ANALYSIS OF NEURITE OUTGROWTH ACTIVITY OF AQUEOUS EXTRACT OF *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN IN PC-12 CELLS

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2015

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES

FACULTY OF SCIENCE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2015

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Regisration/Matric No.: **SGR100042**
Name of Degree: **MASTER OF SCIENCE**
Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

"ANALYSIS OF NEURITE OUTGROWTH ACTIVITY OF AQUEOUS EXTRACT OF *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN IN PC-12 CELLS"

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ABSTRACT

Senescence of neuronal cells reduced the elongation and branching of axons. These lead to neurodegenerative diseases such as Alzheimer's disease. Extracts of selected mushrooms used as folk medicines are being studied, *Lignosus rhinocerotis* (Cooke) Ryvarden, however has been singled out as one of the most potent mushroom with medicinal properties by communities in Malaysia. In this study, aqueous extract was extracted by boiling method mimicking the real cooking method. Activity of *L. rhinocerotis* aqueous extract in stimulating neurite outgrowth and the possible signalling pathway involved were investigated. Neurite outgrowth activity was assessed by number of neurite-bearing cells and immunoreactivity of neurons by indirect immunostaining with neurofilament protein. Aqueous extract of *L. rhinocerotis* gave the maximal stimulation for neurite outgrowth at a lower concentration of 20 µg/mL (w/v) when compared to *H. erinaceus* at 50 µg/mL (w/v) and *T. heimii* at 40 µg/mL (w/v). *Lignosus rhinocerotis* aqueous extract possessed NGF-like activity and up regulated ERK/MAPK signaling pathway for cell differentiation and cell growth.

ABSTRAK

Penuaan sel-sel neuron mengurangkan pemanjangan dan pencabangan akson dari sel neuron yang boleh menyebabkan penyakit neurodegeneratif seperti penyakit Alzheimer. Ekstrak daripada pelbagai cendawan terpilih telah digunakan sebagai ubat-ubatan berikutan kajian dan penyelidikan secara meluas. Tambahan pula, *Lignosus rhinocerotis* (Cooke) Ryvarden telah dikenali sebagai cendawan yang kaya dengan ciri-ciri perubatan oleh masyarakat Malaysia. Dalam kajian ini, ekstrak akueus telah diekstrak dengan kaedah mendidih, ini adalah meniru kaedah memasak yang sebenar. Ekstrak akueus *L. rhinocerotis* merangsang pencabangan neurit dan tapak jalan pengisyaratan yang mungkin terlibat telah disiasat. Aktiviti perkembangan neurite telah dinilai dengan penghitungan sel-sel neurit yang bercabang dan imunoreaktiviti neuron dengan pewarnaan immunofluorescence tidak langsung protein neurofilamen. Ekstrak akueus *L. rhinocerotis* menghasilkan rangsangan maksimum bagi pencabangan neurit pada kepekatan yang lebih rendah, iaitu 20 μg / mL (w/v) berbanding dengan *Hericium erinaceus* pada 50 μg / mL (w/v) dan *Termitomyces heimii* pada 40 μg / mL (w/v). Ekstrak akueus *L. rhinocerotis* mengandungi aktiviti yang serupa dengan NGF dan meningkatkan laluan isyarat ERK/MAPK sebagai isyarat untuk pembezaan dan pertumbuhan sel neuron.

ACKNOWLEDGEMENTS

A research project is never the work of anyone alone. The contributions of many different people, in their different ways, have made this possible. I would like to extend my appreciation especially to the following. Without them, this thesis would not have been completed or written.

First and foremost, my utmost gratitude to Prof. Dr. Vikineswary Sabaratnam and Assoc. Prof. Dr. Murali Naidu, who has supported me throughout my research and thesis with their patience and knowledge whilst allowing me the room to work in my own way.

Besides, I would also like to thank Prof Umah Rani and her students for their kindness to share their laboratory and knowledge to carry out my anti-oxidant research. Dr. Wong Kah Hui for her guidance and advice throughout my project. Dr Khang Tsung Fei for advice and guidance on statistic.

In my daily work I have been blessed with a friendly and cheerful group of fellow students, staffs and seniors in Fungal Biotechnology Laboratory, Mycology and Plant Pathology Laboratory and Anatomy Department to make my research a success.

Thanks to University of Malaya for the generous funding (RG 136/10AFR, TA021-2010, 66-02-03-0074 and F000002-21001) and scholarship (Skim Biasiswa University Malaya - PS311/2010B) to support my studies and conferences attended to widen my knowledge and view. Facilities were provided to conduct my research.

Last but not least, I thank my family, church mates and friends for supporting me throughout my study at University of Malaya. Not to forget my God, my creator and my guardian. Thanks.

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LIST OF SYMBOLS AND ABBREVIATIONS

MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide*
DAPI	4',6-diamidino-2-phenylindole
Abs	Absorbance
ATCC	American Type Culture Collection
APP	amyloid precursor protein
ANOVA	analysis of variance
AOAC	Association of Analytical Communities/Association of Official Agricultural Chemist
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
CO_2	carbon dioxide
CNS	central nervous system
Xg	*centrifugal* force
CGNs	cerebellar granule neurons
JNK	*c-Jun N-terminal kinases*
CI	confidence intervals
Da	Dalton
℃	degree celsius
DNA	deoxyribonucleic acid
DEAE	diethylaminoethyl
DLPE	dilinoleoyl-phosphatidylethanolamine
DMSO	dimethyl sulfoxide
DMR	Duncan’s multiple range
ER	endoplasmic reticulum

ERK	extracellular signal-regulated kinases
FBS	fetal Bovine Serum
FITC	fluorescein isothiocyanate
G. lucidum	*Ganoderma lucidum*
G	gram
>	greater than
H. erinaceus	*Hericium erinaceus*
HE AE	*Hericium erinaceus* aqueous extract
HE EE	*Hericium erinaceus* ethanol extract
HRP	horseradish peroxidase
Hr	hour
Ig	immunoglobulins
IC_{50}	inhibitory concentration 50
F-12K Medium	Kaighn's Modification of Ham's F-12 Medium
K	kilo
Kg	kilogram
<	less than
LC_{50}	lethal concentration 50
L. rhinocerotis	*Lignosus rhinocerotis*
LR AE	*Lignosus rhinocerotis* aqueous extract
LR EE	*Lignosus rhinocerotis* ethanol extract
LR PSP	*Lignosus rhinocerotis* polysaccharides
LA	lipoic acid
L	litre
(LCB)	long-chain base
mRNA	messenger RNA

M	Meter
µ	Micro
mm	Millimeter
min	Minute
MAPK	*mitogen-activated protein kinase*
M	Molar
N	Nano
NGF	nerve growth factor
NF	neurofilament
NT-3	neurotrophin-3
%	Percent
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate buffered saline
PI3K-Akt	phosphatidylinositol-3-kinase-Akt
±	*plus-minus*
PC-12	Rat pheochromocytoma cells
(ROS)	reactive oxygen species
RDA	recommended daily allowance
RT-PCR	reverse transcription polymerase chain reaction
Rpm	*revolutions per minute*
NaOH	rodium hydroxide
T.heimii	*Termitomyces heimii*
UV	Ultraviolet
VGCC	voltage-gated calcium channels
Aβ	*β-amyloid*

INTRODUCTION

An estimated 524 million people equivalent to about 8% of world's population are aged 65 or above. By 2050, the number is estimated to increase by three fold to about 1.5 billion, with most of the increase in developing countries, including Malaysia (World Health Organization, 2011). In 2000, the elderly population in Malaysia was 1.45 million or 6.2% of the total population (The Star Online, 2010). By 2035, Malaysia is likely to be an aging nation with the number of people aged 60 and reaching around 15% of the population (The Star Online, 2010). United Nations categorised an aging nation as any country with 10% of its population above the age of 60 (World Health Organization, 2011). The elderly population face increased risk of traumatic diseases associated with aging that include neurodegenerative disorders such as dementia. Therefore, neurohealth remains one of the concerns for the predicted silver tsunami to hit humans.

At the present, there are no effective treatments or medications available to prevent or to treat neurodegenerative diseases. Neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are crucial proteins that are responsible for the growth, survival, and maintenance of developing neuron and for the maturation of neuronal cells. Absence of NGF in adult brain of mice led to Alzheimer's-like symptoms (Capsoni *et al.*, 2000). As human and mice share some remarkable genetic similarity, the absence of NGF in adult brain may be the cause of Alzheimer's disease. Nerve growth factor may be a hope for preventing, reducing, or treating Alzheimer's disease. In addition to stimulating neuronal growth, NGF and other neurotrophic factors also prevent neuronal death, promote neurite outgrowth, and maintain and organize neuronal functions (Mori *et al.*, 2008). However, due to the large molecular polypeptide structure, NGF cannot be used as an orally administered drug to regenerate brain tissue.

It is too large to cross the blood-brain barrier (BBB). Smaller molecules are preferred candidates in activating neurite outgrowth pathways. There is an intensified search for these small molecules from natural sources such as mushrooms and plants with the ability to prevent or reduce the severity of nerve related diseases that set in with age.

Prevention is better than cure. Traditional regimens are founded on the belief that regular consumption of natural products and herbs is able to increase alertness, enhance immune system, and treat diseases. Many chemicals and biological response modifiers from plants and spices are known to promote *in vitro* morphological, biochemical, and ultrastructural changes to well-differentiated neuroelectrodermal phenotypes (Abemayor & Sidell, 1989). For example, the incidence of Alzheimer's disease among the older generation in India is not in alarming numbers. The regular consumption of spices including turmeric (Mishra & Palanivelu, 2008) and pepper (Chanpathompikunlert *et al.*, 2010) may be the reason. This hypothesis is currently being actively studied.

Besides plants, mushroom is also a favourite dish for its appetizing and highly nutritious property. It is extensively used for cooking Chinese, European and Japanese cuisines. Mushroom is also a natural product with a fleshy, spore-bearing fruiting body of a fungus. Many mushroom species are high in fibre and provide a wide range of vitamins. Historically, a number of mushrooms extracts have been considered as important remedies for prevention and treatment of many diseases by different tribes and are best documented in the Orient (Wasser & Weis, 1999). At least 650 species of mushrooms are known to exhibit various therapeutic properties (Wasser, 2002; Ying *et al.*, 1987).

Abundance of therapeutic properties has been demonstrated for traditionally used mushrooms. Some edible mushrooms possess bioactive properties like anti-inflammatory substances while some are able to induce neuronal differentiation (Shi *et al.*, 2011) and promote neuronal survival (Shi *et al.*, 2011; Wasser & Weis, 1999). Extracts of some mushrooms used as folk medicines are still under intense research. *Hericium erinaceus* (Bull.: Fr.) Pers. (Aphyllophoromycetideae), a mushroom has been shown to possess compounds that are important for neurite outgrowth activity. Compounds of *H. erinaceus* from aqueous (Wong *et al.*, 2007) and ethanol extract-like hericenones (Kawagishi *et al.*, 1991) and erinacine (Shimbo *et al.*, 2005) are able to mimic NGF to stimulate neurite outgrowth. Likewise, *Ganoderma lucidum* contains neuro-active compounds that induce neuronal differentiation (Cheung *et al.*, 2000). The differentiation might be mediated by the ras / extracellular signal-regulated kinase (ERK). *Termitomyces spp.* can occasionally be found in local weekend and farmers' markets. It is highly sought after as food by Temuans, an indigenous community in Malaysia (Chang & Lee, 2004). Compounds isolated from ethanol extract of *Termitomyces albuminosus* (Berk.) Heim, termitomycesphins A-D were reported to demonstrate neuritogenic properties (Qi *et al.*, 2000).

In Malaysia, *Lignosus rhinocerotis* (Cooke) Ryvarden has been singled out as one of the most potent mushroom with medicinal properties by the Malays, Chinese and indigenous communities for the treatment of a variety of ailments. This dates a back to the 1700s as documented by Tuan Haji Mat Yusop, a Malay in Pahang (Tan, 2009; Tan *et al.*, 2010). *Lignosus rhinocerotis* is also known as "cendawan susu rimau" in Malay language or Tiger's Milk mushroom in English. It is a unique "National Treasure" that can only be found in a small geographic region in South China, Thailand, Malaysia, Indonesia, Philippines, Papua New Guinea, New Zealand and Australia (Tan, 2009). The

different indigenous communities in Malaysia use *L. rhinocerotis* for a number of ailments. The Temuans use it as a general tonic to give stamina as well as to treat cancer, food poisoning and other ailments (Tan *et al.*, 2010). However, the medicinal uses recorded by ethano-mycological surveys are yet to be validated scientifically.

Although this mushroom has been recorded to have a number of medicinal properties, its use is limited due to unavailability of the mushroom. The underground tuber or sclerotium is the part with the medicinal value. It can only be noticed when the fruiting body sprouts out from the ground. Unfortunately, when the fruiting bodies are visible, much of the nutrients have been utilised to form the fruiting body, and the sclerotium will shrink in size. Recently, efforts made to cultivate *L. rhinocerotis* have been successful (Tan *et al.*, 2010; Lau *et al.*, 2013) and the mushroom is now available for studies to scientifically validate the ethnomycological uses.

One of the many uses of *L. rhinocerotis* is as general tonic for overall wellness and alertness. This could be related to brain activity and blood circulation. There is a paucity of studies that validate the traditional claims of *L. rhinocerotis.* The neuronal stimulating activity of *L. rhinocerotis* was investigated to validate the activities related to brain.

Rat pheochromocytoma, PC-12 cells were used as an *in vitro* model system to investigate neuronal stimulatory activity (Parmar *et al.*, 2002). These cells responded only to neurotrophin, NGF and differentiated into sympathetic neuron phenotype and extending axon-like processes called neurites. This makes it an appropriate model to investigate the effects of both synthetic and natural molecules that will stimulate neurites outgrowth (Parmar *et al.*, 2002).

1.1 Objectives

The objectives of this study were to:

a. assess neurite outgrowth activity in PC-12 cells with extracts of selected fruiting bodies or sclerotium of edible medicinal mushrooms.

b. evaluate the cytotoxic effects of aqueous and ethanol extracts of the sclerotium of *Lignosus rhinocerotis* in PC-12 cells.

c. investigate possible signaling pathways such as Ras-MAPK involved in neurite outgrowth that maybe activated by extracts of *Lignosus rhinocerotis*.

LITERATURE REVIEW

2.1 Mushroom

Mushrooms have long been appreciated for their flavour and texture. Now, they are becoming more important in our diet as a nutritious food and the source of biologically active compounds with medicinal value (Breene, 1990). High in protein and low in fat or energy content makes them the excellent food in low calorie diets. However, several thousand years ago in the Orient, many edible and certain non-edible mushrooms are believed to have valuable health benefits (Bensky & Gamble, 1993; Hobbs, 1995).

Concerning pharmaceutical potential, such as antimicrobial (Barros *et al.*, 2007), antiviral, antitumor, anti-allergic, immunomodulating, anti-inflammatory, antiatherogenic, hypoglycemic, and hepatoprotective properties (Lindequist *et al.*, 2005), neurite regeneration, mushrooms have also became attractive functional food with physiological beneficial constituents (Vidović *et al.*, 2010). Mushrooms have a promising future as a branch of alternative medicine as it contains rich medicinal values such as, a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids (Lindequist *et al.*, 2005).

Culinary mushrooms usually prepared fresh, cooked or processed to retain its nutrition value (Breene, 1990). Culinary mushrooms are important food source and can be a significant dietary component for vegetarians. The historical decoctions of these essentially scarce, forest obtained culinary medicinal mushrooms were not consumed in its raw state. But has been consumed as hot water extracts or powdered concentrated extracts used in drink or freeze-dried which allows easier handling, transportation and consumption (Mizuno *et al.*, 1995).

Culinary medicinal mushrooms, *Agaricus bisporus, Lentinula edodes* and *Pleurotus ostreatus,* that have been traditionally consumed in many countries have also been widely used to prevent life-threatening diseases such as cancer, diabetic, hyperlipidemia, arteriosclerosis and chronic hepatitis (Bilay *et al.*, 2011). Other mushroom like *Hericium erinaceus* (Kawagishi *et al.,* 2002) act as an inducer of the synthesis of nerve growth factor, an agent to treat gastric ulcers and esophageal carcinoma (Ying *et al.,* 1987).

2.1.1 Hericium erinaceus (Bull.: Fr.) Pers.

Hericium erinaceus is a fleshy edible mushroom under the Basidiomycota division which grows on dead or drying wood. As a well-known medicinal mushroom on neurite stimulating activity, *H. erinaceus* has been proven to have stimulating activity on animal nerve cells (Park *et al.,* 2002), improve cognitive ability in clinical trial (Mori *et al.*, 2009), stimulate NGF by phenol-analogous hericenones (Mori *et al.,* 2008).

Anti-dementia substances can be extracted from *H. erinaceus* which acts as stimulator of NGF-synthesis and may be potential therapeutic agents for degenerative neuronal disorder. The synthesising process of NGF in the cells is activated by benzyl alcohol derivatives, hericenones C to H (Kawagishi *et al.*, 1991; Mori *et al.*, 2008) and diterpenoid derivatives, erinacines A to I (Kawagishi *et al.*, 1996; Shimbo *et al.*, 2005) were derived from fruiting bodies and mycelium of *H. erinaceus*. Exo-polysaccharide derived from *H. erinaceus* mycelium cultivation broth was reported to promote neuronal outgrowth and survival (Park *et al.*, 2002). Furthermore, 5 % of lyophilized *H. erinaceus* powder able to inhibit the cytotoxicity activity of amyloid-β-peptide (Aβ) and is expected to prevent or cure Alzheimer's disease (Mori *et al*., 2011).

A newly isolated compound, dilinoleoyl-phosphatidylethanolamine (DLPE) was found to have neuronal protection to protect cells against cell death caused by Aβ toxicity, endoplasmic reticulum (ER) stress and oxidative stress (Kawagishi & Zhuang, 2008). Results of preliminary clinical trials also showed improvement of the Functional Independence Measure (FIM) score or retarding disease progression in patients with dementia who consumed mushrooms (Kawagishi & Zhuang, 2008).

Figure 2.1: *Hericium erinaceus* is an edible medicinal mushroom. It also known as lion's mane mushroom, bearded tooth mushroom, satyr's beard, bearded hedgehog mushroom, pom pom mushroom or bearded tooth fungus.

2.1.2 Termitomyces sp.

Termitomyces sp. is a tropical culinary mushroom under the genus of Basidiomycete and occurs in symbiosis with termites (Harkonen *et al.*, 2003). A polar fraction α, α,1,1'-trehalose in *Termitomyces* species was previously shown to have α-glucosidase inhibition (Baraza *et al.*, 2007); an anti-diabetic drug (Matsuura *et al.*, 2002) used to suppress postprandial hyperglycemia by preventing digestion of carbohydrates and avoid prolonged high blood glucose levels associated with diabetes (Moordian & Thuman, 1999).

Termitomyces sp. is a rich source of sugar, protein fibre, lipid, vitamin, mineral with added medicinal value in lowering blood pressure, rheumatism, kwashiorkor, obesity and diarrhoea (Apetorgbor *et al.*, 2005; Srivastava *et al.*, 2011). Polysaccharide, fucoglucan was successfully isolated from *Termitomyces robustus* (Mondal *et al.*, 2008).

Four novel cerebrosides termed termitomycesphins A–D isolated from the Chinese mushroom *Termitomyces albuminosus* (Berk.) Heim. ('Jizong' in Chinese) (Qi *et al.*,2000) was examined for neuronal differentiation activity using the PC-12 cell line. Termitomycesphins E and F are cerebrosides that are hydroxylated around the middle of the long-chain base (LCB) of novel cerebrosides. Results showed that major cerebroside which was not hydroxylated was inactive against PC-12 cells (Qi *et al.*, 2000). The di- and tetrahydroxylation of this inactive cerebroside resulted in the enhancement of its neuritogenic activity with the activity (25 % maximum neuronal differentiation at a concentration of 2.5 mM (Qi *et al.*, 2000).

Figure 2.2: *Termitomyces sp.*

2.1.3 Lignosus rhinocerotis (Cooke) Ryvarden

A unique "National Treasure" mushroom, *Lignosus rhinocerotis,* has been one of the most potent medicinal mushroom by Tuan Haji Mat Yusop dating back to the 1700s

(Tan *et al.*, 2010). This mushroom can only be found in South China, Thailand, Malaysia, Indonesia, Philippines, Papua New Guinea, New Zealand and Australia (Tan *et al.*, 2010). *Lignosus rhinocerotis* is named "cendawan susu rimau (tiger)" locally means tiger's milk mushroom and is an important medicinal mushroom found in Malaysia. The local community had been using this mushroom as an important medicinal mushroom since 1930 as described by Tuan Haji Mat Yusop, a local Malay in Pahang (Tan *et al.*, 2010). This mushroom is believed to have more than 15 medicinal uses according to different tribes which were used to treat for fever, cough, asthma, breast cancer, stomach cancer, food poisoning, healing wounds and others (Lee *et al.*, 2012).

Few reports were done on immunomodulatory activities on human innate immune cells stimulation (Wong *et al.*, 2009, 2011) and antipoliferative effects on various leukemic cells (Lai *et al.*, 2008) by sclerotial polysaccharides of *L. rhinocerotis* (*Polyporus rhinocerus*). However, paucity of reports on neurite stimulating activity by *L. rhinocerotis* was reported (Eik *et al.*, 2012). In recent year, Lau *et al.* (2013) studies on the effect of different processing methods of *L. rhinocerotis* aqueous extract as anticancer agents. Preclinical toxicology evaluations of the sclerotium of *L. rhinocerotis* were done and showed no genotoxicity (Lee *et al.*, 2012).

Figure 2.3: *Lignosus rhinocerotis* (Cooke) Ryvarden or known as Tiger milk mushroom.

2.2 Extraction Methods

Different extraction methods with different solvent to extract different chemical compounds were used. The selection of solvent is crucial in extracting the esired chemical components from the mushroom based on the specific nature of the bioactive compound being targeted.

2.2.1 Aqueous extraction

Aqueous extraction or hot water extraction was practiced to prepare decoctions in folk medicine to extract soluble components from the fruiting body. Accordingly, fruiting bodies were crushed or torn into small pieces and boiled and the resulting decoction is consumed. Besides that, raw mushrooms pass largely undigested in human intestine, hence, various processing methods were used to make them more readily assimilated by human digestion system (Stamets, 2005).

Apart from that, heat treatment, particle size, and carriers affect the nutritional impact of ingesting mushrooms. Reducing the particle size and the exposure to high temperature through cooking increase the absorbability, although, some vitamins, particularly vitamins C, might be degraded due to high temperature (Steskova *et al.*, 2006). Hence, the preparation of aqueous extracts is a simulation of cooking conditions—the typical way of consuming edible mushrooms and preparation of decoctions in folk medicine.

Hot water extraction method was widely used to extract high proportion of water soluble substances, primarily polysaccharides that are powerful anti-tumour agents, immune enhancers and strong antioxidants (Boh *et al.*, 2007). Preparation of hot water

aqueous extract have lower cytotoxicity effect than ethanol extraction (Faridur *et al.*, 2010).

2.2.2 Ethanol extraction

Alcoholic preparation which has much higher antioxidant activity is more useful than the aqueous extraction in medical approach (Pietta *et al.*, 1998). Alkaloids and a larger spectrum of biologically active constituents can be extracted by ethanol solvent (Pilarski *et al.*, 2006). Ethanol extraction by a less concentrated ethanol solution (80 ethanol: 20 aqueous) (Wong, 2012) is able to extract a range of polar and non-polar compounds and creates a greater opportunity in medical approaches like medical drugs (Beattie *et al.*, 2011).

Some researchers have reported that triterpenes, an alkaloid with a bitter taste, possesses antioxidant activity (Zhu *et al.*, 1999), hepatoprotection (Kim *et al.*, 2000), anti-hypertension (Kimura & Tamura, 1988) and inhibiting platelet aggregation (Su *et al.*, 1999) Triterpenes are the anti-inflammatory compounds of *Ganoderma lucidum* recommended for arthritis, asthma and allergies (Boh *et al.*, 2007).

Besides, triterpenoids extracted from *Centella asiatica* in ethanol solvent, asiatic acid or madecassic acid has previously been associated with neuroprotective and neurotropic effects (Jew *et al.*, 2000; Soumyanath *et al.*, 2005). Novel diterpenoid, Scabronine A-F (Kita *et al.*, 1998; Ohta *et al.*, 1998), Cyathane diterpenes (Shi *et al.*, 2011) isolated from *Sarcodon scabrosus* in ethanol solvent were reported to have a positive effect on NGF synthesis and neurite-outgrowth promoting activities.

2.2.3 Alkaline extraction

Literature showed that the polysaccharide fraction isolated from *Ganoderma lucidum* could be an important functional factor that has been reported to stimulate the proliferation of mouse spleen lymphocytes (Huang *et al.*, 2010) and exhibit various other bioactivities, including anti-HIV, anti-herpetic, antiviral (Kim *et al.*, 2000), immune regulating (Zhang *et al.*, 2002), anti-tumour properties (Zhang et al., 2007) and neuronal growth and differentiation (Park *et al.*, 2002). Polysaccharides can be extracted via various extraction methods; optimisation of the extraction method is an important process for their application or further research and development.

For polysaccharides, hot water extraction is the most widely used but it is associated with lower yields, long extraction times and high temperatures (Huang & Ning, 2010). In order to obtain higher yields and save time, polysaccharides extracted by alkaline solution consists four types of monosaccharide and 18 types of amino acid and showed good response for anti-tumour activity (Kim *et al.*, 1980).

The alkaline treatment caused the destruction of the coarse and compact fibre structure of cell wall and promote the release of polysaccharides resulting in reduced content of cellulose, hemi-cellulose, acid lignin and silicate from the residue (Huang & Ning, 2010). The major component in the biologically active polysaccharides was identified as β-D-glucan and can provide quite different antitumor effects as their biological activities are influenced by their structures' molecular weight, water solubility, degree of branching and conformation (Mizuno *et al.*, 1995).

A water soluble substance, exo-polysaccharide from the culture broth of *H. erinaceus* was reported to improve neurite outgrowth in PC-12 cells and even more

efficacy than NGF and brain-derived nerve factor (BDNF) (Park *et al.*, 2002). Report stated that NGF and BDNF partially delay the apoptosis of nerve cells (Park *et al.*, 2002).

2.3 Neural Network

Human brain and body consists of a neural network (also known as a network of "nerve cell") that coordinates our body responses and transmits signals to different parts of our body. Active axonal elongation and systematic exploration activity of environment by growing of axons towards their target are required to form the central nervous system (CNS) network (Diez-Revuelta *et al.*, 2010). Axonal elongation of neurones enables them to sense the surrounding environment and form branches. In order to response to molecular information from extracellular environment, the maturation process is instructed in signals form (Diez-Revuelta *et al.*, 2010). Signals are sent to efferent neuron in the form of electrochemical waves (known as impulses) and travel along nerve fibres known as axons.

2.3.1 Neurodegenerative Diseases

Neurodegenerative diseases are traumatic to both patients and their families. Out of these neurodegenerative diseases, Alzheimer's disease is the most common (Tanner and Goldman 1996). Indeed, there has been an exponential increase in our knowledge of disease mechanisms especially during the past decade (Smith *et al.*, 2006). The traditional doctrine has eventually contributed new findings and ideas on this fascinating research. These have changed our understanding of Alzheimer's disease and other neurodegenerative diseases.

Alzheimer's disease causes serious impairment to thinking and memory due to neuronal loss in the brain (Shulman & Jager, 2009). The second most common

neurodegenerative syndrome, Parkinson's disease, is characterised by the classic symptoms of tremors, rigidity and gait impairment (Parkinson, 2002). To understand these neurodegenerative disorders, cell culture models were used to evaluate the process of these diseases processes.

2.4 Cell Culture

Cell culture is dispersal of cells in an artificial environment composed of nutrient solutions, a pre-coated surface to support the growth of cells, with an ideal condition of temperature, humidity and atmosphere gaseous (Freshney, 2005). Cell culture allowed researcher to precisely measure the response of cells alterations. Cell lines used for biological research were usually immortal and cancerous. Many primary neuron cell cultures, such as dorsal root ganglia, cortical neurons, cerebellar granule neurons (CGNs), and established cell lines, such as NG108-15, SH-SY5Y and PC-12 had previuosly been developed and extensively used as models for neurite outgrowth studies (Mitchell *et al.*, 2007).

2.4.1 PC-12 cell line

PC-12 cell line, derived from a rat pheochromocytoma of the rat adrenal medulla served as a good model and has been extensively used for studying the differentiation of neurite. PC-12 in polygonal shape cells stop dividing and terminally respond to neurotrophic factors, NGF, by differentiating into sympathetic neuron-like phenotypes that are characterized by neurite outgrowth and the expression of many neuron-specific proteins (Drubin *et al.*, 1985; Das *et al.*, 2004). Manual examination of individual cells under a microscope was used to measure the neurite outgrowth.

2.5 Cytotoxicity

The measurement of cell viability and growth is a valuable tool in a wide range of pharmacologic research areas. In this study, PC-12 were used to study the cytotoxicity effect of crude *L. rhinocerotis* sclerotial extract on its effect on *in vitro* system for screening purposes involving natural products. The reduction of tetrazolium salts (MTT) is recognised as a safe, fast and accurate test. The applications used for this method are to examine drug sensitivity, cytotoxicity, response to growth factors and cell activation (van de Loosdrecht *et al.*, 1994; Mosmann, 1983).

2.6 Neurite Outgrowth

One of the indication of neuroregeneration potential is via neurite outgrowth in cultured neurons (Mitchell *et al.*, 2007). Cultured neurons from multiple sources are able to extend neurites to be utilised for *in vitro* assays and mimic the mechanism in CNS. Extension of the axon of the neurite can be assessed while screening compounds that exhibit and inhibit the extension of neurite.

