

## FUNCTIONAL RECOVERY ENHANCEMENT BY TIGER MILK MUSHROOM, *LIGNOSUS RHINOCEROTIS* IN A SCIATIC NERVE CRUSH INJURY MODEL AND MORPHOLOGICAL STUDY OF ITS NEUROTOXICITY

M. Farha<sup>1,2</sup>, L. Parkianathan<sup>1</sup>, N.A.I. Abdul Amir<sup>1</sup>, V. Sabaratnam<sup>2,3</sup> and K.-H. Wong<sup>\*1,3</sup>

<sup>1</sup>Department of Anatomy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

<sup>2</sup>Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

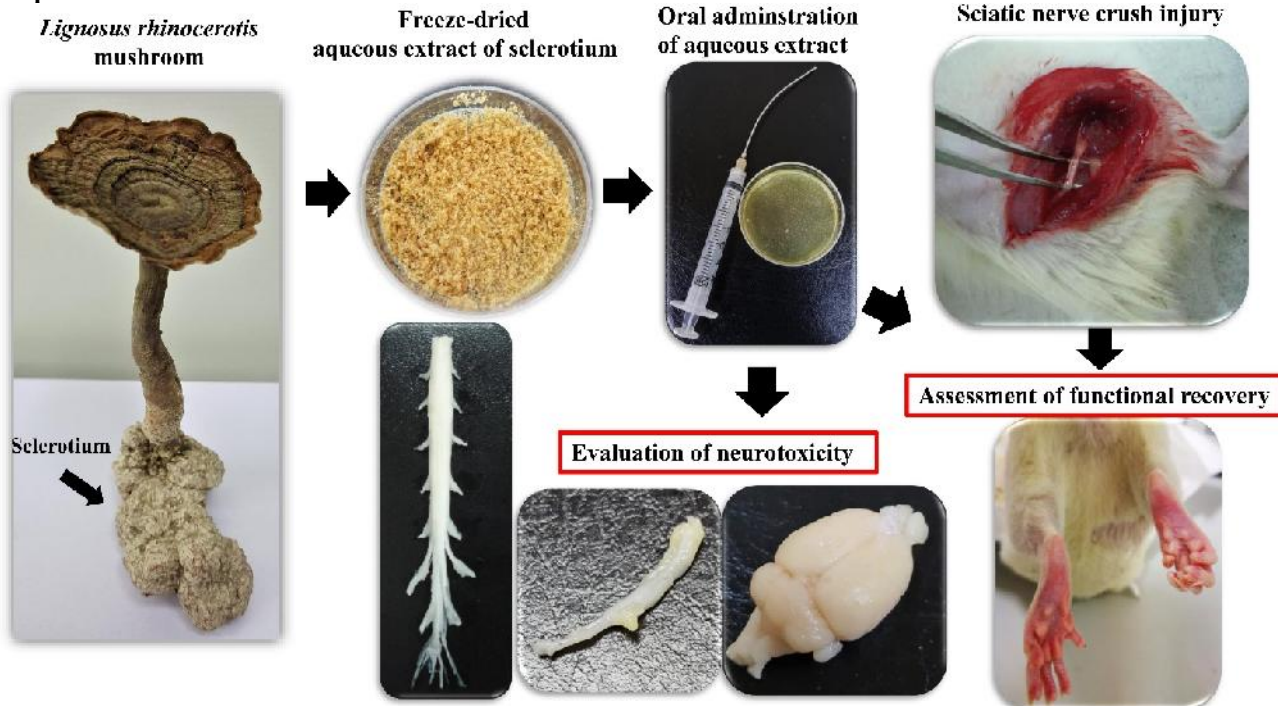
<sup>3</sup>Mushroom Research Centre, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

\*Corresponding author's E-mail address: wkahhui@ummc.edu.my

### ABSTRACT

Insights into the process of peripheral nerve regeneration are essential for the development of novel therapies. We tested the activity of aqueous extract from *Lignosus rhinocerotis* mushroom on nerve regeneration in a rat model of sciatic nerve crush injury and to examine (if any) toxicity effect of the extract on nervous tissues. Activities of aqueous extracts were compared to mecobalamin, a drug for peripheral neuropathies. 24 adult rats were randomly divided to 4 groups, the negative controls, positive controls, low and high dosages of aqueous extracts. Functional recovery was evaluated by sensitivity to thermal pain and toe-spreading reflex. Morphological changes in the nervous tissues was assessed by haematoxylin and eosin stain. Hot plate test showed acceleration of sensory recovery in low dosage group compared to other groups. Return of toe-spreading in the crushed limb was accomplished by day 12 and day 16 in low and high dosage groups, respectively after crush injury. Microscopic examination showed normal features of Peripheral and Central Nervous Tissues. There were no abnormal clinical signs detected. Oral administration of low dosage of *L. rhinocerotis* aqueous extract is capable to enhance motor and sensory functional recovery after nerve injury and had no adverse effects on nervous tissues.

### Graphical Abstract



**Keywords:** *Lignosus rhinocerotis*; aqueous extract; peripheral nerve regeneration; functional recovery; neurotoxicity

## INTRODUCTION

Peripheral nerve injury always results in restricted activity or life-long disability. Traumatic peripheral nerve injury is caused by motor vehicle accidents, falls or penetrating injury. It leads to disruption of intraneural circulation. Sciatic nerve of the lower limb is most often injured followed by peroneal, femoral and tibial nerves. Symptoms experienced would be the numbness of the injured limb, intolerance towards cold, neuropathic pain and in severe cases, paralysis (Grinsell and Keating, 2014). Although microsurgical treatments for nerve injuries have been improved over the past decades, the outcome of peripheral nerve injury repair remains unsatisfactory (Popa *et al.*, 2016). Pre-clinical animal models such as rat, mouse, rabbit, dog, cat, sheep, monkey and pig have been employed for evaluation of peripheral nerve regeneration and repair prior to implementation in clinical practice (Angius *et al.*, 2012).

Mecobalamin, a vitamin B12 derivative is a commonly prescribed medication for peripheral nerve disorders. However, it is associated with side effects such as urticaria, eczematous, exanthematous skin lesions, anaphylactic reactions, polycythemia with peripheral vascular thrombosis, and movement disorders with tremor and myoclonus (Aronson, 2016). Therefore, much emphasis is placed on the potential of using natural resources as neuroprotective and neurotrophic agents (Seow *et al.*, 2013). Natural products that stimulate neurite outgrowth and may substitute nerve growth factor (NGF) or exhibit NGF-like activities that cause neurons and myelin to regrow have been extensively studied.

In Malaysia, *Lignosus rhinocerotis* (Cooke) Ryvarden or Tiger's milk mushroom is hailed as national treasure and has been consumed by the indigenous communities as mycomedicine for multiple diseases. The sclerotium of the mushroom is greyish brown in colour with irregular hard mass appearance. Sclerotial extracts were reported to elicit immunomodulatory effects and tumor suppressor activity (Wong *et al.*, 2009a; Wong *et al.*, 2011a), possessed neuritogenic effects in PC12 cells (Seow *et al.*, 2013) and dissociated cells of brain, spinal cord and retinal cells (Samberkar *et al.*, 2015), and anti-neuroinflammatory effects in BV2 microglia (Nallathambi *et al.*, 2016; Seow *et al.*, 2017).

In addition, Zhou *et al.* (2017) demonstrated that *L. rhinocerotis* was not teratogenic. Subacute toxicity assessment revealed that *L. rhinocerotis* did not have adverse effects on visceral organs namely the liver, kidney, heart, spleen and lungs (Lee *et al.*, 2011). However, there is no information on neurotoxicity in *in vivo* studies. Due to the broad range of ethnomedicinal properties of *L. rhinocerotis*, the neurotoxicity data is vital before it could be developed as a functional food (van der Heijden *et al.*, 1999).

Peripheral Nervous System has an intrinsic growth capacity for repair and regeneration. Therefore it is a widely accepted model for neuroregenerative research (Huebner and Strittmatter, 2009). Based on these promising *in vitro* neuroprotective effects, we investigated the neuroregenerative properties of locally cultivated *L. rhinocerotis* aqueous extract on functional recovery following crush injury to the sciatic nerve in Sprague-Dawley rats and examined the subacute toxicity (if any) of the extract on Peripheral and Central Nervous Tissues.

## MATERIALS AND METHODS

The study was carried out at Department of Anatomy, Faculty of Medicine, University of Malaya, Kuala Lumpur in 2017.

