CHAPTER IV
THE EFFECTS OF NOVEL HERBAL RECIPE (PWCG) ON DIABETIC NEUROPATHY

1. Introduction

Diabetic neuropathy (DN), a common and costly complication of diabetic mellitus (Gordois *et al.*, 2003), affects more than 50% of diabetic patients (Maser *et al.*, 1989). It has been reported that chronic hyperglycemia can destroy sensory, motor, and autonomic fibers especially at distal extremities (Brown and Asbury, 1984). The manifestation of DN is varied depending on the affected nerve ranging from sensory deficit, weakness and autonomic dysfunction. DN is regarded as a leading cause of non-traumatic amputation (Thomson and Jacques, 2009) due to the loss of protective limb mechanical sensations. In addition, approximate 11% of DN cases are associated with chronic painful condition which markedly decreases the quality of life of diabetic patients (Argoff *et al.*, 2006). Since diabetic condition is dramatically increased worldwide, the importance of DN is also increasing its importance. However, the current therapeutic efficiency is still limited.

Recently, it has been demonstrated that hyperglycemia can induce nerve damage by many mechanisms such as the enhanced oxidative stress, the increase of advanced glycation end product formation (Obrosova *et al.*, 2002; van Dam, 2002; Vincent *et al.*, 2004), the accumulation of sorbitol due to the increased aldose reductase activity (Chung *et al.*, 2003) and nerve hypoxia/ischemia (Obrosova *et al.*, 2002; van Dam, 2002; Vincent *et al.*, 2004). However, accumulative lines of evidence have suggested that both the excess oxidative stress and the elevation of aldose reductase may possibly be the important cause for the development of DN. This raises the hypothesis that substances which can suppress oxidative stress and aldose reductase should improve DN.

Herbal medicine is long term used for treating numerous ailments. It has been believed that it is much safer than synthetic drugs. Therefore, herbal medicine is very popular and gains much attention nowadays. To decrease the high medical cost and expenditure, the dietary therapy by using herb-based food has gained much attention.

Recently, it has been reported that *Zingiber officinale* and *Zea mays* (purple color) could improve diabetes and diabetic conditions (Kang *et al.*, 2013). Therefore, the beneficial effect of the novel herbal recipe containing the combined extract of purple waxy corn and ginger (PWCG) might be able to improve DN in streptozotocin (STZ)-diabetic rats. Due to the lack of supported evidence, this study was undertaken to determine the effect of PWCG on DN in STZ-diabetic rats. In addition, the effect of PWCG on oxidative stress markers, aldose reductase activity and axonal change were also investigated.

2. Materials and Methods

2.1 Experimental design

Male Wistar rats, weighing 250-280 g, were used in this study (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 sham operated and administered citrate buffer, a vehicle of streptozotocin (STZ)

Group II DM+ CCI+vehicle of the extract or distilled water

Group III DM+CCI+GABApentine at dose of 50 $mg.kg^{-1}$ BW

Group IV DM+CCI+Ascorbic acid at dose of 100 $mg.kg^{-1}$ BW

Group V-VII DM+CCI+PWCG at doses of 100, 200 and 300 $mg.kg^{-1}$ BW, respectively

All rats in group II-VII were induced diabetes mellitus by a single injection of STZ which was dissolved in citrate buffer (pH 4.5) at dose of 55 $mg.kg^{-1}$ BW. All diabetic rats which showed the blood sugar level levels more than 250 $mg.dL^{-1}$ were selected for further study by inducing peripheral neuropathy with neuropathic pain by using the chronic constriction injury (CCI) of sciatic nerve (Bennett and Xie, 1988; Khongrum *et al.*, 2012). All rats were treated with the assigned interventions once daily after chronic constriction injury throughout the 21 day- study period. The behavioral test including hot plate test, von Frey filament and foot print analysis were evaluated every 3 days until the end of study. At the end of study, nerve conduction velocity was investigated and the lesion nerves were determined histomorphology, aldose reductase and oxidative stress markers including

malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes as shown in figure 4-1.

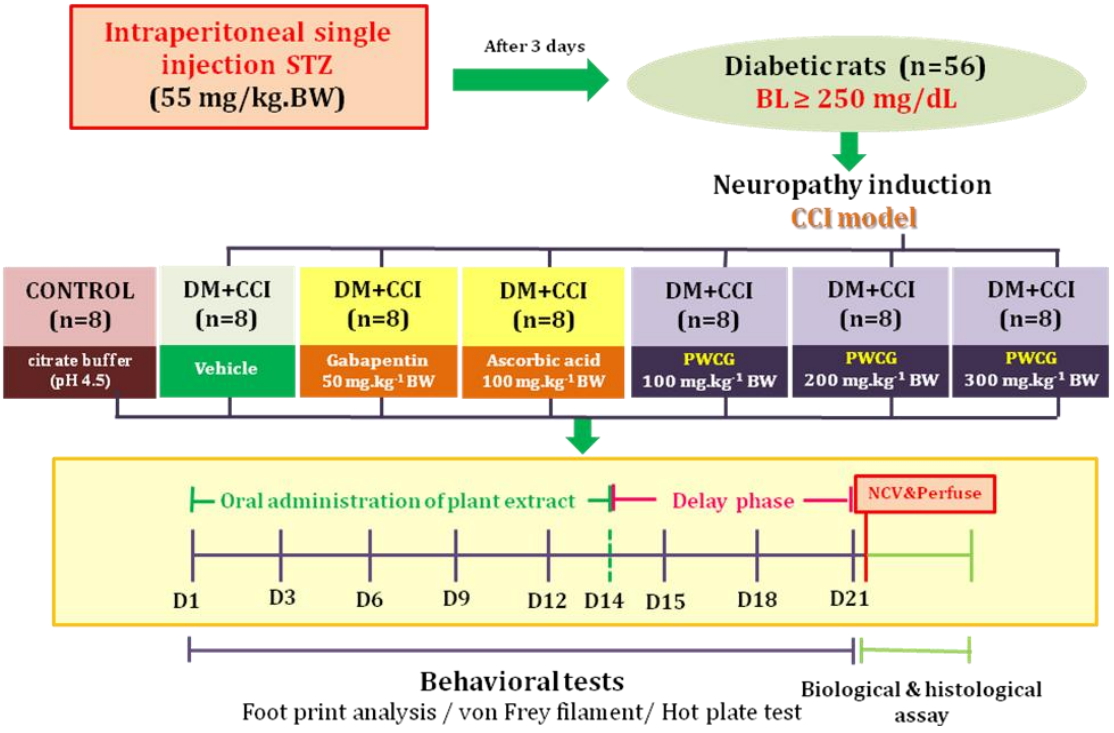

Figure 4-1 Schematic diagram showing experimental protocol for the determination of effect of a novel herbal recipe (PWCG) on diabetic neuropathy model

2.2 Induction of diabetic neuropathy by chronic constriction injury

Diabetic neuropathy was induced as previously described (Bennett and Xie, 1988; Khongrum *et al.*, 2012). After the anesthesia with ethyl ether, the right hind limb was immobilized in extended and slightly elevated position. The Biceps femoris muscle was exposed and the femur bone was used as a landmark for direction of incision. The silk thread number 4.0 was slipped under the sciatic nerve trifurcation and ligated. After the nerve ligation, the muscle and skin layers were sutured. Each animal was allowed to recover for 24 hours before further the investigation was carried out. In this experiment, the contralateral thighs of all treated groups were not subjected to the operation.

2.3 Determination of foot print analysis

Since walking track analysis is one of the commonly used tools to assess the function of innervated target organs after nerve injury, it was used as the evaluation tool in this study. In brief, the hind paws of the animals were dipped in the ink and they were allowed to walk along the wooden walking alley (8.2x42 cm) with one closed end contained the white paper at the floor. From the foot prints, the following parameters were obtained; print length (PL): distance from the heel to the third toe, toe spread (TS): distance from the first to the fifth toe and intermediate toe spread (ITS): distance from the second to the fourth toe. The mean distances of all measurements mentioned earlier were used to calculate the following factors (dynamic and static):

Toe spread factor (TSF) = (OTS – NTS) / NTS

Intermediate toe spread factor (ITSF) = (OITS – NITS) / NITS

Print length factor (PLF) = (OPL – NPL) / NPL

Then, these values were used to calculate the sciatic function index (SFI) by the following formula (Bain *et al.*, 1989):

SFI = (-38.3 x PLF) + (109.5 x TSF) + (13.3 ITSF) - 8.8

According to this calculation, the normal values of SFI were in the range between +11 and -11 and a value of -100 indicated the total impairment (de Medinaceli *et al.*, 1982).

