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Phyllanthus Amarus Facilitates the Recovery of Peripheral Nerve after Injury

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Abstract: Problem statement: Peripheral nerve injuries are associated with morbidity and outcomes of peripheral nerve repair are poor. Moreover, it can subsequently leads to economic or social disability. Recent findings have shown that oxidative stress plays an important role on the functional recovery retardation of peripheral nerve. However, the impairment mentioned earlier is counteracted by antioxidant. Therefore, this study was carried out to determine whether the alcohol-water extract of the aerial parts of *P. amarus* could facilitate the functional recovery in experimental models of peripheral nerve injury induced by sciatic nerve crush injury. **Approach:** Male mice, weighing 30-50 g, were orally given the extract at doses of 50, 100 and 200 mg kg^{-1} BW for 2 weeks before and 4 weeks after crush injury at right sciatic nerve. They were determined the recovery of nerve every 3 days using Sciatic Function Index (SFI) as an index. At the end of the experiment, the injured nerve was determined the level of Malondialdehyde (MDA) and the activities of Superoxide Disease (SOD), Glutathione Peroxidase (GPx) and Catalase (CAT) enzymes. **Results:** The results showed that the extract improved SFI in accompany with the enhanced CAT and the decreased MDA. However, no significant changes in SOD and GPx activities were observed. **Conclusion:** The results obtained from the present study suggest that *P. amarus* extract may enhance the recovery partly via the decreased oxidation stress. It may possibly serve as the natural resource for developing health products to facilitate the recovery of peripheral nerve injury. However, further researches about the possible active ingredient and the precise underlying mechanism are still essential.

Key words: *Phyllantus amarus*, peripheral nerve injury, Sciatic Nerve Function Index (SFI), significant changes, precise underlying, underlying mechanism, glutathione peroxidase

INTRODUCTION

Peripheral nerve injuries frequently occur as a result of accidental trauma, deliberate surgery, or acute compression. Although the incidence of peripheral nerve injury is not very high, it is associated with morbidity and outcomes of peripheral nerve repair are poor. Moreover, it can subsequently lead to economic or social disability. Peripheral nerve injuries frequently occur as a result of accidental trauma, deliberate surgery, or acute compression. Recently, it has been found that the main therapeutic strategy is surgical repair, however, drug administration, a less invasive strategy, is a possible therapy of choice for treating nerve crush injuries in order to accelerate nerve regeneration and target muscle reinnervation.

Recent findings have shown that oxidative stress plays an important role on the functional recovery retardation of peripheral nerve (Fisher and Glass, 2010; Stoll and Muller, 1999). In addition, it is reported that antioxidants can counteract the detrimental effects of Reactive Oxygen Species (ROS) and protect against oxidative injury (Maher, 2006). Numerous studies have demonstrated that substances possessing antioxidant activity such as α-Lipoic Acid (LA), the extract of Gingko, *Achyranthes bidentata* and *Morus alba* are efficacious in the experimental neuropathy (Nagamatsu *et al*., 1995; Cameron *et al*., 1998; Mitsui *et al*., 1999;

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Lin *et al*., 2007; Muchimapura *et al*., 2010). With respect to the role of oxidative stress and the counteract effect of antioxidant mentioned earlier, the development of neuroprotectants for the management of peripheral nerve injuries, including the therapeutic agents derived from natural medicinal plants, has become an active area of intense research.

Phyllanthus amarus Schum (2001) or *Phyllanthus niruri* is belonging to the family of Euphorbiaceae. It is widely used in traditional medicine, in many countries, for the treatment of numerous disturbances, such as dropsy, jaundice, diarrhea, dysentery, intermittent fevers and diseases of the urogenital system, flu, scabies ulcers and wounds (Venkateswaran *et al*., 1987; Calixto *et al*., 1998). It also exhibits pronounced systemic antinociceptive effect, particularly in experimental models of neuropathic pain (Kassuya *et al*., 2003) and antioxidant property Karuna *et al*. (2009). Based on the above mentioned information, this study was carried out to determine whether the alcohol-water extract of the aerial parts of *P. amarus* could facilitate the functional recovery in experimental models of peripheral nerve injury induced by sciatic nerve crush injury.

