HISTOLOGICAL ANALYSIS OF NERVOUS TISSUE OF SPRAGUE-DAWLEY RATS FOLLOWING SUB-ACUTE ORAL ADMINISTRATION OF *Lignosus rhinocerotis* (COOKE) RYVARDEN

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ABSTRACT

Lignosus rhinocerotis Cooke Ryvarden, known as Tiger Milk mushroom and locally knows as *cendawan susu rimau* is one of the medicinal mushroom widely used by the indigenous communities in Malaysia for various ailments. The sclerotium of the mushroom has been reported to possess anti-inflammatory, antioxidant, antimicrobial, anticancer and neuritogenic activities. This study aimed to examine if any, toxicity effect of *L. rhinocerotis* aqueous extract on nervous tissues of Sprague Dawley rats by subacute oral administration. Eighteen female rats were grouped into negative control that received 10 ml/kg body weight/day of distilled water, low dosage group and high dosage group that received 500 mg/kg body weight/day and 1000 mg/kg body weight/day of aqueous extract, respectively. Following the twenty-eight days oral administration, the rats were sacrificed, central and peripheral nervous tissues were harvested and processed for histological analysis. The gross examination of the nervous tissues of rats treated with *L. rhinocerotis* did not display any pathological changes compared to the negative control group. The microscopic features of peripheral nervous tissues namely, sciatic nerve and dorsal root ganglia and the central nervous tissues namely, cerebrum, cerebellum and spinal cord were normal. There were no abnormal clinical signs or body weight changes detected. Signs of toxicity such as neuronal degeneration and swelling or apoptosis of neurons, were not observed. No significant difference in distribution of cell bodies of sensory neurons, multipolar motor neurons, Betz cells and Purkinje cells between treated groups and negative control group were observed. In conclusion, the subacute oral administration of *L. rhinocerotis* aqueous extract had no adverse effects on nervous tissues.

Keywords: *L. rhinocerotis*, subacute, neurotoxicity, central nervous tissues, peripheral nervous tissues.

ANALISIS HISTOLOGI KETOKSIKAN SUBAKUT Lignosus rhinocerotis TERHADAP TISU SARAF TIKUS SPRAGUE DAWLEY MELALUI PENYUAPAN ORAL

ABSTRAK

Lignosus rhinocerotis (Cooke) Ryvarden, juga dikenali sebagai cendawan susu rimau merupakan cendawan ubatan yang digunakan secara meluas oleh masyarakat pribumi di Malaysia sebagai tonik untuk merawat pelbagai penyakit. Bahagian sklerotium adalah berwarna coklat kekelabuan berserta massa keras tidak sekata dan dilaporkan mempunyai aktiviti anti-radang, antioksidan, antimikrob, antikanser dan neuritogenik. Kami mengkaji (jika ada) kesan ketoksikan subakut ekstrak akueus *L. rhinocerotis* terhadap tisu saraf tikus Sprague Dawley melalui penyuapan oral secara paksa harian. Sebanyak lapan belas ekor tikus betina dikelompokkan ke dalam tiga kumpulan iaitu kawalan negatif yang menerima 10 ml/kg/hari air suling, dos rendah ekstrak dan dos tinggi ekstrak yang masing-masing menerima 500 mg/ kg berat badan /hari dan 1000 mg/kg berat badan /hari. Selepas penyuapan subakut selama 28 hari, tisu saraf pusat dan periferi diproses untuk analisis histologi. Tisu saraf periferi iaitu saraf skiatik dan ganglia akar dorsal; dan tisu saraf pusat iaitu serebrum, serebelum dan saraf tunjang menunjukkan ciri-ciri mikroskopik normal. Pemeriksaan secara kasar terhadap tisu saraf tikus yang disuap dengan *L. rhinocerotis* tidak menunjukkan perubahan patologi yang signifikan berbanding dengan kawalan negatif. Kesan ketoksikan, tanda, perubahan dalam berat badan, dan degenerasi, pembengkakan atau apoptosis neuron tidak ditemui. Tiada perbezaan yang signifikan dari segi taburan sel-sel saraf senson, motor, *Betz* dan *Purkinje* dalam kumpulan kawalan negative dan ekstrak. Kesimpulannya, penyuapan oral subakut selama dengan ekstrak akueus *L. rhinocerotis* tidak mempunyai kesan buruk ke atas tisu saraf.

Kata kunci: *L. rhinocerotis*, subacute, neurotoxicity, sistem saraf pusat, sistem saraf peripheral.

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LIST OF SYMBOLS AND ABBREVIATIONS

β	Beta
%	Percentage
ºC	Degree Celsius
µg	Microgram
µm	Micrometre
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
AAPC	American Association of Poison Control Center
A.D	anno Domini
B.C	Before Christ
CNS	Central nervous system
ERK	extracellular signal–regulated kinases
EGFR	epidermal growth factor receptor
FIP	fungal immunomodulatory proteins
IgE	Immunoglobulin E
g	Gram
h	Hour
HIV	human immunodeficiency virus
Kg	Kilogram
kDa	Killo Dalton
L	Litre
ml	Millilitre
Mg	Microgram
MAPK/MEK	Mitogen activated protein kinase
mmol	Millimolar
min	Minute
NOAEL	no observed-adverse-effect-level
N2a	neuroblastoma cell line
NIOSH	the national institute of occupational safety and health
NGF	Nerve growth factor
ORAC	oxygen radical absorption capacity
PC12	Pheochromocytoma
PNS	Peripheral nervous system
PCR	Polymerase chain reaction
RIP	ribosomal inactivating proteins
rpm	Rotation per minute
RNA	Ribonucleic acid
UV	Ultra violet
WHO	World Health Organisation
w/v	Weight/volume

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CHAPTER 1: INTRODUCTION

Mushrooms are used as food and medicine from decades and ever since then the human relationship with mushrooms is captivating. Unique taste and nutritional properties of mushrooms make them essential food for every man's plate. 65-85% of the world population depends on traditional medicine to meet their basic health care needs (Gao & Watanabe, 2011). Greeks believed that mushrooms provided strength for warriors in battle whereas Roman perceived them as "the food of Gods" (Valverde *et al*., 2015).

The mushrooms are referred as nutraceutical food because they contain all three properties of food, nutrition, physiological function and taste, and they are termed as medicinal mushrooms as their extracts may be used as therapeutic agents (Chang & Buswell, 2003). Medicinal mushroom may be an edible mushroom, while not every edible mushroom is a medicinal mushroom. Edible mushrooms are rich source of nutraceutical compounds like carbohydrates (β glucans), glycoproteins, peptides, dietary fibres, terpenes, alcohol, mineral elements, unsaturated fatty acids and antioxidants like ascorbic acid, phenolic compounds, etc (Pardeshi & Pardeshi, 2009). The wide range of biomolecules are responsible for antitumor, antioxidant, antiviral, anticarcinogenic, immunomodulatory, antiinflammation, antiaging, antihyperglycemic and neuritogenic properties (Rathore *et al*., 2017).

According to World Health Organisation (WHO), 80-90 million individuals of age 65 and above are anticipated to be affected by neurodegenerative diseases by year 2050 (Prince & Jackson., 2014). Neurodegenerative diseases are heterogenous disorders characterised by the loss of cognitive and motor function due to progressive loss of function and structure or death of neurons in the central nervous system and peripheral nervous system (Alam *et al*., 2016). The prevalence rate of the spectrum of neurological disorders in India is over 30 million patients (Gourie, 2014). Among the

neurodegenerative diseases, Alzheimer's disease is the most common neurodegenerative disorder, that affected around 35 million individuals around the world in year 2010 (Prince *et al*., 2013). In US alone, it has been marked as the 6th leading cause of death and in year 2012 it affected 5.4 million people (Firoz *et al*., 2015). In Malaysia an estimation of 0.126 % and 0.454 % of population are to be affected by Alzheimer's disease by year 2020 and 2050, respectively due to negligence for medical treatment (Tey *et al*., 2015; Nuri *et al*., 2017). Parkinson's disease is the second leading cause of age-related neurodegenerative disorder throughout the world (Shahnawaz *et al*., 2012). According to Malaysian Parkinson's Disease Association, around 15,000 to 20,000 patients suffer from Parkinson's disease in Malaysia (Oung *et al*., 2015).

The conventional drugs namely, Tacrine marketed under the trade name Cognex [R] by First Horizon and Exelon [R] by Novartis that enhances neurotransmission are used to treat neurodegenerative diseases (Sabaratnam *et al*., 2013a). These drugs do not reverse the damage done to cognitive function but delay the process of deterioration of neurons. These drugs also induce side effects like vomiting, nausea, headache, high blood pressure, kidney problems and liver disorders (Schneider, 2000).

Neuronal health is point of concern, therefore, there is an urge to search for effective drugs, especially of natural origin. Natural products are assumed to be safe and impart less side effects compared to synthetic drugs. Interest was focused on medicinal mushrooms, as neuroprotective agent.

Extensive research demonstrates that out of 14,000 species of mushrooms, approximately 2000 are regarded as edible mushrooms and, few countable edible and medicinal mushrooms do have neurotrophic properties and may promote nerve and brain health. (Chang & Wasser, 2012; Phan *et al*., 2014). The synergism of various active biocomponents in mushroom extracts showed potential in promoting neurite outgrowth

of differentiating neuroblastoma cells and nerve regeneration following injury (Wong *et al*., 2009; Phan *et al*., 2013). *Ganoderma lucidum, Grifola frondosa*, *Sarcodon scabrosus, Hericium erinaceus*, *Lignosus rhinocerotis*, *Pleurotus giganteus* and *Cordyceps militaris,* have been reported for brain and cognitive health benefits (Phan *et al*., 2013; Sabaratnam *et al*., 2013a).

Lignosus rhinocerotis (Cooke) Ryvarden is one medicinal mushroom that has been reported to stimulate neurite outgrowth (Eik *et al*., 2012; Seow *et al*., 2015). *Lignosus rhinocerotis* belonging to Polyporaceae family under Basidiomycota division is also known as tiger milk mushroom (TMM) in English and "cendawan susu rimau" in Malay language. *L. rhinocerotis* is distributed in China, Malaysia, Australia, Philippines, Sri Lanka and East Africa (Jhou *et al*., 2017). *Lignosus rhinocerotis* has the central pileus that is supported by hard woody stipe that emerges from sclerotium, that is spherical or oval or irregular shape (Figure 1.1) with no specific size (Townsend *et al*., 1954). The outer skin of the sclerotium is rough and appears greyish brown in colour, whereas the inner section has an off-white ivory granular texture with a slight milky odour (Yang & Fang, 2008).

Sclerotia is initially developed from aggregated hyphae, as it grows, the central hyphae manifest exceptional dichotomous branching and their cells become swollen and vacuolated, which facilitates in accumulating nutrition from the parental mycelia (Wong & Cheung, 2008). Traditionally, the sclerotia are purportedly effective in treating cancer, coughs, asthma, fever, and other ailments (Lau *et al*., 2015). It has been reported to possess antitumor, anti-inflammatory, antioxidative, immunomodulatory, antimicrobial, antiulcer, antiasthmatic and antiplatelet activities (Sabaratnam *et al*., 2013b; Lau *et al*., 2015; Nyam *et al*., 2016; Nallathamby *et al*., 2018).

Figure 1.1: *Lignosus rhinocerotis* mushroom (Cooke) Ryvarden.

Seow *et al*. (2015) reported for the first time that the sclerotia of *L. rhinocerotis* mimics neuritogenic activity of nerve growth factor via MEK/ERK1/2 signalling pathway in *in vitro* model. The sclerotium of *L. rhinocerotis* induced maximum neurite outgrowth in brain, spinal cord and retinal cells (Samberkar *et al*., 2015). It was shown that *L. rhinocerotis* extract possessed neuroprotective effect by reducing nitric oxide level in treated microglial cells (BV2) and had antineuroinflammatory properties (Nallathamby *et al*., 2018; Seow *et al*., 2017). The neurite outgrowth was enhanced under combination of aqueous extract of *L. rhinocerotis* with 1 µg/ml curcumin and this observation was comparable to neurite outgrowth by curcumin treatment alone (John *et al*., 2013). Owing to the broad range of ethnomedicinal properties of *L. rhinocerotis*, toxicity data is vital

before it can be developed as functional food for neuronal health. In nature, there are possibilities of existing bioactive compound with both medicinal and adverse properties. For example, cannabinoids in *Cannabis sativa* exhibit neuroprotection as well as neurotoxic effect (Sarne *et al*., 2011). This dual opposite effect may be due to different dosages of cannabinoids.

Neurotoxicity is defined as the unintended adverse effects on the structure and function of the central and peripheral nervous system at the result of exposure to various biological, chemical or physical agents. According to the studies, the neurotoxicity is considered as the major cause of neurodegenerative diseases (Soleimani *et al*., 2016).

Earlier studies have demonstrated that *L. rhinocerotis* did not display any mutagenicity, genotoxicity, teratogenicity and subacute and chronic adverse effects on visceral organs (liver, kidney, heart, spleen and lungs) (Lee *et al*., 2011a; Chen *et al*., 2013; Lee *et al*., 2013; Jhou *et al*., 2017).

However, according to our knowledge there is no information available on the neurotoxicity of *L. rhinocerotis*. Histological analysis of nervous tissues will be beneficial to examine the effects of *L. rhinocerotis* on neuronal distribution and degree of toxicity on selected nervous tissues. Research has shown the neuritogenic properties of *L. rhinocerotis*, it is anticipated that *L. rhinocerotis* can be developed as functional food against neurological disorders.

The aim of this study was to examine the possible neurotoxic effect on nervous tissues if any, following subacute oral administration of *L. rhinocerotis* to Sprague-Dawley rats.