2.4 Determination of sensory function in respond to mechanical stimuli by using von Frey filament test

A series of 10 von Frey filaments (0.1 Gr, 0.2 Gr, 0.4 Gr, 0.8 Gr, 1.0 Gr, 1.2 Gr, 1.5 Gr, 2.5 Gr, 3.6 Gr and 4.0 Gr) were used to evoke paw withdrawal response. Testing starts with the lowest filament of the series and each hind paw was stimulated 5 times. The intensity of withdrawal responses of each filament in a 3second- stimulation duration was recorded (Hsu *et al.*, 2014).

2.5 Determination of sensory function in respond to temperature stimuli by using hot plate test

The rats were gently dropped into a plastic box with a metal floor that was preheated to a certain temperature (hot plate) (Liu *et al.*, 2010). The time duration between the initiation of the hot plate exposure and the time which the paw was raised from the floor was recorded and considered as paw withdrawal latency. Each animal

was measured 5 withdrawal latencies per session, with time interval 5 min between tests. The average paw withdrawal latency was calculated and used as index. Minimal and maximal cut offs were assigned at 0.5 to 20 seconds, respectively.

2.6 Determination of sciatic nerve conduction by nerve conduction velocity (NCV)

In this experiment, the sciatic nerve conduction velocity was performed by modifying the measurement of conduction velocity in human. In brief, the sciatic nerve of the animal was stimulated and the response of gastrocnemius which was supplied by this nerve was recorded as nerve activity (van Dam *et al.*, 1999). Each rat was anesthetized with thiopental sodium via intraperitoneal injection. The right sciatic nerve of each rat was exposed and gently dissected away from the surrounding muscle tissue and the electrodes were placed at two different points. The stimulating electrode was placed at proximal site of the lesion whereas the recording electrode was place at distal site of the lesion. The sciatic nerve was stimulated using electrical stimuli at an interval of 0.5 ms, amplitudes between 50-2000 mV. The response latency was recorded. Then the location of recoding electrode was moving from the original site. The change of muscle response latency was then recorded. The nerve conduction velocity was calculated by dividing the distance obtained from the displacement of recording electrode with the change of muscle response latency which occurred as the result of the displacement of recording electrode.

2.7 Fasting blood glucose level

Blood samples were taken from rat tails after overnight fasting. The fasting blood glucose level was monitored at the end of experiment by using ACCU-CHEK active**.**

2.8 Homogenate preparation

At the end of experiment, homogenate of right sciatic nerve was prepared in 1 ml of 0.1 M phosphate buffer, pH 7.4. The obtained nerve homogenate was adjusted to 10% w/v and centrifuged at 10,000 g, 4°C for 1 hour. The supernatant was harvested and processed for the estimation of biochemical parameters.

2.9 Determination of malondialdehyde (MDA)

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 of chilled water and 2500 µl of butanol and pyridine [15:1 v/v] were added into each tube andmixed thoroughly with vortex. Then, the solution was centrifuged at 800 x g for 20 minuts. 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.

2.10 Determination of 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) was prepared at 25ºC. The optical density was determined at 415 nm. A system devoid of enzyme was served as the control and three parallel experiments were conducted (Sun *et al.*, 1988). SOD activity was expressed as U/mg.protein.

2.11 Determination of catalase (CAT) assay

Nerve catalase activity was assessed 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 which contained 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 25º is regarded as one unit (Goth, 1991). The value of CAT activity was expressed as U/mg.protein.

2.12 Determination of 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 25º C 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.

2.13 Determination of aldose reductase (AR) activity

Aldose reductase activity was evaluated using colorimetric 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 nerve supernatant was 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).

2.14 Histomorphology study

After an anesthesia with sodium pentobarbital (60 mg/kg BW), the animals were perfused with 0.9 % normal saline. Sciatic nerve was collected and immersed sequentially for 24 h each in 10 % formaline. The frozen sample was immersed in a stainless steel container which was suitable for the tissue size and filled with Optimal Cutting Temperature (O.C.T.) compound. The specimens were frozen rapidly and 5 µm thick sections were made using cryostat.

2.15 Determination of axonal density

The nerve sample was stained with toluidine blue and determined the axon density in the lesion sciatic nerve. In brief, the sections were stained with 0.1% toluidine blue for 10-30 s, rinsed with running water for about 5 min, and mounted with glycerin under a cover glass. Histological analysis was performed by light microscope and the density of axons was accessed by using Image Pro-plus 5.1 program.

2.16 Statistical Analysis

All parameters were compared using one-way analysis of variance (ANOVA). The post hoc test was used to identify specific mean differences. They were represented as mean ± standard error mean (mean ± S.E.M). Statistical analysis was carried out using SPSS version 15. Differences were considered significant at p-value <0.05.

3. Results

3.1 Effect of a novel herbal recipe (PWCG) on fasting blood sugar level

The average fasting blood glucose levels of all the experimental groups were shown in figure 4-2. It was found that the blood sugar levels of all diabetic rats were more than 250 $mg.dL^{-1}$ throughout the study period. PWCG failed to produce a significant reduction of the fasting blood glucose level in all treated groups.

3.2 Effect of a novel herbal recipe (PWCG) on function recovery in peripheral neuropathy in diabetic rats

Table 4-1 showed that before CCI (baseline data), no significant changes among groups were observed. In addition, all groups still showed normal SFI. Diabetic rats plus CCI which received vehicle showed the significant reduction of SFI (p-value<0.001 all, compared to control group) and showed the impaired SFI throughout the study period. Diabetic rats which subjected to CCI and received GABApentine failed to produce the significant change of SFI throughout the study period. Ascorbic acid significantly improved SFI of diabetic rats which subjected to CCI at 9 and 12 days of treatment (p-value<0.05 and 0.01 respectively; compared with diabetic rats plus CCI which received vehicle). PWCG at doses of 100 and 200 $mg.kg^{-1}$ BW significantly improved SFI of diabetic rats which subjected to CCI at 6 (p-value<0.01 all; compared with diabetic rats plus CCI which received vehicle), 9

(p-value<0.05 and 0.01 respectively; compared with diabetic rats plus CCI which received vehicle) and 12 days of treatment (p-value<0.01 and 0.05 respectively; compared with diabetic rats plus CCI which received vehicle). It was found that diabetic rats plus CCI which were orally given PWCG at dose of 300 $mg.kg^{-1}$ BW produced significant improvement in SFI since day 3 to day 12 of treatment (p-value<0.05,0.05,0.001 and 0.05 respectively; compared with diabetic rats plus CCI which received vehicle). No significant improvement of SFI was observed in any treatment groups at the other treatment duration.

The effect of PWCG on sensory function of sciatic nerve was also evaluated by both von Frey filament test and hot plate test and data were shown in table 4-2 and table 4-3. According to the data shown in table 4-2, it was found that no significant differences among groups were observed at baseline data. Diabetic rats which received CCI and were orally given vehicle showed the significant reduction in paw withdrawal threshold which evaluated via von Frey filament test every 3 days throughout a 21day-experimental period (p-value<0.01, 0.05, 0.01, 0.01, 0.05, 0.05 and 0.05, respectively; compared to control group). GABApentine significantly mitigated the decreased paw withdrawal threshold induced by CCI in diabetic rats since day 3 to day 15 of treatment (p-value<0.01, 0.05, 0.05, 0.01 and 0.05 respectively; compared to diabetic rats plus CCI which received vehicle). Diabetic rats plus CCI which received ascorbic acid showed the significant increase in paw withdrawal threshold in respond to mechanical stimuli in diabetic rats plus CCI only at 12 days of treatment (p-value<0.05; compared to diabetic rats plus CCI which received vehicle). Interestingly, PWCG at dose of 100 $mg.kg^{-1}$ BW significantly mitigated the decrease of paw withdrawal threshold in respond to mechanical stimuli induced by CCI in diabetic rats at 9 and 12 days of treatment (p-value<0.05 all; compared to diabetic rats plus CCI which received vehicle) whereas PWCG at doses of 200 and 300 $mg.kg^{-1}$ BW produced the significant mitigation effect on the reduction of paw withdrawal threshold in respond to mechanical stimuli induced by CCI in diabetic rats since between day 9 and day 15 of treatment (p-value<0.05 all; compared to diabetic rats plus CCI which received vehicle).

