NEURITE OUTGROWTH STIMULATORY ACTIVITY OF AN EDIBLE MUSHROOM *PLEUROTUS GIGANTEUS* IN DIFFERENTIATING NEUROBLASTOMA-2A CELLS

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ABSTRACT

Neurite outgrowth is an important process for the establishment of synaptic connections during development, as well as neuronal regeneration in neuropathological conditions or injury. With growing concerns over neurodegenerative diseases attributed to impairment of neurite outgrowth e.g. dementia and Alzheimer's disease, identification of alternative therapeutics has become paramount. One way to prevent and/or delay the onset of such diseases is by discovering alternative therapeutic molecules from functional foods. One such candidate is the edible mushroom (higher Basidiomycetes). In this study, eight culinary-medicinal mushrooms were evaluated for neurite outgrowth stimulatory effects by using neuroblastoma-2a (N2a) cells as an *in vitro* model. The mushroom extracts were also subjected to *in vitro* neuro- and embryotoxicity tests using N2a and 3T3 fibroblasts cell lines. The preliminary results showed that the aqueous extract of *Pleurotus giganteus* significantly ($p < 0.05$) promoted neurite outgrowth in N2a cells by 33.4 ± 4.6%. The IC_{50} values obtained from tetrazolium (MTT), neutral red uptake (NRU) and lactate dehydrogenase (LDH) release assays showed no toxic effects following 24 hours exposure of N2a and 3T3 cells to the mushroom extract. The basidiocarps of *P. giganteus* were then analysed for various nutritional attributes. The mushroom composed of protein (15.4–19.2 g/100 g), polysaccharides, phenolics, and flavonoids as well as vitamins B1, B2, and B3. The antioxidant properties of the aqueous and ethanol extracts of *P. giganteus* were investigated. The results indicated that the aqueous extract of *P. giganteus* exhibited scavenging of 2,2-diphenyl-1-picrylhyd-razyl (DPPH) radical with an IC_{50} value of 21.46 ± 6.95 mg/mL. Based on the ferric reducing antioxidant power (FRAP) assay, the reducing power of the mushroom extracts was in the range of 1.17–3.88 µM $FeSO_4 \cdot 7H_2O$/g mushroom and the ethanol extract showed lipid peroxidation inhibitory activity of 49.58–49.80%. The efficacy of the chemical constituents of *P. giganteus* (linoleic acid, oleic acid, cinnamic acid,

caffeic acid, *p*-coumaric acid, succinic acid, benzoic acid, and uridine) for neurite outgrowth activity was investigated. Uridine (100 μM) increased the number of neurite bearing cells by 43.1 ± 0.5%, which was about 1.8-fold higher than NGF (50 ng/mL)-treated cells. In this study, we demonstrated that uridine of *P. giganteus* (1.80 ± 0.03 g/100g mushroom extract) increased the phosphorylation of extracellular-signal regulated kinases (ERKs) and protein kinase B (Akt); simultaneously promoting neurite outgrowth in N2a cells. Neurite outgrowth stimulatory activity was inhibited by the inactivation of mitogen-activated protein kinase (MEK)/ERKs and Akt signaling with specific inhibitors. Further, phosphorylation of the mammalian target of rapamycin (mTOR) was also increased. MEK/ERK and PI3K-Akt-mTOR further induced phosphorylation of cAMP-response element binding protein (CREB) and expression of growth associated protein 43 (GAP43), tubulin alpha 4a (TUBA4A), and tubulin beta 1 (TUBb1); all of which promoted neurite outgrowth of N2a cells. In conclusion, these findings demonstrated that *P. giganteus* may enhance neurite outgrowth and one of the key bioactive molecule responsible for neurite outgrowth is uridine.

ABSTRAK

Pengunjuran neurit merupakan satu proses yang penting untuk penubuhan sambungan sinaptik semasa perkembangan, dan regenerasi sel saraf (neuron) selepas keadaan neuropatologi ataupun kecederaan. Memandangkan penyakit neurodegenerasi yang berkaitan dengan kemerosotan pengunjuran neurit (demensia dan penyakit Alzheimer's) semakin ketara, pengenalpastian cara perubatan alternatif menjadi lebih penting. Satu cara untuk menghindari dan/atau melewatkan permulaan penyakit tersebut ialah melalui penemuan molekul terapeutik alternatif daripada makan fungsian. Satu calon makan fungsian ialah cendawan (Basidiomysit tinggi). Dalam kajian ini, sekoleksi cendawan masakan-ubatan telah dinilai dari segi aktiviti perangsangan pengunjuran neurit atas sel neuroblastoma-2a (N2a). Ekstrak cendawan juga dinilai secara *in vitro* dari segi ketoksikan saraf dan embrio. Hasil awalan menunjukkan bahawa ekstrak akueus *Pleurotus giganteus* meningkatkan pengunjuran neurit sel N2a secara signifikan ($p < 0.05$) sebanyak 33.4 ± 4.6%. Nilai perencatan pada 50% kepekatan (IC_{50}) yang didapati daripada asai tetrazolium (MTT), pengambilan neutral red (NRU) dan pembebasan laktat dehidrogenase (LDH) menunjukkan ketidakhadiran kesan toksik selepas pendedahan sel N2a dan 3T3 fibroblas kepada ekstrak cendawan selama 24 jam. Komposisi nutrisi basidiokarp (tubuh buah) *P. giganteus* juga dianalisa. Cendawan ini didapati tinggi dari segi kandungan proteinnya (154–192 g/kg), begitu juga polisakarida, fenolik, flavonoid, vitamin B1, B2, dan B3. Sifat antioksidaan ekstrak akueus dan etanol *P. giganteus* turut dikaji. Ekstrak akueus *P. giganteus* menunjukkan pemulungan radikel 2,2-diphenyl-1-picrylhyd-razyl (DPPH) pada kepekatan IC_{50} 21.46 ± 6.95 mg/mL. Kuasa antioksidaan penurunan ferik ekstrak cendawan adalah dalam lingkungan 1.17–3.88 µM $FeSO_4 \cdot 7H_2O$/g cendawan dan ekstrak etanol menunjukkan aktiviti perencatan pengoksidaan lipid sebanyak 49.58–49.80%. Keberkesanan juzuk kimia daripada *P. giganteus* (asid linoleik, asid oleik, asid cinnamic, asid kafeik, asid p-coumaric, asid

succinic, asid benzoik dan uridin) dalam pengunjuran neurit turut dikaji. Uridin (100 μM) meningkatkan peratusan sel yang mengandungi neurit sebanyak 43.1 ± 0.5%, dan ini adalah kira-kira 1.8 kali ganda daripada sel yang dirawat dengan NGF (50 ng/mL). Dalam kajian ini, uridin daripada *P. giganteus* (1.796 ± 0.032 g/100g ekstrak cendawan) meningkatkan pemfosforilan kinase yang dikawalselia oleh isyarat luaran sel (ERKs) dan protein kinase B (Akt); dan pada masa yang sama ia menggalakkan pengunjuran neurit pada N2a. Penyahaktifan protein kinase yang diaktifkan oleh mitogen (MEK)/ERKs dan Akt dengan perencat khusus merencatkan aktiviti stimulasi pengunjuran neurit. Tambahan pula, pemfosforilan sasaran mamalia rapamycin (mTOR) juga meningkat. MEK/ERK dan PI3K-Akt-mTOR mendorong pemfosforilan protein pengikat elemen tindak balas cAMP dan membawa kepada pengungkapan protein yang berkaitan dengan pertumbuhan 43 (GAP 43), tubulin alfa 4a (TUBA4A), dan tubulin beta 1(TUBb1); semua ini menggalakkan pengunjuran neurite sel N2a. Secara keseluruhannya, kajian ini mencadangkan bahawa *P. giganteus* mungkin dapat meningkatkan kesihatan otak dan persembahan kognitif; dan salah satu molekul bioaktif yang memainkan peranan yang penting dalam aktivi pengunjuran neurit ialah uridin.

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ABBREVIATIONS AND SYMBOLS

%	Percentage
×	Times
µg	Microgram
µL	micro litre
µm	Micrometre
µM	micro molar
Abs	Absorbance
AD	Alzheimer's disease
Akt	protein kinase b
$AlCl_3$	aluminium chloride
ATCC	American type culture collection
AU	absorbance unit
BHT	butylated hydroxytoluene
C	Carbon
CO_2	carbon dioxide
CREB	CAMP-response element binding protein
DAPI	4'-6-diamidino-2- phenylindole
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal regulated kinases
FBS	foetal bovine serum
$FeSO_4$	ferrous sulphate

FITC	fluorescein isothiocyanate
FRAP	ferric reducing antioxidant power
g	Gram
GAE	gallic acid equivalent
GAP43	growth associated protein 43
H_2SO_4	sulphuric acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IC_{50}	half maximal inhibitory concentration
IgG	immunoglobulin G
kD	kilo Dalton
kg	Kilogram
L	Litre
LDH	lactate dehydrogenase
m	Metre
MEK	mitogen-activated protein kinase
MEM	minimum essential medium
mg	Milligram
mL	Millilitre
mm	millimetre
mM	mill molar
mTOR	mammalian target of rapamycin
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
N2a	neuroblastoma-2a
Na_2CO_3	sodium carbonate
ng	nano gram

NGF	nerve growth factor
nm	Nanometre
No.	Number
NR	neutral red
^{o}C	degree Celsius
OD	optical density
PBS	phosphate buffered saline
pg	pico gram
PPADS	pyridoxal phosphate-6-azophenyl-2' 4'-disulfonic acid
RE	rutin equivalent
ROS	reactive oxygen species
SD	standard deviation
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TMB	3,3′,5,5′-tetramethylbenzidine
TPC	total phenolic content
TUBA4A	tubulin alpha 4a
TUBb1	tubulin beta 1
UV	ultraviolet
w/w	weight per weight

CHAPTER I

1.1 INTRODUCTION

Many non-communicable diseases (NCDs) are neglected despite causing a considerable health burden. One of the NCDs identified is neurodegenerative diseases, such as Alzheimer's disease (AD) and dementia (Lopez et al., 2014). The economic cost of neurodegenerative disease is enormous, and is expected to grow rapidly as more people live longer. Worldwide, it was estimated that the global medical cost and cost of care for dementia, of which AD is the major ailment, was USD 604 billion in 2013. The amount is about 1% of the world gross domestic product (Housden et al., 2014). The pathological hallmarks of neurological diseases are characterised by impairment of neurite outgrowth due to amyloidogenic processing and subsequent β-amyloid cascade, neuroinflammation, and free radical generation.

Current drug therapy for neurodegenerative diseases is ineffective with many side effects and it only provides a short term delay in the progression of the disease. Further, the drug development pipeline is drying up and the number of innovative drugs reaching the market has lagged behind the growing need for such drugs. It is therefore of utmost importance to find appropriate solutions to prevent, or perhaps impede, the development of neurodegenerative diseases associated with impaired neuritogenesis.

An alternative approach to prevent or treat such diseases is by complementary health approaches, such as dietary supplementations and functional foods. Functional food is food that has a potentially positive effect on health beyond basic nutrition. Examples of functional food are oatmeal, for its high soluble fibre that can help lower cholesterol levels; and orange juice fortified with calcium for bone health. In general,

functional food is considered to offer additional benefits that may reduce the risk of disease or promote optimal health. Turmeric, green tea, and gingko are the examples of functional food that demonstrate therapeutic effects on brain by exerting neuroprotective and antioxidant effects.

Can mushrooms be functional food for brain? Mushrooms have long been celebrated as a source of powerful nutrients. It is not new that the polysaccharides found in mushrooms are exotic and a broad array of them has been shown to be effective immuno-modulating agents (Wasser, 2002). Several compounds isolated from mushrooms have been shown to promote neurite outgrowth, for example hericenones and erinacines from the lion's mane mushroom, *Hericium erinaceus* (Bull.: Fr.) Pers (Kawagishi et al., 1991; Phan, Lee, et al., 2014). Other mushrooms found to have neurite outgrowth stimulatory activities are *Ganoderma lucidum* (Fr) P. Karst, *Lignosus rhinocerotis* (Cooke) Ryvarden, *Ganoderma neo-japonicum* (Fr) P. Karst, and *Cordyceps militaris* (L.:Fr.) Link (Phan, David, Naidu, Wong, & Sabaratnam, 2014).

The ability of natural product to potentiate neurite outgrowth *in vitro* is considered as the first line screening criteria in order to be regarded as a preventive agent for neurodegenerative diseases. Screening of novel natural products that can induce neurite outgrowth has routinely been performed in a neuronal cell culture-based system. To discover potential neurite outgrowth agents, various neuronal cell models originating from mouse, rat or even human were utilised. The goal of this project was to identify new potential edible and medicinal mushroom(s) that induce neurite outgrowth. Taking the advantage of the tractability and simplicity of the brain neuroblastoma cell (N2a) line as a neuronal cell model, it was used to screen mushroom extracts and their molecules for potential neurite outgrowth activity.

1.2 RESEARCH OBJECTIVES

The mushroom extracts selected for the screening were *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde, *Ganoderma lucidum, Hericium erinaceus, Cordyceps militaris, Lignosus rhinocerotis, Pleurotus pulmonarius* (Fr.) Quél, *Ganoderma neo-japonicum,* and *Grifola frondosa* (Dicks.: Fr.) S.F. Gray. The screen was performed on the premise that the mushrooms were able to enhance neurite outgrowth activity of differentiating N2a cells. The activity was deemed effective if the neurite outgrowth stimulatory effect of the extracts was significantly higher than that induced by nerve growth factor (NGF) which was the positive control. The objectives of this project were to:

a) screen and identify the potential edible and medicinal mushrooms for their neurite outgrowth activity and to perform *in vitro* neuro- and embryo-toxicity of the mushroom extracts
b) investigate the nutritional composition such as the total polysaccharides, phenolics, and flavonoids of the selected mushroom and its antioxidant activity
c) evaluate the neurite outgrowth stimulatory activity of the extracts of the selected mushroom and its chemical constituents
d) elucidate the signaling pathways of neuritogenesis induced by the selected mushroom extract and its chemical constituents; and to investigate the expression of selected neuronal biomarker proteins

CHAPTER II

LITERATURE REVIEW

2.1 NEURITE OUTGROWTH

The structural and functional unit which is the core element of the nervous system is the neuron or nerve cell. Neurons, unlike any other cells, do not undergo cell division (mitosis) and when they die, they are not replaced by new ones. A large number of neurons are generated in early development and the excess numbers are cut down in a process called selection pruning, leaving only a sufficient number to last a life time. Unlike other body cells, neurons in the central nervous system (CNS) are only able to undergo mitosis to generate new cells during development and no new neurons are formed at post-development stage (Bhardwaj et al., 2006).

Neurite outgrowth is a process that occurs following the differentiation of precursor cells to a terminal neuronal phenotype (Radio & Mundy, 2008). During the early stage, the cells develop broad, sheet-like extensions (lamellipodia) which subsequently condense into short processes tipped with growth cones (Craig & Banker, 1994). The processes increase in length and complexity as the cells mature, and they become polarized by developing a single long axon and several shorter dendrites as shown in Figure 2.1. The axon grows rapidly in length and acquires axonal characteristics. The remaining neurites elongate more slowly and develop into dendrites several days after the formation of the axon (Figure 2.1).

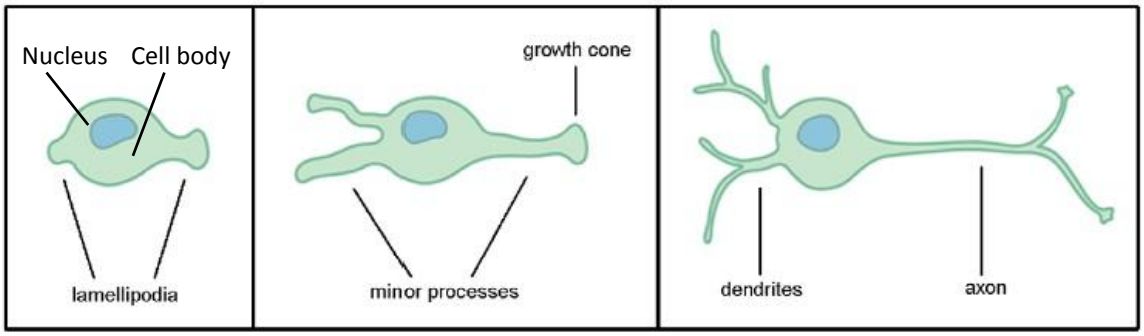


Figure 2.1: Diagram of a sequential event in neurite outgrowth illustrating a cell body with lamellipodia, the development of minor processes (tipped with a growth cone), and transformation of the processes into an axon and dendrites (Radio & Mundy, 2008).

Neurite outgrowth is essential for neuronal path finding and the establishment of synaptic connections during development (Figure 2.2A). It is also an important aspect of neuronal plasticity and neuronal regeneration that occurs after injury or in neurodegenerative conditions (Figure 2.2B). As neurons mature and differentiae, they lose some of their ability to produce neurite, which results in the incapability of neurite regeneration. Thus, finding preventative measures that promote neurite outgrowth and gaining a thorough understanding of the underlying mechanisms regulating neurite outgrowth may facilitate in a health developmental process and for therapeutic management of axonal/neuronal damage.

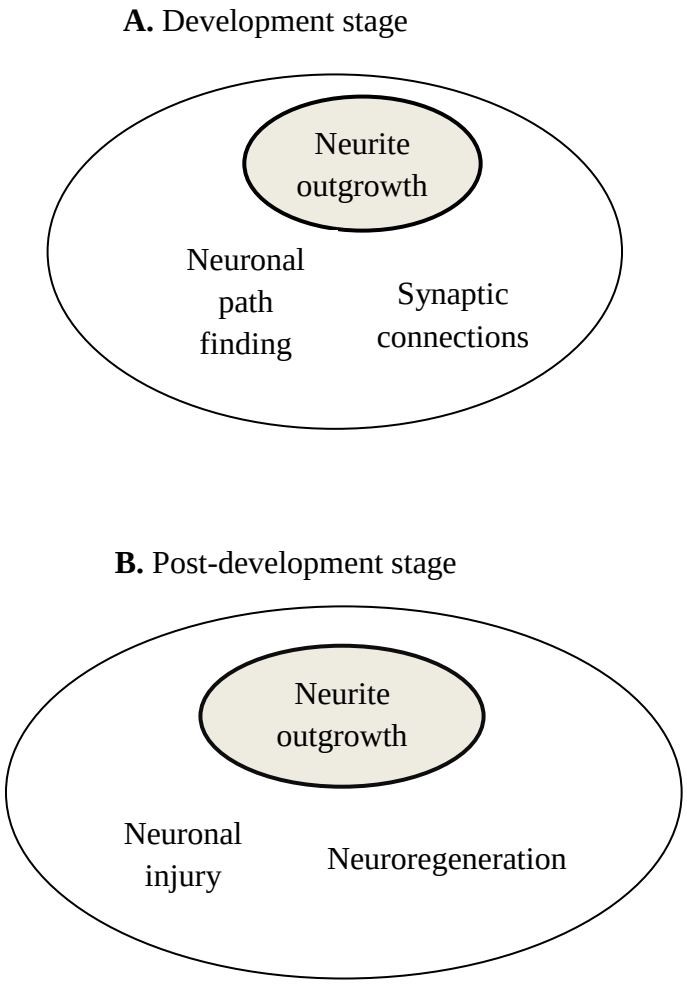


Figure 2.2: The importance of neurite outgrowth in (A) Development stage for neuronal path finding and synaptic connections; and (B) Neuronal repair after injury and regeneration after neuropathological conditions

2.2 NEURITE OUTGROWTH IN DEVELOPMENT

Neuron formation and neurite outgrowth take place during development. By studying neurodevelopment, we can learn how the human brain develops and how brain abnormalities, such as mental retardation and other brain disorders, can be prevented or treated. In the course of development, the human brain and nervous system begin to develop at three weeks of gestation (Zhang, Wernig, Duncan, Brüstle, & Thomson, 2001). By four weeks, the major regions of the human brain can be recognised in

primitive form, including the forebrain, midbrain, hindbrain, and optic vesicle from which eyes develop. Irregular ridges, or convolutions, can be seen clearly by six months of gestation.

The mature nervous system contains a vast array of cell types, which can be divided into two main categories: (1) the neurons, primarily responsible for signaling, and (2) glial cells, the supporting cells in the nervous system (Grosche & Reichenbach, 2013). Neurons of the cerebral cortex are generated in the ventricular zone of the neural tube. Once the neurons have left the cell cycle, the neurons migrate out of the ventricular zone on glial to form the cortical plate (Mahmoudzadeh et al., 2013), which is the gray matter of the cortex. On the cortical plate, neurons become organised into well defined layers. An important step called the "initial wiring" takes place after that. Brain wiring involves the outgrowth of neurons or axons in long distances to find and connect with appropriate partners (Chedotal & Richards, 2010).

Therefore, neurite outgrowth during development is a series of precisely orchestrated events and having said that, stimulation is essential for fine tuning of brain connections. Consumption of certain nutrients can influence brain functions, even when the nutrients are not being used to correct malnutrition syndromes (Wachs, Georgieff, Cusick, & McEwen, 2014). Supplemental docosahexaenoic acid (DHA), an omega-3 fatty acid, is reported to improve cognition in humans (Wurtman, 2014). It also enhances the levels of membrane phosphatides and of specific proteins in synaptic membrane and the density of hippocampal dendritic spines; thus it may enhance synaptogenesis (Wurtman, Cansev, Sakamoto, & Ulus, 2009).

2.3 NEURITE OUTGROWTH IN NEURODEGENERATIVE DISEASES AND NEUROREGENERATION

Life expectancy of humankind had increased to 50-60 years at the beginning of 20th century due to improved medicinal, dietary and sanitation conditions. It is however, foreseen that society will witness an elevated life expectancy of 80-90 years by 21st century (Candore et al., 2006; Troen, 2003). With the increased lifespan of the world's population, it is estimated that approximately 80 million people will suffer from dementia by 2040, whereby AD will account for almost 60% of dementia cases (Bharadwaj, Martins, & Macreadie, 2010).

Impairment in neurite outgrowth will lead to neurodegenerative diseases including dementia, AD and Parkinson's disease (PD) (Martorana et al., 2012). As discussed, the principal morphological characteristics of neuritogenesis are branching of neurites followed by elongation of axons and dendritic arborisation (Kiryushko, Berezin, & Bock, 2004; More et al., 2012). It is believed that pathogenesis of the nervous system may lead to neurite retraction, and AD has been described as a disease of synaptic failure due to brain tissue damage and lack of neurite outgrowth (Wasilewska-Sampaio et al., 2005). Therefore, it has been suggested that reconstruction of the neuronal and synaptic networks in the brains of those suffering from AD, is necessary for the recovery of brain functions (Figure 2.3).