**Preparation of *L. rhinocerotis* aqueous extract:** The freeze-dried cultivated sclerotium of *L. rhinocerotis* marketed as Ligno TM02 (Batch No. PL/16/014-1215) was purchased from Ligno Biotech Sdn Bhd, Selangor, Malaysia. Aqueous extraction was done according to Seow *et al.* (2015). The *L. rhinocerotis* sclerotium powder was weighed and mixed in distilled water with a sample-to-water ratio of 1:10 (w/v). The mixture was agitated in the shaker at 50 rpm at 45°C for 24 h. Following incubation, the mixture was double boiled in a 100°C water bath for 30 min. After cooling to room temperature, the mixture was centrifuged at 10 000 rpm at 4°C for 30 min. Supernatants were freeze dried at -50°C for 48 h (Wong *et al.*, 2009b). The resultant fluffy white product was stored in a -20°C freezer prior to further use.

**Principles of rat grouping:** The *in vivo* study was done with approval by Institutional Animal Care and Use Committee, Faculty of Medicine, University of Malaya (Ethics Ref No 2013-10-08/ANAT/R/WKH). Twenty-four adult female Sprague-Dawley rats with body weight in the range of 180 g to 200 g were randomly assigned to four groups of six rats each; (i) negative control that received distilled water (5 ml/kg body weight/day), (ii) positive control that received 130 µg mecobalamin/kg body weight/day (Methycobal®, 500 µg vitamin B12 in each tablet, Eisai Company Limited, Japan) (iii) low dosage that received 500 mg of *L. rhinocerotis* aqueous extract/kg body weight/day and (iv) high dosage that received 1000 mg of *L. rhinocerotis* aqueous extract/kg body weight/day. All the rats underwent 14 days pre-treatment prior to sciatic nerve injury. The oral administration was continued for the next 28 days after injury. Observations were recorded from day 0 (1 h before injury) and at every 4 days interval until day 28. Food pellet and drinking water were available *ad-libitum*.

**Surgical procedure:** Rats were injected intraperitoneally with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). Incision on skin and gluteal muscle was made by a surgical blade no. 4 (Wong *et al.*, 2015). The sciatic nerve was pinched 1 cm from the bifurcation point by a watchmaker forceps no. 4 for 10 s. Complete crush was indicated by a translucent band on the nerve. The muscle and skin were sutured using a non-absorbable suture and antiseptic cream was applied thereafter. All operations were performed on right limb and the left limb served as an uninjured control.

**Evaluation of functional recovery:** Vibrissae of the rats were observed and ensured to be actively moving. Actively moving vibrissae indicates the readiness of rat for the study (Prescott *et al.*, 2011).

i. **Sensory function:** Hot plate is generally utilized for estimating sensitivity to thermal pain. The hot plate with an open-ended cylindrical space (Panlab, S.L. Spain) was heated to 50°C and the rats were placed on the plate. This study explores behavioural components, namely the paw licking and jumping that are associated with their reaction times. The time taken to elicit a withdrawal response of injured hind limb from the hot plate was recorded by a built-in timer with an external foot switch and termed as withdrawal reflex latency (WRL) (Wong *et al.*, 2015). WRLs were recorded before injury (day 0) and after 4, 8, 12, 16, 20, 24 and 28 of injury. The injured hind limb was tested 3 times and averaged to obtain mean WRL. Non-responsive rat was removed from the hot plate after 30 s of heat stimulation to prevent tissue damage, and therefore was given WRL of 30 s.

ii. **Motor function:** The rats were inspected after 4, 8, 12, 16, 20, 24 and 28 of injury. The toe-spreading reflex was observed by lifting the rat at its tail, the hind limbs were hanging free and forelimbs were resting on the workbench. Each rat was observed for 1-2 min and activities were categorized according to the toe-spreading reflex of injured right hind limb: 0 - no spreading; 1 - minimal spreading; 2 - average spreading; 3 - normal spreading (Wong *et al.*, 2009b).

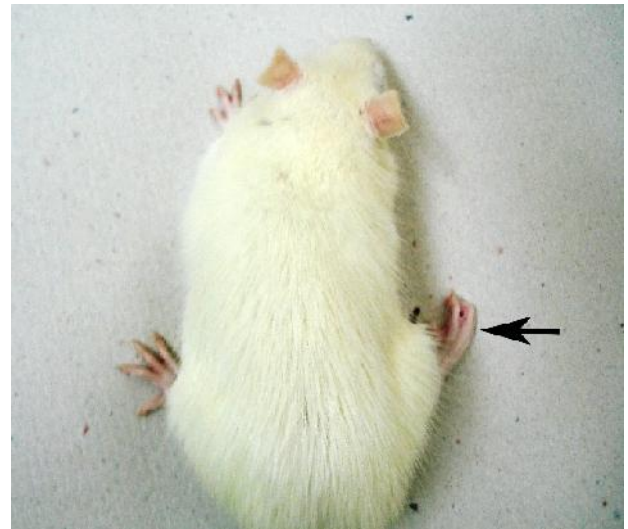
**Histopathological analysis of nervous tissues:** After 28 days of injury, Peripheral Nervous Tissues (sciatic nerve and dorsal root ganglia) and Central Nervous Tissues (cerebrum, cerebellum and spinal cord) were harvested and fixed in Davidson's solution for 24 h followed by 10% neutral buffered formalin solution. The tissues were processed and stained with hematoxylin and eosin. Specimens were examined microscopically for the

evidence of toxicity under a light microscope. Toxicological changes in nervous tissues such as neuronal degeneration and swelling or apoptosis of neurons were examined.

**Statistical analysis:** The means of data (n=6 animals per group) were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by the Duncan's multiple range tests (DMRT) at 95% least significant difference ( $p \leq 0.05$ ).

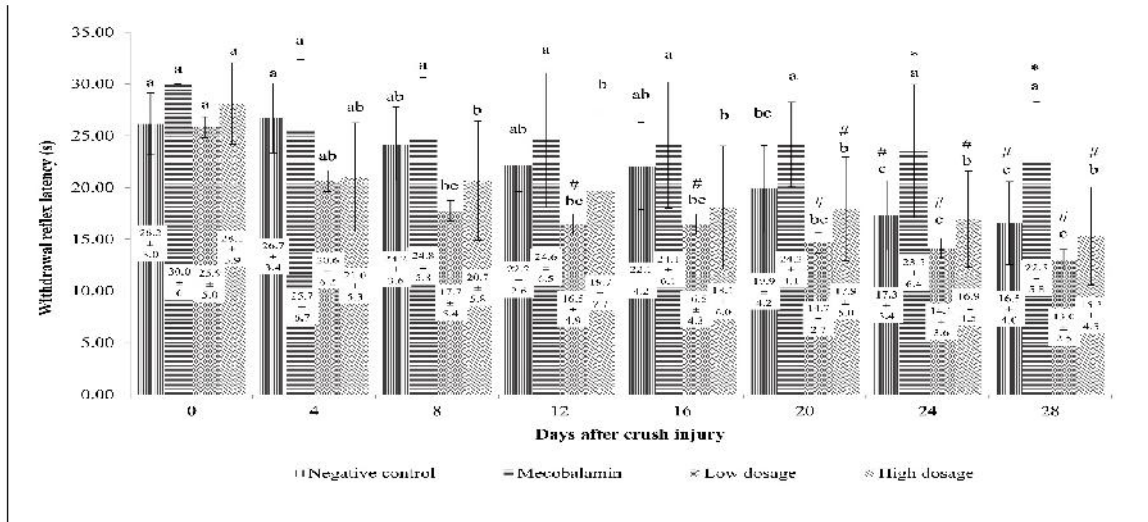
## RESULTS

Following crush injury, there were no signs of self-mutilation. Dragging of feet and clumping of toes of the right hind limb were observed (Fig. 1). The efficacy of the treatments on the sensory and motor function of the right hind limb was compared to the uninjured left hind limb.