Table 4-3 showed that the diabetic rats subjected to CCI and received vehicle also decreased paw withdrawal latency in respond to temperature stimuli evaluated by hot plate test (p-value<0.001 all; compared to control group). Both diabetic rats plus CCI which received GABApentine and diabetic rats plus CCI which received ascorbic acid significantly mitigated the decreased paw withdrawal latency in respond to temperature stimuli since day 3 to day 15 of treatments (p-value<0.01 and 0.05; 0.001 and 0.01; 0.01 all; 0.001 and 0.01; 0.05 all respectively; compared to diabetic rats plus CCI which received vehicle). Low dose of PWCG failed to modulate paw withdrawal latency throughout the study period. However, diabetic rats plus CCI which received medium dose of PWCG significantly mitigated the increased paw withdrawal latency since day 6 to day 12 of treatment (p-value<0.05 all; compared to diabetic rats plus CCI which received vehicle) while those which received high dose of PWCG showed the significant improvement of paw withdrawal latency between day 3 to day 12 of treatment (p-value<0.05 all; compared to diabetic rats plus CCI which received vehicle).

3.3 Effect of a novel herbal recipe (PWCG) on nerve conduction velocity

The effect of PWCG on nerve conduction velocity (NCV) was also evaluated. It was found that diabetic rats which subjected to CCI significantly decreased sciatic nerve conduction velocity as shown in figure 4-3. Diabetic rats which exposed to CCI and received GABApentine failed to show the significant increase in NCV of sciatic nerve. Interestingly, diabetic rats plus CCI which received either ascorbic acid treatment or PWCG at the range used in this study significantly improved sciatic nerve conduction velocity (p-value<0.05, 0.05, 0.01 and 0.01, respectively; compared with diabetic rats plus CCI which received vehicle).

3.4 Effect of a novel herbal recipe (PWCG) on oxidative stress markers and aldose reductase activity

Based on the previous findings that oxidative stress plays a crucial role on diabetic neuropathy, we also investigated the effect of PWCG on oxidative stress markers including the level of MDA and the activities of SOD, CAT and GPx in the lesion nerve and results were shown in figure 4-4-figure 4-7. It was found that diabetic rats plus CCI which received vehicle produced the significant reduction of

MDA level in the lesion sciatic nerve (p-value<0.001; compared to control group). However, the activities of SOD, CAT and GPx in diabetic rats plus CCI which received vehicle were markedly reduced (p-value<0.001 all; compared to control group). Diabetic rats plus CCI which received GABApentine significantly decreased MDA level but increased CAT activity in the lesion sciatic nerve (p-value<.05 and .01 respectively; compared to diabetic rats plus CCI which received vehicle). Ascorbic acid treatment could decrease MDA level but decreased SOD, CAT and GPx activities in the lesion sciatic nerve of diabetic rats plus CCI (p-value<0.001, 0.01, 0.01, and 0.001 respectively; compared to diabetic rats plus CCI which received vehicle). PWCG treatment at dose of 100 $mg.kg^{-1}$ BW significantly decreased MDA level but increased GPx activity in the lesion sciatic nerve. Diabetic rats plus CCI which received PWCG at doses of 200 and 300 $mg.kg^{-1}$ BW produced significant changes of the reduction of MDA level and the increase of SOD activity in the lesion sciatic nerve (p-value<0.001 all and 0.05 all respectively; compared to diabetic rats plus CCI which received vehicle). In addition, diabetic rats plus CCI which received the medium dose of PWCG also decreased GPx activity in the lesion sciatic nerve (p-value<0.01; compared to diabetic rats plus CCI which received vehicle).

The effect of PWCG on aldose reductase, a rate limiting enzyme in polyol pathway, was also investigated and data were shown in figure 4-8. Diabetic rats plus CCI which received vehicle markedly increased aldose reductase activity in the lesion sciatic nerve (p-value<0.001 compared to control group). The only significant reduction of aldose reductase activity in the lesion sciatic nerve was observed only in ascorbic acid treatment group (p-value<0.01; compared to diabetic rats plus CCI which received vehicle). No other groups showed the significant change of this parameter.

3.5 Effect of a novel herbal recipe (PWCG) on density of axon

The effect of PWCG on density of myelinated axon in sciatic nerve evaluated by toluidine blue stain was investigated and results were shown in figure 4-9. Diabetic rats plus CCI which treated with vehicle significantly decreased density of myelinated axon (p-value<0.001; compared to control group). Interestingly, ascorbic acid and all doses of PWCG used in this study mitigated the reduction of density of myelinated axon (p-value<0.001 all; compared to diabetic rats plus CCI which received vehicle).

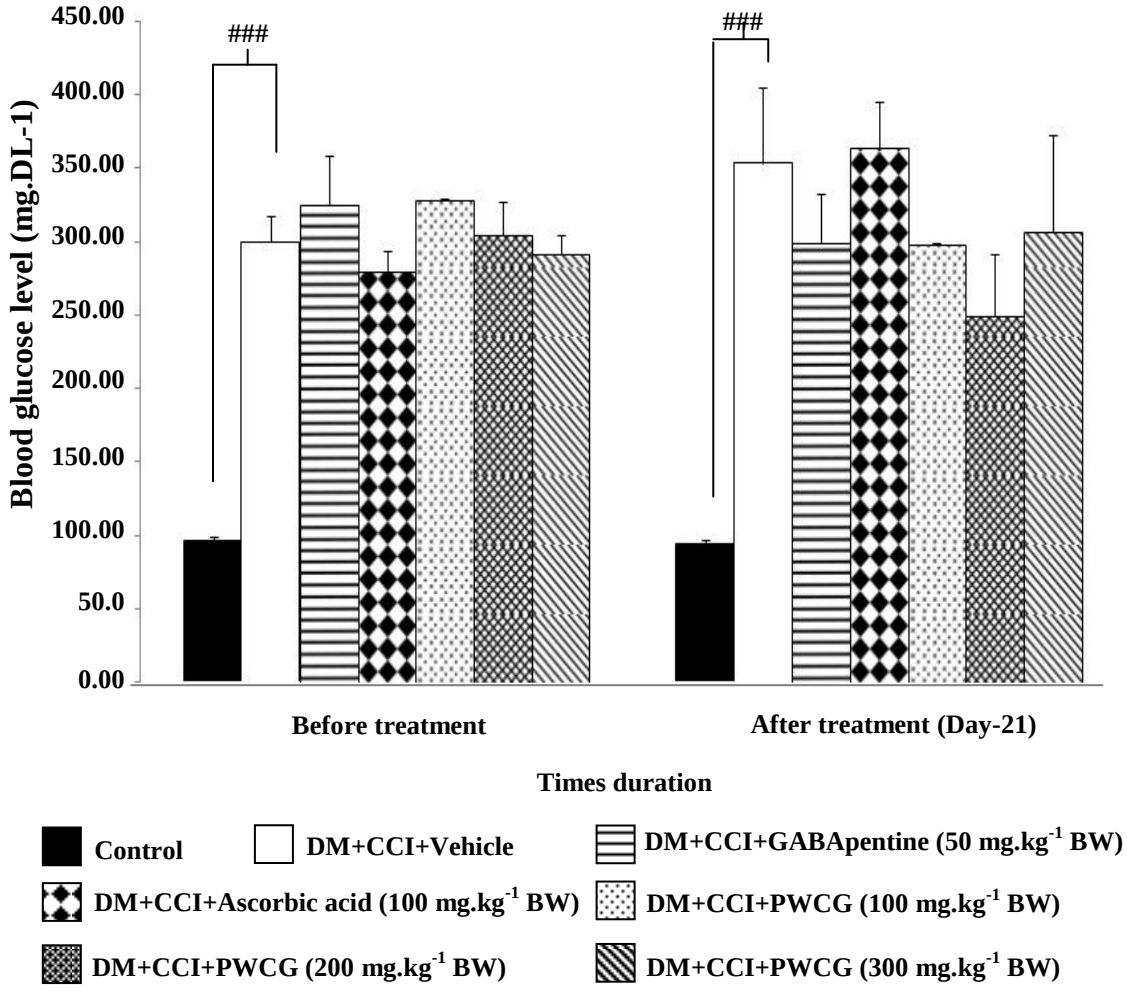

Figure 4-2 The effect of PWCG on fasting blood glucose levels (n=8/group). ### p-value <0.001; compared to control group