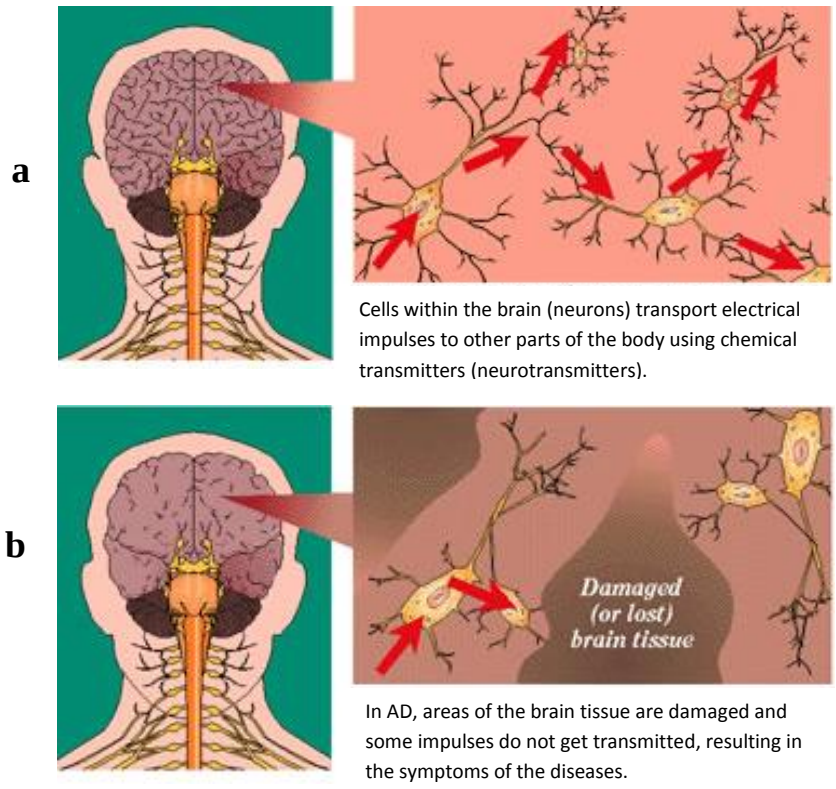


Figure 2.3: The diagram shows the neurons in the non AD condition brain transporting electrical messages to other parts of the body using chemical transmitters (a); whereas, in AD brain (b), the brain tissues are damaged and some messages do not transmit due to lack of neurite outgrowth activity.

The term 'neuroregeneration' describes the sprouting and outgrowth of injured or damaged axons over longer distances and the process is time-consuming, usually taking weeks to months to produce functional improvements (Krieger, 2013). It was once believed that nerve regeneration in the mammalian central nervous system (CNS) was not possible (Filli & Schwab, 2012). However, it has become apparent recently that damaged neurons do regenerate under the presence of stimulatory substances such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Allen, Watson, Shoemark, Barua, & Patel, 2013), lithium (Leeds et al., 2014), and thyroid hormones (Bhumika & Darras, 2014). Peripheral nerve regeneration on the other hand is, believed to be reversible with many neurotrophic factors shown to promote neurite

outgrowth by improving the microenvironment required for nerve regeneration (Allodi, Udina, & Navarro, 2012). Promoters of neurite regrowth are being identified and provide possible therapies to stimulate regeneration. These neuritogenic substances hold the promise of therapeutic efficacy in the treatment of neuronal injuries by the virtue of their ability to stimulate outgrowth of neurites from neuronal cells.

2.4 MEASUREMENTS OF NEURITE OUTGROWTH

The characterisation of neurite outgrowth is an area of intense interest, since this cellular process is essential for interconnection of neuronal cell bodies in neuropathological disorders, neuronal injury, and regeneration (Payne et al., 2014). Major efforts in the nervous system drug discovery research are focused on the identification of compounds that affect neurite outgrowth and/or retraction. However, the study of neurites is held back by difficulties associated with isolating and purifying them (Helmstaedter, Briggman, & Denk, 2011). One of the common practices for measuring neurite outgrowth is by manual microscopic examination of individual cells (Laketa, Simpson, Bechtel, Wiemann, & Pepperkok, 2007). Another way of assessment is by the measurement of total fluorescence from a labelled neuronal cell population using a fluorescence plate reader (Popova & Jacobsson, 2014). The disadvantage of these methods includes the labour intensiveness and subjectivity. Consequently, innovative methods have been actively developed by researchers to compensate the lack of informality and reproducible methodology for neurite quantification.

2.4.1 Semi quantitative method

Assessment of neurite outgrowth in a semi-quantitative fashion does not involve a calibrated measurement of neurite length. Rather, scoring is based on the presence of processes emitting from a cell (Radio & Mundy, 2008). These methods are often preferred as they do not require sophisticated equipment and software for analysis. Observations can be made directly from the microscope or from photomicrographs, thus making these methods rather time saving and simple to perform. However, semi-quantitative methods can be bias and subjective. In general, the endpoint semi-quantitative assessment is the number of cells exhibiting neurites (neurite bearing cells), and the length of a process that qualifies as a neurite is defined as being equal to or greater than one to two times the diameter of the cell body (Das, Freudenrich, & Mundy, 2004).

2.4.2 Quantitative method

Quantitative assessment of neurite outgrowth provides a calibrated measure of neurite length, including the length of the longest neurite, total neurite length, or the average neurite length (Radio & Mundy, 2008). Scoring by the length of the longest neurite is considered the most common practice as it is relatively easy to demarcate from photomicrographs. In some instances, neurite outgrowth can be scored and ranked by counting the number of neurites as well as the number of branch points per neurite. Figure 2.4 summarises the morphologic features used to quantify neurite outgrowth. For quantitative analysis of neurite outgrowth, images are often associated with a scale to be calibrated to the length of the original neurites, in microns, for example. Further, cultures are generally grown under a low cell density to avoid overlap of processes. Measurements can be performed on cultures that have been fixed with 1%

glutaraldehyde in 0.1 M phosphate buffer (Bearer, Swick, O'Riordan, & Cheng, 1999) or 90% (v/v) methanol in phosphate buffered saline (PBS) (Sachana, Flaskos, Alexaki, Glynn, & Hargreaves, 2001). Staining can be done with Coomassie brilliant blue before being examined (De Girolamo, Hargreaves, & Billett, 2001). Nevertheless, analysis of live cultures using phase contrast can be performed without manipulating the cells, thereby decreasing time and costs associated with fixation (Radio & Mundy, 2008). As compared to semi-quantitative assessment, these methods are time-consuming and are always performed with microscopic imaging system (semi- or fully-automated), to facilitate data acquisition.

Assessment by describing the longest neurite, which is sometimes designated as the axon, has been done in PC12 cell line (Das et al., 2004). For primary cultures, neurite length of the rat cerebellar granule cells was measured as the distance between the centre of the cell soma and the tip of its longest neurite (Bearer et al., 1999). A few requirements had to be met: (1) the neurite must emerge from an isolated cell and not a clump of cells, and (2) the neurite must not be in contact with other neurites. Measurement of the longest neurite has also been carried out in the embryonic rat locus coeruleus, a collection of neurons in the pons (Dey, Mactutus, Booze, & Snow, 2006). Meanwhile, measurement of the total neurite length (sum of the lengths of all neurites emanating from a cell) requires more effort and is often carried out using automated analyses. Total neurite length has been assessed in PC12 cell line (Das & Barone, 1999). In another study which employed a primary culture of immature γ-aminobutyric acidergic (GABAergic) interneurons, the total neurite length was measured by drawing all visible processes with a Scion software (Vutskits, Gascon, Tassonyi, & Kiss, 2006).

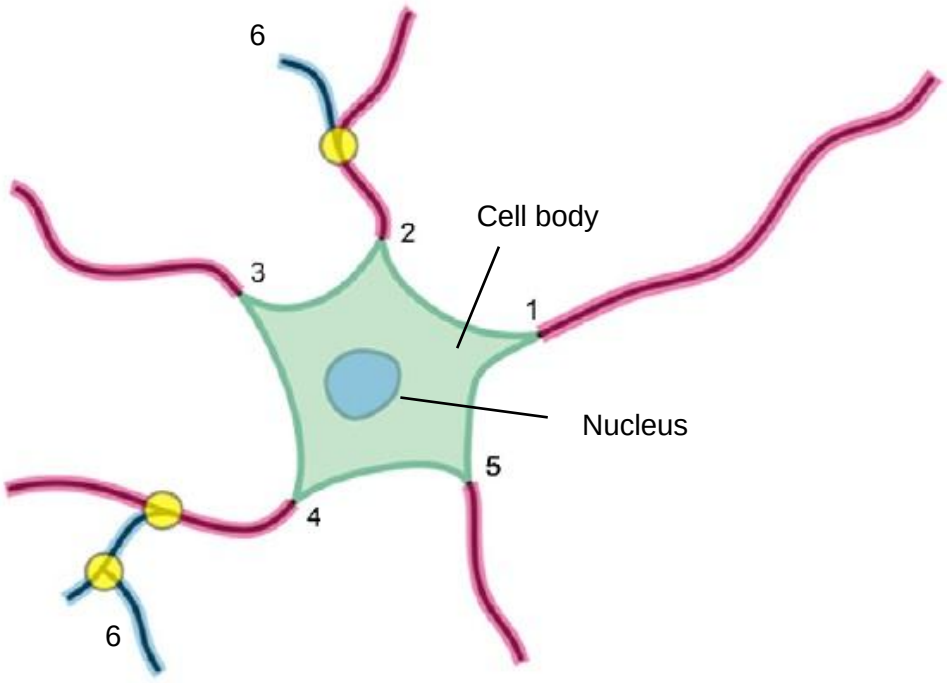


Figure 2.4: Quantitative assessment of neurite outgrowth (Radio & Mundy, 2008). Primary neurites are processes originating directly from the cell body (labelled as 1-5). The longest neurite is labelled as 1. Secondary neurites (labelled as 6) originate from primary neurites. Branch points (circled) indicate branching of neurites. The total neurite length is the sum of the lengths of all primary and secondary neurites (total neurite length = 1+2+3+4+5+6).

2.4.3 Biochemical marker

A variety of biochemical markers have been used to quantify neurite outgrowth. One of the advantages of using biochemical markers is that they correlate to neuronal differentiation and the increase of neurite extension. Biochemical measures, such as immunoblotting and ELISA, provide a higher throughput screening, as compared to morphological analysis.

Phosphorylated microtubule-associated protein, tau was found in axons and dendrites of cortical neurons at all developmental stages and it is present at a higher level during neurite outgrowth (Brion, Octave, & Couck, 1994). Further, phosphorylated tau disappears during the period of neurite stabilisation and synaptogenesis. These suggest that tau protein can be used as a biomarker to study neurite outgrowth. GAP-43 is a growth-associated protein with a well-known role in growth cone formation, axonal elongation, and plasticity (Benowitz & Routtenberg, 1997). A rise in GAP-43 protein and mRNA levels expressed by developing cerebellar granule neurons *in vitro* during neuritogenesis further provided evidence of the role of GAP-43 in neurite outgrowth (Przyborski & Cambray-Deakin, 1994).

Proteins associated with neurons like the cytoskeleton (neurofilaments) have been used for the visualization of axons and dendrites. Neurofilaments belong to the class of intermediate filaments and are one of the most abundant structural proteins in axons, playing a role in axonal calibre regulation, neuronal differentiation and axonal outgrowth and regeneration (Perrot, Berges, Bocquet, & Eyer, 2008). Nitric oxide (NOR4)-induced neurite outgrowth was shown to be accompanied by an increase of the expression of neurofilament 200 kDa subunit (NF200) protein, an axonal marker (Yamazaki, Chiba, & Mohri, 2005). Neurofilament protein levels were also shown to increase upon somatostatin-induced neurite outgrowth in a primary culture, i.e. rat cerebellar granule cells (Taniwaki & Schwartz, 1995). Besides, a correlation between diazinon- and cypermethrin-induced reduction in neurite outgrowth and the decrease in the level of neurofilament protein has also been demonstrated in N2a cells (Flaskos et al., 2007).

2.5 IN VITRO CELL LINE MODEL FOR NEURITE OUTGROWTH

Cell culture is a general term applied to define the removal of cells from a tissue and their subsequent growth in a favourable artificial environment (Sharma, Haber, & Settleman, 2010). Primary culture is a culture derived directly from a tissue; therefore, it best resembles the natural tissue. However, a primary culture often has a limited growth potential and life span (Kretz, Marticke, Happold, Schmeer, & Isenmann, 2007). A cell line, on the other hand, is a population of immortal cells that are used for biological research, as they do not require to be isolated from the host's tissue every time cells are needed (Poulos, Dodson, & Hausman, 2010). Cell lines have a number of advantages that make them useful as *in vitro* models (Radio & Mundy, 2008). First, cell lines provide a clonal and homogenous population of cells. Secondly, they are relatively easy to acquire as compared to the primary culture, and can be stored indefinitely in liquid nitrogen (Maqsood, Matin, Bahrami, & Ghasroldasht, 2013). The protocol to maintain cell line is well established and is easy to follow using standard tissue culture plastic and media. Most importantly, the cells represent an unlimited self-replicating source that can be continuously subcultured (passaged) to provide large numbers of cells in a short period of time (Burdall, Hanby, Lansdown, & Speirs, 2003).A vast variety of neuronal cell lines are available and many have been used as *in vitro* models to examine neurite outgrowth. Table 2.1 lists the most commonly used cell lines for neurite outgrowth study and describe the associated phenotype and agents used to induce neurite outgrowth.

Table 2.1 Different cell lines used for the measurement of neurite outgrowth *in vitro*

Cell line	Source	Phenotype	Inducing agents	References
N2a	Mouse neuroblastoma	Adrenegic, cholinergic, dopaminergic	Dibutyryl cyclic AMP, serum removal, retinoic acid	Chiang, Cheng, Chen, Liang, & Yen (2014)
PC12	Rat pheochromocytoma	Adrenegic, cholinergic, dopaminergic	Nerve growth factor	Eik et al., (2012)
B50	Rat neuroblastoma	Cholinergic	Dibutyryl cyclic AMP, Serum removal	Ibegbu, McBean, Fyfe, & Mullaney (2013)
NB2a	Mouse neuroblastoma	Adrenegic, cholinergic, dopaminergic	Dibutyryl cyclic AMP, retinoic acid	Vural & Tuğlu (2011)
N1E-115	Mouse neuroblastoma	Adrenegic, dopaminergic	Dimethyl sulfoxide, prostaglandin E1 (PGE1), serum removal	Kotake et al., (2014)
SH-SY5Y	Human neuroblastoma	Adrenegic, cholinergic, dopaminergic	Retinoic acid, dibutyryl cyclic AMP, nerve growth factor	Wu et al., (2009)
SK-N-SH	Human neuroblastoma	Dopaminergic	Retinoic acid, nerve growth factor	Olajide, Velagapudi, Okorji, Sarker, & Fiebich (2014)
IMR-32	Human neuroblastoma	Aminergic, cholinergic	5-Bromo-deoxyuridine (BrdUr), nerve growth factor	Tong et al., (2013)
LA-N-5	Human neuroblastoma	Cholinergic	Retinoic acid	Hill & Robertson (1998)
NT2	Human embryonal carcinoma	Cholinergic	Retinoic acid	Tegenge, Roloff, & Bicker (2011)

Different experimental protocols have been employed to investigate the effects of chemical on neurite outgrowth in cell lines. There are two common practices: (1) cells are treated with the chemical of interest at the same time differentiation is being induced; and (2) cells are pre-treated with the inducing factor prior to exposure to the chemical of interest. The first method exposes the cells with the inducing factor and chemical simultaneously. This allows the examination of both initiation of neuronal differentiation and later events such as neurite outgrowth and extension. A classic example is the induction of NGF-differentiated PC12 cells, whereby the cells are co-treated with NGF (the inducing agent) and substances of interest, after which neurite outgrowth is observed after a designated time (Eik et al., 2012). As compared to the first method, the second method focuses on neurite initiation, rather than neurite differentiation. By adapting this protocol, the chemical effects can be examined in cells which have already bore neurites. An example of this procedure is the N2a cell model. The cells are cultured in a complete medium, after which they are re-plated in a serum-free condition with the option of adding the inducing factors (dibutyryl cyclic AMP or retinoic acid) (Wang et al., 2011). The cells which have already been differentiated to the neuronal phenotype, will elaborate neurites in an accelerated manner when treated with neuritogenic substances.

2.6 NEURITOGENIC SUBSTANCES THAT STIMULATE NEURITE OUTGROWTH

2.6.1 Neurotrophic factors

Neurotrophic factors (neurotrophins) such as nerve growth factor (NGF) play an important role in the maintenance of nervous system. They play an integral part in the regulation of development, assembling of neuron-target cell interaction, function and

survival of neurons. Insufficient neurotrophins is believed to result in an array of dysfunctions of the nervous system, which may cause dementia, AD and PD (Allen et al., 2013). However, polypeptides like NGF in therapy are unfavourable as they are unable to cross the blood brain barrier. Therefore, finding small molecules that show neurotrophic properties and/or enhancing the action of endogenous neurotrophic factors, is important.

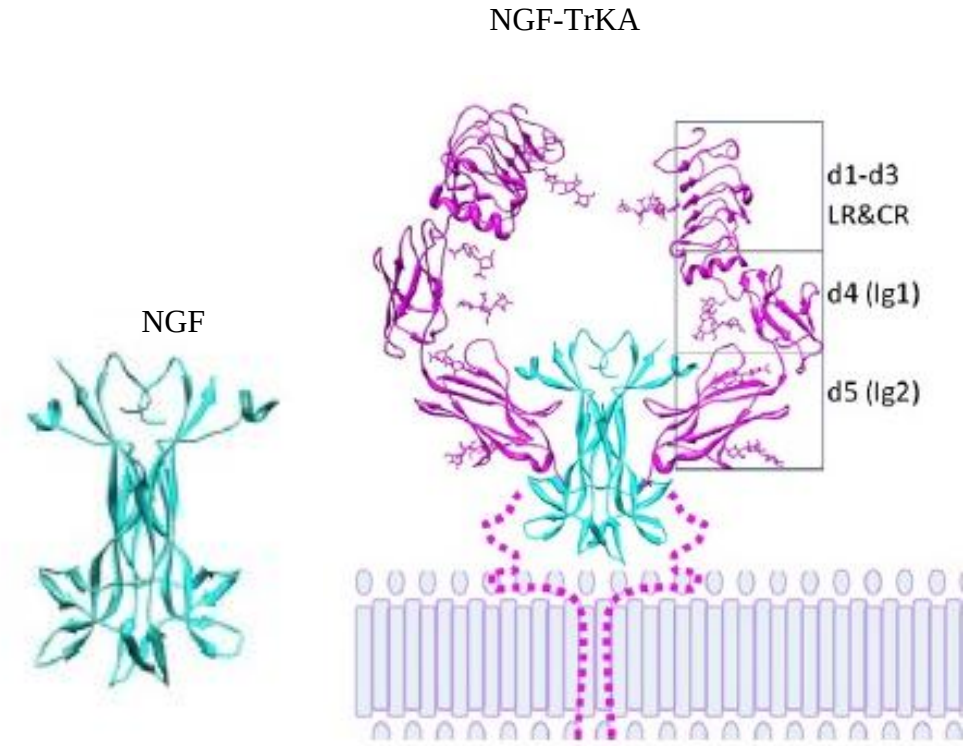


Figure 2.5: X-ray crystal structures of the NGF and its receptor, TrkA. The extracellular region of the Trk receptors can be sub-divided (by amino acid sequence) into different domains (d1–d3 is a leucine-rich, cysteine-rich (LR&CR) region). Domains 4 (d4) and 5 (d5) are immunoglobulin-like domains. d5, the domain closest to the membrane, binds the NGF directly (Allen et al., 2013).

Besides NGF, the members of the neurotrophin family of growth factors include brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4) These are all survival factors, essential for the appropriate development and subsequent survival and maintenance of specific subsets of neurons into adulthood. The mature neurotrophins each bind to their specific tyrosine kinase receptor. NGF binds to TrkA (Figure 2.5), BDNF and NT4 bind to TrkB, and NT3 binds to TrkC (Nagahara et al., 2009).

The use of growth factors such as NGF and BDNF has been proposed. Several *in vitro* and *in vivo* studies revealed the importance of NGF for its ability to enhance the survival of primary basal forebrain cholinergic neurones and to increase the activity of the enzyme choline acetyltransferase (ChAT) (Liu, Lamb, Chou, Liu, & Li, 2007). Intracerebroventricular (ICV) administration of NGF in aged rats was shown to prevent and reverse the cholinergic deficits of AD (Fischer et al., 1987). The concept of NGF protein administration as an AD therapeutic was extended into the first ICV infusion of NGF into early onset AD patients (Olson et al., 1992). The patient's verbal episodic memory was improved after a month. However, ICV infusion of NGF caused weight loss. Other routes of NGF administration have been explored, for example the implant of NGF-transfected fibroblasts into the basal forebrain of primates (Smith, Roberts, Gage, & Tuszynski, 1999). Nasal administration and olfactory bulb injection of radio labelled NGF to the cholinergic basal forebrain has also been explored (Lauer et al., 2000). The NGF therapy was then successfully translated to a Phase I clinical trial in 2001 (Tuszynski et al., 2005). As reported, the Evaluation of the Mini-Mental Status Examination and Alzheimer Disease Assessment Scale-Cognitive subcomponent suggested improvement in the rate of cognitive decline. To date, it is reported that a Phase II multi-centre clinical trial in approximately 50 patients with mild to moderate AD, will be conducted in the United States.

2.6.2 Neuritogenic substances from plants

There has been a recent upsurge of interest in exploring health food to promote neurite outgrowth and improve the overall brain and cognition health (Gunawardena, Shanmugam, et al., 2014). The polyphenol entities found in the vegetables, fruits and nuts were shown to inhibit neuro-inflammation by preventing amyloid precursor protein (APP) signaling and amyloid beta (Aβ) aggregation which is thought to be the culprit of causing AD (Essa et al., 2012). The effect of daily consumption of wild blueberry juice in a sample of nine older adults with early memory changes was investigated (Krikorian et al., 2010). Improvement in the "paired associate learning" and "word list recall" was detected after 12 weeks of consumption of the wild blueberry juice. Blueberries were reported to contain a high level of poly-phenolic compounds, most prominently anthocyanins (Chen, Xin, Yuan, Su, & Liu, 2014). Anthocyanins have been associated with increased neuronal signaling in brain and they were shown to facilitate glucose disposal which ultimately help to mitigate neurodegeneration. Resveratrol (trans-3,4',5-trihydroxystilbene) is a non-flavonoid poly-phenol found abundantly in grapes. Importantly, it was reported that ICV injection of resveratrol reduced neuronal loss in the hippocampus and prevented learning impairment in the p25 transgenic AD mouse model (Vingtdeux, Dreses-Werringloer, Zhao, Davies, & Marambaud, 2008). A variety of phyto-chemical approaches to delay and/or prevent the onset of age-associated neurodegenerative diseases are being investigated, some of which include the galantamine from *Narcissus* sp., lemon balm (*Melissa officinalis*), and periwinkle (*Vinca minor*). Table 2.2 summarises the different medicinal plants which hold the potential in stimulating neurite outgrowth and reducing the occurrence or prevent neurodegenerative diseases.