PC-12 has been extensively used as model for neurite outgrowth model (Mitchell *et al.*, 2007). However, more qualitative results were produced compare to quantitative results has become the limitation of this study. Data was collected quantitatively by manually processing individual images, which can be very time consuming, tedious and susceptible to operator variability (Mitchell *et al.*, 2007). However, this is the most convenient protocol with reproducible results.

2.6.1 Neuronal differentiation assessment

Neuronal differentiation is assessed by evaluating the total number of neuronal process formation, as done by direct measurement of neurite length (Fujii *et al.*, 1982);

counting the number of neurites per cells (Greene & Tischler, 1976); number of neurite-bearing cells with the extending neurites of length double the viable of the cell (Blackman *et al.*, 1993). Another method of studying neuronal differentiation is by investigating immunoreactive neurons for the nonphosphorylated 200-kDa neurofilament (NF) protein (Shepherd *et al.*, 2002).

Neurofilament is the major structural component of neurons. It represents a class of antigenically and biochemically distinct intermediate-sized filaments and is composed of three polypeptides with approximate molecular weights of 68,000, 160,000 and 200,000 (Weber *et al.*, 1983). It has been suggested that NF with molecular weight of 200,000 polypeptide probably has more specialised role in NF architecture and function on the basis of differential expression of NF triplet polypeptides in brain development (Shaw & Weber, 1982). Degree of differentiation of PC-12 can also be evaluated via the amount of neurofilament proteins (NF triplet-proteins), β-tubulin III and cell proliferation (Ohuchi *et al.*, 1994).

2.7 Nerve Growth Factor

Neurotrophins like NGF, brain-derived neurotrophin factor (BDNF) and neurotrophin-3 (NT-3) are factors that response mainly to neurite outgrowth. Whereas NGF acts as the most powerful neurotrophin acting on cholinergic neurons; and was the first identified protein with anti-apoptotic activity on neurons (Dechant & Neumann, 2002). Research was done and has solid support to prove the hypothesis that neurotrophins are able to prevent neuronal death in cell cultures and animal models (Dechant & Neumann, 2002).

These neurotrophic factors belong to the family of proteins, due to their high molecular weights and hydrophilic structure. It is hard for these neurotrophins to cross the blood-brain barrier. Therefore, the new search is now for small molecules that can cross the brain-blood and induce the production of NGF, a family of proteins responsible for the maintenance, survival, and regeneration of neurons during adult life. We may have to switch to nature products to prevent or reduce the severity of nerve-related diseases as we age.

Researchers are discovering natural remedies for ages of their therapeutic properties against various diseases. For example, in India, it is noted that Alzheimer's among the older generation is not in alarming numbers. The regular consumption of spices, including turmeric (Mishra & Palanivelu, 2008; Hishikawa *et al.*, 2012) and piperine, extracted from Thai black pepper (Chanpathompikunlert *et al.*, 2010) may be the reason and is currently being studied actively. Currently, mushrooms are also being investigated as sources of NGF stimulators (Kawagishi *et al.*, 1991, 1997; Cheung *et al.*, 2000).

2.7.1 NGF and neurodegenerative diseases

The connection of NGF deficits and neurodegeneration has been proven with rat model. Nerve growth factor and BDNF are two crucial neurotrophic factors for basal forebrain cholinergic neurons (Hefti *et al.*, 1989). Decrease of NGF gene expression in the nucleus of basal leads to Alzheimer's disease (Mufson *et al.*, 1989). Post mortem samples of hippocampus have revealed a profound decrease in mRNA levels of BDNF in Alzheimer's disease patients as compared with healthy people (Phillips *et al.*, 1991).

Aged anti-NGF mice showed massive and widespread neuronal loss, amyloid deposits and extensive neurofibrillary pathology demonstrated with anti-antibodies. Moreover, these tangle and anti-phosphorylated mice exhibited a severe cholinergic deficit in the basal forebrain and a behavioural impairment in retention and transfer of spatial memory tasks (Capsoni *et al.*, 2000). This finding suggests that the lack of NGF causes Alzheimer's-like disease in adult rat.

Hence, NGF might be useful symptomatic-therapeutic agents and has spurred on hopes in treating neurodegenerative disease. A topical application of NGF into the brain of Alzheimer's patient restores the symptoms (Olson *et al.*, 1992). *Hericium erinaceus* is reported to contain NGF-like compound which is small enough to pass through the blood-brain barrier (Mori *et al.*, 2009). Evidence of oral administration of *H. erinaceus* increases NGF mRNA expression in mouse hippocampus and locus coeruleus proved that the compounds is small enough to be absorbed into blood and delivered into the central nervous system. However, the NGF mRNA expression in cortex was not increased by NGF-like compound in *H. erinaceus* (Mori *et al.*, 2009).

2.7.2 Synergistic effect of NGF and compound from natural sources

Many compounds from natural sources, like mushroom and plants, have been demonstrated to possess neurotrophic and neuroprotective abilities. For instance, alkaloids from *H.erinaceus* (Kawagishi *et al.*, 1991, 1996) and *Sarcodon scabrosus* have the abilities (Ohta *et al.*, 1998). The role of natural products to enhance the neurite outgrowth activity of NGF in various experimental models is also affirmed.

Compounds like hericenones (Kawagishi *et al.*, 1991), curcumin, ginsenoside (More *et al.*, 2012) have shown to have the capability to enhance the action of

neurotrophic factors in stimulating neurite outgrowth and may contribute to the treatment of neurodegeneration disorders like Alzheimer's disease (AD) (Connor & Dragunow, 1998; Kawagishi *et al.*, 2002) Aqueous extract of *H. erinaceus* also proven to help in regeneration in peripheral nerve after a crushing injury (Wong *et al.*, 2011). Literature also suggested that compounds from natural sources in combination with NGF induce the growth of neurites synergistically. Hence, natural products may harmonise very well for the treatment of neuronal injury (Li *et al.*, 2003; Shibata *et al.*, 2008).

2.8 Protein Kinases Signalling Pathway

Protein kinases are ubiquitous enzymes that allow the modification of activities of other proteins by adding phosphate groups to their tyrosine, serine or threonine amino acids (phosphorylation). MAPKs (Mitogen-Activated Protein Kinases), which are activated by many different signals, belong to a large family of serine/threonine protein kinases that are conserved in organisms as diverse as yeast and humans (Schaeffer & Weber, 1999). MAPKs deliver extracellular signals from activated receptors to various cellular compartments, notably the nucleus, where they direct the execution of appropriate genetic programs, where a cell has a physiological change, brought by activation of gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation. Figure 2.1 showed activation of MAPKs activated by extracellular stimuli (Liu *et al.*, 2007).

The MAPK pathway exists in all eukaryotes and acts as controller for fundamental cellular processes like proliferation, differentiation, survival and apoptosis. ERK pathway was activated by growth factor whereas stress and inflammatory cytokines activated JNK and p38 pathways (Liu *et al.*, 2007). Differentiation and survival of PC-12 cells are also activated by growth factor, NGF and require participation of various MAPKs, including

extracellular signal-regulated protein kinase 1/2 (ERK1/2), (Leppa *et al.*, 1998), c-jun N-terminal kinases (JNK) (Leppa & Bohmann, 1999) and p38 MAPKs (Morooka & Nishida, 1998).

2.8.1 Activation of MAPK stimulated neurite outgrowth

Another major growth factor receptor downstream cascade in protein kinase implicated in neuronal survival and neurite outgrowth is phosphatidylinositol-3-kinase-Akt (PI3K-Akt) pathway (Naidu *et al.*, 2009). A cultured sympathetic neuron of the superior cervical ganglion from postnatal rats, showed Ras-MAPK and PI3-Akt pathway. TrkB mutated at the Shc, TrkB-Shc activated the binding site and support in neuronal survival and promote axon outgrowth (Atwal *et al.*, 2000). Even though further details of these two signalling cascades are yet to be discovered, the PI3-Akt cascade has been known in mediating neurotrophin-promoted cell survival, whereas MAPK cascade mediates neurite outgrowth (Crowder & Freeman, 1998).

Nerve growth factor elicit phosphorylation and induce PC-12 cell differentiation through sustained activation of ERK1/2 or p38 MAPK (Morooka & Nishida, 1998). Introduction of inhibitor inhibit phosphorylation of ERK1/2 or p38 MAPK by blocking neurite differentiation in PC-12 cells (Roberson *et al.*, 1999). In a study by Mori et al. (2008) study, JNK acts as predominant kinase involved in enhancement of NGF gene expression induced by *H. erinaceus* by enhancing phosphorylatation of c-Jun and c-fos gene.

A neurotrophic-like factor, Artepillin C, a major component of Brazilian propolis, induces outgrowth of rat PC12m3 cells and activates p38 MAPK through the ERK

signaling pathway (Kano *et al.*, 2008). Alpha-lipoic acid (LA), a therapeutic approach for neural disorders reported that LA administration promotes neurite outgrowthin neuroblastoma N2a cells and primary neurons via phosphorylation of ERK and Akt (Wang *et al.*, 2011).

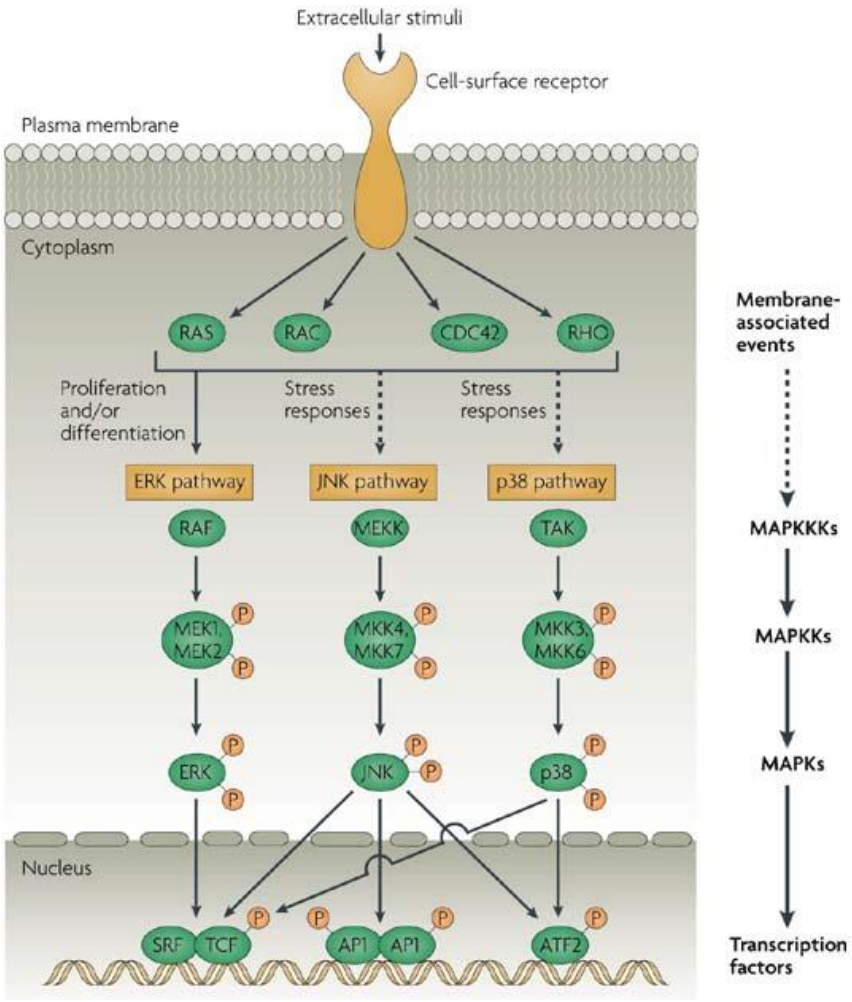


Figure 2.4: Activation of MAPK pathway via extracellular stimuli (Liu *et al.*, 2007).

Besides, cultured neuronal cells, MAPK even phosphor-MAPK is present in the rat sciatic nerve (Johanson *et al.*, 1995), normal dorsal root ganglia and in the rat sciatic nerves even after injury (Naidu *et al.*, 2009). Phosphorylation of synapsin is dependent on MAPK/ERK in the establishment of functional synaptic connections and mobility of synapsin as well as trafficking of synaptic vesicles in nerve terminals upon stimulation

(Chi *et al.*, 2001; Giachello *et al.*, 2010). ERK 1/2 phosphorylation also acts as a key event to the early neuronal differentiation and survival of embryonic stem cells (Li *et al.*, 2006).

MATERIALS AND METHODS

3.1 Mushroom samples

Sclerotium of *L. rhinocerotis* was successfully domesticated and is produced in large scale by Ligno Biotech Sdn Bhd, Malaysia. Freeze-dried sclerotium powder was purchased from Ligno Biotech Sdn Bhd. Fresh fruiting bodies of *H. erinaceus* purchased from Ganofarm *and T. heimii* were collected from the field (identity confirmed by mycologist of Mushroom Research Centre). Fresh mushroom fruiting bodies were initially shredded and freeze-dried. Dried fruiting bodies were then blended in a Waring Commercial Blender (Waring, USA) and the powder was stored at 4 ℃ prior to use. Same batch of mushroom was used throughout the assays.

3.1.1 Aqueous extract

Aqueous extraction of dried fruiting bodies was carried out by the modified method by Wong *et al.* (2007). Freeze-dried powder was weighed and soaked in distilled water at a ratio of 1:10 (w/v) and agitated at 150 rpm for 24 hr at room temperature. The mixture was then double boiled in a water bath for 30 minutes, left to cool and filtered through Whatman's filter paper No. 4 and subsequently through Whatman's filter paper No. 1 (Figure 3.1). Aqueous extract (supernatant) was collected and freeze-dried at -50 ± 2 ℃ for 48 hours. Freeze-dried powder was stored in airtight bottles at 4 ℃ prior to assay.

3.1.2 Ethanol extract

Freeze-dried powder was weighed and soaked with 80% ethanol at a ratio of 1:10 (w/v) for nine days (Wong, 2012). The extract was filtered and the residue was topped with fresh 80% ethanol at three days intervals, for nine days or until the colour turned clear. Cocktails were filtered through Whatman's filter paper No. 1 (Figure 3.1). Filtered

supernatants were collected and ethanol solvent was then subjected to rotary evaporation. Residues were collected and stored in airtight bottles at 4 ℃ prior to assay.

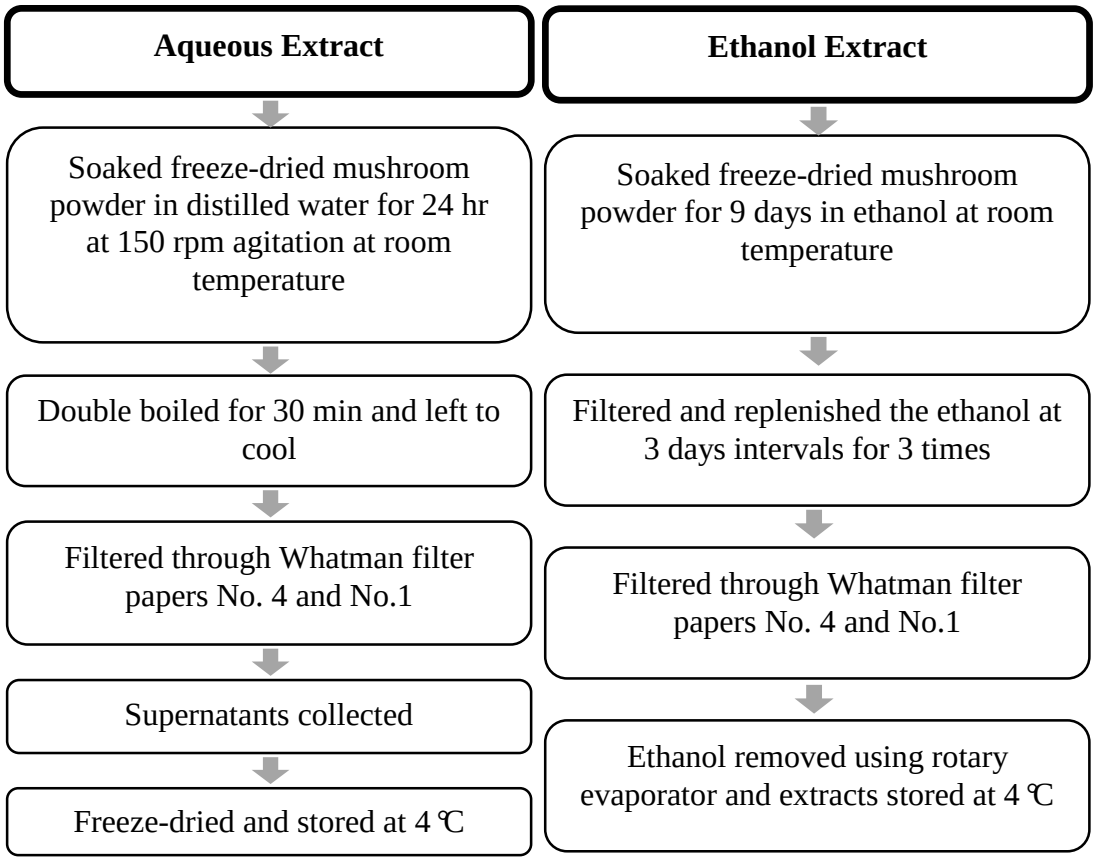


Figure 3.1: The process of aqueous and ethanol extraction of mushroom freeze dried fruiting bodies and sclerotium (Wong *et al.*, 2007; Wong, 2012).

3.1.3 Crude polysaccharides

Crude polysaccharides were extracted according to the alkaline extraction method of Ojha *et al.* (2010). Freeze-dried powder was weighed and soaked in a sufficient amount of 4% (w/v) sodium hydroxide (NaOH) to submerge the mushroom powder. The mixture was heated at 80 ℃ in a water bath for 45 minutes. Alkaline extracts were centrifuged at 7,800 x g for 45 minutes. Supernatant was collected and precipitated at a ratio of 1:5 (v/v) supernatant to absolute ethanol. The mixture was kept for 12 hr at 4 ℃ to precipitate the polysaccharides. The precipitated polysaccharides were centrifuged at 7,800 x g for 45 minutes. The residue was dialysed using a Diethylaminoethyl (DEAE) cellulose bag for

4 hr to obtain the alkaline polysaccharides. The crude polysaccharides were then freeze-dried and stored in airtight bottles at 4 ℃ prior to assay.

3.2 Rat Pheochromocytoma cell line (PC-12)

PC-12 cell line is derived from transplantable rat pheochromocytoma that has been widely used as neuronal model because it proliferates in growth medium, and when NGF is added, the proliferation stops. The cells then differentiate into neuron-like cells (Ohnuma *et al.*, 2006). The cell line was purchased from American Type Culture Collection (ATCC, Manassass, VA, USA) (Cat #: CRL-1721.1TM) and only early passage cells (5^{th} passage to 20^{th} passage) were used in this study.

3.2.1 Culture of PC-12

PC-12 cells were cultured in ATCC-formulated F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) purchased from Sigma-Aldrich (St. Louis, MO, USA). Complete medium was prepared by adding 15% Horse serum (v/v) and 2.5% Fetal Bovine Serum (FBS) (v/v) into F-12K medium. Cells seeded in complete medium were incubated in a 5% CO_2 humidified incubator at 37 ± 2 ℃. Cells were subcultured every two to three days as needed. PC-12 cells tend to form clusters and clumps. Cells were detached from the bottom of the flask by scraping and then forceful aspiration to break the cell clusters.

3.3 Assessment of cytotoxic activity of *L. rhinocerotis* extract in PC-12 cells

3.3.1 Cell viability assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT)

Cell viability assay was carried out according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). MTT [3-(4,5-dimethylthiazolyl-2)-2,5-

diphenyltetrazolium bromide] was used to measure the activity of enzymes in living cells that are able to reduce tetrazolium salt to formazan dyes to produce a purple colour. Formazan dye released was recorded as a means of measuring the cell viability. PC-12 cells were seeded at a density of 1 x 10^5 cells/well in 96-well microtitre plates and were allowed to attach for 24 hours in a 5% CO_2 humidified incubator at 37 ± 2 ℃. Well(s) with the medium alone served as negative control whereas well(s) supplemented with 50 ng/mL (w/v) NGF served as positive control. Aqueous and ethanol extract of *L. rhinocerotis* and *H. erinaceus* was then applied at various concentrations (10-1000 µg/mL). Microtire plates were incubated for 24 hours. Then, MTT at a final concentration of 0.5 mg/mL (w/v) was added into each well and incubated for four hours. The medium with MTT solution was discarded and to each well, 100 µL of DMSO was added to solubilise the intracellular purple formazan crystal.

The absorbance was recorded at 570 nm with background absorbance of 690 nm on Sunrise^{TM} microplate reader (Tecan Group Ltd., Switzerland). Cell viability was expressed as a percentage of untreated cells, which served as the negative control group and was designated as 100%. The results were expressed as a percentage of viability and the half maximal inhibitory concentration (IC_{50}). All assays were performed in quadruplicates. The percentage of cell viability after treatment with extracts was calculated by the formula below:

$$\% \text{ of cell viability} = \frac{\text{Abs Sample}}{\text{(Abs Control)}} \times 100\%$$

3.4 Stimulation of neurite outgrowth by mushroom extracts from PC-12 cells

Cells were cultured for two to three days until 60-70% confluence was achieved. Stimulation of neurite outgrowth was carried out according to Wong *et al.* (2007) method.

Cells were plated at a density of 5 x 10^4 cells per well in complete F-12K medium. Cells grown in complete F-12K medium alone served as negative control. Assay plates were incubated at 37 ±2 ℃ in a 5% CO_2 humidified incubator. Differentiation activity (neurite outgrowth and branching) of cells were observed after 48 hours of incubation unless otherwise stated.

3.4.1 Effects of NGF on neurite outgrowth from PC-12 cells

Nerve growth factor (NGF)-7S from murine submaxillary gland (Sigma, St. Louis, MO, USA) was diluted to concentrations that ranged from 10 ng/ml (w/v) to 100 ng/ml (w/v) in complete F-12K medium. The concentration that showed optimum neurite outgrowth was subsequently used as positive control for all assays unless otherwise stated.

3.4.2 Effects of aqueous extracts of L. rhinocerotis, H. erinaceus and T. heimii on neurite outgrowth from PC-12 cells

Cells were treated with mushroom aqueous extracts. Aqueous extracts were diluted to concentrations that ranged from 10 μg/ml (w/v) to 100 μg/ml (w/v) in distilled water.

3.4.3 Effects of ethanol and aqueous extract on neurite outgrowth from PC-12 cells

According to 3.4.2, *L. rhinocerotis* and *H. erinaceus* aqueous extract showed higher percentage of neurite-bearing cells. Cells were then treated with *L. rhinocerotis* and *H. erinaceus* aqueous and ethanol extracts to compare the neurite outgrowth activity. Aqueous and ethanol extracts were diluted in sterile distilled water or DMSO respectively to concentrations that ranged from 10 μg/ml (w/v) to 100 μg/ml (w/v).

3.4.4 Effects of crude polysaccharide on neurite outgrowth from PC-12 cells

According to 3.4.3, *L. rhinocerotis* aqueous extract showed the highest percentage of neurite-bearing cells. Crude polysaccharides extracted from aqueous extract were tested on neurite outgrowth activity. Polysaccharides diluted with distilled water to the desired concentrations that ranged from 25 µg/ml (w/v) to 100 µg/ml (w/v) was added to PC-12 cells in complete F-12K medium.

3.4.5 Addition effects of aqueous extract with NGF on neurite outgrowth from PC-12 cells

According to 3.4.4, *L. rhinocerotis* aqueous extract still showed the highest percentage of neurite-bearing cells. Cells were treated in a combination of aqueous extracts with NGF at different concentrations. Aqueous extracts were diluted to the optimum concentration, 20 µg/mL (w/v). Concentrations of NGF that ranged from 10 ng/mL (w/v) to 50 ng/mL (w/v) were added to the optimum concentration of aqueous extracts in each well.

3.5 To evaluate the effects of extracts treatment on neurite outgrowth from PC-12

3.5.1 Quantitative assessment of neurite scoring

Neurite outgrowth was recorded after 48 hr. Neurite scorings were recorded with a cell which has a thin neurite extension that was at least double the length of the cell body diameter (Smalheiser and Schwartz, 1987). Morphology of the cells is polygonal. Cells with irregular patterns such as sheet-like spreading cells, rare radially oriented possess, and apparently arising by "shrinkage" were excluded (Smalheiser & Schwartz., 1987; Wong *et al.*, 2007) and cell clumps with more than five cells each were also excluded (Wong *et al.*, 2007). Ten fields per well were randomly examined and

photographed under Nikon Eclipse TS100 with 10 x 10.25 Nikon objective and captured with Nikon DS-Fi1 camera and Nikon's Imaging Software, NIS-Elements. The percentage of neurite-bearing cells were quantified by scoring the total number of neurite-bearing cells and the total number of viable cells in 10 microscopic fields with an average of 200 to 300 cells per well.

3.5.2 Preparation of washing buffer, blocking buffer and antibodies

Washing buffer was prepared by adding 0.3 (v/v) of Triton-X into phosphate buffered saline (PBS).

Blocking buffer was prepared by adding 10% of sheep serum (Sigma, St. Louis, MO, USA) into washing buffer. Blocking buffer was used to constitute primary and secondary antibodies.

Primary antibody, neurofilament 200 produced in rabbit (Sigma, St. Louis, MO, USA) were diluted to ratio of 1:80 in blocking buffer. Secondary antibody, fluorophore-conjugated secondary antibody, anti-rabbit IgG – fluorescein isothiocyanate (FITC) produced in sheep (Sigma, St. Louis, MO, USA) was diluted to ratio of 1:160 in blocking buffer.

3.5.3 Seedling of PC-12 cells

Immunofluorescence staining of neurofilament protein was carried out according to Schimmelpfeng *et al.* (2004). Cells were seeded into 6-well plates with each well containing two sterile coverslips. *Lignosus rhinocerotis* aqueous extract, *H. erinaceus* aqueous extract at 20 µg/mL (w/v) and *L. rhinocerotis* at 20 µg/mL (w/v) along with 30 ng/mL (w/v) of NGF were added into each well with PC-12 cells. This was then incubated for two days at 37 ± 2 ℃ in a 5% CO_2 humidified incubator until a 50% to 70%

confluence level was achieved. Cells were fixed with 4% (v/v) paraformaldehyde for 20 minutes at room temperature.

3.5.4 Localisation of the neurofilament protein in PC-12 by indirect immunofluorescence staining

The cells with extract treatments were incubated with primary antibody at room temperature for two hours and then washed with washing buffer three times at five minute intervals. The secondary antibody was then added and the plates were incubated at room temperature for one hour in the dark and washed with washing buffer three times at five minute intervals.

3.5.5 Detection of the neurofilament protein

After washing with washing buffer, coverslips were removed carefully and mounted with aqueous mounting medium containing 4'-6-Diamidino-2-phenylindole (DAPI) to stain the nuclei. Finally, slides were observed using the Nikon Eclipse 80i microscope under fluorescence illumination and images were captured with the NIS-Elements Imaging Software.

3.6 To elucidate the protein signaling pathway involved in stimulation of neurite outgrowth

3.6.1 To investigate the participation of the TrkA pathway in the induction of neurite outgrowth

Lignosus rhinocerotis aqueous extract, *H. erinaceus* aqueous extract at 20 µg/mL (w/v) and *L. rhinocerotis* at 20 µg/mL (w/v) with 30 ng/mL (w/v) of NGF were used as extracts to stimulate neurite outgrowth in the subsequent assays unless otherwise stated. Cells were plated in 12-well plates (as described in 3.4). PC-12 cells were pre-treated

with inhibitor K252a at 100 nM for 60 min prior to stimulation of neurite outgrowth with extracts. PC-12 cells not pre-treated with inhibitor K252a served as standard to compare the inhibitory activity. Assay plates were incubated at 37 ± 2 ℃ in a 5% CO_2 humidified incubator. The differentiation activity, that is neurite outgrowth and branching of cells were observed after 48 hours of incubation.

3.6.2 To detect mitogen-activated protein kinase (MAPK)

Immunofluorescence staining of protein kinase was carried out by using µ-Chamber 12 well slides (Ibidi, Martinsried, Germany). Each well of the slide was seeded with 4 x 10^4 cells/mL at 100 µL. Cells were treated with the inhibitor cocktail corresponding to the specific protein signaling pathway tested, which was added one hour prior to the addition of extracts. Inhibitor U1026 (Cell Signaling Technology, Danvers, MA, USA) was used to inhibit MEK1/2. Lyophilized inhibitor powder was suspended in DMSO and further diluted to their optimum concentration as suggested by the manufacturer's protocol, 10 µM for U1026 inhibitor. The cells were then treated with extracts (as described in 3.6.1).

The slides were incubated for two days at 37 ± 2 ℃ in a 5% CO_2 humidified incubator. Cells with 50% to 70% confluence levels were fixed with 4% (v/v) paraformaldehyde at room temperature for 20 minutes. Primary antibodies and secondary antibodies were constituted in blocking buffer (as described in 3.5.2). The cells were then incubated with primary antibody (phospho-p44/42, phospho-p38 and phospho-SAPK/JNK) suspended in blocking buffer in their respective ratios at room temperature for two hours.

The primary antibody tested was the family of phospho-MAPK: phospho-p44/42 (Thr202/Tyr204) (1:100 dilution), phospho-p38 (Thr180/Tyr182) (1:800 dilution),

phospho-SAPK/JNK (Thr183/Tyr185) (1:80 dilution) and the family of phospho-Akt pathway: phospho-Akt (Ser473) (1:200 dilution), phosphor-Akt (Thr308) (1:800 dilution), Akt (pan) (1:400 dilution), phospho-c-Raf (Ser259) (1:80 dilution), phosphor-GSK-3β (Ser9) (1:100 dilution), phospho-PTEN (Ser380) (1:80 dilution) and phospho-PDK1 (Ser241) (1:80 dilution) purchased from Cell Signaling Technology (Danvers, MA, USA).

