Research Article

The Effect of Crude-Extracts from *Cleistocalyx nervosum* Var. *Paniala* fruit on Neuropathy in Streptozotocin - Induced Diabetic Rats

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ABSTRACT

Peripheral diabetic neuropathy (PDN) is a harmful diabetic complication. Chronic sustained hyperglycemia induced oxidative stress is known to play a causal role in the nerve dysfunction and delayed repairs of nerve injury. This study investigated the effect of *Cleistocalyx nervosum* var. *paniala* (CP) fruit extract on oxidative stress marker, structural and functional recoveries of sciatic nerve crush injury in a rat model of diabetes mellitus (DM). The crude extract of CP fruit extract at a dose of 125, 250 and 500 mg/kg were daily orally given to streptozotocin (STZ)-diabetic rats which were subjected to a sciatic nerve crush injury for 28 days. The functional recovery and sensory perception of rat were assessed every 7 days using the walking track analysis, rotarod test and Von Frey filament test respectively. At the end of study, the determination of lipid peroxidation level (MDA) and superoxide dismutase (SOD) activity of the lesion nerve were performed. Only the high dose of the crude-extract (500 mg/kg) produced a significant improved the muscle coordination, sensory perception together with the improved oxidative status. These results indicated that daily oral administration of CP fruit extract may be advantageous supplementation to promote nerve function in DM.

Keywords: Antioxidant, diabetes mellitus, *Cleistocalyx nervosum* var. *paniala,* Peripheral diabetic neuropathy, oxidative stress.

INTRODUCTION

Peripheral diabetic neuropathy (PDN) is a serious but common complication of diabetes mellitus (DM). [1] Functional disability related with a peripheral neuropathy can have a profound effect on patient's life and a large socioeconomic impact.[2] Accumulating lines of data reported that oxidative stress has been implicated in initiating, accompanying or causing many disease including PDN.[3] Sciatic nerve conduction deficits and decreased nerve blood flow have been showed in diabetic rats following exposure to oxidative stress.[4,5]

Chronic sustained hyperglycemia, not only generates more reactive oxygen species (ROS) but also activate lipid peroxidation (LPO) in the sciatic nerve.[6,7] Therefore, diabetes-induced oxidative stress may also damage the sciatic nerve similar to damage induced by oxidative stress in diabetic neuropathy. Based on these studies, anti-oxidants supplementation was recommended to protect neuronal damage from the deleterious effects of oxidative stress conditions. *Cleistocalyx nervosum* var. *paniala* (CP), commonly known as Makiang in Thai, is most common found in Southeast Asia, especially in the northern part of Thailand.[8]

The scientific literature strongly supports CP *in vitro* possessed anti-mutagenic, anti-carcinogenic, anti-aging and antioxidant properties.[9-12] Thus, this study was carried out to determine the effect of CP fruit extract on functional recovery of sciatic nerve crush injury in DM rats.

MATERIALS AND METHODS

Plant Materials and Aqueous extract preparation

The fresh fruit of CP at the mature stage were collected from Chiangmai horticulture research center, Thailand. Fruits with peels were washed immediately and carefully with tap water, then dried with hot air oven at 40°C, blend, ground into powder and macerate (CP fruit to water ration, 1:5) for 24 hours. Dried powder (10 g) of CP was extracted in a rotary shaker with 200ml of water at 30◦C for 24 hours, then filtered, concentrated using a rotary evaporator to obtain a crude extract. The residue solid product was dissolved in water, then freeze dried and finally stored in refrigerator until further use. The extraction yield was 17.89%.

Animals and experimental design

The experimental protocols were approved by the Animal Ethic Committee of University of Phayao (No. 5701040012). Young adult male Wistar rats weighing 180 - 250 g were obtained from the National Laboratory Animal Center, Salaya, Nakorn Pathom, Thailand. They were housed at 20±2°C with a 12 hours dark/light cycle. The food in the form of dry tablets and water were made available ad libitum. After 1 week of acclimatization, a total number of 40 animals were randomly divided into five groups (n=8/group).

- Group 1: DM rat + sciatic nerve crush injury + vehicle (distilled water)

- Group 2: DM rat + sciatic nerve crush injury + CP extract (125 mg/kg p.o.)