Specific objectives were:

i. to examine the morphological features of central nervous system (CNS) namely, cerebrum, cerebellum and spinal cord of Sprague-Dawley rats treated with *L. rhinocerotis* aqueous extract.

ii. to examine the morphological features of peripheral nervous system (PNS) namely, dorsal root ganglion and sciatic nerve of Sprague-Dawley rats treated with *L. rhinocerotis* aqueous extract.

CHAPTER 2: LITERATURE REVIEW

2.1 Mushrooms

The word "Mushroom" is derived from the old French *mousseron*, which means moss, representing any fungus having a meaty fruiting body (Baker, 1989). 4600 years ago, the mushrooms were presumed as "the plant of immortality" by the ancient Egyptians according to their hieroglyphics. It's believed that diverse array of mushrooms have possible been eaten by historically humans for nutritional and medical needs for as long as the humans existed on this earth.

A mushroom is defined as a macrofungus with a conspicuous umbrella shaped fruiting body produced above or below the ground. The fruiting body of the mushrooms are large enough to be seen with the naked eyes and to be picked by hand (Chang *et al*., 1992). Mushrooms belong to the Kingdom Fungi and most of the mushrooms fall under the Division Ascomycota, the sac fungi and the Division Basidiomycota, the higher fungi (Hill, 2002).

The mushrooms are chemoheterotrophs, as they lack chlorophyll and cannot synthesis their own food by photosynthesis unlike plants. The Mushrooms take nutrients and utilises chemical energy from outer sources in order to produce energy and synthesis compounds to maintain life.

The different stages in mushroom life cycle leads to the final appearance of mushroom, which is made up of the cap pileus, the stalk and the hyphae. Mushroom propagate by spores that contains half of the genetic material. Spores germinate to form the collective hyphae called mycelium, which accumulates nutrients from the substrate and colonise substrate and develops into young fruiting body called pins primordia, and finally differentiates into cap and stalk forming fruiting bodies (Stamets, 2011).

The mushrooms have been coined to embody both the nutritional and medicinal properties, extractable from either the mycelium or mycelium culture fluids, or fruiting bodies (Chang *et al*., 1996).

2.1.1 Nutritional and medicinal value of mushrooms

Mushrooms contain 50-65% of carbohydrates namely, polysaccharides, monosaccharides, it's derivatives, oligosaccharides and small amount of mannitol and trehalose on dry weight basis (Rathore *et al*., 2017). The polysaccharides are responsible for antitumor activity by initiating the complementary and acute phase responses via activation of the cytotoxic macrophages, monocytes, neutrophils, natural killer cells, dendrites and chemical messengers in thymus-dependent immune mechanism (Wasser, 2011). The polysaccharides have ability of radical scavenging, to reduce lipid peroxidation inhibition, erythrocyte haemolysis and to increase the enzymes activities attributing to antioxidant activity of mushrooms (Kozarski *et al*., 2015). Mushroom polysaccharides also act as antiasthmatic by inhibiting IgE secretion in bronchoalveolar lavage fluid and even pocesses antibiotic, antiviral, antobesity, anti-inflammatory activities and also act as compensate for vitamin D and calcium (Friedman, 2016).

Mushrooms produce bioactive proteins like fungal immunomodulatory proteins, ribosome inactivating proteins, antimicrobial proteins, lectins, ribonucleases, laccases and other proteins (Xu *et al*., 2011). Lectins are nonimmune proteins or glycoproteins binding specifically to cell surface of carbohydrates with immunomodulatory properties, antitumoral, antiviral, antibacterial and antifungal activities (Valverde *et al*., 2015). They contain fatty acids namely, linoleic acid that has the capacity to modulate 5-lipoxygenase activity by reducing the production of 5-hydroxyeicosatetraenoic acid and over expression of five-lipoxygenase activating protein, describing the reduction of tumour growth and metastasis in animal models of breast, prostate and colon cancer (Kim *et al*., 2005).

Mushroom terpenes, well known for anti-inflammatory activity, are basically a group of unsaturated hydrocarbons and generally classified as monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids. They are responsible for pharmaceutical requirements like antimalarial, anticholinesterase, antiviral, antibacterial, antitumor, antioxidant, anti-inflammatory activities and cytotoxic activities (Duru *et al*., 2015).

Sterols from mushrooms play a key role in prevention of cardiovascular diseases and arthritis. The major sterol is ergosterol which displays antioxidant properties in edible mushrooms (Guillamon *et al*., 2010). The phenolic compounds, tocopherol, ascorbic acid and carotenoids extracted from various species of mushroom possess antihypercholesterolemic, antiviral, antimicrobial, antiatherogenic, antithrombotic, cardioprotective and anticancer activities, and ameliorate the toxic effect of chemotherapy and radiotherapy (Valverde *et al*., 2015). They are also rich in potassium, magnesium, calcium and phosphorus, a suitable replacement option for hypersensitive people in their vegetable food diet (Rajarathnam *et al*., 1998).

Many scientific publications have reported on bioactive components and medicinal value of mushrooms namely, *Ganoderma lucidum, Agaricus bisporus, Lentinula edodes, Hericium erinaceus* and *Pleurotus species* (Table 2.1).

Table 2.1: Bioactive components and medicinal values of mushrooms

Mushroom species	Bioactive components	Medicinal value	References
Ganoderma lucidum	Triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids and proteins.	Antimicrobial, antitumor, antidiabetic, antiandrogen, antioxidant, antiaging, antifibrotic, and immunomodulation property.	Tong *et al*. (2008), Sanodiya *et al*. (2009), Weng *et al*. (2009) and Chen *et al*. (2017).
Agaricus bisporus	Carbohydrates, fats, dietary fiber, sugars, protein, water, vitamin B5, vitamin B2, vitamin B3, vitamin C, iron, amino acids and ash contents	Antioxidant, immunomodulatory properties, antitumoral, antiviral, antibacterial, and antifungal activities	Ying *et al*. (1987), Chang *et al*. (2007), Ren *et al*. (2008), Guillamon *et al*. (2010), Jeong *et al*. (2010) and Xu *et al*. (2011).
Lentinula edodes	Carbohydrates, protein, essential amino acids (ergothioneine), dietary fibres, vitamins and free sugars	Antimicrobial, antifungal, antilipidemic, antihypertensive, antitumor, anticancer and immunomodulatory properties.	Dubost *et al*. (2006), Hobbs (2000), Bisen *et al*. (2010) and Gao *et al*. (2018).
Hericium erinaceus	Polysaccharides, proteins, lectins, phenols, hericenones, erinacines and terpenoids	Anticancer, antioxidant, anti-hypertensive, anti-microbial, anti-diabetic, gastro-protective, immuno-modulating, and wound-healing properties.	Mizuno, (1995), Kim *et al*. (2013), Wong *et al*. (2011a), Duru *et al*. (2015), Li *et al*. (2015), Li *et al*. (2017) and Wang *et al*. (2018).
Pleurotus species	Proteins, carbohydrates, fats, ash, crude and dietary fiber and essential amino acids	Antihypertensive, anti-inflammatory, antimicrobial, antinociceptive, antiviral, antiaging, immunomodulatory and cytoprotective.	Kanagasabapathy *et al*. (2014), Al-Bahrani *et al*. (2017), Carrasco *et al*. (2017) and Finimundy *et al*. (2013).

2.1.2 Mushrooms for neuronal health

Neurodegenerative diseases characterised by the loss of cognitive and motor functions due to malfunctioning of neurons in brain and spinal cord are the most widespread diseases (Prince & Jackson, 2014). Searching for an effective drug with less side effects unlike conventional drugs has gained extensive attention. Among the various biological activities, a number of mushrooms are reported to have activities related to brain and nerve health.

The *hericenones* from fruit bodies and *erinacines* from mycelium of *H. erinaceus* exhibit stimulation of nerve growth factor (NGF) (Kawagishi *et al*., 2008). In an *in vitro* study, the extract from fruiting bodies of *H. erinaceus* enhanced normal development of cultivated cerebellar cells as well as regulatory effect on the process of myelin genesis (Kolotushkina *et al*., 2003). Further, in an *in vitro* study, the extract exhibited neurotrophic action and improved myelination process in mature fibers of neuronal cells (Moldavan *et al*., 2007*).* Later, Phan *et al*. (2014) showed the neurotrophic activity of benzyl alcohol derivatives isolated from the fruiting bodies of *H. erinaceus* in rat pheochromocytoma PC-12 cells.

Hericium erinaceus has been extensively tested in *in vivo* for regeneration of peripheral nerve injury. In the behavioural experiment analysed by walking-track and toe-spreading reflex to access the functional recovery enhancement after peripheral nerve injury in rats treated with *H. erinaceus* showed improved peroneal functional index (PFI) and toe spreading reflexes than non-treated group (Wong *et al*., 2009). In an *in vivo* study*,* oral administration of aqueous extract of *H. erinaceus* promoted nerve regeneration via Akt and MAPK signalling pathways in an injured peroneal nerve of rat (Wong *et al*., 2011b). Further, Wong *et al*. (2015) demonstrated that the polysaccharides from fruiting bodies of *H. erinaceus* were capable of accelerating sensory functional recovery after peripheral nerve injury and the effect involves the activation of protein kinase signalling

pathways and restoration of blood-nerve barrier. *Hericium erinaceus* extract triggered neurite outgrowth by 20.1%, 22.4% and 21.7% in brain, spinal cord and retinal cells, respectively (Samberkar *et al*., 2015)

Two new lanostane triterpenes, named methyl ganoderate A acetonide and *n*-butyl ganoderate H extracted from fruiting bodies of *G. lucidum* were examined to exhibit anti acetylcholinesterase activity and may be a suitable drug for treatment of Alzheimer's disease and related neurodegenerative diseases (Lee *et al.*, 2011b). Huang *et al*. (2017) demonstrated that the polysaccharides from *G. lucidum* enhanced neurogenesis therefore, serve as a regenerative therapeutic agent for the treatment of cognitive decline associated with neurodegenerative diseases.

Aqueous extracts of *G. lucidum, Pleurotus giganteus* and *Grifola frondosa,* and ethanol extract of *Cordyceps militaris* exhibited neurite outgrowth in mouse neuroblastoma N2a cells by 38.4%, 33.4%, 33.7% and 35.8%, respectively. Therefore, these mushrooms may be utilised for brain and cognitive health (Phan *et al*., 2013). Vitamin D extracted from the *A. bisporus* enhanced cognitive function and can be used in the treatment of memory-impairment in dementia (Bennett *et al*., 2013).

One of the medicinal mushroom with broad range of medicinal properties, *Lignosus rhinocerotis* is extensively studied as emerging drug in management of neurodegenerative diseases.

2.2 Lignosus rhinocerotis

Lignosus rhinocerotis (Cooke) Ryvarden Tiger milk mushroom: also known as *Polyporus rhinocerus* (Cooke) or *Fomes rhinocerus* (Cooke), belongs to the polyporaceae family (Ainsworth, 2008). *L. rhinocerotis* has different morphology from polyporaceae, it consists of cap on central stem and grows from the sclerotium in the ground rather than

from decaying wood like other polypores. It is widely distributed in Australia, Papua New Guinea, Borneo, Philippines, Indonesia, Malaysia, Sri Lanka and Vanuatu (Ryvarden *et al*., 1980).

Traditionally, the Semang tribe believed that each animal has corresponding soul plant and tiger milk mushroom contained the soul of unborn tiger cub (Toporov., 1985). It was presumed that the *L. rhinocerotis* grows from the place where the prowling tigress has spilled its lactation milk, and it is said that the sclerotia resembled to the coagulated tiger's milk, hence called as tiger milk mushroom (Ridley, 1890).

L. rhinocerotis was first recorded for its usage dating back to 1664, is a traditionally medicinal mushroom in Malaysia, and locally known as "cendawan susu rimau" (Tan *et al*., 2012). Laderman (1987) described the usage of *L. rhinocerotis* as medicine by Malay women during postpartum period.

Based on this historical record and presumption that *Lac tygridis* is referred to *Lignosus* species, then it can be extrapolated that tiger milk mushroom is traditionally been used for more than 350 years. The genus *Lignosus* is composed of six species namely, *Lignosus dimiticus, Lignosus ekombitii, Lignosus goetzii* (Henn.)*, Lignosus. rhinocerotis* (Cooke), *Lignosus sacer* (Afzel. ex Fr.) and *Lignosus hainanensis* (Ryvarden, 1972; Ryvarden, 1975; Douanla & Langer, 2003; Cui *et al*., 2011).

Earlier it was hard to encounter and harvest mushrooms as they were only available in jungle, which served as barrier for investigation on biological properties of mushrooms (Gupta *et al*., 2015). However, now it has been successfully domesticated using readily available lignocellulosic agroresidues and brewery yeast as the nitrogen source (Abdullah *et al*., 2013). The Ligno Biotech Sdn Bhd, Selangor, Malaysia, been successfully cultivating tiger milk mushroom, since 2009 with their own indoor technology using mycelium.

Figure 2.1: Sclerotia of *L. rhinocerotis.*

The indigenous community in Malaysia used to boil the sliced sclerotia (Figure 2.1) and prepared the decoction and drink as medicine to treat various aliments like cough, asthma, fever, cancer, food poisoning, to enhance energy and increase alertness (Lee *et al*., 2009; Tan *et al*., 2009).The Chinese physicians have been using *L. rhinocerotis* sclerotium as expensive folk medicine to treat liver cancer, chronic hepatitis and gastric ulcers (Wong *et al*., 2008). The sclerotia is an aggregated hypha containing nutrition accumulated from mycelium, which is irregular in shape and greyish brown in colour from outside and white colour from inside (Wong & Cheung, 2008). The sclerotium of *L. rhinocerotis* is rich in carbohydrates with low fat content, proteins, water soluble substances and phenolic compounds (Yap *et al*., 2013). Hence, possess broad range of medicinal properties Table 2.2. Moreover, the stipe and the pileus have been reported to contain carbohydrates, proteins, fatty acids, fibre, ash, minerals and vitamins (Lau *et al*., 2013).