**Fig. 1. Gait changes associated with sciatic nerve injury. Right hind foot became everted with weight bearing on the lateral margin. The toes were clumping together. Arrow indicates the operated limb.**

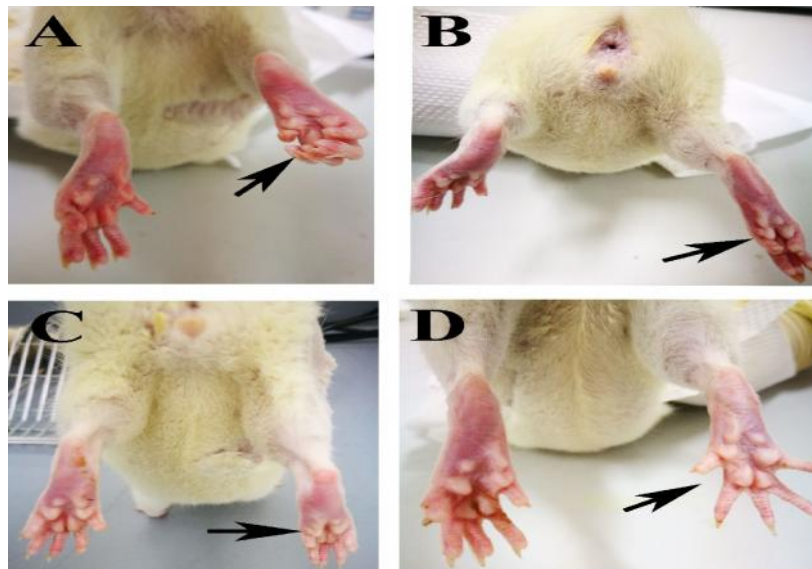
Hot plate test was used to evaluate the sensory functional recovery of the rats. Rats which had the nociceptive sensitisation responded by (i) hind limb withdrawal, (ii) hind paw licking or (iii) jumping off the hot plate. Prior to sciatic nerve injury, the rats responded to heat stimulus within 30 s.



**Fig. 2.** Withdrawal reflex latency (WRL) for injured right hind limb induced by heat. Asterisks (\*) denote significant differences ( $p \leq 0.05$ , DMRT) in WRL compared to negative control on a same experimental day. Hash signs (#) denotes significant differences ( $p \leq 0.05$ , DMRT) in WRL compared to mecobalamin on a same experimental day. Different alphabets denotes significant differences ( $p \leq 0.05$ , ANOVA) between experimental days in a same group.

Fig. 2 shows the data for the WRL over the period of 4 weeks. Rats did not respond to heat stimulus in the first week following sciatic nerve crush indicating loss of temperature and pain sensation on the right foot. Withdrawal response began on day 8 after crush injury and towards the end of week 3 in low dosage and negative control groups, respectively ( $p \leq 0.05$ ). WRL was then improved during the 4-week recovery time. Mecobalamin and high dosage groups did not show any improvement in WRL throughout the recovery period.

WRLs of the low dosage-treated rats were significantly less than the mecobalamin group from day 12 onwards ( $p \leq 0.05$ ). Low dosage group recovered better compared to mecobalamin group ( $p \leq 0.05$ ). There was a significant difference between rats in mecobalamin and negative control groups from day 24 onwards ( $p \leq 0.05$ ). WRLs in low dosage group did not reach statistically significant difference from other groups after 4 and 8 days of injury.



**Fig. 3.** Toe spreading reflex degree. (A) 0, no spreading. (B) 1, minimal spreading. (C) 2, average spreading. (D) 3, normal spreading. Arrows indicate the injured right hind limb.

Fig. 3 shows the toe-spreading reflex degree. Maximal toe-spreading was recorded in low dosage group on day 12 (2 rats), 20 (1 rat), and 28 (1 rat) and in high dosage group on day 16 (1 rat), 24 (1 rat) and 28 (4 rats) after injury. However, rats in the negative control and mecobalamin groups failed to achieve maximal toe-spreading on day 28 after injury.

After 2 weeks of injury, the first signs of functional motor recovery were noticeable. Toe-spreading precedes recovery of sensory function in high dosage group following sciatic nerve crush injury. Jin *et al.* (2012) postulated that an early return of reflex is not a promising sign of nerve regeneration. It manifests an increased sensitivity of denervated muscles to adrenalin. Adrenalin released into systemic circulation during reflex-testing may cause muscle contraction mimicking nerve-induced toe-spreading reflex.

The gross examination of the nervous tissues of rats treated with *L. rhinocerotis* did not display any significant pathological changes compared to the negative control and mecobalamin groups after 28 days of injury.

The microscopic sections of the Peripheral Nervous Tissues namely, the sciatic nerve and dorsal root ganglion and the Central Nervous Tissues namely, the cerebrum, cerebellum, spinal cord are illustrated in Fig. 4-8. In the controls and treated rats, the longitudinal sections of sciatic nerve show parallel bundles of nerve fibres encased by perineurium (Fig. 4) and dorsal root ganglion displayed normal microscopic appearance of cell bodies of sensory neurons surrounded by satellite cells (Fig. 5). As for the Central Nervous Tissues, cerebral cortex of cerebrum consists of the largest pyramidal cells or Betz cells in layer V and cell bodies of other neurons (Fig. 6), cerebellar cortex of cerebellum consists of typical microscopic features of large flask-shaped Purkinje cells in middle layer, scattered basket cells in molecular layer and densely packed granular cells in granular layer (Fig. 7), whereas ventral horn of spinal cord consists of normal architecture of multipolar motor neurons (Fig. 8). Therefore, there was no abnormal changes in terms of neuronal degeneration, swelling or apoptosis was detected.

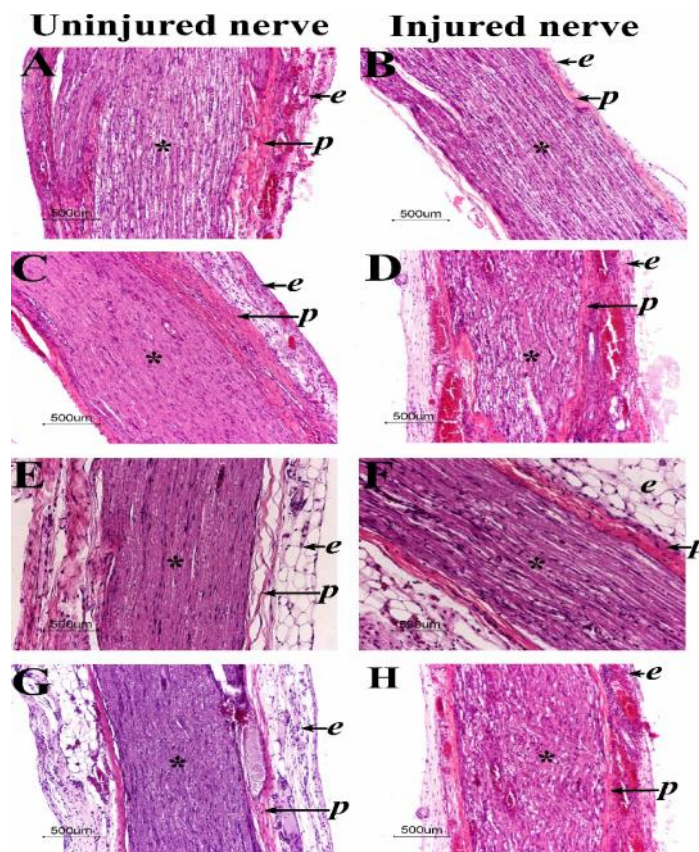


Fig. 4. The longitudinal sections of sciatic nerve. (A) and (B) Negative control group - distilled water (5 ml/kg body weight/day). (C) and (D) Positive control group - mecobalamin (130 µg/kg body weight/day). (E) and (F) Low dosage group - *L. rhinocerotis* aqueous extract (500 mg/kg body weight/day). (G) and (H) High dosage group - *L. rhinocerotis* aqueous extract (1000 mg/kg body weight/day). Asterisks (\*) denote parallel bundles of nerve fibres. Letter *e* denotes epineurium. Letter *p* denotes perineurium. Sections were stained with hematoxylin and eosin. Scale bar = 500µm.

