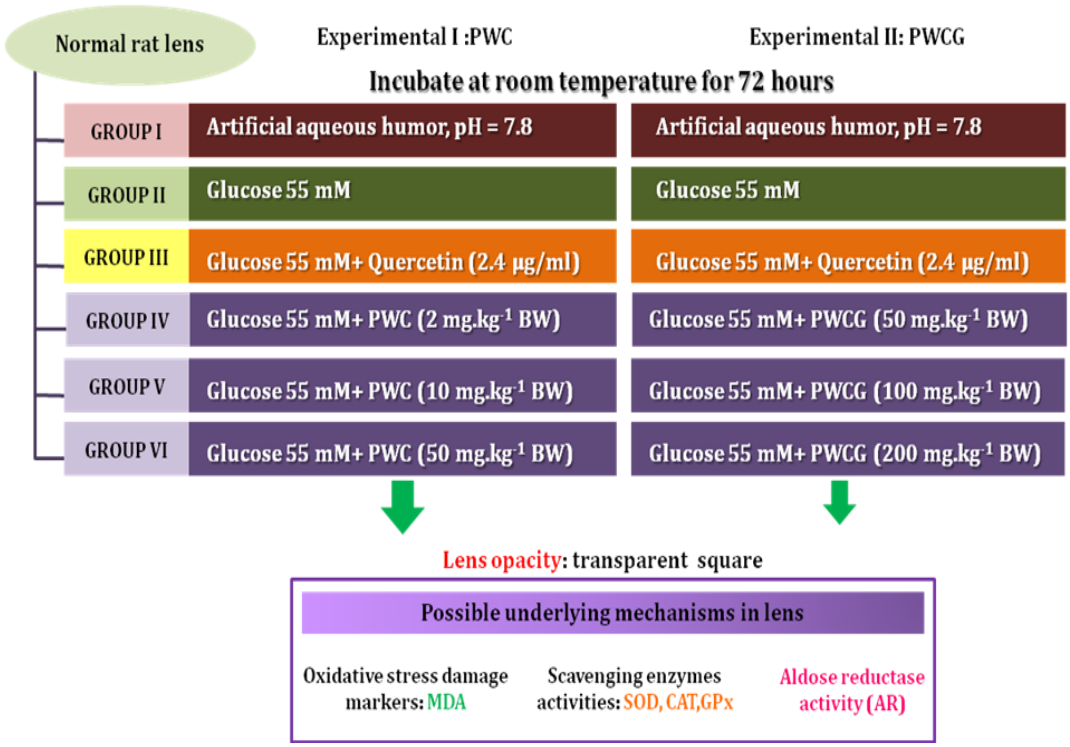

Figure 5-1 Schematic diagram showing experimental protocol for the determination of effect of PWC and PWCG on diabetic cataract in Glucose-induced cataract genesis model

2.2 In vivo study of the anti-cataract effect of PWCG in streptozotocin-induced diabetic rats

The experimental animals used in this study were male Wistar rats at the weight of 200-250 g (n=8 per group). The animals were maintained and treated in accordance with the guideline and approval of the Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU 98/2555). All rats were divided into various groups as following

Group I Control group: all rats in this group were administered citrate buffer, a vehicle of streptozotocin (STZ)

Group II DM+vehicle group: the animals in this group were induced diabetes mellitus via single injection of STZ and received vehicle of the extract or distilled water.

Group III-V DM+the combination extract of purple waxy corn and ginger (PWCG) at doses of 50,100 and 200 mg.kg^{-1} BW respectively.

All rats in group II-V were induced diabetes cataract by a single injection of STZ which was dissolved in citrate buffer (pH 4.5) at dose of 55 $mg.kg^{-1}$ BW. The animals which showed the blood sugar levels more than 250 $mg.dL^{-1}$ were recruited for further study. All rats were treated with the assigned interventions once daily at 3 days after injection of STZ and maintained for 10 weeks. Lens opacity was evaluated every week using slit lamp microscope. At the end of study, lens were collected and determined histomorphology, aldose reductase and oxidative stress including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes in lens as shown in figure 5-2.

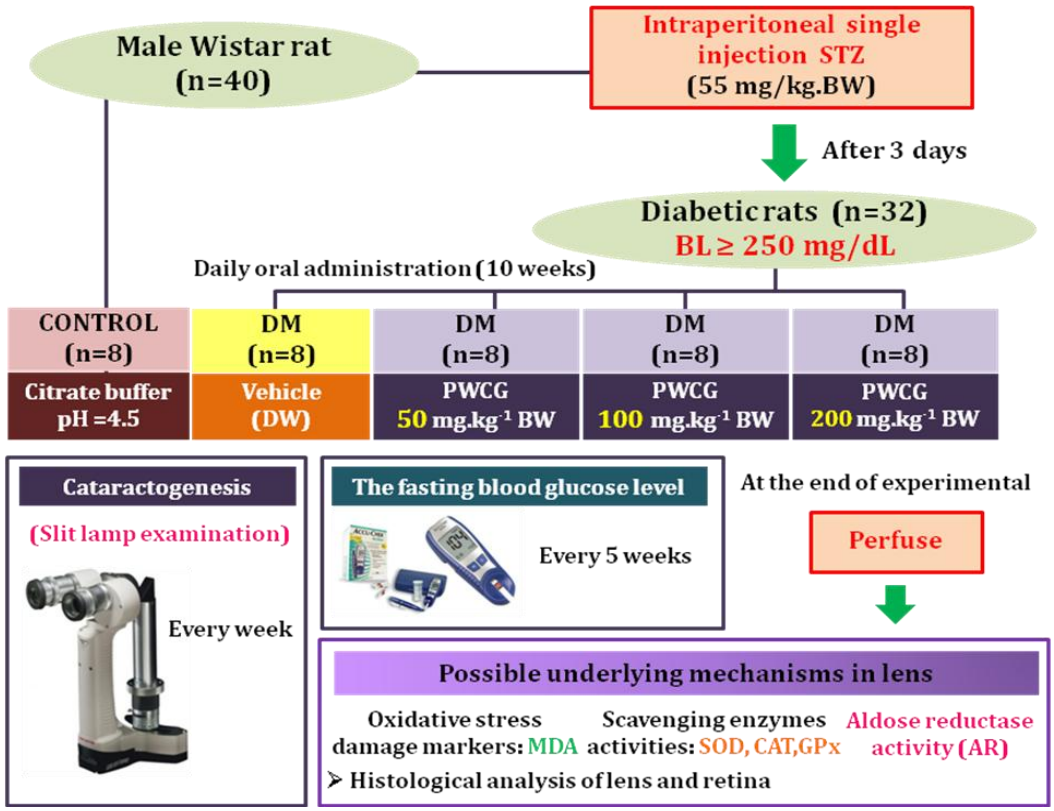

Figure 5-2 Schematic diagram showing experimental protocol for the determination of effect of PWCG on diabetic cataract and retinopathy induced by STZ model

2.3 Determination of fasting blood glucose level

After fasting overnight, blood was collected from rat tails and determined fasting blood glucose levels by using ACCU-CHEK active. This process was performed at the first week and every 5 weeks throughout the study period.

2.4 Cataract evaluation via slit-lamp

Rat eyes were evaluated every weeks using slit lamp microscope (DIOPTRIX-HAWKEYE; France) by trained observer who was blind to the treatment. The severity of cataract was graded as 5 stages according to method Suryanarayana and coworkers (Suryanarayana *et al.*, 2005) as following;

Stage 0 clear lenses with no vacuole

Stage 1 vacuoles cover approximately one-half of the surfaces of the anterior pole forming a sub capsular cataract

Stage 2 some vacuoles have disappeared and the cortex exhibits a hazy opacity

Stage 3 a hazy cortex remains and dense nuclear opacity is present

Stage 4 a mature cataract is observed as a dense opacity in both cortex and nucleus.

Data were presented as opacity index which was calculated from the following formula;

Opacity index = (number of eyes in each stage × stage of the eye) / total number of eyes within group

2.5 Histopathological analysis of rat lens and retina

The eye balls from five rats per group were fixed in 10% formalin overnight, embedded in paraffin, sectioned at 5 µm thick and stained with hematoxylin and eosin. Light microscopic was used to evaluate the histomorphology of lens. The severity of histomorphological change of lens was graded as a 5 grade score according to method of Agrawal and coworkers (Agarwal *et al.*, 2013) as described following;

Grade 0: Presence of anterior epithelium with lens fibers

Grade 1: Presence of anterior epithelium, lens fibers and vacuoles

Grade 2: Presence of anterior epithelium, lens fibers, vacuoles and homogenized area.

Grade 3: Absence of anterior epithelium, presence of lens fibers, vacuoles and homogenized area

Grade 4: Presence of lens fibers and homogenized area only

Histomorphological changes of retina including the total retinal thickness (from inner limiting membrane to Bruch's membrane), the thickness of the retinal outer nuclear layer and the number of cells in the ganglion cell layer were also performed. The average thickness of retina was evaluated using 3 adjacent fields and total five images in each group. The results were showed as mean±SEM

2.6 Determination of the possible underlying mechanism of anti-cataract of PWCG

2.6.1 Homogenate preparation

At the end of experiment, lens were collected and homogenized in 10 weight/volume of 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. Then, the homogenate was centrifuged at 10,000 g at 4°C for 1 hour and the supernatant was separated and used for the determination of biochemical parameters.

2.6.2 Determination of malondialdehyde (MDA) level

Level of malondialdehye (MDA), a lipid peroxidation marker, was monitored by using thiobarbituric acid reacting substances (TBARS) assay. In brief, 100 µl of sample was mixed with the solution containing 100 µl of 8.1% (w/v) sodium dodecyl sulphate, 750 µl 20% (v/v) acetic acid (pH 3.5), and 750 µl of 0.8% thiobarbituric acid (TBA). The solution was heated in a water bath at 95°C for one hour and cooled immediately under running tap water. Then, 500 µl chilled water and 2500 µl of butanol and pyridine [15:1 v/v] were added into each tube and mixed thoroughly with vortex. Then, the solution was centrifuged at 800 x g for 20 min. The upper layer was separated and measured absorbance at 532 nm. 1,3,3-tetra ethoxy propane (TEP) was used as the reference (Ohkawa *et al.*, 1979). The level of MDA was expressed as U/mg.protein.

2.6.3 Superoxide dismutase (SOD) assay

The determination of SOD activity was carried out via nitrobluetetrazolium (NBT) reduction assay. In this assay, the xanthine - xanthine oxidase system was used as a superoxide generator. In brief, the reaction mixture contained 20 µl of sample and 200 µl of reaction mixture consisting of 57 mM

phosphate buffer solution (KH_2PO_4), 0.1 mM EDTA, 10 mM cytochrome C solution and 50 µM of xanthine solution and 20 µl of xanthine oxidase solution (0.90mU/ml) were prepared at 25ºC. The optical density was measured at 415 nm. A system devoid of enzyme served as the control and three parallel experiments were conducted (Sun *et al.*, 1988). SOD activity was expressed as U/mg.protein.

2.6.4 Catalase (CAT) assay

Lens catalase activity was determined based on the ability of the enzyme to break down H_2O_2. In brief, 10 µl of sample was mixed with the reaction mixture containing 50 µl of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0), 25 µl of H_2SO_4 and 150 µl of $KMnO_4$. After mixing thoroughly, the optical density was measured at 490 nm. A system devoid of the substrate (hydrogen peroxide) was served as the control. The difference in absorbance per unit time was expressed as the activity. An amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25ºC is regarded as one unit (Goth, 1991). The value of CAT activity was expressed as U/mg.protein.

2.6.5 Glutathione peroxidase (GPx) assay

This assay was performed based on the glutathione recycling method by using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reductase. According to this method, the reaction between DTNB and GSH gave rise to the generation of 2-nitro-5-thiobenzoic acid and GSSG. Since 2-nitro-5-thiobenzoic acid was a yellow colored product, GSH concentration could be determined by measuring absorbance at 412 nm. In brief, a mixture containing a 20 µl of sample and the reaction mixture consisting of 10 µl of dithiothreitol (DTT) in 6.67 mM potassium phosphate buffer (pH 7), 100 µl of sodium azide in 6.67 mM potassium phosphate buffer (pH 7), 10 µl of glutathione solution and 100 µl of hydrogen peroxide, was mixed thoroughly and incubated at room temperature for 5-10 minutes. Then, 10 µl of DTNB (5,5-dithiobis-2-nitrobenzoic acid) was added and the optical density at 412 nm was recorded at 25 ºC over a period of 5 min. Activities were expressed as nmoles/min/mg lens protein (Rotruck *et al.*, 1973). GPx activity was expressed as U/mg.protein.

2.6.6 Aldose reductase (AR) activity assay

Aldose reductase activity was evaluated using spectrophotometric method. An assay mixture containing 0.7 mL of phosphate buffer (0.067 mol), 0.1 mL of NADPH (25×10^{-5} mol), 0.1 mL of DL-glyceraldehyde (substrate, 5×10^{-4} mol) and 0.1 mL of lens supernatant were prepared. Absorbance was recorded against a reference cuvette containing all other components except the substrate, DL-glyceraldehyde. The final pH of the reaction mixture was adjusted to pH = 6.2. The determination was performed after adding the substrate or DL-glyceraldehydeby measuring the decrease in NADPH absorbance at 390 nm over a 4- minute period (Patel and Mishra, 2009). The enzyme activity was expressed as (nmol/min/mg).

2.7 Statistical Analysis

All data were presented as mean ± standard error mean (mean ± S.E.M). The analysis of data was performed using one-way analysis of variance (ANOVA) followed by the post hoc test of LSD via SPSS version 15. Statistical differences were considered at p-value <0.05.

3. Results

3.1 Effect of purple waxy corn grains extract (PWC) in glucose induced cataractogenesis model

3.1.1 Anti-cataract effect of PWC

Table 5-1 showed that before the exposure to high concentration of glucose (glucose 55 mM), no significant difference in lens opacity among various groups was observed. The incubation of lens with 55 mM of glucose for 72 hours clearly revealed the increased lens opacity (p-value<0.001; compared to normal lens) as shown in figure 5-3 and table 5-1. Interestingly, quercetin and both medium and high doses of PWC could decrease the enhanced lens opacity in model of diabetic cataract induced by high glucose concentration (p-value<0.001; compared to experimental diabetic cataract group).

3.1.2 Effect of PWC on oxidative stress markers

Based on the previous finding that oxidative stress plays the crucial role on cataractogenesis, the effect of PWC on oxidative stress markers including level of MDA and the activities of SOD, CAT and GPx in lens were

determined and results were shown in figure 5-4-figure 5-7 and summarized in table 5-2. Glucose significantly increased MDA level (p-value<0.01; compared to normal lens) but decreased the activities of SOD (p-value<0.01; compared to normal lens), CAT (p-value<0.001; compared to normal lens) and GPx (p-value<0.01; compared to normal lens) in lens. Quercetin, a phenolic compound previously showed anti-cataractogenesis effect, also mitigated the elevation of MDA level (p-value<0.05; compared to experimental diabetic cataract group) and the decreased SOD (p-value<0.05; compared to experimental diabetic cataract group) and GPx (p-value<0.05; compared to experimental diabetic cataract group) induced by high glucose concentration. However, quercetin failed to show the significant change of CAT activity in the lens. It was found that both medium and high doses of purple waxy corn seeds extract could mitigate the elevation of MDA induced by high glucose exposure which used as experimental diabetic cataract. Surprisingly, only the medium dose of extract mitigated the decreased GPx activity in lens and no other significant changes were observed.