Subsequently, a further reaction with the secondary antibody, Fluorophore-conjugated secondary antibody, Anti-Rabbit IgG-Fluorescein isothiocyanate (FITC) antibody produced in sheep was at room temperature for one hour in the dark. Detection method was as described in 3.5.5.

3.7 To quantify protein expression level by ELISA method

The p38 MAPK protein expression was quantified according to the manufacturer protocol by Invitrogen, (Camarillo, CA). The total p44/42 and phosphor-p44/42 MAPK protein were expressed according to the manufacturer protocol by Cell signaling Technology (Danvers, MA). Protein was extracted with extracting buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM of phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF). The stability of protease inhibitor supplemented cell extracting buffer was 24 hours at 4 ℃.

3.7.1 Extraction of protein in PC-12

PC-12 cells treated with extracts (as described in 3.6.1) were cultured in 75 cm^3 of cell culture flask for 2 days until a 70-90% confluence level was achieved. Cells were collected in PBS by scrapping. The cell pellets were collected after centrifugation at 10,000 xg for ten minutes at 4 ℃. The pellets were lysed in protease inhibited and PMSF

supplemented cell extraction buffer for 30 minutes on ice with vortexing at ten minutes intervals. The volume of cell extraction buffer added depends on the cell number in the pellet. One millilitre of cell extract buffer was added to 10^8 of cells to extract the cellular proteins. Extracted proteins were then transferred to microcentrifuge tubes and were pelleted at 10,000 xg for ten minutes at 4 ℃. Supernatants were aliquoted into clean microcentrifuge tubes prior to assay.

3.7.2 Detection of target protein

Primary antibody standard was constituted and diluted with standard diluent buffer. Secondary antibody, anti-rabbit IgG HRP was diluted to 1X with HRP diluent as anti-rabbit IgG HRP working solution. Every 8-well strip required 1 mL of 1X Anti-Rabbit IgG HRP Working Solution. Next, washing buffer was prepared by diluting the wash buffer concentrate to 1X and storage at 4 ℃ for a maximum of two weeks.

Standards, samples or controls (100 µL) were added to the appropriate microtiter wells. Protein samples prepared in cell extraction buffer were diluted to 1:10 or greater in a standard diluent buffer. The side of the plate was tapped to mix the well contents and the plate was covered with a plate cover and incubated for two hours at room temperature. The solution in the wells were then decanted and washed four times with washing buffer. Subsequently, Anti-Rabbit IgG working solution was added to each well except the chromagen blank(s). The plate was covered and incubated for 30 minutes at room temperature.

After 30 minutes of incubation, the solution was decanted and the wells were washed again four times with washing solution. Hundred microliter of stabilised chromogen was then added into each well. Chromogen acted as a substrate for the bound

enzyme to produce a blue coloured product. The plate was incubated for 30 minutes in the dark. Next, the stop solution (100 µL) was added to each well. The solution changed from blue to yellow and its absorbance was recorded at 450 nm using the Sunrise^{TM} microplate reader (Tecan Group Ltd., Switzerland) within two hours of the addition of stop solution.

The calibration graph of absorbance against the standard concentration of the antibody standard was plotted (Appendix B.11). Concentrations of protein antibodies of each treatment were quantified according to the standard graph and multiplied by the appropriate dilution factor.

3.8 To elucidate the nutritional components in *L. rhinocerotis*

Based on the result of the neurite outgrowth activity, *L. rhinocerotis* was selected for further studies. Fifty grams sample of *L. rhinocerotis* sclerotium powder was sent to Consolidated Laboratory (M) Sdn. Bhd. for nutritional analysis.

3.9 Statistical analysis

Statistical analysis was performed using the SPSS program (SPSS Inc. IL, USA). Analysis of variance (ANOVA) was used to examine whether differences observed were statistically significant. In assessing the significance of the results, confidence intervals (CI) provided information on the direction and strength of the effect of the treatments (Murphy *et al.*, 1993; Shakespeare, *et al.*, 2001). Therefore, the CI for all analyses was set at 95% ($p < 0.05$).

RESULTS

4.1 Preparation of mushroom extracts

Lignosus rhinocerotis sclerotium powder extracted in hot water for 30 minutes yielded 4.82 g of crude aqueous extract (48.20 %, w/w to sclerotium powder). Meanwhile, 1.43 g of extract was collected (7.15 % w/w to sclerotium powder) using ethanol extraction. Furthermore, from 50 g of *L. rhinocerotis* sclerotium powder, 21.58 g of crude polysaccharide (43.16% w/w to sclerotium powder) was extracted by alkaline extraction method (Table 4.1).

Aqueous extract contained a high proportion (90% of aqueous extract) of polysaccharide (Table 4.1). Chemical classes extracted from the mushroom were determined by the solvent used. High polarity phytochemicals like sugar and amino acid were extracted in aqueous and aqueous alkaline solvents whereas lower polarity phytochemicals like glycosides were extracted using ethanol.

Table 4.1: Extraction yields from *L. rhinocerotis* freeze-dried powder by different extraction methods

Extraction method	Yield (g/10 g of powder)	Percentage of yield (%)
Aqueous	4.82	48.20
Ethanol	1.43	14.30
Alkaline (for polysaccharides)	4.32	43.16

4.2 Assessment of cytotoxic activity of *L. rhinocerotis* extract in PC-12 cells

The control used was cells with only medium (untreated group) and the viability of the PC-12 cells of the control was considered as 100%. The percentages of viable cells were determined by recording the absorbance of purple formazan formed.

All the concentrations evaluated presented some degree of cytotoxicity to the PC-12 cells. The percentage of cell viability decreased with increasing extract concentration [Figure 4.2 (a) and Figure 4.3 (a)]. At the highest concentration, 1000 µg/ml (w/v) of aqueous and ethanol extracts, the reduction in cell viability recorded was 50.4 % and 57.4% respectively. The half maximal inhibitory concentration (IC_{50}) was obtained from the intercept on the x-axis [Figure 4.2 (b) and Figure 4.3 (b)]. A regression line using the linear part of the curve crossing the y-axis was used to obtain a more precise estimation of the IC_{50}. In this case, value of IC_{50} for *L. rhinocerotis* aqueous extract (y=0): (log IC50) = (1.3759 / 0.4279) = 3.2155, giving an IC_{50} of 1,642.37 µg/mL (w/v) [Figure 4.2 (b)]. Value of IC_{50} for *L. rhinocerotis* ethanol extract (y=0): (log IC_{50}) = (1.1584 / 0.4132) = 2.8035, giving an IC_{50} of 636.04 µg/mL (w/v) [Figure 4.3 (b)]. R^2 values of Figure 4.2 (b) and Figure 4.3 (b) were recorded to be 0.96 and 0.99 respectively. The data fitted well into the plot. Also, there was an observed positive relationship between percentage of inhibition and concentrations of extract.

4.3 Stimulation of neurite outgrowth by mushroom extracts in PC-12 cells

4.3.1 Effect of NGF on neurite outgrowth in PC-12 cells

Visible neurite outgrowths from PC-12 were observed when cells treated with NGF were incubated for 48 hours. Figure 4.1 showed the morphology of PC-12 cells with and without NGF treatment. Table 4.2 shows the effect of NGF concentrations on the percentage of neurite-bearing manner from a NGF concentration of 10 ng/mL (w/v) to 50 ng/mL (w/v). Increasing the concentrations of NGF from 60 ng/mL (w/v) to 100 ng/mL (w/v) significantly decreased ($p<0.05$) the percentage of neurite-bearing cells (Table 4.2). Maximum neurite outgrowth was recorded at 50 ng/ml (w/v).

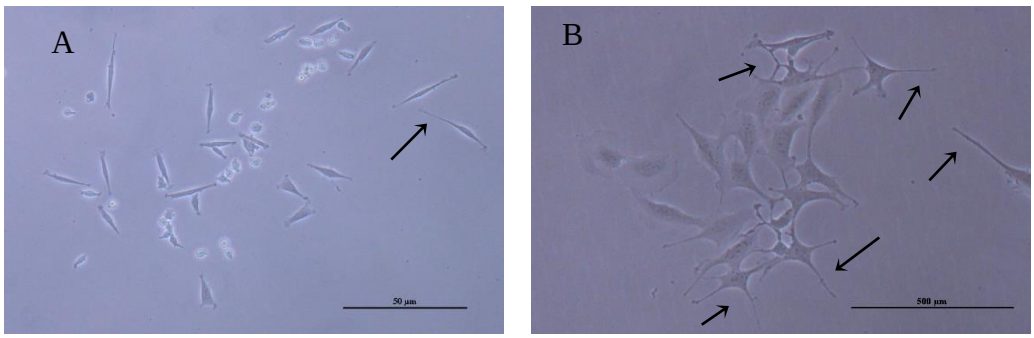
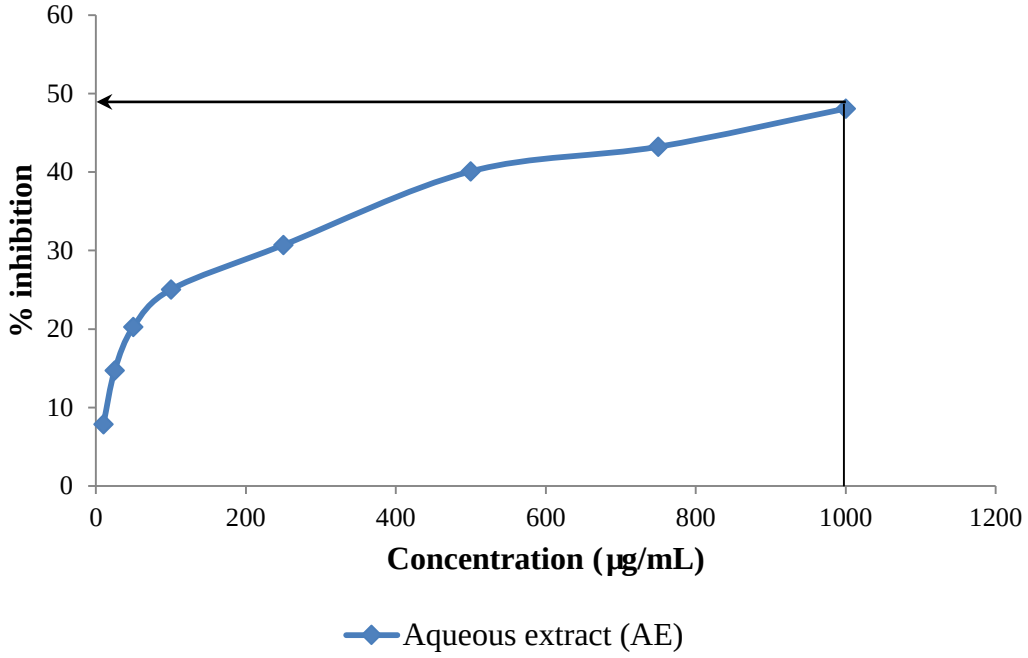


Figure 4.1: PC-12 cells with or without NGF treatment after 48 hrs of incubation at 37 ± 2 ℃ in a 5% humidified CO_2 incubator. Arrows indicate neurite outgrowth. (A) PC-12 cells with medium alone served as negative control. (B) PC-12 cells treated with 50 ng/mL (w/v) of NGF.

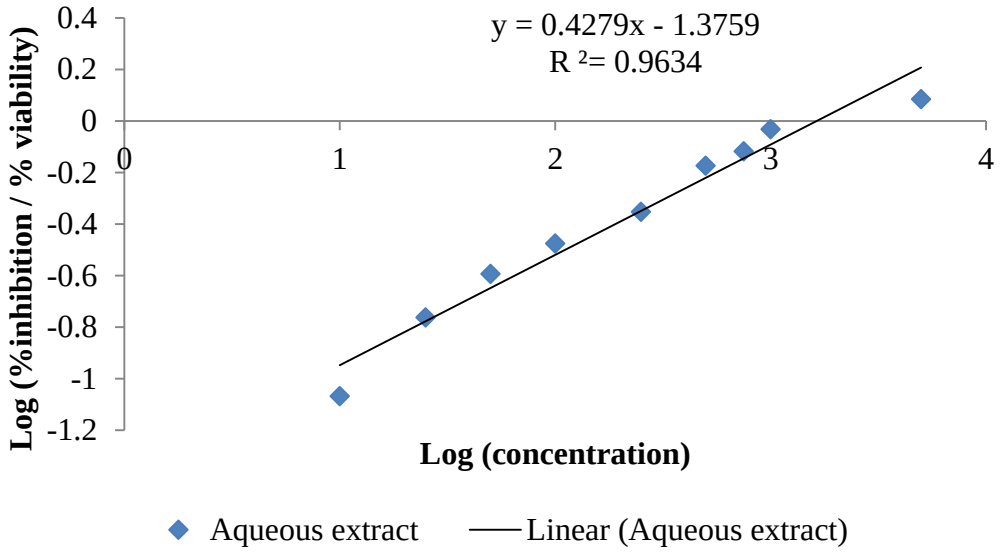
When compared to medium alone or negative control [0 ng/mL (w/v)], an increase of 33% (95% CI: 31-34) (Table 4.2) or almost five-fold increment in proportion of neurite-bearing cells was observed when PC-12 cells were treated with 50 ng/mL of NGF. When PC-12 cells were treated with 100 ng/mL (w/v), the percentage of neurite-bearing cells was comparable to the negative control. Therefore, PC-12 cells treated with 50 ng/mL (w/v) of NGF served as positive control whereas treatment with medium alone served as negative control for the subsequent neurite outgrowth assays.

4.3.2 Effects of aqueous extracts of L. rhinocerotis, H. erinaceus and T. heimii on neurite outgrowth from PC-12 cells

Figure 4.4 shows the effect of aqueous extracts of *L. rhinocerotis*, *H. erinaceus* and *T. heimii* on neurite outgrowth of PC-12 cells. The percentage of neurite-bearing cells increased in a dose-dependent manner when the concentration of the aqueous extract of *H. erinaceus* was increased from 10 µg/mL (w/v) to 50 µg/mL (w/v). The percentage of neurite-bearing cells, however, significantly decreased ($p<0.05$) as

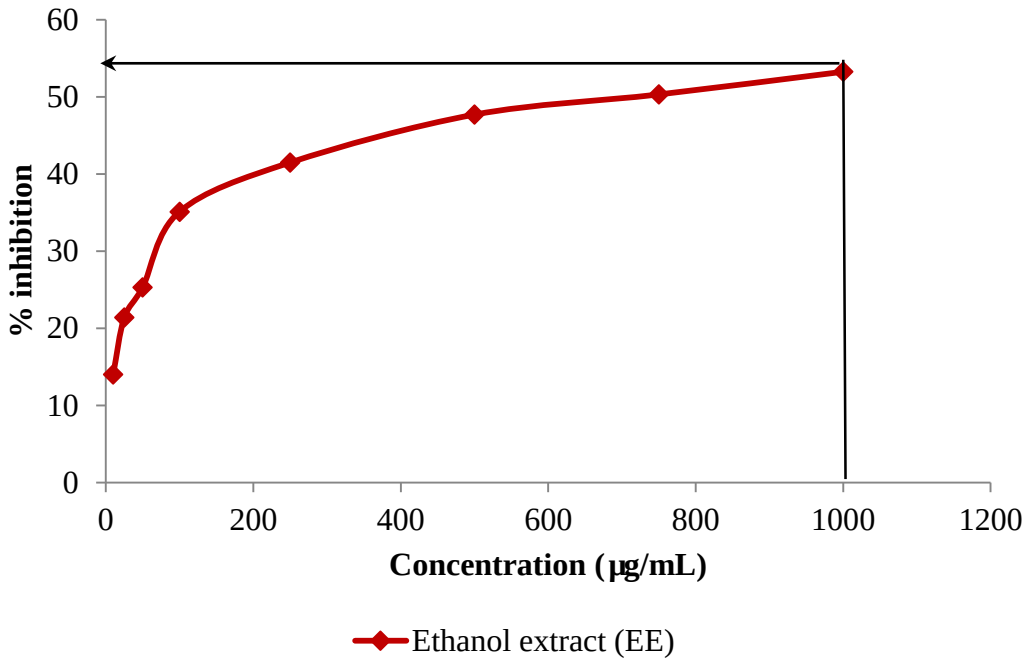


(a)

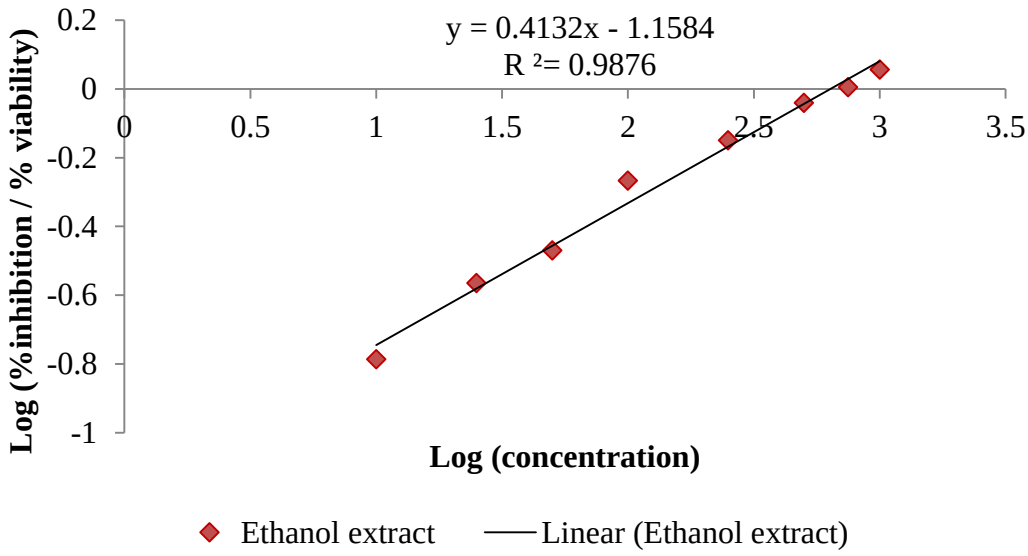


(b)

Figure 4.2: Cytotoxic effect of PC-12 treated with various concentrations of *L. rhinocerotis* aqueous extract. Data are expressed as mean ± SD. (a) Effect of concentration of *L. rhinocerotis* aqueous extract on PC-12 cell viability. (b) The IC_{50} is obtained from the intercept on the x-axis. Differences were tested for significance using ANOVA (analysis of variance) and Duncan multiple range test ($p<0.05$).



(a)



(b)

Figure 4.3: Cytotoxic effect of PC-12 treated with various concentrations of *L. rhinocerotis* ethanol extract using the MTT assay. Data are expressed by mean ± SD. (a) Effect of concentration of *L. rhinocerotis* ethanol extract on PC-12 cell viability. (b) The IC_{50} is obtained from the intercept on the x-axis. Differences were tested for significance using ANOVA (analysis of variance) and Duncan multiple range test ($p<0.05$).

concentrations of *H. erinaceus* aqueous extract was increased from 60 µg/mL (w/v) to 100 µg/mL (Figure 4.4). As the concentration of aqueous extract of *L. rhinocerotis* increased from 10 µg/mL (w/v) to 20 µg/mL (w/v), there was an observed increase in the number of neurite-bearing cells. At 20 µg/mL (w/v), there was no significant difference in number of neurite bearing cells as compared to the NGF positive control at 50 ng/mL. Increasing the concentrations of *L. rhinocerotis* aqueous extract from 30 µg/mL to 100 µg/mL (w/v), however, significantly decreased ($p<0.05$) the percentage of neurite-bearing cells (Figure 4.4) compared to positive control. Concentrations of aqueous extract of *T. heimii* from 10 µg/mL (w/v) to 40 µg /mL significantly increased ($p<0.05$) the percentage of neurite bearing cells while the percentage of nerurite bearing cells were significantly ($p<0.05$) decreased from when concentrations between 50 µg/mL and 100 µg/mL (w/v) was applied (Figure 4.4). The number of neurite bearing cells were, however, much lower when compared to the positive control. Maximum number of neurite bearing cells were recorded at 50 µg/mL (w/v), 20 µg/mL (w/v) and 40 µg/mL (w/v) concentrations of aqueous extracts of *H. erinaceus*, *L. rhinocerotis* and *T. heimii* respectively (Figure 4.4).

When compared to the negative control, a significant increase ($p<0.05$) of 18.4 % (95% CI: 17-21), 15.3 % (95% CI: 12-19) and 13.4 % (95% CI: 11-15) in the proportion of neurite-bearing cells were recorded when PC-12 cells were treated with 50 µg/mL (w/v) of *H. erinaceus,* 20 µg/mL (w/v) of *L. rhinocerotis,* and 40 µg/mL (w/v) of *T. heimii* respectively. When aqueous extract of *H. erinaceus* at 50 µg/mL (w/v) was applied, 27.1 % (95% CI: 25-29) of PC-12 cells produced neurite outgrowth. This was 1.8 % (95% CI: 1-4) higher than the positive control. Also, it was the only extract that stimulated a statistically significant difference ($p<0.05$) in the number of neurite bearing cells when compared to the positive control (Figure 4.4). When PC-12 cells

Table 4.2: Effect of various concentration of NGF of neurite outgrowth of PC-12 cells after 48 h of incubation in 5% CO_2

NGF concentration (ng/mL)	Neurite-bearing cells (%) (Mean ± SD)	Increase compared to negative control (%)
Negative	7.94 ±0.38	0
10	13.70 ±1.12*	5.76
20	20.17 ±0.63*	12.23
30	24.64 ±1.46*	16.70
40	30.92 ±0.60*	22.98
50	40.53 ±1.91*	32.59
60	30.22 ±1.18*	22.28
70	22.66 ±0.96*	14.72
80	19.34 ±0.34*	11.40
90	12.31 ±0.56*	4.37
100	8.69 ±0.46	0.75

Data are expressed as mean of three replicates (n=3) ± standard deviation. *Significant differences between the effects of extracts were determined by independent t-test ($p < 0.05$).

were treated with 90 µg/mL (w/v) and 100 µg/mL (w/v) concentrations of aqueous extract of *L. rhinocerotis*, the percentage of neurite-bearing cells were comparable to the percentage of neurite bearing cells of the negative control. When PC-12 cells were treated with 100 µg/mL (w/v) concentration of aqueous extract of *T. heimii*, there was no significant difference in the number of neurite bearing cells as compared to negative control. Based on the results, since aqueous extracts generally presented higher neurite

outgrowth stimulatory activity, aqueous extracts of *H. erinaceus* and *L. rhinocerotis* were selected for subsequent assays.

4.3.3 Effect of ethanol and aqueous extract on neurite outgrowth from PC-12 cells

Hericium erinaceus extracts served as control to compare the effect of other mushroom extracts on neurite outgrowth activity. Figure 4.5 shows the effect of aqueous and ethanol extract of *L. rhinocerotis* and *H. erinaceus* on PC-12 cells. The percentage of neurite-bearing cells treated with *L. rhinocerotis* aqueous extract (LR AE) and *L. rhinocerotis* ethanol extract (LR EE) increased at concentrations from 10 µg/mL (w/v) to 20 µg/mL (w/v) but decreased significantly ($p<0.05$) as concentrations increased from 30 µg/mL (w/v) to 100 µg/mL (w/v). As concentration of *H. erinaceus* aqueous extract (HE AE) increased from 10 µg/mL (w/v) to 50 µg/mL (w/v), the percentage of neurite-bearing cells were significantly increased ($p<0.05$). However, the percentage of neurite-bearing cells decreased significantly ($p<0.05$) as the concentration of *H. erinaceus* aqueous extract increased from 60 µg/mL (w/v) to 100 µg/mL (w/v).

When PC-12 cells were treated with ethanol extract of *H. erinaceus* (HE EE), percentage of neurite-bearing cells were significantly increased ($p<0.05$) as treatment concentration ranged from 10 µg/mL (w/v) to 70 µg/mL (w/v) while higher concentrations ranging from 80 µg/mL (w/v) to 100 µg/mL (w/v), produced a significant decrease in the percentage of neurite-bearing cells ($p<0.05$).

When compared to the negative control, significant increases ($p<0.05$) of 15.08 % (95% CI: 14-16) and 10.12% (95% CI: 10-11) of neurite-bearing cells were recorded when PC-12 were treated with 20 µg/mL (w/v) aqueous and ethanol extract respectively of *L. rhinocerotis*. However, when PC-12 cells were treated with 50 µg/ml (w/v)

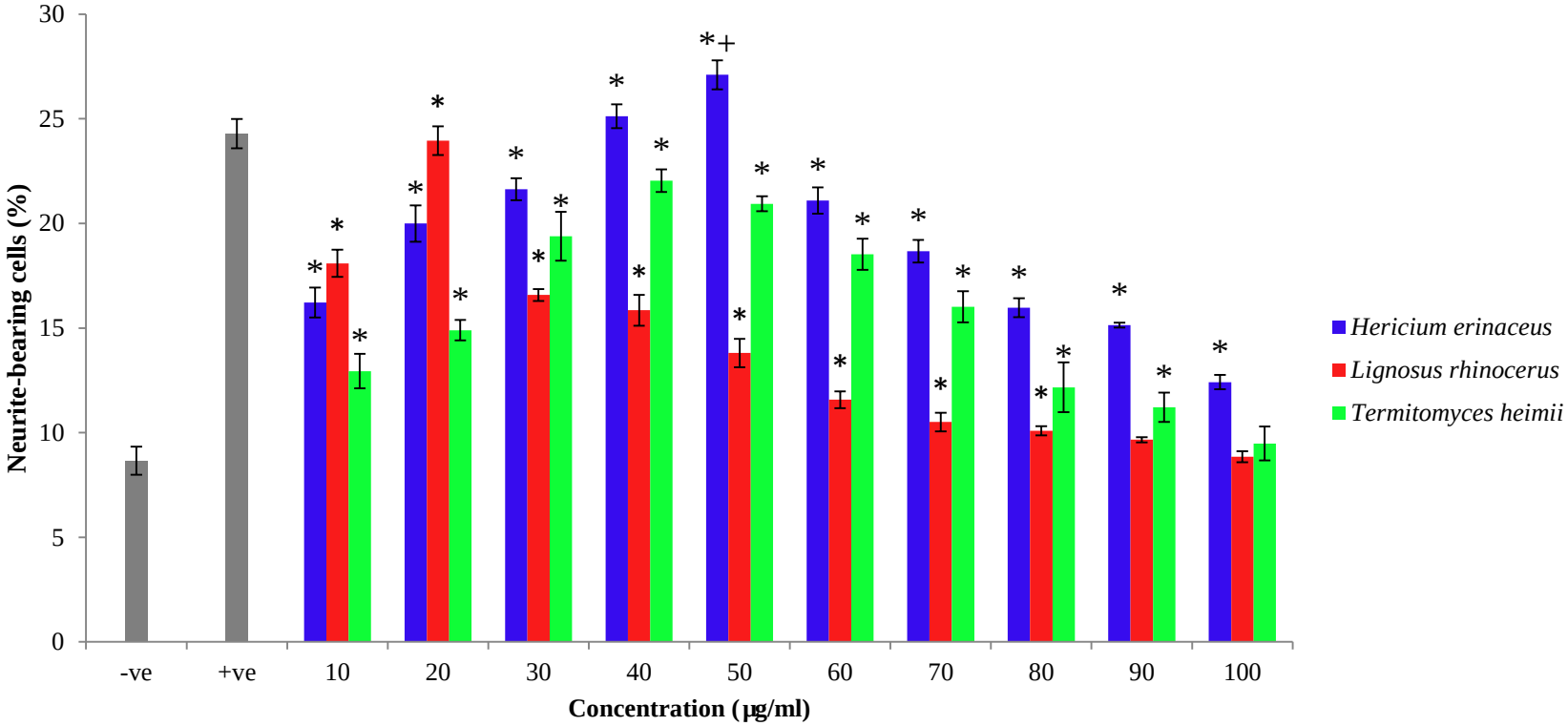


Figure 4.4: Effects of various concentrations of aqueous extracts (*L. rhinocerotis*, *H. erinaceus and T. heimii*) on neurite outgrowth of PC-12 cells after 48 h of incubation in 5% CO_2. Data are expressed as mean of three replicate (n=3) ± standard deviation. ANOVA and Duncan's Multiple Range Test were done to test the significance of the treatment. * $p < 0.05$ compared to the negative control; $^{+}p < 0.05$ compared to the positive control; -ve: negative control (medium alone); +ve: positive control (50 ng/mL NGF).

aqueous extract and 70 µg/ml (w/v) ethanol extracts concentrations of *H. erinaceus*, 18.65% (95% CI: 17-21) and 16.08% (95% CI: 13-15) increase respectively, in the proportion of neurite-bearing cells was observed when compared to the negative control. Aqueous extract of *H. erinaceus* at 50 µg/mL (w/v) concentration was the only extract that showed a stimulation which was comparable to the positive control. An increase of 2.3 % (95% CI: 2-3) as compared to the positive control was recorded. When PC-12 cells were treated with either 20 µg/mL (w/v) aqueous extract concentration of *L. rhinocerotis* or 40 µg/mL (w/v) aqueous extract concentration of *H. erinaceus*, there were no significant differences in the number of neurite bearing cells, as compared to the positive control. When PC-12 cells were treated with 100 µg/mL (w/v) aqueous extract concentration of *L. rhinocerotis*, the percentage of neurite-bearing cells was comparable to the percentage of neurite bearing cells observed for the negative control. Aqueous extracts were thus selected for subsequent assays.