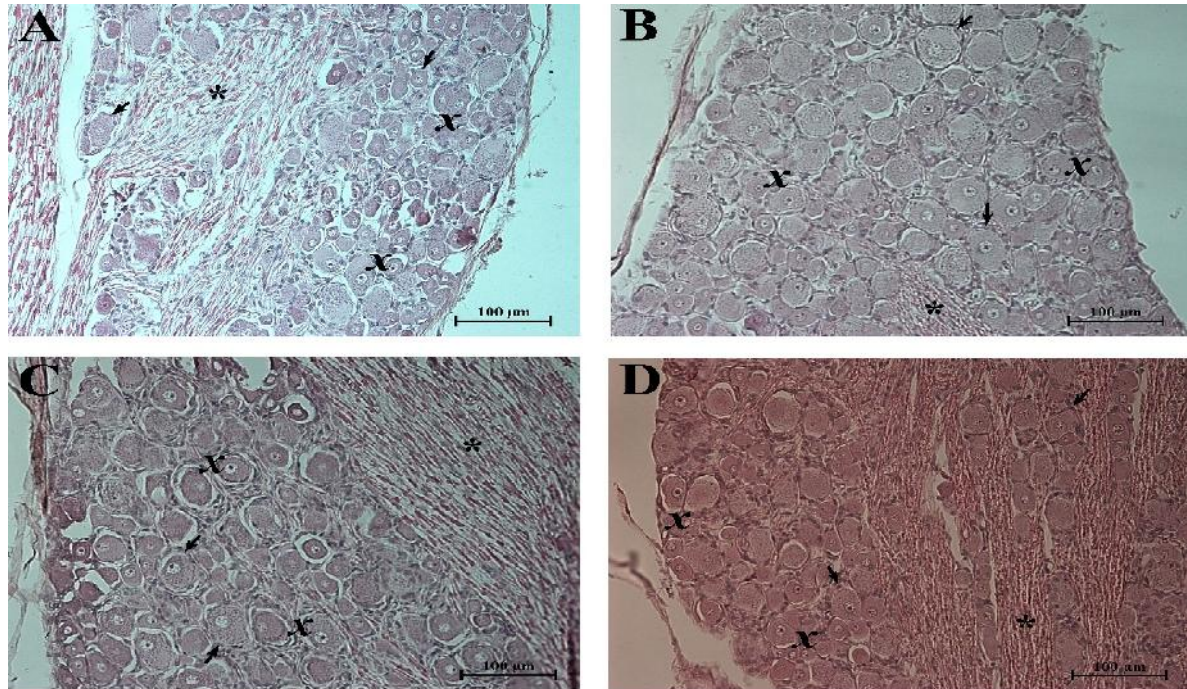


Fig. 5. The longitudinal sections of dorsal root ganglion from uninjured side. (A) Negative control group – distilled water (5 ml/kg body weight/day). (B) Positive control group – mecobalamin (130 µg/kg body weight/day). (C) Low dosage group – *L. rhinocerotis* aqueous extract (500 mg/kg body weight/day). (D) High dosage group – *L. rhinocerotis* aqueous extract (1000 mg/kg body weight/day). Asterisks (\*) denote nerve fibres. Arrows denote satellite cells. Letter x denotes cell bodies of sensory neurons. Sections were stained with hematoxylin and eosin. Scale bar = 100µm.

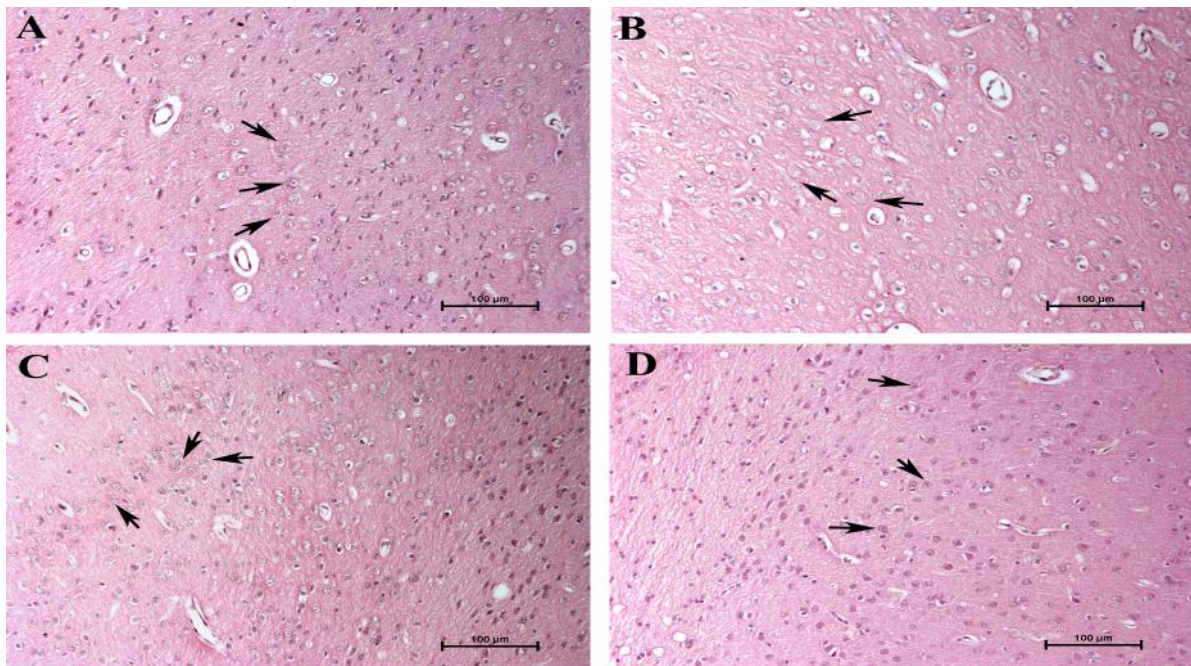


Fig. 6. The cross sections of cerebrum. (A) Negative control group – distilled water (5 ml/kg body weight/day). (B) Positive control group – mecobalamin (130 µg/kg body weight/day). (C) Low dosage group – *L. rhinocerotis* aqueous extract (500 mg/kg body weight/day). (D) High dosage group – *L. rhinocerotis* aqueous extract (1000 mg/kg body weight/day). Arrows denote Betz cells. Sections were stained with hematoxylin and eosin. Scale bar = 100µm.

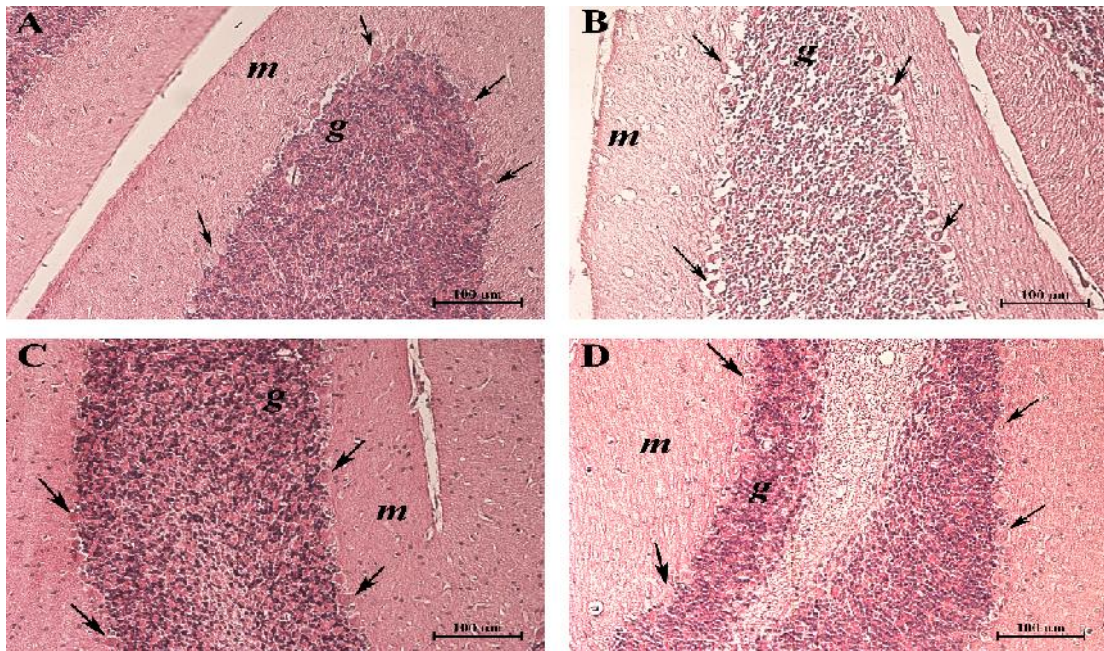


Fig. 7. The cross sections of cerebellum. (A) Negative control group – distilled water (5 ml/kg body weight/day). (B) Positive control group – mecobalamin (130  $\mu$ g/kg body weight/day). (C) Low dosage group – *L. rhinocerotis* aqueous extract (500 mg/kg body weight/day). (D) High dosage group – *L. rhinocerotis* aqueous extract (1000 mg/kg body weight/day). Arrows denote single layer of Purkinje cells in middle layer. Letter *m* denotes molecular layer. Letter *g* denotes granular layer. Sections were stained with hematoxylin and eosin. Scale bar = 100 $\mu$ m.