Table 4-1 Effect of PWCG on the sciatic function index evaluated by walking track analysis (n=8/group) $^{###}$p-value<0.001; compared between diabetic rats plus CCI which received vehicle with control rats *,**,***p-value<0.05,0.01 and 0.001 respectively; compared between diabetic rats plus CCI which received various treatments with diabetic rats plus CCI which received vehicle with control rats

Time	Sciatic function index (SFI)						
	Control	**DM+CCI+ vehicle**	**DM+CCI+GABApentine (50 mg.kg^{-1} BW)**	**DM+CCI+Ascorbic acid (100 mg.kg^{-1} BW)**	**DM+CCI+PWCG (100 mg.kg^{-1} BW)**	**DM+CCI+PWCG (200 mg.kg^{-1} BW)**	**DM+CCI+PWCG (300 mg.$^{kg-1}$ BW)**
Baseline	-9.29±3.51	-10.49±3.80	-7.40±4.56	-8.25±1.82	-8.65±2.95	-10.56±3.89	-7.59±6.86
Day-1	-7.98±1.35	-91.72±1.58$^{###}$	-84.36±7.12	-87.93±2.04	-85.03±2.45	-90.76±1.69	-88.86±2.21
Day-3	-7.86±2.00	-91.94±1.36$^{###}$	-90.59±1.75	-86.28±1.93	-89.02±2.09	-90.45±1.46	-80.81±3.79*
Day-6	-7.74±1.73	-91.82±1.48$^{###}$	-89.86±2.28	-86.28±1.93	-83.69±1.82**	-83.56±1.54**	-84.93±2.31*
Day-9	-9.98±1.89	-91.53±1.77$^{###}$	-91.72±1.58	-86.73±1.79*	-86.54±2.22*	-84.41±0.62**	-83.54±1.71***
Day-12	-9.78±2.40	-92.19±1.11$^{###}$	-90.24±2.05	-84.32±1.79**	-84.01±2.17**	-87.14±1.50*	-86.38±1.94*
Day-15	-6.75±2.54	-91.53±1.77$^{###}$	-91.89±1.41	-85.50±1.57	-89.65±5.21	-90.56±4.40	-85.52±1.76
Day-18	-7.19±1.37	-91.69±1.61$^{###}$	-90.88±2.42	-82.42±9.04	-93.91±5.52	-84.20±1.52	-88.24±2.00
Day-21	-7.48±1.75	-91.53±1.77$^{###}$	-89.15±2.74	-81.45±10.32	-89.85±3.63	-83.03±2.37	-87.57±1.88

Table 4-2 Effect of PWCG on the paw withdrawal threshold intensity in respond to mechanical stimuli evaluated by von Frey filament test (n=8/group) [#,##]p-value<0.05 and .01 respectively; compared between diabetic rats plus CCI which received vehicle with control rats [*,**]p-value<0.05and.01respectively; compared between diabetic rats plus CCI which received various treatments with diabetic rats plus CCI which received vehicle with control rats

Time	Paw withdrawal threshold (g)						
	Control	**DM+CCI+ vehicle**	**DM+CCI+GABApentine (50 mg.kg-1 BW)**	**DM+CCI+Ascorbic acid (100 mg.kg-1 BW)**	**DM+CCI+PWCG (100 mg.kg-1 BW)**	**DM+CCI+PWCG (200mg.kg-1 BW)**	**DM+CCI+PWCG (300 mg.kg-1 BW)**
Baseline	3.92±0.08	3.92±0.08	3.92±0.08	3.92±0.08	3.950.05	3.95±0.05	3.90±0.02
Day-3	3.92±0.08	3.02±0.32[##]	3.84±0.10**	3.70±0.30	3.61±0.17	3.58±0.24	3.66±0.08*
Day-6	3.84±0.10	2.70±0.56[#]	3.84±0.10*	3.70±0.30	3.54±0.30	3.31±0.3	3.40±0.13
Day-9	4.00±0.00	2.80±0.56[##]	3.84±0.10*	3.50±0.50	3.66±0.18*	3.71±0.18*	3.71±0.11*
Day-12	3.92±0.08	2.90±0.48[##]	3.84±0.10**	3.70±0.30*	3.61±0.17*	3.71±0.18*	3.71±0.09*
Day-15	3.92±0.08	3.10±0.37[#]	3.92±0.08*	3.70±0.30	3.66±0.22	3.76±0.23*	3.71±0.10*
Day-18	4.00±0.00	3.12±0.49[#]	3.92±0.08	3.70±0.30	3.48±0.22	3.53±0.23	3.53±0.10
Day-21	4.00±0.00	3.24±0.31[#]	3.84±0.10	3.70±0.30	3.54±0.30	3.61±0.17	3.71±0.18

Table 4-3 Effect of PWCG on the paw withdrawal threshold latency in respond to temperature stimuli evaluated by hot plate test (n=8/group) $^{###}$p-value<0.001; compared between diabetic rats plus CCI which received vehicle with control rats *,**,*** p-value<0.05, 0.01and.001respectively; compared between diabetic rats plus CCI which received various treatments with diabetic rats plus CCI which received vehicle with control rats

Time	Paw withdrawal latency (sec)						
	Control	**DM+CCI+ vehicle**	**DM+CCI+GABApentine (50 mg.kg^{-1} BW)**	**DM+CCI+Ascorbic acid (100 mg.kg^{-1} BW)**	**DM+CCI+PWCG (100 mg.kg^{-1} BW)**	**DM+CCI+PWCG (200 mg.kg^{-1} BW)**	**DM+CCI+PWCG (300 mg.$^{kg-1}$ BW)**
Baseline	4.47±0.33	4.53±0.53	4.60±0.38	4.40±0.32	4.54±0.41	4.25±0.26	4.21±0.36
Day-3	4.20±0.25	1.40±0.16$^{###}$	2.87±0.17**	2.40±0.07*	2.00±0.15	2.17±0.14	2.50±0.51*
Day-6	4.86±0.29	1.60±0.13$^{###}$	2.70±0.32***	2.47±0.23**	1.87±0.18	2.21±0.14*	2.17±0.09*
Day-9	4.20±0.23	1.70±0.13$^{###}$	2.93±0.07**	2.73±0.30**	2.33±0.34	2.42±0.14*	2.46±0.20*
Day-12	4.07±0.40	1.33±0.18$^{###}$	2.66±0.21***	2.40±0.29**	1.90±0.16	2.00±0.22*	2.04±0.10*
Day-15	3.53±0.45	1.53±0.20$^{###}$	2.33±0.15*	2.27±0.13*	1.46±0.13	1.67±0.14	1.92±0.10
Day-18	4.33±0.40	1.40±0.13$^{###}$	1.93±0.12	1.90±0.14	1.33±0.13	1.58±0.12	1.67±0.13
Day-21	4.40±0.45	1.60±0.13$^{###}$	2.27±0.19	2.09±0.21	1.38±0.17	1.46±0.15	1.50±0.15