3.1.3 Effect of PWC on aldose reductase

In this study, we also determined the effect of purple waxy corn seeds extract on aldose reductase activity in lens and data were shown in figure 5-8. Lens which exposed to high glucose concentration or experimental diabetic cataract showed the elevation of aldose reductase (p-value<0.001; compared to normal lens). Both quercetin and high dose of purple waxy corn seeds extract significantly attenuated the decreased aldose reductase activity induced by high glucose exposure (p-value<0.001 and 0.05 respectively; compared to experimental diabetic cataract).

3.2 Effect of the combination extract of purple waxy corn and ginger (PWCG) in glucose induced cataractogenesis model

3.2.1 Anti-cataract effect of PWCG

The incubation of lens with 55 mM of glucose for 72 hours clearly revealed the increased lens opacity (p-value<0.001; compared to normal lens) as shown in figure 5-9 Interestingly, quercetin could decrease the enhanced lens opacity in model of diabetic cataract induced by high glucose concentration (p-value<0.001; compared to experimental diabetic cataract group). Nevertheless, all doses of PWCG could not investigate because the color from this extract hides the transparent squares.

3.2.2 Effect of PWCG on oxidative stress markers

The effect of PWCG on oxidative stress markers including level of MDA and the activities of SOD, CAT and GPx in lens were evaluated and results were shown in figure 5-10-figure 5-13 and were summarized in table 5-3. Glucose significantly increased MDA level (p-value<0.001; compared to normal lens) but decreased the activities of SOD (p-value<0.05; compared to normal lens), CAT (p-value<0.001; compared to normal lens) and GPx (p-value<0.001; compared to normal lens) in lens. Quercetin, a phenolic compound previously showed anti-cataractogenesis effect, also mitigated the elevation of MDA level (p-value<0.001; compared to experimental diabetic cataract group) and the decreased CAT (p-value<0.001; compared to experimental diabetic cataract group) and GPx (p-value<0.001; compared to experimental diabetic cataract group) induced by high glucose concentration. However, quercetin failed to show the significant change of SOD activity in the lens. It was found that all doses of PWCG could mitigate the elevation of MDA induced by high glucose exposure which used as experimental diabetic cataract. Surprisingly, both the medium and high dose of extract mitigated the decreased GPx and CAT activity in lens and no other significant changes were observed.

3.2.3 Effect of PWCG on aldose reductase

In this study, the effect of PWCG on aldose reductase activity in lens was also assessed and data were shown in figure 5-14. Lens which exposed to high glucose concentration or experimental diabetic cataract showed the elevation of aldose reductase (p-value<0.01; compared to normal lens). Both quercetin and high dose of PWCG significantly attenuated the decreased aldose reductase activity induced by high glucose exposure (p-value<0.05; compared to experimental diabetic cataract).

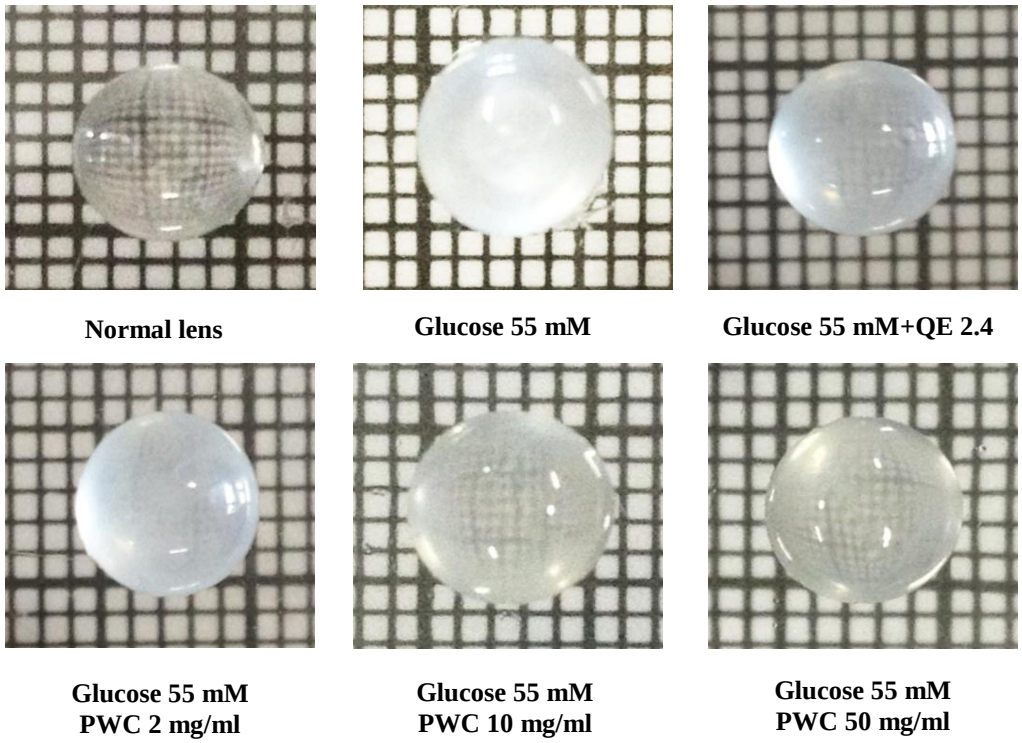

Figure 5-3 Anti-cataract effect of the extract of purple waxy corn grains after 72 hours incubation with glucose

Table 5-1 Effect of purple waxy corn grains extract (PWC) on lens opacity in normal and experimental groups before and after the 72 hours incubation period

Groups	Transparent squares (before incubated)	Transparent squares (after incubated)	p-value
1) Normal lens (artificial aqueous humor, pH= 7.8)	151.00±10.00	140.00±2.00	
2) Glucose 55 mM	141.50±3.50	0.50±0.50$^{###}$	0.000
3) Glucose 55 mM+quercetin 2.4 µg/ml	152.50±1.50	77.00±2.00***	0.000
4) Glucose 55 mM+PWC 2 mg/ml	141.50±3.50	14.00±2.00	0.069
5) Glucose 55 mM+PWC 10 mg/ml	149.50±6.50	71.50±2.50***	0.000
6) Glucose 55 mM+PWC 50 mg/ml	138.50±3.50	86.00±1.00***	0.000

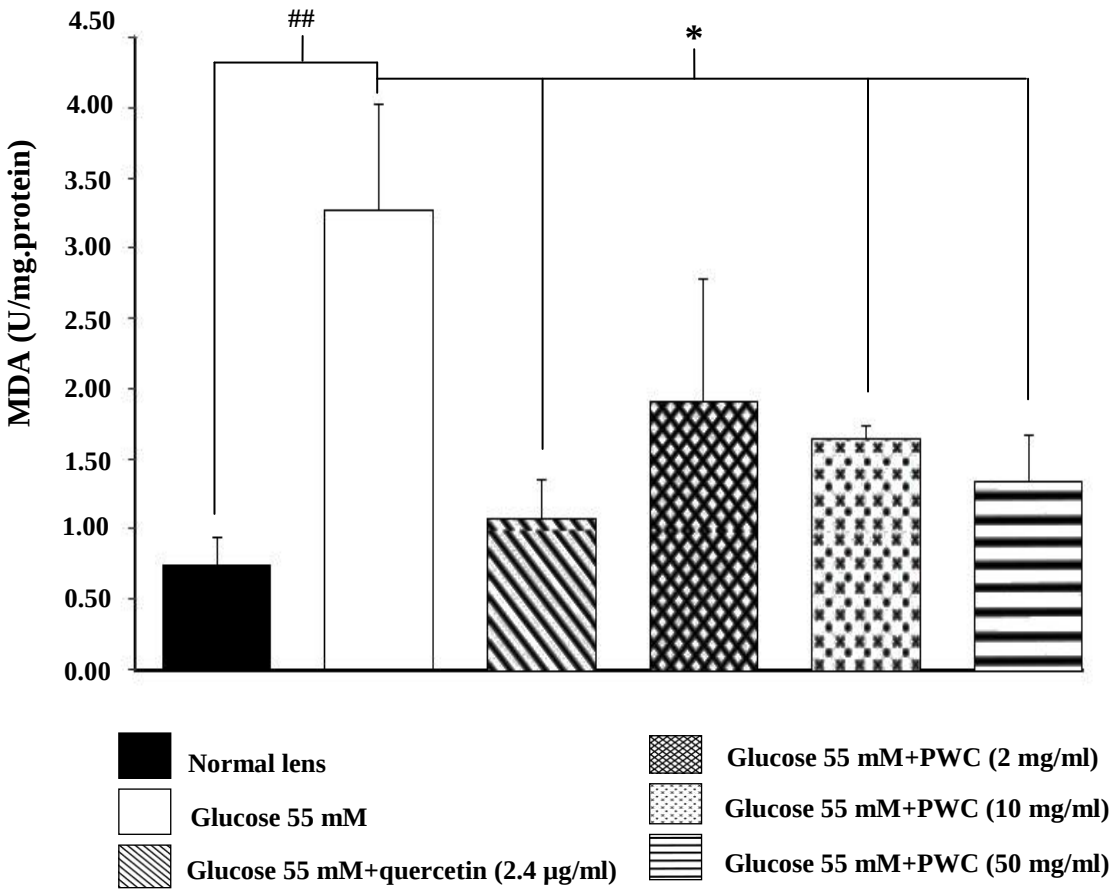

Figure 5-4 The effect of purple waxy corn grains extract (PWC) at doses of 2, 10 and 50 mg/ml on the level of malondialdehyde (MDA) level in lens exposed to high concentration of glucose. $^{\#\#}$p-value < 0.01; compared to normal lens *p-value < 0.05; compared to experimental diabetic cataract group

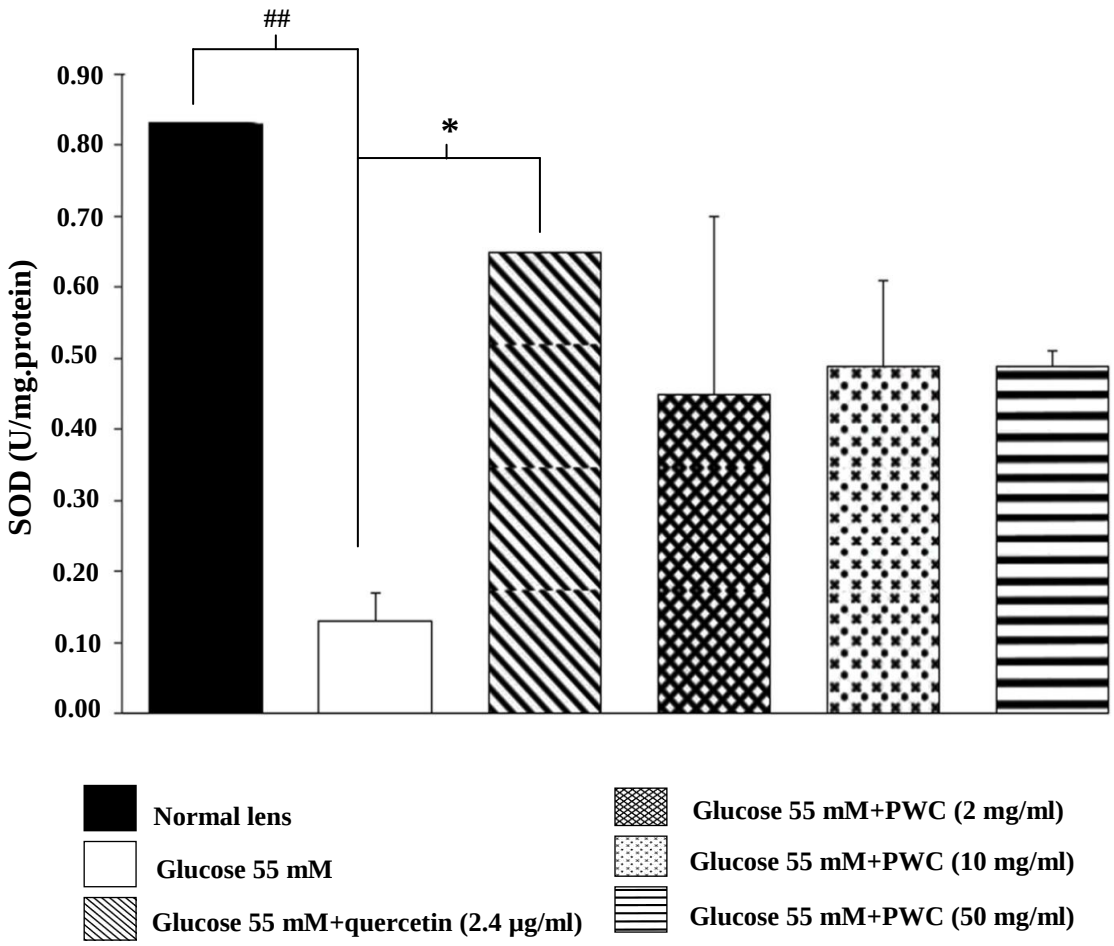

Figure 5-5 The effect of purple waxy corn grains extract (PWC) at doses of 2, 10 and 50 mg/ml on superoxide dismutase (SOD) activity in lens exposed to high concentration of glucose. $^{###}$p-value<0.001; compared to normal lens *p-value<0.05; compared to experimental diabetic cataract group

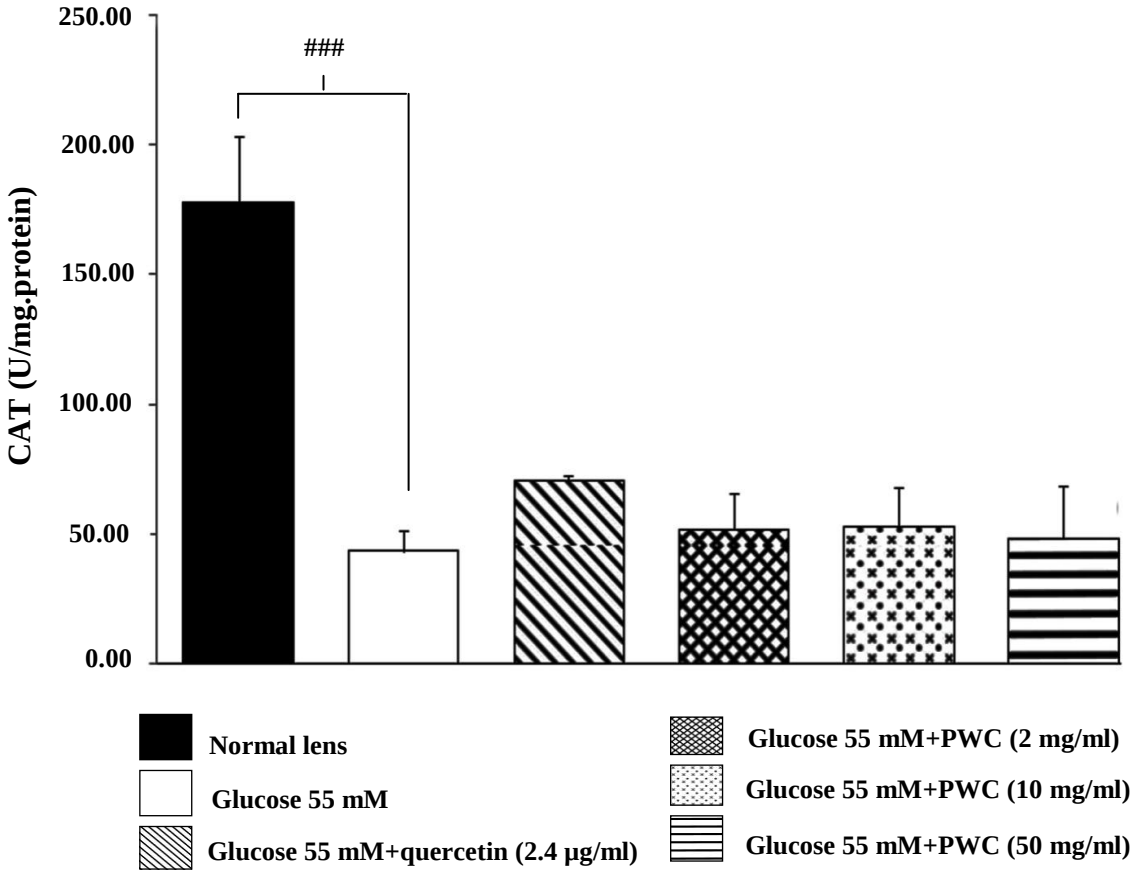

Figure 5-6 The effect of purple waxy corn grains extract (PWC) at doses of 2, 10 and 50 mg/ml on catalase (CAT) activity in lens exposed to high concentration of glucose. $^{###}$p-value < 0.001; compared to normal lens