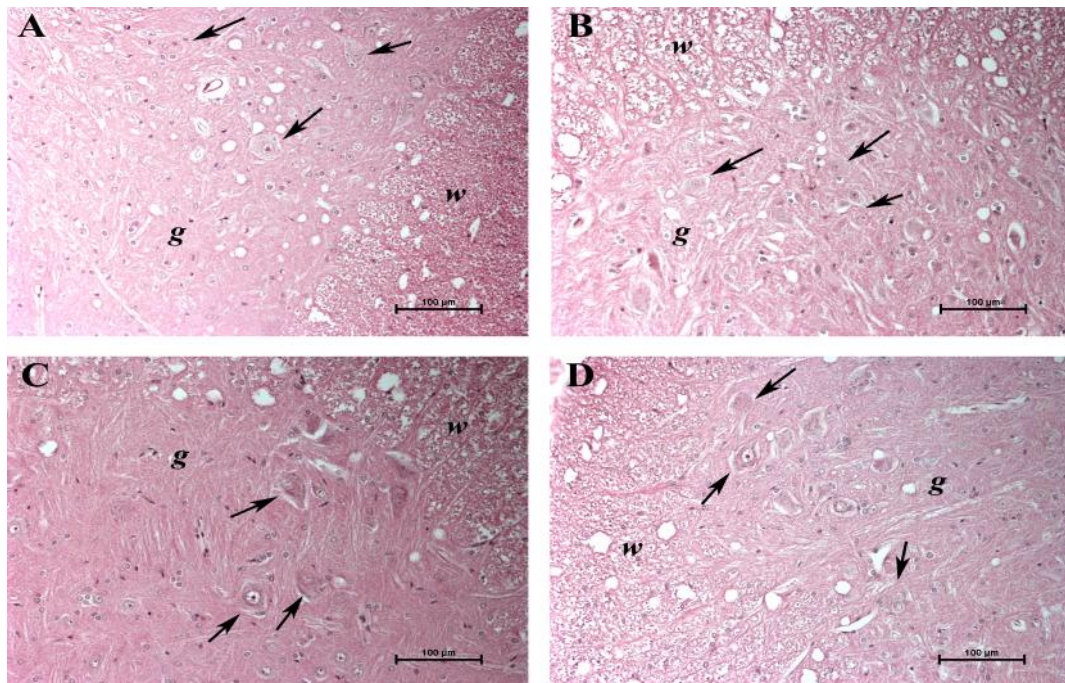


Fig. 8. The cross sections of spinal cord. (A) Negative control group – distilled water (5 ml/kg body weight/day). (B) Positive control group – mecobalamin (130  $\mu$ g/kg body weight/day). (C) Low dosage group – *L. rhinocerotis* aqueous extract (500 mg/kg body weight/day). (D) High dosage group – *L. rhinocerotis* aqueous extract (1000 mg/kg body weight/day). Arrows denote multipolar motor neurons in the ventral horn of grey matter. Letter *g* denotes grey matter. Letter *w* denotes white matter. Sections were stained with hematoxylin and eosin. Scale bar = 100 $\mu$ m.

## DISCUSSION

Nerve crush or axonotmesis is characterized by a disruptive lesion of the axon and its myelin sheath. The nerve segment distal to the injury site undergoes cellular alterations that characterise Wallerian degeneration. However, the connective tissue coverings namely the endoneurium, perineurium and epineurium remain intact to guide and redirect the growth of axonal buds during the regeneration process. Crush injury to the sciatic nerve causes impaired extension at the hip joint, impaired flexion at the knee joint, loss of dorsiflexion and plantar flexion at the ankle joint and loss of inversion and eversion of the foot (Drake *et al.*, 2014).

The rationale for the utilization of crush injury over transection is due to successful regeneration guided by Schwann cells and maintenance of endoneurial tubes that enhances axonal elongation and subsequently reinnervation of skeletal muscle fibres (Valero-Cabr e *et al.*, 2004). Crush injuries are being employed in the studies of molecular events that participate in peripheral nerve regeneration. Withdrawal latency stimulated by nociception and cutaneous stimulation are functionally unadapted in neonatal rats (Miranda *et al.*, 2006). The task-specific nociceptive withdrawal reflex organization then gradually emerges over the first three postnatal weeks. The reflex patterns in postnatal maturation reflects a tuning of spinal connectivity.

Mecobalamin is widely used for the treatment of peripheral and diabetic neuropathies (Lawrence, 2016). Concentrations in the range of 100 to 130  $\mu\text{g}/\text{kg}$  body weight were recommended in peripheral nerve crush regeneration study using rat sciatic nerve model (Jiang *et al.*, 2009; Ma *et al.*, 2010). In our study, mecobalamin at 130  $\mu\text{g}/\text{kg}$  body weight did not improve functional recovery over the period of study, and therefore higher dosages than 130  $\mu\text{g}/\text{kg}$  body weight warrant further testing. Ultra-high dosage of mecobalamin at 500  $\mu\text{g}/\text{kg}$  body weight has been reported to promote regeneration in a Wistar rat model of acrylamide neuropathy (Watanabe *et al.*, 1994) and neuroprotection in a Wistar rat model of sciatic nerve ligation and crush injury (Muthal *et al.*, 2008), whereas 30 mg/kg body weight has been found to delay the progression of motor and neuropathological symptoms in a Wobbler mouse model of amyotrophic lateral sclerosis (Ikeda *et al.*, 2015).

Compounds that exhibit neuroprotective and neuroregenerative activities are the cordycepin from *Cordyceps militaris* (Cheng *et al.*, 2011), complex carbohydrates and sterols from *Ganoderma lucidum* (Zhao *et al.*, 2005), lysophosphatidylethanolamine from *Grifola frondosa* (Nishina *et al.*, 2006), phenol derivatives and diterpene xylosides from *Hericium erinaceus* (Kawagishi *et al.*, 2008), alkaloids and diterpenes from *L. rhinocerotis* (Seow *et al.*, 2015) and cerebrosides from *Termitomyces albuminosus* (Qu *et al.*,

2012). With these findings, it interests us to explore the effects of *L. rhinocerotis* on functional recovery by using an *in vivo* model of sciatic nerve crush injury.

*Lignosus rhinocerotis* hot and cold aqueous extracts have been found to possess a broad spectrum of cytotoxic activities, immunodulatory, antimicrobial, antiviral, fibrinolytic and neurotogenic activities attributed to the fatty acids, polysaccharides, polysaccharide-protein complexes, glucans, peptides, proteases and proteins (Lau *et al.*, 2015; Nallathamby *et al.*, 2018). Cold aqueous extract was cytotoxic against human cell lines, namely the acute promyelocytic leukaemia, breast adenocarcinoma, colorectal carcinoma, prostate adenocarcinoma, lung carcinoma, lung fibroblast, hepatocellular carcinoma, embryonic liver, squamous carcinoma and nasopharyngeal carcinoma (Lau *et al.*, 2013).

Our results showed that daily oral administration of low dosage of *L. rhinocerotis* aqueous extract enhanced functional recovery of damaged sciatic nerve. We revealed a neuroregenerative role of polysaccharide (Wong *et al.*, 2015) and  $\beta$ -glucan from *H. erinaceus* aqueous extract in the Peripheral Nervous System (Wong *et al.*, 2016). Thus the polysaccharide component of *L. rhinocerotis* aqueous extract plays a role in its neuroregenerative effect. On the other hand, water-soluble polysaccharides of *Tremella fuciformis* has also been shown to trigger NGF production, and ameliorate memory and learning deficits (Kim *et al.*, 2007).

Low dosage of *L. rhinocerotis* aqueous extract increased the pain sensitivity to heat and enhanced the reflex response of injured right hind limb after 8 to 12 days of injury. These significant changes were observed subsequently during the study period of 28 days. Even when promising sensory recovery occurs, motor deficits may impair the functional outcome. Recovery of motor function depends on the integrity of skeletal muscle upon arrival of regenerating axons. Reinnervation of denervated skeletal muscles occurs after 18 to 24 months of injury with the exception of hand intrinsic muscles at 6 months after injury. However, Ma *et al.* (2011) postulated that lacking of motor function after injury is due to failure of synapse or formation of neuromuscular junction after extended denervation rather than attenuation of axonal growth. Further, a functional motor performance depends on the interactions between primary motor cortices or inter-hemispheric functional coupling (Kajal *et al.*, 2017).