Table 2.2: Medicinal properties of sclerotia of *L. rhinocerotis*

Medicinal properties	**Experiment**	**References**
Antiplatelet	*in vitro*, fibrin plate assay	Sabaratnam *et al*. (2013b)
Antimicrobial	disk diffusion test	Mohanarji *et al*. (2012) .
Antiviral	plague reduction assay	Kavithambigai *et al*. (2013)
Immunomodulatory effect	*in vivo* study on BALB/c mice	Wong *et al*. (2011a).
Antitumor, antifungal, anti- HIV and anticancer.	*in vitro* study on cancer cell line; *in vitro* on promyelocytic leukemic cells (HL-60) and breast cancer cell line.	Lai *et al*. (2008), Wong *et al*. (2010) and Lee *et al*. (2012).
Gastrointestinal health, Antiobesity, hepatoprotective properties and antiulcer	*in vitro* culture; *in vivo* study on hamsters; *in vivo* study on rats	Gao *et al*. (2009) and Hoe (2014).
Antidiabetic and antiasthmatic	*in vivo* study on rats induced with streptozotocin; asthma induced rats	Nyam *et al*. (2017) and Nallathamby *et al*. (2018).
Antiproliferation, anti-inflammatory and antioxidant	*in vitro* study on human colorectal cancer cells (HCT 116) and antioxidant test.	Suziana *et al*. (2013), Yap *et al*. (2013), Zaila *et al*. (2013) and Keong *et al*. (2016).
Neuritogenic properties	*in vitro* study	Eik *et al*. (2012), Phan *et al*. (2013), Seow *et al*. (2015), Seow *et al*. (2017) and Wong *et al*. (2017).

2.2.1 *L. rhinocerotis* on neuronal health

Eik *et al*. (2012) reported for the first time that the sclerotia of *L. rhinocerotis* contained NGF like compounds that induced 24.4% and 42.1% of neurite outgrowth at 20 µg/ml (w/v) of aqueous extract alone and a combination of 20 µg/ml (w/v) aqueous extract and 30 ng/ml (w/v) of NGF in rat PC-12Adh cell line, respectively. The neurite outgrowth was enhanced to 27.2% with combination of aqueous extract with 1 µg/ml curcumin and this observation was comparable to 29.5% of neurite outgrowth by 10 µg/ml curcumin treatment alone (John *et al*., 2013). Also, Phan *et al*. (2013) observed that the aqueous extract of *L. rhinocerotis* promoted neurite outgrowth in differentiating N2a cells by 38.1%.

The cell viability and neuritogenic effects of sclerotia of *L. rhinocerotis* hot aqueous and ethanolic extracts, and crude polysaccharides were explored (Seow *et al*., 2015). The hot aqueous and ethanolic extract, and crude polysaccharides stimulated neuritogenesis but did not stimulate the production of NGF in rat PC-12 cells and were non-cytotoxic to PC-12 cells. It was also noticed that the hot aqueous extract (25 µg/ml) exhibited neuritogenic activity comparable to NGF (50 ng/ml) in rat PC-12 cells.

In an *in vitro* study on BV2 microglial cell line, 500 µg/ml of hot aqueous extract of *L. rhinocerotis* sclerotium inhibited the production of nitric oxide by 88.95% and no cytotoxic effects were observed (Seow *et al*., 2017; Wong *et al*., 2017).

Owing to the broad range of medicinal properties of *L. rhinocerotis*, toxicity data is important before it can be developed as functional food for treatment of neuronal and other disorders.

2.2.2 Importance of toxicity screening

World Health Organisation (WHO) has proclaimed that 72.5% of world's population especially Asian and African countries depends on the folk medicine to meet their basic health care needs like cough, cold, headache and gastroenteritis (Gao & Watanabe, 2011). Folk medicines generally do not produce immediate relief to the symptoms of the disorder but hold a long history of use due to perception of its safety and no side effects.

Not only mushrooms hold popularity as folk medicines, but the medicinal herbs are also used for prevention, improvement and treatment of diseases from thousands of years (Firenzuoli & Gori, 2007). Even in the modern world, herbs have gained popularity, for instance, there were around 30,917 hits on herbal medical product with 2,700 published data on PubMed in year 2013 (Pelkonen *et al*., 2014).

There are emerging issues in safety administration of folk medicines with the increased use of the folk medicine by people. Despite the beneficial properties of natural products, there are also few toxic natural products with harmful effects. Toxicity from natural product is mostly due to the following reasons (Nasri & Shizad, 2013):

i. Misidentification of edible natural products from toxic products.

The ingestion of toxic herbs or mushrooms is mostly due to careless harvesting, accidental consumption by children or confusion between edible and toxic herbs or mushrooms. The presences of aristolochic acid in medicinal herbs has resulted in adverse renal effects and renal toxicity (Asif, 2012). Patients with chronic illness taking combination of drug like warfarin with herbs like ginger or garlic containing salicylate for long duration of time may lead to serious consequences like intracranial hematoma (Izzo & Ernst, 2001).

The scientific definition of the toxic mushroom is any mushroom containing amatoxins, orellanine and gyromitrin, where amatoxin is a cyclic octapeptides that inhibits transcription by blocking RNA polymerase II, resulting in impaired protein formation and cell death (Garcia *et al*., 2015). Examples of toxic mushrooms are *Clitocybe* and *Inocybe* species, and *Psilocybe* and *Panaeolus* species (magic mushrooms), these mushrooms contain neurotoxins like muscarine and psilocybin which may lead to neurotoxicity (Persson, 2012).

Horibe *et al*. (2010) isolated toxic isolectins from the toxic mushroom *Boletus venenatus* which causes severe gastrointestinal problems such as nausea, repetitive vomiting, diarrhoea and stomach-ache

ii. Presence of toxic compounds in combination with bioactive compounds.

Not only toxic mushrooms but also edible mushroom contain toxic ingredients. Like, *Termitomyces microcarpus* widely consumed by the people of Kenya for its good flavour and in treatment of cancer and HIV/AIDS, also contains cytotoxic ergostane in minute concentration (Nakalembe *et al*., 2015; Njue *et al*., 2018).

Administration of *A. bisporus* at 3, 6 or 9 g/kg body mass of mice for 5 days resulted in increase of plasma bilirubin concentrations though there were no pathological changes observed in microscopic sections of liver and muscles. This result indicates that *A. bisporus* may cause hepatotoxic effects and should be taken care while using as functional food (Nieminen *et al*., 2009).

iii. Incorrect preparation

Storage, processing and extraction are one of the important factors that influences the qualitative and quantitative chemical profile of folk medicines (Shaw *et al*., 2012). Incorrect preparation of herbal extracts has been reported for unexpected toxicity of herbal products (Shaw, 2010). Several toxicity reports on Chinese herbal medicines are either due to incorrect usage of herb (overdosage

and incorrect herbal combination) or inadequate processing of herbs (Zhang *et al*., 2009). Gawlikowski *et al*. (2015) reported on toxicity of edible mushrooms due to incorrect storage and preparation.

iv. Excess dosage of administration

The history of toxicology begins with Paracelsus (1493-1541) also well known as "the father of toxicology" had once stated that "Poison is in everything; no thing is without poison, the dosage makes it either a poison or a remedy" (Dolan *et al*., 2010).

Even the water if consumed in large amount (4-5 litres) in short duration of time (2-3 hours) may result in water intoxication including hyponatraemia following cerebral edema, seizure, coma and death (Farrell & Bower, 2003).

Agaricus bisporus well known for immunomodulating and anti-tumour activities is toxic at higher concentration 4000 mg/kg body weight (Jeong *et al*., 2012; Dhamodharan *et al*., 2013).

The toxicities of herbs and mushrooms associated with promising medicinal and nutritional value indicates the importance of systematic evaluation of the toxicity data of the any folk medicines. The safety assessment of several edible mushrooms: were performed using analytical data combined with in silico toxicology evaluation for safety consumption of fungal dietary ingredients (Vander *et al*., 2015). The toxicology report on the chiefly consumed medicinal mushrooms are listed in Table 2.3.

Table 2.3: Toxicity data on mushrooms

Mushroom	Duration	Dosage	Toxicity	References
G. lucidum	90 days	300, 600 and 1200 mg/kg	No adverse effects on renal function and other organs	Wihastuti *et al*. (2015).
	13 weeks	2000 mg/kg/day	No adverse effect on growth, ophthalmoscopy, haematology, clinical investigation and non-mutagenicity	Chen *et al*. (2011).
A. bisporus	Acute	2500 mg/kg body weight	No adverse effect on general behaviour and non-mutagenicity	Dhamodharan *et al*. (2013) and Von *et al*. (1982).
L. edodes	-	-	non-genotoxicity	De Lima *et al*. (2001).
	Acute	2000 mg/kg/day	No adverse effects and no mortality rate	Yoshioka *et al*. (2009).
H. erinaceus	Acute	5 g/kg	No adverse effects	Wong *et al*. (2013).
	Subacute	3 g/kg	No adverse effects on urinalysis, haematology, serum biochemistry parameters a	Li *et al*, (2014a).
	Sub-chronic	1000 mg/kg/ body weight	No adverse effect on the general behaviour, body weight, haematology, clinical biochemistry and no histopathological changes	Li *et al*. (2014b) and Lakshmanan *et al*. (2016).
Pleurotus ostreatus	Acute	5000 mg/kg/body weight	No adverse effects	Deepalakshmi *et al*. (2014)
Pleurotus sajor-caju	Acute	400 mg/kg	No adverse effects	Ademola *et al*. (2017)

2.2.3 Toxicity data on *L. rhinocerotis*

Lee *et al*. (2011a) pioneered the toxicity study of *L. rhinocerotis*. Oral administration of sclerotia of *L. rhinocerotis* (TM02) had no adverse effects on haematological, growth rate and clinical biochemical parameters, and micrographs of liver, spleen, lung, kidney and heart did not show any pathological alterations. Further, in a chronic toxicity test on female and male rats following oral administration of 250, 500, 1000 mg/kg of sclerotia of *L. rhinocerotis* (TM02) had no observed-adverse-effect-level (NOAEL) (Lee *et al*., 2013).

In an *in vitro* study for cytotoxicity screening of *L. rhinocerotis* using mouse embryonic fibroblast (BALB/3T3) and N2a cell lines, the results expressed as IC_{50} values (concentration resulting in 50% inhibition of cell growth and proliferation after 24 h exposure) did not show cytotoxicity effect on cell lines (Phan *et al*., 2013). In a comparison study of hot aqueous and cold aqueous extracts of sclerotia of *L. rhinocerotis* on cellular toxicity, the cold aqueous extract exerted stronger cytotoxicity on solid tumour cell line but also on normal cell line, whereas the hot aqueous extract was non-cytotoxic to normal cell line and exhibited comparable cytotoxicity to solid tumour cell line (Lau *et al*., 2013).

The mutagenicity and genotoxicity effect of *L. rhinocerotis* was studied in Ames test using *Salmonella typhimurium* strains, an *in vitro* chromosome aberration test in Chinese hamster ovary (CHO-K1) cells and an *in vivo* erythrocyte micronucleus test in ICR mice, the results confirmed that *L. rhinocerotis* neither displayed mutagenicity nor genotoxicity (Chen *et al*., 2013).

For teratogenicity, pregnant Sprague-Dawley female rats were orally administrated with *L. rhinocerotis* mycelium at dosages of 850, 1700 and 3400 mg/kg/day and parameters like mortality, bodyweight and clinical signs were observed in pregnant rats

and foetus following gross necropsy on gestation day 20 (Jhou *et al*., 2017). The results showed that *L. rhinocerotis* at 3400 mg/kg/day neither displayed teratogenicity in foetus nor toxicity in dams. However, there is no information on neurotoxicity of *L. rhinocerotis*.

2.2.4 Relevance of histology study for toxicity

Histology is the field of study of the microscopic structure of cells and tissues and the ways in which individual biological components are structurally and functionally related (Lowe & Anderson, 2014). Fixation, embedding and staining are various steps involved in preparation of histological sections that can be studied with the aid of light microscope.

Histological examination of the tissues is an essential technique to evaluate the safety of novel drugs (Greaves, 2011). Karikoski, (2011) reported that histological examination of placenta helps to reveal ischemic changes, inflammation of the placenta and fetal membranes, which may be a threat to that foetus and cause later complications during childhood. Histology was one of the fundamental parameter used to evaluate the *in vivo* toxicity effect of *H. erinaceous* and *L. rhinocerotis* (Lee *et al*., 2011a; Lakshmanan *et al*., 2016).

The first step in histology is fixation, a process of using chemical methods to preserve tissues (Carter & Shieh, 2015). The simple and most commonly used fixative is 10% neutral buffer formalin which forms cross linkage between proteins (Junqueira *et al*., 1992). Davidson's fixative solution which is composed of ethyl alcohol, 10% neutral buffer formalin and glacial acetic acid is widely used in preservation of tissues for toxicological analysis (Latendresse *et al*., 2002). Davidson's fixative solution has rapid rate of reaction and is simple to use without disposable problems like formalin, therefore provides no additional safety hazards (Kelder *et al*., 2008).

In comparison to formalin fixative, the Davidson's fixative solution has been proven to be the best fixative for staining, producing sharp cellular morphology of tissues and

easy sectioning without breakages in tissue areas (McKay *et al*., 2009). Davidson's solution was proven to be excellent preservative for retinal tissues than 10% formalin and Bouin's fluid (Latendresse *et al*., 2002; Shariati *et al*., 2008).