- Group 3: DM rat + sciatic nerve crush injury + CP extract (250 mg/kg p.o.)

- Group 4: DM rat + sciatic nerve crush injury + CP extract (500 mg/kg p.o.)

- Group 5: DM rat + sciatic nerve crush injury + Vitamin C (Vit C; 100 mg/kg p.o.)

All rats were orally assigned substances via the intragastric feeding tube once daily for 28 days after sciatic nerve crush injury. On 7th, 14th, 21st and 28th day, behavioral parameters including the motor coordination and sensory perception were determined. All rats were killed on 29th day and oxidative marker in the sciatic nerve such as, superoxide dismutase (SOD) activity and lipid peroxidation (malondialdehyde; MDA) were assessed.

Induction of DM and sciatic nerve crush injury

Diabetes was induced by intraperitoneal (i.p.) injection of a single dose of streptozotocin (STZ) (65 mg/kg body weight; Sigma-Aldrich: Darmstadt, Germany). STZ was dissolved in a 0.05% sodium citrate buffer (pH 4.5) before i.p. administration. Fasting blood glucose (FBS) level of all rats were determined at 72 h after the STZ injection. Rats with a glucose levels exceeding 250 mg/dl were considered to be diabetic and were enrolled for further nerve crush injury. Blood glucose concentrations in rats were collected in tail vein blood samples and measured using Accutrend Alpha (Roche Diagnostics Inc., Somerville, MA, USA), with gluco strips. A sciatic nerve crush surgery was performed following anesthetized with i.p. injection of sodium pentobarbital (50 mg /kg body weight), shaved and washed with 70% alcohol before surgery. The right sciatic nerve was exposed through a gluteus maximus muscle-splitting incision. Then the nerve was crushed at a distances of 5 mm from the sciatic notch for 30 sec using hemostatic forceps before wound closure. The wound area was sutured and infiltrated with 10% povidine solution for anti-septic postoperative care.

Calculation of sciatic functional index (SFI) from walking track analysis

SFI derived from walking track analysis in rats proved a reliable method of assessed motor function recovery. Evaluation of SFI was performed on days 7, 14, 21 and 28 after surgery and measured as previously described by Matsuoka *et al.*[13] Rats were allowed conditioning trials in a confined walkway (10 × 60 cm) darkened at one end. White papers were placed on the bottom of the track. The hind paws were dipped in blue ink and footprints were appeared immediately on the papers when the rats walked down the track. The following measurements were taken from the footprints: print length (distance from the heel to the third toe, PL), toe spread (distance from the first to fifth toe, TS), and intermediary toe spread (distance from the second to the fourth toe, IT). PL, TS, and IT were accumulated in both the left normal (N) and the right experimental (E) hind legs. The SFI were calculated by the following formula derived by Bain *et al.*[14]

$$SFI = -38.3 \times (EPL - NPL) + 109.5 \times (ETS - NTS) / NTS + 13.3 \times (EIT - NIT) / NIT - 8.8$$

An SFI equal to -100 indicates significant impairment, whereas an SFI value close to 0 was considered normal function.

Motor coordination measurement

A rotarod apparatus model Acela Rota-rod for rats (Ugo-Basile, Monvalle, Varese, Italy) was used to investigated motor coordination. All rats underwent a 3 day training program. During that period, animals were trained to walk against the motion of a rotating drum at a constant speed of 10 RPM (rotations per minute) for a maximum of 2 min. Each rat received three training trials per day with an interval trial time of 10 min. The initial speed of the rotarod was set at 5 rpm and gradually increased from 5 to 40 rpm over 5 minutes. The latency time (sec) of the rats falling off the rotarod onto the sensor platform was recorded.

Assessment of sensory perception (mechanical hyperalgesia)

The von Frey filament test as described by Stuesse *et al.*[15] Rats were placed in a plexiglass cage on an elevated wire mesh openings. Von Frey filaments (Stoelting, Wood Dale, Illinois, U.S.A) were used to determine the sensitivity of the skin to tactile stimulation and applied with increasing strengths (2-60 g) consecutively to the plantar surface of the left hind paw of the rat. The paw withdrawal threshold (PWT) was decided as the minimum gram strength producing two sequential responses at 3 min intervals (withdrawal from pressure).