MATERIALS AND METHODS

Preparation of plant extract: The aerial part of *Phyllanthus amarus* Schum (2001) was collected from Khon Kaen province during May-June, 2009. The plant was authenticated and prepared as the alcoholic extract by Associate Professor Dr. Bungorn Sripanidsakulchai, Center for Research and Development of Herbal Health Product, Khon Kaen University, Khon Kaen, Thailand. The voucher specimen was also deposited there. Fresh aerial parts were dried thoroughly in the oven at temperature of 45°C. Then, they were cut into small pieces and extracted with 50% ethanol with a maceration method for 7 days. The extract was filtered through a 3-4 gauze cloth and Whatman paper number 1 respectively. Then, the filtrate was evaporated with rotatory evaporator (EYELA, N-100, RIKAKIKAI CO., LTD) at 50°C. Finally, the extract was freeze-dried and kept on-20°C prior to use. The resulting extract contained the phenolic compound at a concentration of 567.18±0.01 mg of Gallic Acid Equivalent (GAE) /g of extract.

Animals: Male mice, weighing 30-50 g, were obtained from National Laboratory Animal Center, Salaya, Nakhon Pathom. They were housed 5 per cage and maintained in 12:12 light: Dark cycle and given the excess to food and water *ad libitum*. The experiments were performed to minimize animal suffering in accordance with the internationally accepted principles for laboratory use and care of the European Community (EEC directive of 1986; 86/609/EEC). The experimental protocols were approved by the Institutional Animal Care and Use Committee.

Experimental protocol: The animals were randomly divided into 4 groups (N = 8/group) as follows; (1) Vehicle treated group) (2-4) *Phyllanthus amarus* treated groups. All animals were orally given the assigned substance for a period of 14 days before and 28 days after the induction of crush injury in the upper one third of the right sciatic nerve. They were assessed the recovery of nerve after injury every 3 days via De Medinacelli method using Sciatic Function Index (SFI) as an index. At the end of the experiment, they were sacrificed, the unilateral sciatic nerves were isolated and determined oxidation damage markers including the level of malondialdehyde and the activities of Supersede Disease (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx). Moreover, the axon density was also evaluated.

Surgical procedure: The rats were anesthetized with 50 mg kg^{-1} (i.p.) Sodium pentobarbital. After induction of anesthesia, the hair around the mid thigh was shaved and the sciatic nerve was exposed through an incision in the mid-thigh of the right hind limb and it was crushed at a duration of 60 seconds by a smooth-jaw micro forceps.

Walking track analysis (De Medinacelli method): In order to examine functional recovery, walking track analysis was performed on animals every 3 days throughout the 28-day experimental period. This technique is used to detect whether the animal supports its weight upon the paw while walking. The paw-prints of mice were recorded by with red ink and having them walk along a 6×40 cm corridor lined with white paper. After the paw-prints were scanned, the toe spread and paw length were measured. The Sciatic Functional Index (SFI) was calculated according to the formula:

SFI =-38.3([EPL-NPL]/NPL) + 109.5([ETS-NTS]/NTS) +13.3([EIT-NIT]/NIT)-8.8

Paw Print Length (PL), total Toe Spread (TS) and Intermediary Toe spread (IT) on both the Normal (N) and Experimental (E) sides were measured and used for calculation of the SFI.

Determination of the superoxide dismutase, glutathione peroxidase and catalase activities and

the malondialdehyde level: The rats were divided into various groups as previously described in the experimental protocol. After the last dose of administration, all rats were sacrificed. The sciatic nerve of the lesion side was isolated and prepared as homogeneous for the determination of MDA level and the activities of SOD, CAT and GPx. MDA was estimated by determining the accumulation of Thiobarbituric Acid Reactive Substances (TBARS) (Ohkawa *et al*., 1979) in the nerve homogenate whereas the activities of superoxide dismutase (McCord and Fridovich, 1969), glutathione peroxides (Jakoby, 1980) and catalase (Goldblith and Proctor, 1950) were determined using the colorimetric method.