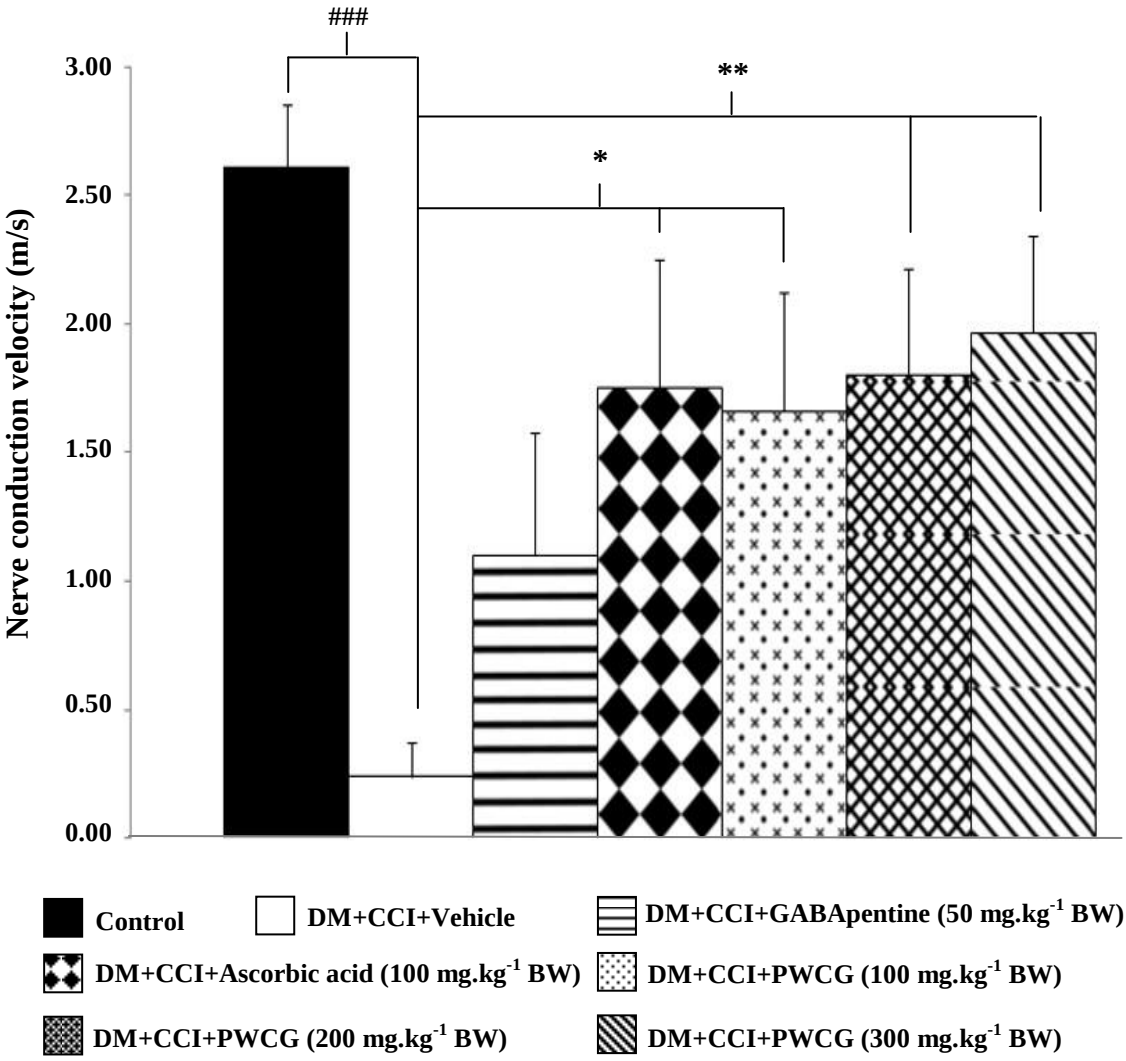

Figure 4-3 Effect of PWCG on sciatic nerve conduction velocity (n=8/group) ### p-value<0.001; compared to control group. *,** p-value<0.05 and 0.01 respectively; compared to diabetic rats which subjected to CCI and received vehicle

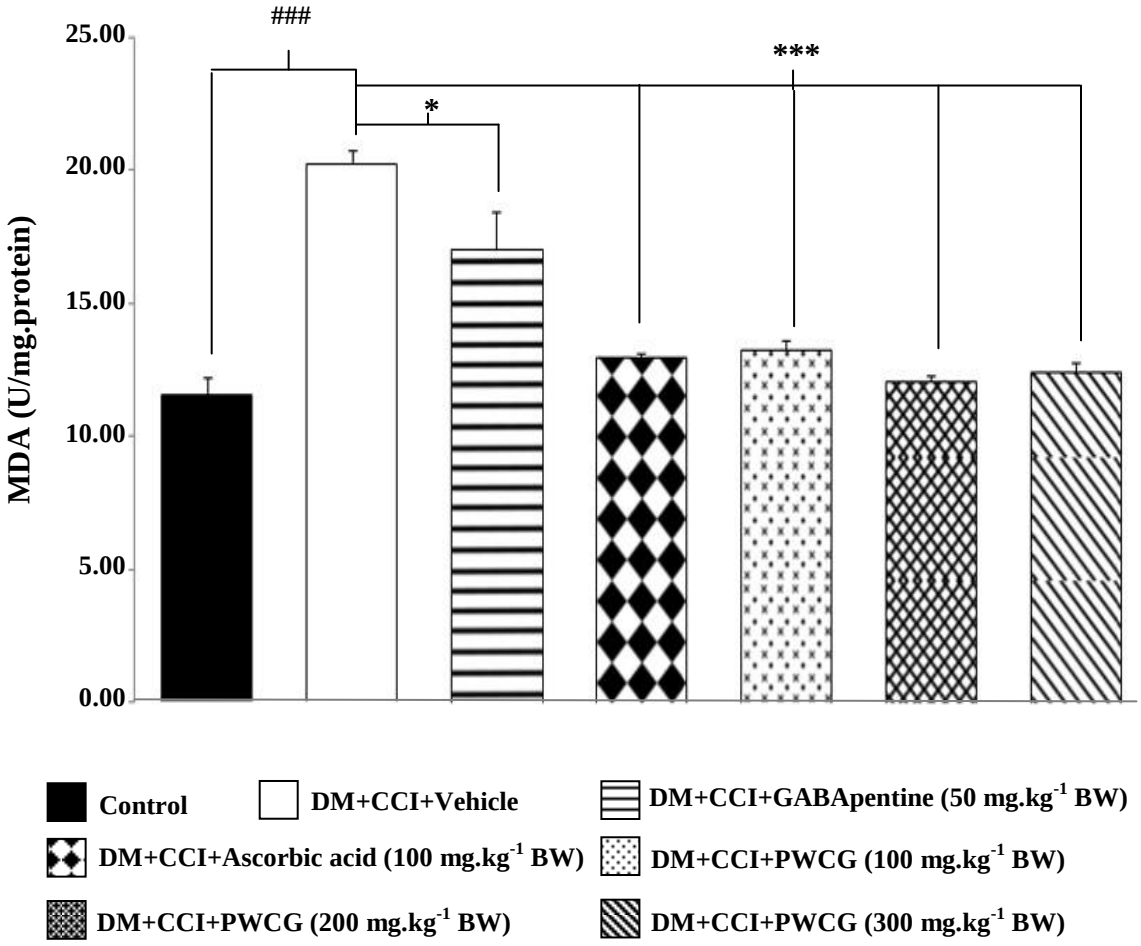

Figure 4-4 Effect of PWCG on malondialdehyde (MDA) level in the lesion sciatic nerve (n=8/group) $^{###}$p-value<0.001; compared to control group. *,*** p-value<0.05 and 0.001 respectively; compared to diabetic rats which subjected to CCI and received vehicle