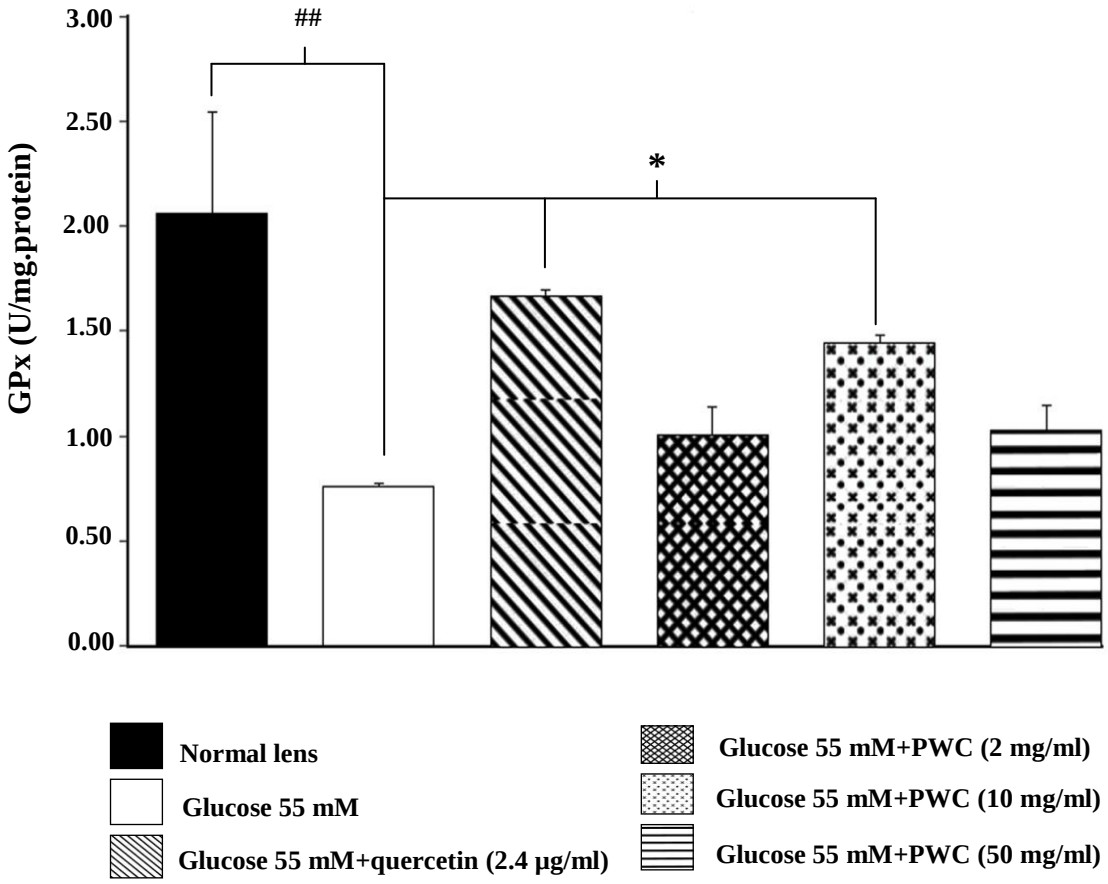

Figure 5-7 The effect of purple waxy corn grains extract (PWC) at doses of 2, 10 and 50 mg/ml on glutathione peroxidase (GPx) in lens exposed to high concentration of glucose. ##p-value < 0.01; compared to normal lens *p-value < 0.05; compared to experimental diabetic cataract group

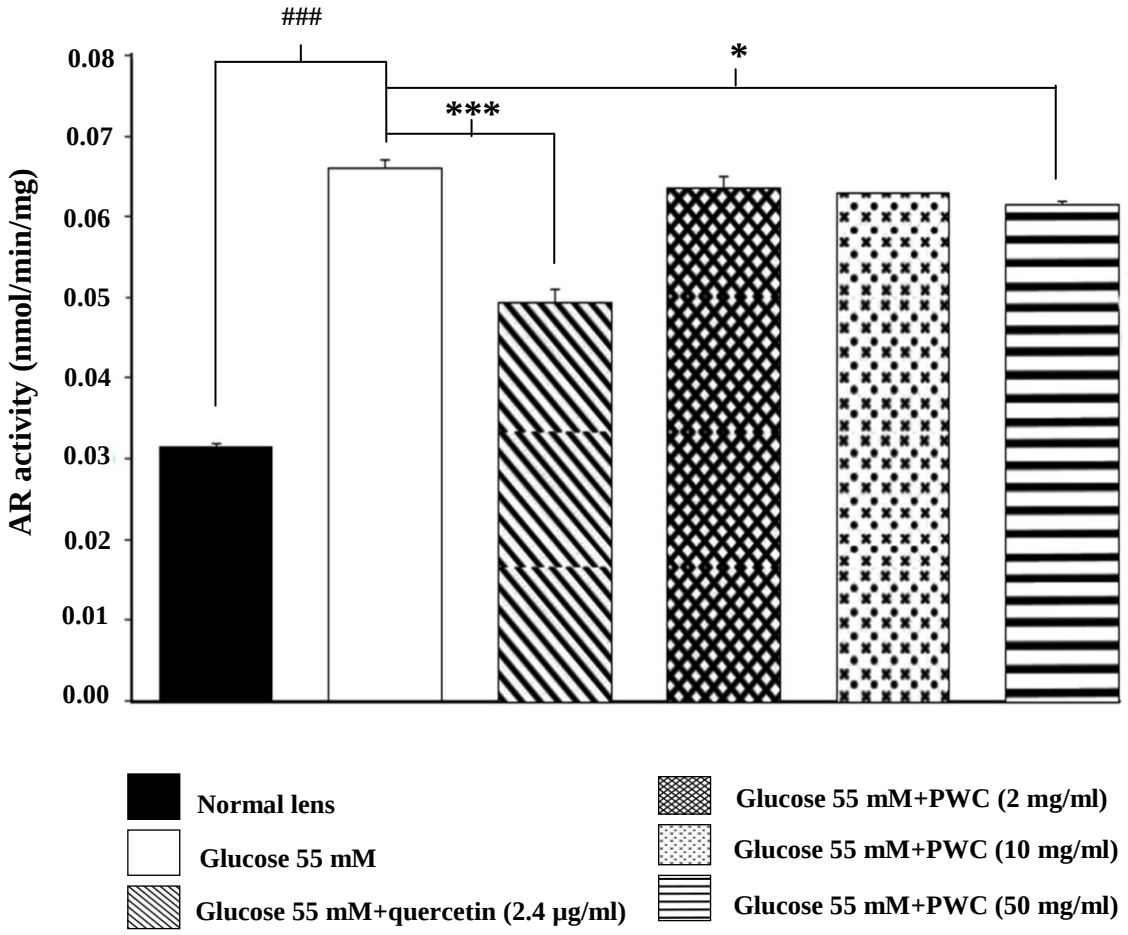

Figure 5-8 The effect of purple waxy corn grains extract (PWC) at doses of 2, 10 and 50 mg/ml on aldose reductase (AR) activity in lens exposed to high concentration of glucose. $^{###}$p-value < 0.001; compared to normal lens *,***p-value < 0.05, 0.001; compared to experimental diabetic cataract group

Table 5-2 Effect of PWC on aldose reductase and oxidative stress including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes in rat lens after the exposure to a 72 h glucose incubation. $^{\#\#,\#\#\#}$p-value <0.01,0.01 and 0.001 respectively; compared to normal lens*,***p-value <0.05 and 0.001 respectively; compared to experimental diabetic cataract group

Groups	**Effects of PWC on oxidative stress markers and enzymatic activity**				
	MDA (U/mg.protein)	AR activity (nmol/min/mg)	SOD (U/mg.protein)	CAT (U/mg.protein)	GPx (U/mg.protein)
Normal lens	0.75±0.20	0.0315±0.0005	0.83±0.00	178.05±25.45	2.06±0.49
Glucose 55 mM	3.27±0.76$^{\#\#}$	0.0660±0.0010$^{\#\#\#}$	0.13±0.04$^{\#\#}$	43.18±8.13$^{\#\#\#}$	0.76±0.02$^{\#\#}$
Glucose 55mM+quercetin (2.4 µg/ml)	1.08±0.28*	0.0495±0.0015***	0.65±0.00*	70.53±1.78	1.67±0.03*
Glucose 55 mM+PWC seed extract (2 mg/ml)	1.91±0.88	0.0635±0.0015	0.45±0.25	51.82±13.45	1.01±0.13
Glucose 55 mM+PWC seed extract (10 mg/ml)	1.64±0.10*	0.0630±0.000	0.49±0.12	53.03±14.72	1.45±0.32*
Glucose 55 mM+PWC seed extract (50 mg/ml)	1.34±0.34*	0.0615±0.0005*	0.49±0.02	48.55±19.60	1.03±0.12

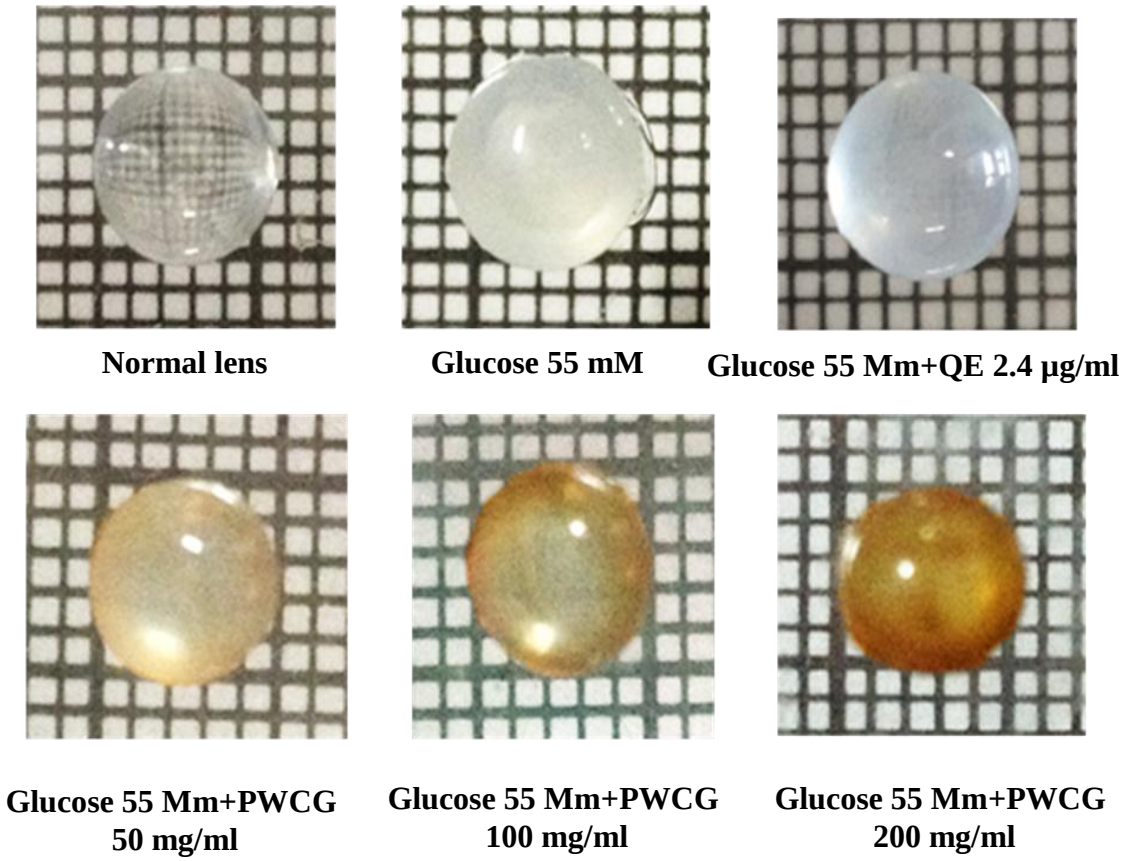

Figure 5-9 Anti-cataract effect of PWCG after 72 hrs incubation with glucose

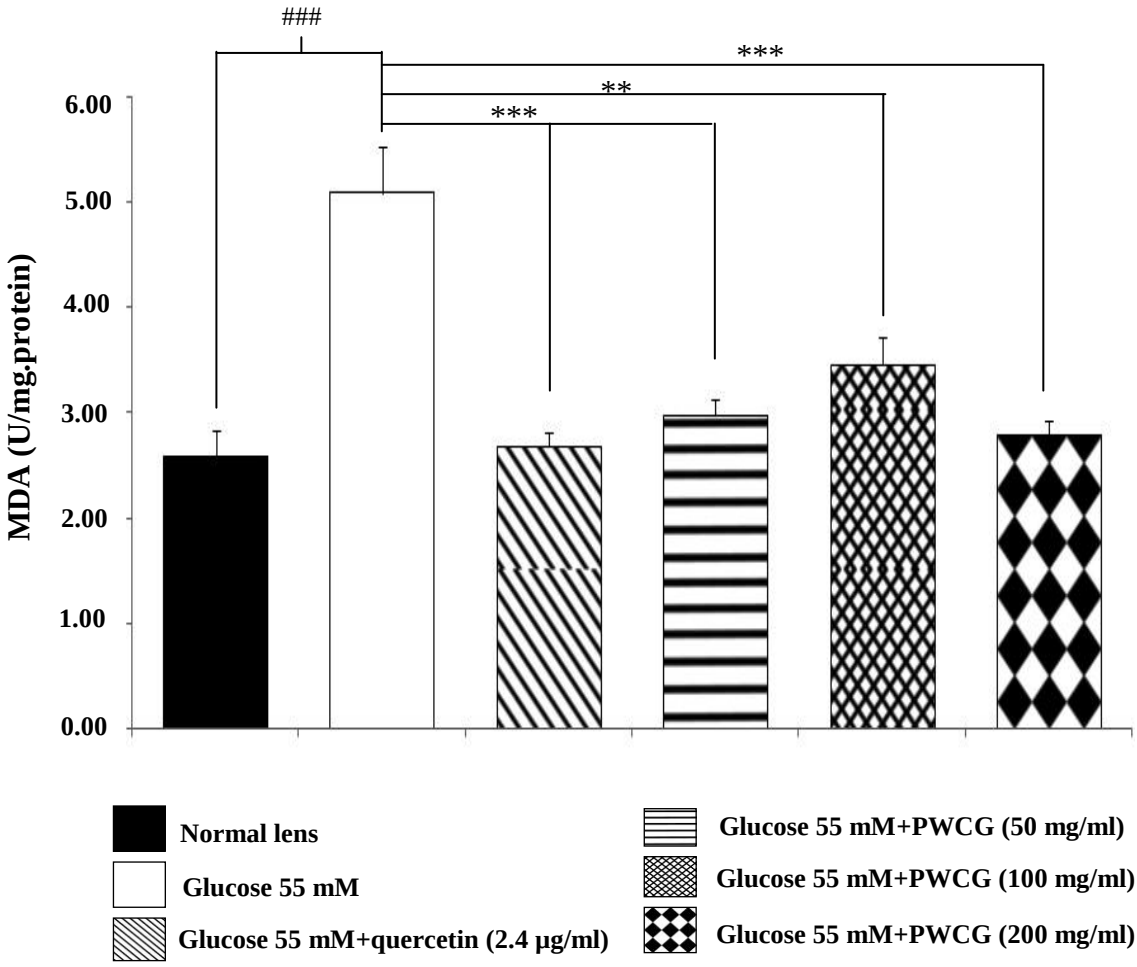

Figure 5-10 The level of malondialdehyde (MDA) (U/mg.protein) in lens after incubated 72 hours in the combination extract of purple waxy corn and ginger (PWCG) at doses of 50, 100 and 200 mg/ml. (N=6/group) $^{###}$ p-value < 0.001; compared to normal lens **,***p-value < 0.01, 0.001; compared to experimental diabetic cataract group