Histological evaluation: At the end of 28-day experimental period, mice were transcardially sequentially perfused with saline and 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS, pH 7.4). The sciatic nerves were dissected and stained for H and E to determine the morphometry and density of axons.

Statistical analysis: Data are expressed as means ± S.E.M. and were analyzed statistically by one-way ANOVA, followed by Post-hoc (LSD) test. The results were considered statistically significant at P - values <0.05.

RESULTS

The SFI value characterizes crucial aspects of locomotion activities involving recovery of hind limb sensory and motor function and it varies from 0-100, with 0 corresponding to normal function and-100 to complete dysfunction. It was found that the animals which subjected to the extract treatment at all dosage range used in this study could improve the Sciatic Function Index (SFI) at the period between day 15-day 18 after crush injuries as shown in Fig. 1 (p-value<0.05, compared to vehicle treated group).

Our data also showed the significant decrease in MDA level after the crush injury in all groups subjected to the extract treatment. To accompany either the change mentioned earlier, the increase in CAT activity was also observed. However, the significant changes were observed only at the doses of 50 and 200 mg kg^{-1} BW (p-value<0.05 all, compared to vehicle treated group) while changes in SOD and GPx were observed as shown in Fig. 2-5.

In addition, we also determined the morphometry and density of axons. It was found that the animals which received the plant extract showed less degenerated axon but higher axon density than vehicle Le treated group as shown in Fig. 6.

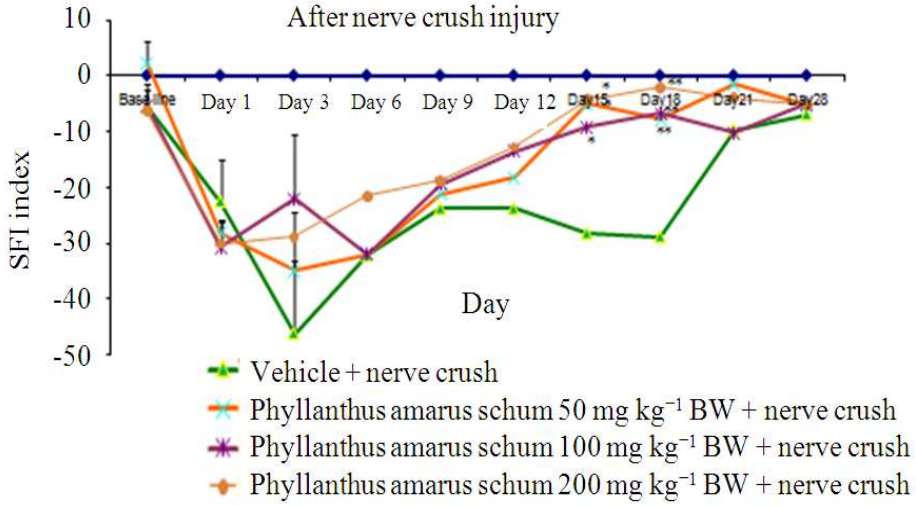

Fig. 1: The effect of *Phyllanthus amarus* extract on the Sciatic Function Index (SFI). Mice were orally given treated either with the plant extract at doses of 50, 100 and 200 mg kg^{-1} BW or with the vehicle at a period of 14 days before and 28 days after the induction of crush injury at right sciatic nerve. Then, they were assessed SFI every 3 days throughout the experimental period. (N = 8. group) All data were expressed as mean ± SEM. *=p-value<0.05 compared to vehicle treated group