Table 2.2: Medicinal plants with neurite outgrowth stimulatory activity and their bioactive compounds (More et al., 2012)

Plant	Compound	Dosage	Biological effects	References
Panax ginseng	Ginsenoside Rb1	40 mg	Neuritogenesis in rats	Gao et al., (2010)
	Ginsenoside Rg1	10 mM	Survival of dopaminergic neurons	Radad et al., (2004)
Curcuma longa	Curcumin	10-20 μM/ 0.2 mg	Neurite outgrowth in PC12 cells, neuritogenesis in mouse	Liao et al., (2012)
Withania somnifera	Withanoside IV and VI	1 μM	Axon and dendritic extension in rat cortical neurons	Tohda, Kuboyama, & Komatsu (2005)
Camellia sinensis	Epigallocatechin gallate (EGCG)	0.1-1 μM	Neurite outgrowth in PC12 cells	Gundimeda, McNeill, Schiffman, Hinton, & Gopalakrishna (2010)
Picrorhiza scrophulariiflora	Picroside I & II	60 μM	Potentiating NGF induced neurite outgrowth in PC12D cells	P. Li, Matsunaga, Yamakuni, & Ohizumi (2000)
Rehmannia glutinosa	Catalpol	5, 15 and 50 mg, 100 μM	Increase in the number of mouse tyrosine hydroxylase positive cells	Xu et al., (2010)
Citrus depressa	Nobiletin	100 μM	Neurite outgrowth in PC12 cells	Nagase et al., (2005)
Sargassum macrocarpum	Sargaquinoic acid	1.25–100 ng	Potentiating NGF induced neurite outgrowth in PC12D cells	Tsang & Kamei (2004)
Tripterygium wilfordii	Tripchlorolide	10^{-10} M	Neurite outgrowth & survival of dopaminergic neurons	F.-Q. Li et al., (2003)
Scutellaria baicalensis	Baicalein	5 μg; 50 and 200 mg	Neurite outgrowth in PC12 cells/Increase and survival of rat TH-positive cells	Mu et al., (2009)

2.7 MEDICINAL MUSHROOMS FOR NEURITE OUTGROWTH

Mushroom offers great potential as a poly-pharmaceutic drug because of the complexity of chemical contents and different variety of bioactivities. Available evidence suggests that mushrooms exhibit anti-oxidants, anti-tumour, anti-virus, anti-cancer, anti-inflammatory, immunomodulating, anti-microbial and anti-diabetic activities (Roupas, Keogh, Noakes, Margetts, & Taylor, 2012). Mushrooms with anti-inflammatory properties can be used as functional foods to suppress inflammation which contributes to many age-related chronic diseases (Gunawardena, Bennett, et al., 2014). Contrary to plant and herbal medicine which is widely explored and relatively more advanced, the brain and cognition health effects of mushrooms are in early stages of research.

2.7.1 *Sarcodon cyrneus* Maas Geest and *Sarcodon scabrosus* (Fr.) P. Karst

Sarcodon spp., also called "bitter tooth", are widely distributed in Europe, North America and Asia (Figure 2.6a). *Sarcodon* mushrooms are considered inedible due to their bitter taste. Cyrneines A **(1)** and B **(2)** (Figure 2.7) isolated from *Sarcodon cyrneus* Maas Geest stimulated neurite outgrowth in PC12 cells at 100 μM with no cytotoxicity as indicated by lactate dehydrogenase (LDH) analysis (Marcotullio, Pagiott, et al., 2006) (Table 2.3). Later, it was shown that both cyrneines A and B promoted NGF production in 1321N1 cells (Marcotullio et al., 2007). Neurite outgrowth activity was also observed in NG108-15 cells, a hybrid neuronal cell line derived from mouse neuroblastoma and rat glioma (Yutaro Obara, Hoshino, Marcotullio, Pagiotti, & Nakahata, 2007). On the other hand, cyrneines C **(3)** and D **(4)** failed to induce neurite outgrowth. In addition, glaucopine C **(5)**, isolated from the hexane extract of *Sarcodon glaucopus* (Marcotullio, Pagiotti, et al., 2006), did not significantly promote neurite

outgrowth in PC12 cells but induced NGF gene expression in a lesser extent when compared to cyrneines A and B. It seemed that the presence of the hydroxyl cycloheptadienyl carbaldehyde system in cyrneines could be important for neuritogenesis (Marcotullio et al., 2007). In other words, minor differences in functional groups on cyathane structures in cyrneines A, B, C and D can influence the responses in neuronal cells. Figure 2.7 shows the chemical structures of different cyrneines **(1-5)**.

a

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Thelephorales
Family	:	Bankeraceae
Genus	:	*Sarcodon*
Species	:	*cyrneus*

b

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Thelephorales
Family	:	Bankeraceae
Genus	:	*Sarcodon*
Species	:	*scabrosus*

Figure 2.6: (a) The basidiocarps of wild *Sarcodon cyrneus* and its taxonomy. (b) The basidiocarps of wild *Sarcodon scabrosus* and its taxonomy. Source: http://www.mycobank.org/

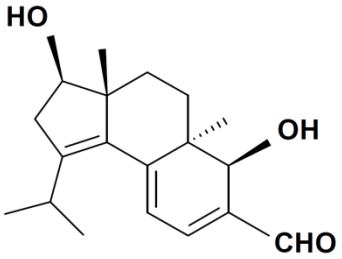
Scabronine A **(6)** (Table 2.3) isolated from *Sarcodon scabrosus* (Figure 2.6b) showed potent inductive activity of NGF synthesis in 1321N1 human astrocytoma cells (Ohta et al., 1998). Further investigation led to the isolation of novel cyathane diterpenoids named scabronines B-F **(7-11)** (Kita, Takaya, & Oshima, 1998), G **(12)** (Obara et al., 1999), K **(13)** and L **(14)** (Shi, Liu, Gao, & Zhang, 2011). However, only scabronines B, C, E and G (Figure 7) showed NGF-synthesis stimulating activity. It appeared that the presence of the α,β-unsaturated aldehyde system in the seven-membered ring could be crucial for the bioactivity. Recently, the first synthesis of scabronine G in an optically pure form, has been reported and the neurite outgrowth activity was comparable to NGF and natural scabronine G (Waters, Tian, Li, & Danishefsky, 2005). Meanwhile, scabronine G-methyl-ester **(15)** synthesised from scabronine G also potently promoted the secretion of NGF and interleukin-6 (IL-6), another major neurotrophic factor released from astrocytes. Most recently, secoscabronine M **(16)**, a hemiacetal cyathane diterpenoid was isolated from *S. scabrosus* but neuritogenesis was not reported for this compound. Figure 2.7 shows the structures of scabronines and secoscabronines **(6-16)** isolated from *S. scabrosus*.

Table 2.3: The effects of *Sarcodon cyrneus, S. glaucopus, S. scabrosus*; and their chemical constituents with neurite outgrowth activity

No	Mushroom	Compound	*In vitro* study	Neurite outgrowth activity	References
1	***Sarcodon cyrneus***	Cyrneine A	PC12; NG108-15; 1321N1	Neurite outgrowth ↑ NGF ↑	Marcotullio, Pagiott, et al., (2006); Yutaro Obara et al., (2007)
2		Cyrneine B	PC12	Neurite outgrowth ↑; NGF ↑	Marcotullio et al., (2007); Marcotullio, Pagiott, et al., (2006)

No	Mushroom	Compound	*In vitro* study	Neurite outgrowth activity	References
3	***Sarcodon cyrneus***	Cyrneine C	PC12	-	Marcotullio et al., (2007)
4		Cyrneine D	PC12	-	Marcotullio et al., (2007)
5	***Sarcodon glaucopus***	Glaucopine C	PC12	NGF gene expression ↑	Marcotullio et al., (2007); Marcotullio, Pagiotti, et al., (2006)
6	***Sarcodon scabrosus***	Scabronine A	1321N1	Neurite outgrowth ↑	Ohta et al., (1998)
8		Scabronine C	Rat astroglial cells	NGF ↑	Kita et al., (1998)
9		Scabronine D	Rat astroglial cells	-	Kita et al., (1998)
10		Scabronine E	Rat astroglial cells	NGF ↑	Kita et al., (1998)
11		Scabronine F	Rat astroglial cells	-	Kita et al., (1998)
12		Scabronine G	1321N1	Neurite outgrowth ↑	Y Obara et al., (1999); Waters et al., (2005)
13		Scabronine G-methyl ester	PC12	NGF and IL-6 ↑	Y Obara, Kobayashi, Ohta, Ohizumi, & Nakahata (2001)
14		Scabronine K	PC12	-	Shi et al., (2011)
15		Scabronine L	PC12	-	Shi et al., (2011)
16		Secoscabronine M	-	-	Shi, Zhang, Pescitelli, & Gao (2012)

Note: -: No effect on neurite outgrowth; NGF: nerve growth factor; ↑: Promoted/increased



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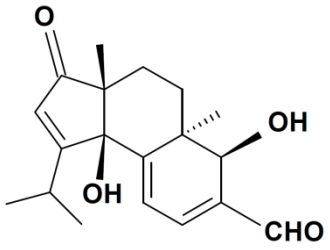

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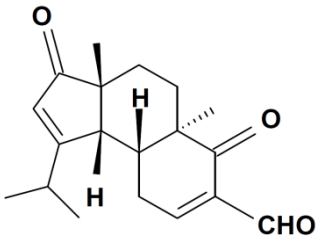

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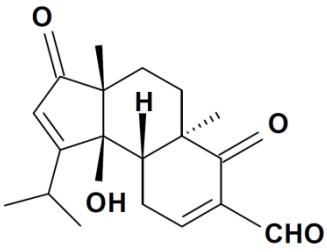

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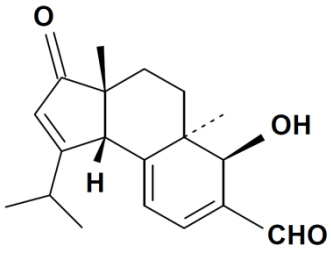

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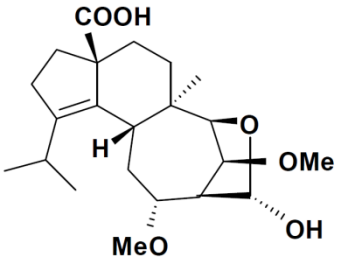

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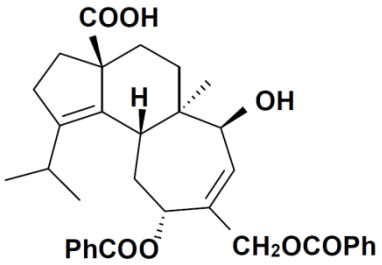

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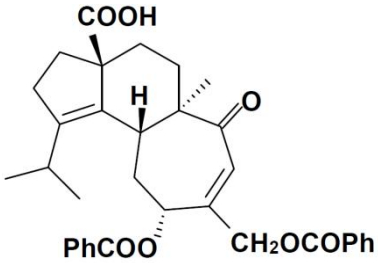

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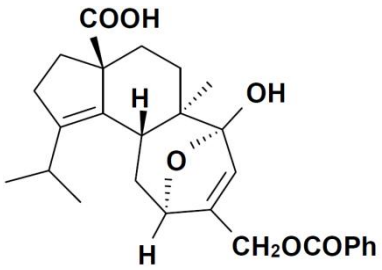

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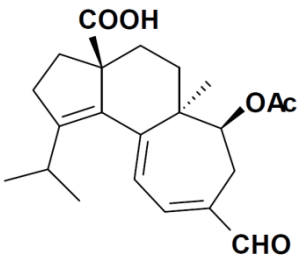

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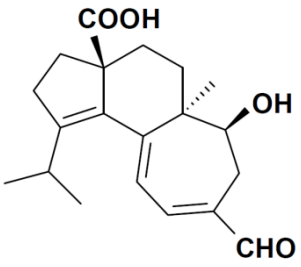

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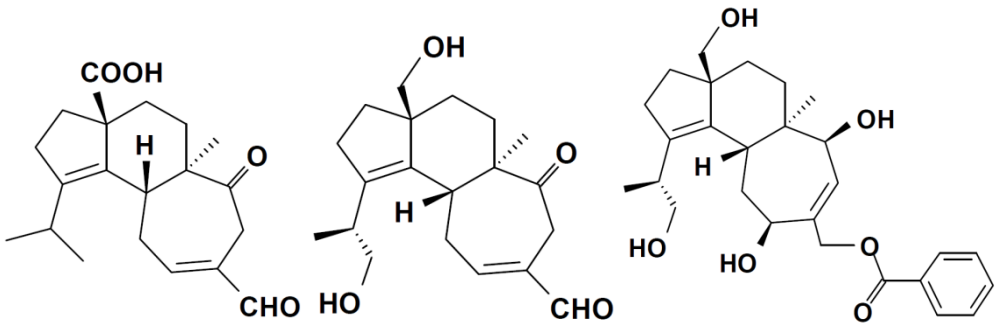
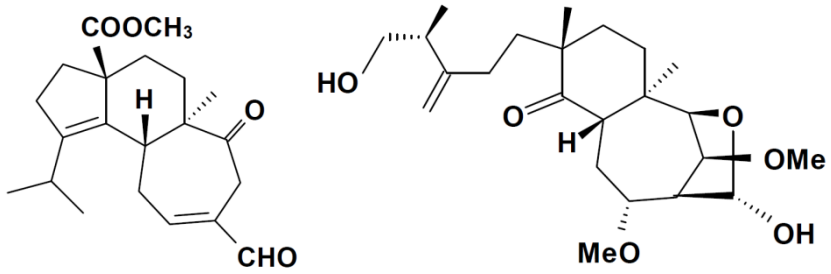


(12) **(13)** **(14)**



(15) **(16)**

Figure 2.7: Cyrneines A (1), B (2), C (3) and D (4) from *Sarcodon cyrneus*; and glaucopine C (5), isolated from the hexane extract of *Sarcodon glaucopus*. Scabronines A-G (6-12), K (13), L (14), scabronine G-methyl-ester (15), and secoscabronine M (16), isolated from *Sarcodon scabrosus*.

2.7.2 *Hericium erinaceus* (Bull.: Fr.) Pers.

Hericium erinaceus is also called the lion's mane mushroom, monkey's head mushroom, hedgehog mushroom, satyr's beard, pom pom, bearded tooth, and Yamabushitake. The basidiocarp is often white to creamy white in colour and with icicle-like projections (Figure 2.8).

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Russulales
Family	:	Hericiaceae
Genus	:	*Hericium*
Species	:	*erinaceus*

Figure 2.8: The basidiocarps of *Hericium erinaceus* and its taxonomy. Source: Mushroom Research Centre, University of Malaya, 2014.

There is a possible use of *Hericium erinaceus* (Bull.: Fr.) Pers. in the treatment of neurological disorders and dementia as reported by Kawagishi and Zhuang (2008). In a study by Wong et al., (2007) , the extracts of *H. erinaceus* basidiocarp and mycelium induced neurite outgrowth of neuronal cells NG108-15 *in vitro*. Besides, ethanol extract of *H. erinaceus* promoted the neurite outgrowth of PC12 cells, enhanced NGF mRNA expression and secretion of NGF from 1321N1 human astrocytoma cells (Mori et al., 2008). Further, *in vivo* functional recovery of axonotmetic peroneal nerve injury in Sprague-Dawley rats was assessed by walking-track analysis and toe-spreading reflex (Wong et al., 2009). The peroneal functional index (PFI) and toe-spreading reflex improved more rapidly in the group treated with daily administration of *H. erinaceus* extract. These data suggested that *H. erinaceus* could promote the regeneration of nerve injury in the early stage of recovery (Wong et al., 2010).

Although preliminary, it was demonstrated that the *H. erinaceus* extract exerted neurotrophic action and improved the myelination process in the rat brain without affecting nerve cell growth and toxicity (Moldavan et al., 2007). There was an attempt to isolate a polysaccharide from the mycelium of *H. erinaceus* and the polysaccharide

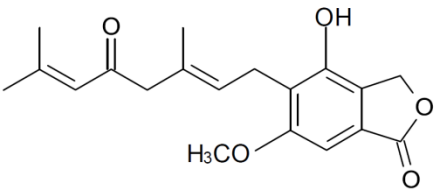
(molar ratio of 1.5: 1.7: 1.2: 0.6: 0.9; glucose: galactose: xylose: mannose: fructose) promoted neurite outgrowth in PC12 cells *in vitro* (Park et al., 2002).

Hericenones (benzyl alcohol derivatives) were isolated from the fruiting bodies of *H. erinaceus* (Table 2.4). Hericenones A **(17)** and B **(18)** (Figure 2.9) were first reported in 1990 but no neurite outgrowth activity was reported (Kawagishi, Ando, & Mizuno, 1990). Hericenones C **(19)**, D **(20),** E **(21)**, F **(22)**, G **(23)**, and H **(24)** exhibited stimulating activity for the biosynthesis of NGF *in vitro* (Kawagishi & Ando, 1993; Kawagishi et al., 1991). Hericenone E isolated from *H. erinaceus* cultivated under tropical conditions in Malaysia was able to stimulate NGF secretion which was two-fold higher than that of the positive control (50 ng/mL of NGF) (Phan, Lee, et al., 2014). Hericenone E also increased the phosphorylation of extracellular-signal regulated kinases (ERKs) and protein kinase B (Akt) responsible for neurite outgrowth activity. On the other hand, diterpenoid derivatives (named erinacines) were isolated from the mycelium of *H. erinaceus* (Figure 2.9). Erinacines A-I **(25-33)** significantly induced the synthesis of nerve growth factor (NGF) *in vitro* (Kawagishi, Shimada, Sakamoto, Bordner, & Kojima, 1996; Kawagishi et al., 1994; Kawagishi, Simada, et al., 1996; Lee et al., 2000) and *in vivo* (Shimbo, Kawagishi, & Yokogoshi, 2005). Isolation of new compounds from this mushroom continued with the discovery of erinacines J **(34)**, K **(35)**, P-R **(36-38)**, as well as erinacol **(39)**, a novel cyathadien-14*β*-ol (Kawagishi, Masui, Tokuyama, & Nakamura, 2006; Kenmoku, Sassa, & Kato, 2000; Kenmoku, Shimai, Toyomasu, Kato, & Sassa, 2002; Kenmoku, Tanaka, Okada, Kato, & Sassa, 2004; Ma, Zhou, Li, & Li, 2008; Ma et al., 2010). Structures of hericenones **(17-24)** and erinacines **(25-39)** can be found in Figure 2.9.

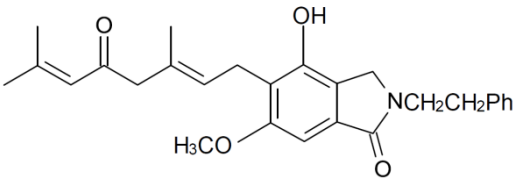
Table 2.4: List of hericenones and erinacines in *Hericium erinaceus*, some of which showed neurite outgrowth activity

No	Mushroom component	Compound	*In vitro* study	Neurite outgrowth activity	Reference
17	F	Hericenone A	-	-	Kawagishi et al., (1990)
18	F	Hericenone B	-	-	Kawagishi et al., (1990)
19	F	Hericenone C	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1991)
20	F	Hericenone D	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1991)
21	F	Hericenone E	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1991)
22	F	Hericenone F	Mouse astroglial cells	NGF ↑	Kawagishi & Ando (1993)
23	F	Hericenone G	Mouse astroglial cells	NGF ↑	Kawagishi & Ando (1993)
24	F	Hericenone H	Mouse astroglial cells	NGF ↑	Kawagishi & Ando (1993)
25	M	Erinacine A	Mouse astroglial cells Rat (*in vivo*)	NGF ↑; Catecholamine ↑ in the CNS of rats	Kawagishi et al., (1994); Shimbo et al,. (2005)
26	M	Erinacine B	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1994)
27	M	Erinacine C	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1994)
28	M	Erinacine D	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1996)
29	M	Erinacine E	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1996)
30	M	Erinacine F	Mouse astroglial cells	NGF↑	Kawagishi et al., (1996)
31	M	Erinacine G	Mouse astroglial cells	NGF ↑	Kawagishi et al., (1996)
32	M	Erinacine H	Rat astroglial cells	NGF ↑	Lee et al., (2000)
33	M	Erinacine I	Rat astroglial cells	NGF ↑	Lee et al., (2000)
34	M	Erinacine J	MRSA	-	Kawagishi et al., (2006)
35	M	Erinacine K	MRSA	-	Kawagishi et al., (2006)
36	M	Erinacine P	-	Biosynthesis of erinacines	Kenmoku et al., (2000)
37	M	Erinacine Q	-	Biosynthesis of erinacine C	Kenmoku et al., (2002)
38	M	Erinacine R	-	-	Ma et al., (2010) Ma et al., (2008)
39	M	Erinacol	-	Biosynthesis of erinacine Q	Kenmoku et al., (2004)

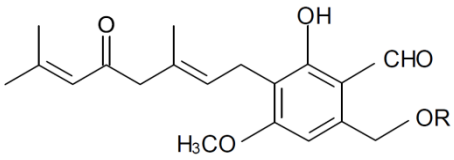
Note: F: fruiting body; M: mycelium; -: none; NGF: nerve growth factor; CNS: central nervous system; MRSA: Methicillin-resistant *Staphylococcus aureus*



(17)



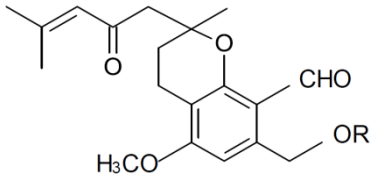
(18)



(19) R = palmtoyl

(20) R = stearoyl

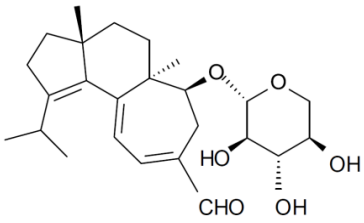
(21) R = linoleoyl



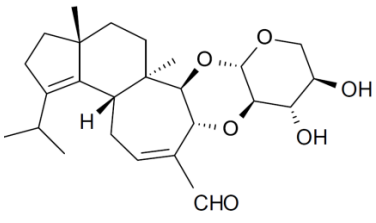
(22) R = palmtoyl

(23) R = stearoyl

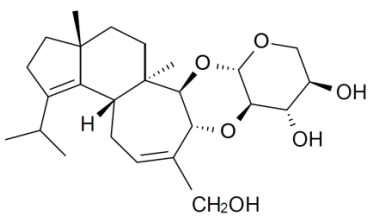
(24) R = linoleoyl



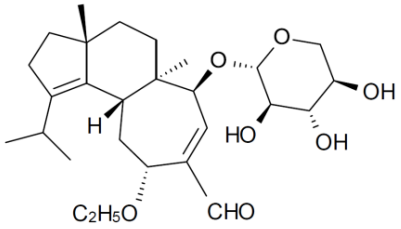
(25)