Once the tissues are fixed, embedded and fixed, they have to be stained. Tissues are colourless, with very few exceptions, which makes it difficult to observe under the light microscope without staining. The haematoxylin and eosin stain the nucleus or nucleic acids with dark blue colour and proteins or cytoplasm with pink or orange colour, respectively (Cardiff *et al*., 2014).

The haematoxylin and eosin technique has been the most universal and traditional staining method for examination of formalin fixed and paraffin embedded tissue sections (Sarnat & Carpenter, 2014). This is centrally used staining technique to identify the gross structure of the tumor and to evaluate major parameters such as the nuclear/cytoplasmic ratio of lymphoma tumor cells (Amalraj & Naeim, 2012). The haematoxylin and eosin staining is the most commonly used to staining technique for toxicology study. The toxicity effects of mushrooms on organs namely, heart, liver, kidney, spleen, lung and brain were studied with the aid of haematoxylin and eosin staining (Lakshmanan *et al*., 2016; Lee *et al*., 2011a). Therefore, histological analysis will be valuable to assess the neurotoxicity effect of *L. rhinocerotis*.

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of aqueous extract

The freeze-dried cultivated sclerotium of *L. rhinocerotis* (Figure 3.1) marketed as Ligno TM02 (Batch No. PL/16/014-1215) was purchased from Ligno Biotech Sdn Bhd, Selangor, Malaysia.

Figure 3.1: Freeze-dried powder of sclerotia of *L. rhinocerotis*.

Aqueous extraction was done according to the protocol (Seow *et al*., 2015) with slight modifications in terms of duration of centrifugation. The *L. rhinocerotis* sclerotium powder was weighed and mixed in distilled water with a sample-to-water ratio of 1:5…0 (w/v). The mixture was agitated in the shaker at 50 rpm at 45°C for 24 h (Seow *et al*., 2015). Following incubation, the mixture was double boiled in a 100°C water bath for 30 min. After cooling at 24°C, the mixture was centrifuged at 10,000 rpm at 4°C for 30 min. The vacuum filtration was conducted using Whatman filter paper No.4 to collect supernatants. Finally, the supernatants were freeze dried at -50°C for 48 h. The resultant fluffy white product was stored in a -20°C freezer prior to further use.

3.2 Principals of rat grouping

The *in vivo* rat experiment study was done with approval by the Institutional Animal Care and Use Committee, Faculty of Medicine, University of Malaya (Ethics Ref No 2013-10-08/ANAT/R/WKH). Eighteen adult female Sprague-Dawley rats with body weight ranging from 180 to 200 g (Figure 3.2) were purchased from Animal Experimental Unit (AEU), Faculty of Medicine, University of Malaya. The rats had *ad-libitum* access to food pellet and drinking water. Oral administration of distilled water and aqueous extract of *L. rhinocerotis* was carried out for 28 days.

Figure 3.2: Female Sprague-Dawley rats weighing 180-200 g.

The subacute and chronic toxicity test of *L. rhinocerotis* at dosages 250, 500 and 1000 mg/kg did not display any adverse effects on other tissues namely, liver, spleen, lung, kidney and heart (Lee *et al*., 2011a; Lee *et al*., 2013). Considering the previous toxicity data, 500 and 1000 mg/kg body weight/day were selected as the two dosages to observe any adverse effects if any of the aqueous extract on nervous tissues of rats administrated orally for a period of 28 days.

Eighteen rats were randomly assigned to three groups of six rats each.

i. Negative control group that received oral administration of 5 ml distilled water/kg body weight/day.
ii. Low dosage group that received oral administration of 500 mg *L. rhinocerotis* aqueous extract/kg body weight/day.
iii. High dosage group that received 1000 mg *L. rhinocerotis* aqueous extract/kg body weight/day.

After weighing the extract to feed 6 rats per group that is 0.6 mg for low dosage group and 1.2 mg for high dosage group, the extract was dissolved in 6 ml of distilled water. So, every individual rat received 1 ml of extract solution which was orally administrated with the aid of oral gavage feeding needle.

3.3 Effect of *L. rhinocerotis* on body weight

The body weight (g) of each rat was measured on the first day of treatment with distilled water and aqueous extract of *L. rhinocerotis* at dosage of 500 and 1000 mg/kg body weight/day. The measurement of the body weight was continued on weekly basis during 28 days of treatment.

3.4 Effect of *L. rhinocerotis* on nervous tissues

3.4.1 Perfusion

After 28 days of oral administration, the rats were anesthetized by intraperitoneal injection with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). The anesthetized rat was placed on surgical board and incision was made with scalpel through abdomen the length of diaphragm. The rib cage was cut to expose heart. Then, perfusion was carried out by introducing the butterfly catheter with needle in the left ventricle of the heart (Morawietz *et al*., 2004). Simultaneously, a cut was made in the right atrium with sharp

scissor and phosphate buffer saline (Appendix B.1) was allowed to flow freely. Finally, fixed with Davidson's solution (Appendix B.3).

3.4.2 Harvesting of nervous tissues

The nervous tissues namely, the dorsal root ganglion and sciatic nerve of the peripheral nervous system and the cerebrum, cerebellum and spinal cord of the central nervous system were harvested after perfusion. The brain and spinal cord (Figure 3.3) were fixed with Davidson's solution after removing skull. The dorsal root ganglion was obtained at lumbar 4 and lumbar 5. The tissues were fixed in the Davidson's solution (Latendresse *et al*., 2002) for 24 h followed by 10% neutral buffer formalin (Appendix B.2).

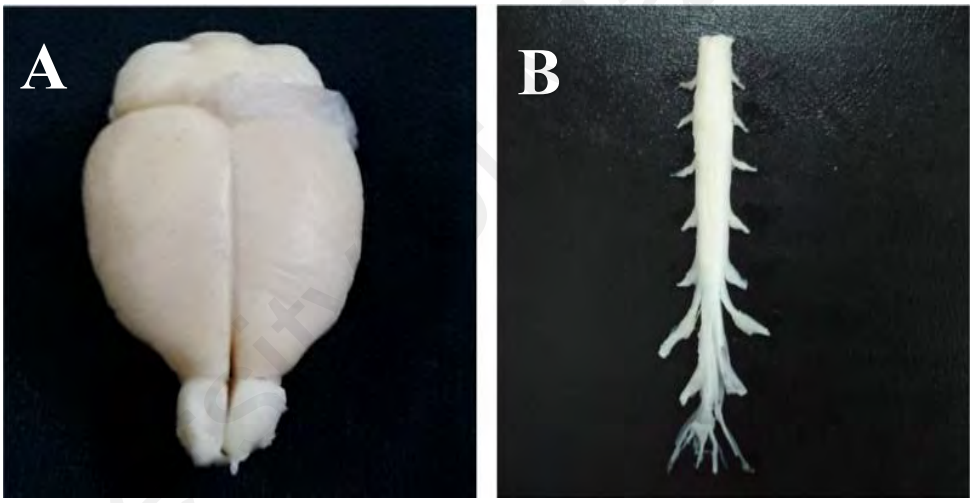

Figure 3.3: Harvested (A) Brain (superior view) and (B) Spinal cord with DRG.

3.4.3 Tissue processing of nervous tissues

The tissues were dehydrated with series of increasing concentration of alcohol (50 – 100 %) at different time duration depending on the size of tissues (Carleton *et al*., 1980). The smaller size tissues were dehydrated (dorsal root ganglion and sciatic nerve) for 30 min each and larger tissues (cerebrum, cerebellum and spinal cord) for 1 h each, respectively. Then the tissues were immersed in a solution containing equal part of cedar wood oil and alcohol for an interval of 1 h and 2 h for smaller and larger tissues,

respectively. The tissues were placed in pure cedar wood oil overnight for clearing purpose.

3.4.4 Embedding and sectioning

The processed tissues were infiltered in histological wax (PARAPLAST™). The tissues were immersed in equal part of solution containing paraffin wax and benzene followed by series of four paraffin wax jars at melting point 50º - 60ºC (Norton & Isaacson, 1989). The tissues are placed in four different wax jars for proper impregnation. The smaller size tissues were immersed for 30 min each and larger tissues for 1 h each. Later, the tissues were embedded in paraffin wax blocks and microtome sectioned at 5 µm thickness (Figure 3.4).

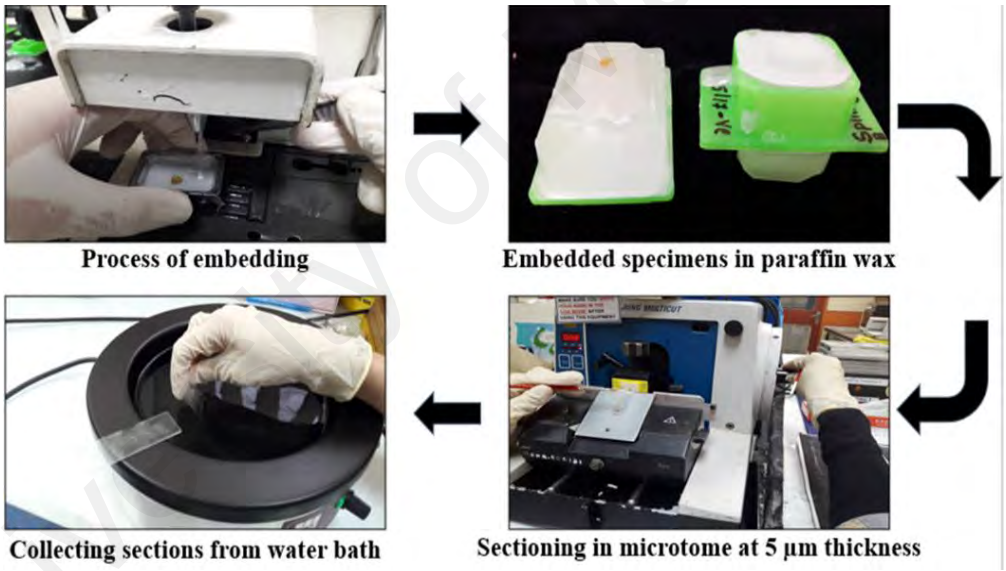

Figure 3.4: Tissue processing and embedding. Pictures captured during laboratory work.

3.4.5 Hematoxylin and eosin staining

The tissue sections were stained in hematoxylin (Appendix B.4) and eosin (Appendix B.5) staining in accordance with Bancroft & Cook (1984). The tissue sections were deparaffinised with series of three xylene for 3 min each and hydrated with decreasing concentration of alcohol (100% - 50%) for 2 min each and water for 3 min. The tissue sections were immersed in hematoxylin for 20 min and then washed in water to remove

excess of hematoxylin. Then, the tissue sections were differentiated by dipping in acid alcohol.

For bluing step (the conversion of initial soluble red colour of hematoxylin within the nucleus to insoluble blue colour in alkaline medium), the tissue sections were washed in running tap water for 5 min. Later, the tissue sections were placed in eosin for 5 min and dehydrated with increasing concentration of alcohol (95%-100%) for 2 min each. Finally, the tissue sections were cleared in series of two xylene for 3 min each and the tissue sections on glass slides were mounted with DPX (a mixture of distyrene, a plasticizer, and xylene). The prepared slides were examined for the evidence of toxicity using a light microscope.

3.5 Effect of *L. rhinocerotis* on distribution of neuronal cells

The total number of specific cells namely, cell bodies of sensory neurons, Purkinje cells, Betz cells and multipolar motor neurons in tissue section of fixed area were counted manually. A section of tissues on a microscope slide is assumed to be a two-dimensional sample plane cut though a three-dimensional structure (Saladin, 2007).

Images were captured and analysed with Nikon Eclipse 80i upright microscope equipped with a digital colour camera controller (DS-5Mc-U2), neutral density filter and NIS-Elements algorithm that enables maximum accuracy, resulting in ultra-high-resolution images (NIS-Elements Advanced Research, Nikon, Japan). Images were captured at 40X magnification.

The total number of cell bodies of sensory neurons in dorsal root ganglion, Betz cells in cerebrum cortex, Purkinje cells in cerebellum cortex and motor neurons in ventral horn of spinal cord were counted manually in the randomly selected area of fixed dimension 217.426 × 162.542 µm. The average number of cells in five sections of tissues per group was noted and compared with average number of cells in five sections of other groups to determine the quantitative difference.

3.6 Statistical analysis

Statistical analysis was performed using IBM SPSS (Statistical Package for Social Sciences) statistics 25. All data was expressed as mean ± standard deviation. Body weight data of six rats per group and histological data of five tissue sections per group were subjected to one-way analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) was used to determine the statistical difference between means of negative control and treatment groups. All differences were considered statistically significant at the $P < 0.05$ level.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Preparation of aqueous extract

In present study for evaluating the subacute toxic effect if any of *L. rhinocerotis*, the hot water extraction of sclerotia of *L. rhinocerotis* was prepared. The freeze dried hot aqueous extract was fluffy light brown product (Figure 4.1). The yield was 4 g of aqueous extract per 25 g of *L. rhinocerotis* freeze-dried powder.

Figure 4.1: Hot aqueous extract of freeze-dried powder of sclerotia of *L. rhinocerotis.*

The cell wall of the mushroom composed of chitin is indigestible by humans yet contains potent immune stimulating compounds the glucans (Stengler, 2005). Hot water extraction is the only proven and traditionally used method for breaking down the chitinous cell wall and releasing the bioactive compounds structurally intact and undamaged, and removes indigestible fibre releasing the bioactive compounds (Stengler, 2005; Lakshmanan *et al*., 2016).