Estimation of malondialdehyde (MDA) levels

After sacrifice, the right sciatic nerve of each rat was rapidly isolated to assess the MDA level. Nerve tissue homogenate was prepared in 1 ml of 0.1 M phosphate buffer (pH 7.4) then the obtained nerve homogenate was adjusted to 10% w/v and centrifuged for 60 min at 4 °C 1,000 g. The supernatant was processed for determination of biochemical parameters. As previously described by Ohkawa *et al.*[16] MDA level was measured using thiobarbituric acid reactive substances (TBARS) method. For this, 0.1 ml of sciatic nerve tissue homogenate sample was dissolved in 0.75 ml of acetic acid, and added to a mixture containing 0.1 ml of 8.1% sodium dodecyl sulfate (SDS), 0.75 ml 0.8% aqueous solution of thiobarbituric acid (TBA), and boiled in a temperature controlled water bath for 60 min at 100°C. After cooling on ice-water, 500 µl of chilled water and 2.5 ml of butanol and pyridine was added to the reaction mixtures, and the samples were centrifuged for 20 min at 800 g. The absorbance of the upper pink layer was measured at a wavelength of 532 nm using a spectrophotometer. In this case, using tetraethoxypropane as the standard MDA. Plasma level of MDA was expressed as nmol/mg protein.

Estimation of superoxide dismutase (SOD) activity

The SOD activity in sciatic nerve was determined according to the method described of Wattanathorn *et al.*[17] Briefly, 20 µl of right sciatic nerve homogenate solution and 200 µl of reaction mixture contained 57 mM phosphate buffer solution (KH_2PO_4), 0.1 µM of xanthine solution, 20 µl of xanthine oxidase solution was prepared at 25 °C for 5 min. the absorbance was reading at a wavelength of 415 nm and the SOD activity was expressed in U/mg protein.

Statistical analysis

Statistical evaluations were performed by ANOVA, expressed as mean ± Standard Error of Mean (SEM.) followed by Turkey's post hoc test for multiple comparisons. P-value less than 0.05 were considered as significance.

RESULTS

Effect of CP fruit extract on motor functional recovery

At the beginning of the study, no significant differences in baseline values on SFI scores in all experimental groups. After the nerve crush surgery, SFI scores were progressively decreased in all groups of treatment throughout the periods of experiment which indicated that a sciatic nerve crush significantly motor dysfunction. Compared with the vehicle treated group subjected to crush injury, oral administration of Vit C significantly ($p<0.01$) increased SFI on day 21 and 28. Interestingly, only a high dose of CP fruit extract (500 mg/kg) showed the successful and significantly ($p< 0.01$) enhanced SFI score on postoperative day 14 and this phenomenon still existed when the treatment was prolonged further to 21-28 days ($p< 0.001$ all; compared with the vehicle treated group), while the low and medium doses of CP fruit extract did not produce the significant changes on this parameter. However, this crude extract showed no dose-dependent effect on motor functional recovery (Table 1).

Effect of CP fruit extract on motor coordination

Time latencies reduced significantly ($p<0.01$) in crushed sciatic nerve groups 7 days after treatment with vehicle, Vit C or different doses CP fruit extract when compared with baseline values. However, 21 days treatment with CP fruit extract 500 mg/kg significantly ($p < 0.01$) improved the latency of falling time on a rotarod as compared to vehicle treated groups, whereas the time latency of DM rats treated with Vit C (100 mg/kg) caused significant increased in this latency ($p<0.05$) when compared vehicle treated group for 28 days (Table 2).

Effect of CP fruit extract on sensory perception (Mechanical hypersensitivity)

In response to von Frey test, the results showed that STZ leading to the significant development of mechanical hyperalgesis which indicated that a decrease in the withdrawal threshold after nerve crush injury as compare to the baseline. Treatment with CP fruit extract at dose of 500 mg/kg leading to a significantly longer paw withdrawal threshold (PWT) on postoperative since day 14 ($p < 0.01$), this effect was remained until the end of the experiment, whereas the Vit C treated group revealed a significantly longer PWT after nerve crush injury day 21 ($p < 0.05$) as compared to vehicle treated group (Table 3).

Table 1: Effect of CP extract on the sciatic function index (SFI) at 7, 14, 21, 28 days after nerve crush injury.