4.3.4 Effect of crude polysaccharide on neurite outgrowth from PC-12 cells

The activity of *L. rhinocerotis* crude polysaccharide (LR PSP) was compared with its aqueous extract (LR AE) (Figure 4.6). The percentage of neurite-bearing cells decreased when PC-12 cells were treated with LR PSP and LR AE at dose-dependent concentrations that ranged from 25 µg/mL (w/v) to 100 µg/mL (w/v). From the results obtained, LR AE produced a higher percentage of neurite-bearing cells as compared to LR PSP at all the tested concentrations. Maximal neurite outgrowth for both LR AE and LR PSP were observed at 25 µg/mL (w/v).

PC-12 cells when treated with LR AE and LR PSP at 25 µg/mL (w/v) concentration showed increments of 19.2% (95% CI: 17-21) and 16.2% (95% CI: 13-19) in the

proportion of neurite-bearing cells when compared to the negative and positive controls respectively.

4.3.5 Addition effects of aqueous extract with NGF on neurite outgrowth from PC-12 cells

Figure 4.7 shows the effect of *L. rhinocerotis* aqueous extract (LR AE) at 20 µg/mL (w/v) concentration in addition to various concentrations of NGF. The percentage of neurite bearing cells significantly ($p<0.05$) increased as concentration of NGF increased from 10 ng/mL (w/v) to 30 ng/mL (w/v). However, the percentage of neurite bearing cells decreased in a dose-dependent manner at concentrations of NGF ranging from 40 ng/mL (w/v) to 50 ng/mL (w/v). Maximal neurite outgrowth was recorded when 20 µg/mL (w/v) concentration of LR AE was complemented with 30 ng/mL (w/v) of NGF, producing an observed 42.12% (95% CI: 38-46) of neurite-bearing cells.

Addition effect of 20 µg/mL (w/v) of LR AE with 30 ng/mL (w/v) of NGF showed an increase of 17.4% (95% CI: 14-20) of neurite-bearing cells when compared to the negative control. When 20 µg/mL (w/v) concentration of LR AE was complemented with 50 ng/mL (w/v) of NGF, the percentage of nerurite-bearing cells was comparable to the negative control.

4.4 Qualitative assessment of neurofilaments by Immunofluorescene staining

Extract-treated and non-treated PC-12 cells were incubated for 48 hours for neurite stimulation after which anti-neurofilament staining was conducted. Plate 4.1 shows immunofluorescence staining of neurofilament of PC-12 cells. Anti-neurofilament antibody stained the axon green while DAPI stained the nuclei blue. Extract treated PC-12 cells with neurite-bearing were stained in green with a thin

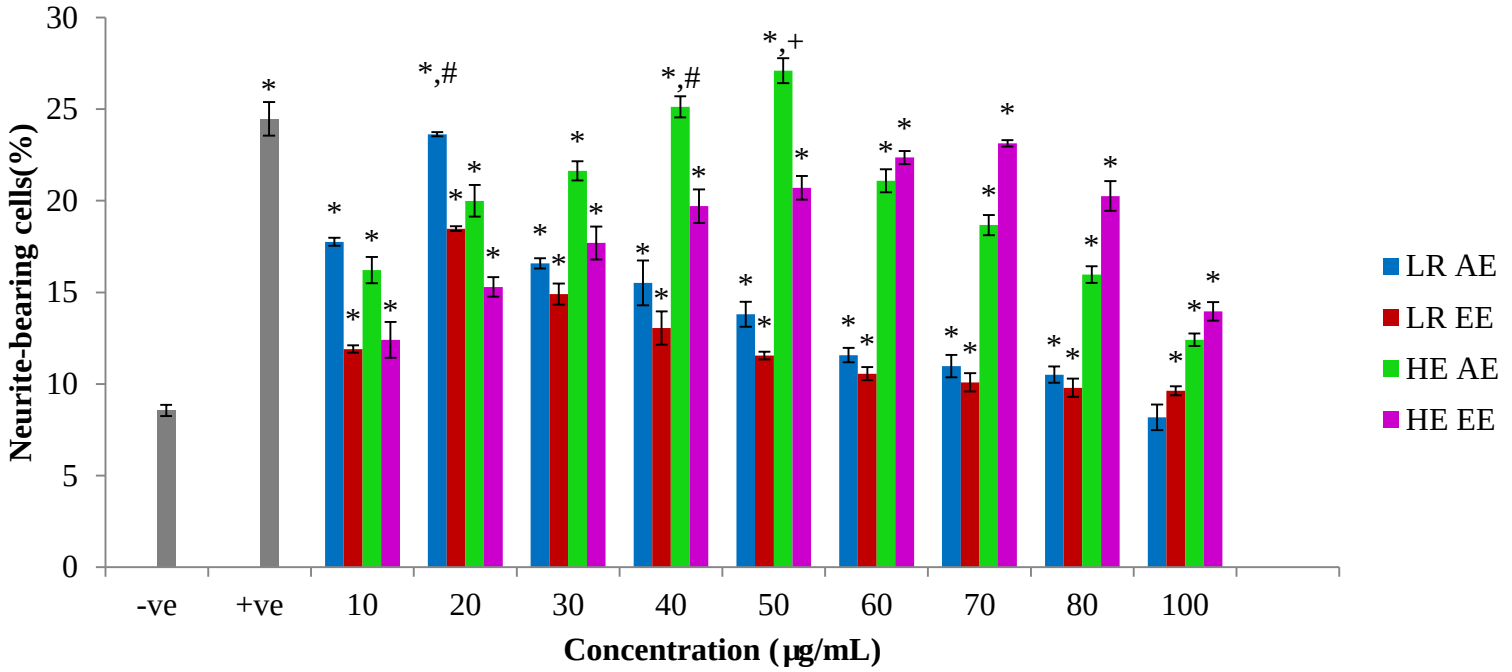


Figure 4.5: Percentage of neurite-bearing cells in the cell line PC-12 in response to treatments with extracts of mushrooms (µg/mL) (w/v). Data are expressed as means ± standard deviation (n =3). Repeated measures of ANOVA of the neurite-bearing cells among treatment groups were followed by Duncan's Multiple Range Test (* p <0.05 compared to the negative control, +p <0.05 compared to the positive control.) (-ve: negative control (medium alone); +ve: positive control (50 ng/mL NGF); LR AE: *L. rhinocerotis* aqueous extract; LR EE: *L. rhinocerotis* ethanol extract; HE AE: *H. erinaceus* aqueous extract; HE EE: *H. erinaceus* ethanol extract).

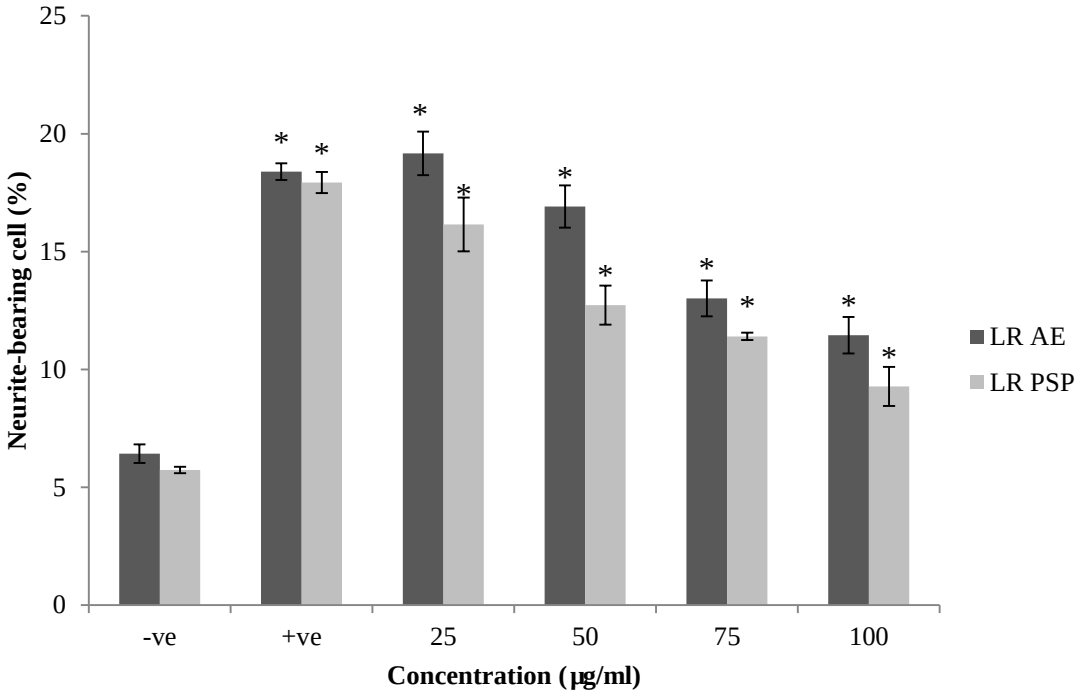


Figure 4.6: Percentage of neurite-bearing cells in the cell lines of PC-12 in response to treatment with aqueous extracts (LR AE) and polysaccharide (LR PSP) (w/v). Data are expressed in triplicates (n =3). Repeated measures of ANOVA of the neurite-bearing cells among treatment groups were followed by DMR analysis (*p <0.05 compared to the negative control) (-ve: negative control (medium alone); +ve: positive control (50 ng/mL NGF)

green fluorescence filament that doubles the length of the cell body. Based on Plate 4.1, PC-12 cells treated with 20 µg/mL (w/v) concentration of *L. rhinocerotis* aqueous extract in addition to 30 ng/mL concentration of NGF showed the most neurite-bearing cells. This was followed by the NGF treatment alone, *L. rhinocerotis* aqueous extract alone and the negative control.

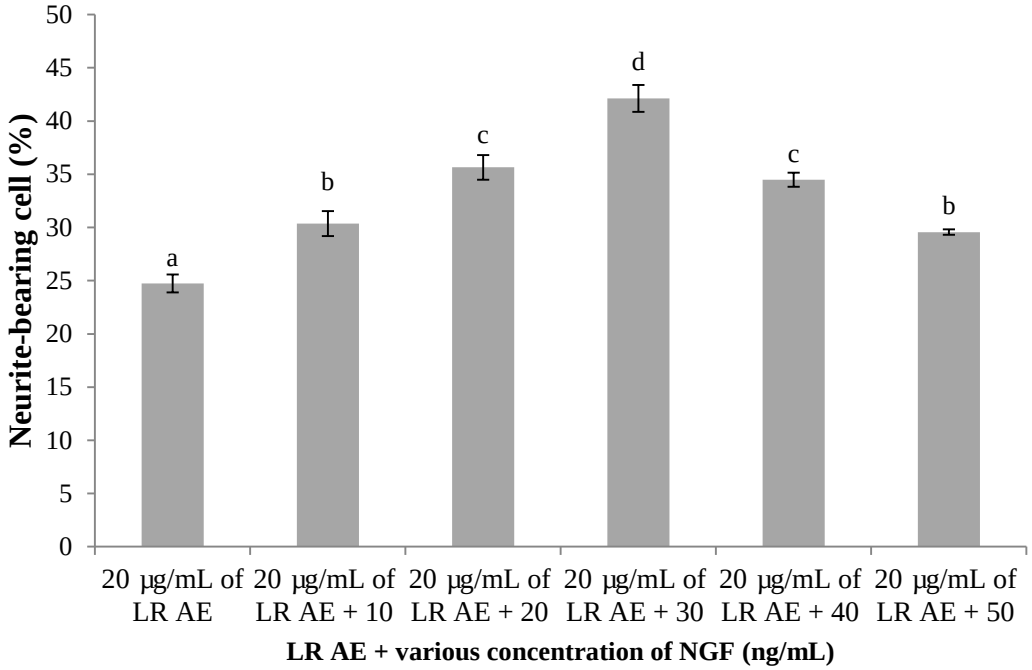


Figure 4.7: Percentage of neurite-bearing cells in the cell line PC-12 in response to treatment with a range of NGF concentrations in addition to the optimum concentration of *L. rhinocerotis* aqueous extract, 20 µg/ml (w/v). Data are expressed in triplicates (n =3). Means with different alphabets showed significant difference (DMR analysis)] ($p < 0.05$). LR AE: aqueous extract of *L. rhinocerotis*.

4.5 Elucidation of protein signaling pathway involved in stimulation of neurite outgrowth

4.5.1 The inhibitory effect of K252a on the TrkA pathway in the induction of neurite outgrowth

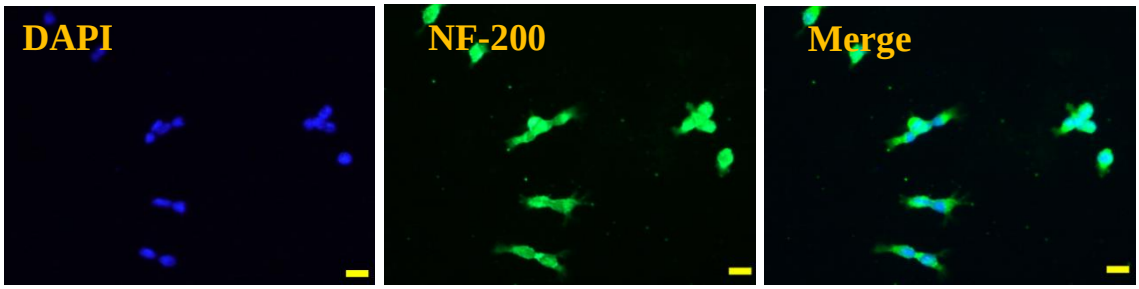
Inhibitory effect of K252a on neurite outgrowth was recorded based on the percentage of neurite bearing cells inhibited. PC-12 inhibited group were pre-treated with K252a prior to treatment with extracts at their optimum concentration. PC-12 cells treated with extracts alone served as a standard to compare the inhibitory effect of K252a.

Results showed that the outgrowth of neurite induced by NGF, *L. rhinocerotis* aqueous extract and *H. erinaceus* aqueous extract was markedly inhibited ($p < 0.05$) by

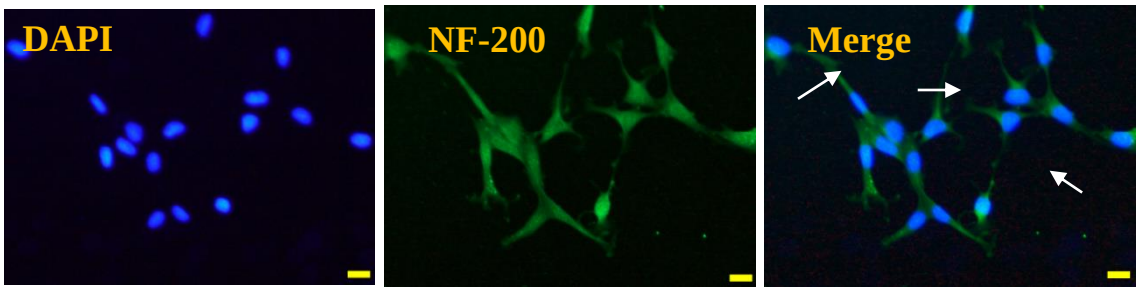
K252a (Figure 4.8). Apart from *H. erinaceus* aqueous extract (HE AE), PC-12 cells pre-treated with K252a inhibitor generally presented no significant difference ($p < 0.05$) when compared to the negative control. However, the percentage of neurite-bearing cells pre-treated with K252a was relatively lower (3.2 times lower) than the non-treated cells. A generally low level (8.8 – 12.1%) of PC-12 cells pretreated with K252a showed neurite bearing activity. From this result, we propose that aqueous extract of *L. rhinocerotis* and *H. erinaceus* possess NGF-like activity.

4.5.2 Detection of protein kinase: mitogen-activated protein kinase (MAPK) - The activation of Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) in PC-12 cells

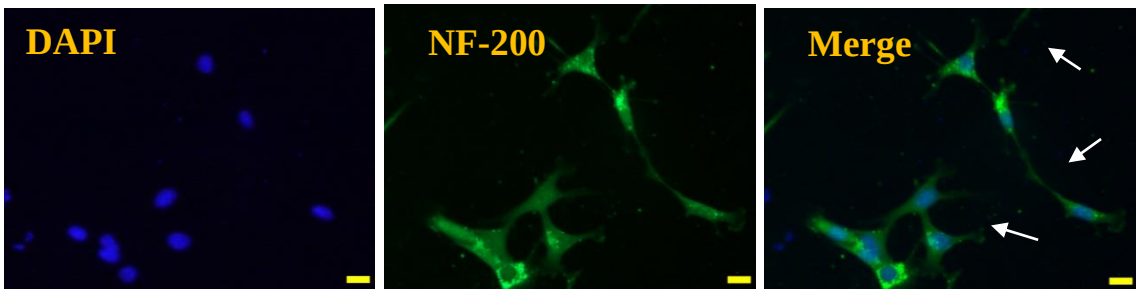
In-vitro expression of phospho-p44/42 MAPK in PC-12 cells was detected after 48 hours of incubation (Plate 4.2a, 4.2b and Figure 4.9). In this study, immunofluorescence demonstrating neurite outgrowth was inhibited by U1026. The outgrowth of neurite was dependent on the activation of ERK (Plate 4.2a). PC-12 cells without inhibitor treatments served as control to compare the inhibitory activity demonstrated by U1026 (Plate 4.2b). The immunoreactivity of phospho-p44/42 MAPK was recorded as fluorescence intensity unit as shown in Figure 4.9. The fluorescence intensity unit of cells stained with phospho-p44/42 MAPK (in green) was the highest when cells were treated with NGF and *L. rhinocerotis* aqueous extract (LR+NGF) (Figure 4.9). In comparison, fluorescence intensity unit of cells treated with *H. erinaceus* aqueous extract (HE AE), *L. rhinocerotis* aqueous extract (LR AE) and positive control showed no significant difference ($p<0.05$).



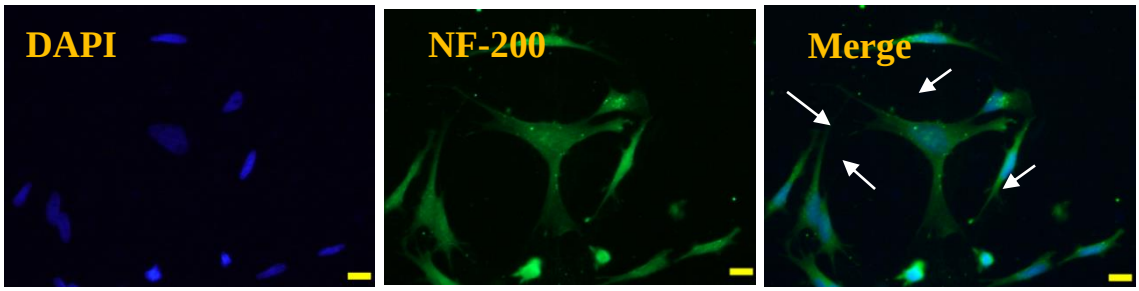
PC-12 cells with medium alone



PC-12 cells with 50 ng/mL (w/v) NGF



PC-12 cells with 20 µg/mL (w/v) *L. rhinocerotis* aqueous extract (LR AE)



PC-12 cells with 20 µg/mL (w/v) *L. rhinocerotis* aqueous extract (LR AE) with 30 ng/mL (w/v) NGF

Plate 4.1: Neurofilament stain on PC-12 cells with various treatments. Nuclei were stained blue and PC-12 cells with neurofilament were stained green. Arrows indicate neurite outgrowth. Scale bar =20 µm.

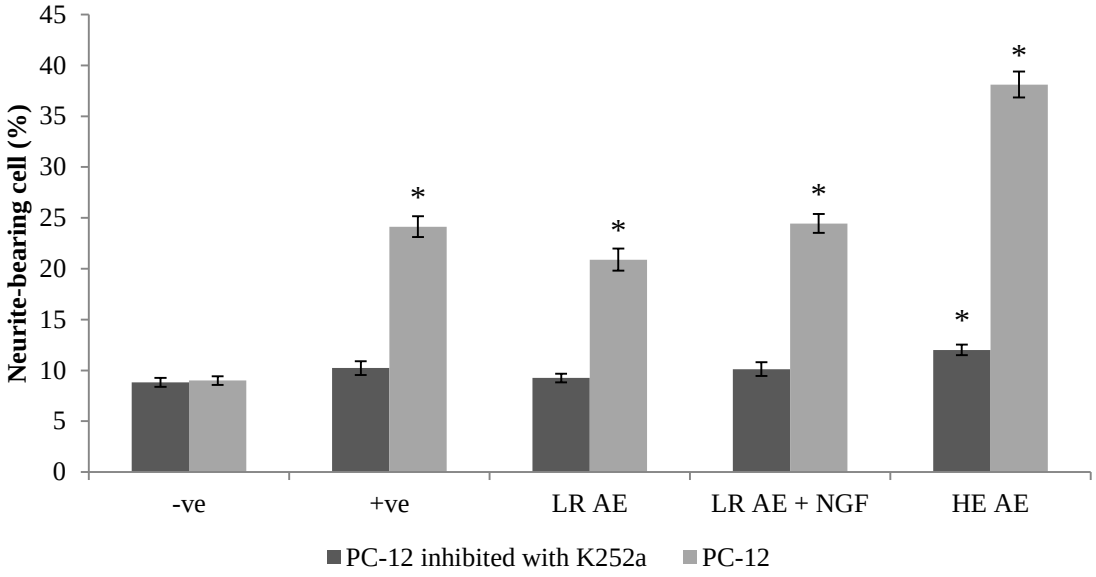


Figure 4.8: Percentage of neurite-bearing cells in the cell line PC-12 with and without inhibitor (K252a) treatment. Data are expressed in triplicates (n=3). Repeated measures of ANOVA of the neurite-bearing cells among treatment groups were followed by Duncan test (*p <0.05 compared to the negative control.)(-ve: negative control; +ve: positive control; LR AE: *L. rhinocerotis* aqueous extract; HE AE: *H. erinaceus* aqueous extract)

Fluorescence intensity of the addition effect of NGF and *L. rhinocerotis* aqueous extract (LR AE+NGF) were significantly ($p<0.05$) higher compared to the negative control, positive control, treatments with *L. rhinocerotis* aqueous extract (LR AE) and *H. erinaceus* aqueous extract HE AE.

The fluorescence intensity of cells without inhibition treatment was recorded and values obtained followed the following order: addition effect of NGF and LR AE (LR AE+NGF) > *H. erinaceus* aqueous extract (HE AE) > *L. rhinocerotis* aqueous extract (LR AE) > positive control > negative control.

4.5.3 Detection of protein kinase: mitogen-activated protein kinase (MAPK) - Activations of phospho-SAPK/JNK (Thr183/Tyr185) in PC-12 cells

Plate 4.3a and 4.3b show the in-vitro expression of phospho-SAPK/JNK in PC-12 cells after various treatment and 48 hours of incubation. In this study, immunofluorescence demonstrating neurite outgrowth was inhibited by U1026 indicating that neurite outgrowth was dependent on the activation of phospho-SAPK/JNK (Plate 4.3a). PC-12 cells without inhibitor treatment served as control to compare the inhibitory activity demonstrated by U1026 (Plate 4.3b).

The immunoreactivity of phospho-SAPK/JNK was measured in fluorescence intensity unit as shown in Figure 4.10. The fluorescence intensity unit of phospho-SAPK/JNK activation in the cells was observed to be the highest when cells were treated with *H. erinaceus* aqueous extract (HE AE) (Figure 4.10). Fluorescence intensity unit was however, significantly lower in *L. rhinocerotis* aqueous extract (LR AE) as compared to the negative control, positive control, *H. erinaceus* aqueous extract (HE AE) and *L. rhinocerotis* aqueous extract combined with NGF (LR AE+NGF) ($p<0.05$).

Fluorescence intensity of treatment with various extracts without inhibition was recorded and the values were found to be in the order: *H. erinaceus* aqueous extract (HE AE) > addition effect of NGF and LR AE (LR AE+NGF) > positive control > negative control > *L. rhinocerotis* aqueous extract (LR AE).

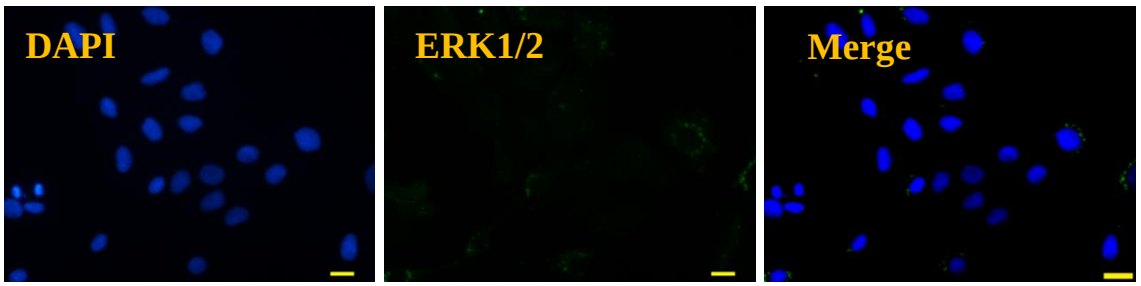
4.5.4 Detection of protein kinase: mitogen-activated protein kinase (MAPK) - Activations of phospho-p38 MAPK (Thr180/Tyr182) in PC-12 cells

Plate 4.4a and 4.4b show the in-vitro expression of phospho-p38 in PC-12 cells after various treatments and 48 hours of incubation. In this study, immunofluorescence

Negative control (medium alone)

Positive control (50 ng/mL NGF)

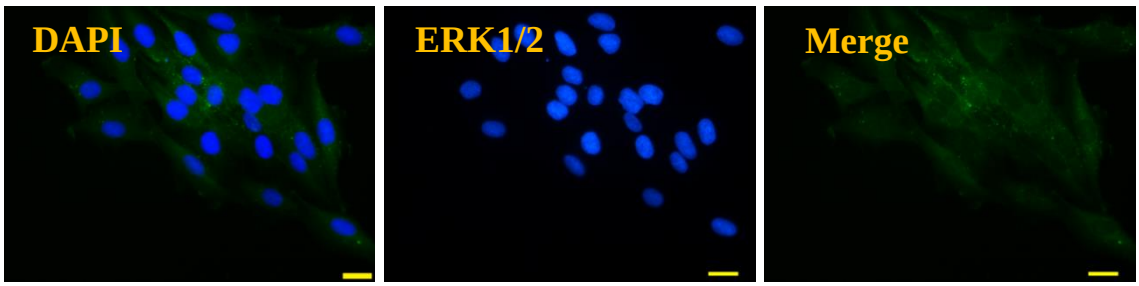
L. rhinocerotis aqueous extract (20 µg/mL)



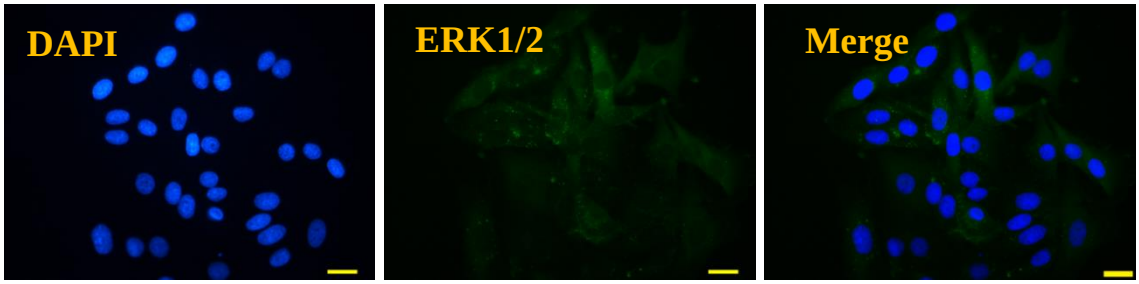
L. rhinocerotis aqueous extract (20 µg/mL) with NGF (30 ng/mL)

H. erinaceus aqueous extract (50 µg/mL)

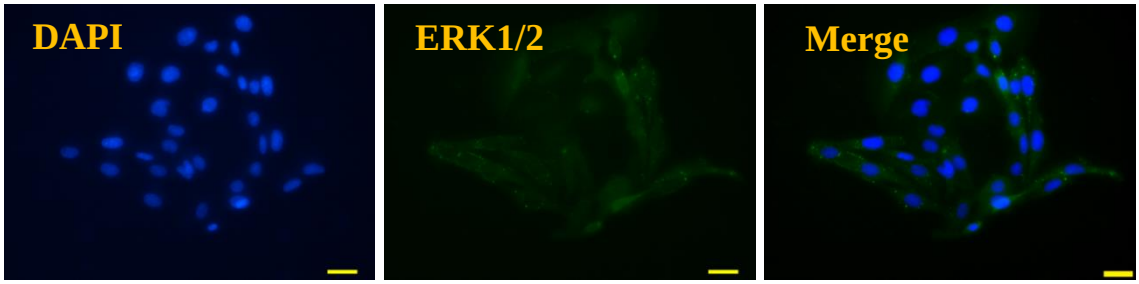
Plate 4.2a: Effects of various treatments and inhibitor U1026 on PC-12 cells after 48 hr of incubation in a 5% humidified CO_2 incubator. Expression of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) stained PC-12 cells green. Nuclei were stained blue. Scale bar = 50 µm.