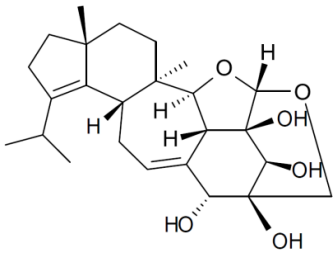
(26)



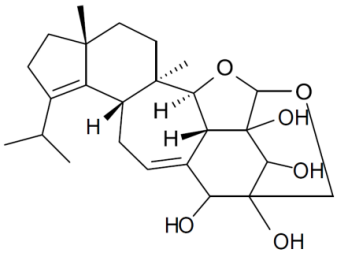
(27)



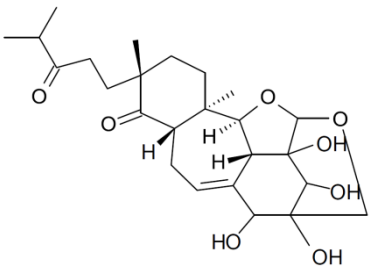
(28)



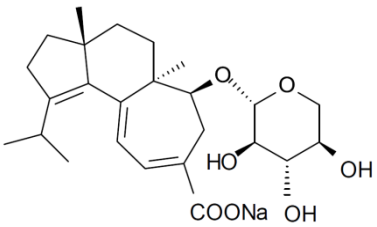
(29)



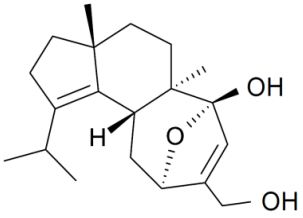
(30)



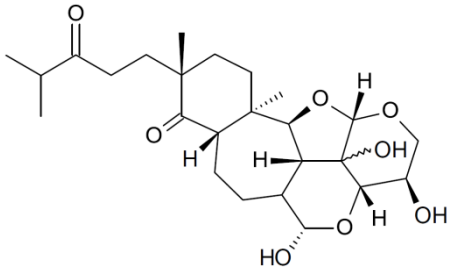
(31)



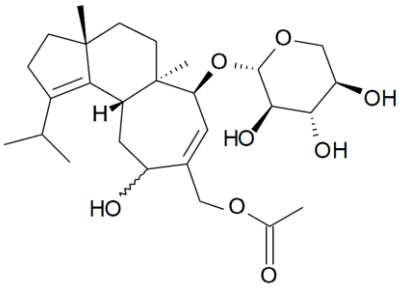
(32)



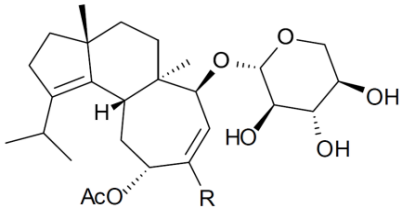
(33)



(34)

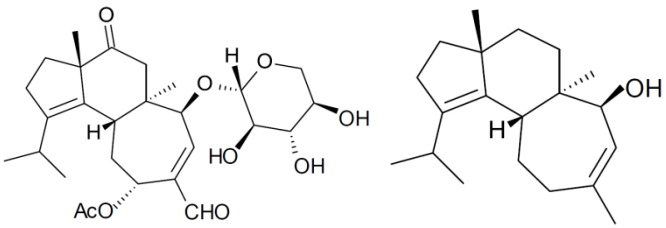


(35)



(36) R = CHO

(37) R = CH_2OH



(38) **(39)**

Figure 2.9: Hericenones A-H (17-24); erinacine A-K (25-35), P-R (36-38) and erinacol (39) isolated from *H. erinaceus.*

2.7.3 *Ganoderma lucidum* (Fr) P. Karst, *Dictyophora indusiata* (Vent.) Desv., and *Grifola frondosa* (Dicks.: Fr.) S.F. Gray

Ganoderma lucidum (Ling Zhi), a famous medicinal mushroom, is used widely in traditional Chinese medicine. The basidiocarp is corky, almost flat, red-varnished, and kidney shaped (Figure 2.10a). Cheung et al., (2000) reported that *G. lucidum* extract reduced PC12 cell proliferation and induced neuronal differentiation and neurite outgrowth *via* the activation of MAP kinases and cAMP-response element binding protein (CREB) signaling pathways. In addition, a lipophilic fraction of *G. lucidum* (125 and 500 mg/L) was also shown to induce neurite outgrowth of PC12 cells (Zhang et al., 2005).

Dictyophora indusiata is a famous edible mushroom used in Chinese cuisine and medicine (Figure 2.10b). It is called the "Queen of the mushrooms'', bamboo mushroom and Kinugasatake in Japanese. Two eudesmane-type sesquiterpenes, dictyophorines A **(40)** and B **(41)** (Figure 2.11), were isolated from the mushroom and were found to promote NGF synthesis by astroglial cells (Kawagishi et al., 1997). It was shown that NGF secreted into the medium in the presence of 3.3 mM of dictyophorines A was four times higher than the negative control. *Grifola frondosa* (hen of the woods, dancing mushroom, Maitake) is a mushroom with manifold curled or spoon-shaped

gray-brown caps (Figure 2.10c). It also has a tuber-like sclerotium. Lysophosphatidylethanolamine (LPE) isolated from *G. frondosa* was found to induce neurite outgrowth and it upregulated the neurofilament M expression in cultured PC12 cells (Nishina et al., 2006). The study also showed suppressive effect of *G. frondosa* on serum deprivation-induced apoptosis of the PC12 cells.

a

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Polyporales
Family	:	Ganodermataceae
Genus	:	*Ganoderma*
Species	:	*lucidum*

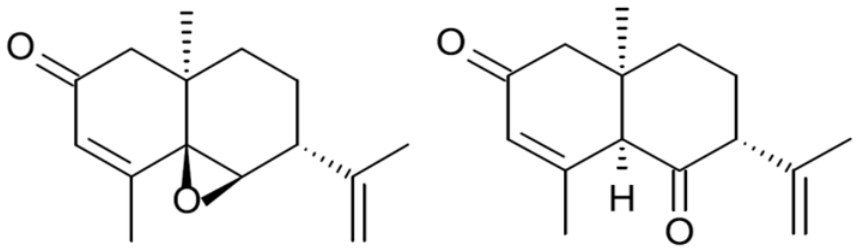
b

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Phallales
Family	:	Phallaceae
Genus	:	*Dictyophora*
Species	:	*indusiata*

c

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Polyporales
Family	:	Fomitopsidaceae
Genus	:	*Grifola*
Species	:	*frondosa*

Figure 2.10: Basidiocarps of (a) *Ganoderma lucidum*, (b) *Dictyophora indusiata, and* (c) *Grifola frondosa*. Source: http://www.mycobank.org/



(40) **(41)**

Figure 2.11: Dictyophorines A (40) and B (41), isolated from *Dictyophora indusiata*.

2.7.4 *Tremella fuciformis* Berk, *Tricholoma* sp., and *Termitomyces albuminosus* (Berk.) R. Heim

Tremella fuciformis is also known as Yin Er, white jelly fungus, silver ear mushroom, and snow mushroom (Figure 2.12a). It has a white, frond-like, and gelatinous basidiocarp. The aqueous extract of *T. fuciformis* not only promoted the neurite outgrowth of PC12 cells, but significantly reversed the scopolamine- and trimethyltin-induced memory deficit in rats, as revealed by the Morris water maze test and choline acetyltransferase (ChAT) immunohistochemistry (Kim et al., 2007; Park et al., 2012). *Tricholoma* is mycorrhizal, and it has a gilled cap and fleshy stem (Figure 2.12b). Neuritogenic compounds named tricholomalides A-C **(42-44)** (Figure 2.13) were also isolated from *Tricholoma* sp. and neurite outgrowth in PC-12 cells was significantly induced at concentrations of 100 μM (Tsukamoto et al., 2003). *Termitomyces albuminosus* is a wild and edible mushroom with long stem, and it often emerges from a termite nest (Figure 2.12c). The cerebrosides named termitomycesphins A-D **(45-48)** (Qi, Ojika, & Sakagami, 2000), E-F **(49-50)** (Qi, Ojika, & Sakagami, 2001), and G-H **(51-52)** (Qu et al., 2012) (Figure 2.13) were identified to potentiate neuritogenesis in PC12 cells. It is interesting that termitomycesphin with a 16-carbon-chain fatty acid (A, C, and G) showed higher neuritogenic activity than that of

termitomycesphin with an 18-carbon-chain fatty acid (B, D, and H), suggesting that the chain length of the fatty acyl moiety played a determining role in neuritogenesis.

a

Kingdom : Fungi
Phylum : Basidiomycota
Class : Tremellomycetes
Order : Tremellales
Family : Tremellaceae
Genus : *Tremella*
Species : *fuciformis*

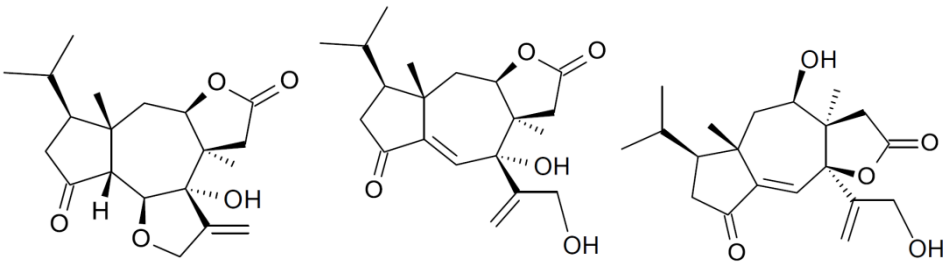
b

Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Agaricales
Family : Tricholomataceae
Genus : *Tricholoma*
Species : *Sp.*

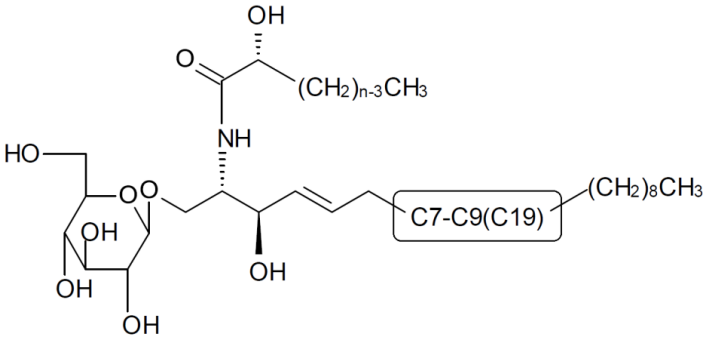
c

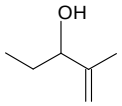
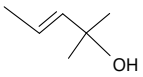
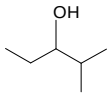
Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Agaricales
Family : Lyophyllaceae
Genus : *Termitomyces*
Species : *albuminosus*

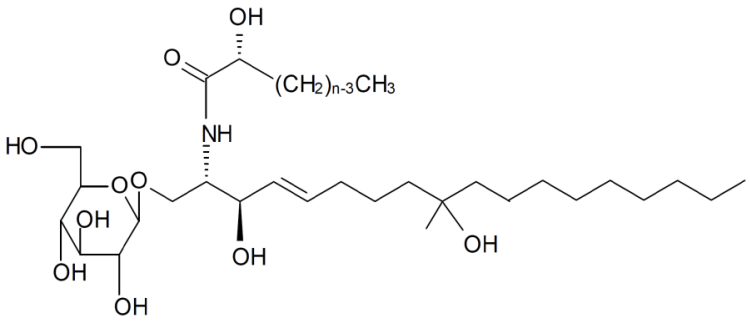
Figure 2.12: Basidiocarps of (a) *Tremella fuciformis*, (b) *Tricholoma* sp., and (c) *Termitomyces albuminosus*. Source: http://www.mycobank.org/



(42) **(43)** **(44)**



No	Termitomycesphin	C7-C9(C19)	n
45	A		16
46	B		18
47	C		16
48	D		18
49	E		16
50	F		18



(51) n = 16

(52) n = 18

Figure 2.13: Tricholomalides A-C (42-44) and termitomycesphins (45-52) isolated from *Tremella fuciformis* and *Termitomyces albuminosus*; respectively.

2.7.5 *Lignosus rhinocerotis* (Cooke) Ryvarden, *Ganoderma neo-japonicum* (Fr) P. Karst, and *Cordyceps militaris* (L.:Fr.) Link

Known as the tiger's milk mushroom, *Lignosus rhinocerotis* poses underground tuber-like sclerotium and has a solitary basidiocarp (Figure 2.14a). Aqueous extract of *L. rhinocerotis* sclerotium (Eik et al., 2012) and *L. rhinocerotis* mycelium (John, Wong, Naidu, & Sabaratnam, 2013) exhibited neurite outgrowth activity in PC12 cells. *Ganoderma neo-japonicum* (Figure 2.14b) grows on dead hardwoods or bamboos. The indigenous people in Malaysia drink the water infusion of this wild as a tonic to strengthen their body. *Ganoderma neo-japonicum* was shown to induce neuronal differentiation and stimulate neurite outgrowth of PC12 cells (Seow et al., 2013). Meanwhile, 5-20 μg/mL of methanol extract of *Cordyceps militaris* (Figure 2.14c) was found to increase primary neurite sprouting and choline acetyltransferase expression in differentiated N2a cells (Lee et al., 2011). Administration of *C. militaris* also restored the scopolamine-induced memory deficit in rat.

a

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Polyporales
Family	:	Polyporaceae
Genus	:	*Lignosus*
Species	:	*rhinocerotis*

b

Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Polyporales
Family : Ganodermataceae
Genus : *Ganoderma*
Species : *neo-japonicum*

c

Kingdom : Fungi
Phylum : Basidiomycota
Class : Sordariomycetes
Order : Hypocreales
Family : Clavicipitaceae
Genus : *Cordyceps*
Species : *militaris*

Figure 2.14: Basidiocarps of (a) *Lignosus rhinocerotis*, (b) *Ganoderma neo-japonicum*, and (c) *Cordyceps militaris*. Source: http://www.mycobank.org/

2.8 *PLEUROTUS GIGANTEUS* (BERK.) KARUNARATHNA & K.D. HYDE

Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde (Figure 2.15) is a saprobe and one of the largest edible mushrooms which grows on the ground. It is solitary or can be found in groups, often around stumps, wood, dead roots, in the open and in lowland, and mountain forest up to 3000 masl (Mortimer et al., 2012). *Pleurotus giganteus* is gaining popularity for its organoleptic properties and commercial prospects. Consumption of this wild mushroom has long been a tradition in the indigenous villages in Peninsular Malaysia (Lee, Chang, & Noraswati, 2009). A variety of *P. giganteus* from China is now being cultivated in Malaysia and the common commercial name in Malay language for *P. giganteus* is "*Seri Pagi*" (morning glory). In China, *P. giganteus* is widely referred as "Zhudugu" (swine's stomach) (Deng et al.,2006).

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Agaricales
Family	:	Pleurotaceae
Genus	:	*Pleurotus*
Species	:	*giganteus*

Figure 2.15: The basidiocarps of *Pleurotus giganteus.*

In mushroom biology, species boundaries are always indistinct and many mushrooms are subsumed under erroneous names (Hallenberg, Nilsson, & Robledo, 2012). The "*Panus-Pleurotus-Lentinus"* confusion of this mushroom has long existed and has resulted in the perplexity of nomenclature and taxonomy of these three species. Briefly, *Panus giganteus* (Polyporaceae, Polyporales) is characterised by its unbranched skeletal hyphae that usually grows on buried woody substrates (Corner, 1981). While Pegler has merged *Panus* as a subgenus within *Lentinus*, Corner, on the other hand

grouped the genus *Panus* to species with skeletal hyphae and separated those species with ligative hyphae in *Lentinus*. Hence, *Lentinus giganteus* is regarded as synonym for *Panus giganteus* and *Lentinus giganteus* should be used if recommendation of Pegler is ever adopted. However, Karunarathna and colleagues have revisited this issue and concluded that *Panus*/*Lentinus giganteus* should be unified and positioned in *Pleurotus* as supported by molecular evidences (Karunarathna et al., 2011).

Studies on *P. giganteus* include domestication and cultivation of the mushroom (Klomklung, Karunarathna, Chukeatirote, & Hyde, 2012). Accordingly, the optimal growth of *P. giganteus* was reported to be obtained on soybean agar within a pH range of 5.0-6.5 and the optimal temperature of 30 ℃ (Klomklung, Karunarathna, Hyde, & Chukeatirote, 2014). In a recent study on the liver protective activity of *P. giganteus*, rats were injected intraperitoneally with thioacetamide (TAA) thrice a week and were orally administered with freeze-dried basidiocarps of *P. giganteus* daily for two months (Wong et al., 2012). After 60 days, the rats administered with *P. giganteus* showed lower liver body weight ratio, restored levels of serum liver biomarkers and oxidative stress parameters comparable to treatment with the standard drug i.e. silymarin. Gross necropsy and histopathological examination further confirmed that *P. giganteus* was able to prevent or reduce the severity of TAA-induced liver injury in rats.

Pleurotus giganteus also exhibited anti-*Candida* activity (Phan et al., 2013). In the study, *Candida albicans* WM1172, *C. albicans* ATCC90028, *C. dubliniensis*, *C. glabrata* CBS138, *C. glabrata* ATCC90030, *C. krusei* ATCC6258, *C. pseudotropicalis*, and *C. tropicalis* WM30 were used. The minimum inhibitory concentration (MIC) of the ethyl acetate extract and its fractions against all the tested yeasts were determined. The MIC values for all the *Candida* spp. tested, ranged from 2.0 ± 1.0 to 10.3 ± 2.5 µg/mL for fraction A; and 9.3 ± 2.3 to 34.3 ± 10.8 µg/mL for fraction B. Constituents of both the fractions from ethyl acetate extract were elucidated by using gas

chromatography mass spectrometry (GCMS). As a result, 12 compounds were identified in both the fractions:, i.e. methyl palmitate, ethyl palmitate, methyl linoleate, methyl oleate, methyl stearate, and ethyl oleate, palmitic acid and oleic acid, ergosterol, ergosta-5,7,9 (11),22-tetraen-3β-ol, ergost-5,8(14)-dien-3-ol, and γ-ergostenol.

CHAPTER III

IDENTIFICATION OF MUSHROOMS WITH NEURITE OUTGROWTH STIMULATORY EFFECTS USING DIFFERENTIATING NEUROBLASTOMA-2A CELLS

3.1 INTRODUCTION

Neurite outgrowth is an important event in neuronal path finding and the establishment of synaptic connections during development (Bernd, 2008; Fornasiero, Bonanomi, Benfenati, & Valtorta, 2010). It is also essential in neuronal plasticity, neuronal regeneration after injury (Loers & Schachner, 2007) and neurodegenerative conditions such as Alzheimer's and Parkinson's diseases (Shulman & Jager, 2009). Therefore, treatments aiming at promoting neurite outgrowth and preserving the neurite network and synaptic connections are needed.

The potential use of culinary-medicinal mushrooms in neurodegenerative diseases is being explored (Sabaratnam, Wong, Naidu, & David, 2011). On-going research shows that *Hericium erinaceus* (Bull.: Fr) Pers. (monkey's head mushroom, lion's mane mushroom and Yamabushitake) (Wong et al., 2007) and *Lignosus rhinocerotis* (Cooke) Ryvarden (tiger milk mushroom) (Eik et al., 2012; Seow et al., 2013) exhibit neurite outgrowth stimulatory effects in NG108-15 and PC12 cell lines. This observation raised a question with respect to the neurodevelopmental effects, if any, of culinary-medicinal mushrooms. Congenital diseases are present in 2–3% of human newborns (Genschow et al., 2004). About 20% of the birth defects are due to genetic anomaly and 10% are caused by environmental factors during pregnancy (Seiler & Spielmann, 2011). Therefore, toxicological safety assessments of food, chemicals and drugs to evaluate the effects on reproductive health and for embryotoxicity have become

important requirements. Thus, the aims of the present study were (a) to evaluate neurite outgrowth stimulatory effects of selected culinary-medicinal mushrooms using neuroblastoma-2a (N2a) cells and (b) to assess the neuro- and embryotoxicity of the mushroom extracts using N2a and embryonic 3T3 fibroblasts.

3.2 MATERIALS AND MEETHODS

3.2.1 Mushrooms

The mushrooms were authenticated by experts in the Mushroom Research Centre, University of Malaya and voucher specimens were deposited in the University of Malaya (Table 3.1). Fresh basidiocarps of *Ganoderma lucidum* (Fr) P. Karst (KLU-M 1233) and *H. erinaceus* (KLU-M 1232) were obtained from Ganofarm Sdn Bhd. *Pleurotus giganteus* (KLU-M 1227) was provided by Nas Agro Farm and Dong Foong Biotech. Freeze dried powder of *Cordyceps militaris* (L.:Fr.) Link and *L. rhinocerotis* were purchased from BioFact Life and Ligno Biotech Sdn Bhd, respectively. *Pleurotus pulmonarius* (Fr.) Quél. fruiting bodies (KLU-M 1309) and *Gingko biloba* extracts were obtained from Reishilab Sdn Bhd, Selangor. Wild *Ganoderma neo-japonicum* Imazeki 1939 (KLU-M 1231) was collected from a forest in Ulu Grik, Perak, Malaysia. *Grifola frondosa* (Dicks.: Fr.) S.F. Gray (KLU-M 1229) imported from Japan was obtained from supermarkets in Selangor, Malaysia. *Lycium barbarum (*wolfberry), a traditional Chinese medicine was purchased from a Chinese medicine shop in Selangor, Malaysia. Curcumin was purchased (NatXtra, Synthite Co., India).