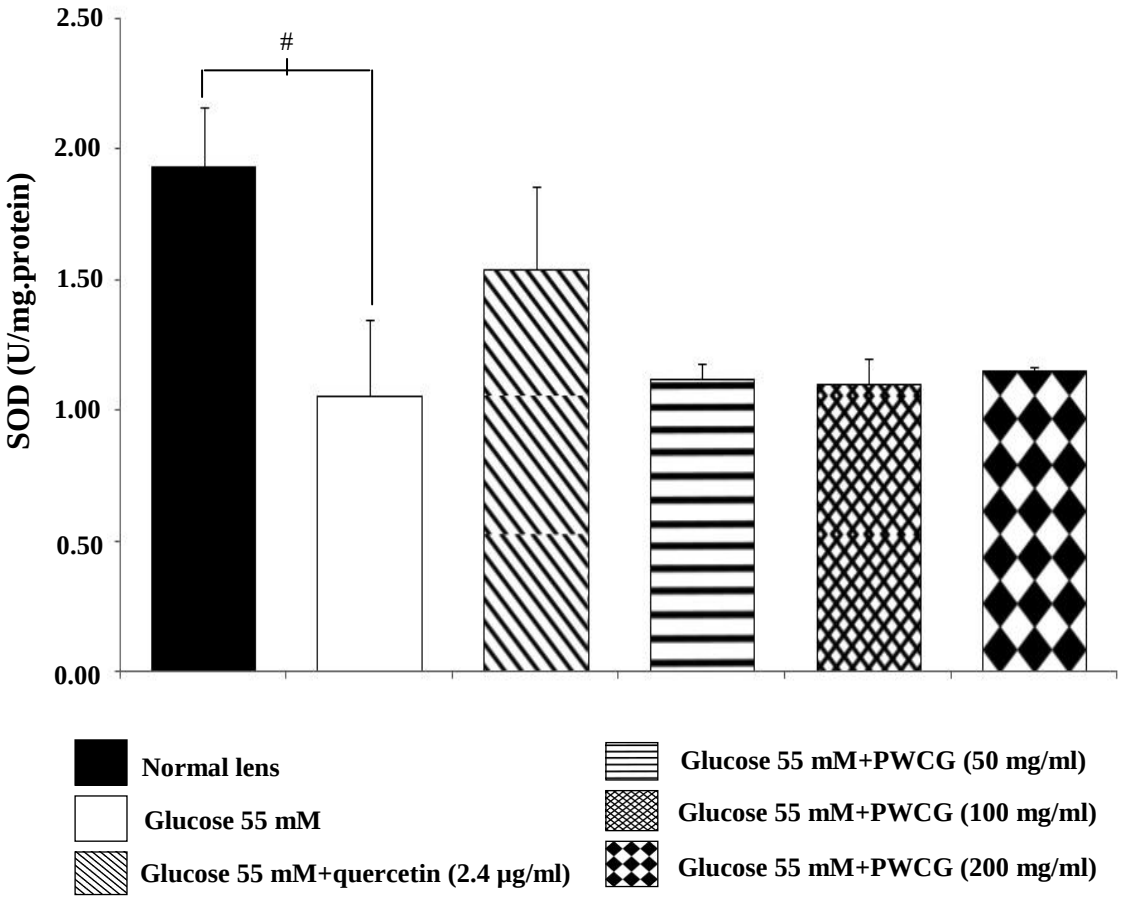

Figure 5-11 The activity of superoxide dismutase (SOD) (U/mg.protein) in lens after incubated 72 hours in the combination extract of purple waxy corn and ginger (PWCG) at doses of 50, 100 and 200 mg/ml. (N=6/group) # p-value < 0.05; compared to normal lens

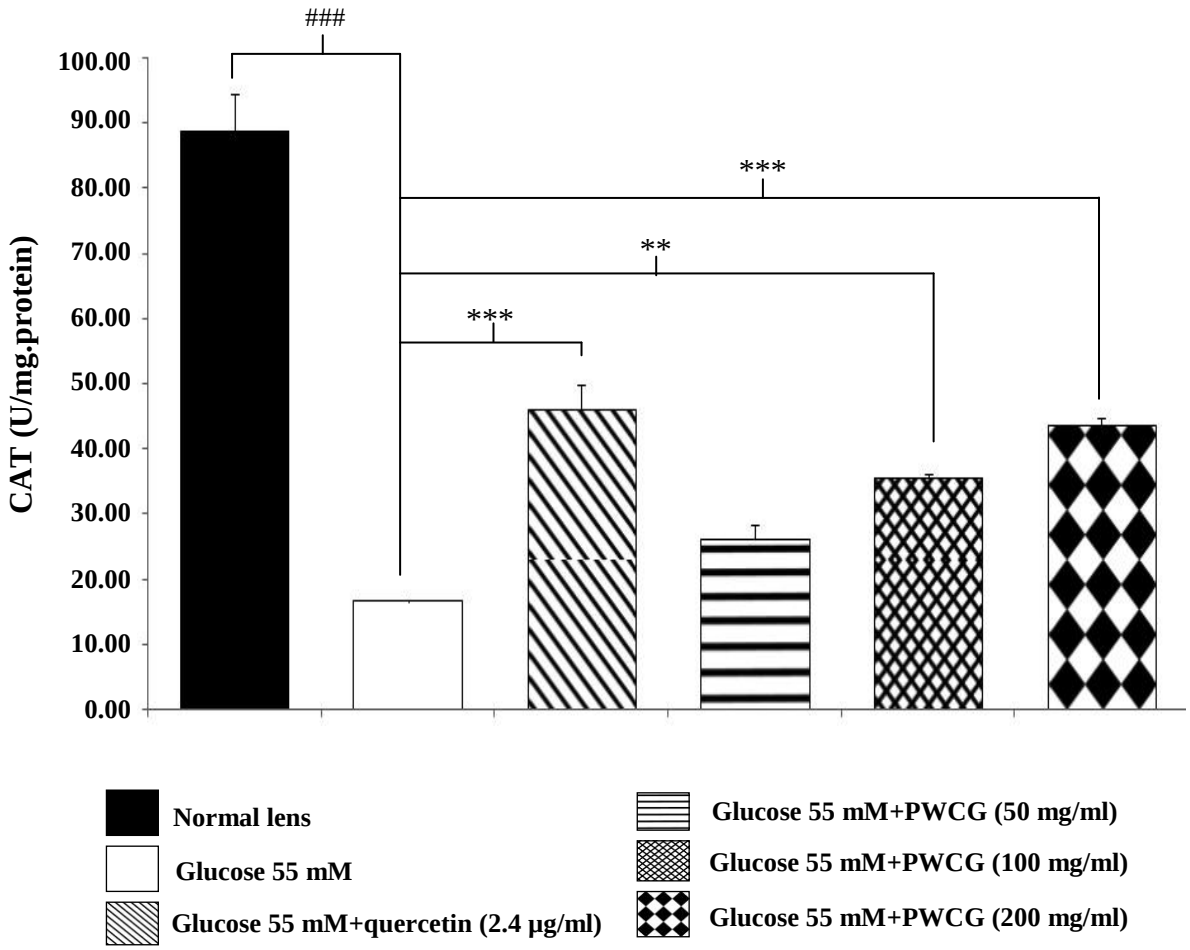

Figure 5-12 The activity of catalase (CAT) (U/mg.protein) in lens after incubated 72 hours in the combination extract of purple waxy corn and ginger (PWCG) at doses of 50, 100 and 200 mg/ml. (N=6/group) $^{###}$p-value < 0.001; compared to normal lens **,***p-value < 0.01, 0.001; compared to experimental diabetic cataract group

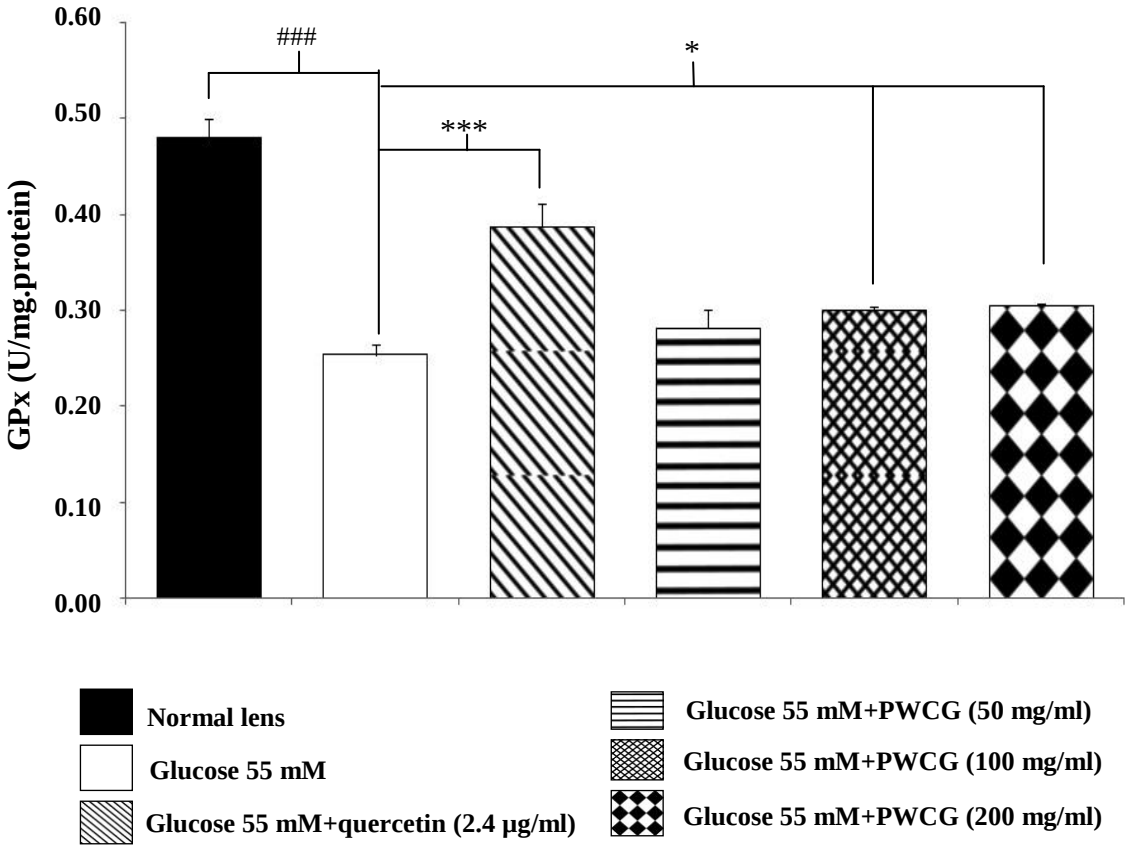

Figure 5-13 The activity of glutathione peroxidase (GPx) (U/mg.protein) in lens after incubated 72 hours in the combination extract of purple waxy corn and ginger (PWCG) at doses of 50, 100 and 200 mg/ml. (N=6/group) ### p-value < 0.001; compared to normal lens *,*** p-value < 0.05, 0.001; compared to experimental diabetic cataract group

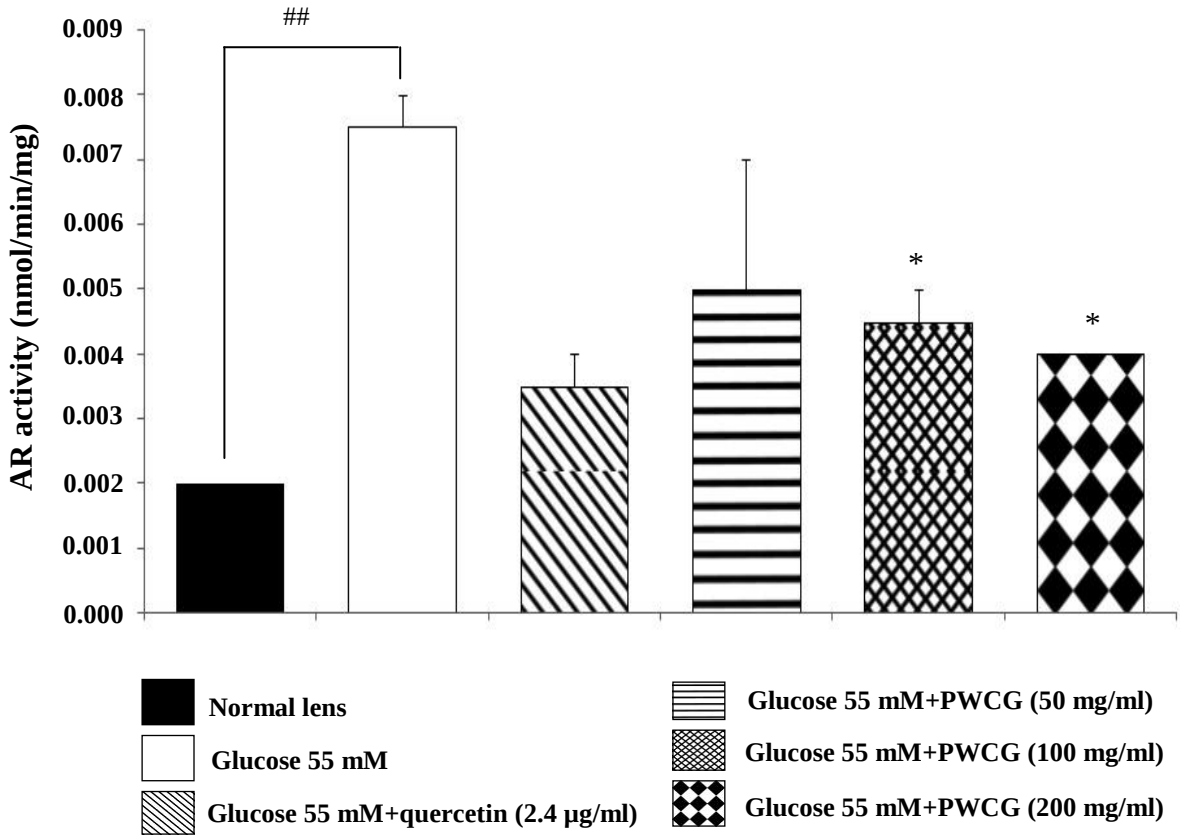

Figure 5-14 The activity of aldose reductase (AR) (nmol/min/mg) in lens after incubated 72 hours in the combination extract of purple waxy corn and ginger (PWCG) at doses of 50, 100 and 200 mg/ml. (N=6/group) $^{\#\#}$ p-value < 0.01; compared to normal lens *p-value < 0.05; compared to experimental diabetic cataract group