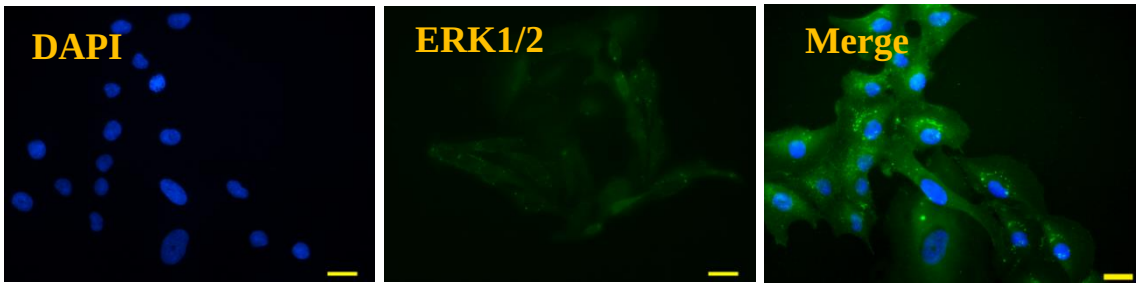
Negative control (medium alone)



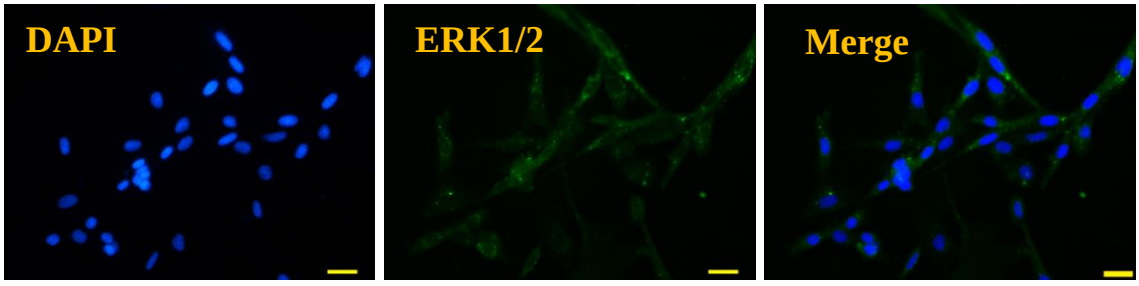
Positive control (50 ng/mL NGF)



L. rhinocerotis aqueous extract (20 µg/mL)



L. rhinocerotis aqueous extract (20 µg/mL) with NGF (30 ng/mL)



H. erinaceus aqueous extract (50 µg/mL)

Plate 4.2b: Effects of various treatments on PC-12 cells after 48 hr of incubation in a 5% humidified CO_2 incubator. Expression of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) stained PC-12 cells green. Nuclei were stained blue. Scale bar = 50 µm.

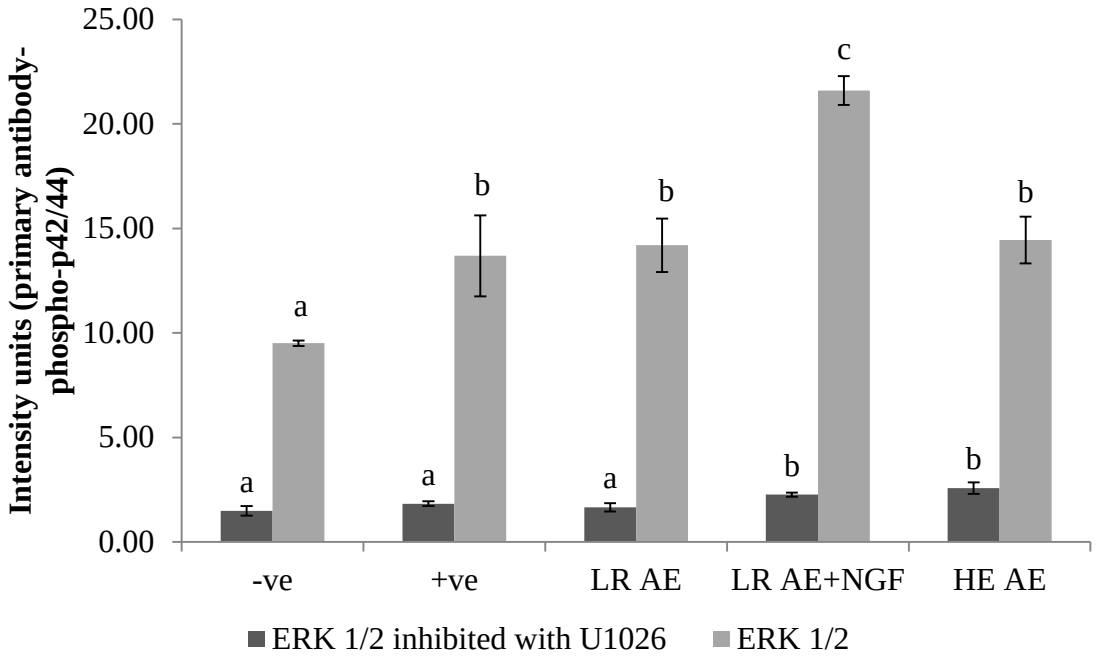


Figure 4.9: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) activation in PC-12 after 48 hr of treatment with various extracts. Level of Erk1/2 was measured by the intensity of immunoreactivity. Means with different alphabet letters in different treatment groups were significantly different (DMR analysis) ($p < 0.05$).

demonstrating neurite outgrowth was inhibited by U1026 indicating that neurite outgrowth was dependent on the activation of phospho-p38 (Plate 4.4a). PC-12 cells with inhibitor treatment served as the control to compare the inhibitory activity demonstrated by U1026 (Plate 4.4b).

The immunoreactivity of phospho-p38 was measured in fluorescence intensity unit as shown in Figure 4.11. Fluorescence intensity unit for phospho-p38 was the highest when cells were treated with *H. erinaceus* aqueous extract (HE AE). Fluorescence intensity unit was however lower in *L. rhinocerotis* aqueous extract (LR AE) as compared to the negative control, positive control, *L. rhinocerotis* aqueous extract with NGF (LR AE+NGF) and *H. erinaceus* aqueous extract (HE AE)($p<0.05$).

Negative control (medium alone)

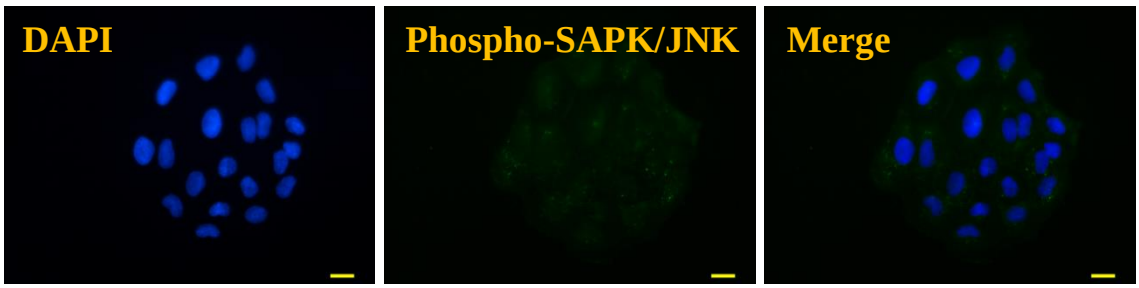
Positive control (50 ng/mL NGF)

L. rhinocerotis aqueous extract (20 µg/mL)

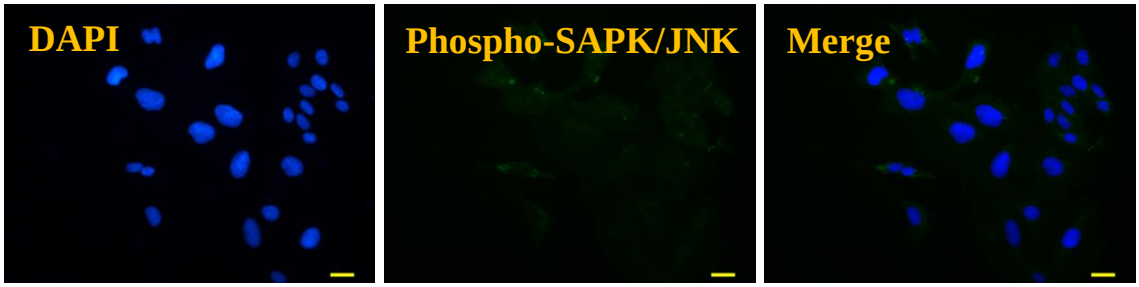
L. rhinocerotis aqueous extract (20 µg/mL) with NGF (30 ng/mL)

H. erinaceus aqueous extract (50 µg/mL)

Plate 4.3a: Effects of various treatments and inhibitor U1026 in PC-12 cells after 48 hr of incubation at 5% humidified CO_2 incubator. Expression of phospho-SAPK/JNK (Thr183/Tyr185) stained PC-12 cells green. Nuclei were stained blue. Scale bar = 50 µm.

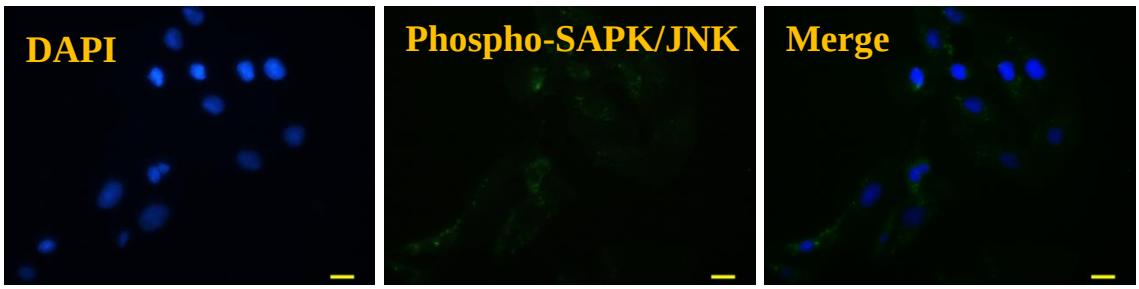


Negative control (medium alone)

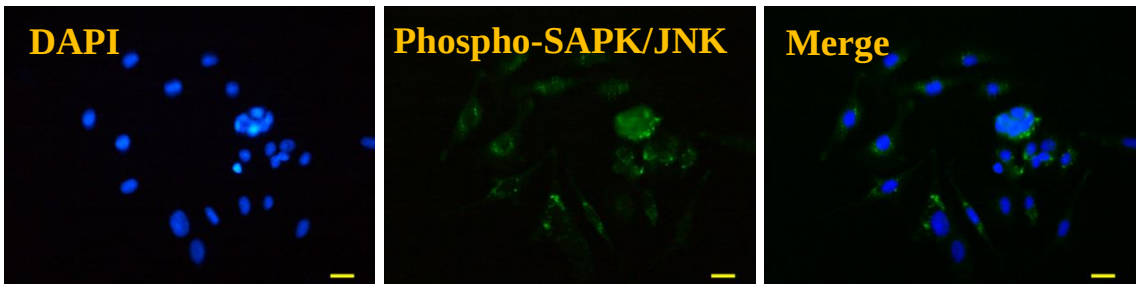


Positive control (50 ng/mL NGF)

L. rhinocerotis aqueous extract (20 µg/mL)



L. rhinocerotis aqueous extract (20 µg/mL) with NGF (30 ng/mL)



H. erinaceus aqueous extract (50 µg/mL)

Plate 4.3b: Effects of various treatments in PC-12 cells after 48 hr of incubation at 5% humidified CO_2 incubator Expression of phospho-SAPK/JNK (Thr183/Tyr185) stained PC-12 cells green. Nuclei were stained blue. Scale bar = 50 µm.

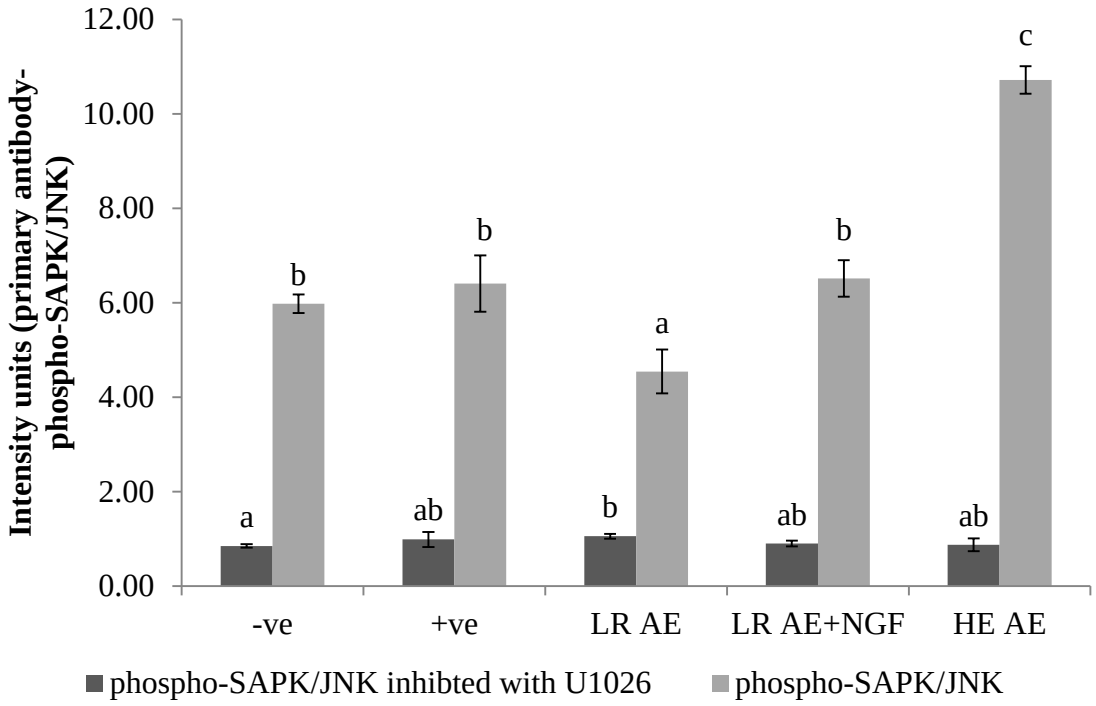


Figure 4.10: Phospho-SAPK/JNK (Thr183/Tyr185) activation in PC-12 cells after 48 hr of treatment with various extracts. Phospho-SAPK/JNK level was measured by the intensity of immunoreactivity. Means with different alphabets in different treatment groups were significantly different (DMR analysis) ($p < 0.05$) (-ve: negative control medium alone); +ve: positive control (50 ng/mL NGF).

The fluorescence intensity of treatment without inhibition was recorded and the values obtained were in the order: *H. erinaceus* aqueous extract (HE AE) > NGF with *L. rhinocerotis* aqueous extract (LR AE+NGF) > negative control > positive control > *L. rhinocerotis* aqueous extract (LR AE).

4.6 Quantification of protein expression level by ELISA method

4.6.1 Expression of p38 MAPK (Total) signaling pathway

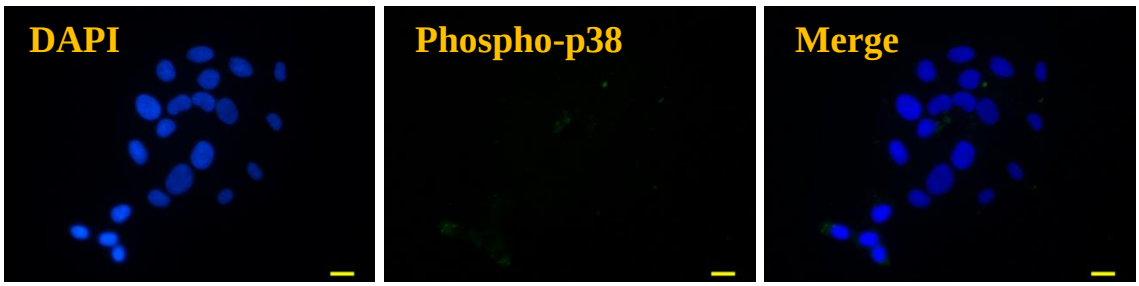
The magnitude of absorbance for the colour developed is proportional to the quantity of p38 MAPK protein expressed. Optical densities of *H. erinaceus* aqueous extract (HE AE) and the positive control were significantly different ($p<0.05$) when

compared to the negative control (Figure 4.12). However, optical density of *L. rhinocerotis* aqueous extract (LR AE) was observed to be lower as compared to the negative control. Total p38 protein in the cells treated with various extracts was recorded and the values were found to be in the order: HE AE > positive control > addition effect of NGF and LR AE (LR AE+NGF) > LR AE > negative control.

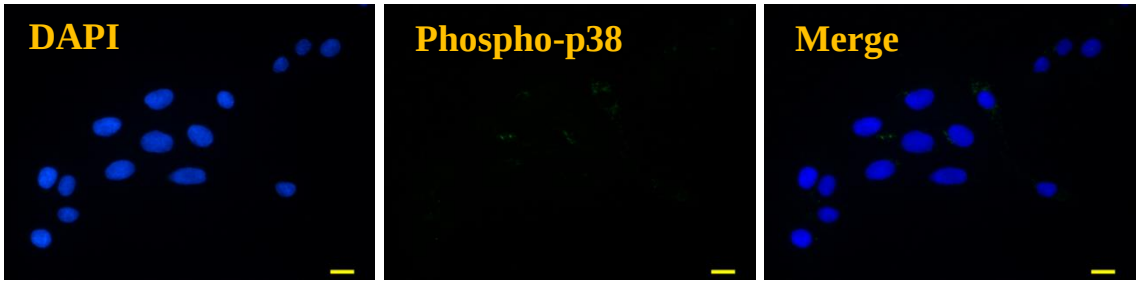
4.6.2 Expression of total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) signaling pathway

Sandwich ELISA Kit was used to detect endogenous levels of p44/42 MAPK (Erk1/2) when phosphorylated at Thr202/Tyr204. Coated antibodies were captured by cell lysate extracted from the cell with various treatments after incubation. Anti-Rabbit IgG, HRP-linked antibody was then used to recognise the bound detection antibody. HRP substrate, TMB, was added to develop colour. The magnitude of absorbance for this developed colour is proportional to the quantity of MAPK protein expressed by PC-12. Among the cells treated with NGF (positive control), *L. rhinocerotis* aqueous extract (LR AE), NGF and LR AE (LR AE+NGF) and *H. erinaceus* aqueous extract (HE AE), both total p44/42 MAPK protein and phospho-p44/42 MAPK protein expression were significantly different when compared to the negative control (Figure 4.13).

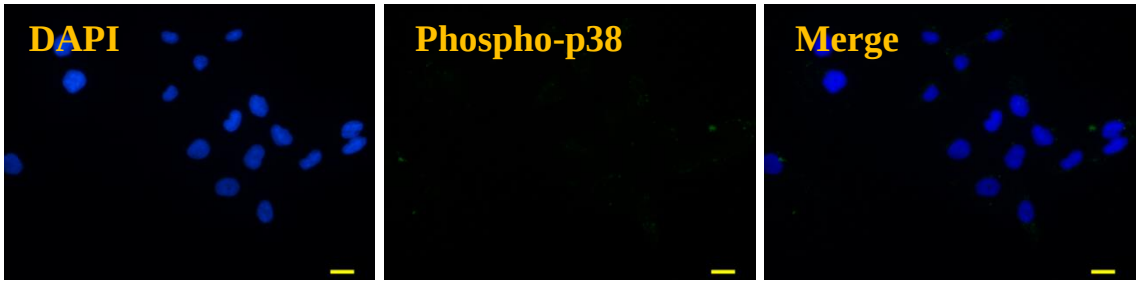
Total p44/42 MAPK (Erk1/2) protein expressed in PC-12 cells treated with extracts was in the order: *L. rhinocerotis* aqueous extract with NGF (LR AE+NGF) > *H. erinaceus* aqueous extract (HE AE) > positive control > *L. rhinocerotis* aqueous extract (LR AE) > negative control. Whereas the phosphorylated p44/42 MAPK (Erk1/2) protein was expressed in extract treated PC-12 cells in the order: *L. rhinocerotis* aqueous extract with NGF (LR AE+NGF) > positive control > *L.*



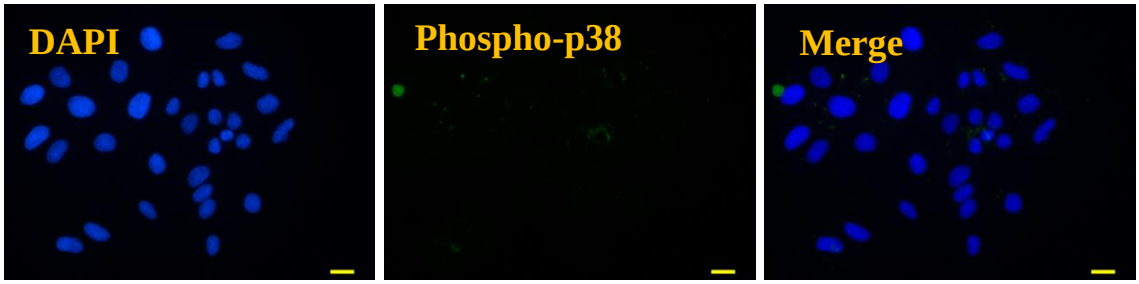
Negative control (medium alone)



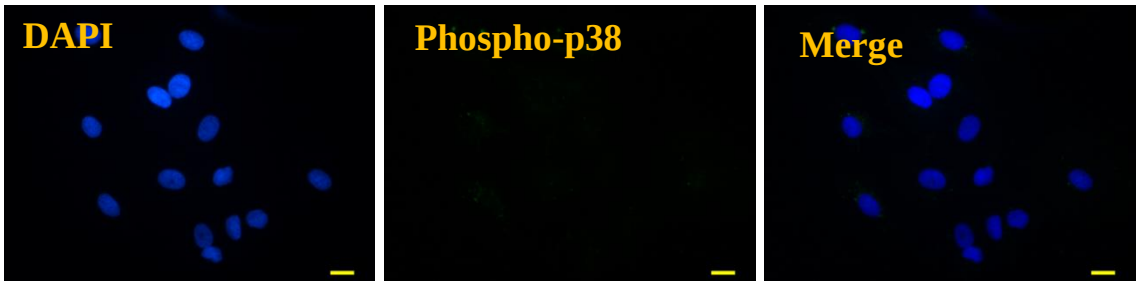
Positive control (50 ng/mL NGF)



L. rhinocerotis aqueous extract (20 µg/mL)

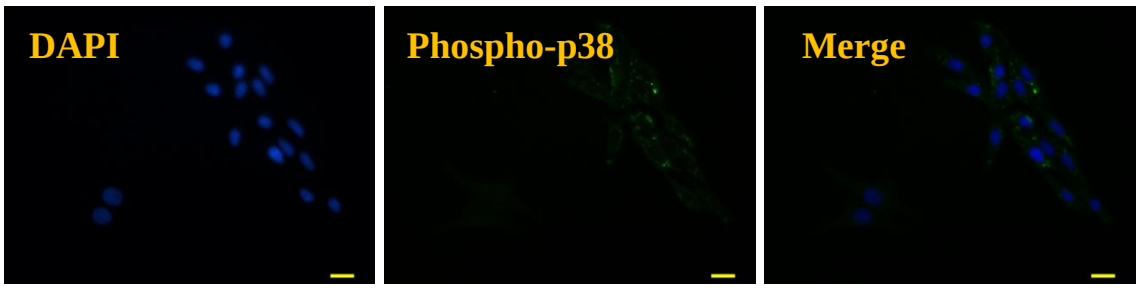


L. rhinocerotis aqueous extract (20 µg/mL) with NGF (30 ng/mL)



H. erinaceus aqueous extract (50 µg/mL)

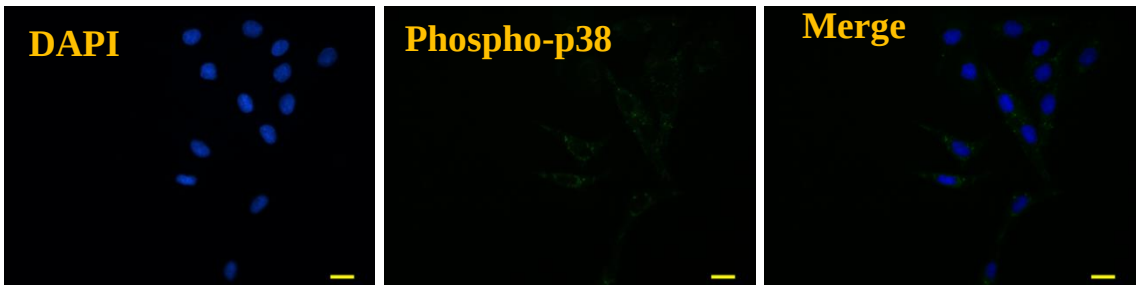
Plate 4.4a: PC-12 cells with various treatments and inhibited with U1026 after 48 hr of incubation in a 5% humidified CO_2 incubator. Expressed phospho-p38 MAPK (Thr180/Tyr182) stained PC-12 cells green. Nuclei were stained blue. Scale bar = 50 µm.



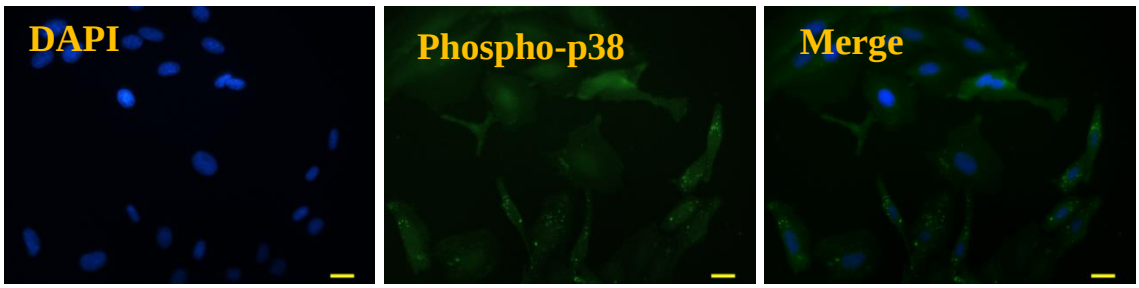
Negative control (medium alone)

Positive control (50 ng/mL NGF)

L. rhinocerotis aqueous extract (20 µg/mL)



L. rhinocerotis aqueous extract (20 µg/mL) with NGF (30 ng/mL)



H. erinaceus aqueous extract (50 µg/mL)

Plate 4.4b: PC-12 cells with treatments after 48 hr of incubation at 5% humidified CO_2 incubator. Expression of phospho-p38 MAPK (Thr180/Tyr182) stained PC-12 cells in green. Nuclei were stained blue. Scale bar = 50 µm.

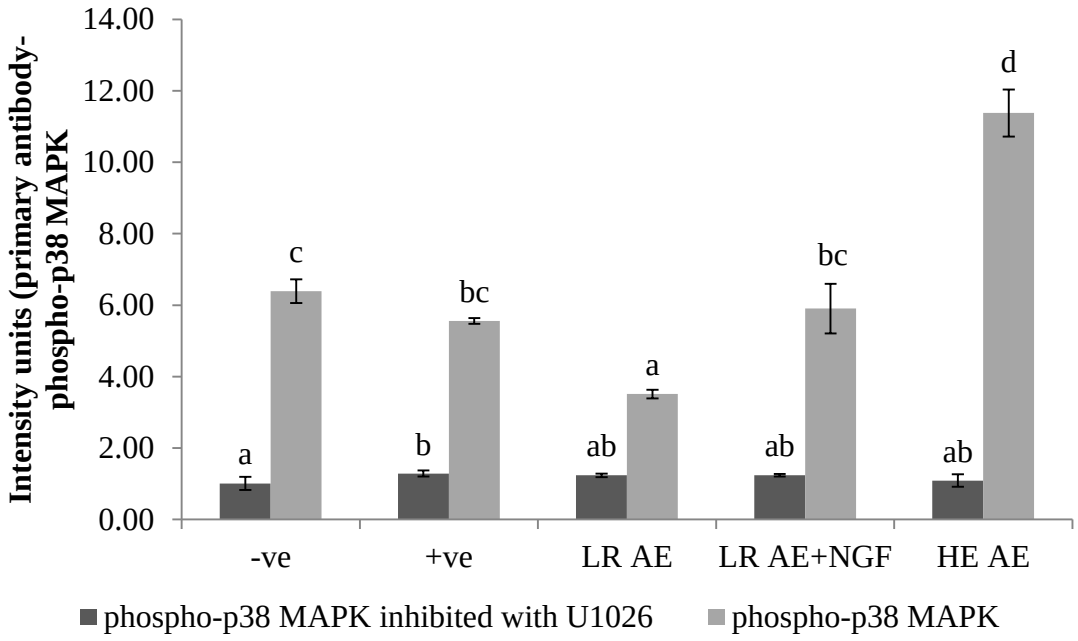


Figure 4.11: Phospho-p38 MAPK (Thr180/Tyr182) activation of PC-12 cells after 48 hr of treatment with various extracts. Phospho-p38 level was measured by the intensity of immunoreactivity. Means with different alphabets in different treatment groups were significantly different (DMR analysis) ($p < 0.05$); -ve: negative control (medium alone); +ve: positive control (50 ng/mL NGF).

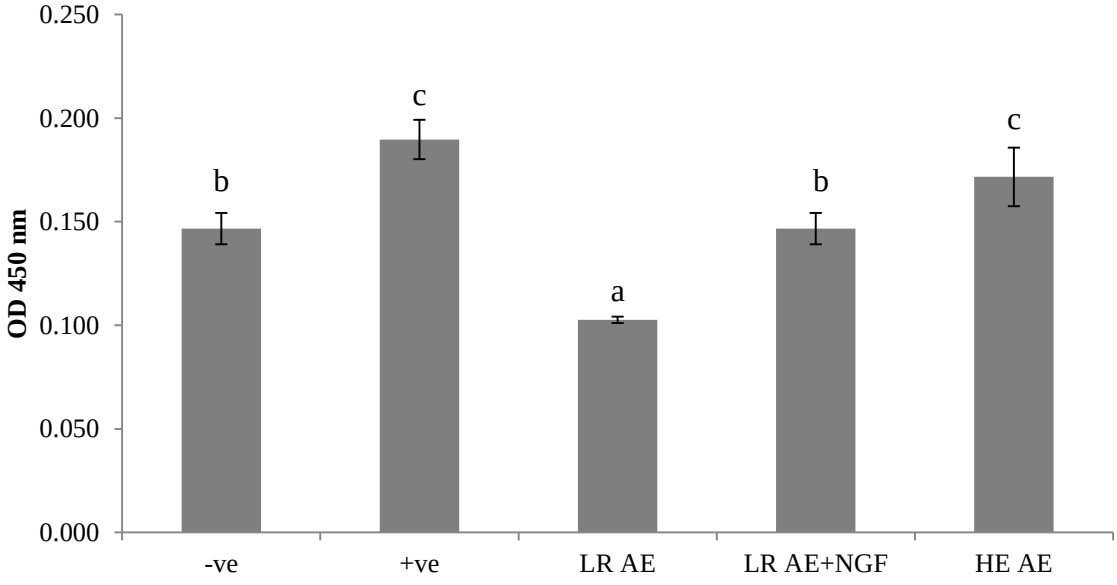


Figure 4.12: Total p38 MAPK activation in PC-12 after 48 hr of treatment with various extracts. Phospho-p38 level was measured using a determination of the optical intensity. Means with different alphabets in different treatment groups were found to be significantly different (DMR analysis) ($p < 0.05$) -ve: negative control (medium alone); +ve: positive control (50 ng/mL NGF).

rhinocerotis aqueous extract (LR AE) and *H. erinaceus* aqueous extract HE AE > negative control (Figure 4.13).