Table 3.1: Mushrooms used in this study, their common names, medicinal and culinary nature

Mushroom species	Voucher number	Common names	Local names (in Malay)	Part used	Edible/ culinary	Wild/ cultivated	Medicinal properties	References
Pleurotus giganteus (Panus giganteus)	KLU-M 1227	Zhudugu, cow's stomach mushroom	*Cendawan seri pagi* (morning glory), *perut lembu* (cow's stomach)	Fruiting body	Culinary	Cultivated	Antioxidant, neurite outgrowth simulation	Phan, Wong, David, Naidu, & Sabaratnam (2012); Wong et al., (2012)
Pleurotus pulmonarius (Pleurotus sajor-caju)	KLU-M 1309	Grey oyster mushroom	*Cendawan tiram kelabu* (grey oyster)	Fruiting body	Culinary	Cultivated	Antioxidant, anti-diabetic	Kanagasabapathy, Malek, Kuppusamy, & Vikineswary, (2011)
Lignosus rhinocerotis	Ligno Biotech Sdn Bhd	Tiger milk mushroom	*Cendawan susu rimau* (tiger's milk)	Sclerotium and mycelium	Non-culinary but edible	Cultivated	Anticancer, neurite outgrowth stimulation	Eik et al., (2012); Lee, Tan, Fung, Tan, & Ng (2012)
Hericium erinaceus	KLU-M 1232	Monkey's head mushroom, lion's mane mushroom, Yamabushitake	*Cendawan bunga kubis* (cauliflower)	Fruiting body	Culinary	Cultivated	Anti-ulcer, neurite outgrowth stimulation	Wong, Sabaratnam, Abdullah, Kuppusamy, & Naidu, (2009); Wong et al., (2010); Wong et al., (2009, 2007)
Ganoderma lucidum	KLU-M 1233	Lingzhi, reishi	*Cendawan merah* (red mushroom)	Fruiting body	Non-culinary but edible	Cultivated	Anticancer, neuroprotection	Zhou et al., (2010)
Ganoderma neo-japonicum	KLU-M 1231	Purple reishi	*Cendawan senduk* (cobra mushroom)	Fruiting body	Non-culinary but edible	Wild	Antioxidant, neurite outgrowth stimulation	Lin, Lin, Chen, Ujiie, & Takada, (1995)

Mushroom species	Voucher number	Common names	Local names (in Malay)	Part used	Edible/ culinary	Wild/ cultivated	Medicinal properties	References
Cordyceps militaris	Purchased from BioFact Life Sdn Bhd	Winter worm summer grass, caterpillar fungus	-	Fruiting body (ascocarp)	Non-culinary but edible	Cultivated	Anti-inflammatory, anticancer, relief respiratory disorders	Lee et al., (2011)
Grifola frondosa	KLU- M 1229	Maitake, hen of the woods	*Cendawan maitake*	Fruiting body	Culinary	Cultivated	Anti-inflammatory, anti-cholesterol, neurite outgrowth stimulation	Nishina et al., (2006)

3.2.2 Cell culture

Mouse neuroblastoma cells (N2a, ATCC CCL-131) and mouse embryonic fibroblast cells (BALB/c 3T3, ATCC clone A31) were purchased from American Type Culture Collection (ATCC; MD, USA). N2a cells were cultured in Eagle's minimum essential medium (MEM) with L-glutamine (PAA) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (PAA), 100 U/ml penicillin, and 100 µg/ml streptomycin. 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (high glucose at 4.5 g/l) supplemented with 10% FBS. All the cells were maintained at 37 ℃ and 5% CO_2 in a humidified atmosphere. N2a cells were subcultured at 3 – 4 days intervals while 3T3 cells were routinely passaged every 2 – 3 days. For preservation, the cells were frozen at -70 ℃ liquid nitrogen in complete medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO; Sigma) as a cryoprotecting agent.

3.2.3 Preparation of mushroom extracts

The fresh fruiting bodies of *P. giganteus*, *P. pulmonarius*, *H. erinaceus*, and *G. frondosa* were sliced, frozen and then freeze-dried for two days. The freeze-dried fruiting bodies were then ground to powder and kept at 4 - 8 ℃. For aqueous extraction, the freeze dried powder was soaked in distilled water (1:20, w/v) at room temperature and at 200 rpm in a shaker for 24 h. The mixture was then double boiled in water bath for 30 min, cooled and then filtered (Whatman No. 4). The resulting aqueous extracts were freeze-dried and kept at -20 ℃ prior to use. The process was repeated for the freeze-dried powder of *C. militaris* and *L. rhinocerotis* sclerotia. For ethanol extraction, the freeze dried powder was soaked in 95% ethanol at room temperature for three days and the process was repeated three times. The solvent was then evaporated using a rotary evaporator (Eyela N-1000, USA) to give a brownish viscous extract.

3.2.4 Neurite outgrowth assay

N2a cells were seeded in a 24-well culture plate at an initial density of 5,000 cells per well containing complete growth medium (1 ml/well) and incubated overnight. Concentrations of NGF ranging from 5 - 100 ng/ml (w/v) were tested to determine the optimum concentration that stimulates maximum neurite outgrowth. The optimum concentration was then used as a positive control throughout the subsequent assays. Aqueous and ethanol mushroom extracts were stocked at 10 mg/ml and were subsequently dissolved in sterile distilled water or dimethyl sulfoxide (DMSO), to the appropriate concentrations. The final concentration of DMSO in the assays was 0.1 - 0.25%. To induce cell differentiation, the complete medium was carefully replaced with 5% serum medium before exposure to mushroom extracts at 10 - 50 µg/ml. Cells with medium only served as a negative control. All the cells were incubated for 48 h at 37 ℃, 95% air and 5% CO_2 to observe neuritogenic activity, if any. Curcumin, *G. biloba* and *L. barbarum* extracts were also tested to compare the neurite outgrowth activities with those of mushroom extracts.

3.2.5 Quantification of neurite bearing cells

Five random fields (100 – 200 cells/well) were examined in each well by using a phase contrast microscope (20× magnifications) equipped with QImaging Go-3 camera (QImaging, Canada). Neurite length was measured in at least 30 cells in randomly chosen fields by using image processor system Image-Pro Insight (Media Cybernetics, MD). The number of neurite outgrowths, defined as axon-like extensions that were double or more than the length of the cell body diameter was recorded. The percentage of neurite bearing cells (%) is the number of neurite bearing cells divided by the total number of cells in a field and then multiplied by 100%. At least three independent

experiments were conducted and results were expressed as mean ± standard deviation (S.D).

3.2.6. Fluorescence immunocytochemistry study

The axon-like extensions were confirmed as neurite outgrowth by immunofluorescence staining. N2a cells were seeded in 12-well µ-dishes (ibidi, Martinsried, Germany) and were exposed to treatments for 48 h. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7) for 20 min. After two washes with PBS, the cells were incubated with rabbit anti-neurofilament 200 polyclonal antibody (1:80 in 10% sheep serum as blocking buffer) for 1 h. The cells were washed and then incubated in a mixture of fluorescein isothiocyanate (FITC)-conjugated secondary antibody and sheep anti-rabbit IgG (1:160) in blocking buffer) for 2 h at room temperature in the dark. The cells were then washed three times. 4'-6-diamidino-2- phenylindole (DAPI) was used to counter stain the nuclei. Images were observed under a fluorescent microscope (Nikon Eclipse 80i microscope).

3.2.7 Evaluation of embryo- and/or neurotoxic effects of mushroom extracts

3.2.7.1 *MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay*

N2a and 3T3 cells (1×10^4) per well were seeded in 96-well plates. After incubation for 24 h, different concentrations of mushroom extracts (0 – 5 mg/ml) dissolved in phenol red free culture medium were added to each well. The samples were incubated for 24 h at 37 ℃ under humidified atmosphere of 5% CO_2 and 20 µl MTT (5 mg/ml) was added to each well. The crystal dyes taken up by the cells were then dissolved with DMSO. Absorbance was measured at 570 nm in a microplate reader (Tecan, Austria) using 630 nm as a reference wavelength. All measurements were done in triplicates, and at least three independent experiments were carried out. To calculate IC_{50} values which

estimated the concentration of mushroom extract that caused 50% inhibition of proliferation (viability) in N2a and 3T3 cells, Probit analysis was conducted using SPSS 17.0 (SPSS Science Inc., Chicago, IL).

3.2.7.2 *Neutral red uptake assay*

Neutral red medium (40 µg/ml) was prepared fresh before use by diluting the neutral red stock (4 mg/ml) with phenol red free culture medium. Neutral red medium was centrifuged at 1800 rpm for 10 min to remove any precipitated dye crystals before use. After cell seeding and treatment (0 – 5 mg/ml), the medium was discarded and replaced with equal amount of neutral red medium to each well of the plate. The plate was then incubated for 2 h. The neutral red medium was then removed and the cells were washed quickly with adequate amount of PBS. A total of 100 µl of neutral red solubilising solution (1% acetic acid in 50% ethanol) was added to each well and allowed to stand for 10 minutes at room temperature until the neutral red extracted from the cells reached a homogeneous solution. The absorbance at a wavelength of 540 nm with 690 nm of background absorbance was spectrophotometrically measured (Tecan, Austria). The experiment was repeated at least three different times.

3.2.7.3 *Lactate dehydrogenase (LDH) release assay*

After cell seeding and treatment with mushroom extracts (0 – 5 mg/ml), the culture plates were centrifuged at 1500 rpm for 5 minutes and 50 µl of supernatant was then transferred to a new plate for LDH analysis according to manufacturer instruction (Sigma). To each well, 100 µl of LDH mixture solution comprising of LDH assay substrate, dye and cofactor was added and incubated at room temperature for 30 min. The reaction was stopped by adding 10 µl of HCl (1 N) to each well. Absorbance was measured spectrophotometrically at 490 nm with background absorbance at 690 nm.

Triton X-100 (0.5%, Scharlau) was used as a positive control and was thus set to 0% viability representing a 100% cell death.

3.2.8 Statistical Analysis

All the experimental data are expressed as mean ± standard deviation (S.D). Statistical differences between groups were analysed and calculated by one-way analysis of variance (ANOVA) from at least three independent experiments. This was followed by Duncan's multiple range tests. $P < 0.05$ was considered to be significant between groups.

3.3 RESULTS

3.3.1 Determination of the optimum NGF concentration in neurite outgrowth of differentiating N2a cells

Nerve growth factor induced neurite outgrowth of N2a cells in a dose-dependent manner (Figure 3.1). After 48 h, the percentage of neurite bearing cells increased significantly ($p < 0.05$) to 26.1 ± 1.8% in N2a cells treated with 50 ng/ml NGF when compared to negative control (7.6 ± 2.5 %). At 60 ng/ml of NGF, the percentage of neurite bearing cells significantly decreased to 12.2 ± 2.1% ($p < 0.05$). Based on these findings, the optimised concentration of NGF of 50 ng/ml was selected as a positive control in the following studies unless otherwise stated.

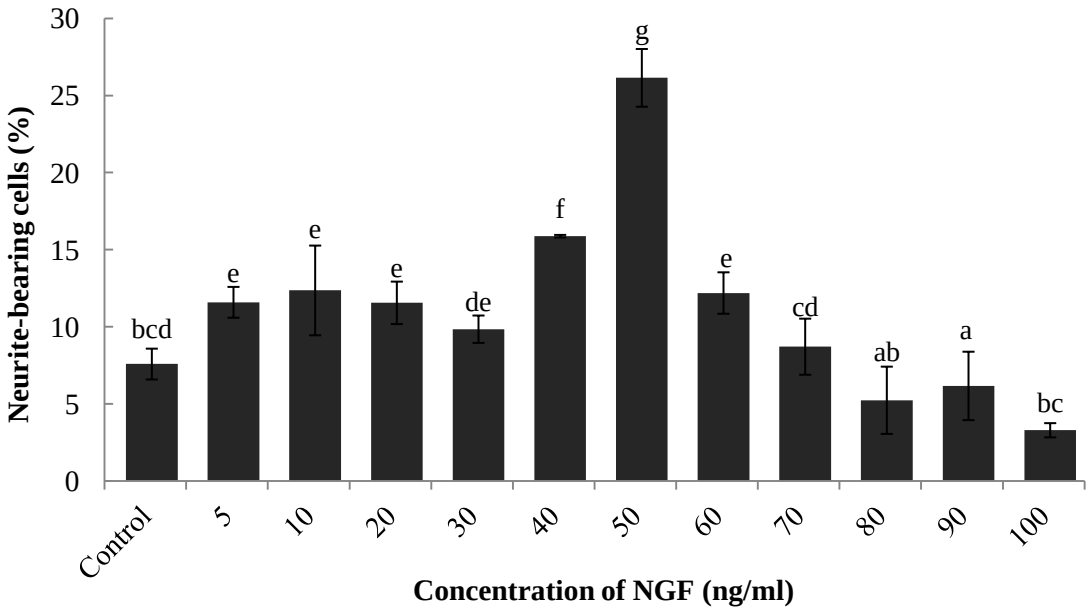


Figure 3.1: The effects of different NGF concentrations (5 – 100 ng/ml) on stimulation of neurite outgrowth using differentiating N2a cells as an *in vitro* model. The results shown represent the mean ± SD; n = 3. Means not sharing a common letter were significantly different at $p < 0.05$.

3.3.2 Neurite outgrowth of differentiating N2a cells promoted by different mushroom extracts

The yield of extracts from the mushrooms are summarised in Table 3.2. The positive control (NGF) recorded 26.4 ± 3.6% of neurite-bearing cells (Figure 3.2). The percentage of neurite bearing cells after treatment with aqueous extracts of *G. lucidum* (38.4 ± 4.2%), *L. rhinocerotis* (38.1 ± 2.6%), and ethanol extract of *C. militaris* (35.8 ± 3.4%) were significantly higher ($p < 0.01$) than NGF control by approximately 1.45-, 1.44- and 1.35-fold, respectively. Aqueous extracts of *G. frondosa* (33.7 ± 1.5%) and *P. giganteus* (33.4 ± 4.6%) were also shown to induce significantly ($p < 0.05$) higher neurite bearing cells compared to the NGF control. Meanwhile, the aqueous extracts of *L. rhinocerotis* mycelium, *H. erinaceus, G. neo- japonicum*, *P. pulmonarius*, as well as

ethanol extracts of *H. erinaceus*, *P. pulmonarius* and *P. giganteus* showed varied neurite outgrowth stimulatory effects with average neurite bearing cells ranging from 26.4 ± 5.4% to 29.6 ± 2.2%. Further, these extracts showed no significant difference when compared to NGF control. Among the plant extracts tested, wolfberry extract did not show any neurite outgrowth activity. The percentage of neurite bearing cells obtained after treatment with 20 μg/ml of ethanol extract of *G. biloba* (30.3 ± 2.5%) was higher than curcumin treated cells which yielded 26.4 ± 5.4% at 20 μg/ml.

Table 3.2: Yield of aqueous and ethanol extracts from the mushrooms and plants studied

Mushrooms	**Extract**	**Yield (%, w/w)**
Lignosus rhinocerotis (S)	Aqueous	3.56
Lignosus rhinocerotis (M)	Aqueous	4.65
Hericium erinaceus	Aqueous	8.56
Ganoderma lucidum	Aqueous	3.67
Ganoderma neo-japonicum	Aqueous	5.75
Grifola frondosa	Aqueous	3.54
Pleurotus pulmonarius	Aqueous	3.60
Pleurotus giganteus	Aqueous	6.70
Hericium erinaceus	Ethanol	4.50
Cordyceps militaris	Ethanol	3.45
Pleurotus pulmonarius	Ethanol	6.70
Pleurotus giganteus	Ethanol	5.30
Lycium barbarum (wolfberry)	Aqueous	9.76
Gingko biloba	Ethanol	n.d

(S) = sclerotium, (M) = mycelium. n.d. = not determined

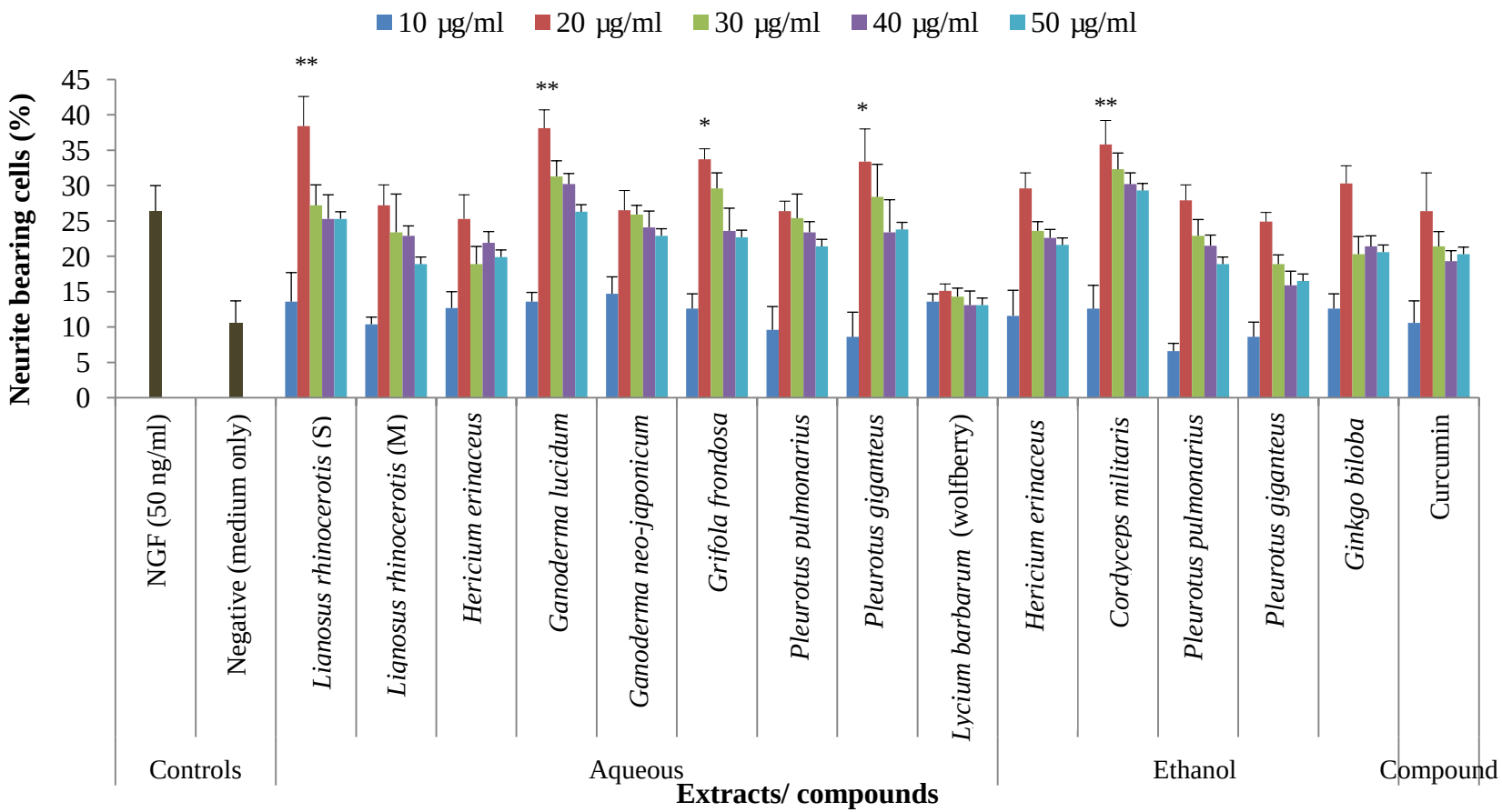


Figure 3.2: Percentage of neurite bearing cells after treatment with different concentrations of mushroom and plant extracts. * $p < 0.05$, ** $p < 0.01$ compared to the positive control (NGF). (S) = sclerotium, (M) = mycelium.

3.3.3 Mushroom extracts treatment increased neurite length in N2a

To qualify as a "neurite", the axon-like extension needs to be double or more than the cell body length of N2a. The average neurite length of NGF-stimulated cells was 78.58 ± 18.6 µm (Figure 3.3), which is approximately 4-time longer than the cell body. The mean diameter of N2a cell body was found to be 19.45 ± 0.72 µm. Cells treated with aqueous extract of *G. lucidum* were found to develop the longest mean neurite length i.e. 121.51 ± 28.6 µm (6.25-time longer than cell body), followed by aqueous extract of *P. giganteus* which recorded mean neurite length of 116.72 ± 29.5 µm (5.99-time longer than cell body).

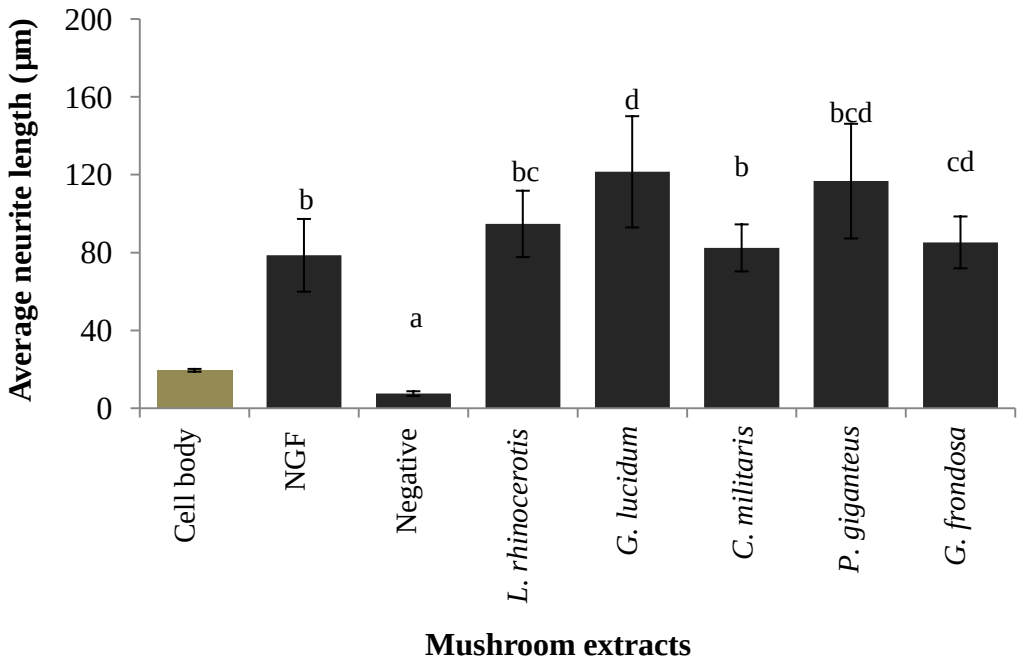


Figure 3.3: The mean neurite length of N2a cells treated with different mushroom extracts at 20 µg/ml. The results shown represent the mean ± SD; n = 3. Means not sharing a common letter were significantly different at $p < 0.05$.

3.3.4 Immunofluorescence staining of neurofilament

Figure 3.4 shows the morphology of differentiating N2a cells with neurites after 48 h of treatment with 50 ng/ml NGF (a) and 20 μg/ml of aqueous extracts of *G. lucidum* (b), *L. rhinocerotis* (c), *P. giganteus* (d) and *G. frondosa* (e); as well as ethanol extract of *C. militaris* (f). Neurofilament belongs to a class of intermediate filament found in neuronal cells that provides specific support for axons. There is a direct relationship between neurite outgrowth and neurofilament expression as neurofilament protein levels increase with differentiation of cell lines (Flaskos, Fowler, Teurtrie, & Hargreaves, 1999). While Plate 1A shows the phase contrast photomicrograph, Plate 1B shows the immunocytochemical labeling of neurons. The expression of neurofilament protein during neurite outgrowth was stained green while nuclei were stained blue.