Studies have also shown that peripheral nerve injury produces neuropathic pain and memory deficits in the hippocampal CA3 area (Joshi *et al.*, 2017), parahippocampal cortex, posterior hippocampus, retrosplenial cortex, angular gyrus, posterior cingulate cortex and precuneus (Robin *et al.*, 2018). Brain-derived neurotrophic factor (BDNF) plays a crucial role in regulating synaptic plasticity and neuronal activity. Histone deacetylase inhibition in the aged brain has been

shown to improve spatial memory in a BDNF-dependent manner (Snigdha *et al.*, 2016). Multiple lines of evidence have documented that free radicals generated after tissue injury delay functional recovery. However, antioxidants and anti-inflammatory agents that reduce ischemic injury accelerates functional recovery (Algora *et al.*, 1996). Owing to this, *L. rhinocerotis* has been shown to possess anti-inflammatory (Lee *et al.*, 2014; Nallathamby *et al.*, 2016), neurotogenic (Seow *et al.*, 2013) and free radical scavenging (Lau *et al.*, 2015) activities and therefore could relieve neuropathic pain and multiple effects of peripheral nerve injury.

Generally, the issue of low bioavailability requires the use of very high dosages to obtain therapeutic effects *in vivo* and therefore are more likely to cause side effects (Caillaud *et al.*, 2018). Although high dosage of aqueous extract at 1000 mg/kg body weight/day did not produce any signs of toxicity or mortality (as discussed in the following sections), it did not improve WRL and had minimal effect on the return of toe-spreading over the period of study. Administration of high concentration of undesirable substances that may also act as inhibitory substances can mask the effect of the actual components such as polysaccharides or  $\beta$ -glucans responsible for functional recovery enhancement (Turner *et al.*, 2011).

Our observation is in accordance with several studies in animal models. *Morus alba* aqueous extract at 0.1 mg/kg improved motor and sensory functions after crush injury. Increased dosages of 1.0 and 10 mg/kg failed to show any significant recovery (Mucimapura *et al.*, 2010). Huang *et al.* (2011) revealed that low dosage of *Paeoniae alba* Radix water extract at 1.25 and 12.5 mg/ml in silicone rubber chambers promoted nerve fibres to grow across a 10-mm gap lesion in sciatic nerve. The effect was reversed by a high dosage of 125 mg/ml. Chang *et al.* (2011) demonstrated a nerve growth-suppressing action by high dosages of earthworm extract at 250 to 1000 mg/ml. Further, local infusion of low-dosage curcumin at the injury site at 0.2 mg/day for 4 weeks has been demonstrated to improve functional recovery, nerve conduction velocity and remyelination after sciatic nerve injury (Caillaud *et al.*, 2018). Therefore, an optimum dosage of *L. rhinocerotis* aqueous extract should be determined to achieve maximal potential of peripheral nerve regeneration. In other words, there are threshold dosages above and below a certain effective dose.

Various studies on peripheral nerve regeneration by natural products are being actively conducted worldwide. Buyang Huanwu decoction promoted regeneration of a 10-mm gap formed between the proximal and distal stumps of rat sciatic nerve (Cheng *et al.*, 2001). Jiang *et al.* (2009) verified that chitooligosaccharides enhanced sciatic nerve regeneration in a rat model of crush injury through functional and

morphological assessment. Ding *et al.* (2008) reported the therapeutic effect of *Achyranthes bidentata* root aqueous extract in accelerating nerve regeneration in a rabbit model of crushed common peroneal nerve. The polypeptides of aqueous extract has also been shown to enhance peripheral nerve regeneration and functional recovery after crush injury in mice (Yuan *et al.*, 2010), rats (Wang *et al.*, 2013) and rabbits (Cheng *et al.*, 2014). Astragaloside IV (AS-IV), a purified compound of Radix astragali has been found to accelerate remyelination in a mice model of sciatic nerve injury. The effect was modulated by upregulation of GAP-43 (Lu *et al.*, 2010). In addition, Ramli *et al.* (2017) had reported the efficacy of evening primrose oil at 6000 mg/day in accelerating the rate of sciatic nerve regeneration after crush injury in rat. Wong *et al.* (2011b; 2015; 2016) explored the role of local protein synthesis in regenerating axons, combined acetylcholinesterase and silver method for demonstrating motor endplates, and expression of Akt, MAPK, c-Jun and c-Fos in the dorsal root ganglia in accelerating motor and sensory functional recovery after crush injury by *H. erinaceus*.

The neurotoxicity test of *L. rhinocerotis* is crucial before developing it as a functional food. Several substances have been reported to interfere with functional recovery. Greensmith *et al.* (1994) postulated that N-methyl-D-aspartate, a glutamate agonist in developing spinal cord has resulted in apoptosis of motor neuron following sciatic nerve injury during postnatal period but not in adult motor neurons. Montañez *et al.* (2000) revealed an interaction between seizures and anticonvulsants administered simultaneously following a brain injury. Application of phenobarbital before initiation of subclinical seizures was found to be more destructive in the process of recovery than seizures alone, or treatment with phenobarbital after the seizures. The impact depends on timing of phenobarbital administration, its mechanism of action and frequency of seizures. In a study by Nakamura *et al.* (2001), intravenous administration of vincristine, a chemotherapy medication has been shown to cause a dose-dependent delay in functional recovery in a mouse model of sciatic nerve crush although weight loss or disruption of grooming and social behaviour were not observed throughout the 4-week experimental period. Generally, medicinal mushrooms exhibit minimal adverse effects compared to the drugs, nevertheless scientific toxicity data is required. Pertaining to the previous studies on the toxic effect of *L. rhinocerotis*, lyophilized mycelium up to 3400 mg/kg did not cause either mutagenicity in an *in vivo* ICR mice model of micronucleus assay (Chen *et al.*, 2013) nor did it provoke toxicity in dams or teratogenicity in fetal development during the critical period of embryonic organogenesis (Jhou *et al.*, 2017)

Further, in a subacute toxicity test for 28 days, oral administration of two cultivars of *L. rhinocerotis*,

namely TM02 in the range of 250 to 1000 mg/kg and TM03 at 1000 mg/kg, and wild type at 1000 mg/kg did not produce any signs of toxicity in adult rats as confirmed by haematological analysis, clinical biochemistry, urinalysis and histological analysis of vital organs (Lee *et al.*, 2011). Lee *et al.* (2013) extended their investigations into the chronic toxicity of cultivar TM02. Administration of sclerotial powder over the same range of dosages for 180 days did not show adverse effects. In addition, sclerotial powder administered at 100 mg/kg over the period of 7–8 weeks also did not reveal fertility disorders nor causing teratogenic effect in the offsprings.

Our toxicity study on nervous tissues following subacute oral administration of *L. rhinocerotis* showed no adverse effects. The microscopic evaluation of the nervous tissues treated with aqueous extracts did not show any difference in histological structure compared to the negative control and mecobalamin. The gross examination showed no changes in the architecture of sciatic nerve, dorsal root ganglion, cerebrum, cerebellum, and spinal cord. Parameters indicating signs of toxicity were not observed. Therefore, this confirms the safety profile of *L. rhinocerotis*. These dosages can be further tested in human trials for the development as functional food.

**Conclusion:** The oral administration of *L. rhinocerotis* aqueous extract at 500 mg/kg body weight/day has been demonstrated to enhance functional recovery following peripheral nerve injury. Dosages at 500 and 1000 mg/kg body weight/day had no toxicity effects on nervous tissues. Therefore it could be developed as a functional food with minimal adverse effects.

**Acknowledgements:** Fundamental Research Grant Scheme FP011-2016 from the Ministry of Higher Education Malaysia and University of Malaya High Impact Research Grant (UM.C/625/1/HIR/MOE/ASH/01) from the Ministry of Education Malaysia.