4.7 Nutritional composition of freeze-dried sclerotium of *L. rhinocerotis*

Nutritional composition of sclerotium of *L. rhinocerotis* is shown in Table 4.3. Every 100 g of freeze-dried sclerotium of *L.rhinocerotis* contained 64.7 % carbohydrates, 19.2 % protein and 32.5 % dietary fibre. It is also rich in minerals such as calcium (39.25 mg), magnesium (51.67 mg), potassium (180.41 mg) and phosphorus (176.41 mg) (Table 4.3).

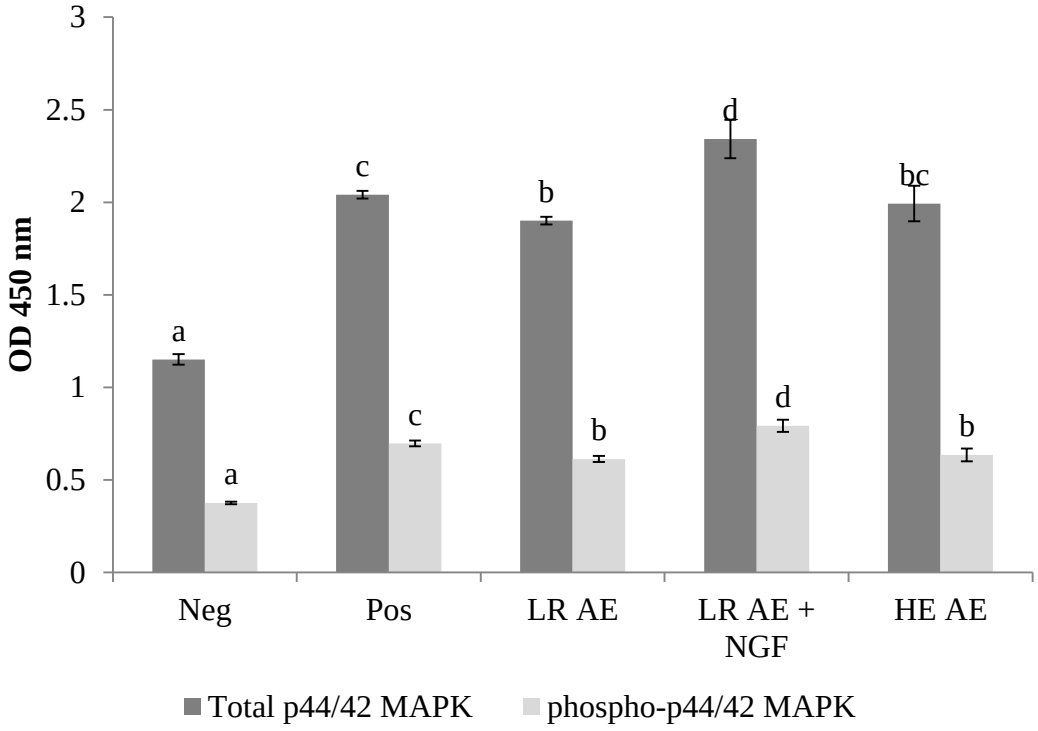


Figure 4.13: Total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) activation in PC-12 after 48 hr of treatment with various extracts. Total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) level were measured via the determination of the optical intensity. Means with different alphabets in different treatment groups were found to be significantly different (DMR analysis) ($p < 0.05$).

Table 4.3: The proximate analysis of nutritional components of freeze dried sclerotium of *L. rhinocerotis*

Parameter	**Result ***	**Recommended daily allowance (RDA) (≥ 4 years)**	**% of RDA**
Energy (kcal)	364	-	-
Protein (g)	19.2	50	38.4
Fat (g)	3.1	65	4.8
Monosaturated fat	0.28	-	-
Polysaturated fat	0.36	-	-
Saturated fat	0.22	-	-
Trans fat	<0.01	-	-
Cholesterol (mg)	<0.001	300	-
Carbohydrate (g)	64.7	300	21.6
Dietary Fibre (g)	32.5	25	130
Mineral composition			
Sodium (mg)	11.78	2400	0.5
Calcium (mg)	39.25	1000	3.9
Magnesium (mg)	51.67	400	12.9
Iron (mg)	1.09	18	6.1
Zinc (mg)	2.28	15	15.2
Phosphorus (mg)	176.41	700	25.2
Potassium (mg)	180.41	3500	5.2
Copper (mg)	0.27	2.0	0.14
Manganese (mg)	1.41	2.0	0.71
Selenium (mg)	<0.02 mg/kg	0.07	<28.6

* g or mg/100 g of freeze-dried fruiting bodies; test method was according to AOAC (Association of Analytical Communities/Association of Official Agricultural Chemist)

DISCUSSION AND CONCLUSION

5.1 Preparation of mushroom extracts

The total yield of extracts varied, depending on the extraction methods used. Compared to aqueous extract, ethanol extraction was generally known to extract more of the bioactive compounds (Ibrahim *et al.*, 2010). Bioactive compounds like *hericinones* (Kawagishi *et al.*, 1990; Kawagishi *et al.*1991; Kawagishi *et al.* 1993) and erinacines (Kawagishi *et al.*, 1994; Kawagishi *et al.*1996; Lee *et al.* 2000) which are examples of bioactive compounds that can be extracted by ethanol extraction from *H. erinaceus.* However, aqueous extraction has been historically applied for decades to extract water soluble components from mushroom fruiting bodies, particularly in folk medicine (Mizuno *et al.*, 1995). This study was thus designed to mimic the preparation methodology as reported in ethno-knowledge.

Aqueous extracts of mushrooms' fruiting bodies and mycelium have been demonstrated to guarantee the availability of bioactive components and their potential use for therapeutic applications (Stengler, 2005). Hot aqueous extraction of *curcuma aromatica* (Lee *et al.*, 2007) and mushrooms (*Russula vesca*, *Auricularia auricular-judae*, *Volvariaella vulvacea* and *Pleurotus squarrosolus*) (Nwachukwu *et al.*, 2010) gave a higher yield than ethanol extraction (65-70% higher) due to the great proportions of water-soluble constituents in mushrooms (Ijeh *et al.*, 2005).

Polysaccharides (including beta glucans) of *L. rhinocerotis* are found inside indigestible cell walls. In order to solubilize and to obtain the bioactive compounds while maintaining their structural integrity, the only feasible approach is via boiling (Sahoo *et al.*, 2010). Besides, Yap *et al.* (2013) also reported that hot aqueous extract of wild *L.*

rhinocerotis sclerotial extract contained more phenolic compounds (29.42 mg GAE g^{-1}), compared to cold aqueous extract (28.38 mg GAE g^{-1}) and methanol extract (19.32 mg GAE g^{-1}). Apparently, higher temperature used in hot aqueous extract was able to solubilize large amount of water-soluble polysaccharides and cellular structural / strorage protein of *L. rhinocerotis*.

Hot aqueous extract of *L. rhinocerotis* also contained equal amount of carbohydrates (37.4%) and protein (41.3%) compared to cold aqueous extract contains mainly carbohydrate (82.3%) and small amount of protein (1.3%) (Lai *et al*, 2013). Report of Lai *et al.* (2013) also stated that carbohydrate content in sclerotium of *L. rhinocerotis* (51.30%) was higher compared to *Ganoderma spp.* (50.3%) (Aremu *et al.*, 2009) and Pleurotus tuber-regium (36.25%) (Ezeibekwe *et al.*, 2009).

Researchers have shown that polysaccharides extracted with 5.1 % NaOH solution comprised of more types of monosaccharides and amino acids and showed significant anti-tumour activity (Kim *et al.*, 1980). *Lignosus rhinocerotis* contains rich amounts of β-glucan, polysaccharides of D-glucose monomers linked by β-glycosidic bonds (Wong, 2011). Previously published results also showed that, 43.16% of the freeze-dried sclerotium of *L. rhinocerotis* was made up of crude polysaccharides. This level was notably higher than the percentage of polysaccharides contained in *G. lucidum* (12.3%) when is extracted by alkaline extraction (Huang & Ning, 2010).

In this study, we hypothesize that polysaccharides, triterpenoids, peptide rather than phenolic compounds were involved in the neurite stimulatory activity. Therefore, hot aqueous extraction method was employed.

5.2 Assessment of cytotoxic activity of *L. rhinocerotis* extract in PC-12 cells

It is important to carry out safety assessment on functional food and health food remedies before being recommended for prevention or treatment of diseases. In recent years, numbers of potential biochemical compounds have been introduced. However, some could be toxic when administrated in high concentration or combination with other medication (Maria *et al.*, 1997). This assay was done to narrow down the test dosage range of *L. rhinocerotis* aqueous extract which will have the highest viability of cells stimulate the highest number of neurite-bearing cells. In this study, MTT assay was used to evaluate the cytotoxicity of aqueous and ethanol extract of *L. rhinocerotis.* Neither aqueous nor ethanol extracts of *L. rhinocerotis* triggered cytotoxic effects in PC-12 cell lines at low concentrations (100 µg/mL (w/v) followed by 48 hr of incubation.

Aqueous extracts of oyster mushroom extracts (*Pleurotus ostreatus*) were reported to have a lower cytotoxic effect, with a lower LC_{50} value of 20.89 µg/mL when compared to methanol chloroform extracts, with a LC_{50} value of 18.62 µg/mL (Faridur *et al.*, 2010). This was also supported by Phan *et al.* (2013) that ethanol extracts of *P. gigantus, P. pulmonarius and H. erinaceus* have a lower IC_{50} ($p < 0.05$) value against Neuroblastoma 2a cells compared to aqueous extract. This is consistent with Lau *et al.*, (2013), that *L. rhinocerotis* hot aqueous extract (IC_{50}: >500 µg/mL) were less cytotoxic than *L. rhinocerotis* cold aqueous extract (IC_{50}: 37-120 µg/mL) against the most susceptible normal cell lines compared to cancer cell lines (Lau *et al.*, 2013).

Furthermore, oral administration of the sclerotial powder at a daily dose of up to 1 g/kg for 28 days was given to Sprague Dawley rats (Lee et al., 2011). Lee *et al.* (2011) had shown that there were no adverse effects on the growth rate and the haematological

and clinical biochemical parameters (including renal and liver function parameters) in the experimental rats.

5.3 Assessment of neurite outgrowth in PC-12 cells

The present study supported recent findings that aqueous extracts of selected mushroom fruiting bodies contained components that stimulate neurite outgrowth. Three different species of medicinal mushrooms, *H. erinaceus, L. rhinocerotis* and *T. heimii* which are appreciated for their pharmaceutical merits were investigated. It is important to repeat the neurite outgrowth activity between batches of processed *L. rhinocerotis*. Nutritional composition of *L. rhinocerotis* may vary from batch to batch due to the substrate formulation, genetic strain, age of mycelium and environmental factors (Chang and Miles, 2004). However, in this study, studies were done with the same batch of processed *L. rhinocerotis.*

Hericium erinaceus (also known as Lion's Mane Mushroom or "monkey head mushroom" in Chinese) has been widely investigated for its anti-dementia bioactive compounds (Mori *et al.*, 2009). Researchers had reported that extract of *H. erinaceus* had neurite stimulatory activity (Wong *et al.,* 2007), promoting functional recovery of peripheral nerve injury (Wong *et al.,* 2012) and as a potent stimulators for the production of NGF (Mori *et al.*, 2008). In this study, neurite outgrowth activity was recorded at 27.5% of neurite-bearing cells when treated with 50 µg/mL (w/v) of *H. erinaceus* aqueous extract in PC-12. Wong *et al.* (2007) also reported that, treatment of 20 µg/mL (w/v) of aqueous extracts of freeze-dried fruiting bodies of *H. erinaceus* in NG108-15 stimulated 17.3% neurite-bearing cells, an 88.2% increase when compared to the negative control. The concentration and percentage of neurite-bearing cells result in differences due to types of cell line.

Bioactive compounds isolated from the fruiting bodies and mycelium of *H. erinaceus* have been reported to have neurite outgrowth activities include hericenones C-H (Kawagishi *et al.*, 1990; Kawagishi *et al.*, 1991; Kawagishi *et al.*, 1992; Kawagishi *et al.*, 1993) and erinacines A—I (Kawagishi *et al.*, 1994; Kawagishi *et al.*, 1996; Lee *et al.*, 2000).

Terimite mushroom (*Termitomyces heimii*) has extensively been used as human food and also to lower or reduce blood pressure, rheumatism, kwashiorkor, obesity and diarrhea (Synytsya *et al.*, 2009). Two cerebrosides and termitomycesphins, which successfully isolated from *Termitomyces albuminosus* were able to promote neurite outgrowth in PC-12 (Qi *et al.*, 2000; Qi *et al.*, 2001).

Lignosus rhinocerotis has been described as the national treasure of Malaysia as it is rare and often used as folk medicine. Aqueous extracts of sclerotia of *L. rhinocerotis* were reported able to stimulate neurite outgrowth in PC-12 (Eik *et al.*, 2012). This study was consistent with studies by Phan *et al.* (2013), that the sclerotia of *L. rhinocerotis* had improved neurite outgrowth in N2a (38.4 ± 4.2% of neurite bearing cells) and performed better than the mycelial extract (27.2 ± 2.9% of neurite bearing cells).

In this study, aqueous extracts of *H. erinaceus* stimulated a higher percentage of neurite-bearing cells of PC-12 cells (27.1 %), when compared to *L. rhinocerotis* (24.0 %) and *T. heimii* (22.4 %) (Figure 4.3). However, higher concentrations of *H. erinaceus* aqueous extracts [50 µg/mL (w/v)] were required to reach the maximal stimulation for neurite outgrowth, when compared to the 20 µg/mL (w/v) of *L. rhinocerotis* aqueous extracts, and 40 µg/mL (w/v) of *T. heimii* aqueous extracts.

As reported, the percentage of cell viability also decreased as the concentrations of extracts increased (Figure 4.1, 4.2). Oyster mushroom extracts (*P. ostreatus*) including hot aqueous extract do contain terpenoids and steroids like compounds and have potent cytotoxic effect in cancer cell as well as brine shrimp nauplii (Faridur *et al.*, 2010). Apparently, increase in concentration of extracts eventually increased the risk of cytotoxic effects. Furthermore, result of phytotoxicity test of 5 plants (*Parkia biglobosa*, *Vitelaria paradoxa*, *Azadrachta indica*, *Chromolaena odorata* and *Lippia multiflora*) showed that all plants extracts are toxic at high concentration (100 mg/mL and 10 mg/mL) with 100% larval mortility (Malau & James, 2007). Therefore, *L. rhinocerotis* aqueous extract were selected for further investigation.

Lignosus rhinocerotis contains a rich amount of carbohydrate (64.7%). This study also showed that about 90% of aqueous extract are polysaccharides. Water-soluble polysaccharides were said to help in neurite outgrowth. *Lignosus rhinocerotis* contained higher contents of polysaccharide, 43.16 % (w/w) (Table 4.1) compared to *G. lucidum*, 40.6 % (w/w) (Cheung *et al.*, 2000). PC-12 cells incubated with *G. lucidum* polysaccharides at concentration higher than 16.65 µg/µl (w/v), resulted in the reduction in cell proliferation rate and induction of neuronal differentiation at the same time protected PC-12 neurons from apoptosis (Cheung *et al.*, 2000).

Upon treatment with an exo-biopolymer, 0.8 mg/L purified from the liquid culture broth of *H. erinaceus* mycelium, cell growth of PC-12 cells was enhanced by 25.8% (Park *et al.*, 2002). This polymer also improved the number of neurite-bearing cells (26% compared to control) and with maximum length of neurites up to 235 µm and were better when compared to neurotrophic NGF and BDNF. The number of neurite bearing cells treated with polymer were 25.3% and 6.3% higher compared to NGF and BDNF

respectively. In addition, the cell viability in PC-12 cells treated with polymer against apoptosis was also higher (89%) when compared to NGF (57.5%) and BDNF (73%) (Park *et al*., 2002). In this study, *L. rhinocertis* aqueous extract (1,642.37 µg/mL) also showed higher IC_{50} in compared to *P. giganteus* aqueous extract at 806.39 µg/mL (w/v) (Phan *et al.*, 2013) and *Ganoderma lucidum* fruiting bodies aqueous extract at 1000 µg/mL (w/v) (Lu *et al.*, 2004).

Aqueous extract nor NGF alone did not induce neurite outgrowth to an appreciable extent. Even at its optimum concentrations of either, there was only a 16.2 % and 15.6 % increase respectively in neurite-bearing cells when compared to negative control (Figure 4.3). Interestingly, it was evident from the results of this study that the aqueous extracts of *L. rhinocerotis* had an additive effect when combined with neurotrophic factors, 30 ng/mL of NGF, in the promotion of neurite outgrowth in PC-12 cells with an observed, 42.1% of neurite-bearing cells. A recent study investigated a combination of *Lignosus rhinocerotis* mycelium aqueous extract and curcumin (27.2% neurite bearing cells) and found that a combination was more effective than *L. rhinocerotis* mycelium (21.2% neurite bearing cells) at 20 µg/mL (w/v) or curcumin alone (29.5%) at 10 pg/mL in the stimulation of neurite outgrowth in PC-12. Another report from More *et al.* (2012) also reported that a combination of plant extract - green tea polyphenol and NGF, had synergistically increased the number, length and branching of neurites.

5.4 Qualitative assessment by immunofluorescene staining of neurofilaments

Indirect immunofluorescence staining was used to localize neurofilament triplet protein in PC-12 cells. Aggregation of neurofilaments are responsible for the radial growth of axon (Rao *et al*., 1998). Neurofilament was aggregated in the body of the cells

that when treated with the *L. rhinocerotis* aqueous extracts, *H. erinaceus* aqueous extract and NGF. An increase in the area and length of axonal staining as compared to negative control was observed.

During axonal growth, new neurofilament antibodies were incorporated along the axons in a dynamic manner. This involves the elongation of axons, as well as the branching of neurites (Alberts *et al.*, 2002). The high affinity of antibody binding feature showed evidence that *L. rhinocerotis* aqueous extract and *H. erinaceus* aqueous extract possess compounds that able to stimulate the extension of axons in PC-12 cells through the staining of the neurofilament antibody. This is parallel to Xu *et al.* (2012) study, the expression of neurofilaments was markedly increased in the cotreatment of NGF and isorhamnetin from *Ginko biloba* in PC-12 cells.

5.5 Assessment of protein signaling pathways involved in stimulating neurite outgrowth

TrkA is a receptor tyrosine kinase for NGF. Many of biological effects like differentiation, development and neuronal survival were mediated via the binding of NGF to TrkA receptor (Katzir *et al.*, 2002). K252a is a specific and potent serine/threonine protein kinase inhibitor of NGF-induced tyrosine phosphorylation of TrkA (Knusel & Hefti, 1992; Koizumi *et al.*, 1988). To investigate the possibility that the effect of mushroom extracts on neurite outgrowth in PC-12 is mediated through TrkA activation, PC-12 cells were pre-treated with K252a.

Exposure of PC-12 cells to K252a led to a significant decrease in neurite outgrowth at 100 nM concentration. K252a compete with NGF and inhibite TrkA receptor. A significant suppression of TrkA was observed on cells by the reducing of

percentage of neurite-bearing cells (Figure 4.7). The results therefore showed that extracts of *L. rhinocerotis* and *H. erinaceus* possessed NGF-like compounds which stimulate neurite outgrowth.

However, discrepancy was recorded in a report by Cheung *et al.* (2000) report, that *Ganoderma lucidium* has no direct involvement of TrkA in stimulating neurite outgrowth. Similarity, isorhamnetin from *Ginko biloba* initiated neurite outgrowth of PC-12 neither through directly activate signaling molecules by phosphorylation, nor NGF-induced activation of the signaling pathways (Xu *et al.*, 2012). Besides, α-Phenyl-N-tert-butylnitron was also found to induce neurite outgrowth in PC-12 independent of TrkA (Tsuji *et al.*, 2001). Neurite stimulatory activity in PC-12 was initiated by *L. rhinocerotis* aqueous extract even without NGF. This suggested that activation of TrKA receptor tyrosine kinase may not be necessary in neurite outgrowth activity.

According to Vaudry *et al.* (2002), mitogen-activated protein kinase (MAPK) is thought to be a potent cascade mediating neurite outgrowth in PC-12 cells. Activation of MAPK pathway by the extracts is also crucial in the preliminary search for effective therapeutic agents in the management of human diseases including neurogenerative diseases (Kim & Choi, 2010). The intracellular transmission of extracellular signals are usually mediated by a network of interacting proteins (Seger & Krebs, 1995). Growth factors such as NGF act as stimuli to initiate the signaling pathway. It allows the elucidation of pathway from the growth factor receptors to effector molecules in the cytoplasm and nucleus of cells. An overview of MAPK pathway was summarized in Figure 5.1. Three major MAPK are c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK).

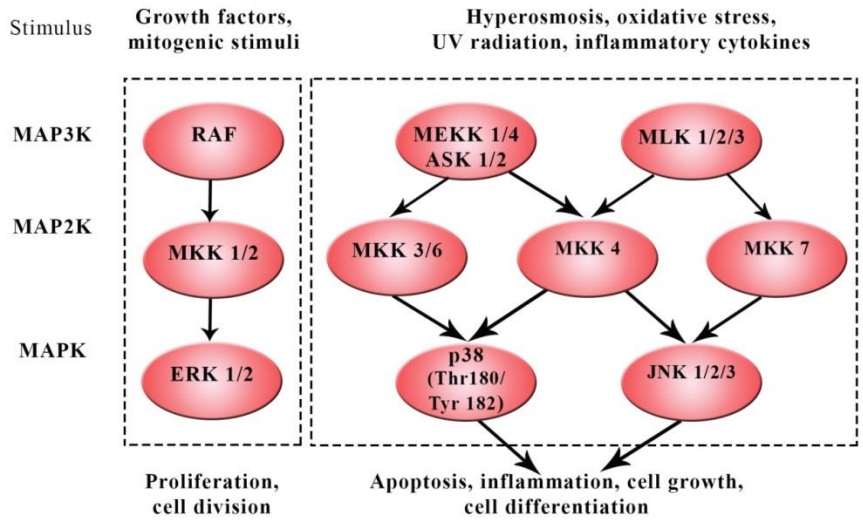


Figure 5.1: Overviewed of MAPK pathway (Cargnello and Roux, 2011).

JNK kinase, also known as stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) is potently and preferentially activated by a variety of environmental stresses, including UV, gamma radiation (Ichijo, 1999), and inflammatory cytokines (Davis, 1999; Kyriakis & Avruch, 2001). Extracts of *H. erinaceus* induced phosphorylation of c-Jun N-terminal kinase (JNK) and its downstream substrate c-Jun, had increased c-Fos expression, suggesting that *H. erinaceus* promotes nerve growth factor gene expression *via* JNK signalling (Wong *et al.*, 2012). *Lignosus rhinocerotis aqueous extract* was found to have less efficacy in inducing JNK activation when compared to negative control, NGF and *H. erinaceus* aqueous extract (Figure 4.9).

However, there are some argumentative statements on the c-Jun activation (phosphorylated of JNK). There are evidences that c-Jun activation could be a step leading to cell apoptosis. However, there are also many arguments in support of contrary view, under certain circumstances, c-Jun can promote proliferation or differentiation of cells (Leppa & Bohmann, 1999). Neurite outgrowth of PC-12 cells induced by d-

limonene (orange oil) revealed that little activity of JNK were recorded (Shinomiyaa *et al.*, 2012).

Another participant in the signaling cascade, p38 MAP kinase (MAPK), is activated by a variety of cellular stresses. These include osmotic shock (Rouse *et al.*, 1994), inflammatory cytokines (Lee *et al.*, 1994), lipopolysaccharides (LPS) (Han *et al.*, UV radiation and growth factors (Rouse *et al.*, 1994). Activation of p38 occurs by phosphorylation both at Thr180 and Tyr182. The p38 MAPK subfamily plays an important role in cytokine production and stress response in mammalian cells (Han *et al.*, 1994).

Major component of essential oils from oranges, d-limonene induced neurite outgrowth PC-12 and revealed that p38 MAPK was strongly activated (Shinomiya *et al.*, 2012). Expression of phosphor-p38 was high when treated with *H. erinaceus* aqueous extracts as compared to other treatments like positive control (NGF), *L. rhinocerotis* aqueous extract alone and *L. rhinocerotis* aqueous extract with NGF (Figure 4.10 and Figure 4.12). Kano *et al.* (2008) stated, that artepillin C in propolis induced activation of p38 MAPK through the ERK signaling pathway is responsible for the neurite outgrowth of PC-12m3 cells.

Both JNK and p38 signaling pathways respond to stress stimuli and caused cells to be involved in neuronal cell differentiation and apoptosis. Treatment with aqueous extracts of *H. erinaceus* up regulated ERK, JNK, and p38. JNK and p38 triggered oxidative stress to the neuronal cell, in order to perform cell differentiation (neurite outgrowth) by phosphorylation of enzymes. However, the latter phosphorylation in the nucleus may either cause the cell to differentiate or lead to inflammation and

subsequently, apoptosis. This is supported by Kim *et al.*, 2012 that Cheongja 3 of black soybean seed coat anthocyanins have brain neuroprotective effects against oxidative stress by inhibiting the activation of JNK and p38 pathways.

Protein MEK1/2 are protein kinases that participate in RAS-RAF-MEK-ERK signal transduction cascade (Roskoski, 2011). This cascade is involved in large variety of processes like apoptosis, cell cycle progression, cell migration, differentiation, metabolism and proliferation (Roskoski, 2011). It has been demonstrated that ERK-cascade was necessary and sufficient enough for NGF-induced neuronal differentiation of PC-12 cells.

In this study, with the elevated fluorescence intensity in PC-12 cells treated with mushroom extracted, suggested that ERK1/2 phosphorylation was affected. This indirectly implied that activation of ERK1/2 is necessary for *L. rhinocerotis* in stimulating neurite outgrowth. Inhibitor U1026 is recognised as a highly selective inhibitor of MEK1 and MEK2. Outgrowth of neurites on PC-12 cells treated with *L. rhinocerotis* was inhibited by U1026 (Figure 4.8, Figure 4.9 and Figure 4.10), via the inhibition of MEK1 and MEK2 phosphorylation. Involvement of ERK signaling pathway was also found in neurite outgrowth by stimulated by curcuminoids in curcumin (Liao *et al.*, 2012).

Aqueous extracts of *L. rhinocerotis* up regulated ERK signaling pathways for cell differentiation and cell growth. It has been demonstrated that ERK-cascade is necessary and adequate for NGF-induced neuronal differentiation of PC-12 cells (Phan *et al.*, 2012). Ganoderma extract was also reported to possess neuroactive compounds that mediate the neuronal differentiation and neuroprotection in PC-12 cells (Cheung *et al.*, 2000).

Figure 5.2 shows a model of stimulation of neurite outgrowth by the Ras-MAPK pathway activated by NGF and *L. rhinocerotis* aqueous extracts. Nerve growth factor initiates cell differentiation and neurite outgrowth by the binding and activation of the TrkA receptor. Tyrosine kinase induces phosphorylation of Shc, followed by activation of the mitogen-activated protein kinase kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1/2).

Neurite outgrowth stimulated by NGF-like extract from: *L. rhinocerotis* and *H. erinaceus* was inhibited when ERK1/2 activation was blocked by U0126. Data suggested that ERK1/2 activities play a role in neurite outgrowth from PC-12 cells. It has been demonstrated that ERK-cascade was necessary and adequate for NGF-induced neuronal differentiation of PC-12 cells.

Upon inhibition by MEK-selective inhibitor U0126, the intensity unit of ERK1/2 stained with FITC has decreased significantly (Figure 4.10). Therefore, the results suggested that phosphorylation of ERK1/2 was activated and this reaffirmed that activation of ERK-cascade was necessary for *L. rhinocerotis* aqueous extract to mediate neurite outgrowth activity in PC-12 cells.

Therefore, aqueous extracts of *L. rhinocerotis* could be a tonic that promotes health of neuronal cells. It could thus be a potential candidate that provides nutrition to neuronal cells in order for them to exhibit neurite outgrowth.