Various extract preparations display different biological activities due to difference in their chemical composition. In antioxidant study of *Inonotus obliquus,* hot water extract

exhibited strong radical-scavenging activity than ethanol extract (Hu *et al*., 2009). The polysaccharides extracted with hot water extraction method from *Pleurotus tuber regium* showed high degree of *in vivo* and *in vitro* antitumor activities than that obtained by ultrasonication (Zhang *et al*., 2004)

Lau *et al*. (2013) reported that hot aqueous extract preparation released more carbohydrates and proteins than cold water extract and displayed selective cytotoxicity against cancer cell lines unlike cold water extract. However, the other extracts namely, cold water extract showed high degree of cytotoxicity against cancer cell lines and cytotoxicity components were thermolabile and water-soluble peptides (Lau *et al*., 2013; Cheung & Cheung, 2005).

4.2 Effect of aqueous extract of *L. rhinocerotis* on body weight

Body weight was measured continually on weekly basis during 28 days of treatment (Appendix D, Table D.1). Normal body weight gain was observed in both treated and negative control groups throughout the experimental period (Figure 4.2). On the day 0 the rats in low dosage and high dosage groups weighed 216 ± 18.51 g and 208 ± 28.21g, which consequently increased. On the 28th day the rats in low dosage and high dosage groups weighed 260 ± 46.13 g and 261.3 ± 20.47 g. In the negative control group, the rats weighed 210 ± 36.54 g and 256 ± 27.89 g on the first day and the 28th day, respectively. The rats treated with aqueous extract of *L. rhinocerotis* at 500 and 1000 mg/kg body weight/day and negative control group showed normal weight gain. No significant difference ($p > 0.05$) in the body weight gain was observed in control and treated rats (Figure 4.2)

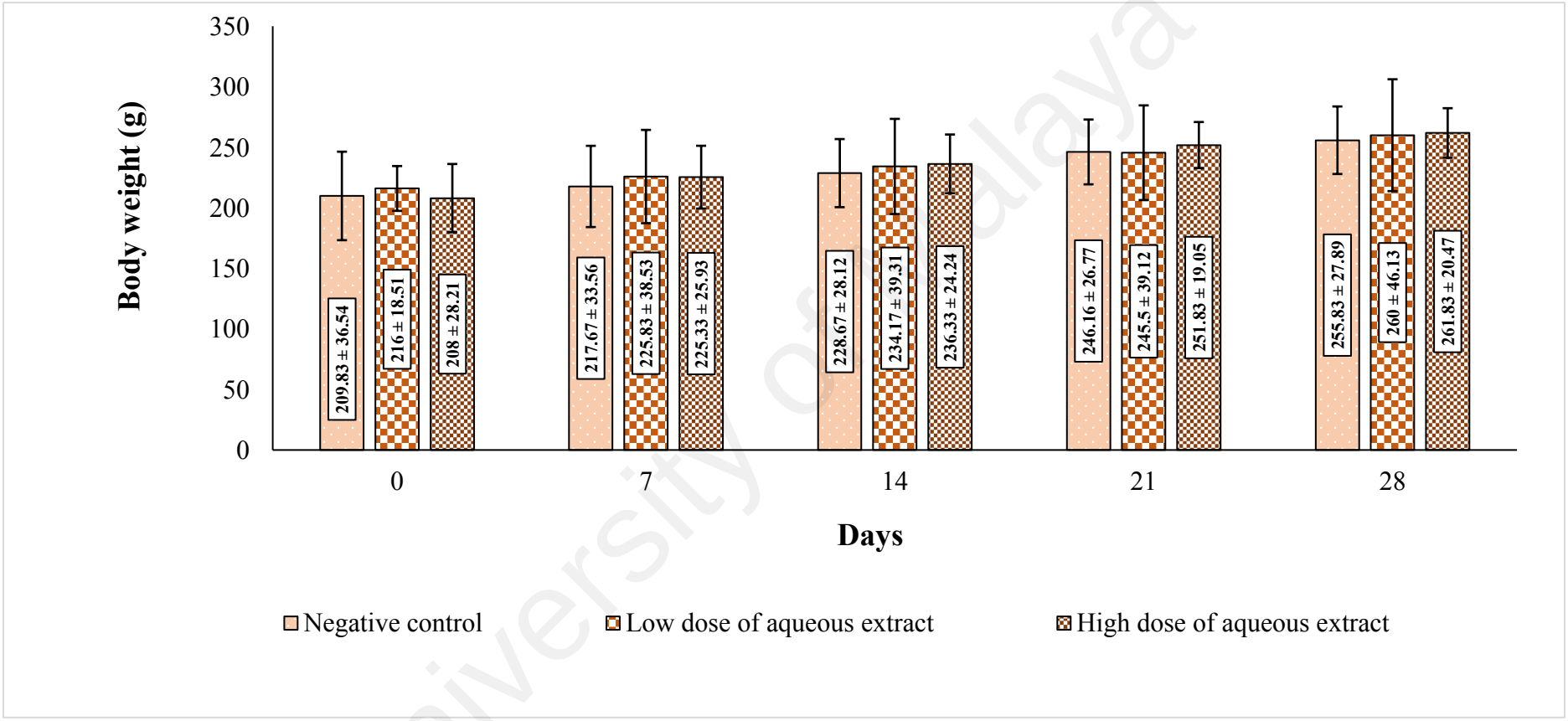

Figure 4.2: Effect of *L. rhinocerotis* aqueous extract on body weight. Negative control group - 5 ml/kg body weight/day of distilled water; Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract; High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Values are mean of six rats per treatment group. Data was analysed using SPSS, one-way ANOVA following Duncan's Multiple Range Test (Appendix C). No significant difference in body weight and weight gain between control and treatment groups ($p > 0.05$) was observed.

In this study, all the rats treated at dosages 500 mg/kg body weight/day and 1000 mg/kg body weight/day of aqueous extract respectively, showed a similar growth pattern and continued normal weight gain throughout the 28-day toxicological study. 20.37%, 25.83% and 21.9% were percentage of body weight increment observed on day 28 in low dosage, high dosage and negative control groups, respectively.

Abnormal changes in body weight is used as critical reference to indicate early signs of adverse effects from exposure to any potential toxic chemical or drug (Sireeratawong *et al*., 2008; Mukinda & Eagles, 2010). In this study, all the rats treated with aqueous extract of *L. rhinocerotis* at two dosages 500 and 1000 mg/kg body weight/day and distilled water displayed similar growth pattern and did not show any abnormal weight gain. This indicates that there is no gross toxic effect due to oral administration of *L. rhinocerotis* aqueous.

The subacute oral administration of *L. rhinocerotis* aqueous extract at 500 and 1000 mg/kg body weight/day did not produce any mortality or abnormal clinical signs throughout the course of the study.

4.3 Effect of aqueous extract of *L. rhinocerotis* on nervous tissues

4.3.1 Dorsal root ganglion

The microscopic features of dorsal root ganglion from rats treated with aqueous extract of *L. rhinocerotis* at low (Figure 4.3 B) and high dosages (Figure 4.3 C) did not show any microscopic difference with dorsal root ganglion from rats of negative control group (Figure 4.3 A). Normal cell bodies of sensory neurons surrounded by satellite cells in the longitudinal sections of dorsal root ganglion were observed.

The Dorsal root ganglion contains small, medium and large sizes cell bodies of sensory neurons, which are responsible for nociception and non-nociception stimuli, respectively.

These neurons are of pseudounipolar type. The satellite cells are more intensely stained, whereas the cell bodies of sensory neurons are lightly stained. The centrally located nuclei and nucleoli may or may not be included in the cross sections due to the large size of cell bodies. The nerve fibres associated with the cell bodies of sensory neurons were also observed. The sensory fibres carry the information from the periphery through the dorsal horn to the spinal cord. Toxicological changes like swelling or loss of cell bodies and degeneration of sensory fibres were not observed (Figure 4.3).

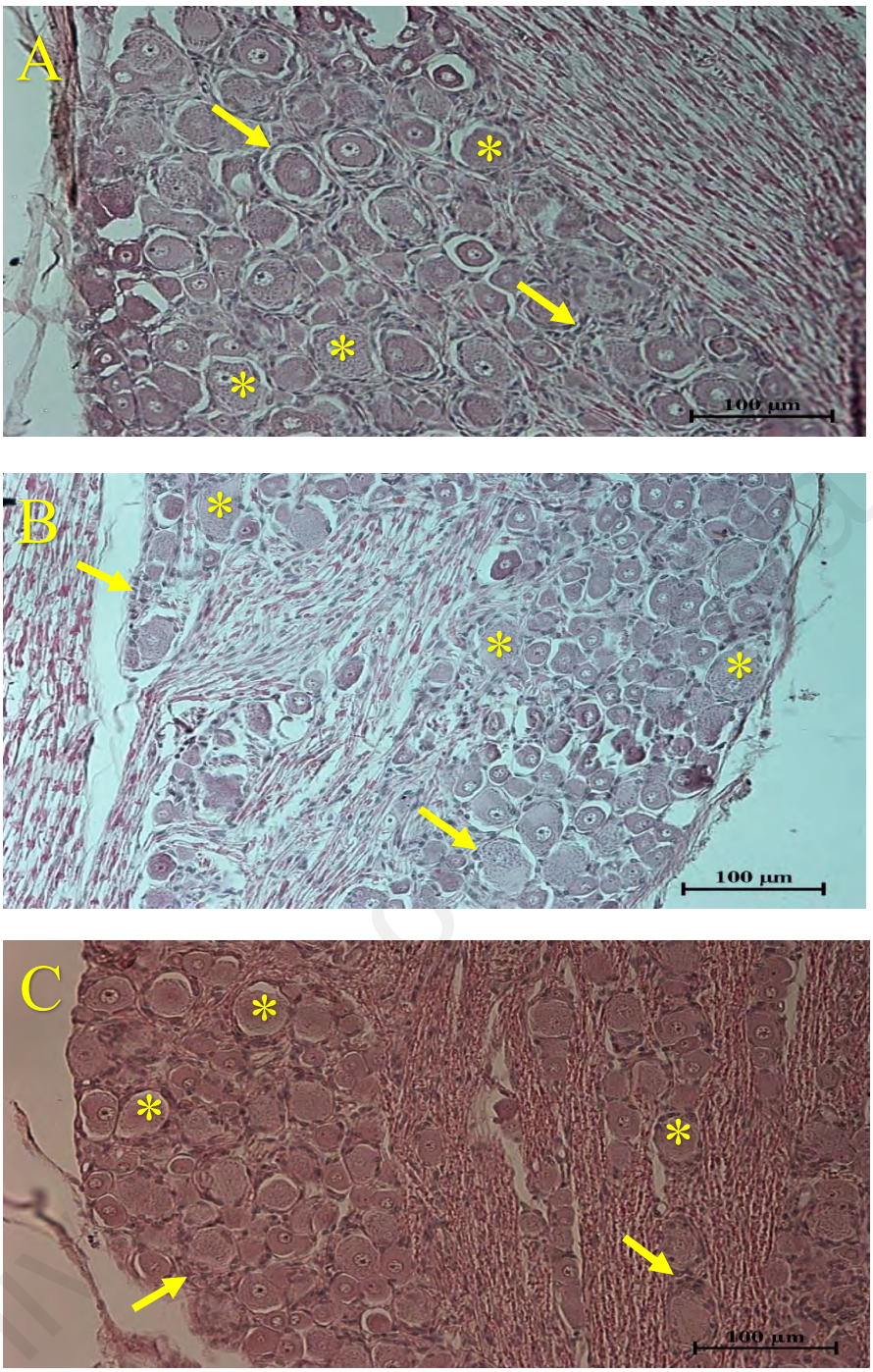

Figure 4.3: The longitudinal sections of dorsal root ganglion. (A) Negative control group - 5 ml/kg body weight/day of distilled water. (B) Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. (C) High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Sections were stained with hematoxylin and eosin. Arrows show cell bodies of sensory neurons and asterisks (*) show satellite cells. Scale bar indicates 100 µm, 2maginification.

The distribution of cell bodies of sensory neurons in dorsal root ganglion from rats treated with *L. rhinocerotis* aqueous extract at both low and high dosages showed similar distribution with cell bodies of sensory neurons in negative control group (Figure 4.4). The findings show that treatment with *L. rhinocerotis* aqueous extract did not show toxic effects on cell bodies of sensory neurons. Even within the groups the total number of cell bodies of sensory neurons (Appendix D, Table D.2) was not significant.

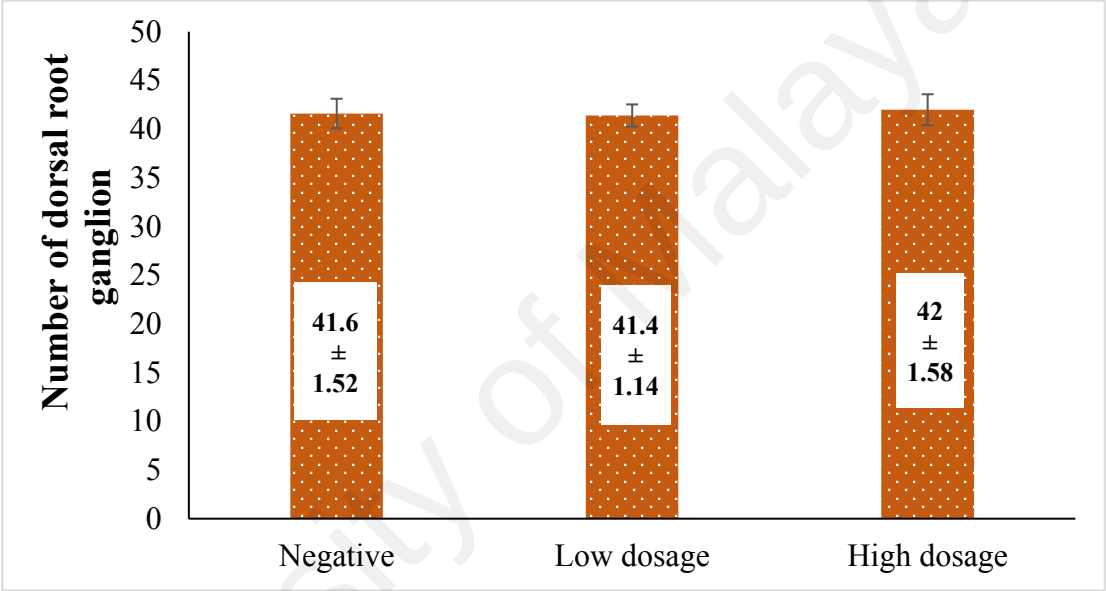

Figure 4.4: Effect of *L. rhinocerotis* aqueous extract on distribution of cell bodies of sensory neurons in dorsal root ganglion. Negative control group - 5 ml/kg body weight/day of distilled water; Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract; High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Values are mean of five tissue sections per treatment group. Data was analysed using SPSS, one-way ANOVA following Duncan's Multiple Range Test (Appendix C). No significant difference in distribution of cell bodies of sensory neurons between control and treatment groups ($p > 0.05$) was observed.