## REFERENCES

- Algora, J., L.E. Chen, A.V. Seaber, G.H. Wong, and J.R. Urbaniak (1996). Functional effects of lymphotoxin on crushed peripheral nerve. *Microsurgery* 17(3): 131-135.
- Angius, D., H. Wang, R.J. Spinner, Y. Gutierrez-Cotto, M.J. Yaszemski, and A.J. Windebank (2012). A systematic review of animal models used to study nerve regeneration in tissue-engineered scaffolds. *Biomaterials* 33(32): 8034-8039.
- Aronson, J.K. (2016). B<sub>12</sub> (cobalamins). In *Meyler's Side Effects of Drugs: The International Encyclopedia of Adverse Drug Reactions and Interactions*. 16th Ed. Elsevier Science; Amsterdam (Netherlands). pp. 475-477.
- Caillaud, M., B. Chantemargue, L. Richard, L. Vignaud, F. Favreau, P.A. Faye, P. Vignoles, F. Sturtz, P. Trouillas, J.M. Vallat, A. Desmoulière, and F. Billet (2018). Local low dose curcumin treatment improves functional recovery and remyelination in a rat model of sciatic nerve crush through inhibition of oxidative stress. *Neuropharmacology* 139: 98-116.
- Chang, Y.M., Y.T. Shih, Y.S. Chen, C.L. Liu, W.K. Fang, F.J. Tsai, W.W. Kuo, T.Y. Lai, and C.Y. Huang (2011). Schwann cell migration induced by earthworm extract via activation of PAs and MMP2/9 mediated through ERK1/2 and p38. *Evid. Based Complement. Alternat. Med.* 2011: 395458.
- Chen, T., H.W. Zhuang, Y.C. Chiao, and C.C. Chen (2013). Mutagenicity and genotoxicity effects of *Lignosus rhinocerotis* mushroom mycelium. *J. Ethnopharmacol.* 149(1): 70-74.
- Cheng, Q., C. Jiang, C. Wang, S. Yu, Q. Zhang, X. Gu, and F. Ding (2014). The *Achyranthes bidentata* polypeptide k fraction enhances neuronal growth *in vitro* and promotes peripheral nerve regeneration after crush injury *in vivo*. *Neural Regen. Res.* 9(24): 2142-2150.
- Cheng, Y.S., W.C. Cheng, C.H. Yao, C.L. Hsieh, J.G. Lin, T.Y. Lai, C.C. Lin, and C.C. Tsai (2001). Effects of buyang huanwu decoction on peripheral nerve regeneration using silicone rubber chambers. *Am. J. Chin. Med.* 29(3-4): 423-432.
- Cheng, Z., W. He, X. Zhou, Q. Lv, X. Xu, S. Yang, and L. Guo (2011). Cordycepin protects against cerebral ischemia/reperfusion injury *in vivo* and *in vitro*. *Eur. J. Pharmacol.* 664(1-3): 20-28.
- Ding, F., Q. Cheng, and X. Gu (2008). The repair effects of *Achyranthes bidentata* extract on the crushed common peroneal nerve of rabbits. *Fitoterapia* 79(3): 161-167.
- Drake, R.L., A.W. Vogl, and A.W.M. Mitchell (2014). *Anatomy for Students: With Student Consult Online Access*. 3rd Ed. Elsevier Health Science; London (United Kingdom).
- Greensmith, L., H.I. Hasan, and G. Vrbová (1994). Nerve injury increases the susceptibility of motoneurons to N-methyl-D-aspartate-induced neurotoxicity in the developing rat. *Neuroscience* 58(4): 727-733.
- Grinsell, D. and C.P. Keating (2014). Peripheral nerve reconstruction after injury: A review of clinical and experimental therapies. *Biomed. Res. Int.* 2014: 698256.
- Huang, K.S., J.G. Lin, H.C. Lee, F.J. Tsai, D.T. Bau, C.Y. Huang, C.H. Yao, and Y.S. Chen (2011). *Paeoniae alba* Radix promotes peripheral nerve

- regeneration. Evid. Based Complement. Alternat. Med. 2011: 109809.
- Huebner, E.A. and S.M. Strittmatter (2009). Axon regeneration in the peripheral and central nervous systems. Results Probl. Cell. Differ. 48: 339-351.
- Ikedo, K., Y. Iwasaki, and R. Kaji (2015). Neuroprotective effect of ultra-high dose methylcobalamin in wobbler mouse model of amyotrophic lateral sclerosis. J. Neurol. Sci. 354(1-2): 70-74.
- Jhou, B.Y., H.H. Liu, S.H. Yeh, and C.C. Chen (2017). Oral reproductive and developmental toxicity of *Lignosus rhinocerotis* mycelium in rat. J. Ethnopharmacol. 208: 66-71.
- Jiang, M., X. Zhuge, Y. Yang, X. Gu, and F. Ding (2009). The promotion of peripheral nerve regeneration by chitooligosaccharides in the rat nerve crush injury model. Neurosci. Lett. 454(3): 239-243.
- Jin, J., M. Park, A. Rengarajan, Q. Zhang, S. Limburg, S.K. Joshi, S. Patel, H.T. Kim, and A.C. Kuo. (2012). Functional motor recovery after peripheral nerve repair with an aligned nanofiber tubular conduit in a rat model. Regen. Med. 7(6): 799-806.
- Joshi, A., M. Salib, T.J. Viney, D. Dupret, and P. Somogyi (2017). Behavior-dependent activity and synaptic organization of septo-hippocampal GABAergic neurons selectively targeting the hippocampal CA3 area. Neuron 96(6): 1342-1357.
- Kajal, D.S., C. Braun, J. Mellinger, M.D. Sacchet, S. Ruiz, E. Fetz, N. Birbaumer, and R. Sitaram (2017). Learned control of inter-hemispheric connectivity: Effects on bimanual motor performance. Hum. Brain Mapp. 38(9): 4353-4369.
- Kawagishi, H., C. Zhuang, and R. Yunoki (2008). Compounds for dementia from *Hericium erinaceum*. Drugs Future 33(2): 149-155.
- Kim, J.H., H.C. Ha, M.S. Lee, J.I. Kang, H.S. Kim, S.Y. Lee, K.H. Pyun, and I. Shim (2007). Effect of *Tremella fuciformis* on the neurite outgrowth of PC12h cells and the improvement of memory in rats. Biol. Pharm. Bull. 30(4): 708-714.
- Lau, B.F., N. Abdullah, N. Aminudin, and H.B. Lee (2013). Chemical composition and cellular toxicity of ethnobotanical-based hot and cold aqueous preparations of the tiger's milk mushroom (*Lignosus rhinocerotis*). J. Ethnopharmacol. 150(1): 252-262.
- Lau, B.F., N. Abdullah, N. Aminudin, H.B. Lee, and P.J. Tan (2015). Ethnomedicinal uses, pharmacological activities, and cultivation of *Lignosus* spp. (tiger's milk mushrooms) in Malaysia- A review. J. Ethnopharmacol. 169: 441-458.
- Lawrence, R.S. (2016). Vitamin B12-responsive neuropathies: A case series. Nutr. Neurosci. 19(4): 162-168.
- Lee, S.S., F.K. Enchang, N.H. Tan, S.Y. Fung, and J. Pailoor (2013). Preclinical toxicological evaluations of the sclerotium of *Lignosus rhinocerus* (Cooke), the Tiger Milk mushroom. J. Ethnopharmacol. 147(1): 157-163.
- Lee, S.S., N.H. Tan, S.Y. Fung, J. Pailoor, and S.M. Sim (2011). Evaluation of the sub-acute toxicity of the sclerotium of *Lignosus rhinocerus* (Cooke), the Tiger Milk mushroom. J. Ethnopharmacol. 138(1): 192-200.
- Lee, S.S., N.H. Tan, S.Y. Fung, S.M. Sim, C.S. Tan, and S.T. Ng (2014). Anti-inflammatory effect of the sclerotium of *Lignosus rhinocerotis* (Cooke) Ryvarden, the Tiger Milk mushroom. BMC Complement. Altern. Med. 14: 359.
- Lu, M.C., C.H. Yao, S.H. Wang, Y.L. Lai, C.C. Tsai, and Y.S. Chen (2010). Effect of *Astragalus membranaceus* in rats on peripheral nerve regeneration: *in vitro* and *in vivo* studies. J. Trauma Acute Care Surg. 68(2): 434-440.
- Ma, C.H.E., T. Omura, E.J. Cobos, A. Latrémolière, N. Ghasemlou, G.J. Brenner, E. van Veen, L. Barrett, T. Sawada, F. Gao, G. Coppola, F. Gertler, M. Costigan, D. Geschwind, and C.J. Woolf (2011). Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. J. Clin. Invest. 121(11): 4332-4347.
- Ma, J., W. Li, R. Tian, and W. Lei. (2010). Ginsenoside Rg1 promotes peripheral nerve regeneration in rat model of nerve crush injury. Neurosci. Lett. 478(2): 66-71.
- Miranda, A., S. Peles, R. Shaker, C. Rudolph, and J.N. Sengupta (2006). Neonatal nociceptive somatic stimulation differentially modifies the activity of spinal neurons in rats and results in altered somatic and visceral sensation. J. Physiol. 572(Pt 3): 775-787.
- Montañez, S., A.E. Kline, T.A. Gasser, and T.D. Hernandez (2000). Phenobarbital administration directed against kindled seizures delays functional recovery following brain insult. Brain Res. 860(1-2): 29-40.
- Mucimapura, S., J. Wattanathorn, S. Thongrong, K. Chaisiwamongkol, and B. Sripanidkulchai (2010). *Morus alba* enhanced functional recovery after sciatic nerve crush injury. Am. J. Agri. Biol. Sci. 5(3): 294-300.
- Muthal, A.P., A.S. Morani, and S.L. Bodhankar (2008). Neuroprotective effect of ultra-high dose of