Table 5-3 Effect of PWCG on aldose reductase and oxidative stress markers including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes in rat lens after the exposure to a 72 h- glucose incubation. $^{\#,\#\#,\#\#\#}$p-value <0.05,0.01 and 0.001 respectively; compared to normal lens *,**,***p-value <0.05, 0.01 and 0.001 respectively; compared to experimental diabetic cataract group

Groups	**Effects of PWCG on oxidative stress markers and enzymatic activity**				
	MDA (U/mg.protein)	AR activity (nmol/min/mg)	SOD (U/mg.protein)	CAT (U/mg.protein)	GPx (U/mg.protein)
Normal lens	2.58±0.26	0.0020±0.0000	1.93±0.23	88.74±5.73	0.48±0.02
Glucose 55 mM	5.09±0.44$^{\#\#\#}$	0.0075±0.0005$^{\#\#}$	1.06±0.30$^{\#}$	16.54±0.27$^{\#\#\#}$	0.25±0.01$^{\#\#\#}$
Glucose 55mM+quercetin (2.4 µg/ml)	2.69±0.12***	0.0035±0.0005	1.54±0.32	46.01±3.72***	0.39±0.02***
Glucose 55 mM+PWCG (50 mg/ml)	2.98±0.15***	0.0050±0.0020	1.13±0.05	26.28±2.00	0.28±0.02
Glucose 55 mM+PWCG (100 mg/ml)	3.47±0.25**	0.0045±0.0005*	1.11±0.10	35.59±0.57**	0.30±0.00*
Glucose 55 mM+PWCG (200 mg/ml)	2.80±0.13***	0.0040±0.0000*	1.16±0.02	43.52±1.17***	0.31±0.00*

3.3 Effect of the combination extract of purple waxy corn and ginger (PWCG) in STZ-induced DM cataract model.

3.3.1 Effect of PWCG on fasting blood sugar level

The effect of PWCG on the average fasting blood glucose levels was determined and the results were shown in Figure 5-15. The results showed that diabetic rats which received distilled water or vehicle used in this study significantly enhanced the blood sugar levels throughout the study period (p-value<0.001 all; compared with control rats which received distilled water). It was found that the blood sugar levels of all diabetic rats were more than 300 $mg.dL^{-1}$ throughout the study period. PWCG at all doses used in this study failed to decrease the fasting blood glucose level in diabetic rats.

3.3.2 Anti-cataract effect of PWCG

The effect of PWCG on lens opacity was shown in Figure 5-16. The lens opacity in control rats also developed cataract during 6-10 weeks period. At 10-week study period, all lens from both right (1A and 1B) and left eyes (1a and 1b) of control rats were clear and normal. Both right (2A and 2B) and left eyes (2a and 2b) of diabetic rats which received vehicle showed lens opacity. However, the increased lens opacity induced by diabetic condition was attenuated in diabetic rats which received all doses of PWCG (3A-5A, 3B-5B, 3a-5a and 3b-5b).

Table 5-4 showed that the lens opacity indices of diabetic rats which received vehicle started to increase at 3-week treatment duration (p-value<0.01; compared with control rats). This change was still observed until the end of study (p-value<0.001; compared with control rats). Diabetic rats which received PWCG at dose of 200 $mg.kg^{-1}$ significantly mitigated the elevation of lens opacity index induced by diabetes at 5-wk of treatment (p-value<0.05; compared with diabetic rats which received vehicle). This change was persisted until the end of study (p-value<0.01, 0.05, 0.001, 0.001, 0.001 respectively; compared with diabetic rats which received vehicle). Diabetic rats which received PWCG at low dose (50 $mg.kg^{-1}$ BW) also mitigated the elevation of lens opacity index induced by diabetes but the significant changes were observed at 3-week, 5 week, 8-week, 9- week and 10-week treatment duration (p-value<0.05, 0.05, 0.05, 0.001 and 0.001 respectively; compared with diabetic rats which received vehicle). However, the medium dose of

PWCG produced the significant mitigation effect on lens opacity index induced by diabetes only at 8-week, 9-week and 10-week treatment duration (p-value<0.05, 0.001 and 0.001 respectively; compared with diabetic rats which received vehicle). The effects of PWCG on lens opacity were confirmed by histopathological analysis of lens as shown in Figure 5-17. It was found that at 10-week study period, the len capsule thickness and the density of epithelial cells of diabetic rat subjected to vehicle treatment were decreased.

In addition, the reduction of differentiated lens fibers, vacuoles and homogenized area were also observed (5-17B). Diabetic rats which received PWCG at doses of 50,100 and 200 $mg.kg^{-1}$ BW showed the increased density of epithelial cells and differentiated lens fiber thickness together with the decreased vacuoles and homogenized area (5-17C, 5-17D and 5-17E).

Figure 5-18 showed the effect of PWCG on the severity of lens damage. Our results showed that the diabetic rats which received vehicle showed the increased severity of cataract (p-value<0.001 compared with control rats). All doses of PWCG significantly mitigated the enhanced cataract severity induced by diabetes mellitus (p-value<0.001 all; compared with diabetic rats which received vehicle).

3.3.3 Effect of PWCG on retinopathy in diabetic rats

The effect of PWCG on histopathological change of retina was shown in Figure 5-19. At 10-week study period, the total retinal thickness (TRT), the thickness of the retinal outer nuclear layer (ROT) and the number of cells in the ganglion cell layer (NG) of diabetic rats which received vehicle significantly decreased when compared to control. However, the decreased TRT, ROT and NG induced by diabetic condition were attenuated in diabetic rats which received all doses of PWCG. Figure 5-20-figure 5-22 showed that diabetic rats which received PWCG at doses of 50, 100 and 200 $mg.kg^{-1}$ BW significantly increased the TRT (p-value<0.001 all; compared with diabetic rats which received vehicle), ROT (p-value<0.001 all; compared with diabetic rats which received vehicle) and NG of retina (p-value<0.001, 0.05 and 0.001 respectively; compared with diabetic rats which received vehicle).

3.3.4 Effect of PWCG on oxidative stress markers

Based on the crucial role of oxidative stress on cataract and retinopathy, the effects of PWCG on oxidative stress markers including level of MDA and the activities of SOD, CAT and GPx in lens were carried out. The results were shown in figure 5-23-figure 5-26. It was found that diabetic rats which received vehicle significantly increased MDA level (p-value<0.001; compared with control) but decreased SOD, CAT and GPx activities (p-value<0.001 all; compared with control) in lens. PWCG at doses of 50, 100 and 200 $mg.kg^{-1}$ BW significantly mitigated the elevation of MDA level induced by diabetes mellitus (p-value<0.001 all; compared with diabetic rats which received vehicle). Low dose of PWCG also mitigated the reduction of GPx activity in lens of diabetic rats (p-value<0.05; compared with diabetic rats which received vehicle) whereas high dose of PWCG mitigated the reduction of both CAT and GPx activities of lens of diabetic rats (p-value<0.01 and 0.001 respectively; compared with diabetic rats which received vehicle). However, no significant changes of scavenger enzymes activities were observed in the diabetic rats which received medium dose of PWCG.

3.3.5 Effect of PWCG on aldose reductase activity

In addition to the oxidative stress, the polyol pathway also plays the crucial role on the cataractogenesis. Therefore, the effect of PWCG on aldose reductase enzyme activity in lens was also investigated and data were shown in Figure 5-27. It was demonstrated that aldose reductase activity in lens of diabetic rats was increased (p-value<0.05; compared with control). PWCG at dose of 200 $mg.kg^{-1}$ BW could significantly decrease the elevation of aldose reductase induced by diabetes mellitus in rat lens. No other significant changes were observed.

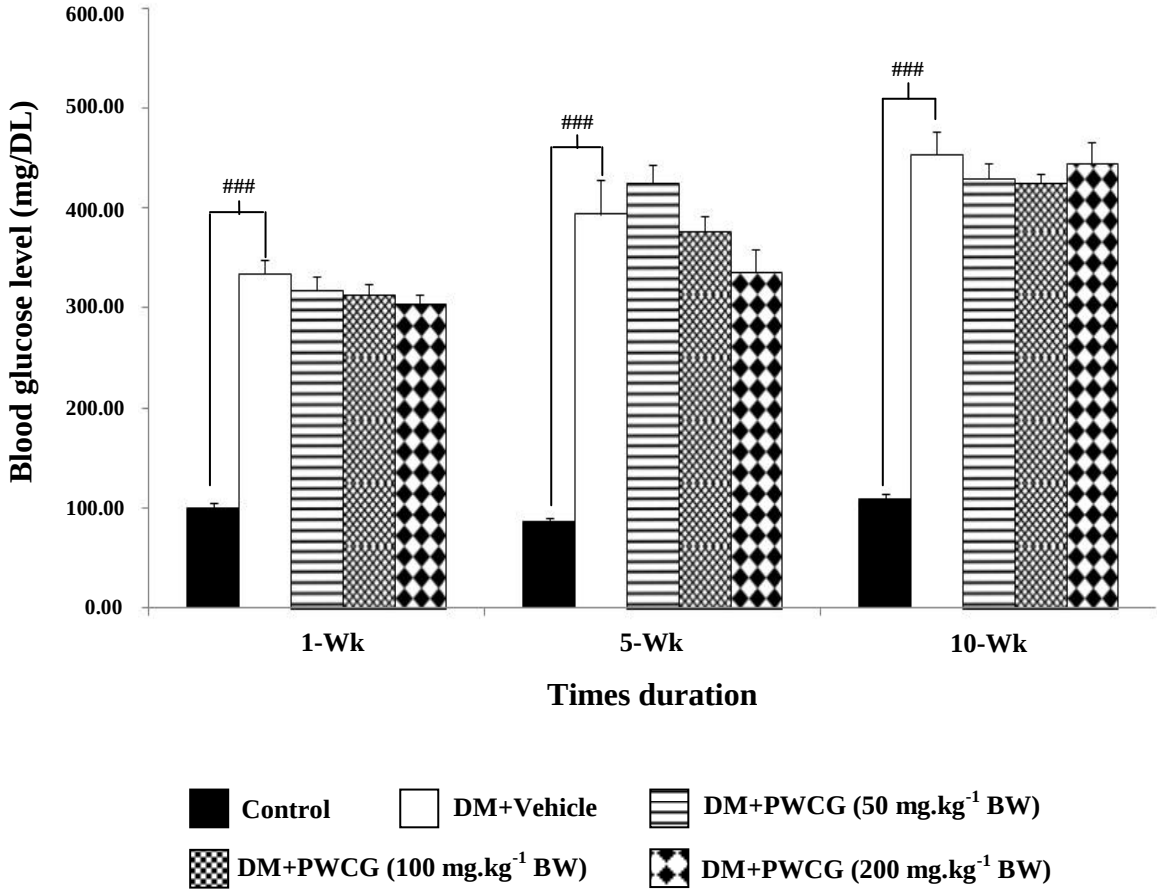

Figure 5-15 The average fasting blood glucose levels of all the experimental groups at 1-week, 5- week and 10-week intervention period. (N=8/ group) ### p-value< 0.001; compared with control group

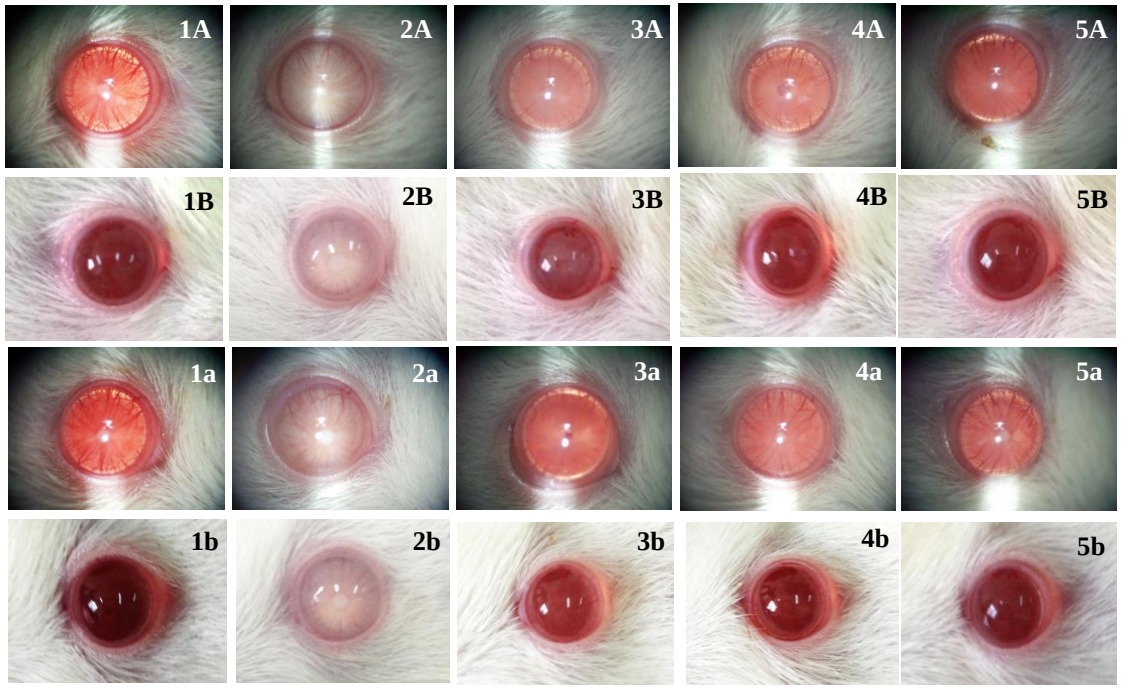

Figure 5-16 Representative photographs showing the effects of a 10-week treatment of PWCG on cataractogenesis. A, a: Photographs via slit lamp, B, b: Photographs via camera; 1: Control rat; 2: DM+vehicle; 3: DM+PWCG 50 mg.kg^{-1} BW; 4: DM+PWCG 100 mg.kg^{-1} BW; 5: DM+PWCG 200 mg.kg^{-1}BW

Table 5-4 Opacity index of the lens of normal and diabetic rats (DM) which received either vehicle or PWCG at doses of 50, 100 and 200 mg.kg^{-1} BW for 10 weeks. (N=8/group) $^{\#\#, \#\#\#}$p-value<0.01 and 0.001 respectively; compared with control group.*, **, *** p-value<0.05, 0.01 and 0.001 respectively; compared with diabetic rats which received vehicle (DM+Vehicle)