3.3.5 The cytotoxic effects of mushroom extracts on 3T3 and N2a cells

Table 3.3 shows the results of cytotoxicity screening of mushroom and plant extracts to N2a cells and 3T3 fibroblasts. The cytotoxicity determination are expressed as IC_{50} values, which is the concentration resulting in 50% inhibition of cell growth and proliferation after 24 h exposure. All the extracts of mushrooms and plants did not show cytotoxic effects to the two tested cell lines ($IC_{50} \geq 1$ mg/ml in all cases). Three cytotoxicity assessments were used in this experiment, which include MTT, NR uptake assay and LDH release assay. Interestingly, IC_{50} determined by NRU test and LDH test was higher than that of MTT. In a general term, the ethanol extracts showed lower IC_{50} too.

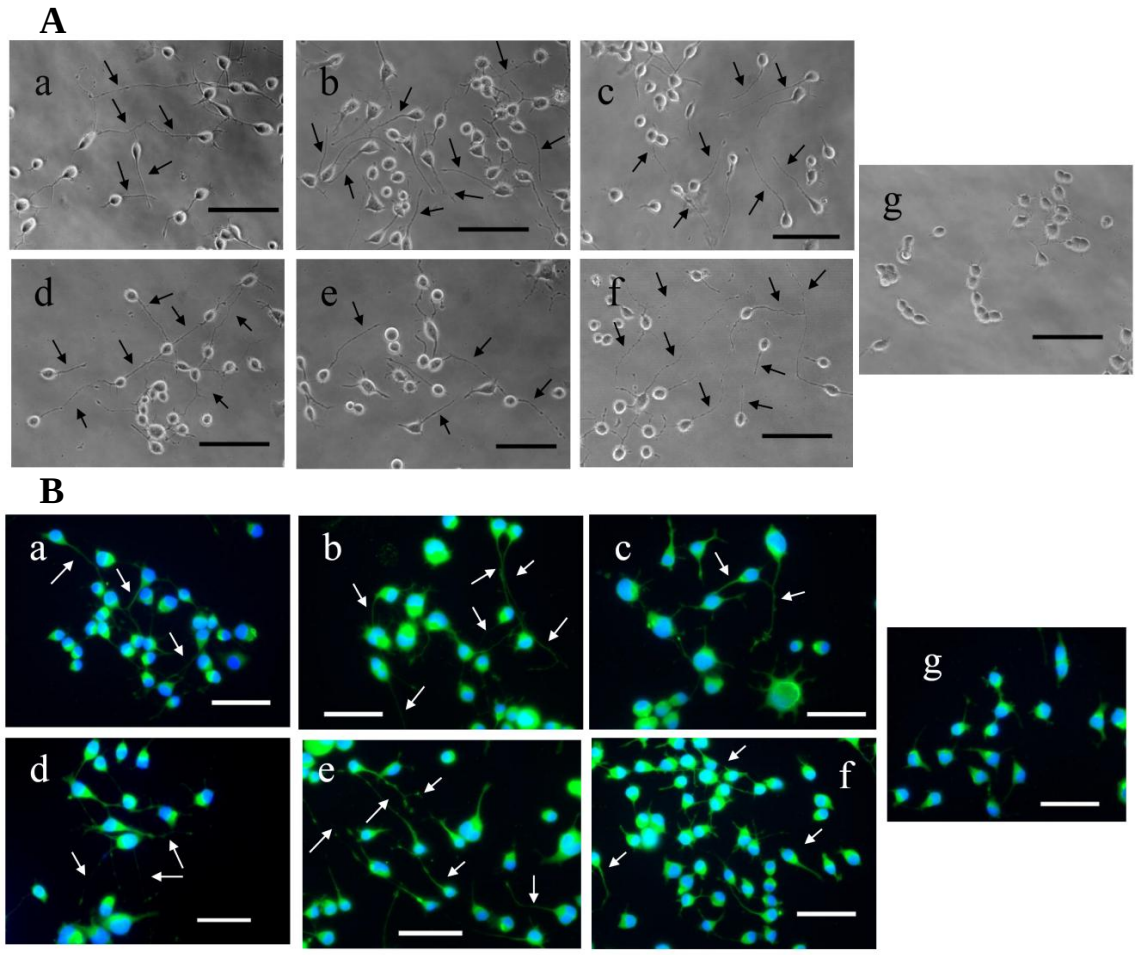


Figure 3.4: (A) Phase contrast photomicrographs showing the effects of (a) NGF, (b) *G. lucidum*, (c) *L. rhinocerotis*, (d) *P. giganteus*, (e) *G. frondosa*, and (f) *C. militaris* on the morphology of differentiating N2a cells after 48 h. Untreated cells serve as control (g) and only contained 5% FBS as vehicle. (B) Immunocytochemical staining of neurofilament in N2a cells. DAPI stains nuclei blue, while anti-neurofilament 200 kD labeled with FITC stains neuronal cells green. Arrows indicate neurite outgrowth. Photomicrographs of representative microscope fields were taken with a 20× objective. Scale bar represents 20 µm.

Table 3.3: Cytotoxic effects of extracts of mushrooms and plants assessed by different cytotoxicity assays- MTT, NRU and LDH release

Mushroom/Plant	Species	Extracts	Neuroblastoma 2a cells IC_{50} (mg/ml) after 24 h			3T3 Embryonic Fibroblast IC_{50} (mg/ml) after 24 h		
			MTT	NRU	LDH	MTT	NRU	LDH
Culinary/medicinal mushroom	*Pleurotus giganteus*	Aqueous	4.07 ± 0.67^{e}	6.95 ± 1.00^{g}	-	2.16 ± 0.05^{c}	2.67 ± 0.47^{d}	-
	Pleurotus pulmonarius	Aqueous	2.85 ± 0.06^{cd}	2.13 ± 0.87^{cdef}	-	1.75 ± 0.14^{bc}	2.60 ± 0.55^{d}	-
	Lignosus rhinocerotis (sclerotium)	Aqueous	3.27 ± 0.77^{d}	2.72 ± 0.65^{ef}	-	5.63 ± 0.06^{e}	5.93 ± 0.05^{f}	-
	Lignosus rhinocerotis (mycelium)	Aqueous	2.43 ± 0.12^{c}	1.75 ± 0.18^{abc}	-	5.23 ± 0.17^{e}	5.60 ± 0.1^{f}	-
	Hericium erinaceus	Aqueous	2.60 ± 0.52^{cd}	2.85 ± 0.56^{e}	-	3.43 ± 0.29^{d}	3.53 ± 0.06^{e}	-
	Ganoderma lucidum	Aqueous	1.35 ± 0.03^{a}	1.52 ± 0.17^{ab}	2.20 ± 0.52^{ab}	1.19 ± 0.06^{ab}	1.35 ± 0.02^{ab}	1.50 ± 0.08^{a}
	Ganoderma neo-japonicum	Aqueous	1.17 ± 0.006^{a}	1.11 ± 0.03^{a}	2.00 ± 0.53^{ab}	1.47 ± 0.37^{ab}	1.78 ± 0.56^{bc}	1.58 ± 0.44^{a}
	Grifola frondosa	Aqueous	2.72 ± 0.08^{cd}	4.60 ± 1.10^{f}	-	6.67 ± 0.87^{f}	7.60 ± 0.1^{g}	-
	Pleurotus giganteus	Ethanol	2.43 ± 0.10^{c}	2.81 ± 0.15^{e}	5.8 ± 0.10^{c}	1.66 ± 0.56^{bc}	1.96 ± 0.42^{c}	1.73 ± 0.48^{a}
	Pleurotus pulmonarius	Ethanol	2.30 ± 0.72^{bc}	2.64 ± 0.16^{def}	2.53 ± 0.96^{bc}	1.27 ± 0.06^{ab}	1.48 ± 0.05^{abc}	1.67 ± 0.12^{a}
	Hericium erinaceus	Ethanol	2.47 ± 0.50^{c}	1.61 ± 0.51^{ab}	2.10 ± 0.72^{ab}	1.31 ± 0.03^{ab}	1.84 ± 0.57^{bc}	1.47 ± 0.31^{a}
	Cordyceps militaris	Ethanol	1.65 ± 0.015^{ab}	1.81 ± 0.10^{abcd}	2.37 ± 0.21^{b}	1.02 ± 0.08^{a}	1.10 ± 0.02^{a}	1.40 ± 0.29^{a}
Plants	*Lycium barbarum*	Aqueous	4.37 ± 1.00^{e}	6.96 ± 0.73^{g}	-	8.40 ± 0.72^{g}	7.86 ± 0.06^{g}	-
	Gingko biloba	Ethanol	1.56 ± 0.08^{ab}	1.66 ± 0.11^{ab}	1.43 ± 0.39^{a}	1.56 ± 0.58^{abc}	1.37 ± 0.07^{ab}	1.78 ± 0.07^{a}
	Curcumin	Compound	1.13 ± 0.01^{a}	1.15 ± 0.01^{a}	1.30 ± 0.16^{a}	1.16 ± 0.06^{ab}	1.23 ± 0.03^{ab}	1.60 ± 0.22^{a}

The data represent the mean $\pm$ SD of three determinations. Means not sharing a common letter were significantly different at $p < 0.05$.

3.4 DISCUSSIONS

Eight species of medicinal mushrooms were investigated and categorised into two groups: culinary and non-culinary. The former group represents mushrooms that can be used for culinary purposes like preparing meal and cooking, especially from the basidiocarps. This group (culinary-medicinal mushrooms) comprised of *P. giganteus*, *P. pulmonarius, H. erinaceus*, and *G. frondosa*. Medicinal mushrooms that do not exhibit culinary properties are appreciated for their pharmacological merits and are not cooked as a meal. The basidiocarps or sclerotia are often handpicked, ground to powder and subjected to various extraction methods before being used as a traditional medication (Tibuhwa, 2012). *Lignosus rhinocerotis, G. lucidum, G. neo-japonicum,* and *C. militaris* are examples of non-culinary medicinal mushrooms.

Ganoderma lucidum (also known as Lingzhi in Chinese or Reishi in Japanese) has been widely investigated for its potential therapeutic benefits and longevity. The results showed that the aqueous extract of *G. lucidum* promoted neurite outgrowth in N2a cells with 38.4 ± 4.2% increase in neurite bearing cells. This agrees with the finding of Cheung et al (2000) who showed that *Ganoderma* extract contained NGF-like compounds that mediated the neuronal differentiation and elongation of rat pheochromocytoma (PC12) cells. The *Ganoderma* neuroactive constituents that accounted for neurite outgrowth activity are triterpenoids, such as lucidenic acid (Connolly & Hill, 2003), 7-oxo-ganoderic acid Z, ganolucidic acid A, methyl ganoderic acid A, ganoderic acid S1, and 4,4,14α-trimethyl-5α-chol-7,9(11)-dien-3-oxo-24-oic acid (Zhang et al., 2011). Further, the water-soluble polysaccharides of *G. lucidum* were shown to significantly ($p < 0.05$) reduce neuronal cell death and apoptosis of rat primary cortical neurons (model of brain cerebral ischemia) induced by oxygen/glucose deprivation treatment (Zhou et al., 2010). *Ganoderma* extracts may also

provide mitigation to Parkinson's disease as it was shown to prevent dopaminergic neuron degeneration by attenuating the pro-inflammatory response of microglial cells (R. Zhang et al., 2011).

The tiger's milk mushroom, *L. rhinocerotis*, has been described as the national treasure of Malaysia since this macrofungus is rare and is often used as folk remedy to treat a variety of diseases. Consistent with the previous study (Eik et al., 2012), the sclerotia of *L. rhinocerotis* improved neurite outgrowth in N2a cells. Interestingly, in the present study, the sclerotial extract (38.4 ± 4.2% of neurite bearing cells) performed better than the mycelial extract (27.2 ± 2.9%). However, the active components in *L. rhinocerotis* that play the role in neurite outgrowth activity need further investigation. The protein or carbohydrate/protein complex of the cold water scerotial extract (4^{o}C) is responsible for the antiproliferative activity against human breast and lung carcinoma (M. L. Lee et al., 2012). In this study, a hot water extraction approach was employed and it is hypothesised that polysaccharides or triterpenoids rather than peptides are involved in the neurite outgrowth stimulatory activity of *L. rhinocerotis.*

The medicinal properties of *P. giganteus* are comparatively unknown when compared to *P. pulmonarius* (grey oyster mushroom). *Cordyceps militaris* is a parasitic fungus that colonises moth larvae (Lepidoptera) and has been valued in Traditional Chinese Medicine for more than 2000 years. The major bioactive components in *C. militaris* include adenosine, cordycepin and polysaccharides (Yu, Wang, Huang, & Duh, 2006). The finding is in agreement with the work by Lee et al (2011) where methanol extract of *C. militaris* was shown to significantly reverse the scopolamine-induced deficit in the memory of rat and improve neurite outgrowth in N2a cells. Similar to the results in this study, lysophosphatidylethanolamine isolated from *G. frondosa* (Maitake) was also reported to induce neuronal differentiation in PC12 cells,

causing up-regulation of neurofilament M expression of PC12 cells (Nishina et al., 2006).

It is well known that N2a cells, upon the withdrawal of serum, differentiate and elaborate neurites (X. Wang et al., 2011; Z. Wang et al., 2011). This well-defined neuronal model is often employed for studies relating to neuronal differentiation (Wasilewska-Sampaio et al., 2005). It is also a popular cell line in studying neurotoxicity as the brain is the first target in situations such as ageing and neurodegenerative diseases (Radio & Mundy, 2008). NGF is the most appropriate positive control in neurite outgrowth assays as its role in neural development have been characterised extensively (Sofroniew, Howe, & Mobley, 2001). The therapeutic application of neurotrophins like nerve growth factor (NGF) is not possible as NGF cannot penetrate the blood-brain barrier. Studies indicate that lower-molecular-weight molecules may be a promising alternative for therapeutic intervention, for example, α-phenyl-N-tert-butylnitron (Tsuji, Inanami, & Kuwabara, 2000). However, most of the experiments testing natural products have been conducted *in vitro*, and few studies evaluated these compounds in the brain *in vivo*. It is anticipated that mushroom extracts (comprising of neuroactive molecules) under certain condition (serum deprivation) participate in triggering NGF signals, hence activating the downstream neuronal responses to axonal growth (W. Zhang & Liu, 2002).

It is important that functional and health food remedies recommended for the prevention or management of diseases undergo safety assessment. Today, a number of natural products with potential biomedical application are being launched, although some could be potentially toxic when ingested at high doses or in combination with other medications (Maria, Lopez, Diaz, & Alba, 1997). The results indicated that at the concentrations tested, there were no cytotoxic effects to the cells. Notably, ethanol extract of *C. militaris* showed the lowest IC_{50} ($p < 0.05$) value against 3T3 fibroblast.

Similarly, curcumin although was reported beneficial in neuroprotection (F. Yang et al., 2005), the IC_{50} value detected against N2a was the lowest by means of MTT, NRU and LDH assays. Elsewhere, *in vivo* toxicity evaluation of *Ganoderma boninense* (Pat.) was carried out (Sasidharan, Jinxuan, Latha, & Amutha, 2011) and a significant toxicity (IC_{50} = 640 μg/ml) against *Artemia salina* (brine shrimp) was demonstrated after 24 h. However, *Ganoderma* extract is granted safe on short-term exposure. Conversely, *in vivo* toxicity profiling of total triterpene fraction from *G. lucidum* against Swiss albino mice showed that ganoderma triterpenes did not possess significant toxicity (Smina, Mathew, Janardhanan, & Devasagayam, 2011) and administration of *G. lucidum* β–glucan (2000 mg/kg body weight/day) to Sprague Dawley rats did not cause toxicological abnormality (S. N. Chen et al., 2011). Mutagenicity studies by means of *Salmonella typhimurium* also did not reveal any genotoxicity. Meanwhile, sub-acute toxicity study of the sclerotial powder of *L. rhinocerotis* by using rat model showed no treatment-related toxicity at 1000 mg/kg (Shien, Hong, Yee, Pailoor, & Mui, 2011).

More than one cytotoxicity assay namely MTT, NRU, and LDH release assay was chosen in this study to determine the *in vitro* cell viability in order to increase the reliability of the results obtained and also to avoid over- or underestimation of the mushroom or plant toxicity. The mechanisms of each of the chosen assays are different. While MTT is based on the enzymatic conversion of MTT in the mitochondria, NRU assay is based on the dye uptake capability by lysosomes (Weyermann, Lochmann, & Zimmer, 2005). Both served as colorimetric assays, whereby viable and uninjured cells stain blue and red, for MTT and NRU assays, respectively. LDH release assay, on the other hand is based on the release of the enzyme into the culture medium after the disruption of cell membrane (Fotakis & Timbrell, 2006). It is noteworthy that the toxicity profiles detected by the three different assays generally followed a similar trend although some results were not in agreement. For instance, no IC_{50} values were

recorded for some mushroom extracts by using LDH assays, suggesting that LDH may be the least sensitive method among the three.

3.5 CONCLUSION

The extracts of *G. lucidum*, *L. rhinocerotis*, *P. giganteus*, *G. frondosa* and *C. militaris* showed potential in promoting neurite outgrowth of differentiating N2a cells. The synergism of the various active entities in these mushroom extracts may be responsible for the neurite outgrowth activity. This study also showed the absence of embryotoxic and neurototoxic effects of the various mushroom extracts in 3T3 and N2a cells, respectively. *Pleurotus giganteus* was selected for further study since the study has shown for the first time its neurite outgrowth activity which was 33.4 ± 4.6% of neurite bearing cells. The mushroom extract also triggered mean N2a neurite length of 116.72 ± 29.5 µm. Further, *P. giganteus* was less studied when compared to the other mushrooms for its neuritogenic properties. Coming from a *Pleurotus* genus, this mushroom is edible and culinary; therefore, it would be interesting to diversify what is on the table and to develop *P. giganteus* as a functional food which may serve as a brain food.

CHAPTER IV

NUTRITIONAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF *PLEUROTUS GIGANTEUS*, A POTENT NEURONAL-HEALTH PROMOTING MUSHROOM

4.1 INTRODUCTION

Age-related neurodegenerative diseases such as Alzheimer's involved a programmed apoptotic neuronal death triggered as a result of NGF depletion and oxidative stress exerted by reactive oxygen species (ROS) (Liochev, 2013). Strategies aimed at preventing neuronal loss and preserving and/or restoring the neurite network, are crucial in preventing and treating neurodegenerative diseases. Dietary intake of antioxidants, therefore, is important to improve the intrinsic antioxidant mechanisms (superoxide dismutase, catalase, and glutathione peroxide) to avoid an environment where pro-oxidant species overwhelm antioxidant species.

Studies have shown that there is a positive correlation between the neurite outgrowth stimulatory effects of a molecule and its antioxidant capacity. Curcumin is a natural product widely used in India since ancient days to treat various ailments. The high antioxidant activity has made it an effective neuroprotective and neuritogenic agent to prevent and/or treat multiple neurological disorders (Liao et al., 2012). Green tea represents a popular beverage across Asian countries. The polyphenols in green tea possess radical scavenging and iron chelating properties (Biasibetti et al., 2013). Evidence indicates that the antioxidants, e.g. catechin, epicatechin, and epigallocatechin gallate (EGCG) present in green tea is able to regulate APP processing, protect neurons from apoptosis, and stimulate neurite outgrowth *via* various signaling pathways (Jeon, Bae, Seong, & Song, 2003; Reznichenko, Amit, Youdim, & Mandel, 2005).

In recent times, amounts of consumed mushrooms have risen greatly. Mushrooms are valued not only for their nutritional properties, but their health properties. Recently, the phenolics in mushrooms have attracted much attention due to their strong antioxidant activity. The phenolics includes caffeic acid, catechin, chlorogenic acid, *p*-coumaric acid, ferulic acid, gallic acid, gentisic acid, p-hydroxybenzoic acid, homogentisic acid, myricetin, protocatechuic acid, and pyrogallol (Palacios et al., 2011). Further, mushrooms (*Boletus edulis*, *Pleurotus ostreatus,* among others), are known to contain high concentrations of ergothioneine which was reported to have a high antioxidant potency (Ey, Schömig, & Taubert, 2007).

There are ample studies available in the literature regarding the chemical composition of different mushroom species from all over the world. However, such information on *P. giganteus*, an edible mushroom found in China, Thailand, and Sri Lanka is scanty. Moreover, in this study *P. giganteus* has been shown to promote neurite outgrowth (Phan, David, Naidu, Wong, & Sabaratnam, 2013; Phan, Wong, David, Naidu, & Sabaratnam, 2012), it would be interesting to analyse its various nutritional attributes and its antioxidant properties. Besides, it is important to ensure a consistency of nutritional composition between the wild mushroom and the domesticated one. In this study, an intra-strain comparison (the domesticated wild strain versus the commercial strain of *P. giganteus*) on their various nutritional components including protein, carbohydrates, sugar profile, vitamins, fatty acids, and amino acids; was carried out. The cultivation conditions were kept constant. Besides, the phenolic and flavonoid contents, as well as antioxidant activities of the extracts, were evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Mushroom

Fresh basidiocarps of *P. giganteus* (commercial strain KLU-M 1227) were collected from Nas Agro Farm and Dong Foong Biotech. Wild *P. giganteus* (KLU-M 1228) collected from Ayer Hitam Forest Reserve, Puchong, Malaysia was domesticated and cultivated. The mushroom identity (molecular finger-printing) was authenticated by Dr. Yee-Shin Tan from Mushroom Research Centre, University of Malaya. Voucher specimens were deposited in the Herbarium of University of Malaya, Kuala Lumpur.

4.2.2 Cultivation of *P. giganteus*

Pleurotus giganteus (KUM61102) was maintained on potato dextrose agar (PDA) at 4 - 10 ℃ and regularly subcultured. The substrate formulation for the cultivation of *P. giganteus* is similar to that for oyster mushroom cultivation, i.e. 89 - 94% (w/w) rubber wood sawdust, 5 - 10% (w/w) rice bran and 1% (w/w) calcium carbonate. Polypropylene bags are used for substrate bagging and the moisture content in the substrate was kept at 60% - 65%. The temperature for mycelia growth, spawn run, and fruiting body formation is 26 - 32 ℃. Relative humidity of 70% and 80 - 90% during mycelia growth and fruiting; respectively, should be maintained. Direct illumination should be avoided as it has been reported to inhibit the fruiting body formation. A 20-day cycle after complete colonization of the artificial log is needed for each harvest and about four harvests (a total yield of 280 g) can be obtained from each bag of 900 g (Nas Agro Farm, personal communication).