4.3.2 Sciatic nerve

The microscopic features of sciatic nerve from rats treated with aqueous extract of *L. rhinocerotis* at low (Figure 4.5 B) and high dosages (Figure 4.5 C) did not show any microscopic difference with sciatic nerve from rats treated with distilled water (Figure 4.5 A). Normal parallel bundles of nerve fibres were observed in longitudinal section of sciatic nerve. Toxicological changes namely, the loosening of nerve fibres, abnormal architecture of Schwann cells and axonal degeneration were absent in longitudinal section of sciatic nerve (Figure 4.5).

The parallel bundles of nerve fibres are tightly packed. The fibres are enclosed by connective tissues. The dark blue spot in longitudinal sections of sciatic nerve are nuclei of Schwann cells and fibroblast (Figure 4.5) which are not demarcated by routine staining. Schwann cells are supporting cells that forms the myelin sheath around the axons. The bundles of nerve fibers are grouped in fascicles and surrounded by a thick layer of dense irregular connective tissue called epineurium. Each fascicle is surrounded intimately by the perineurium, which is a densely stained layer. Group of fibres are bound in fascicles by perineurium. The epineurium and perineurium are indicated by the letter "*e*" and "*p*" (Figure 4.5). The endoneurium is a delicate layer connective tissues surrounding each axon. The endoneurium is not clearly observed, whereas individual axons can be observed under lower magnification.

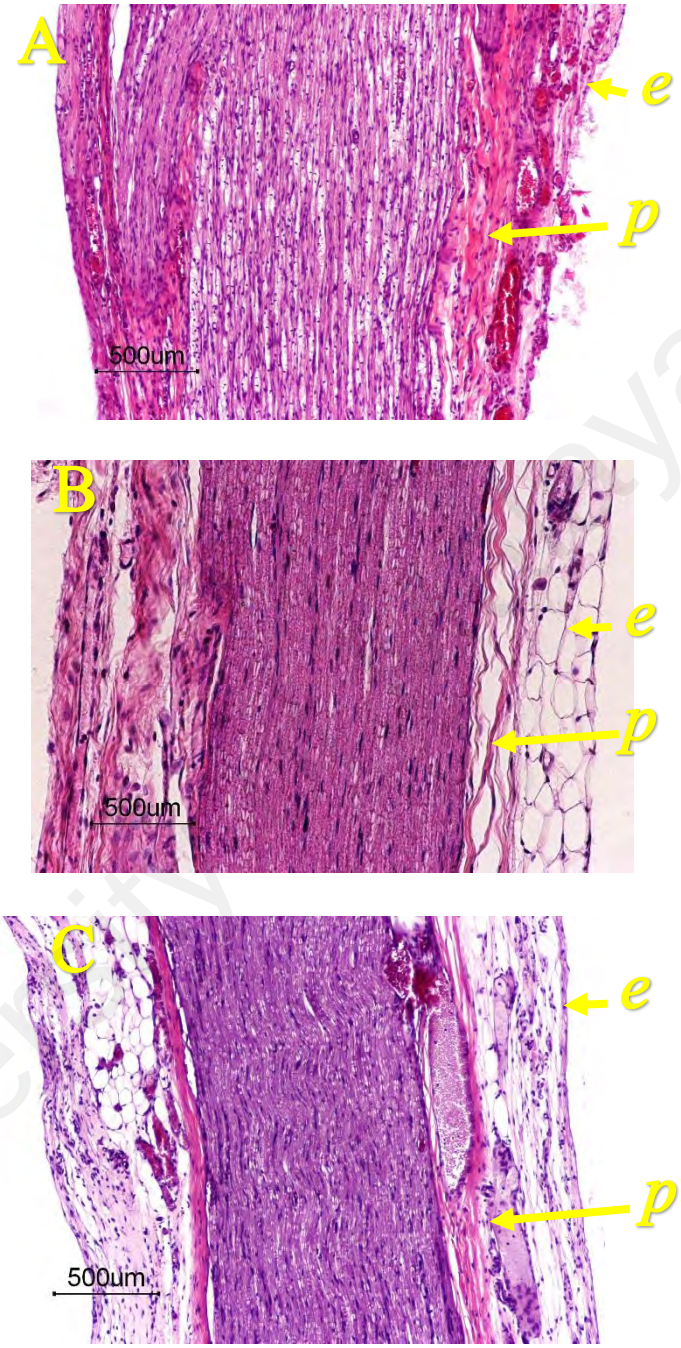

Figure 4.5: The longitudinal sections of sciatic nerve. (A) Negative control group - 5 ml/kg body weight/day of distilled water. (B) Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. (C) High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Parallel bundles of nerve fibres were observed in longitudinal sections of sciatic nerve. Letter *e* denotes epineurium. Letter *p* denotes perineurium. Scale bar indicates 100 µm, magnification 20x.

4.3.3 Cerebrum

At lower magnification (10x) cell bodies of neurons distributed in six layers of cerebral cortex can be seen in low dosage (Figure 4.6 A) and high dosage (Figure 4.6 B) group treated with *L. rhinocerotis* and negative control group (Figure 4.6 C). The six layers are different in characteristic neuron morphology, size and population density, and the layers are merged with one another than being highly distinct, however vary in thickness and function.

At higher magnification (40x) normal Betz cells in layer V and cell bodies of other neurons were observed in cerebral cortex treated with *L. rhinocerotis* at low (Figure 4.6 B) and high dosages (Figure 4.6 C), and distilled water (Figure 4.6 A). Betz cells are indicated by arrows (Figure 4.6). The Betz cells are large cells with branching dendrite extending from the apex of the pyramid toward the cortical surface, and with an axon extending downward from the base of the pyramid. Toxicological changes like swelling, shrinkage and loss of Betz cells were not observed (Figure 4.6).

The microscopic features of cerebral cortex from rats treated with aqueous extract of *L. rhinocerotis* at low (Figure 4.6 B) and high dosage (Figure 4.6 C) did not show any microscopic difference with cerebral cortex from rats of negative control group (Figure 4.6 A).

The cerebral cortex is the most integrated area of the nervous system and less well organised compared to the cerebellar cortex (Young *et al*., 2013). The sensory information is collected and processed into meaningful perceptual images, and cognitive function and skeleton muscle activity (Widmaier *et al*., 2004). The cortical neurons are composed of pyramidal and non-pyramidal cells.

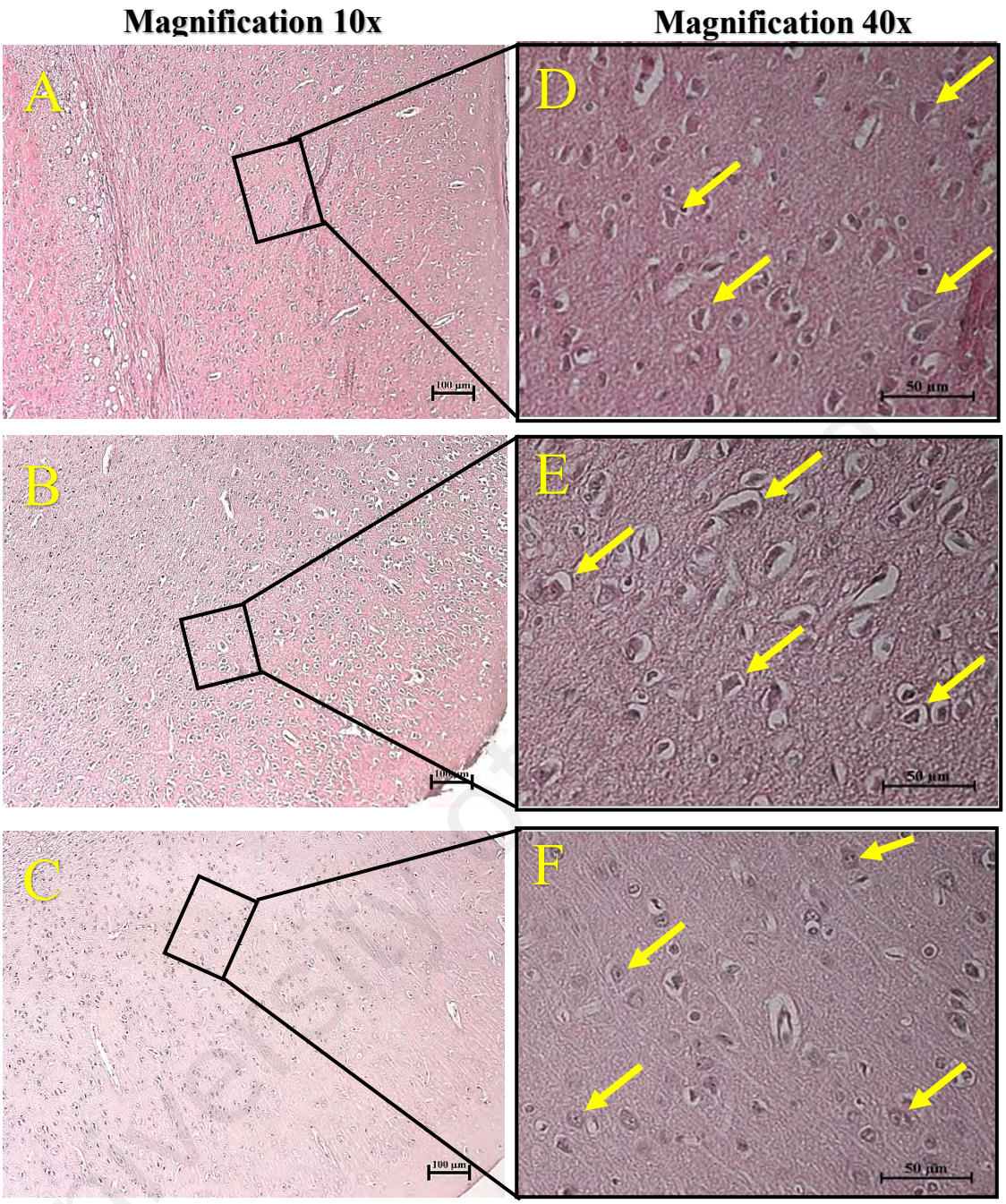

Figure 4.6: The cross sections of cerebral cortex. (A) and (D) Negative control group - 5 ml/kg body weight/day of distilled water. (B) and (E) Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. (C) and (F) High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Sections were stained with hematoxylin and eosin. Arrows show the Betz cells in fifth layer of cerebral cortex at magnification 40x. A-C: Scale bar indicates 100 µm, magnification 10x. D-F: Scale bar indicates 50 µm, magnification 40x.

The following are six layers of cerebral cortex was observed:

i. Molecular layer: The top most layer containing axons and dendrites of cortical neurons making synapses with one another.
ii. Outer granular layer: A densely packed thin layer composed of small pyramidal cells and stellate cells
iii. Pyramidal layer: Composed of moderate size pyramidal cells.
iv. Inner granular layer: Narrow layer consisting mainly of densely packed stellate cells.
v. Ganglionic layer: Composed of largest pyramidal cells and smaller stellate cells and cells of Martinotti (Young *et al*., 2013). The name of the layer "ganglionic" is referred to the huge pyramidal Betz cells of cerebral cortex.
vi. Multiform cell layer: Composed of varying morphological forms of neurons namely, small pyramidal cells and cells of Martinotti, and stellate cells in exterior part, and fusiform cells in the deeper part.

The distribution of Betz cells in cerebral cortex from rats treated with *L. rhinocerotis* aqueous extract at both low and high dosages showed similar distribution with Betz cells in negative control group (Figure 4.7). The findings show that treatment with *L. rhinocerotis* aqueous extract did not cause damage or loss of the Betz cells. Even within the groups the total number of Betz cells (Appendix D, Table D.3) was not significant.