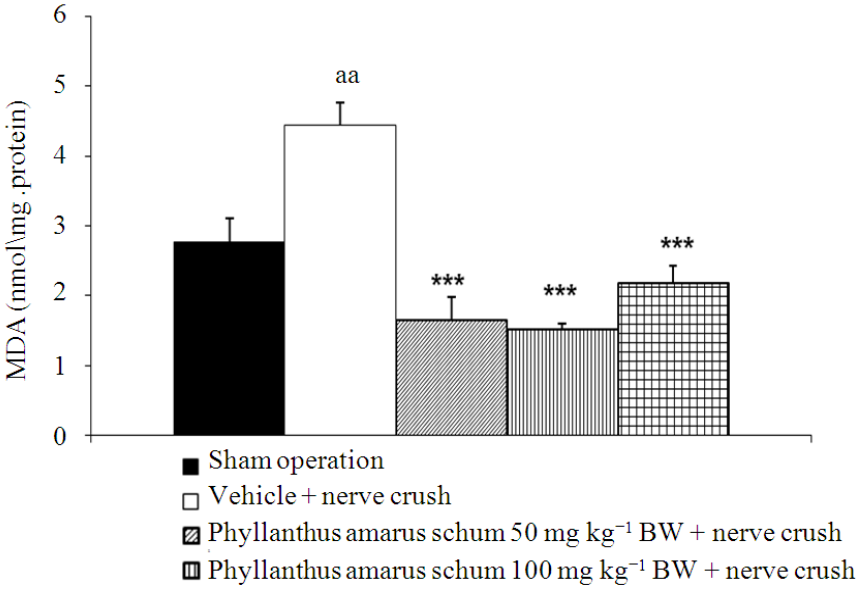

Fig. 2: Sffect of *Phyllanthus amarus* extract on the level of Malondialdehyde (MDA) in the nerve. Mice were orally given treated either with the plant extract at doses of 50, 100 and 200 mg kg^{-1} BW or with the vehicle at a period of 14 days before and 28 days after the induction of crush injury at right sciatic nerve. At the end of the experiment, the right sciatic nerve was isolated and determined the level of MDA. (N = 8. group) All data were expressed as mean ± SEM. *= p-value<0.05 compared to vehicle treated group

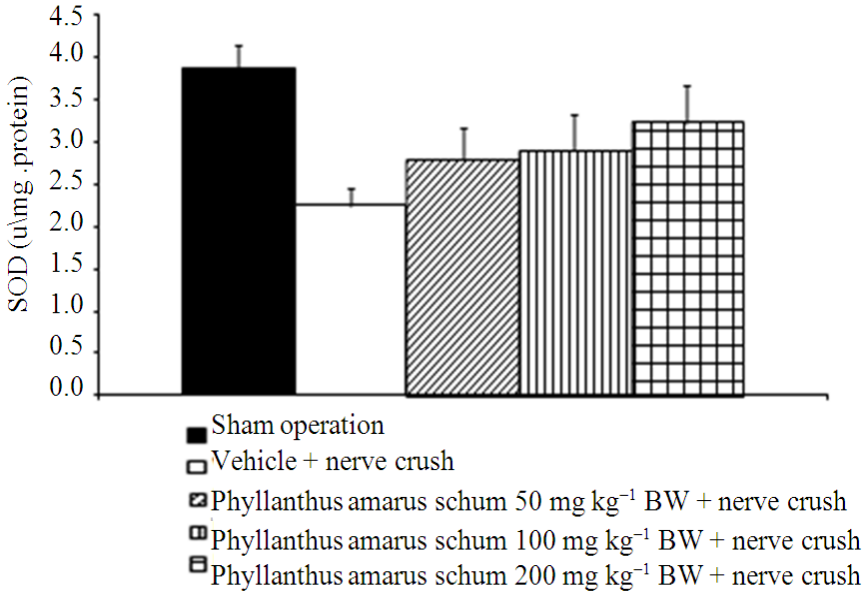

Fig. 3: Effect of *Phyllanthus amarus* extract on the activity of Superoxide Dismutase (SOD) in the nerve. Mice were orally given treated either with the plant extract at doses of 50, 100 and 200 mg kg^{-1} BW or with the vehicle at a period of 14 days before and 28 days after the induction of crush injury at right sciatic nerve. At the end of the experiment, the right sciatic nerve was isolated and determined the activity of SOD. (N = 8. group) All data were expressed as mean ± SEM