- methylcobalamin in models of mononeuropathy in rats. *Pharmacologyonline* 2: 282-295.
- Nakamura, Y., H. Shimizu, C. Nishijima, M. Ueno, and Y. Arakawa (2001). Delayed functional recovery by vincristine after sciatic nerve crush injury: A mouse model of vincristine neurotoxicity. *Neurosci. Lett.* 304(1-2): 5-8.
- Nallathamby, N., G.S. Lee, J. Raman, N. Abd Malek, S. Vidyadaran, M. Naidu, and V. Sabaratnam (2016). Identification and *in vitro* evaluation of lipids from sclerotia of *Lignosus rhinocerotis* for antioxidant and anti-neuroinflammatory activities. *Nat. Prod. Commun.* 11(10): 1485-1490.
- Nallathamby, N., C.W. Phan, S.L.S. Seow, A. Baskaran, H. Lakshmanan, S.N. Abd Malek, and V. Sabaratnam. (2018). A status review of the bioactive activities of tiger milk mushroom *Lignosus rhinocerotis* (Cooke) Ryvarden. *Front. Pharmacol.* 8: 998.
- Nishina, A., H. Kimura, A. Sekiguchi, R.H. Fukumoto, S. Nakajima, and S. Furukawa (2006). Lysophosphatidylethanolamine in *Grifola frondosa* as a neurotrophic activator via activation of MAPK. *J. Lipid Res.* 47(7): 1434-1443.
- Popa, V.I., O.F. Racasan, A.C. Margina, S. Cortan, P. Neagu, and I.T. Sebe (2016). Advances in peripheral nerve regeneration: Materials, methods, techniques. *Modern Med.* 23(4):257-269.
- Prescott, T.J., B. Mitchinson, and R.A. Grant (2011). Vibrissal behavior and function. *Scholarpedia* 6(10): 6642.
- Qu, Y., K. Sun, L. Gao, Y. Sakagami, H. Kawagishi, M. Ojika, and J. Qi (2012). Termitomycesphins G and H, additional cerebrosides from the edible Chinese mushroom *Termitomyces albuminosus*. *Biosci. Biotechnol. Biochem.* 76(3): 791-793.
- Ramli, D., I. Aziz, M. Mohamad, D. Abdulahi, and J. Sanusi (2017). The changes in rats with sciatic nerve crush injury supplemented with evening primrose oil: Behavioural, morphologic, and morphometric analysis. *Evid. Based Complement. Alternat. Med.* 2017: 3476407.
- Robin, J., R. Bradley, B.R. Buchsbaum, and M. Moscovitch (2018). The primacy of spatial context in the neural representation of events. *J. Neurosci.* 38(11): 2755-2765.
- Samberkar, S., S. Gandhi, M. Naidu, K.H. Wong, J. Raman, and V. Sabaratnam (2015). Lion's Mane, *Hericium erinaceus* and Tiger's milk, *Lignosus rhinocerotis* (higher basidiomycetes) medicinal mushrooms stimulate neurite outgrowth in dissociated cells of brain, spinal cord, and retina: An *in vitro* study. *Int. J. Med. Mushrooms* 17(11): 1047-1054.
- Seow, S.L.S., L.F. Eik, M. Naidu, P. David, K.H. Wong, and V. Sabaratnam (2015). *Lignosus rhinocerotis* (Cooke) Ryvarden mimics the neuritogenic activity of nerve growth factor via MEK/ERK1/2 signaling pathway in PC-12 cells. *Sci. Rep.* 5: 16349.
- Seow, S.L.S., M. Naidu, P. David, K.H. Wong, and V. Sabaratnam (2013). Potentiation of neuritogenic activity of medicinal mushrooms in rat pheochromocytoma cells. *BMC Complem. Alt. Med.* 13: 157.
- Seow, S.L.S., M. Naidu, V. Sabaratnam, S. Vidyadaran, and K.H. Wong (2017). Tiger's milk medicinal mushroom, *Lignosus rhinocerotis* (Agaricomycetes) sclerotium inhibits nitric oxide production in LPS-stimulated BV2 microglia. *Int. J. Med. Mushrooms* 19(5): 405-418.
- Snigdha, S., G.A. Prieto, A. Petrosyan, B.M. Loertscher, A.P. Dieskau, L.E. Overman, and C.W. Cotman (2016). H3K9me3 inhibition improves memory, promotes spine formation, and increases BDNF levels in the aged hippocampus. *J. Neurosci.* 36(12): 3611-3622.
- Turner, P.V., T. Brabb, C. Pekow, and M.A. Vasbinder (2011). Administration of substances to laboratory animals: Routes of administration and factors to consider. *J. Am. Assoc. Lab. Anim. Sci.* 50(5): 600-613.
- Valero-Cabr e, A., K. Tsironis, E. Skouras, X. Navarro, and W.F. Neiss (2004). Peripheral and spinal motor reorganization after nerve injury and repair. *J. Neurotrauma* 21(1): 95-108.
- van der Heijden, K., M. Younes, L. Fishbein, and S. Miller (1999). *International Food Safety Handbook: Science, International Regulation, and Control.* 1st Ed. Marcel Dekker, Inc; New York (The United States of America).
- Wang, Y., W. Shen, L. Yang, H. Zhao, W. Gu, and Y. Yuan (2013). The protective effects of *Achyranthes bidentata* polypeptides on rat sciatic nerve crush injury causes modulation of neurotrophic factors. *Neurochem. Res.* 38(3): 538-546.
- Watanabe, T., R. Kaji, N. Oka, W. Bara, and J. Kimura. (1994). Ultra-high dose methylcobalamin promotes nerve regeneration in experimental acrylamide neuropathy. *J. Neurol. Sci.* 122(2): 140-143.
- Wong, K.H., C.K.M. Lai, and P.C.K. Cheung (2009a). Stimulation of human innate immune cells by mushroom sclerotial polysaccharides. *Int. J. Med. Mushrooms* 11(3): 215-223.

- Wong, K.H., C.K.M. Lai, and P.C.K. Cheung (2011a). Immunomodulatory activities of mushroom sclerotial polysaccharides. *Food Hydrocoll.* 25(2): 150-158.
- Wong, K.H., G. Kanagasabapathy, M. Naidu, P. David, and V. Sabaratnam (2016). *Hericiium erinaceus* (Bull.: Fr.) Pers., a medicinal mushroom, activates peripheral nerve regeneration. *Chin. J. Integr. Med.* 22(10): 759-767.
- Wong, K.H., G. Kanagasabapathy, R. Bakar, C.W. Phan, and V. Sabaratnam (2015). Restoration of sensory dysfunction following peripheral nerve injury by the polysaccharide from culinary and medicinal mushroom, *Hericiium erinaceus* (Bull.: Fr.) Pers. through its neuroregenerative action. *Food Sci. Technol. (Campinas)* 35(4): 712-721.
- Wong, K.H., M. Naidu, P. David, M.A. Abdulla, N. Abdullah, U.R. Kuppasamy, and V. Sabaratnam (2009b). Functional recovery enhancement following injury to rodent peroneal nerve by Lion's Mane mushroom, *Hericiium erinaceus* (Bull.: Fr.) Pers. (Aphylophoromycetidae). *Int. J. Med. Mushrooms* 11(3): 225-236.
- Wong, K.H., M. Naidu, P. David, M.A. Abdulla, N. Abdullah, U.R. Kuppasamy, and V. Sabaratnam (2011b). Peripheral nerve regeneration following crush injury to rat peroneal nerve by aqueous extract of medicinal mushroom *Hericiium erinaceus* (Bull.: Fr.) Pers. (Aphylophoromycetidae). *Evid. Based Complement. Alternat. Med.* 2011: 580752.
- Yuan, Y., H. Shen, J. Yao, N. Hu, F. Ding, and X. Gu (2010). The protective effects of *Achyranthes bidentata* polypeptides in an experimental model of mouse sciatic nerve crush injury. *Brain Res. Bull.* 81(1): 25-32.
- Zhao, H.B., S.Z. Wang, Q.H. He, L. Yuan, A.F. Chen, and Z.B. Lin (2005). *Ganoderma* total sterol (GS) and GS 1 protect rat cerebral cortical neurons from hypoxia/reoxygenation injury. *Life Sci.* 76(9): 1027-1037.