Times duration	**Opacity index of the lens (mean±SEM)**				
	Control	**DM+Vehicle**	**DM+PWCG (50 mg.kg^{-1}BW)**	**DM+PWCG (100 mg.kg^{-1}BW)**	**DM+PWCG (200 mg.kg^{-1}BW)**
Baseline	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Week-1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Week-2	0.00±0.00	0.06±0.06	0.00±0.00	0.00±0.00	0.00±0.00
Week-3	0.00±0.00	0.50±0.16$^{\#\#}$	0.14±0.10*	0.50±0.13	0.22±0.10
Week-4	0.00±0.00	1.00±0.20$^{\#\#\#}$	0.79±0.24	1.00±0.18	0.72±0.23
Week-5	0.00±0.00	1.81±0.31$^{\#\#\#}$	1.00±0.21*	1.25±0.23	1.00±0.28*
Week-6	0.08±0.08	2.25±0.32$^{\#\#\#}$	1.50±0.29	1.50±0.22	1.11±0.31**
Week-7	0.17±0.11	2.50±0.34$^{\#\#\#}$	1.71±0.29	1.88±0.29	1.44±0.41*
Week-8	0.25±0.13	3.06±0.23$^{\#\#\#}$	2.00±0.30*	2.19±0.25*	1.67±0.40***
Week-9	0.50±0.15	3.81±0.10$^{\#\#\#}$	2.29±0.35***	2.38±0.26***	1.78±0.39***
Week-10	0.50±0.15	4.00±0.00$^{\#\#\#}$	2.21±0.37***	2.63±0.26***	1.89±0.40***

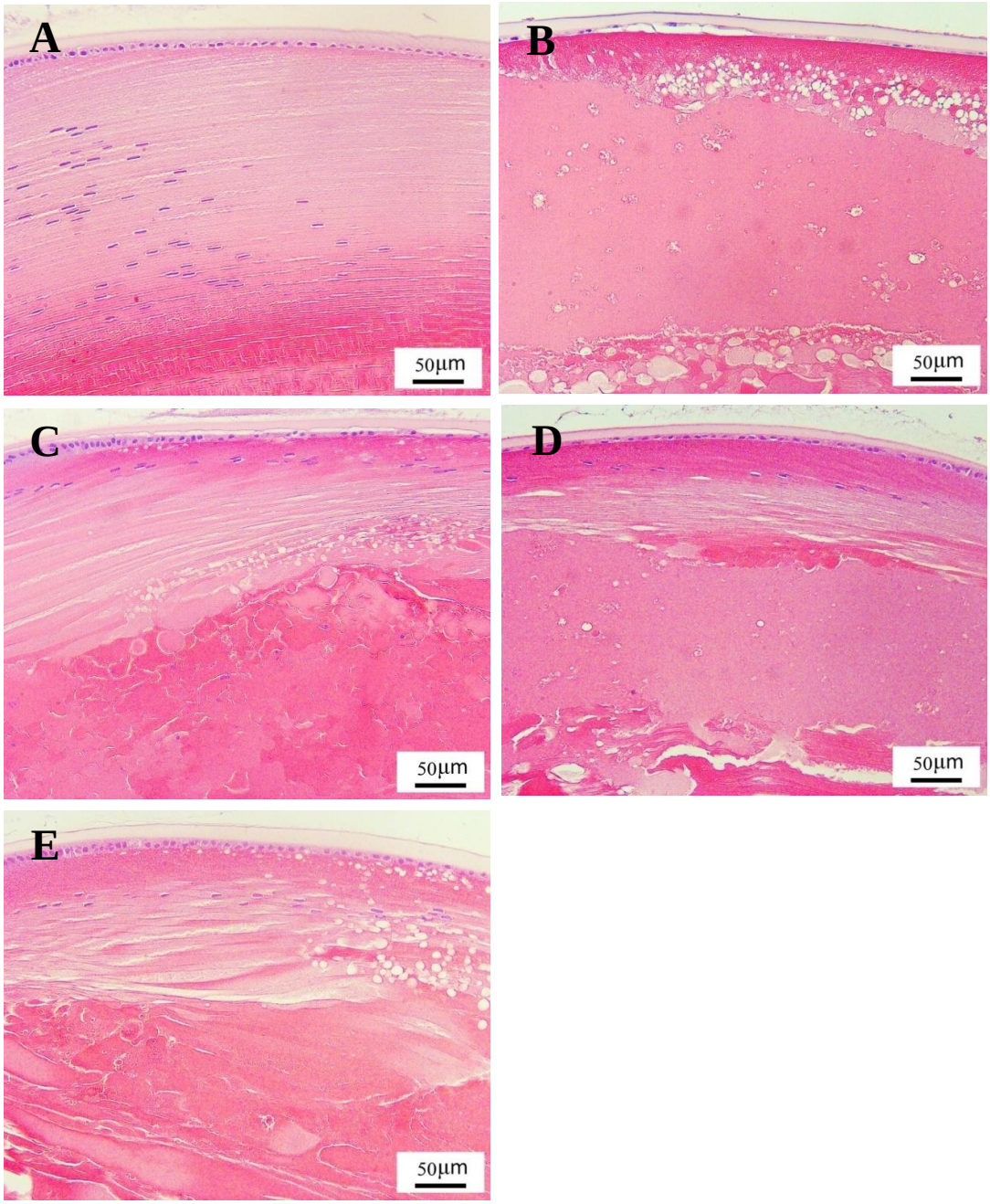

Figure 5-17 Photographs of transverse sections of eye balls (5 µm thickness), stained with haematoxylin and eosin-stained sections were determined the cataract severity at the end of experiment using light microscope (Week-10). (A): control, (B): DM+Vehicle, (C): DM+PWCG (50 mg.kg^{-1} BW), (D): DM+PWCG (100 mg.kg^{-1}BW), (E): DM+PWCG (200 mg.kg^{-1}BW)

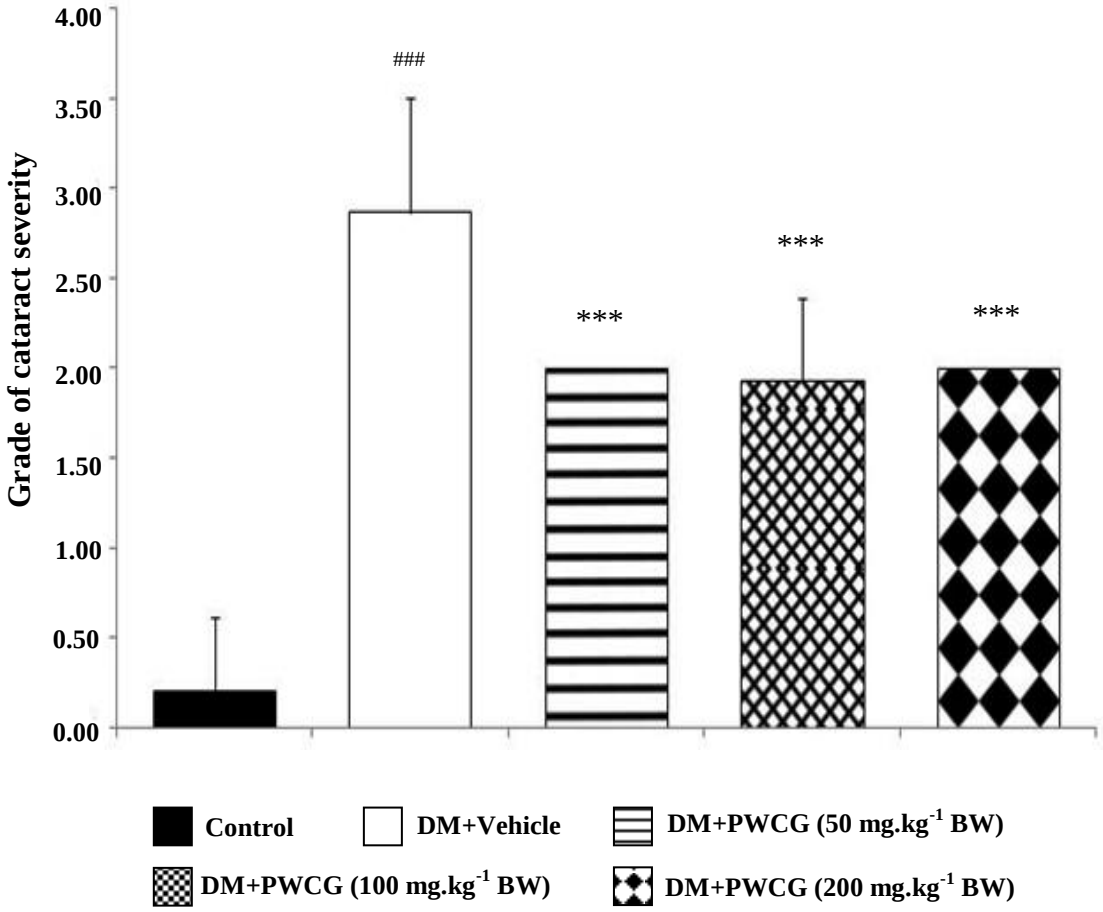

Figure 5-18 The effect of a 10-week treatment of PWCG at doses of 50, 100 and 200 mg.kg^{-1} BW on cataract in STZ-induced diabetic rats. $^{###}$p-value < 0.001; compared with control and ***p-value < 0.001; compared with vehicle.

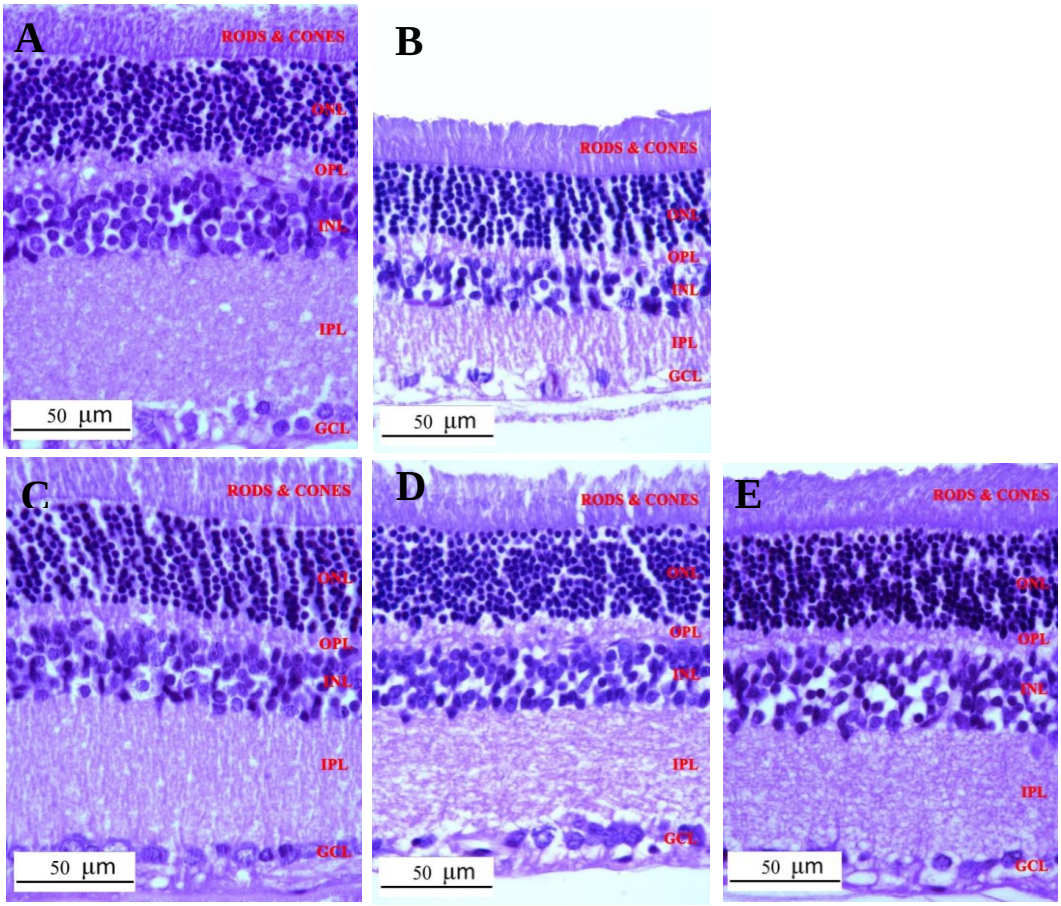

Figure 5-19 Effect of a 10-week treatment with PWCG on retinal thickening and extracellular matrix in STZ-treated rats evaluated by haematoxylin and eosin-stained sections. A: Control rat; B: DM+vehicle; C: DM+PWCG 50 mg.kg^{-1} BW; D: DM+PWCG 100 mg.kg^{-1} BW; E: DM+PWCG 200 mg.kg^{-1} BW

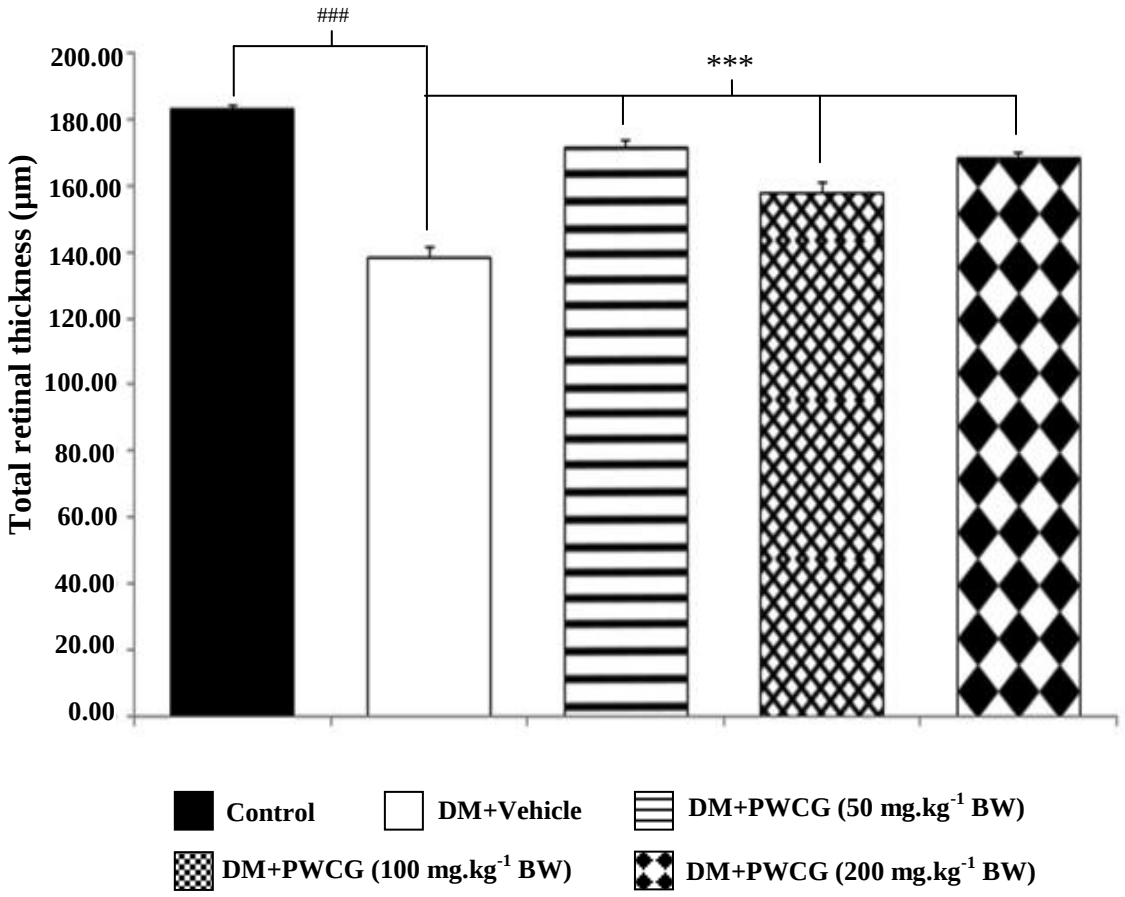

Figure 5-20 The effect of a 10- week treatment of PWCG on total retinal thickness of retina (TRT). $^{###}$p-value < 0.001; compared with control and ***p-value < 0.001; compared with vehicle.