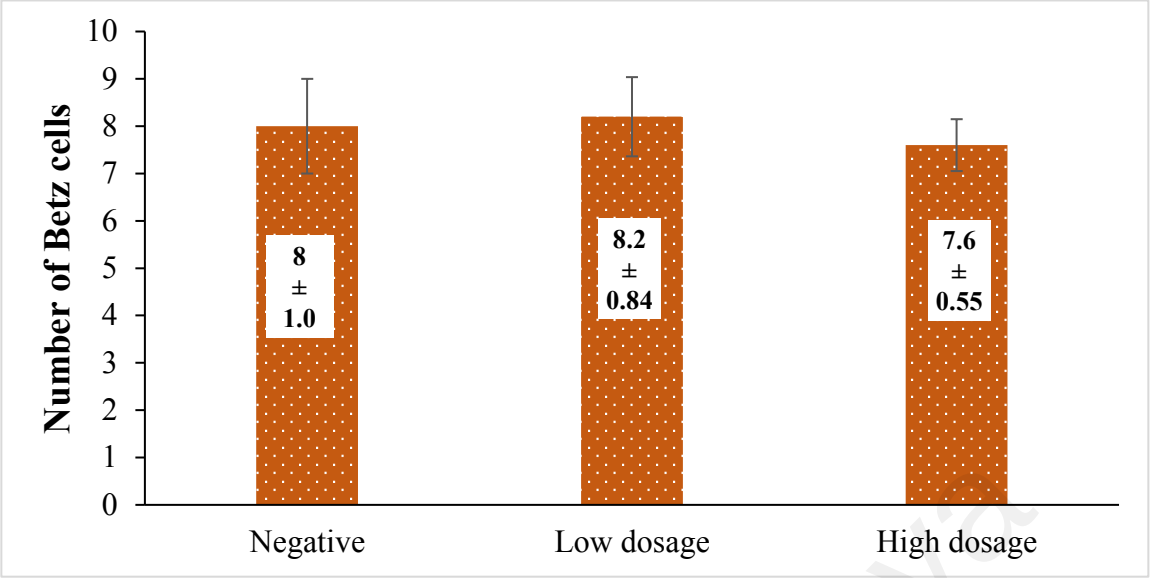

Figure 4.7: Effect of *L. rhinocerotis* aqueous extract on distribution of Betz cells in fifth layer of cerebral cortex. Negative control group - 5 ml/kg body weight/day of distilled water; Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract; High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Values are mean of five tissue sections per treatment group. Data was analysed using SPSS, one-way ANOVA following Duncan's Multiple Range Test (Appendix C). No significant difference in distribution of Betz cells between control and treatment groups ($p > 0.05$) was observed.

4.3.4 Cerebellum

Normal histological structures of cerebellar cortex from rats treated with aqueous extract of *L. rhinocerotis* at low (Figure 4.8 B) and high (Figure 4.8 C) dosages and distilled water were observed (Figure 4.8 A). The large flask-shaped Purkinje cells in the middle layer are indicated by the arrow. Scarcely scattered basket cells in molecular layer and densely packed granular cells in the granular layer were also seen in cerebellar cortex. Toxicological changes like swelling or loss of Purkinje cells and vacuolization was absent (Figure 4.8). Highly branched dendrites that extends from the Purkinje cell bodies into the molecular layer were not seen, nor we can see their axons, which extend down through the granular layer into deeper parts of the cerebellum under routine staining.

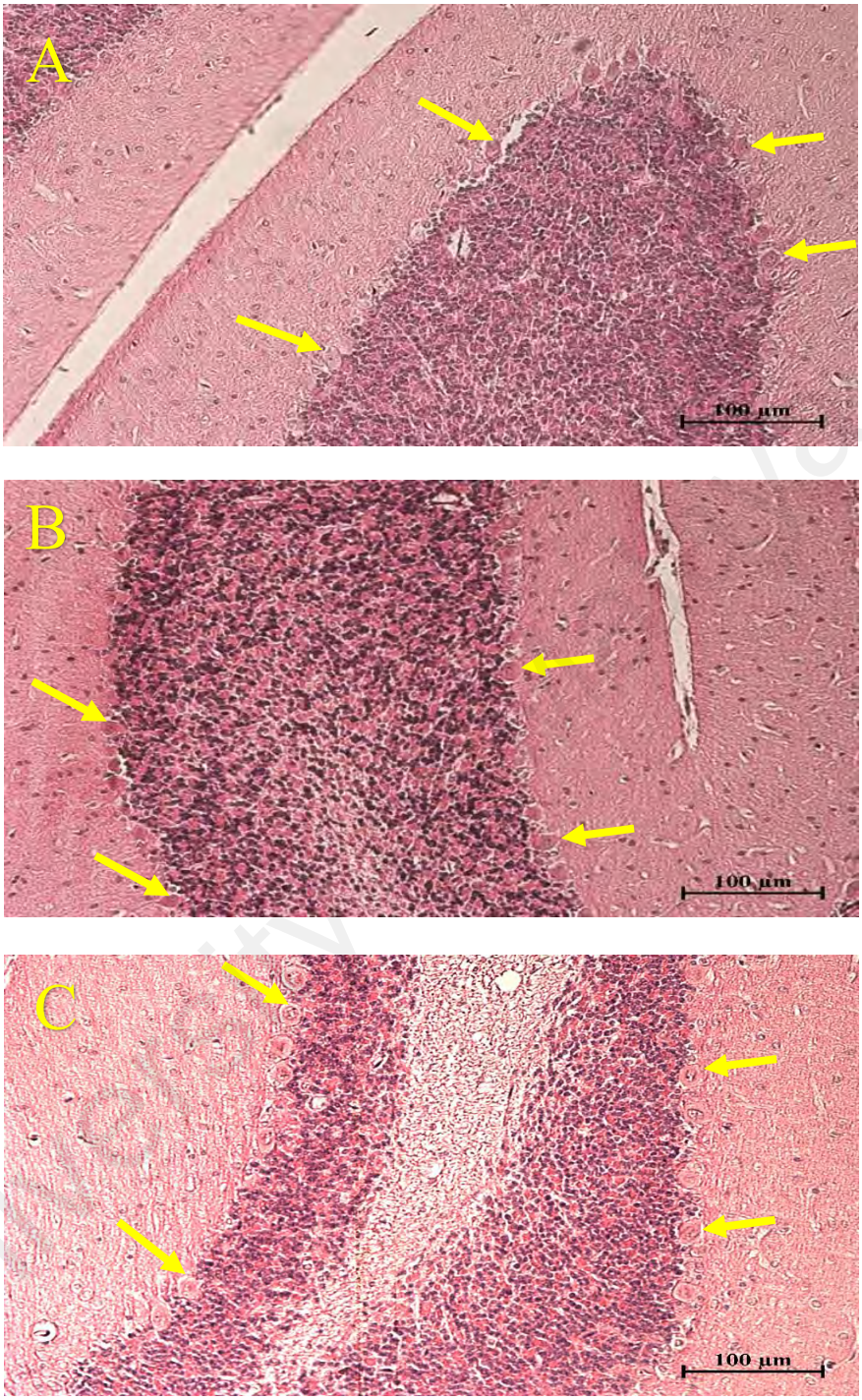

Figure 4.8: The cross sections of cerebellar cortex. (A) Negative control group - 5 ml/kg body weight/day of distilled water. (B) Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. (C) High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Sections were stained with hematoxylin and eosin. Arrows show single layer of Purkinje cells in middle layer. Scale bar indicates 100 µm, magnification 20x.

Cerebellum is important centre for coordinating movements and controlling posture and balance (Widmaier *et al*., 2004). The Purkinje cells have huge cell body of diameter 40 - 60 microns, relatively fine axons extending down granular layer and highly branched dendrites extending in the top layer (Young *et al*., 2013).

The distribution of Purkinje cells in cerebral cortex from rats treated with *L. rhinocerotis* aqueous extract at both low and high dosages showed similar distribution with Purkinje cells in negative control group (Figure 4.9). The findings show that treatment with *L. rhinocerotis* aqueous extract did not cause damage or loss of the Purkinje cells. Even within the groups the total number of Purkinje cells (Appendix D, Table D.4) was not significant.

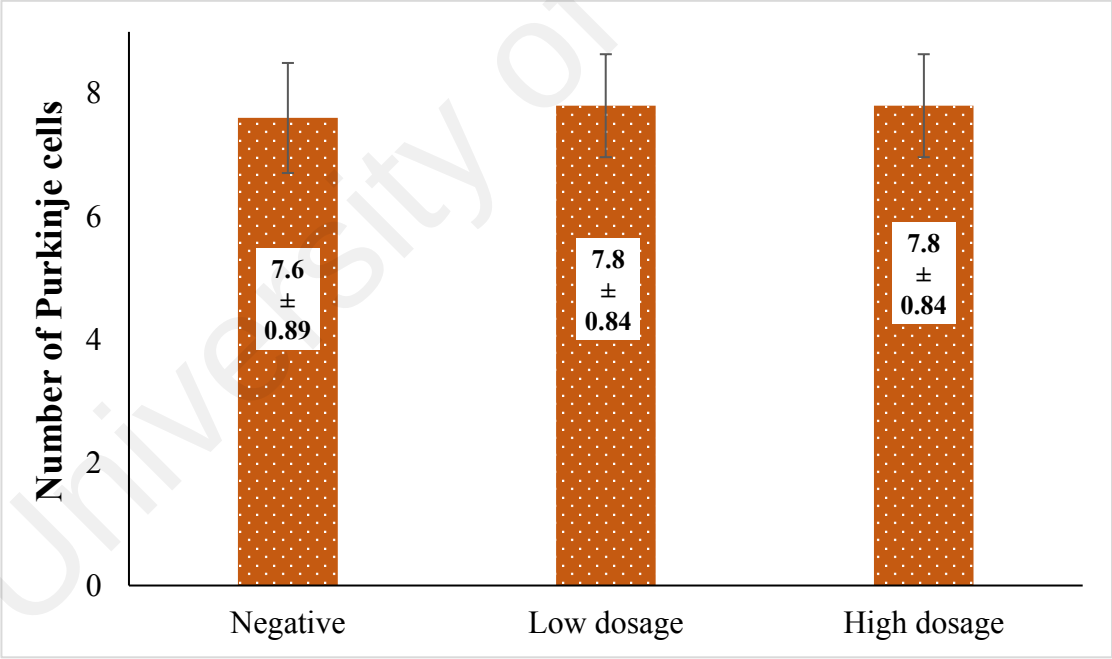

Figure 4.9: Effect of *L. rhinocerotis* aqueous extract on distribution of Purkinje cells in the middle layer of cerebellar cortex. Negative control group - 5 ml/kg body weight/day of distilled water; Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract; High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Values are mean of five tissue sections per treatment group. Data was analysed using SPSS, one-way ANOVA following Duncan's Multiple Range Test (Appendix C). No significant difference in distribution of Purkinje cells between control and treatment groups ($p > 0.05$) was observed.

4.3.5 Spinal cord

Normal architecture of multipolar motor neurons in the ventral horn of spinal cord was observed in all the rats treated with aqueous extract of *L. rhinocerotis* at low (Figure 4.10 B) and high (Figure 4.10 C) dosages and distilled water (Figure 4.10 A).

Under lower magnification of cross sections, the butterfly shape of the central mass of grey matter was observed under lower magnification (Figure 4.10). The central canal is also seen under lower magnification. At higher magnification multipolar motor neurons are observed.

Toxicological changes like axon degeneration, swelling or loss of multipolar motor neurons and vacuolization was absent (Figure 4.10).

The grey matter of spinal cord is composed of interneurons, cell bodies and dendrites of afferent and efferent neurons, and glial cells. The grey matter is wrapped by white matter which consists of myelinated axons (Young *et al*., 2013). The central canal is the cerebrospinal fluid-filled space that runs throughout the spinal cord (Widmaier *et al*., 2004). The dorsal horn is composed of cell bodies of small second order sensory neurons. Ventral horn of the spinal cord is composed of multipolar motor neurons in the grey matter, which have two or more dendrites and single axon, that may have one or more collateral branches.

Motor neurons are responsible to carry the information from brain to the effector organs. The myelinated axons in the white matter is also seen. These are the ascending and descending fibres that run longitudinally between the brain and the spinal cord (Moore *et al*., 2018).

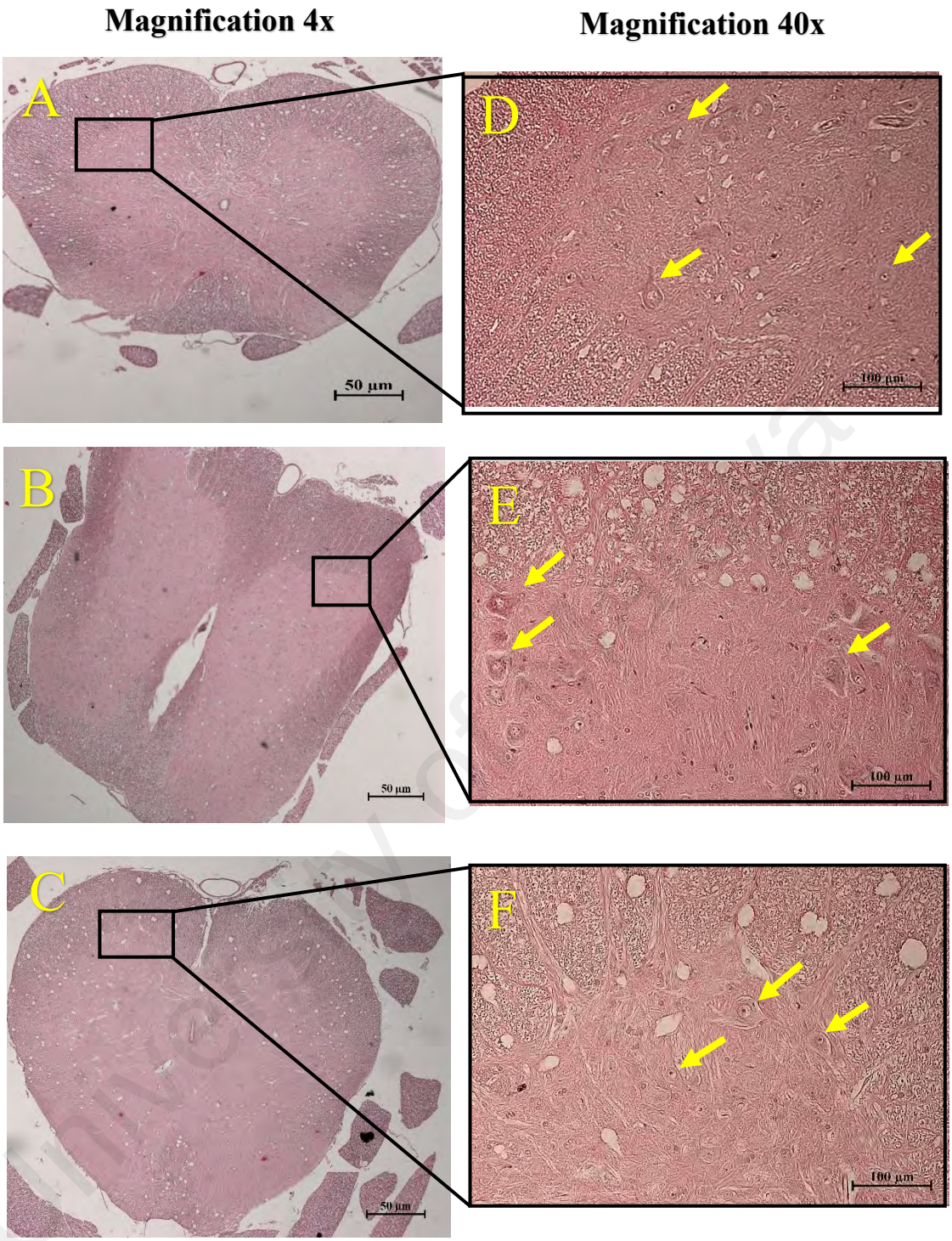

Figure 4.10: The cross sections of spinal cord. (A) and (D) Negative control group - 5 ml/kg body weight/day of distilled water. (B) and (E) Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. (C) and (F) High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Sections were stained with hematoxylin and eosin. The butterfly shape of the spinal cord can be observed at magnification 4x. Arrows show multipolar motor neurons in the ventral horn of grey matter at magnification 40x. A-C: Scale bar indicates 50 µm, magnification 4x. D-F: Scale bar indicates 100 µm, magnification 40x.