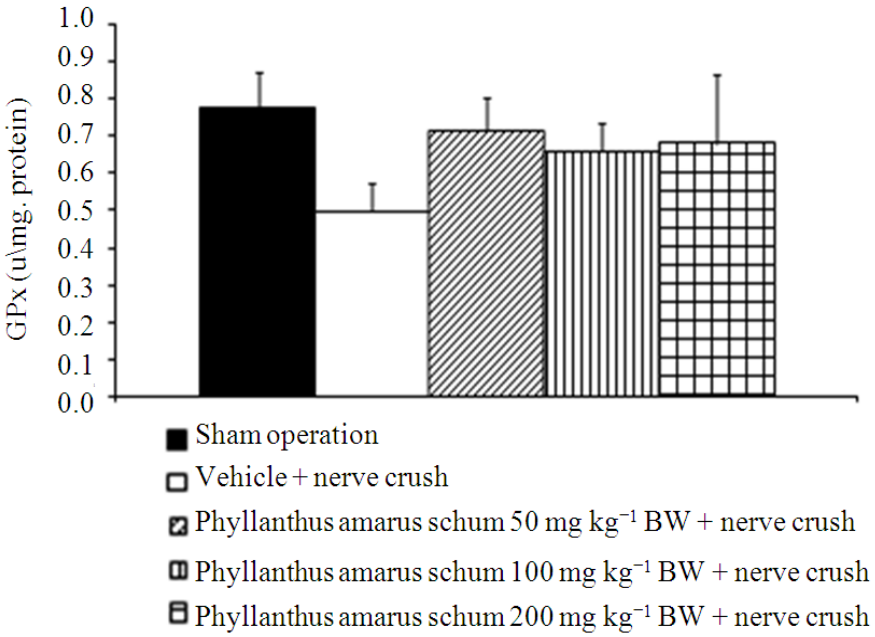

Fig. 4: Effect of *Phyllanthus amarus* extract on the activity of Glutathione Peroxidase (GPx) in the nerve. Mice were orally given treated either with the plant extract at doses of 50, 100 and 200 mg kg^{-1} BW or with the vehicle at a period of 14 days before and 28 days after the induction of crush injury at right sciatic nerve. At the end of the experiment, the right sciatic nerve was isolated and determined the activity of GPx. (N = 8. group) All data were expressed as mean ± SEM

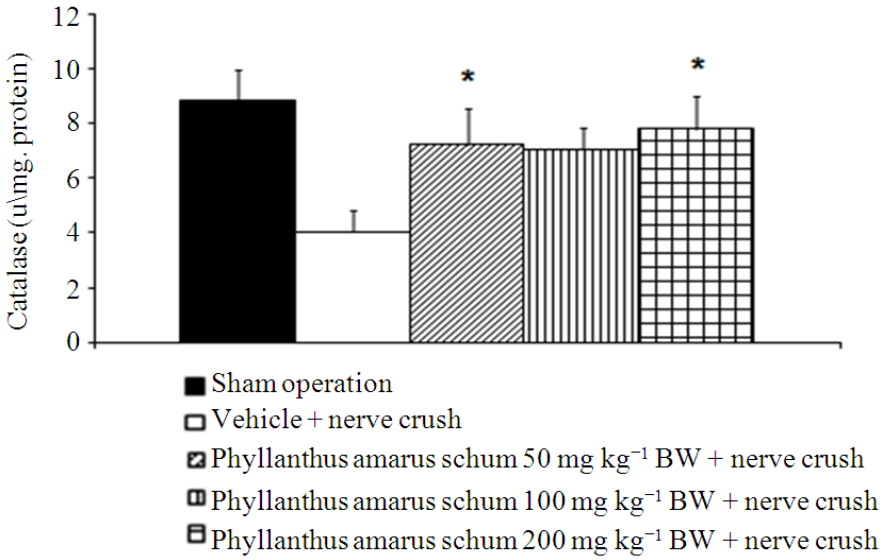

Fig. 5: Effect of *Phyllanthus amarus* extract on the activity of Catalase (CAT) in the nerve. Mice were orally given treated either with the plant extract at doses of 50, 100 and 200 mg kg^{-1} BW or with the vehicle at a period of 14 days before and 28 days after the induction of crush injury at right sciatic nerve. At the end of the experiment, the right sciatic nerve was isolated and determined the activity of CAT. (N = 8. group) All data were expressed as mean ± SEM. *= p-value<. 05 compared to vehicle treated group