Treatment	SFI scores				
	Baseline	Day 7	Day 14	Day 21	Day 28
DM + crush + vehicle	- 4.44 ± 1.80	- 68.73 ± 1.66[a]	- 61.57 ± 2.96[a]	- 57.73 ± 2.25[a]	- 45.70 ± 2.11[a]
DM + crush + CP 125	- 5.02 ± 1.65	- 69.05 ± 1.18[a]	- 61.40 ± 2.22[a]	- 53.15±2.05[a]	- 41.88 ± 1.96[a]
DM + crush + CP 250	- 4.97 ± 1.37	- 68.04 ± 1.54[a]	- 59.66 ± 2.44[a]	- 51.76 ± 1.98[a]	- 42.13 ± 2.23[a]
DM + crush + CP 500	- 5.11 ± 1.25	-67.55 ± 1.32[a]	- 52.77 ±1.85[a,b]	- 47.58±0.57[a,c]	- 32.44 ± 1.27[a,c]
DM + crush + Vit C 100	- 4.53 ± 1.59	-67.68 ± 1.51[a]	- 59.24 ± 3.02[a]	- 48.75 ± 0.73[a,b]	- 35.97 ± 1.32[a,b]

Each value represented the mean ± SEM (n=8). [a]$p < 0.001$ vs. baseline; [b]$p <0.01$ vs. the control negative (DM + crush + vehicle); [c]$p <0.001$ vs. the control negative (DM + crush + vehicle).

Table 2: Effect of CP fruit extract on falling latency in rotarod test at 7, 14, 21, 28 days after nerve crush injury.

Treatment	Falling latency (sec)				
	Baseline	Day 7	Day 14	Day 21	Day 28
DM + crush + vehicle	24.64±1.65	12.83±1.52[a]	15.52±2.47[a]	17.77±1.12[a]	18.80±1.71[a]
DM + crush + CP 125	23.81±1.70	13.45±1.28[a]	16.15±2.13[a]	18.28±1.95[a]	19.84±1.56[a]
DM + crush + CP 250	23.78±1.52	12.14±1.31[a]	17.03±2.80[a]	17.77±2.08[a]	18.19±1.72[a]
DM + crush + CP 500	24.14±1.45	11.57±1.02[a]	17.97±2.54[a]	20.59±0.53[a,b]	22.59±0.44[a,b]
DM + crush + Vit C 100	24.33±1.33	12.63±1.06[a]	16.59±2.72[a]	20.18±0.45[a,b]	21.17±0.52[a,c]

Each value represented the mean ± SEM (n=8). [a]$p < 0.01$ vs. baseline; [b]$p <0.01$ vs. the control negative (DM + crush + vehicle); [c]$p <0.05$ vs. the control negative (DM + crush + vehicle).

Table 3: Effect of CP extract on von Frey test at 7, 14, 21, 28 days after nerve crush injury

Treatment	Paw withdrawal threshold (PWT) (g)				
	Baseline	Day 7	Day 14	Day 21	Day 28
DM + crush + vehicle	42.68±0.34	17.83±0.22[a]	16.72±0.41[a]	16.17±0.12[a]	17.40±0.27[a]
DM + crush + CP 125	41.31±0.50	16.62±0.58[a]	17.49±0.53[a]	18.07±0.98[a]	18.89±0.46[a]
DM + crush + CP 250	39.98±0.52	15.74±0.61[a]	18.08±1.00[a]	17.87±1.06[a]	18.77±0.68[a]
DM + crush + CP 500	41.14±0.26	15.17±0.92[a]	22.35±0.42[a,b]	24.57±0.33[a,b]	25.32±0.26[a,b]
DM + crush + Vit C 100	41.33±0.31	16.18±1.04[a]	19.51±1.71[a]	23.33±0.29[a,c]	24.16±0.22[a,c]

Each value represented the mean ± SEM (n=8). [a]p < 0.01 vs. baseline; [b]p <0.01 vs. the control negative (DM + crush + vehicle); [c]p <0.05 vs. the control negative (DM + crush + vehicle).

Effect of CP fruit extract on sciatic nerve oxidative stress biomarkers

Vit C, a standard drug used as positive control in this study significantly (both p < 0.01) decreased MDA level and increased the SOD activity in sciatic nerve when compared to vehicle treated rats. Again, treatment with CP fruit extract at a dose of 500 mg/kg, the MDA level and SOD activity was significantly (both p < 0.001) restored compared with vehicle treated group, while the low and high doses of this extract did not produce the significant changes on this parameter (Table 4).