4.2.3 Proximate analysis of basidiocarps of *P. giganteus*

The freeze-dried powder of basidiocarps of *P. giganteus* was analyzed for the nutritional components using the standard American Oil Chemists' Society (AOCS) procedures (AOAC, 1995). Total fat, dietary fibre, and protein content were analyzed using AOAC 989.05, AOAC 985.25, and Kjeldahl method with boric acid modification; respectively. Carbohydrates were calculated using the formula carbohydrates (g) = 100 - (protein+ fat + ash). Energy was calculated using the formula energy (kcal) = 4 × (protein + carbohydrate) + 9 × (fat) (AOAC, 1995).

4.2.4 Determination of free sugars, minerals, vitamins, fatty acids, and amino acids

Free sugars and amino acids were analysed by high performance liquid chromatography (HPLC). Minerals were determined using inductively coupled plasma optical emission spectrometry ICP-OES following the AOAC 985.01 and 922.02, as well as American Association of Cereal Chemists (AACC 40-70) procedures. Vitamin C was determined by the AOAC 967.21 method. Preparation of methyl esters of long-chain fatty acids was carried out based on AOCS Ce-2-66 test for further analysis by gas chromatography (GC). Chromatography system (Agilent Technologies 6890N) equipped with an Inert Mass Selective Detector (Agilent Technologies 5975) (70eV direct inlet) on a HP-5ms (5% phenyl methyl siloxane) capillary column (30 m × 250 µm × 0.25 µm) initially set at 150 ℃, then increased at 5 ℃ per min to 300 ℃ and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL per min. The total ion chromatogram obtained was autointegrated by chemstation and the constituents were identified by comparison with the accompanying mass-spectra database (Wiley 9th edition with NIST 11 Mass Spectral Library, USA) wherever possible. Omega-3 and Omega-6 fatty acids were analyzed using AOCS 1d-91 methods by capillary gas-liquid chromatography (GLC).

4.2.5 Preparation of *P. giganteus* extracts

The aqueous and ethanol extracts were prepared as described in section 3.2.3.

4.2.6 Determination of total polysaccharides in *P. giganteus* extracts

The total polysaccharide content of the aqueous and ethanol extracts was determined using the phenol-sulphuric acid method with $_D$-glucose as a reference (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). Briefly, 1 mL of 5% phenol was added to 1 mL of sample solution, followed by 5 mL of concentrated H_2SO_4. The absorbance was measured after 10 min at 497 nm.

4.2.7 Determination of total phenolic contents (TPC) in *P. giganteus* extracts

The total phenolic contents in the mushroom extracts, expressed as gallic acid equivalents (GAEs), were determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). Fifty microliter of sample was mixed with an equal volume of Folin–Ciocalteu phenol reagent. After 3 min, 100 µL of Na_2CO_3 (10%) was added to the mixture. The reaction was kept in the dark for 90 min, after which the absorbance was read at 750 nm using a spectrophotometer. A calibration curve was prepared with different concentrations of gallic acid (0 - 100 µg/mL) as standard. TPC was expressed as mg GAE/ g of extract.

4.2.8. Determination of total flavonoids in *P. giganteus* extracts

Total flavonoids in the mushroom extract were estimated by using the aluminum calorimetric method (Iqbal Ismail, Chan, Adam Mariod, & Ismail, 2010). Mushroom extract of 150 µL was mixed with an equal volume of aluminium chloride, $AlCl_3$ (2%). After 10 min, the absorbance of the supernatant was measured at 435 nm by using an ELISA microplate reader (Sunrise, Austria). The total flavonoid content of the

mushroom extract was expressed as rutin equivalents in microgram per gram extract (mg RE/g extract).

4.2.9 Evaluation of antioxidant activity of *P. giganteus* extracts

4.2.9.1 DPPH scavenging activity assay

The DPPH free radical scavenging activity was determined as previously described (Gorinstein et al., 2005). Mushroom extracts of various concentrations (5 µL) was mixed with 195 µL of DPPH reagent. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm using a microtiterplate reader (Tecan, Austria). The radical scavenging activity was calculated using the following equation: % radical scavenging activity = (Abs $_{Blank}$ - Abs $_{Sample}$) / Abs $_{Blank}$ × 100%] where Abs $_{Sample}$ is the absorbance of the sample whereas Abs $_{Blank}$ is the absorbance of the DPPH solution. The antioxidant property of the extracts was expressed in terms of IC_{50} value that is the concentration to quench 50% of available DPPH content. L-ascorbic acid (0-25µM) was used as standard and butylated hydroxytoluene (BHT) was used as control.

4.2.9.2 FRAP (ferric reducing antioxidant power) assay

The reducing power of mushrooms extracts was determined by the ferric reducing antioxidant potential (FRAP) assay as previously described (Benzie & Strain, 1999). To prepare FRAP reagent, 2.5 mL of FeCl3•6H_2O solution (20 mM in 40 mM HCl) was mixed with 2.5 mL of 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) (10 mmol/L in 300 mmol/L acetate buffer). Then, 10 µL of mushroom extract was added to 300 µL of the FRAP reagent after which the absorbance of the product of the reaction between Fe^{2+} and TPTZ was measured at 593 nm against a blank for each sample. Ferrous sulfate

($FeSO_4$) of concentrations 0-20 mM was used as standard and BHT as control. The FRAP value was expressed as μM of $FeSO_4$ equivalents/g mushroom.

4.2.9.3 Inhibition of lipid peroxidation

The assay was based on the thiobarbituric acid reaction method (Kuppusamy, Indran, & Balraj, 2002). Mushroom extracts of different concentrations (0 - 20 mg/mL) was mixed with 0.5 mL of egg yolk suspension and 0.5 mL of $FeSO_4$. The mixture was incubated at 37 ℃ for an hour after which 0.5 mL of 20% trichloroacetic acid (TCA) and 1 mL of 0.8% thiobarbituric acid (TBA) were added. The mixture was then heated in boiling water for 15 min and centrifuged at 3500 rpm for 20 min. The absorbance of thiobarbituric acid reactive substances (TBARS) present in the supernatant was measured at 532 nm using a microtiterplate reader (Tecan, Austria). Result was expressed as percentage inhibition of lipid peroxidation at extract concentration of 10 mg/ml. BHT was used as a control in this assay.

4.3 RESULTS

4.3.1 Proximate analysis, determination of sugars, minerals; and vitamins in *P. giganteus*

The proximate nutritional components of commercial *P. giganteus* (KLU-M 1227) and domesticated wild *P. giganteus* (KLU-M 1228) are shown in Table 4.1. The commercial strain presented a significantly higher ($p < 0.05$) carbohydrate, dietary fibre, total fat, and monosaturated fat content compared to the domesticated wild strain by 3.7, 2.7, 16, and 2.1%; respectively. Both strains showed no difference in the gross energy value and saturated fat contents. However, the wild strain presented a significantly higher ($p < 0.05$) crude protein and polyunsaturated fat content.

The sugar composition of *P. giganteus* basidiocarps is also given in Table 4.1. Glucose and fructose were detected in the basidiocarps of both strains. The glucose and fructose content of the domesticated wild strain was approximately 47% and 26% higher ($p < 0.05$) than that of the commercial strain. For macroelements composition, potassium in the basidiocarps of commercial strain (13.46 $\pm$0.0 g kg^{-1}) was significantly higher ($p < 0.05$) than the domesticated wild strain (11.71 $\pm$0.32 g kg^{-1}). On the other hand, the calcium level in the domesticated wild strain (0.087 $\pm$0.01 g kg^{-1}) was higher than that of the commercial strain (0.058 $\pm$ 0.0 g kg^{-1}). The vitamin profiles showed similarity but the concentrations in the two strains were different. Vitamin C (ascorbic acid) in the wild strain was almost 3.9-fold higher than that of the commercial strain (Table 4.1). Vitamin B3 (niacin) is the most abundant vitamin found in this mushroom with 0.09 $\pm$0.10 and 0.06 $\pm$0.02 g kg^{-1} in the commercial and domesticated wild strain; respectively.

Table 4.1: Chemical compositions, sugars, macro-, microelements, and vitamins of commercial *Pleurotus giganteus* (KLU-M 1227) and wild *P. giganteus* (KLU-M 1228)

Parameter	*Pleurotus giganteus* (commercial strain)	*Pleurotus giganteus* (wild strain)	Recommended daily allowance (RDA)
Carbohydrate (g kg^{-1})	672 ± 0.00[a]	647 ± 0.00[b]	300 g
Protein (g kg^{-1})	154 ± 0.00 [c]	192 ± 0.00 [d]	50 g
Energy (kcal kg^{-1})	3640 ± 0.00 [e]	3640 ± 0.00 [e]	-
Dietary fiber (g kg^{-1})	333.5 ± 0.07 [f]	324.5 ± 0.07 [g]	25 g
Total fat (g kg^{-1})	37.0 ± 0.00 [h]	31.0 ± 0.00 [i]	65 g
Saturated fat	9.7 ± 0.00 [j]	9.5 ± 0.00 [j]	-
Monosaturated fat	19.7 ± 0.00 [k]	13.2 ± 0.00 [l]	-
Polyunsaturated fat	7.8 ± 0.00 [m]	8.3 ± 0.00 [n]	-
Trans fat	ND	ND	-
Cholesterol (g kg^{-1})	ND	ND	300 mg
Free sugars (g kg^{-1})			
Fructose	7.2 ± 0.2 [a]	10.6 ± 0.4 [b]	-
Glucose	31.4 ± 0.7 [c]	39.7 ± 0.2 [d]	-
Sucrose	ND	ND	-
Lactose	ND	ND	-
Maltose	ND	ND	-
Maltotriose	ND	ND	-
Macroelements (g kg^{-1})			
Potassium (as K)	13.46 ± 0.00 [a]	11.71 ± 0.32 [b]	3.5 g
Phosphorus (as P)	5.27 ± 0.39 [c]	4.01 ± 0.04 [d]	0.7 g
Magnesium (as Mg)	0.67 ± 0.00 [e]	0.65 ± 0.00 [f]	0.4 g
Calcium (as Ca)	0.058 ± 0.00 [g]	0.087 ± 0.01[h]	1.0 g
Sodium (as Na)	0.058 ± 0.07 [g]	0.047 ± 0.00 [i]	2.4 g
Microelements (g kg^{-1})			
Iron (as Fe)	0.019 ± 0.04 [a]	0.014 ± 0.00 [b]	18 mg
Zinc (as Zn)	0.027 ± 0.01 [c]	0.042 ± 0.00 [d]	15 mg
Manganese (as Mn)	0.041 ± 0.01 [e]	0.043 ± 0.00 [e]	2.0 mg
Copper (as Cu) (mg kg^{-1})	0.60 ± 0.01 [f]	0.228 ± 0.00 [f]	2.0 mg
Selenium (as Se) (mg kg^{-1})	ND	ND	70 µg
Vitamins (g kg^{-1})			
Vitamin B1	0.004 ± 0.01 [a]	0.002 ± 0.00 [b]	1.4 mg
Vitamin B2	0.009 ± 0.00 [c]	0.009 ± 0.00 [c]	1.6 mg
Vitamin B3	0.09 ± 0.10 [d]	0.06 ± 0.02 [e]	14 - 16 mg
Vitamin C	0.003 ± 0.00 [a]	0.001 ± 0.01 [f]	75 - 90 mg

Each values are expressed as mean ± SD (n = 3). In each row, the different letters represent significant differences between samples ($p < 0.05$). ND: Not detectable.

4.3.2 Determination of amino acids in *P. giganteus*

All the essential amino acids (threonine, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan, lysine, histidine, and arginine) were detected in this mushroom with the exception of tryptophan (Table 4.2). Among the essential amino acids, leucine (commercial strain 20.4 ± 0.10 g kg^{-1}; domesticated wild strain 19.4 ± 0.0 g kg^{-1}) was found to be the largest constituent, followed by phenylalanine, and histidine. The methionine, isoleucine and valine were present in small amounts ranging from 0.31-0.80% for both the *P. giganteus* strains. In particular, *P. giganteus* had the highest concentration of glycine, which is categorized as the non-essential amino acid. Also, the wild strains exhibited higher amount of glutamine, alanine, and asparagine as compared to the commercial strains.

4.3.3 Determination of fatty acids in *P. giganteus*

Table 4.3 presents the fatty acid profile of basidiocarps of *P. giganteus*. Among the saturated fatty acids, palmitic acid (C16:0) was predominant with 3.8 ± 0.01 and 3.3 ± 0.0 g kg^{-1} in the commercial strain and wild strain; respectively. Amongst the polyunsaturated fatty acids, oleic acid was the predominant fatty acid in this species. Oleic acid (C18: 1n9c) in the commercial strain (10.3 ± 0.06 g kg^{-1}) was significant ($p < 0.05$) higher than that in the wild strain (8.9 ± 0.0 g kg^{-1}). Linoleic acid was also present in significant quantities (4.5 – 5.0 g kg^{-1}) in *P. giganteus*. The medium chain fatty acid, caprylic acid (C8:0) and the long chain fatty acid, stearic acid (C18:0) were also present. Capric, undecanoic, palmitoleic and eicosadienoic acids were detected in trace amounts in *P. giganteus.*

Table 4.2: Amino acid content (g kg^{-1} dry weight) of *Pleurotus giganteus* KLU-M 1227 and KLU-M 1228

Amino acids	*Pleurotus giganteus* (commercial strain)	*Pleurotus giganteus* (wild strain)
Asp	6.9 ± 0.00 ^{c}	7.0 ± 0.00^{c}
Glu	15.7 ± 0.00 ^{jk}	17.2 ± 0.10 ^{l}
Ser	11.1 ± 0.01 ^{e}	11.0 ± 0.01 ^{e}
His*	15.4 ± 0.04 ^{ji}	15.1 ± 0.05 ^{j}
Gly	29.0 ± 0.11 ^{p}	29.5 ± 0.04 ^{p}
Thr*	5.3 ± 0.01 ^{b}	5.2 ± 0.00 ^{b}
Arg*	11.4 ± 0.04 ^{ef}	12.1 ± 0.00 ^{fg}
Ala	11.1 ± 0.01 ^{e}	13.5 ± 0.00 ^{h}
Tyr	5.8 ± 0.06 ^{b}	5.9 ± 0.00 ^{b}
Cys	ND	ND
Val*	8.0 ± 0.01 ^{d}	7.1 ± 0.00 ^{c}
Met*	3.1 ± 0.01 ^{a}	3.1 ± 0.00 ^{a}
Phe*	16.4 ± 0.09 ^{kl}	16.5 ± 0.00 ^{kl}
Ile*	7.7 ± 0.02 ^{cd}	7.3 ± 0.00 ^{cd}
Leu*	20.4 ± 0.10 ^{n}	19.4 ± 0.00 ^{m}
Lys*	14.5 ± 0.01 ^{i}	12.8 ± 0.00 ^{gh}
Asn	15.2 ± 0.07 ^{ij}	24.6 ± 0.00 ^{o}
Gln	ND	ND
Nva	ND	ND
Trp*	ND	ND

Asp (Aspartate); Glu (Glutamate); Ser (Serine); His (Histidine); Gly (Glycine); Thr (Threonine); Arg (Arginine); Ala (Alanine); Tyr (Tyrosine); Cys (Cystine); Val (Valine); Met (Methionine); Phe (Phenylalanine); Ile (Isoleucine); Leu (Leucine); Lys (Lysine); Asn (Asparagine); Gln (Glutamine); Nva (Norvaline); Trp (Tryptophan). Each values are expressed as mean ± SD (n = 3). In each row, the different letters represent significant differences between samples ($p < 0.05$). ND: Not detected. * Essential amino acids.

Tabe 4.3: Fatty acids (g kg^{-1} dry weight) detected in the basidiocarps of *Pleurotus giganteus*

	Fatty acid	*Pleurotus giganteus* (commercial strain)	*Pleurotus giganteus* (wild strain)
C8:0	Caprylic	1.3 ± 0.00 [a]	1.1 ± 0.00 [ab]
C10:0	Capric	0.4 ± 0.00 [c]	0.4 ± 0.00 [c]
C11:0	Undecanoic	0.8 ± 0.00 [b]	0.8 ± 0.00 [b]
C16:0	Palmitic	3.8 ± 0.00 [d]	3.3 ± 0.00 [e]
C16:1	Palmitoleic	0.1 ± 0.00 [c]	0.1 ± 0.00 [c]
C18:0	Strearic	1.1 ± 0.03 [ab]	0.8 ± 0.00 [b]
C18:1n9c	Oleic ***	10.3 ± 0.06 [g]	8.9 ± 0.00 [h]
C18:2n6c	Linoleic **	5.0 ± 0.01 [f]	4.5 ± 0.00 [f]
C20:2	Eicosadienoic **	0.3 ± 0.01 [c]	0.3 ± 0.00 [c]
C22:0	Beheric	0.1 ± 0.0 [c]	0.1 ± 0.00 [c]
C24:0	Lignoceric	0.4 ± 0.01 [c]	0.4 ± 0.00 [c]

** Omega-6 PUFAs; *** Omega-9 PUFAs. Each values are expressed as mean ± SD (n = 3). In each row, the different letters represent significant differences between samples ($P < 0.05$).

4.3.4 Determination of total polysaccharides, total phenolic, and total flavonoids in *P. giganteus* extracts

The total polysaccharides of the commercial and wild *P. giganteus* were comparable. The aqueous extract had the highest amount of polysaccharides as shown in Table 4.4. Notably, the total polysaccharides in aqueous extract of the wild strains (17.91 ± 0.05%, w/w) was significantly ($p > 0.05$) higher than that of the commercial strain (14.93 ± 0.04%). On the other hand, the total phenolic content in the ethanol extracts were higher. The quantity of phenolics in the mushrooms extracts was in descending order: commercial strain ethanol extract > wild strain ethanol extract > commercial extract aqueous extract > wild strain aqueous extract. There was also a significant difference ($p < 0.05$) in the total flavonoids between the basidiocarps of the commercial and wild

strains. The wild *P. giganteus* had approximately 2.09% higher flavonoids than the commercial strains.

Table 4.4: Total polysaccharides, phenolics, and flavonoids present in the crude aqueous and ethanol extracts of basidiocarps of *Pleurotus giganteus*

	***Pleurotus giganteus* (commercial strain)**		***Pleurotus giganteus* (wild strain)**	
	Aqueous	**Ethanol**	**Aqueous**	**Ethanol**
Extraction yield (%, w/w)	15.60 ± 2.20 [a]	12.00 ± 1.00 [b]	13.77 ± 1.68 [ab]	6.67 ± 1.06 [c]
Total polysaccharides (%, w/w)	14.93 ± 0.04 [a]	11.31 ± 0.16 [b]	17.91 ± 0.05 [c]	13.72 ± 0.04 [d]
Total phenolic content (mg GAE/g)	12.14 ± 1.89 [a]	24.08 ± 1.04 [b]	9.58 ± 0.18 [c]	21.61 ± 1.47 [d]
Total flavonoids (mg RE/g)	ND	2.94 ± 0.00 [a]	ND	6.14 ± 0.01 [b]

In each row, the different letters represent significant differences between samples ($p < 0.05$). ND = not detected.

4.3.5 Evaluation of antioxidant activity in *P. giganteus* extracts

4.3.5.1 DPPH scavenging activity assay

The scavenging effect of the mushroom extracts on DPPH radicals increased with sample concentration, depending on the extraction solvent and strain type. In general, the ethanol extracts showed higher scavenging activity, hence lower IC_{50} (mg/mL) when compared to the aqueous extracts (Table 4.5). The scavenging activity obtained in descending order was: wild strain ethanol extract > commercial strain ethanol extract > wild strain aqueous extract > commercial strain aqueous extract.

4.3.5.2 FRAP (ferric reducing antioxidant power) assay

In the ferric reducing power assay, the reducers present in the mushroom extracts lead to the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The reducing capacity of the mushroom extract serves as an index of antioxidant activity. The reducing ability of the different extracts was in the range of 1.17 – 3.88 µM $FeSO_4.7H_2O$/ g mushroom (Table 4.5). The antioxidant activity obtained in descending order was: commercial strain ethanol extract ≥ wild strain ethanol extract ≥ commercial strain aqueous extract ≥ commercial strain aqueous extract.

4.3.5.3 Inhibition of lipid peroxidation

The study of lipid peroxidation (LPO) inhibition is based on the measurement of malondialdehyde (MDA) generated by the polyunsaturated fatty acid peroxides upon decomposition. As a result of LPO, destruction of cellular components occurs and brings about oxidative stress in biological systems. As shown in Table 4.5, there was no significant difference ($p > 0.05$) in terms of lipid peroxidation inhibition between the commercial and domesticated wild mushroom extracts. However, the ethanol extracts of both the strains showed significantly higher ($p < 0.05$) lipid peroxidation inhibitory ability (49.58 – 49.80%) when compared to the aqueous extracts (44.41 – 44.61%). Therefore, the extraction methods and solvent used instead of mushroom strains played a more prevailing role in lipid peroxidation inhibition.

Table 4.5: Antioxidant activities of the aqueous and ethanol extracts from the commercial strain of *Pleurotus giganteus* (KLU-M 1227) and the wild strain (KLU-M 1228)

Antioxidant properties	Test method	Positive control (BHT)	*Pleurotus giganteus* (commercial strain)		*Pleurotus giganteus* (wild strain)	
			Aqueous	Ethanol	Aqueous	Ethanol
Free radical scavenging	DPPH (IC_{50}; mg/mL)	0.09 ± 0.01	21.46 ± 6.95 [a]	11.28 ± 3.54 [bc]	16.18 ± 1.76 [ab]	8.10 ± 2.15 [c]
Reducing power	FRAP (μM $FeSO_4.7H_2O$/ g)	780.29 ± 13.40	2.26 ± 0.29 [ab]	2.99 ± 0.14 [b]	2.04 ± 0.32 [a]	2.69 ± 0.71 [b]
Lipid peroxidation inhibition	Inhibition of lipid peroxidation at extract concentration of 10 mg/mL (%)	79.07 ± 2.25	44.41 ± 1.00 [a]	49.58 ± 1.87 [b]	44.61 ± 1.42 [a]	49.80 ± 3.27 [b]

In each row, the different letters represent significant differences between samples ($p < 0.05$).

4.3.6 Correlation between total polysaccharides, total phenolics, and antioxidant activities

The TPC in the mushroom extract was positively correlated to the DPPH scavenging capacity (r = +0.827) and FRAP reducing power (r = +0.820). This indicated that the antioxidant effects increased with increasing concentrations of the total phenolics present in the mushroom extracts. However, a weak correlation was found between the lipid peroxidation inhibition activity and the TPC (r = +0.321). Nevertheless, the DPPH scavenging capacity, FRAP reducing power, and lipid peroxidation inhibition

showed a strong positive correlation (r = +0.806 to +0.820) between each antioxidant activity.