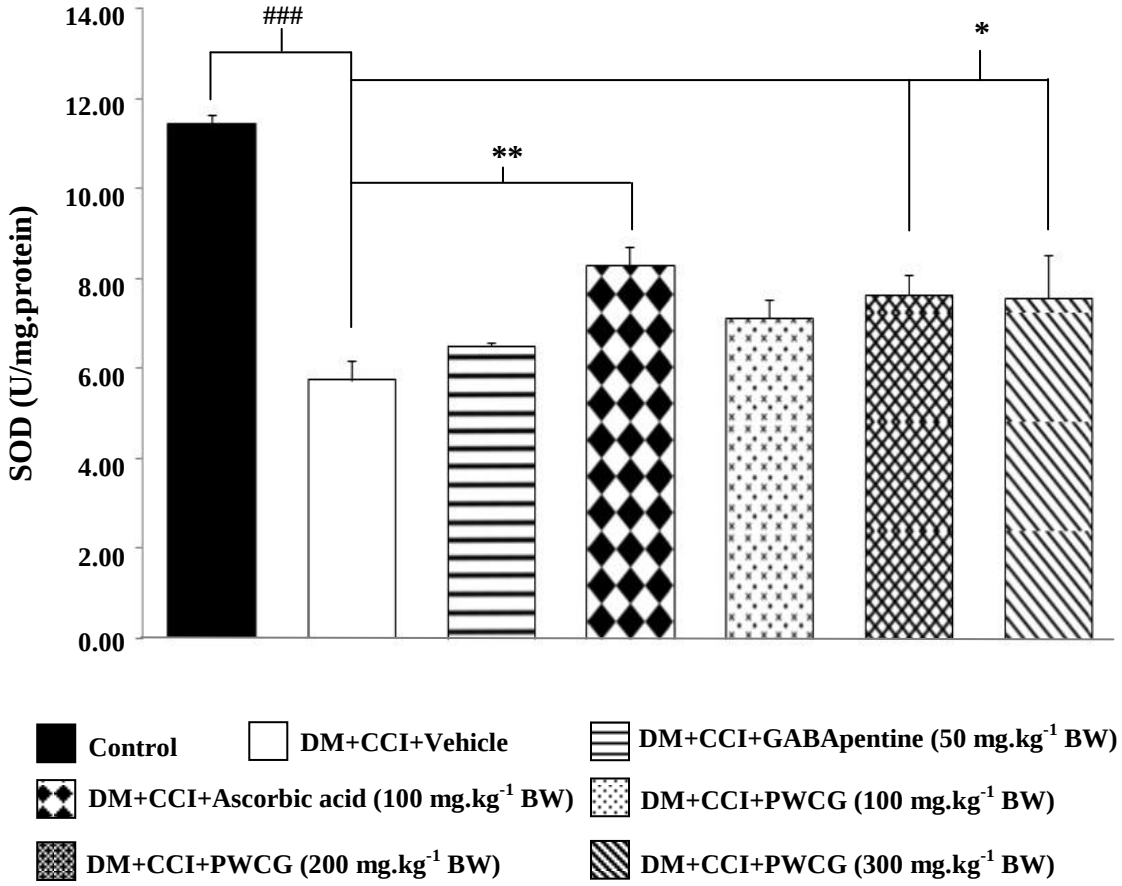

Figure 4-5 Effect of PWCG on superoxide dismutase (SOD) activity in the lesion sciatic nerve (n=8/group) $^{###}$p-value<0.001; compared to control group. *,**p-value<0.05 and 0.01 respectively; compared to diabetic rats which subjected to CCI and received vehicle

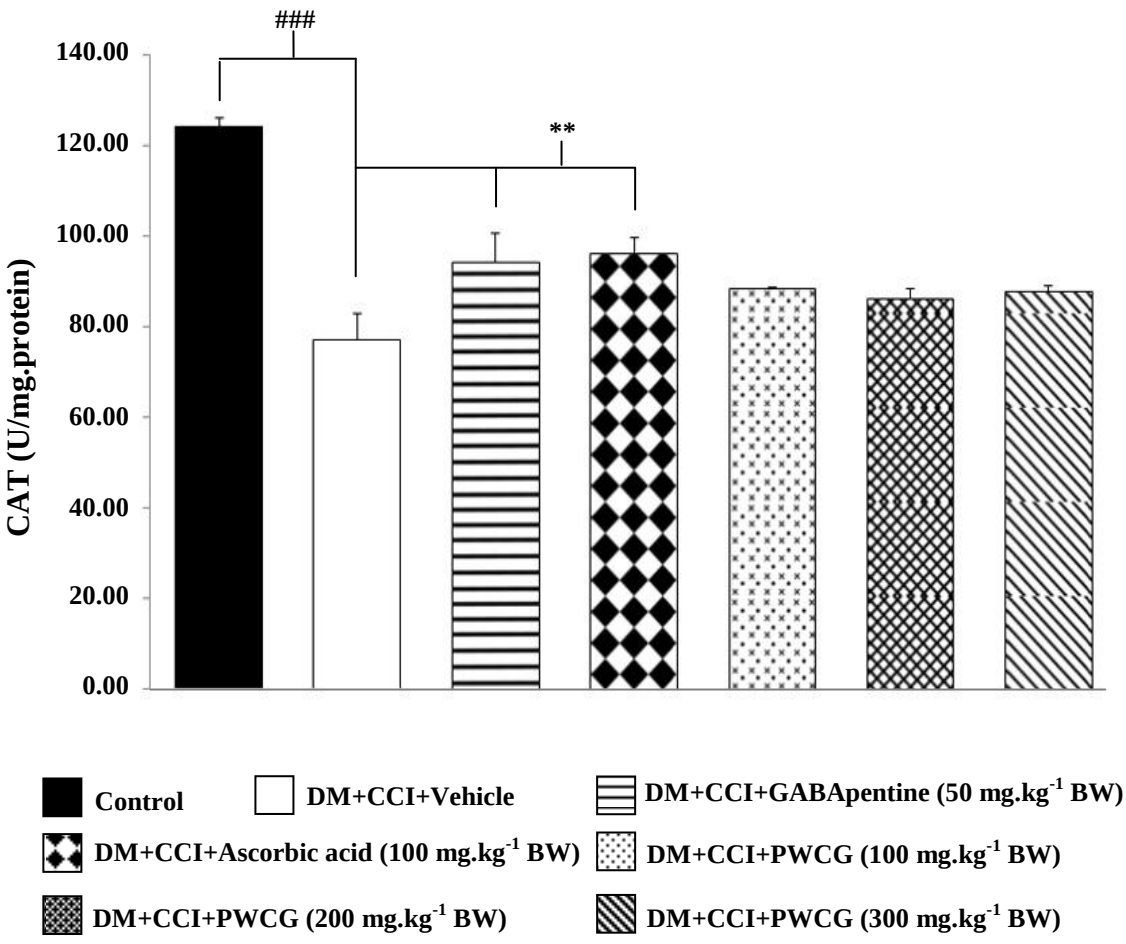

Figure 4-6 Effect of PWCG on catalase (CAT) activity in the lesion sciatic nerve (n=8/group) $^{###}$p-value<0.001; compared to control group. **p-value<0.01; compared to diabetic rats which subjected to CCI and received vehicle