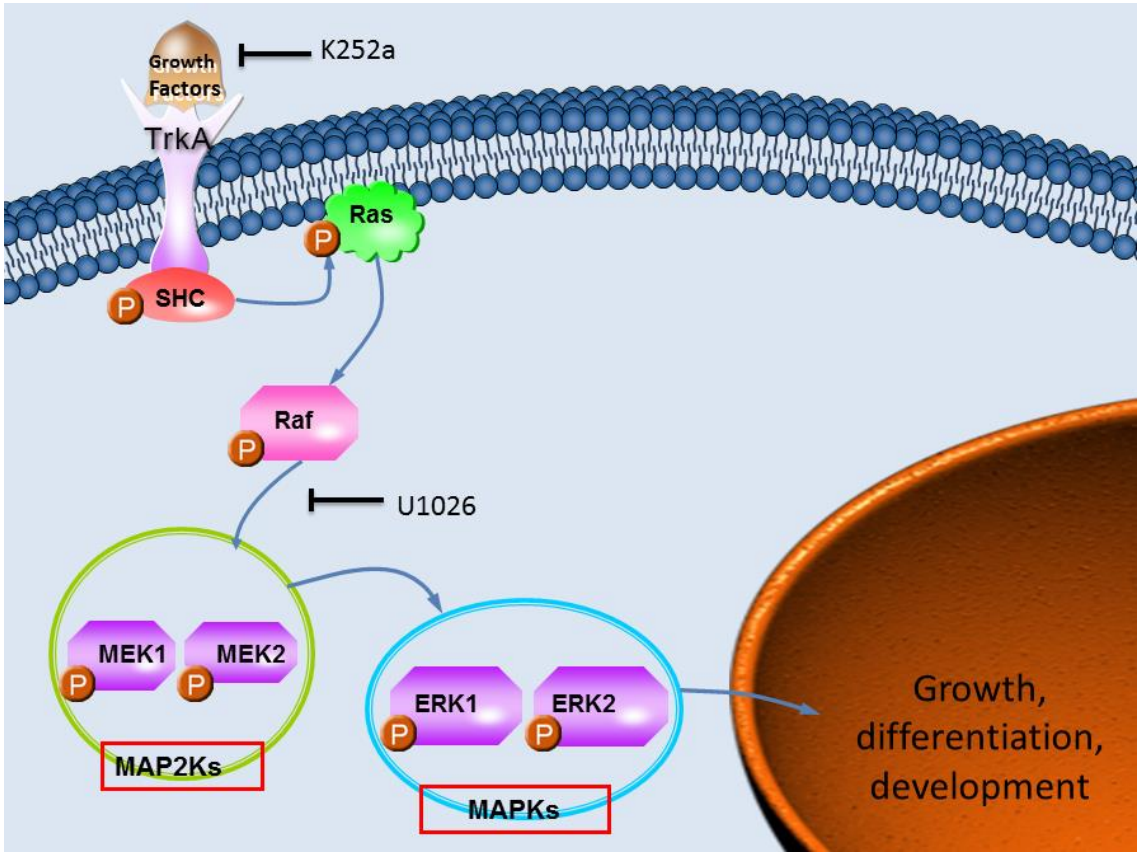


Figure 5.2: Model for neurite outgrowth by the Ras-MAPK pathway activated by NGF and extracts. Nerve growth factor initiates cell differentiation and neurite outgrowth by the binding and activation of the TrkA receptor. Tyrosine kinase induces phosphorylation of SHC, followed by activation of the mitogen-activated protein kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1/2) cascades. TrkA and MEK 1/2 are inhibited by K252a and U1026 respectively.

5.6 Nutritional composition of freeze dried sclerotium of *L. rhinocerotis*

Data available on the nutrient content of *L. rhinocerotis* is limited. Generally, mushrooms are regarded as good sources of proteins (Omar *et al.*, 2011). However, it also highly depends on the substrates used during mushroom cultivation and methods of extraction (Lau *et al.*, 2013). Protein content of fruiting bodies of *P. ostreatus*, *P. sajor-caju* (*culti*vated in lignocellulosic wastes) and *P. giganteus (*cultivated in wheat grain) were reported to range from 13.1 % to 18.4 %, (Bonatti *et al.*, 2004, *Phan et al., 2012*).

This study showed that the protein level in *L. rhinocerotis* (19.2%) was higher when compared to *P. ostreatus*, *P. sajor-caju* and *P. giganteus.*

The carbohydrate content in *L. rhinocerotis (64.7 g/100 g)* was at least two times higher than other edible mushrooms such as *Lentinula edodes* (17.12 g/100 g), *Flammulina velutipes* (10.57 g/100 g), *P. ostreatus* (9.30 g/100 g), *P. eryngii* (8.95 g/100 g), *Agaricus bisporus* (8.25 g/100 g) (Barros *et al.*, 2008), and *A. bisporus* (5.98 g/100 g) (Reis *et al.*, 2012).

This suggested that the sclerotium of *L. rhinocerotis* contained mainly carbohydrates which included simple and complex sugar. Examples of simple and complex sugars are glucose, mannitol, trehalose, oligosaccharide groups, and reserved polysaccharides like glycogen. Besides polysaccharides and protein, other bioactive compounds like phenolics, triterpenes and alkaloids may be present in *L. rhinocerotis* aqueous extract (Lau *et al.*, 2013).

The high potassium and calcium levels from the sclerotium of cultivated *L. rhinocerotis* play a role in the development of the CNS by promoting proximo-distal development (neurite outgrowth in sensory neurons, spinal cord neurons and sympathetic neurons) as these levels increased in the growth development (Sussdorf & Campenot, 1986). Benquet *et al.* (2002) suggested that the calcium influx through voltage-gated calcium channels (VGCC), plays an important role in the survival of cells and neurite outgrowth in vertebrates as well as the neurons in the brain of insects.

In a study by Cohen-Cory *et al.* (1991), cell numbers of Purkinje cells, the major efferent neurons of the brain cerebellum were increased by 40% when treated with

potassium. Besides, potassium or calcium alone or coupled with NGF are able to enhance the cell survival, cell differentiation and neurite outgrowth. Due to the high contents of potassium and calcium present, *L. rhinocerotis* extracts may act as a depolarising agent in the regulation of the morphological differentiation of PC12 cells and the promotion of neurite outgrowth. The high contents of potassium and calcium could also be due to the high levels of potassium and calcium in the culture medium. Yet the nutrient values may vary due to the type of mushroom, strain of mushroom, environmental factors, and composition of growth media (Manzi *et al.*, 2001).

5.7 Recommendation for future work

Based on the results, the aqueous extract of *L. rhinocerotis* was shown to stimulate neurite outgrowth in PC12 cell line via ERK/MAPK protein kinase signaling pathways. Furthermore, it showed no cytotoxic effects *in vivo* (Lee *et al.*, 2011) and *in vitro* (Eik *et al.*, 2012; Lee *et al.*, 2012). More efforts should be focussed on the cellular antioxidants of *in vitro* models after treatment with *L. rhinocerotis*. Measurement of antioxidant activities in cell cultures is important in the screening of natural products for potential health benefits to be validated biologically. This is necessary to evaluate the efficiency and the potential for long-term usage of this mushroom as therapeutic drugs for neurodegenerative diseases.

Lignosus rhinocerotis is a new medicinal mushroom and was first reported for its neurite outgrowth activity in PC12 cells by Eik *et al.* (2012). It is also crucial to test the neurite-outgrowth between the batches of processed *L. rhinocerotis*. Nutritional composition of *L. rhinocerotis* might be varies from batch to batch. The neurite outgrowth properties are, however, based only on the data obtained from *in vitro* studies. Detailed *in vivo* mechanism on outgrowth and regeneration of neurites needs to be further

investigated. Further clinical assessments of *in vivo* activities are needed to evaluate the efficiency of the potential bioactive compounds like polysaccharide prior to their application in the therapeutic management of disease condition. Long term *in vivo* and *in vitro* trials may be needed. There is a need to conduct gene expression studies at NGF mRNA levels in cell lines and mouse brain tissue in order to assess the potentials of the mushroom in the treatment of Alzheimer's disease.

More efforts should be focussed on isolating pure bioactive compounds and validating the findings in the present study. This treasured mushroom has been widely used in folk medicine but reports are scarce that validate its therapeutic effects. To the best of our knowledge, very few bioactive compounds have been successfully isolated and characterised. An ethanol fraction of aqueous extract was found to perform better neurite outgrowth activity compared to aqueous extract and possess anti-inflammatory activity (unpublished data).

According to folk medicine, several natural sources are combined to prepare the decoction. Hence, a combination with other mushrooms or with other natural products that may have neurite outgrowth activity may harmonise very well for the treatment of neurodegenerative diseases (Priscilla *et al.*, 2013). It is common to have multiple bioactive compounds in mushrooms with medicinal value. It will be interesting to find out which of these compounds could act independently or synergistically to elicit their pharmacological effects.

In this study, it was noted that JNK and p38 that are responsive to stress stimuli were not upregulated by *L. rhinocerotis.* Both of these pathways were activated by stress stimuli and may led to cell differentiation. Prolonged stress stimulation may led to

apoptosis. Aqueous extracts of *L. rhinocerotis* up regulated ERK signaling pathways and down regulated JNK and p38 pathway. Up regulated ERK signaling pathways exhibited cell differentiation and cell growth. ERK-cascade was necessary and adequate for NGF-induced neuronal differentiation of PC12 cells. Therefore, there is a possibility that *L. rhinocerotis* may exert neuroprotective effects through the inhibition of the cell apoptosis pathway.

5.8 Conclusions

Aqueous extract of *Lignosus rhinocerotis* a treasured medicinal mushroom of Malaysia was investigated for neurohealth properties. In this study it was observed that

a. *Lignosus rhinocerotis* aqueous extract which was not cytotoxic to PC12 cells at 445 µg/mL possessed neurite stimulatory agents
b. *Lignosus rhinocerotis* aqueous extract triggered maximal neurite outgrowth in PC12 cells at 20 µg/mL with 23.96 % of neurite bearing cells
c. an additive effect was recorded when PC-12 cells were treated with 20 µg/mL of *L. rhinocerotis* aqueous extract and 30 ng/mL of NGF, 42.12 % neurite bearing cells were recorded
d. ERK-cascade was necessary and sufficient for neurite outgrowth in PC12 cells
e. the treatment of PC12 cells with 20 µg/mL *L. rhinocerotis* aqueous extract stimulated neurite outgrowth by activating ERK/MAPK signaling pathways

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APPENDIX

APPENDIX A: CELL CULTURE MEDIA AND STORAGE OF CELL

1. F-12K Medium preparation

Powdered media are hygroscopic and should be protected from moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form. Supplements can be added prior to filtration or introduced aseptically to sterile medium.

i. Measure out 90% of final required volume of water. Water temperature should be 15-20 ℃.
ii. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
iii. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
iv. To the solution in step 3, add 2.5 g sodium bicarbonate or 33.3 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
v. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
vi. Add additional water to bring the solution to final volume.
vii. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
viii. Aseptically dispense medium into sterile container.

2. Storage and Stability

Store the dry powdered medium at 2-8℃ under dry conditions and liquid medium at 2-8 ℃ in the dark. Deterioration of the powdered medium may be recognized by any or all of the following: [1] colour change, [2] granulation/clumping, [3] insolubility. Deterioration of the liquid medium may be recognized by any or all of the following: [1] pH change, [2] precipitate or particulates, [3] cloudy appearance [4] colour change. The nature of supplements added may affect storage conditions and shelf life of the medium. Product label bears expiration date.

APPENDIX B: DATA AND STATISTICAL TABLES

Table B.1: MTT of *L. rhinocerotis* extract treatment on PC12

	Aqueous extract			Ethanol extract		
Conc(µg/ml)	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
Blank	0.033	0.029	0.035	0.035	0.034	0.039
Control	0.213	0.200	0.199	0.290	0.287	0.286
10	0.189	0.162	0.175	0.255	0.257	0.253
25	0.168	0.163	0.150	0.231	0.242	0.233
50	0.145	0.150	0.162	0.226	0.211	0.221
100	0.137	0.132	0.128	0.210	0.188	0.209
250	0.118	0.128	0.112	0.191	0.184	0.193
500	0.110	0.117	0.093	0.167	0.158	0.162
750	0.107	0.108	0.089	0.142	0.139	0.152
1000	0.100	0.088	0.098	0.122	0.134	0.142

Table B.2: Neurite bearing cells (%) on PC12 treated with various concentration of NGF

NGF	dup 1 (%)	dup 2 (%)	dup 3 (%)
Negative	8.07	7.51	8.23
10	14.45	12.42	14.24
20	19.64	20.86	20
30	23.44	26.27	24.2
40	31.21	31.32	30.23
50	38.69	42.51	40.4
60	28.9	31.19	30.57
70	21.84	22.41	23.72
80	19.02	19.29	19.7
90	11.66	12.69	12.57
100	8.29	9.19	8.59

Table B.2.1: Descriptives: Neurite bearing cells (%) on PC12 treated with various concentration of NGF

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	7.9367	.37807	.21828	6.9975	8.8758	7.51	8.23
10ng/ml	3	13.7033	1.11635	.64452	10.9302	16.4765	12.42	14.45
20ng/ml	3	20.1667	.62684	.36191	18.6095	21.7238	19.64	20.86
30ng/ml	3	24.6367	1.46466	.84562	20.9982	28.2751	23.44	26.27
40ng/ml	3	30.9200	.60008	.34646	29.4293	32.4107	30.23	31.32
50ng/ml	3	40.5333	1.91349	1.10475	35.7800	45.2867	38.69	42.51
60ng/ml	3	30.2200	1.18444	.68384	27.2777	33.1623	28.90	31.19
70ng/ml	3	22.6567	.96397	.55655	20.2620	25.0513	21.84	23.72
80ng/ml	3	19.3367	.34239	.19768	18.4861	20.1872	19.02	19.70
90ng/ml	3	12.3067	.56323	.32518	10.9075	13.7058	11.66	12.69
100ng/ml	3	8.6900	.45826	.26458	7.5516	9.8284	8.29	9.19
Total	33	21.0097	9.88693	1.72109	17.5039	24.5155	7.51	42.51

Table B.2.2: One way analysis of variance (ANOVA): Neurite bearing cells (%) on PC12 treated with various concentration of NGF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3106.194	10	310.619	312.738	.000
Within Groups	21.851	22	.993		
Total	3128.045	32			

Table B.2.3: Duncan: Neurite bearing cells (%) on PC12 treated with various concentration of NGF

Concentration	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
Negative	3	7.9367						
100ng/ml	3	8.6900						
90ng/ml	3		12.3067					
10ng/ml	3		13.7033					
80ng/ml	3			19.3367				
20ng/ml	3			20.1667				
70ng/ml	3				22.6567			
30ng/ml	3					24.6367		
60ng/ml	3						30.2200	
40ng/ml	3						30.9200	
50ng/ml	3							40.5333
Sig.		.365	.100	.319	1.000	1.000	.399	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B. 3: Effects of various concentrations of aqueous extracts (*L. rhinocerotis, H. erinaceus and T. heimii*) on neurite outgrowth of PC12 cells

Aqueous extract

	Hericium erinaceus			*Lignosus rhinocerus*			*Termitomyces sp.*		
	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
Neg	8.55	7.95	8.86	7.94	9.55	8.97	8.76	8.96	8.36
Pos	23.87	25.66	24.69	23.52	24.52	23.01	24.31	23.15	25.86
10	16.95	15.52	16.18	18.8	17.95	17.52	13.03	12.07	13.72
20	19.17	20.9	19.91	24.75	23.52	23.61	14.95	15.36	14.38

30	21.22	22.22	21.46	16.88	16.53	16.32	19.19	20.63	18.32
40	24.63	25	25.75	16.67	15.65	15.23	22.1	21.48	22.55
50	26.33	27.31	27.66	14.1	14.29	13.03	20.7	21.35	20.76
60	20.76	20.69	21.81	12.02	11.26	11.44	17.99	19.38	18.21
70	18.14	19.23	18.64	10.84	10.68	10.00	16.67	16.17	15.21
80	16.23	15.45	16.24	9.92	10.00	10.34	13.43	12.00	11.07
90	15.26	15.02	15.13	9.54	9.66	9.79	11.66	10.41	11.57
100	12.61	12.02	12.61	8.94	8.56	9.05	8.57	10.12	9.76

B.3.1: Descriptives: Neurite bearing cells (%) on PC12 treated with with various aqueous extract (a) *H. erinaceus* (b) *L. rhinocerotis* and (c) *T. heimii*

(a) *H. erinaceus* aqueous extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	8.4533	.46264	.26710	7.3041	9.6026	7.95	8.86
Positive	3	24.7400	.89605	.51733	22.5141	26.9659	23.87	25.66
10ug/ml HEAE	3	16.2167	.71570	.41321	14.4388	17.9946	15.52	16.95
20ug/ml HEAE	3	19.9933	.86801	.50114	17.8371	22.1496	19.17	20.90
30ug/ml HEAE	3	21.6333	.52205	.30140	20.3365	22.9302	21.22	22.22
40ug/ml HEAE	3	25.1267	.57064	.32946	23.7091	26.5442	24.63	25.75
50ug/ml HEAE	3	27.1000	.68942	.39804	25.3874	28.8126	26.33	27.66
60ug/ml HEAE	3	21.0867	.62740	.36223	19.5281	22.6452	20.69	21.81
70ug/ml HEAE	3	18.6700	.54562	.31501	17.3146	20.0254	18.14	19.23
80ug/ml HEAE	3	15.9733	.45325	.26168	14.8474	17.0993	15.45	16.24
90ug/ml HEAE	3	15.1367	.12014	.06936	14.8382	15.4351	15.02	15.26
100ug/ml HEAE	3	12.4133	.34064	.19667	11.5671	13.2595	12.02	12.61
Total	36	18.8786	5.37990	.89665	17.0583	20.6989	7.95	27.66

(b) *L. rhinocerotis* aqueous extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	8.8200	.81541	.47078	6.7944	10.8456	7.94	9.55
Positive	3	23.6833	.76814	.44348	21.7752	25.5915	23.01	24.52
10ug/ml LRAE	3	18.0900	.65138	.37608	16.4719	19.7081	17.52	18.80
20ug/ml LRAE	3	23.9600	.68564	.39585	22.2568	25.6632	23.52	24.75
30ug/ml LRAE	3	16.5767	.28290	.16333	15.8739	17.2794	16.32	16.88
40ug/ml LRAE	3	15.8500	.74054	.42755	14.0104	17.6896	15.23	16.67
50ug/ml LRAE	3	13.8067	.67929	.39219	12.1192	15.4941	13.03	14.29
60ug/ml LRAE	3	11.5733	.39716	.22930	10.5867	12.5599	11.26	12.02
70ug/ml LRAE	3	10.5067	.44602	.25751	9.3987	11.6146	10.00	10.84
80ug/ml LRAE	3	10.0867	.22301	.12875	9.5327	10.6407	9.92	10.34
90ug/ml LRAE	3	9.6633	.12503	.07219	9.3527	9.9739	9.54	9.79
100ug/ml LRAE	3	8.8500	.25710	.14844	8.2113	9.4887	8.56	9.05
Total	36	14.2889	5.29296	.88216	12.4980	16.0798	7.94	24.75

(c) *T. heimii* aqueous extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	8.6933	.30551	.17638	7.9344	9.4522	8.36	8.96
Positive	3	24.4400	1.35967	.78501	21.0624	27.8176	23.15	25.86
10ug/ml TERAE	3	12.9400	.82867	.47843	10.8815	14.9985	12.07	13.72
20ug/ml TERAE	3	14.8967	.49217	.28416	13.6740	16.1193	14.38	15.36
30ug/ml TERAE	3	19.3800	1.16666	.67357	16.4819	22.2781	18.32	20.63
40ug/ml TERAE	3	22.0433	.53725	.31018	20.7087	23.3779	21.48	22.55
50ug/ml TERAE	3	20.9367	.35921	.20739	20.0443	21.8290	20.70	21.35
60ug/ml TERAE	3	18.5267	.74715	.43137	16.6706	20.3827	17.99	19.38
70ug/ml TERAE	3	16.0167	.74198	.42838	14.1735	17.8598	15.21	16.67
80ug/ml TERAE	3	12.1667	1.18879	.68635	9.2135	15.1198	11.07	13.43
90ug/ml TERAE	3	11.2133	.69716	.40251	9.4815	12.9452	10.41	11.66
100ug/ml TERAE	3	9.4833	.81119	.46834	7.4682	11.4984	8.57	10.12
Total	36	15.8947	5.06575	.84429	14.1807	17.6087	8.36	25.86

Table B.3.2: One way analysis of variance (ANOVA): Neurite bearing cells (%) on PC12 treated with various aqueous extract (a) *H. erinaceus* (b) *L. rhinocerotis* (c) *T. heimii*

(a) *H. erinaceus*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1004.251	11	91.296	249.937	.000
Within Groups	8.767	24	.365		
Total	1013.018	35			

(b) *L. rhinocerotis*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	973.086	11	88.462	284.801	.000
Within Groups	7.455	24	.311		
Total	980.541	35			

(c) *T. heimii*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	881.531	11	80.139	115.644	.000
Within Groups	16.632	24	.693		
Total	898.163	35			

Table B.3.3: Duncan: Neurite bearing cells (%) on PC12 treated with various aqueous extract (a) *H. erinaceus* (b) *L. rhinocerotis* and (c) *T. heimii*

(a) *H. erinaceus*

		Subset for alpha = 0.05								
Concentration	N	1	2	3	4	5	6	7	8	9
Negative	3	8.4533								
100ug/ml HEAE	3		12.4133							
90ug/ml HEAE	3			15.1367						
80ug/ml HEAE	3			15.9733	15.9733					
10ug/ml HE	3				16.2167					
70ug/ml HEAE	3					18.6700				
20ug/ml HEAE	3						19.9933			
60ug/ml HEAE	3							21.0867		
30ug/ml HEAE	3							21.6333		
Positive	3								24.7400	
40ug/ml HEAE	3								25.1267	
50ug/ml HEAE	3									27.1000
Sig.		1.000	1.000	.103	.626	1.000	1.000	.279	.441	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(b) *L. rhinocerotis*

		Subset for alpha = 0.05						
Concentration	N	1	2	3	4	5	6	7
Negative	3	8.8200						
100ug/ml LRAE	3	8.8500						
90ug/ml LRAE	3	9.6633	9.6633					
80ug/ml LRAE	3		10.0867					
70ug/ml LRAE	3		10.5067					
60ug/ml LRAE	3			11.5733				
50ug/ml LRAE	3				13.8067			
40ug/ml LRAE	3					15.8500		
30ug/ml LRAE	3					16.5767		
10ug/ml LRAE	3						18.0900	
Positive	3							23.6833
20ug/ml LRAE	3							23.9600
Sig.		.091	.091	1.000	1.000	.123	1.000	.549

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.

(c) *T. heimii*

Concentration	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
Negative	3	8.6933						
100ug/ml TERAE	3	9.4833						
90ug/ml TERAE	3		11.2133					
80ug/ml TERAE	3		12.1667	12.1667				
10ug/ml TERAE	3			12.9400				
20ug/ml TERAE	3				14.8967			
70ug/ml TERAE	3				16.0167			
60ug/ml TERAE	3					18.5267		
30ug/ml TERAE	3					19.3800		
50ug/ml TERAE	3						20.9367	
40ug/ml TERAE	3						22.0433	
Positive	3							24.4400
Sig.		.257	.174	.266	.112	.221	.117	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.4: Percentage of neurite bearing cells in the cell line PC12 in response to treatments with aqueous and ethanol extracts of *Lignosus rhinocerotis* and *H. erinaceus* (µg/mL)

	Lignosus rhinocerus						*Hericium erinaceus*					
	Aqueous Extract			Ethanol Extract			Aqueous Extract			Ethanol Extract		
	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
Neg	7.83	9.17	8.64	8.38	8.30	8.39	8.55	7.95	8.86	8.87	9.21	9.06
Pos	24.24	23.10	23.40	26.43	24.65	25.92	23.87	25.66	24.69	24.73	23.63	23.32
10	18.80	17.95	17.52	11.99	12.06	11.67	16.95	15.52	16.18	12.79	11.30	13.13
20	25.75	21.52	23.61	18.55	18.55	18.33	19.17	20.90	19.91	15.52	14.69	15.68
30	16.88	16.53	16.32	15.44	14.29	14.96	21.22	22.22	21.46	17.12	18.73	17.23
40	16.67	15.65	14.23	12.02	13.76	13.36	24.63	25.00	25.75	19.26	20.76	19.10
50	14.10	14.29	13.03	11.39	11.79	11.48	26.33	27.31	27.66	20.07	21.35	20.69
60	12.02	11.26	11.44	10.17	10.65	10.86	20.76	20.69	21.81	21.96	22.43	22.68
70	11.56	11.01	10.34	10.03	10.62	9.62	18.14	19.23	18.64	23.33	23.02	23.05
80	10.84	10.68	10.00	9.87	9.26	10.24	16.23	15.45	16.24	20.75	20.70	19.32
100	8.94	7.56	8.05	9.51	9.47	9.91	12.61	12.02	12.61	13.71	13.64	14.54

Table B.4.1: Descriptives: Neurite bearing cells (%) on PC12 treated with aqueous and ethanol extracts of *Lignosus rhinocerotis* and *H. erinaceus*

(a) *H. erinaceus* aqueous extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	8.4533	.46264	.26710	7.3041	9.6026	7.95	8.86
Positive	3	24.7400	.89605	.51733	22.5141	26.9659	23.87	25.66
10ug/ml HEAE	3	16.2167	.71570	.41321	14.4388	17.9946	15.52	16.95
20ug/ml HEAE	3	19.9933	.86801	.50114	17.8371	22.1496	19.17	20.90
30ug/ml HEAE	3	21.6333	.52205	.30140	20.3365	22.9302	21.22	22.22
40ug/ml HEAE	3	25.1267	.57064	.32946	23.7091	26.5442	24.63	25.75
50ug/ml HEAE	3	27.1000	.68942	.39804	25.3874	28.8126	26.33	27.66
60ug/ml HEAE	3	21.0867	.62740	.36223	19.5281	22.6452	20.69	21.81
70ug/ml HEAE	3	18.6700	.54562	.31501	17.3146	20.0254	18.14	19.23
80ug/ml HEAE	3	15.9733	.45325	.26168	14.8474	17.0993	15.45	16.24
100ug/ml HEAE	3	12.4133	.34064	.19667	11.5671	13.2595	12.02	12.61
Total	33	19.2188	5.49762	.95701	17.2694	21.1682	7.95	27.66

(b) *H. erinaceus* ethanol extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	9.0467	.17039	.09838	8.6234	9.4699	8.87	9.21
Positive	3	23.8933	.74097	.42780	22.0527	25.7340	23.32	24.73
10ug/ml HEEE	3	12.4067	.97336	.56197	9.9887	14.8246	11.30	13.13
20ug/ml HEEE	3	15.2967	.53144	.30683	13.9765	16.6168	14.69	15.68
30ug/ml HEEE	3	17.6933	.89946	.51931	15.4589	19.9277	17.12	18.73
40ug/ml HEEE	3	19.7067	.91571	.52869	17.4319	21.9814	19.10	20.76
50ug/ml HEEE	3	20.7033	.64010	.36956	19.1132	22.2934	20.07	21.35
60ug/ml HEEE	3	22.3567	.36556	.21106	21.4486	23.2648	21.96	22.68
70ug/ml HEEE	3	23.1333	.17098	.09871	22.7086	23.5581	23.02	23.33
80ug/ml HEEE	3	20.2567	.81156	.46856	18.2406	22.2727	19.32	20.75
100ug/ml HEEE	3	13.9633	.50063	.28904	12.7197	15.2070	13.64	14.54
Total	33	18.0415	4.68168	.81498	16.3815	19.7016	8.87	24.73

(c) *L. rhinocerotis* aqueous extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	8.5467	.67486	.38963	6.8702	10.2231	7.83	9.17
Positive	3	23.5800	.59093	.34117	22.1120	25.0480	23.10	24.24
10ug/ml LRAE	3	18.0900	.65138	.37608	16.4719	19.7081	17.52	18.80
20ug/ml LRAE	3	23.6267	2.11505	1.22112	18.3726	28.8807	21.52	25.75
30ug/ml LRAE	3	16.5767	.28290	.16333	15.8739	17.2794	16.32	16.88
40ug/ml LRAE	3	15.5167	1.22545	.70752	12.4725	18.5609	14.23	16.67
50ug/ml LRAE	3	13.8067	.67929	.39219	12.1192	15.4941	13.03	14.29
60ug/ml LRAE	3	11.5733	.39716	.22930	10.5867	12.5599	11.26	12.02
70ug/ml LRAE	3	10.9700	.61098	.35275	9.4522	12.4878	10.34	11.56
80ug/ml LRAE	3	10.5067	.44602	.25751	9.3987	11.6146	10.00	10.84
100ug/ml LRAE	3	8.1833	.69960	.40391	6.4454	9.9212	7.56	8.94
Total	33	14.6342	5.32580	.92710	12.7458	16.5227	7.56	25.75

(d) *L. rhinocerotis* ethanol extract

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	8.3567	.04933	.02848	8.2341	8.4792	8.30	8.39
Positive	3	25.6667	.91664	.52922	23.3896	27.9437	24.65	26.43
10ug/ml LREE	3	11.9067	.20793	.12005	11.3901	12.4232	11.67	12.06
20ug/ml LREE	3	18.4767	.12702	.07333	18.1611	18.7922	18.33	18.55
30ug/ml LREE	3	14.8967	.57761	.33348	13.4618	16.3315	14.29	15.44
40ug/ml LREE	3	13.0467	.91134	.52616	10.7828	15.3106	12.02	13.76
50ug/ml LREE	3	11.5533	.20984	.12115	11.0321	12.0746	11.39	11.79
60ug/ml LREE	3	10.5600	.35369	.20421	9.6814	11.4386	10.17	10.86
70ug/ml LREE	3	10.0900	.50269	.29023	8.8412	11.3388	9.62	10.62
80ug/ml LREE	3	9.7900	.49487	.28572	8.5607	11.0193	9.26	10.24
100ug/ml LREE	3	9.6300	.24331	.14048	9.0256	10.2344	9.47	9.91
Total	33	13.0885	4.90610	.85404	11.3489	14.8281	8.30	26.43

Table B.4.2: One way analysis of variance (ANOVA): Neurite bearing cells (%) on PC12 treated with aqueous and ethanol extracts of *Lignosus rhinocerotis* and *H. erinaceus*

(a) *H. erinaceus* aqueous extract

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	958.426	10	95.843	241.314	.000
Within Groups	8.738	22	.397		
Total	967.164	32			

(b) *H. erinaceus* ethanol extract

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	691.505	10	69.150	154.061	.000
Within Groups	9.875	22	.449		
Total	701.380	32			

(c) *L. rhinocerotis* aqueous extract

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	889.724	10	88.972	109.169	.000
Within Groups	17.930	22	.815		
Total	907.654	32			

(d) *L. rhinocerotis* ethanol extract

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	764.650	10	76.465	301.244	.000
Within Groups	5.584	22	.254		
Total	770.234	32			

Table B.4.3: Duncan: Neurite bearing cells (%) on PC12 treated with aqueous and ethanol extracts of *Lignosus rhinocerotis* and *H. erinaceus*

(a) *H. erinaceus* aqueous extract

		Subset for alpha = 0.05							
Concentration	N	1	2	3	4	5	6	7	8
Negative	3	8.4533							
100ug/ml HEAE	3		12.4133						
80ug/ml HEAE	3			15.9733					
10ug/ml HEAE	3			16.2167					
70ug/ml HEAE	3				18.6700				
20ug/ml HEAE	3					19.9933			
60ug/ml HEAE	3						21.0867		
30ug/ml HEAE	3						21.6333		
Positive	3							24.7400	
40ug/ml HEAE	3							25.1267	
50ug/ml HEAE	3								27.1000
Sig.		1.000	1.000	.641	1.000	1.000	.300	.460	1.000

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.