The distribution of multipolar motor neurons in the spinal cord from rats treated with *L. rhinocerotis* aqueous extract at both low and high dosages showed similar distribution with multipolar motor neurons in negative control group (Figure 4.11). The findings show that treatment with *L. rhinocerotis* aqueous extract did not cause damage or loss of the multipolar motor neurons. Even within the groups the total number of Purkinje cells (Appendix D, Table D.5) was not significant.

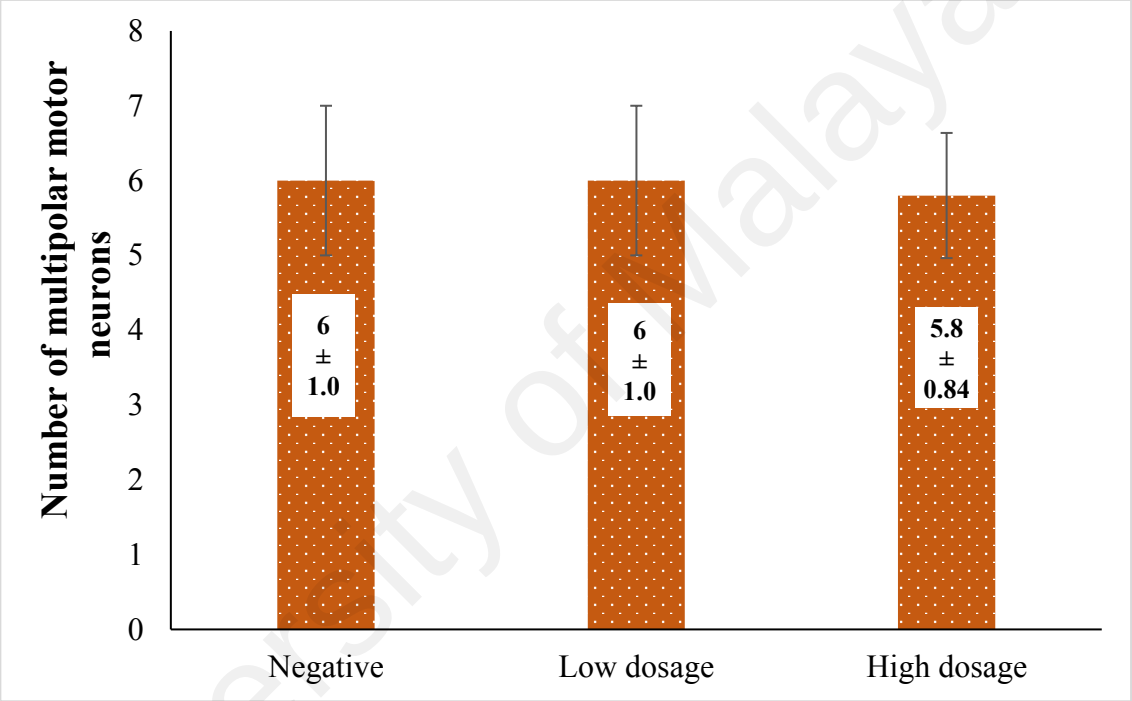

Figure 4.11: Effect of *L. rhinocerotis* aqueous extract on distribution of multipolar motor neurons in ventral horn of spinal cord. Negative control group - 5 ml/kg body weight/day of distilled water; Low dosage group - 500 mg/kg body weight/day of *L. rhinocerotis* aqueous extract; High dosage group - 1000 mg/kg body weight/day of *L. rhinocerotis* aqueous extract. Values are mean of five tissue sections per treatment group. Data was analysed using SPSS, one-way ANOVA following Duncan's Multiple Range Test (Appendix C). No significant difference in distribution of multipolar motor neurons between control and treatment groups ($p > 0.05$) was observed.

Human nervous system is the control centre of the body involved in cognitive and motor function, communication with the external environment and coordinating the activities of all other organ systems. Even minimal damage to the nervous system can result in massive neuronal problems like loss of memory, coma, paralysis, disturbed

communication, loss of attention and alertness, dementia, convulsions, and motor disorder (National Research Council, 1992).

A person can be affected by any neurotoxicant chemical at any time of the life cycle, even during gestation period as the nervous system is developing, it is more vulnerable to the foreign chemical and the abnormal changes can manifest with the age (Slikker & Bowyer, 2005). The clinical manifestation of neurotoxic disorders depends on the vulnerability of the various cells towards the exposed neurotoxicant and the normal susceptibility of the cells. For example, degeneration of dopamine neurons due to exposure to N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, results in Parkinson's disease (National Research Council, 1992). In another example, ginseng interacts with phenelzine and other monoamines inhibitors causing central nervous system stimulant effect (Tomlinson *et al*., 2000). The medical knowledge about any drug is vital to avoid any unnecessary health problems or loss of the lives.

The increased popularity of the folk medicine among people due to broad range of beneficial properties implies the necessity of toxicity data. Toxicity from natural product is mostly due to misidentification of edible natural products from toxic products, presence of toxic compound in combination with bioactive compounds, incorrect preparation and excess dosage of administration (Nasri & Shizad, 2013). The toxicities of natural products associated with promising medicinal and nutritional value indicates the importance of systematic evaluation of the toxicity data of the any natural product.

From 1664, *L. rhinocerotis* is used to enhance energy, increase alertness, and overall wellness of the individual (Tan *et al*., 2009). The wide range of bioactive compounds namely, carbohydrates, proteins, phenolic compounds and fatty acids in *L. rhinocerotis* and its medicinal value has attracted the doctors and researchers (Yap *et al*., 2013). The toxicity data is vital before it could be developed as a functional food.

Histological examination of the tissues is an essential technique to evaluate the safety of novel drugs (Greaves, 2011). Knowledge of the normal histology of the tissue structures is necessary for recognition and understanding the adverse effects caused by drugs.

For fixation, the Davidson's fixative solution proved to be best fixative for studying tissue structures. For example, the toxicological screening of male reproductive organs requires special nonroutine fixatives. Davidson's solution is reported as better fixative for testes for histopathological analysis (Creasy, 2002; Latendresse *et al*., 2002).

The haematoxylin and eosin technique has been the traditional staining method for examination tissue sections This is centrally used staining technique to identify the gross structure of the tumor, such as to differentiate between adenocarcinoma and neuroendocrine solid tumor and to evaluate major parameters such as the nuclear/cytoplasmic ratio of lymphoma tumor cells (Amalraj & Naeim, 2012).

Previous studies have demonstrated that the oral administration of sclerotia of *L. rhinocerotis* (TM02) had no adverse effects on hematological, growth rate parameters and, micrographs of liver, spleen, lung, kidney and heart did not show any pathological alterations (Lee *et al*., 2011a). Lee *et al*. (2013) extended their studies into a chronic toxicity test on rats following oral administration of 250, 500, 1000 mg/kg of sclerotia of *L. rhinocerotis* had no observed-adverse-effect-level (NOAEL) on body weight, haematological, clinical biochemistry, urine and organ weight parameters.

In an *in vitro* study for cytotoxicity screening of *L. rhinocerotis* using mouse embryonic fibroblast (BALB/3T3) and N2a cell lines, the results expressed as IC_{50} values did not show cytotoxicity effect on cell lines (Phan *et al*., 2013). The Ames test, an *in vitro* chromosome aberration test and an *in vivo* erythrocyte micronucleus test in ICR mice, conformed that *L. rhinocerotis* neither displayed mutagenicity nor genotoxicity

(Chen *et al*., 2013). Jhou *et al*. (2017) showed that the oral feeding of 3400 mg/kg/day of *L. rhinocerotis* to pregnant Sprague-Dawley female rats neither displayed teratogenicity in foetus nor toxicity in dams. However, there was no information on neurotoxicity.

In present study, all the rats treated with aqueous extract of *L. rhinocerotis* at selected dosages 500 and 1000 mg/kg body weight/day did not show any abnormal weight gain. The gross examination of the peripheral nervous tissues namely, dorsal root ganglion and sciatic nerve, and, the central nervous tissues namely, cerebrum, cerebellum and spinal cord of rats treated with *L. rhinocerotis* did not display any significant pathological changes compared to the negative control group. Therefore, there were no abnormal changes like neuronal degeneration, swelling or apoptosis of neurons, vacuolization and loss of cells were observed either in peripheral nervous tissue nor in central nervous tissues.

Further, normal distribution of cell bodies of sensory neurons, Betz cells, Purkinje cells and multipolar motor neurons in dorsal root ganglion, fifth layer of cerebral cortex, middle layer of cerebellar cortex and ventral horn of spinal cord, respectively were observed. The statistical results indicate that there is no significant loss of cells between rats of low dosage and high dosage groups treated with aqueous extract of *L. rhinocerotis* at dosages 500 and 1000 mg/kg body weight/day, and negative control group treated with 10 ml/kg body weight/day, respectively. Therefore, there were no toxic effect of oral administration of aqueous extract of *L. rhinocerotis* on cells of the nervous tissue for 28 days consecutively.

4.4 Recommendation for future studies

The subacute neurotoxicity test can be further performed at various higher dosages, as in present subacute toxicity test of aqueous extract of *L. rhinocerotis* at highest dosage 1000 mg/kg body weight/day did not display any adverse effect on nervous

tissues. Further, the subacute toxicity study of *L. rhinocerotis* at dosages 500 mg/kg body weight/day and 1000 mg/kg body weight/day on nervous tissue can be extended to subchronic and chronic toxicity test.

Once the neurotoxicity data of *L. rhinocerotis* is thoroughly evaluated at different dosages and different periods of time then it can be efficiently used in the treatment of neurological disorders. As it has been reported that *L. rhinocerotis* mimics neuritogenic activity of nerve growth factor via MEK/ERK1/2 signalling pathway, possessed neuroprotective and neuroinflammatory activity, and displayed maximal neurite outgrowth in dissociated cells of brain, spinal cord and retinal cells (Samberkar *et al*., 2015; Nallathamby *et al*., 2018; Seow *et al*., 2017). This implies that in future *L. rhinocerotis* can be used in treatment of neurodegenerative disorders like Alzheimer's and Parkinson's disease, once the chronic neurotoxicity data is evaluated.

CHAPTER 5: CONCLUSION

The results presented in this study showed that the subacute oral administration of aqueous extract of *L. rhinocerotis* at dosages 500 and 1000 mg/kg body weight/day did not show any significant changes ($p > 0.05$) in body weight of the rats compared to the negative control rats.

Further, the histopathological analysis showed normal microscopic structure of peripheral nervous tissues namely, sciatic nerve and dorsal root ganglion, and central nervous tissues namely, cerebrum, cerebellum and spinal cord. There were no abnormal changes like neuronal degeneration, swelling or apoptosis of neurons, vacuolization and loss of cells were not observed in peripheral nervous tissues and also central nervous tissues.

Finally, no significant difference ($p > 0.05$) between treated and negative control group was observed in distribution of cell bodies of sensory neurons, Betz cells, Purkinje cells and multipolar motor neurons in dorsal root ganglion, fifth layer of cerebral cortex, middle layer of cerebellar cortex and ventral horn of spinal cord, respectively.

The present histological analysis of nervous tissues following subacute oral administration of *L. rhinocerotis* reveals nontoxic effect of mushroom on nervous tissues, thus has the potential to be developed as a functional food for neuronal health.

The nervous system is the control centre of the human body. Even minimal damage can result in massive neuronal problems. Neurotoxicity can result in damage to the structure and function of nervous system at any time of the life cycle. The consumption of more medicinal mushrooms in our daily diet could be an initiative and effective step to reduce the risk of neuronal problems. Especially, *L. rhinocerotis* that has been reported

to possess neuroprotective effect, has no toxic effect on nervous tissues and can be easily cultivated nowadays.

However, to support the safety assessment of *L. rhinocerotis* sclerotia as a functional food for human neuronal health, there is a limited information available on the higher dosages and on long term usage of the mushroom. The study can be further extended to subchronic and chronic neurotoxicity test with similar and different dosages.

Once the complete safety data of *L. rhinocerotis* on nervous tissue is obtained, it can be used in future for treatment of neurodegenerative disorders like Alzheimer's and Parkinson's diseases.

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