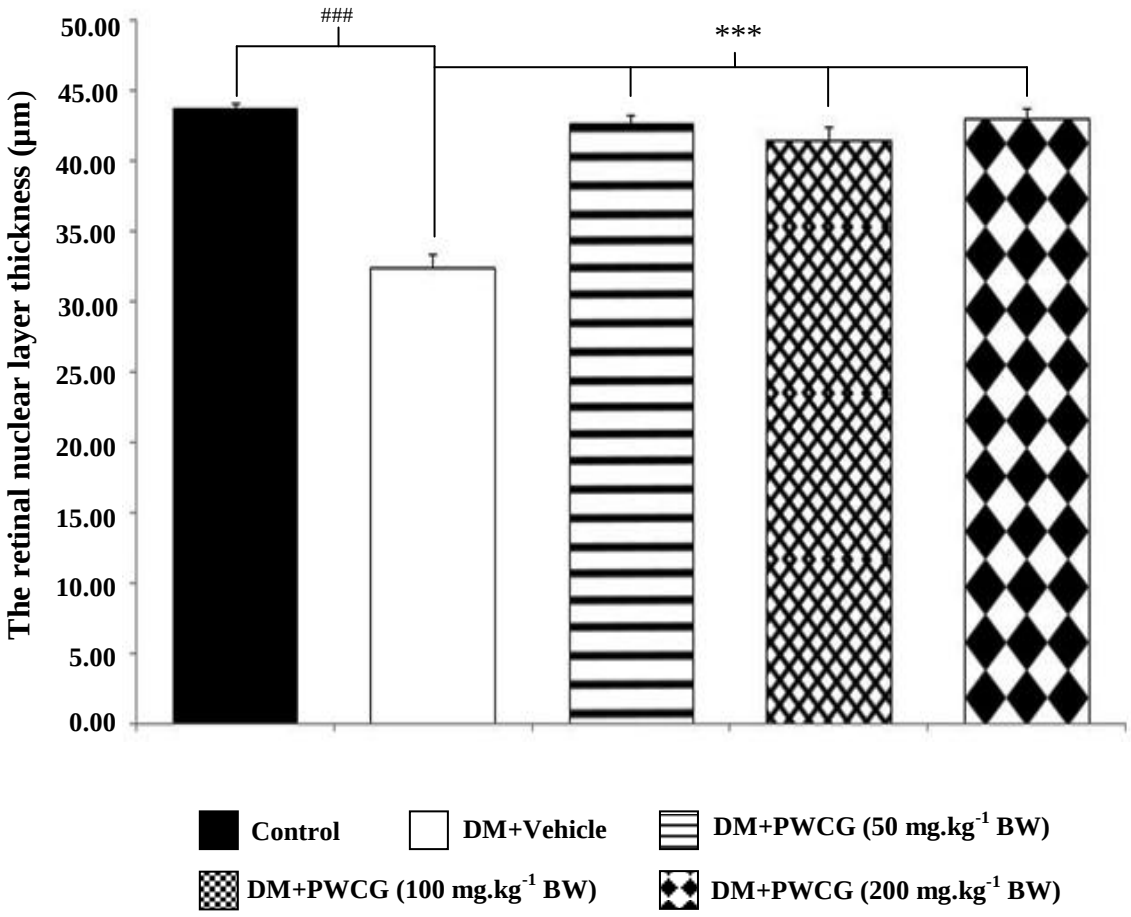

Figure 5-21 The effect of a 10- week treatment of PWCG on the thickness of the retinal outer nuclear layer (ROT). $^{###}$p-value < 0.001; compared with control and ***p-value < 0.001; compared with vehicle.

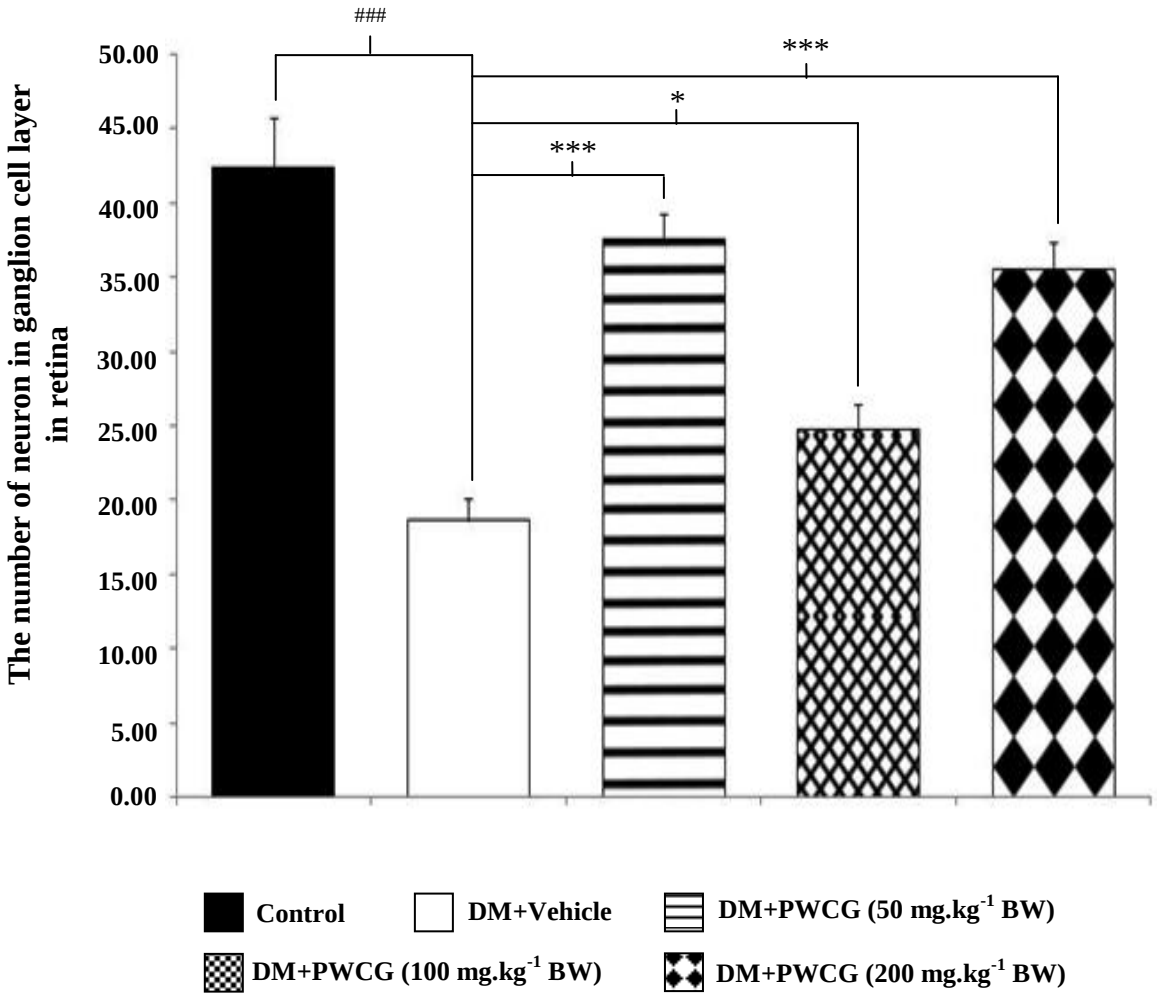

Figure 5-22 The effect of a 10- week treatment of PWCG on the number of cells in the ganglion cell layer (NG) in retina. $^{###}$p-value < 0.001; compared with control and $^{*, ***}$p-value <0.05 and 0.001; compared with vehicle

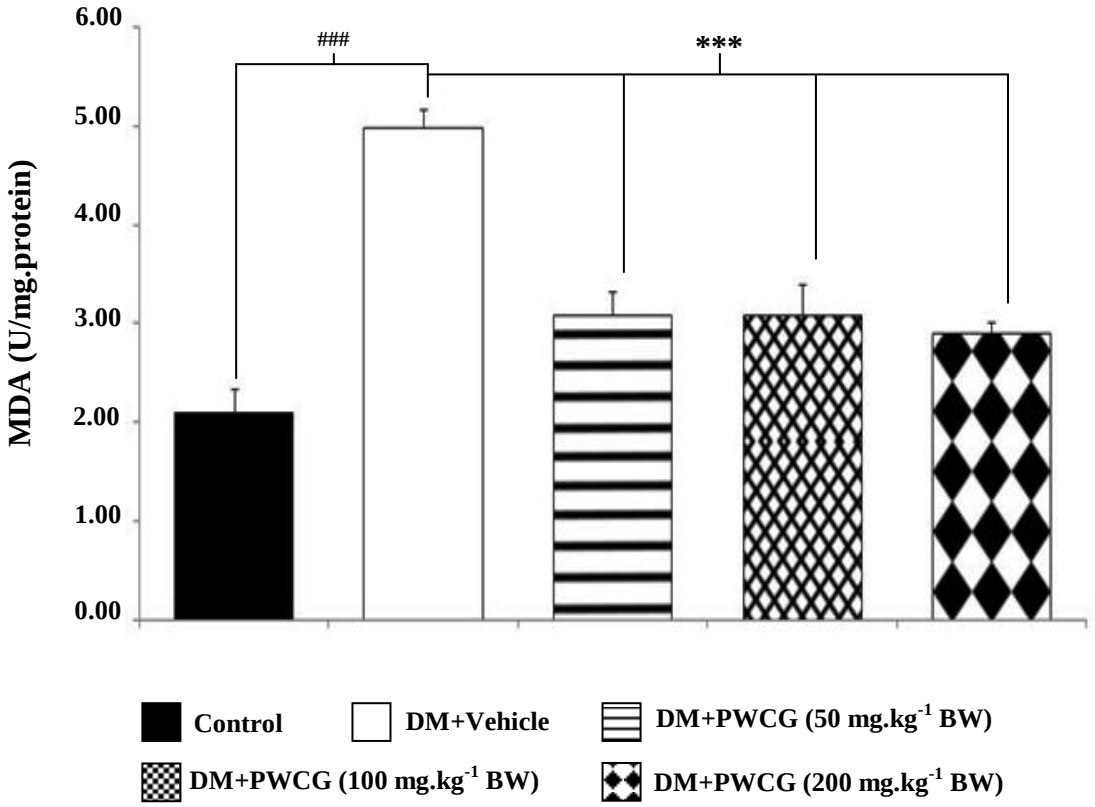

Figure 5-23 The effect of a 10-week treatment of PWCG on malondialdehyde (MDA) level in lens of STZ-induced diabetic rats. (N=8/group) ### p-value < 0.001; compared with control ***p-value < 0.001; compared with diabetic rats which received vehicle (DM+Vehicle)

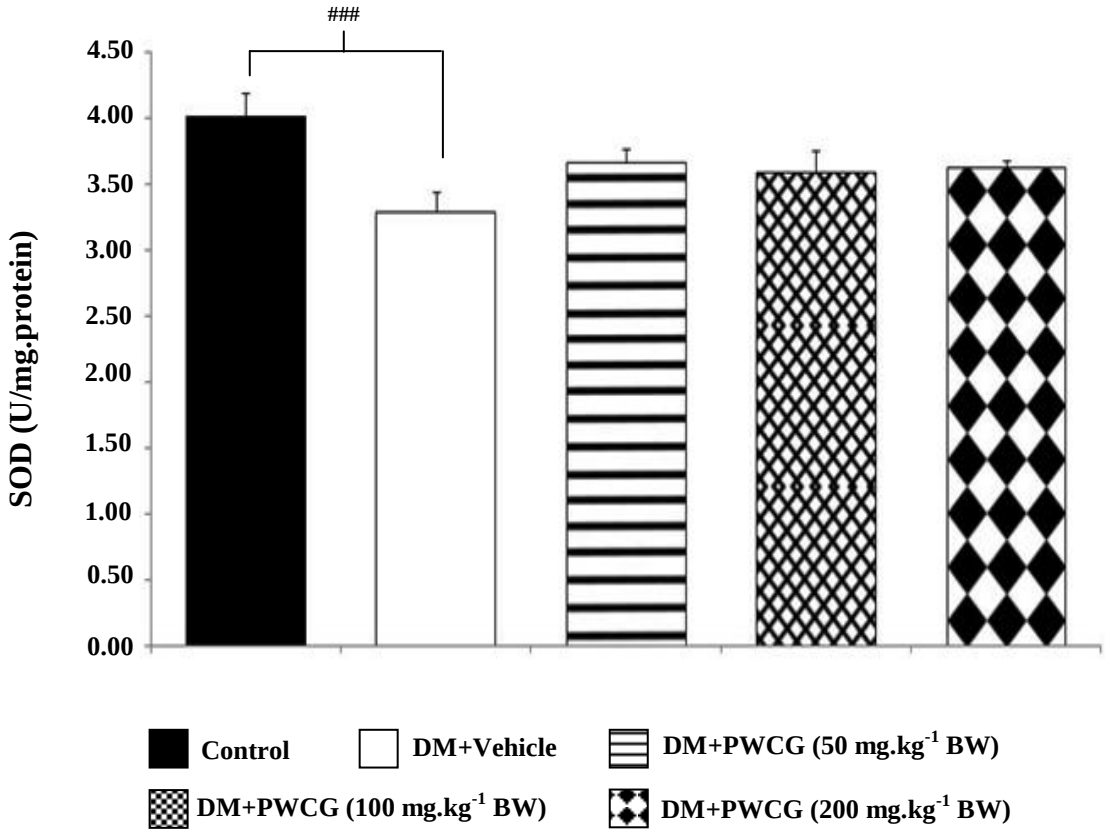

Figure 5-24 The effect of a 10- week treatment of PWCG on superoxide dismutase (SOD) activity in lens of STZ-induced diabetic rats. (N=8/group) ### p-value < 0.001; compared with control