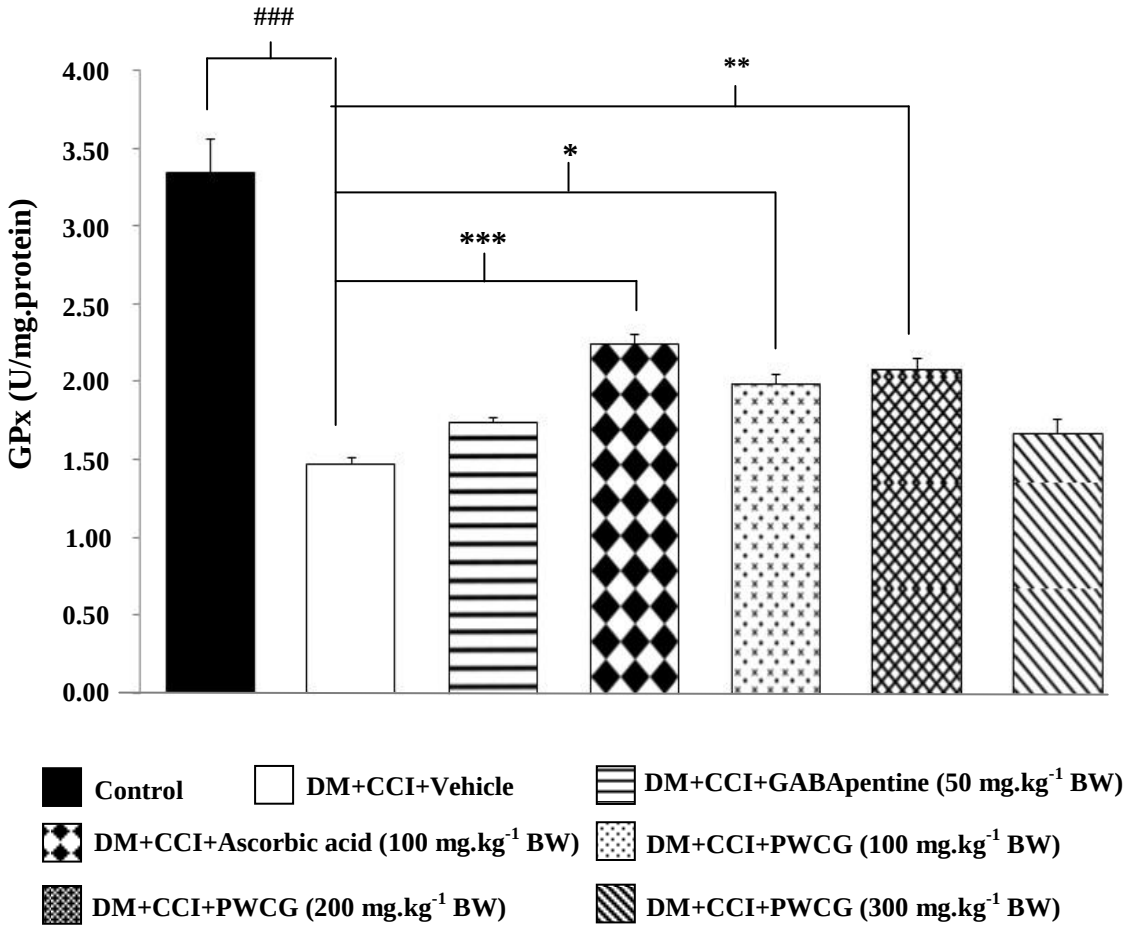

Figure 4-7 Effect of PWCG on glutathione peroxidase (GPx) activity in the lesion sciatic nerve (n=8/group) $^{###}$p-value<0.001; compared to control group. *,**,***p-value<0.05, 0.01 and 0.001 respectively ; compared to diabetic rats which subjected to CCI and received vehicle

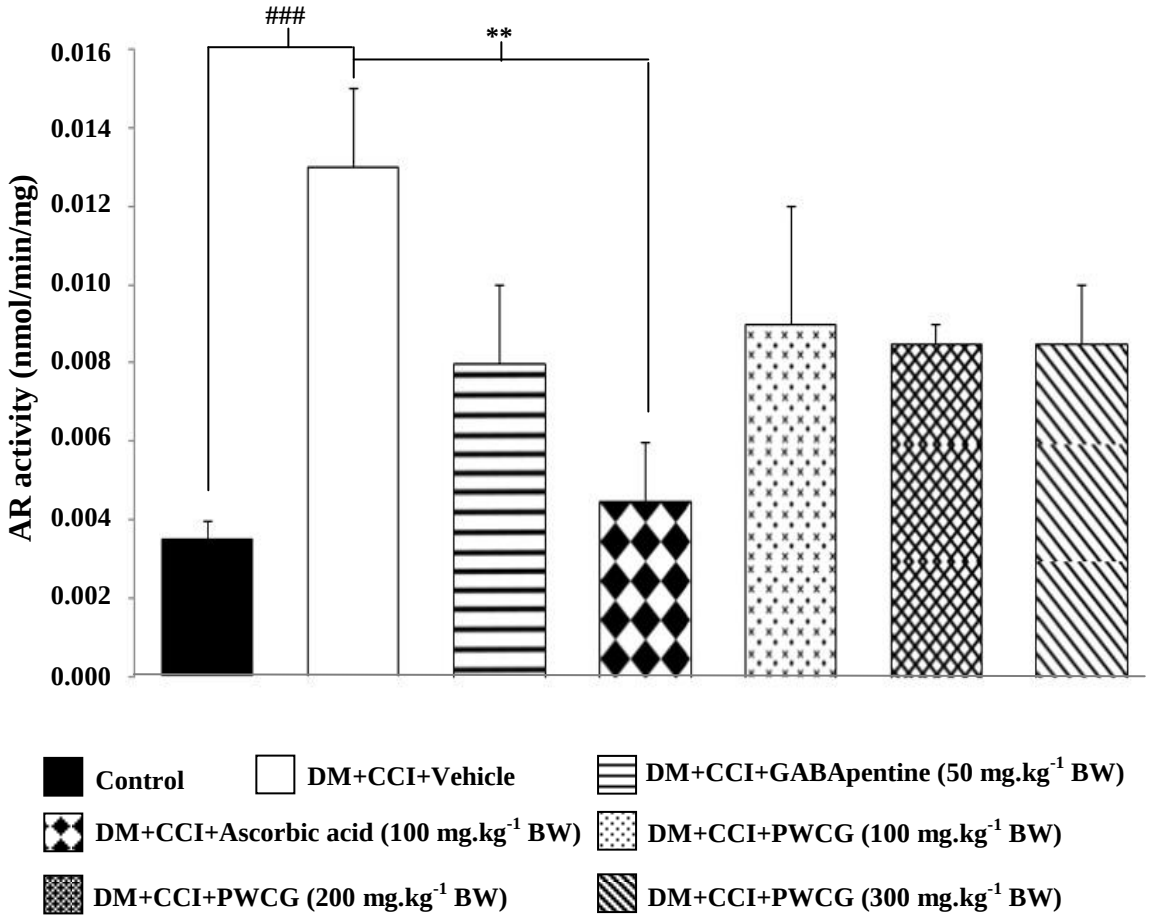

Figure 4-8 Effect of PWCG on aldose reductase (AR) activity in the lesion sciatic nerve (n=8/group) $^{###}$p-value<0.001; compared to control group. **p-value<0.01; compared to diabetic rats which subjected to CCI and received vehicle