(b) *H. erinaceus* ethanol extract

		Subset for alpha = 0.05							
Concentration	N	1	2	3	4	5	6	7	8
Negative	3	9.0467							
10ug/ml HEEE	3		12.4067						
100ug/ml HEEE	3			13.9633					
20ug/ml HEEE	3				15.2967				
30ug/ml HEEE	3					17.6933			
40ug/ml HEEE	3						19.7067		
80ug/ml HEEE	3						20.2567		
50ug/ml HEEE	3						20.7033		
60ug/ml HEEE	3							22.3567	
70ug/ml HEEE	3							23.1333	23.1333
Positive	3								23.8933
Sig.		1.000	1.000	1.000	1.000	1.000	.098	.170	.179

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.

(c) *L. rhinocerotis* aqueous extract

Concentration	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
100ug/ml LRAE	3	8.1833					
Negative	3	8.5467					
80ug/ml LRAE	3		10.5067				
70ug/ml LRAE	3		10.9700				
60ug/ml LRAE	3		11.5733				
50ug/ml LRAE	3			13.8067			
40ug/ml LRAE	3				15.5167		
30ug/ml LRAE	3				16.5767	16.5767	
10ug/ml LRAE	3					18.0900	
Positive	3						23.5800
20ug/ml LRAE	3						23.6267
Sig.		.627	.185	1.000	.164	.052	.950

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(d) *L. rhinocerotis* ethanol extract

Concentration	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
Negative	3	8.3567							
100ug/ml LREE	3		9.6300						
80ug/ml LREE	3		9.7900	9.7900					
70ug/ml LREE	3		10.0900	10.0900					
60ug/ml LREE	3			10.5600					
50ug/ml LREE	3				11.5533				
10ug/ml LREE	3				11.9067				
40ug/ml LREE	3					13.0467			
30ug/ml LREE	3						14.8967		
20ug/ml LREE	3							18.4767	
Positive	3								25.6667
Sig.		1.000	.302	.089	.400	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.5: Percentage of neurite bearing cells in the cell line PC12 in response to treatment of aqueous extracts (LR AE) and polysaccharide (LR PSP) (w/v).

Concentration (µg/ml)	LR AE			LR PSP		
	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
-ve	6.02	6.44	6.80	5.68	5.88	5.62
+ve	18.15	18.22	18.80	17.42	18.15	18.22
25	19.02	18.32	20.15	14.94	16.32	17.20
50	16.41	16.37	17.94	12.36	12.14	13.67
75	13.28	13.61	12.16	11.35	11.58	11.27
100	11.88	11.91	10.55	9.65	8.33	9.85

Table B.5.1: Descriptives: Neurite bearing cells (%) on PC12 treated with (a) aqueous extracts (LR AE) and (b)polysaccharide (LR PSP)

(a) *L. rhinocerotis* aqueous extracts

Descriptives

Percentage

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
-ve	3	6.42	.390	.225	5.45	7.39	6	7
+ve	3	18.39	.357	.206	17.50	19.28	18	19
LR AE 25 ug/ml	3	19.16	.923	.533	16.87	21.46	18	20
LR AE 50 ug/ml	3	16.91	.895	.517	14.68	19.13	16	18
LR AE 75 ug/ml	3	13.02	.760	.439	11.13	14.90	12	14
LR AE 100 ug/ml	3	11.45	.777	.448	9.52	13.38	11	12
Total	18	14.22	4.623	1.090	11.93	16.52	6	20

(b) *L. rhinocerotis* polysaccharide

Descriptives

Percentage

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
-ve	3	5.7267	.13614	.07860	5.3885	6.0649	5.62	5.88
+ve	3	17.9300	.44306	.25580	16.8294	19.0306	17.42	18.22
LR PSP 25 ug/ml	3	16.1533	1.13918	.65771	13.3235	18.9832	14.94	17.20
LR PSP 50 ug/ml	3	12.7233	.82718	.47757	10.6685	14.7782	12.14	13.67
LR PSP 75 ug/ml	3	11.4000	.16093	.09292	11.0002	11.7998	11.27	11.58
LR PSP 100 ug/ml	3	9.2767	.82591	.47684	7.2250	11.3283	8.33	9.85
Total	18	12.2017	4.23769	.99883	10.0943	14.3090	5.62	18.22

Table B.5.2: One way analysis of variance (ANOVA): Neurite bearing cells (%) on PC12 treated with aqueous extracts (LR AE) and polysaccharide (LR PSP)

(a) *L. rhinocerotis* aqueous extracts

ANOVA

Percentage

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	357.069	5	71.414	137.580	.000
Within Groups	6.229	12	.519		
Total	363.298	17			

(b) *L. rhinocerotis* polysaccharide

ANOVA

Percentage

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	299.477	5	59.895	123.715	.000
Within Groups	5.810	12	.484		
Total	305.286	17			

Table B.5.3: Duncan: Neurite bearing cells (%) on PC12 treated with aqueous extracts (LR AE) and polysaccharide (LR PSP)

(a) *L. rhinocerotis* aqueous extracts

Percentage

Duncan[a]

Concentration	N	Subset for alpha = 0.05				
		1	2	3	4	5
-ve	3	6.42				
LR AE 100 ug/ml	3		11.45			
LR AE 75 ug/ml	3			13.02		
LR AE 50 ug/ml	3				16.91	
+ve	3					18.39
LR AE 25 ug/ml	3					19.16
Sig.		1.000	1.000	1.000	1.000	.213

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(b) *L. rhinocerotis* polysaccharide

Percentage

Duncan[a]

Concentration	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
-ve	3	5.7267					
LR PSP 100 ug/ml	3		9.2767				
LR PSP 75 ug/ml	3			11.4000			
LR PSP 50 ug/ml	3				12.7233		
LR PSP 25 ug/ml	3					16.1533	
+ve	3						17.9300
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.6: Percentage of neurite bearing cells in the cell line PC12 in response to addition effect of *L. rhinocerotis* aqueous extracts (LR AE) and various concentration of NGF (w/v).

Concentration (20 µg/mL of LRAE + concentrations of NGF)	dup 1 (%)	dup 2 (%)	dup 3 (%)
0 µg/mL of LRAE	24.17	24.13	25.94
20 µg/mL of LRAE + 10 ng/mL of NGF	30.52	28.85	31.73
20 µg/mL of LRAE + 20 ng/mL of NGF	36.18	34.02	36.73
20 µg/mL of LRAE + 30 ng/mL of NGF	42.73	40.35	43.27
20 µg/mL of LRAE + 40 ng/mL of NGF	34.63	33.62	35.21
20 µg/mL of LRAE + 50 ng/mL of NGF	29.56	29.88	29.25

B.6.1: Descriptives: Neurite bearing cells (%) of addition effect on PC12

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	24.7467	1.03365	.59678	22.1789	27.3144	24.13	25.94
LR+10 ng/ml NGF	3	30.3667	1.44611	.83491	26.7743	33.9590	28.85	31.73
LR+20 ng/ml NGF	3	35.6433	1.43249	.82705	32.0848	39.2018	34.02	36.73
LR+30 ng/ml NGF	3	42.1167	1.55362	.89698	38.2573	45.9761	40.35	43.27
LR+40 ng/ml NGF	3	34.4867	.80463	.46455	32.4878	36.4855	33.62	35.21
LR+50 ng/ml NGF	3	29.5633	.31501	.18187	28.7808	30.3459	29.25	29.88
Total	18	32.8206	5.70699	1.34515	29.9825	35.6586	24.13	43.27

B.6.2 One way analysis of variance (ANOVA): Neurite bearing cells (%) of addition effect on PC12

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	536.941	5	107.388	76.962	.000
Within Groups	16.744	12	1.395		
Total	553.685	17			

B.6.3 Duncan: Neurite bearing cells (%) of addition effect on PC12

	Treatment	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	negative	3	24.7467			
	LR+50 ng/ml NGF	3		29.5633		
	LR+10 ng/ml NGF	3		30.3667		
	LR+40 ng/ml NGF	3			34.4867	
	LR+20 ng/ml NGF	3			35.6433	
	LR+30 ng/ml NGF	3				42.1167
	Sig.		1.000	.421	.254	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.7: Percentage of neurite bearing cells in the cell line PC12 with and without inhibitor, K252a treatment.

	without K252a			With K252a		
	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
-ve	8.670	9.460	8.860	8.330	7.550	7.590
+ve	25.320	23.600	23.490	8.220	7.560	7.900
LR AE	20.590	20.000	22.090	7.780	8.560	8.400
LR AE + NGF	25.120	23.380	24.840	0.830	8.890	8.640
HE AE	38.560	36.690	39.110	8.560	9.800	8.590

B.7.1: Descriptives: Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor, K252a treatment and (b) without inhibitor, K252a treatment.

(a) without inhibitor, K252a treatment

Descriptives

Percentage

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
-ve	3	8.9967	.41235	.23807	7.9723	10.0210	8.67	9.46
+ve	3	24.1367	1.02627	.59252	21.5873	26.6861	23.49	25.32
LR AE	3	20.8933	1.07751	.62210	18.2166	23.5700	20.00	22.09
LR AE + NGF	3	24.4467	.93431	.53942	22.1257	26.7676	23.38	25.12
HE AE	3	38.1200	1.26858	.73242	34.9687	41.2713	36.69	39.11
Total	15	23.3187	9.65772	2.49361	17.9704	28.6669	8.67	39.11

(b) with inhibitor, K252a treatment

Descriptives

Percentage

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
-ve	3	7.8233	.43924	.25360	6.7322	8.9145	7.55	8.33
+ve	3	7.8933	.33005	.19055	7.0734	8.7132	7.56	8.22
LR AE	3	8.2467	.41199	.23786	7.2232	9.2701	7.78	8.56
LR AE + NGF	3	6.1200	4.58298	2.64598	-5.2648	17.5048	.83	8.89
HE AE	3	8.9833	.70741	.40843	7.2260	10.7406	8.56	9.80
Total	15	7.8133	2.02208	.52210	6.6935	8.9331	.83	9.80

Table B.7.2: One way analysis of variance (ANOVA): Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor, K252a treatment and (b) without inhibitor, K252a treatment.

(a) without inhibitor, K252a treatment treatment

ANOVA

Percentage

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1296.069	4	324.017	332.903	.000
Within Groups	9.733	10	.973		
Total	1305.802	14			

(b) with inhibitor, K252a treatment treatment

ANOVA

Percentage

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.292	4	3.323	.756	.577
Within Groups	43.951	10	4.395		
Total	57.243	14			

Table B.7.3: Duncan: Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment.

(a) without inhibitor treatment

Percentage

Duncan[a]

Concentration	N	Subset for alpha = 0.05			
		1	2	3	4
-ve	3	8.9967			
LR AE	3		20.8933		
+ve	3			24.1367	
LR AE + NGF	3			24.4467	
HE AE	3				38.1200
Sig.		1.000	1.000	.708	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(b) with inhibitor treatment

Percentage

Duncan[a]

Concentration	N	Subset for alpha = 0.05
		1
LR AE + NGF	3	6.1200
-ve	3	7.8233
+ve	3	7.8933
LR AE	3	8.2467
HE AE	3	8.9833
Sig.		.155

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B.8: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) activation in PC12 after 48 hr of treatment with various extracts. (a) with inhibitor treatment and (b) without inhibitor treatment

	inhibited			Not inhibited		
ERK	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
-ve	1.58	1.23	1.66	9.38	9.5	9.64
+ve	1.84	1.72	1.94	12.89	15.89	12.28
LR AE	1.56	1.88	1.53	13.95	13.06	15.58
LR AE+NGF	2.16	2.28	2.35	22.33	21.47	20.97
HE AE	2.88	2.32	2.53	14.25	15.65	13.43

B.8.1: Descriptives: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) activation in PC12 after 48 hr of treatment with various extracts. (a) with inhibitor treatment and (b) without inhibitor treatment

(a) **with** inhibitor treatment

Descriptives

Intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
negative	3	1.4900	.22869	.13204	.9219	2.0581	1.23	1.66
positive	3	1.8333	.11015	.06360	1.5597	2.1070	1.72	1.94
LR AE	3	1.6567	.19399	.11200	1.1748	2.1386	1.53	1.88
LR AE + NGF	3	2.2633	.09609	.05548	2.0246	2.5020	2.16	2.35
HE AE	3	2.5767	.28290	.16333	1.8739	3.2794	2.32	2.88
Total	15	1.9640	.44611	.11518	1.7170	2.2110	1.23	2.88

(b) without inhibitor treatment

Descriptives

Intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
negative	3	9.5067	.13013	.07513	9.1834	9.8299	9.38	9.64
positive	3	13.6867	1.93236	1.11565	8.8864	18.4869	12.28	15.89
LR AE	3	14.1967	1.27798	.73784	11.0220	17.3713	13.06	15.58
LR AE + NGF	3	21.5900	.68790	.39716	19.8812	23.2988	20.97	22.33
HE AE	3	14.4433	1.12256	.64811	11.6547	17.2319	13.43	15.65
Total	15	14.6847	4.15416	1.07260	12.3842	16.9852	9.38	22.33

Table B.8.2: One way analysis of variance (ANOVA): Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment

(a) with inhibitor treatment

ANOVA

Intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.403	4	.601	15.702	.000
Within Groups	.383	10	.038		
Total	2.786	14			

(b) **without** inhibitor treatment

ANOVA

Intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	227.363	4	56.841	39.930	.000
Within Groups	14.235	10	1.424		
Total	241.598	14			

Table B.8.3: Duncan: Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment.

(a) with inhibitor treatment

Intensity

Duncan[a]

Sample	N	Subset for alpha = 0.05	
		1	2
negative	3	1.4900	
LR AE	3	1.6567	
positive	3	1.8333	
LR AE + NGF	3		2.2633
HE AE	3		2.5767
Sig.		.067	.078

Means for groups in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000.

(b) without inhibitor treatment

Intensity

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
negative	3	9.5067		
positive	3		13.6867	
LR AE	3		14.1967	
HE AE	3		14.4433	
LR AE + NGF	3			21.5900
Sig.		1.000	.476	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000

B.9: Phospho-SAPK/JNK (Thr183/Tyr185) activation in PC12 after 48 hr of treatment with various extracts.

	Inhibited			Not inhibited		
JNK	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
-ve	0.82	0.83	0.89	6.17	5.98	5.78
+ve	0.81	1.12	1.03	6.08	6.04	7.09
LR AE	1.06	1	1.1	4.29	4.26	5.08
LR AE+NGF	0.88	0.97	0.85	6.48	6.14	6.91
HE AE	0.98	0.72	0.92	11.05	10.51	10.59

B.9.1: Descriptives: Phospho-SAPK/JNK (Thr183/Tyr185) activation in PC12 after 48 hr of treatment with various extracts. (a) with inhibitor treatment and (b) without inhibitor treatment

with inhibitor treatment

Descriptives

Intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	.8467	.03786	.02186	.7526	.9407	.82	.89
Positive	3	.9867	.15948	.09207	.5905	1.3828	.81	1.12
LR AE	3	1.0533	.05033	.02906	.9283	1.1784	1.00	1.10
LR AE + NGF	3	.9000	.06245	.03606	.7449	1.0551	.85	.97
HE AE	3	.8733	.13614	.07860	.5351	1.2115	.72	.98
Total	15	.9320	.11712	.03024	.8671	.9969	.72	1.12

(b) without inhibitor treatment

Descriptives

Intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Negative	3	5.9767	.19502	.11260	5.4922	6.4611	5.78	6.17
Positive	3	6.4033	.59501	.34353	4.9253	7.8814	6.04	7.09
LR AE	3	4.5433	.46501	.26847	3.3882	5.6985	4.26	5.08
LR AE + NGF	3	6.5100	.38588	.22279	5.5514	7.4686	6.14	6.91
HE AE	3	10.7167	.29143	.16826	9.9927	11.4406	10.51	11.05
Total	15	6.8300	2.16675	.55945	5.6301	8.0299	4.26	11.05

Table B.9.2: One way analysis of variance (ANOVA): Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment

(a) with inhibitor treatment

ANOVA

Intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.088	4	.022	2.131	.151
Within Groups	.104	10	.010		
Total	.192	14			

(b) without inhibitor treatment

ANOVA

Intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	64.043	4	16.011	95.061	.000

Within Groups	1.684	10	.168		
Total	65.727	14			

Table B.9.3: Duncan: Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment.

(a) with inhibitor treatment

Intensity

Duncan[a]

Sample	N	Subset for alpha = 0.05	
		1	2
Negative	3	.8467	
HE AE	3	.8733	.8733
LR AE + NGF	3	.9000	.9000
Positive	3	.9867	.9867
LR AE	3		1.0533
Sig.		.147	.071

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(b) without inhibitor treatment

Intensity

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
LR AE	3	4.5433		
Negative	3		5.9767	
Positive	3		6.4033	
LR AE + NGF	3		6.5100	
HE AE	3			10.7167
Sig.		1.000	.159	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

B.10: Phospho-p38 MAPK (Thr180/Tyr182) activation of PC12 after 48 hr of treatment with various extracts.

	inhibited			Not inhibited		
P38	dup 1 (%)	dup 2 (%)	dup 3 (%)	dup 1 (%)	dup 2 (%)	dup 3 (%)
-ve	1.17	1.05	0.81	6.54	6.01	6.62
+ve	1.22	1.38	1.26	5.64	5.48	5.55
LR AE	1.18	1.25	1.28	3.47	3.65	3.42
LR AE+NGF	1.27	1.2	1.24	6.34	5.1	6.27
HE AE	1.05	0.94	1.28	11.43	10.69	12.01

B.10.1: Descriptives: Phospho-p38 MAPK (Thr180/Tyr182) activation of PC12 after 48 hr of treatment with various extracts. (a) with inhibitor treatment and (b) without inhibitor treatment

(a) with inhibitor treatment

Descriptives

Intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
negative	3	1.0100	.18330	.10583	.5547	1.4653	.81	1.17
positive	3	1.2867	.08327	.04807	1.0798	1.4935	1.22	1.38
LR AE	3	1.2367	.05132	.02963	1.1092	1.3641	1.18	1.28
LR AE + NGF	3	1.2367	.03512	.02028	1.1494	1.3239	1.20	1.27
HE AE	3	1.0900	.17349	.10017	.6590	1.5210	.94	1.28
Total	15	1.1720	.14939	.03857	1.0893	1.2547	.81	1.38

(b) without inhibitor treatment

Descriptives

Intensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
negative	3	6.3900	.33151	.19140	5.5665	7.2135	6.01	6.62
positive	3	5.5567	.08021	.04631	5.3574	5.7559	5.48	5.64
LR AE	3	3.5133	.12097	.06984	3.2128	3.8138	3.42	3.65
LR AE + NGF	3	5.9033	.69659	.40217	4.1729	7.6338	5.10	6.34
HE AE	3	11.3767	.66161	.38198	9.7331	13.0202	10.69	12.01
Total	15	6.5480	2.72493	.70357	5.0390	8.0570	3.42	12.01

Table B.10.2: One way analysis of variance (ANOVA): Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment

(a) with inhibitor treatment

ANOVA

Intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.163	4	.041	2.742	.089
Within Groups	.149	10	.015		
Total	.312	14			

(b) without inhibitor treatment

ANOVA

Intensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	101.846	4	25.461	120.792	.000
Within Groups	2.108	10	.211		
Total	103.953	14			

Table B.10.3: Duncan: Percentage of neurite bearing cells in the cell line PC12 (a) with inhibitor treatment and (b) without inhibitor treatment.

(a) with inhibitor treatment

Intensity

Duncan[a]

Sample	N	Subset for alpha = 0.05	
		1	2
Negative	3	1.0100	
HE AE	3	1.0900	1.0900
LR AE	3	1.2367	1.2367
LR AE + NGF	3	1.2367	1.2367
Positive	3		1.2867
Sig.		.060	.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(b) without inhibitor treatment

Intensity

Duncan[a]

Sample	N	Subset for alpha = 0.05		
		1	2	3
LR AE	3	3.5133		
Positive	3		5.5567	
LR AE + NGF	3		5.9033	
Negative	3		6.3900	
HE AE	3			11.3767
Sig.		1.000	.059	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.11: Standard graph of Total p38 MAPK.

Standard (pg/mL)	R1	R2	R3	blank-R1	blank-R2	blank-R3	Mean
0	0.108	0.106	0.124	0.077	0.075	0.092	0.081333
31.2	0.132	0.137	0.158	0.101	0.106	0.126	0.111
62.5	0.141	0.142	0.163	0.11	0.111	0.131	0.117333
125	0.184	0.169	0.187	0.153	0.138	0.155	0.148667
250	0.227	0.239	0.237	0.196	0.208	0.205	0.203
500	0.338	0.332	0.314	0.307	0.301	0.282	0.296667
1000	0.506	0.479	0.44	0.475	0.448	0.408	0.443667
blank	0.031	0.031	0.032				

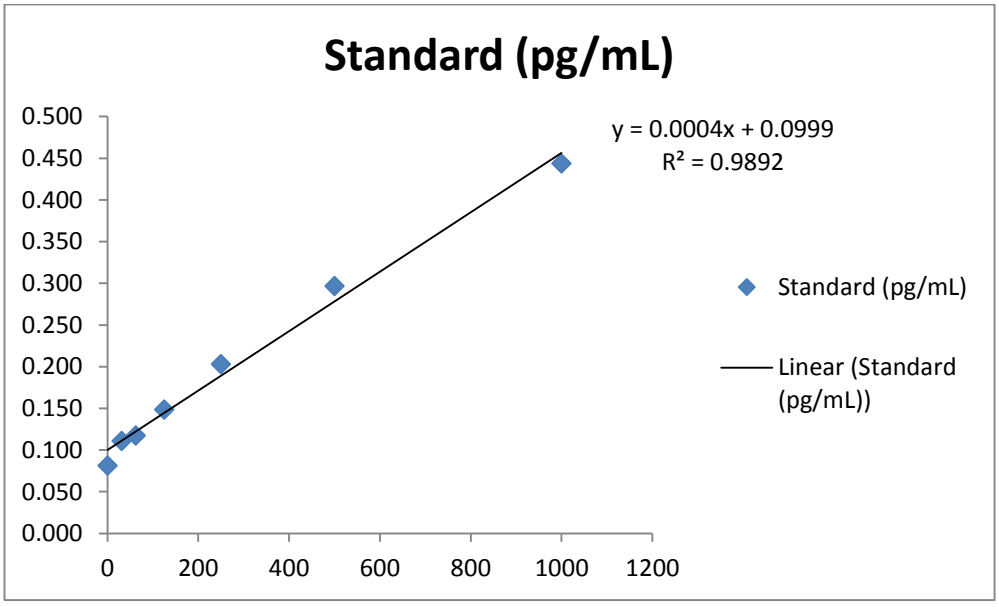

Figure B.1: Standard curve

Table B.11.1: Total p38 MAPK activation in PC12 after 48 hr of treatment with various extracts.

	R1	R2	R3	blank-R1	blank-R2	blank-R3	Mean	Total content of p38
-ve	0.176	0.186	0.172	0.145	0.155	0.14	0.147	116.917
+ve	0.211	0.23	0.222	0.18	0.199	0.19	0.190	224.417
LR AE	0.132	0.135	0.135	0.101	0.104	0.103	0.103	6.917

LR								
AE+NGF	0.186	0.171	0.177	0.155	0.14	0.145	0.147	116.917
HE AE	0.195	0.194	0.22	0.164	0.163	0.188	0.172	179.417

Table B.11.2: Descriptive: Total p38 MAPK activation in PC12 after 48 hr of treatment with various extracts.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
neg-inhibited	3	.1787	.01026	.00593	.1532	.2042	.17	.19
pos-inhibited	3	.2203	.00950	.00549	.1967	.2439	.21	.23
LR AE-inhibited	3	.1373	.00462	.00267	.1259	.1488	.13	.14
ADD-inhibited	3	.1787	.00808	.00467	.1586	.1987	.17	.19
HE AE inhibited	3	.2017	.01607	.00928	.1617	.2416	.19	.22
Total	15	.1833	.03009	.00777	.1667	.2000	.13	.23

Table B.11.3: One way analysis of variance (ANOVA): Total p38 MAPK activation in PC12 after 48 hr of treatment with various extracts.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	4	.003	26.805	.000
Within Groups	.001	10	.000		
Total	.013	14			

Table B.11.4: Duncan: Total p38 MAPK activation in PC12 after 48 hr of treatment with various extracts.

		N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	LR AE-inhibited	3	.1373		
	neg-inhibited	3		.1787	
	ADD-inhibited	3		.1787	
	HE AE inhibited	3			.2017
	pos-inhibited	3			.2203
	Sig.		1.000	1.000	.053

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.12: Total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) activation in PC12 after 48 hr of treatment with various extracts.

p42/44 MAPK	dup 1 (%)	dup 2 (%)	dup 3 (%)	Mean	sd
Neg	2.787	2.787	2.804	2.792667	0.009815
Pos	3.092	3.022	3.07	3.061333	0.035796
LR AE	2.883	2.901	2.957	2.913667	0.038592
ASS	2.899	2.882	3.024	2.935	0.077544
HE AE	3.085	3.109	3.107	3.100333	0.013317

phospho-p42/44 MAPK	dup 1 (%)	dup 2 (%)	dup 3 (%)	Mean	sd
Neg	0.413	0.399	0.39	0.400667	0.01159
Pos	0.432	0.422	0.398	0.417333	0.017474
LR AE	0.403	0.394	0.383	0.393333	0.010017

ADD	0.454	0.445	0.477	0.458667	0.016503
HE AE	0.378	0.38	0.39	0.382667	0.006429

Table B.12.1: Descriptives: Total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) activation in PC12 after 48 hr of treatment with various extracts. (a) Total p44/42 MAPK (b) phosphor-p44/42 MAPK

(a) Total p44/42 MAPK

Descriptives

Neurite

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Neg	3	2.7927	.00981	.00567	2.7683	2.8170	2.79	2.80
pos	3	3.0613	.03580	.02067	2.9724	3.1503	3.02	3.09
p42/44 LR AE	3	2.9137	.03859	.02228	2.8178	3.0095	2.88	2.96
p42/44 ADD	3	2.9350	.07754	.04477	2.7424	3.1276	2.88	3.02
p42/44 HE AE	3	3.1003	.01332	.00769	3.0673	3.1334	3.09	3.11
Total	15	2.9606	.11962	.03088	2.8944	3.0268	2.79	3.11

(b) phosphor-p44/42 MAPK

Descriptives

Neurite

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Neg	3	.4007	.01159	.00669	.3719	.4295	.39	.41
pos	3	.4173	.01747	.01009	.3739	.4607	.40	.43
phospho-p42/44 LR AE	3	.3933	.01002	.00578	.3685	.4182	.38	.40
phospho-p42/44 ADD	3	.4587	.01650	.00953	.4177	.4997	.45	.48
phospho-p42/44 HE AE	3	.3827	.00643	.00371	.3667	.3986	.38	.39
Total	15	.4105	.02965	.00765	.3941	.4270	.38	.48

Table B.12.2: One way analysis of variance (ANOVA): Total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) activation in PC12 after 48 hr of treatment with various extracts. (a) Total p44/42 MAPK (b) phosphor-p44/42 MAPK

(a) Total p44/42 MAPK

ANOVA

Neurite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.182	4	.046	25.145	.000
Within Groups	.018	10	.002		
Total	.200	14			

(b) phosphor-p44/42 MAPK

ANOVA

Neurite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.011	4	.003	15.519	.000
Within Groups	.002	10	.000		
Total	.012	14			

Table B.12.3: Duncan: Total p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Thr202/Tyr204) activation in PC12 after 48 hr of treatment with various extracts. (a) Total p44/42 MAPK (b) phosphor-p44/42 MAPK

(a) Total p44/42 MAPK

Neurite

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Neg	3	2.7927		
p42/44 LR AE	3		2.9137	
p42/44 ADD	3		2.9350	
pos	3			3.0613
p42/44 HE AE	3			3.1003
Sig.		1.000	.553	.288

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

(b) phosphor-p44/42 MAPK

Neurite

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
phospho-p42/44 HE AE	3	.3827		
phospho-p42/44 LR AE	3	.3933	.3933	
Neg	3	.4007	.4007	
pos	3		.4173	
phospho-p42/44 ADD	3			.4587
Sig.		.138	.057	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.