Table 4: Effect of CP extract on sciatic nerve oxidative stress biomarkers in rats

Treatment	MDA (nmol/mg protein)	SOD activity (U/mg protein)
DM + crush + vehicle	0.14 ± 0.13	28.97 ± 1.63
DM + crush + CP 125	0.13 ± 0.08	32.14 ± 1.04
DM + crush + CP 250	0.12 ± 0.14	34.06 ± 1.21
DM + crush + CP 500	0.08 ± 0.01***	35.82 ± 0.61***
DM + crush + Vit C 100	0.11 ± 0.06**	33.49 ± 1.30**

Each value represented the mean ± SEM (n=8). **p < 0.01 and ***p < 0.001 vs. the vehicle treated group.

DISCUSSION

The current study revealed that daily oral administration of high doses of CP fruit extract for 28 days successfully improved the nerve dysfunction of a sciatic nerve crush injury and also oxidative stress status in diabetic condition.

No unexpected mortality of any rats occurred after oral administration of CP fruit extract used in this study. As a result, this crude extract was considered to be safe at the dosing schedule used.

STZ produced reactive oxygen or nitrogen species (ROS or RNS) and lipid peroxidation, with an accompanying reduce antioxidant enzymes protection, which in turn induced pancreatic beta cell toxicity and destruction.[18, 19] Insulin deficit and/or hyperglycemia are principally involved in the pathogenesis of PDN.[20,21,22]

PDN is a peripheral diabetic neuropathy in which sensory and motor dysfunction. In addition, previous studies proposed that the pathophysiology of PDN involves the interplay of hyperglycemia, ischemia, and oxidative stress status.[23,24,25] The increased ischemic condition and oxidative stress status can exacerbate the functional and morphological changes in the diabetic peripheral nerve.[26, 27] Compared with the baseline, all groups of DM rats subjected to the crush injury showed lower SFI scores, shorter falling latency in rotarod and PWT time in Von Frey test, which indicated that motor function, sensorimotor coordination and sensory perception were deficits. Furthermore, our study demonstrated that rats with DM subjected to sciatic nerve crush injury and treated with vehicle showed enhanced MDA but reduced SOD enzyme activity. These results confirms the conclusion of Sellamuthu *et al*.[28], which showed that STZ induced oxidative damage as indicated by the rise in MDA levels and reduced cellular anti-oxidant defense system such as SOD activity.

Accumulating evidences suggest that increased in the free radical activity is the possible mechanism involved pathophysiological phenomenon in PDN.[29] The results of the present study indicated that oral administration of CP fruit extract at a dose of 500 mg/kg induced SFI score and increased the PWT time in Von Frey test since day 14, while enhanced the fall latency in a rotarod test at day 21. Moreover, our results revealed that the rats with DM subjected to sciatic nerve crush injury and treated with a high dose of CP fruit extract significantly reduced MDA and increased anti-oxidant SOD activity in the sciatic nerve homogenate. The same observations were made among rats treated with Vit C, suggesting that CP fruit extract may improve sensory and motor functional recoveries by its antioxidant effects. Many mechanisms involved in oxidative induced nerve damage, thus the precise underline mechanisms involved in the oxidative stress pathway need to further study.

Notably, low and medium dose of CP fruit extract did not produce this effect. One possible explanation for this phenomenon might be related to the insufficient concentrations of active ingredients of CP fruit extract to

reach the therapeutic or a plateau level. Previous study of Jansom *et al.*[30] reported that the main essential constituent in *C. nervosum* or CP is anthocyanin especially cyaniding 3-glucoside. Therefore, cyaniding 3-glucoside, the most active antioxidant anthocyanin may be the active component which promoted the functional recovery of the sciatic nerve following the crush injury induced by STZ in the current study.

CONCLUSION

With the above data, it can provided support to the role of CP in the protection against oxidative neuropathy damage in DM, possibly through quenching the ROS or by acting as a defense armor to protect the antioxidant defense mechanism. However, further researches about possible active constituents and pharmacokinetic of this crude extract are still needed before moving forward to clinical trial research.

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