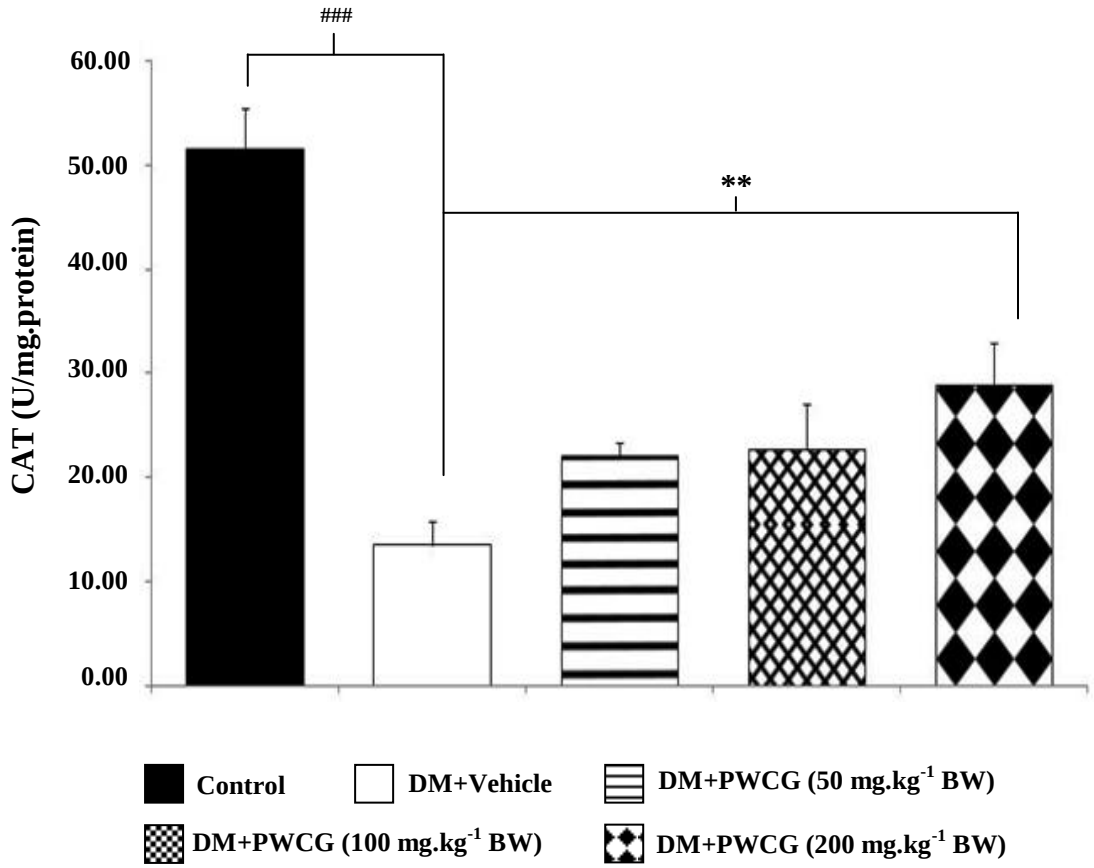

Figure 5-25 The effect of a 10- week treatment of PWCG on catalase (CAT) activity in lens of STZ-induced diabetic rats. (N=8/group) $^{###}$p-value < 0.001; compared with control******p-value < 0.01; compared with diabetic rats which received vehicle (DM+Vehicle)

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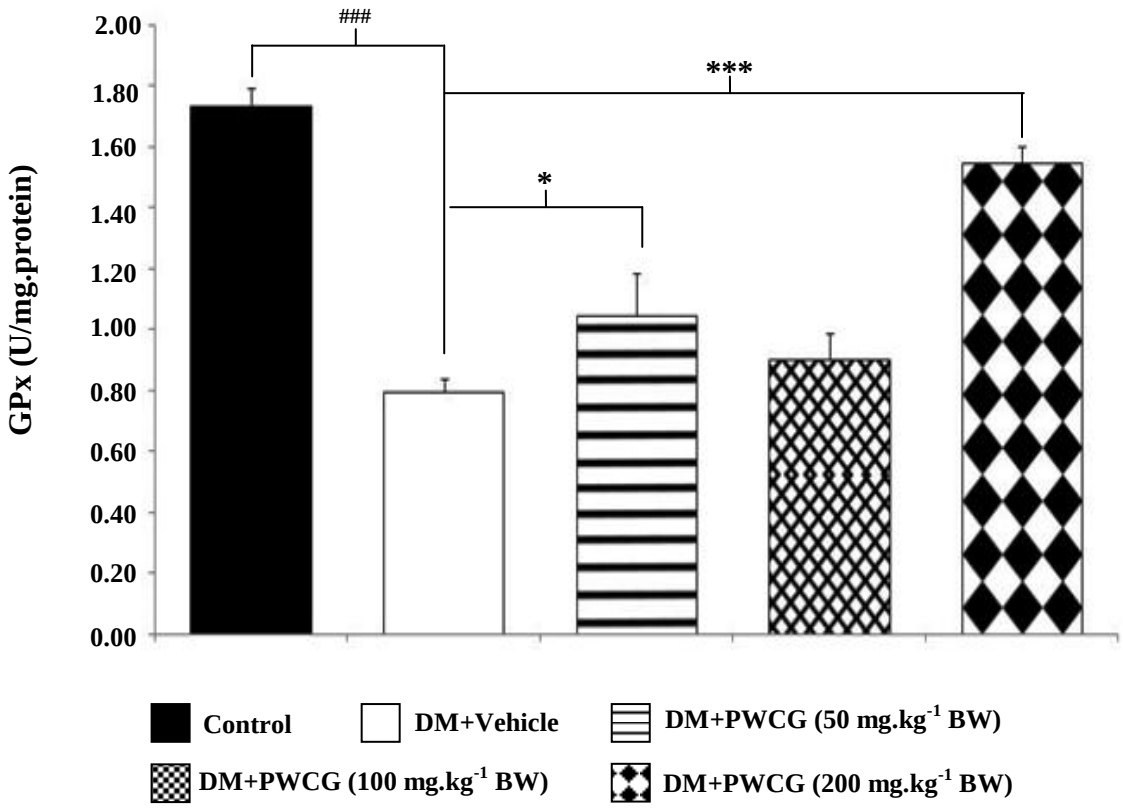

Figure 5-26 The effect of a 10-week treatment of PWCG on glutathione peroxidase (GPx) activity in lens of STZ-induced diabetic rats (N=8/group). ### p-value<0.001; compared with control ***,***** p-value<0.05, 0.001 respectively; compared with diabetic rats which received vehicle (DM+Vehicle)

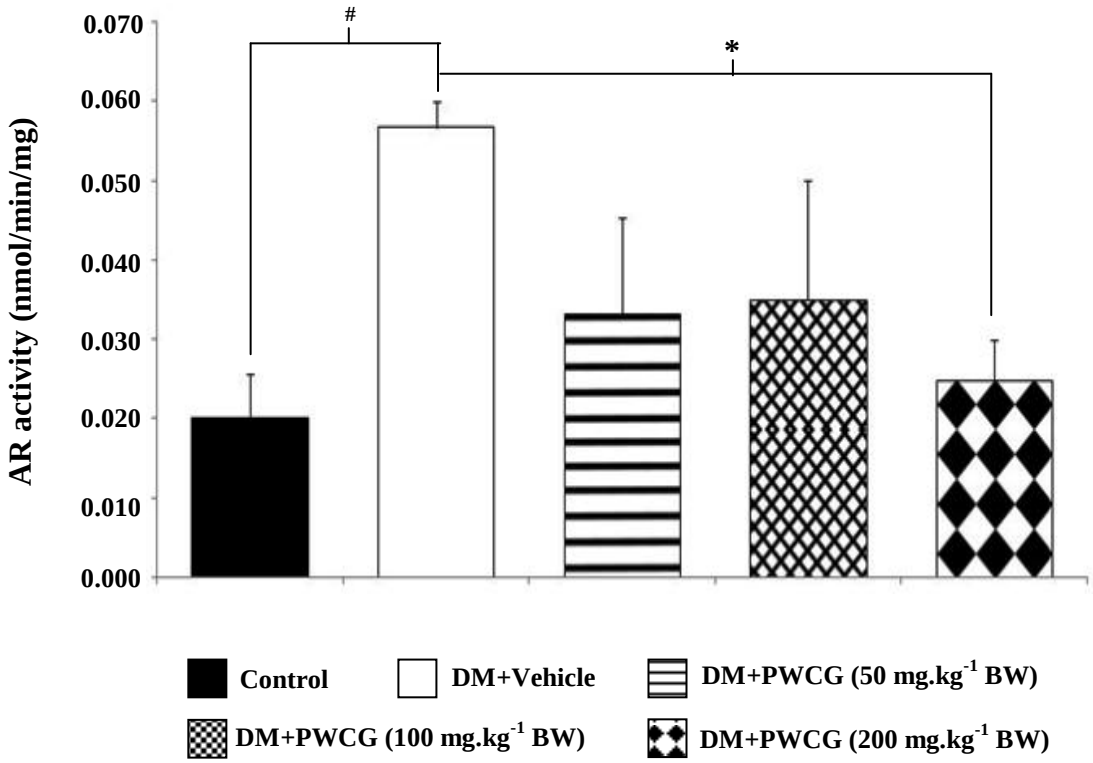

Figure 5-27 The effect of a 10-week treatment of PWCG on aldose reductase (AR) activity in lens of STZ-induced diabetic rats. (N=8/group) #p-value <0.05; compared with control *****p-value <0.05; compared with diabetic rats which received vehicle (DM+Vehicle)

4. Discussion

Diabetic cataract and retinopathy are the important causes of blindness so the prevention of these conditions is very much important. It has been well known that oxidative stress is one of the possible causes of diabetic cataractogenesis. High glucose concentration enhanced the formation of superoxide (O2.-) radicals and H_2O_2 (Singh, 2011) but decreased antioxidant enzymes such as SOD, CAT and GPx (Ozmen *et al.*, 2002) resulting in the excess oxidative stress reflecting by an elevation of lipid peroxidation product or MDA level. The excess oxidative stress was previously reported to induce extensive oxidative modifications on lens proteins especially α-crystalline protein, a major protein component of the mammalian eye lens resulting in the enhanced lens opacity (Xu *et al.*, 2011; Zhao *et al.*, 1998). In addition, the markedly elevation of aldose reductase in the lens was also observed.

Numerous lines of evidence demonstrated that the AR-mediated intracellular accumulation of polyols induced the collapse and liquefaction of lens fibers, which led to the formation of lens opacities (Kinoshita, 1965; Kinoshita, 1974).

Recently, considerable attention has been paid to the survey for novel intervention against diabetic cataract. Epidemiologic data have shown that consumption of vitamins, minerals, fiber, and numerous phytochemicals, including flavonoids can lower the risk of cataract in humans (Taylor, 1999). Phenolic compounds, the potential anti-cataract agents, could reduce the risk of cataract formation by affecting multiple key pathways pertinent to eye lens opacification, including oxidative stress and polyol pathway. It has been reported that numerous types of phenolic compounds such as Quercetin and Anthocyanin showed anti-cataract activity (Ghosh and Konishi, 2007; Stefek, 2011). Moreover, anthocyanins and anthocyanins-rich substance have the potential to protect against retinopathy (Nabavi *et al.*, 2015) and diabetic cataract. In addition, ginger and its constituents also show the potential to protect against diabetic retinopathy (Rahmani *et al.*, 2014). Therefore, the anti-cataractogenesis and anti-retinopathy of PWC and PWCG may be due to the flavonoids such as anthocyanins in them. Other types of flavonoid may also play a role. However, further researches are required to confirm the possible active ingredient.

In glucose induced cataractogenesis study, the medium dose of the PWC might exert its effect mainly via the increased GPx activity resulting in the decreased oxidative stress reflecting by the decreased MDA level in lens. The decreased oxidative stress in turn decreased the oxidation of lens proteins and finally decreased the lens opacification. However, the high dose of the extract appeared to exert the primarily effect via the suppression of aldose reductase resulting in the decreased polyol accumulation in the lens which in turn decreased the swelling of the lens epithelial cells and in the central lens region. This phenomenon was followed by swelling of the superficial cortical fibers, which eventually ruptured to form visible vacuoles (Kador *et al.*, 2000). As the degeneration of lenticular fibers progressed, the entire cortex became opaque and the formation of nuclear opacity occurred. Moreover, the medium and high dose of the PWCG might exert its effect mainly via the increased GPx activity resulting in the decreased oxidative stress reflecting by the decreased MDA level in lens. The decreased oxidative stress in turn decreased the

oxidation of lens proteins and finally decreased the lens opacification. Moreover, the medium and high dose of the extract appeared to exert the primarily effect via the suppression of aldose reductase resulting in the decreased polyol accumulation in the lens

In STZ-induced cataract and retinopathy, our results clearly demonstrated that PWCG significantly mitigated the reduction of ganglia in outer nuclear layer (ONL), OUL thickness and total retina thickness. In addition, PWCG also showed anti-cataract effect. Together with the changes mentioned earlier, the decreased oxidative stress and aldose reductase activity in rat lens were also observed.

It has been reported that the neurodegeneration of ganglion cells plays the crucial role on the reduction of OUL and total retina thickness (van Dijk *et al.*, 2010). The degeneration of retinal ganglion cells is under the influence of many factors such as inflammation, oxidative stress or exposure to advanced glycation end products (Kern and Barber, 2008) and polyol pathway (Ramadan, 2007). Our data also demonstrates the increased oxidative stress markers such as MDA level and the enhanced aldose reductase activity in lens of diabetic rats. These changes are in agreement with the previous study (Kern and Barber, 2008; Ramadan, 2007). Therefore, we suggested that the effect of PWCG to attenuate retinopathy induced by diabetes mellitus might be partly related to the decreased oxidative stress and aldose reductase activity in rat lens. It was found that the enhanced of GPx activity in rat lens might be responsible in part for the reduction of oxidative stress in rat lens of diabetic rats especially at low and high doses of PWCG. In addition to the increased GPx activity, the high dose of PWCG also enhanced CAT in rat lens. Therefore, the elevation of both CAT and GPx might be responsible for the decreased oxidative stress which in turn enhanced the survival of ganglion cells in OUL leading to the increased total retina thickness. However, the decreased oxidative stress in rat lens induced by medium dose of PWCG might be associated with other mechanism besides the increased the enzymatic scavenger system. Since the excess oxidative stress occurs as the result of imbalance between oxidative stress formation and oxidative stress buffering capacities, we suggested that the decreased oxidative stress formation might play a role on the reduction of oxidative stress induced by the PWCG especially at the medium concentration. Furthermore, we do suggest that the effect of

PWCG to improve retinopathy especially at high dose should also relate with the aldose reductase inhibitory effect.

In this study, the slit lamp examination clearly provided the changes of lens characteristic. However, no pupil was dilated during the grading of severity as that was performed during the slit lamp examination in human. This may possibly influence on the precision of grading scores which reflect the real severity of cataract. To obtain the precise severity of cataract the slit lamp examination should be performed while pupil was dilated. However, all rats were assessed under the condition without the pupil dilator so no confounding error about the beneficial effect of PWCG on cataract was achieved.

The lenticular opacification in control rats was also observed during the 6-10 weeks of study period. Based on the previous study that stress hormone or glucocorticoids can also induce cataract (James, 2007; Watanabe *et al.*, 2000), we do suggest that the development of cataract in control group may possibly occur as the exposure to stress condition leading to the elevation of glucocorticoids hormone resulting in the cataractogenesis. Unfortunately, the level of corticosterone has not been determined in this study. Therefore, the precise understanding mechanism of this change is required further investigation. In addition, spontaneous age-related cataract can also occur in laboratory rodents. The prevalence is varied depending on species. Even young adolescent rodents can be found cataract approximately 20% (Greaves, 2007). Therefore, the cataract observed in normal rats might occur either via stress or via spontaneous cataractogenesis of the laboratory rodents mentioned earlier.

5. Conclusion

The present results suggest that PWC and PWCG is the potential beneficial food to protect against diabetic cataract and diabetic retinopathy. The possible underlying mechanism may occur partly via the decreased oxidative stress and the suppression of aldose reductase activity in lens. The possible active ingredient may be associated with flavonoids such as anthocyanin and other flavonoids. Further researches are still essential to understand the precise underlying mechanism and possible active ingredient.