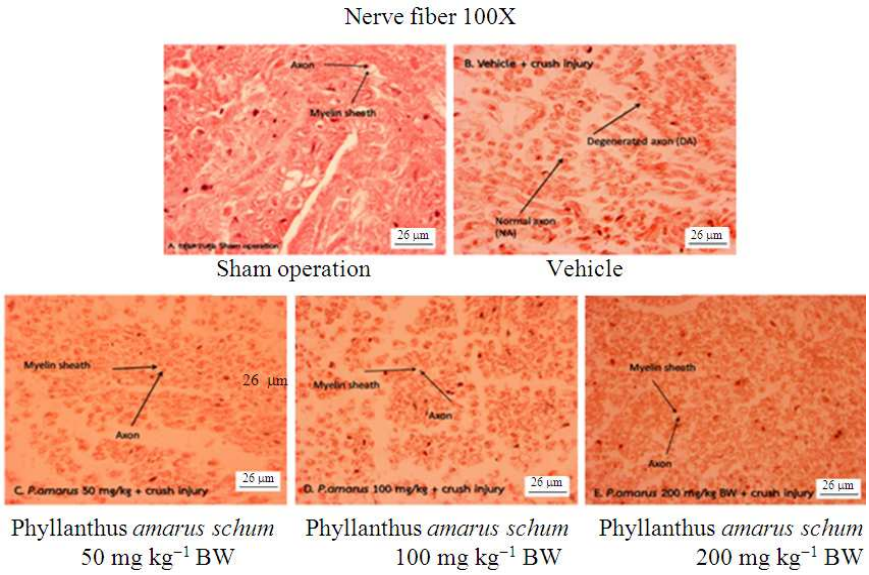

Fig. 6: Photomicrograph demonstrates the density and morphometry of the axon. At the end of the experimental period, the sciatic nerve were determined the density and morphometry of axon via H and E staining at 100× magnification

DISCUSSION

Peripheral nerves may be subjected to crush injuries in a variety of circumstances, including motor vehicle accidents, fractures, dislocations and natural disasters such as earthquakes (Chen *et al*., 1992). Despite significant advances in therapeutic strategies over the past years the management of peripheral nerve injuries remains a major clinical challenge by virtue of the unsatisfactory functional outcomes achieved (Fu and Gordon, 1995; 1997).

Herbal products have been traditionally accepted as remedies due to popular belief that they present minor adverse effects (Barbisan *et al*., 2002). In this study, we had determined the effect of *P. amarus* extract on the recovery of nerve after injury by using sciatic nerve crush injury, one of the validated and popular models for assessing the recovery of nerve after injury. Previous studies had demonstrated that the SFI obtained from the walking track analysis showed the close relationship with the function of hind limb and their footprints (Dinh *et al*., 2009). In addition, the footprints also indicated the co-ordination of motor signals from cortical area and the sensory feedback (Bain *et al*., 1989).

Our data demonstrated the enhanced recovery function of the sciatic nerve in accompany with the increased axon density and the elevation of CAT but decreased MDA. These findings were in agreement with previous studies which demonstrated that the substances possessing anti-oxidant could facilitate the functional recovery of nerve (Maher, 2006; Serarslan *et al*., 2009). Although, the elevation of GPx was previously reported to play the crucial role in the neuroprotection against nerve injury, no change was observed. The possible explanation for the loss of the significant change of GPx might be due to the appropriate time window effect selected in this study. It was found that the maximum peak of GPx was observed at 2 days after injury and then, they became to decrease to normal level (Serarslan *et al*., 2009). Moreover, the enhanced axon density was also observed. Therefore, *P. amarus* might facilitate the nerve recovery partly via the improved oxidative stress and the enhanced axon density.

Besides oxidation stress, other factors including Schwann cell, neurotrophic factor and extracellular matrix were also contributed the important role in the recovery of nerve after injury (Ma *et al*., 2010). Since numerous factors were involved in the recovery of nerve after injury, the dose dependent effect was not observed.

CONCLUSION

Phyllanthus amarus extract is capable of accelerating the nerve recovery after peripheral nerve injury, indicating that it has the potential to be a neuroprotective agent for nerve injury repair applications. However, further researches about the

possible active ingredient and the precise underlying mechanism are still essential.

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