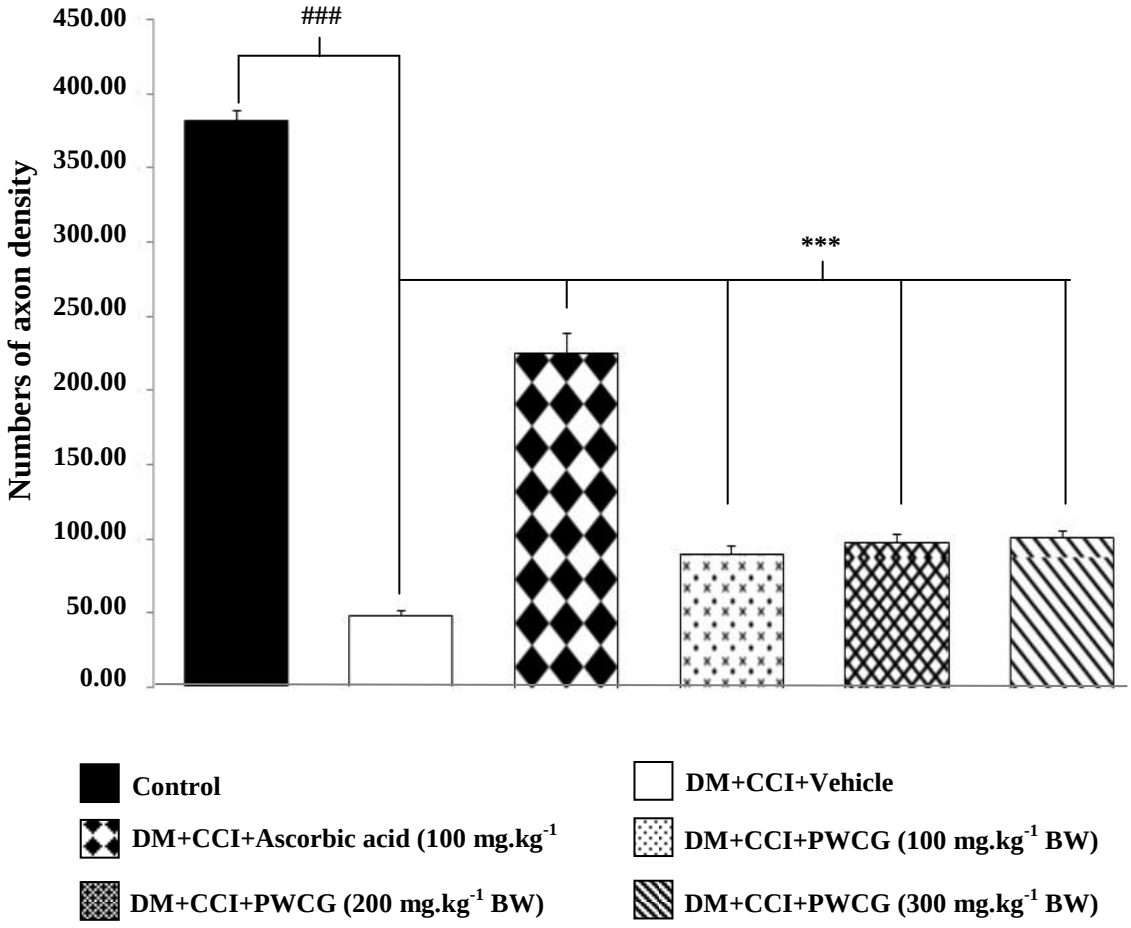

Figure 4-9 Effect of PWCG on the density of myelinated fibers evaluated by using toluidine blue stain (n=8/group) $^{###}$p-value<0.001; compared to control group. ***p-value<0.001; compared to diabetic rats which subjected to CCI and received vehicle

4. Discussion

The current data has demonstrated that PWCG successfully improves the myelinated nerve fiber loss and nerve dysfunction in neuropathy with neuropathic pain in diabetic condition. PWCG also improves oxidative stress status in the lesion nerve while no suppression of aldose reductase activity is observed. In addition, no change of blood sugar was observed in PWCG treated rats.

It has been reported that the pathophysiology of diabetic neuropathy involves the interplay of hyperglycemia, ischemia, and oxidative stress. The increase ischemic

condition and oxidative stress status can exacerbate morphological changes of peripheral nerve in diabetic rat (Wang *et al.*, 2004) and reduce the time duration to induce the damage of peripheral nerve and the clinical manifestation development. Therefore, the ischemic condition of nerve by using CCI in STZ-diabetic rats was performed (Khongrum *et al.*, 2012). Based on the previous finding, it has been reported that oxidative stress and the accumulation of sorbitol via the enhanced polyol pathway are responsible for the development of peripheral diabetic neuropathy (Dobretsov *et al.*, 2007; Sundkvist *et al.*, 2000). In this study, CCI can develop the impairment of motor function especially walking pattern together with the hyperalgesia conditions which are in agreement with the clinical manifestation observed in patients with diabetic neuropathy (Courtemanche *et al.*, 1996; Gooch and Podwall, 2004).

Oxidative stress and the accumulation of sorbitol via the enhanced polyol pathway are reported to be responsible for the development of peripheral diabetic neuropathy (Dobretsov *et al.*, 2007; Sundkvist *et al.*, 2000). Both oxidative stress and sorbitol can damage the myelin sheath leading to the myelin degradation and the decreased NCV. In addition, both factors can decrease the myelinated nerve fiber density. It has been well known that myelinated fibers play the important role on the transmission of proprioception information which in turn plays the crucial role on postural stability. The abnormal gait which reflects the postural instability observed in this study may be due to the loss of myelinated fiber resulting in the decreased myelinated nerve density which in turn disturbs gait stability as shown by the impairment of SFI. Although hyperglycemia has been previously reported to be the important cause of nerve dysfunction, our data show that PWCG improve nerve dysfunction in diabetic condition without the change of hyperglycemic. Our finding is in agreement with previous study which showed that the nerve dysfunction is associated with the decreased myelinated nerve fiber density (Eriksson *et al.*, 1994; Sundkvist *et al.*, 2000).

Accumulative lines of evidence also demonstrate that oxidative stress plays an important role on neuropathic pain (Kim *et al.*, 2004; Naik *et al.*, 2006; Tal, 1996; Wang *et al.*, 2004). In addition, neuropathic pain can be attenuated by substances possessing antioxidant activity (Eriksson *et al.*, 1994; Tal, 1996). Since the data

obtained from our study showed that PWCG significantly increased SOD and GPx activities which in turn decreased the oxidative stress reflecting by the decreased MDA level, therefore, we suggested that the anti-hyperalgesic effect of PWCG might occur partly via the decreased oxidative stress which in turn decreased the damage of peripheral nerve fiber resulting in the decreased pain fiber stimulation from potassium ion and prostaglandin which had been released from nerve injury. In addition to oxidative stress, neuropathic pain is also under the influence of many factors including polyol pathway (Hosseini and Abdollahi, 2013). The increased polyol pathway activity can induce nerve damage directly via the accumulation of sorbitol, a toxic substance, or indirectly via the increased oxidative stress. However, our data failed to show the suppression of aldose reductase in the lesion nerve. Therefore, the improved hyperalgesia in diabetic rats plus CCI which received PWCG might not be associated with the suppression of aldose reductase.

The inhibition of γ-aminobutyric acid or GABA pathway also plays a crucial role on the modulating the response of peripheral nerve injury (Sivilotti and Woolf, 1994). It has been reported that the activity of GABA pathway is decreased in neuroapathic pain condition (Stiller *et al.*, 1996). Therefore, the improved GABA activity might contribute a role on the improved neuropathic pain. However, it has been reported that quercetin, the main flavonoids in PWCG was the GABA antagonist so it was less likely to produce antineuropathic pain via the increased GABA activity (Goutman and Calvo, 2004). Unfortunately, the determination of GABA activity had not been performed in this study. Therefore, this was the limitation of our study and required further study to confirm the exclusion of the modulation of GABA pathway by PWCG.

In this study, no dose dependent manner of PWCG was observed. The possible explanation might be due to the masking effect of the other ingredients and the interaction effect among the ingredients on the effect of active ingredient (s). In addition, PWCG might not exert the effect directly on the observed parameters but it might exert the modulation effect via other mediators such as the signal molecules.

Taken all data together, PWCG might increase SOD and GPx activities which in turn decreased oxidative stress status in the lesion nerve giving rise to the improved nerve damage leading to the increased myelinated nerve fiber density and the

improved SFI. In addition, the decreased nerve damage due to the decreased oxidative stress also decreased the stimulation of pain fiber leading to the improved paw withdrawal threshold intensity in respond to mechanical stimuli and the improved paw withdrawal latency in respond to thermal stimuli. However, other mechanisms such as the involvement of GABA pathway may also play a role on the improvement of hyperalgesia and this requires further investigation. In addition, the improved oxidative stress might improve the damage of myelin sheath giving rise to the increased NCV of sciatic motor function. Although PWCG mitigated the neuropathy in diabetic condition, no reduction of blood glucose and the suppression of aldose reductase activity in the lesion nerve were observed. Therefore, both hypoglycemic effect and the suppression of polyol pathway may not involve in anti-neuropathy effect of PWCG.

5. Conclusion

PWCG is the potential candidate is the potential candidate to serve as functional food to improve peripheral neuropathy in diabetic patient. However, further researches about the pharmacokinetic and interactions with diabetic drugs are very much necessary before serving as the adjuvant therapy.