4.4 DISCUSSION

The nutritional components found in the present work are in accordance with the literature. A study in Italy showed that the protein content in *Pleurotus ostreatus* (grey oyster mushroom) was 1.61 ± 0.02 g/100 g (Manzi, Aguzzi, & Pizzoferrato, 2001), which is lower than the protein content of *P. giganteus* in this study. *Pleurotus giganteus* also showed a higher protein content when compared to *Pleurotus sajor-caju* (13.0-18.4 g/100 g) (Bonatti, Karnopp, Soares, & Furlan, 2004). The protein content of mushrooms is dependent on the strain, substrate chemical composition, pileus size, and cultivation time (Nunes, Maria, Paes, Júlio, & Ribeiro, 2012; Shashirekha, Rajarathnam, & Bano, 2005). In this study, rice bran supplementation may have increased the soluble protein content present in both strains as the protein content in rice bran is about 10-15% of the total weight of the mushroom growth substrate (Fabian & Ju, 2011).

The carbohydrate content of *Pleurotus ostreatus* was mainly composed of glucose (14.29 g kg^{-1}) and mannose (10.55 g kg^{-1}) (M.-Y. Kim et al., 2009). While fructose was not detected in *P. ostreatus*, 27.81 g kg^{-1} of glucose was detected in *P. eryngii* with trace amounts of ribose and xylose. *Pleurotus* spp. in particular are rich in calcium, potassium, magnesium, iron, and phosphorus (Ragunathan & Swaminathan, 2003). *Pleurotus sajor-caju*, *P. platypus*, and *P. citrinopileatus* were reported to contain 16.3 ± 0.22, 11.2 ± 0.3, and 10.3 ± 0.2 g kg^{-1} of potassium; respectively. The concentrations of vitamin B3 in mushrooms were highly species dependent and it varies from 34 to 109 mg/100 g dry weight for *P. ostreatus* (Cheung, 2010). Mushrooms contain higher vitamin B2 (riboflavin) when compared to vegetables. Some varieties of

Agaricus bisporus (white button mushroom) have also been reported to contain vitamin B2 levels as high as those found in eggs and cheese (Mattila et al., 2001). In this study, vitamin B1 (thiamin) was higher in the commercial strain by 1.9-fold, while vitamin B2 level was not significantly different when compared to that of the wild strain.

The amino acids in *P. giganteus* were comparable with reported literature values. Tanzanian wild mushrooms *Lactarius* sp. (milky cap), *Boletus pruinatus* (Matt Bolete mushroom) and *Boletinus cavipes* were reported to have leucine as high as 15.9%, 10.6% and 8.40%; respectively (Mdachi, Nkunya, Nyigo, & Urasa, 2004). The fresh *P. ostreatus* was found to have 2.74, 1.76, and 1.43 g kg^{-1} of glutamine, asparagine, and arginine; respectively (Jaworska, Bernaś, Mickowska, & Bernas, 2011). Similar to the present study, other edible mushrooms also contained high levels of oleic acid (% total fatty acid methyl esters): *Auricularia polytricha* (27.1), *Lentinus sajor-caju* (23.5), *Lentinus squarrosulus* (5.8), *Pleurotus djamor* (28.8), *P. sajor-caju* (16.4), and *Russula brevepis* (39.2) (Kavishree, Hemavathy, Lokesh, Shashirekha, & Rajarathnam, 2008). The presence of caprylic and capric acids in mushrooms is rare. *Calocybe gambosa* (St. George's mushroom) and *Clitocybe odora* (Aniseed funnel mushroom) were reported to have 0.25 ± 0.02 and 0.03 ± 0.00% of caprylic acid; respectively; while *Coprinus comatus* (shaggy mane mushroom) registered 0.09 ± 0.00% of capric acid (Vaz et al., 2011).

Extraction with boiling water is used to obtain extracts with high molecular weight compounds, such as polysaccharides which play an important medicinal role in mushrooms (Ferreira, Vaz, Vasconcelos, & Martins, 2010). Low molecular weight compounds, such as phenolic compounds were usually from ethanol extraction (Vaz et al., 2011). Although flavonoid was not detected in *P. sajor-caju* (Kanagasabapathy et al., 2011), its concentration ranging from 0.24 - 0.32 mg/g was reported in *P. ostreatus* (Vamanu, 2013). Flavonoids were also present in other mushroom species like

Clitocybe gibba (3.56 mg chatequin/g extract) and *Boletus armeniacus* (8.59 mg chatequin/g extract) (Pereira, Barros, Martins, & Ferreira, 2012). The rubber wood sawdust which is rich in lignin could have been depolymerized by the lignocellulosic enzymes of mushrooms into phenolic units and further dimerized or polymerized, creating flavonoids (Dubost, Ou, & Beelman, 2007).

DPPH radical scavenging effects of the mycelia extracts of *Pleurotus* spp. (*P. citrinopileatus*, *P. djamor*, *P. eryngii*, *P. flabellatus*, *P. florida*, *P. ostreatus*, and *P. sajor-caju*) have been reported (Mishra et al., 2013; Vamanu, 2013). The methanol and hot water extracts (10 mg/mL) of *P. eous* were found to scavenge DPPH radical by 85.19% and 70.21%, respectively (Sudha, Vadivukkarasi, Shree, & Lakshmanan, 2012). The higher DPPH scavenging ability of ethanol extracts might be due to more hydrogen-donating components including phenolic compounds extracted from the mushroom. The higher phenolic content of ethanol extracts might account for the better results found in reducing power as compared to the aqueous extract (Singdevsachan, Patra, & Thatoi, 2013). When comparing the FRAP values of *P. giganteus* with *P. sajor-caju*, FRAP values of aqueous extract of *P. giganteus* was lower than that of *P. sajor-caju* (35.06 ± 0.86 µM $FeSO_4.7H_2O$/ g mushroom) (Kanagasabapathy et al., 2011). The higher phenolic content in the ethanol extracts might contribute to a higher inhibitory effect on lipid peroxidation. *Pleurotus florida* was reported to display 57% of lipid peroxidation inhibition, while *P. flabellatus*, *P. cystidiosus*, *P. eryngii*, and *P. sajor-caju* showed 50%, 49.8%, 48% and 43% of lipid peroxidation inhibition, respectively (Abdullah, Ismail, Aminudin, Shuib, & Lau, 2012). Meanwhile, *P. ostreatus* at a concentration of 10 mg/ml inhibited LPO activity in rat liver homogenate by 56.20% (Jayakumar, Thomas, & Geraldine, 2009).

4.5 CONCLUSION

It was shown that *P. giganteus* markedly promoted the neurite outgrowth activity in differentiating N2a cells but the nutritional profile of *P. giganteus* is yet to be determined. This is the first report on the nutritional profile and chemical compositions of *P. giganteus.* The extracts of the commercial and domesticated wild strains showed similar nutritional profile (carbohydrate, protein, and dietary fibre), monosaccharides (fructose and glucose), macroelements (potassium, phosphorus, magnesium, calcium, and sodium), microelements (iron, zinc, manganese, and copper), and vitamins (B2, B2, B3, and C). The amino acid and fatty acid composition of both the strains are also similar. This study suggests that the high linoleic acid (4.5-5.0%) present in the mushroom extract could be linked to the neurite outgrowth activity as this polyunsaturated fatty acid was reported to induce neurite outgrowth. Further, the neurite outgrowth activity may be attributed to the vitamins B2 (0.009 g/kg) and B3 (0.09 g/kg) which were reported to be beneficial to brain as they help to carry neurotransmitters. On the other hand, the commercial and domesticated wild strains of *P. giganteus* showed distinctive antioxidant activities due to the differential distributions of total phenolics and flavonoids. The aqueous extract did not contain flavonoids and had a lower phenolic content, hence explaining its lower antioxidative capacity. The new data presented here are useful and will support future studies on the effects of *P. giganteus* supplementation in individuals at high risk of age-related disease. Further studies will consider the direct effect of the chemical compounds in the mushroom extract on differentiating N2a cells.

CHAPTER V

EFFICACY OF THE CHEMICAL CONSTITUENTS OF *PLEUROTUS GIGANTEUS* EXTRACTS ON NEURITE OUTGROWTH IN N2A CELLS AND THE UNDERLYING MECHANISMS

5.1 INTRODUCTION

As discussed earlier, functional food plays a significant role in preventing or reducing the severity of lifestyle associated diseases and improve physical and mental well-being of consumers. Edible and medicinal mushrooms are gaining recognition as preventative agents for age-related diseases including neurodegenerative diseases such as AD, Parkinson's disease, and dementia (Sabaratnam et al., 2011). Currently, medications are prescribed for mild, moderate, to severe Alzheimer's disease to help delay or prevent behavioural symptoms but that too, seems to be effective for only a limited time frame. Trials with NGF for Alzheimer's disease had gained some degree of success but the high molecular weight of NGF protein reduces its ability to cross the blood-brain barrier (Allen et al., 2013). Considering the limitation of the above, early intervention strategy using mushrooms as functional food may be helpful. Edible and medicinal mushrooms are packed with a wide array of bioactive and nutritional components that could scavenge reactive oxygen species (ROS). They are able to exert neuroprotective effects and promote neuritogenesis as well as play a role in neuroregeneration (Roupas et al., 2012).

Pleurotus giganteus is one of the edible mushrooms which have been shown to have neurite outgrowth stimulatory effects (Chapter III). In Chapter IV, the mushroom extracts exhibit high antioxidant activities (Phan, David, et al., 2014). There is a need to

elucidate the chemical compounds which contribute to the neuritogenic properties of this mushroom. Therefore, the objective of this chapter was to determine the efficacy of the individual chemical constituents of *P. giganteus* in stimulating neurite outgrowth of N2a cells. The chemical constituents selected were linoleic acid, oleic acid, cinnamic acid, caffeic acid, *p*-coumaric acid, succinic acid, benzoic acid, and uridine based on a previous study (Moroney, 2012). The compound which showed the most promising neurite outgrowth activity will be subjected to finger printing by using HPLC for another round of verification. The compound will then be used for the subsequent tests along with the standardised extracts. In order to confirm the underlying mechanisms of the neurite outgrowth activity induced by the chemical compounds and the standardised extracts, treatment of cells with specific inhibitors was carried out followed by measurement of the phosphorylated kinases using enzyme-linked immunosorbent assays.

5.2 MATERIALS AND METHODS

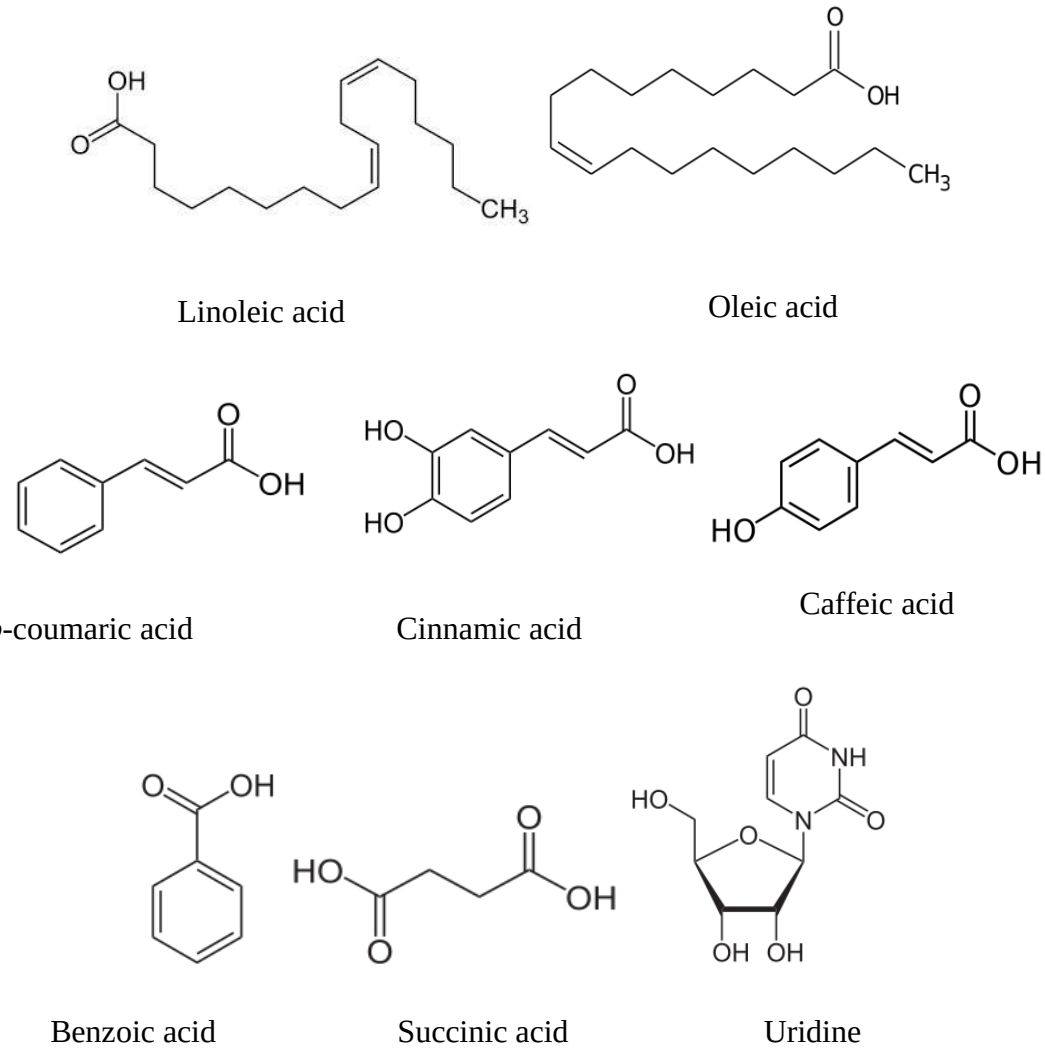
5.2.1 General methodology

Preparation of the aqueous and ethanol extracts of *P. giganteus* is given in Section 3.2.3. Cell culture method and neurite outgrowth assay by quantification of neurite bearing cells can be found in Sections 3.3.2, 3.2.4, and 3.2.5.

5.2.2 Compounds detected in the basidiocarps of *P. giganteus*

The compounds in the basidiocarps of *P. giganteus* were previously identified by GCMS and LCMS/MS (Moroney, 2012). Linoleic acid (5.00%) and oleic acid (10.30%) were present in the ethanol extract of *P. giganteus*; while cinnamic acid (0.1%), caffeic acid (1.31%), *p*-coumaric acid (0.07%), succinic acid (12.57%), benzoic acid (2.14),

and uridine (0.55%) were present in the aqueous extract. All the compounds were purchased from Sigma (St. Louis, MO, USA). Figure 5.1 shows the structure of the compounds.



Linoleic acid

Oleic acid

p-coumaric acid

Cinnamic acid

Caffeic acid

Benzoic acid

Succinic acid

Uridine

Figure 5.1: Compounds detected in the aqueous and ethanol extracts of *P. giganteus.*

5.2.3 Neurite outgrowth assay using chromogenic method

5.2.3.1 Preparation of cell suspension, treatment, coating and fixation

N2a cells were seeded in culture flask and grown to 70% confluence. The growth media was replaced with 5%-serum differentiation media for 24 hours to initiate cell differentiation and neurite extension. The insert membrane surface of the NS220 Neurite Outgrowth Assay Kit (Merck Millipore, Germany) was then prepared. Ten µg/mL of collagen Type 1 from rat tail (Sigma) which acts as an extracellular matrix (ECM) protein was prepared freshly with 1X phosphate buffered saline (PBS). The ECM protein solution (400 µL) was added to the bottom of an empty well of the 24-well plate. The Millicell insert (Merck Millipore) provided in the assembly kit was placed into the well containing the ECM protein and allowed to coat the underside of the membrane for 2 hours at 37℃ (Figure 5.2). While membranes were being coated, N2a cells were detached from culture flasks and resuspended in 5%-serum media at a concentration of 100,000 cells per 100 µL. Following coating, the insert was removed from its coating solution and placed into a new well containing 600 µL of 5%-serum media with the mushroom extracts or compounds. Cell suspension (100 µL) was added onto the top of the membrane and allowed to sit at room temperature for 15 minutes to enable even cell distribution. The plate assembly was then transferred to a 37℃ incubator to allow neurites to extend on the underside of the membrane for 48 hours (Figure 5.2). After 48 hours, each insert was removed from its culture well and the liquid from the top of the membrane was gently removed. PBS (1X, 800 µL) was placed in the well of a 24-well assay plate and insert was allowed to sit in the PBS briefly to rinse. The insert was then transferred to a new well containing 400 µL of - 20℃ methanol and allowed to fix for 20 minutes at room temperature. Then, it was rinsed again briefly in 800 µL of fresh PBS.

5.2.3.2 Staining of neurites for visualisation and quantification

Following fixation, the insert was placed into 400 µL of Neurite Stain Solution (Merck Millipore) in a new well. The neurites were allowed to stain for 15-30 minutes at room temperature, and then rinsed in 800 µL of fresh PBS. All the cell bodies were removed from the top of the membrane (particularly at the edge) by gently wiping with the cotton swab moistened with PBS (Figure 5.2).

The stained neurite extensions were visualized on intact inserts in a 24-well plate using an inverted microscope focused on the underside of the membrane. For quantification, 200 µL drop of Neurite Stain Extraction Buffer (Merck Millipore) was added to each insert which was placed onto a piece of Parafilm that has been taped to a flat surface. The underside of the membrane was placed over the drop of Extraction Buffer so that the entire membrane surface is covered. The stain was allowed to extract for 5 minutes at room temperature. The buffer from around and inside of the insert was collected and placed in 96-well plate. The neurite extension was then quantified using spectrophotometer by reading the absorbance at 560 nm.

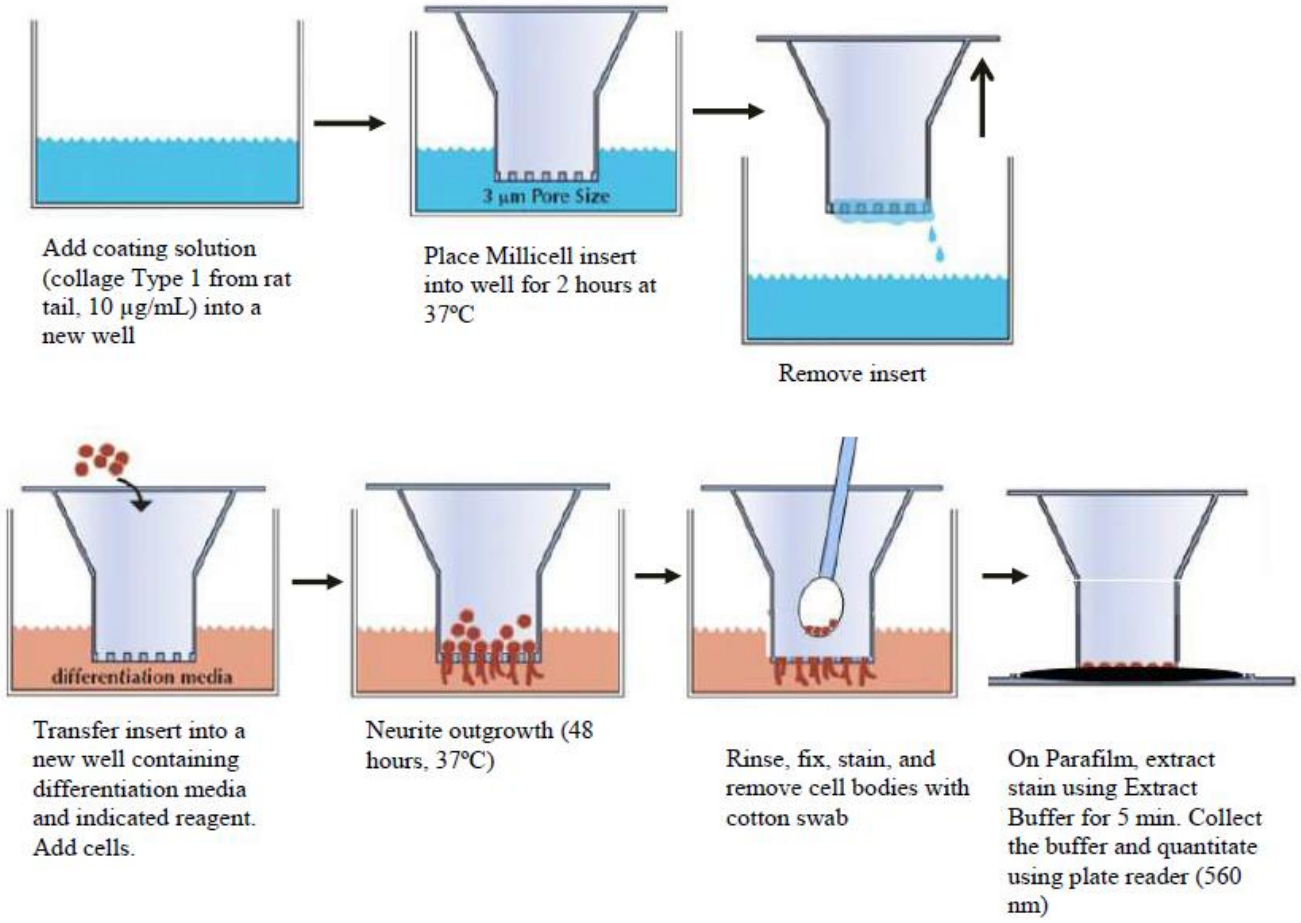


Figure 5.2: Overview of neurite outgrowth assay using chromogenic method (NS220) Neurite Outgrowth Assay Kit from Merck Millipore, Germany. Diagram was modified from NS220 Neurite Outgrowth Assay Kit guide book.

5.2.4 Quantification of uridine in *P. giganteus* extracts

For quantification of uridine, uridine reference compound was accurately weighed and dissolved in water to prepare the standard chemical solutions (1 mg/mL). Perkin Elmer Series 200 liquid chromatography equipped with a Perkin Elmer Series 200 UV detector was used. The detector signal was recorded by the Turbochrom workstation software. The column used was Hypersil BDS C18 (4.6 × 250 mm) with Alltech refillable C18 Guard column (10 × 4.6 mm) (Alltech, USA). The mobile phase consisted of methanol: 10 mM monobasic potassium phosphate (15:85; pH 5.0) and the flow rate was 1.5 mL/min. The calibration curve was prepared by injecting a series of uridine (Sigma) standard dilutions. Uridine was quantified by means of calibration curves obtained from standard from Sigma.

5.2.5 Treatment with specific inhibitors

Stock solution (10 mM) of MEK inhibitors (U0126, PD98059) and PI3K inhibitor (LY294002) were prepared in DMSO and stored in −20 °C in the dark. Each inhibitor i.e. U0126 (10 μM), PD98059 (40 μM), and LY294002 (30 μM) was then prepared by diluting in medium prior to use. P2Y receptor antagonists, suramin (30 μM) and pyridoxal phosphate-6-azophenyl-2' 4'-disulfonic acid (PPADS; 30 μM) were prepared in double distilled water and protected from light. Cells were either incubated with or without the treatment of inhibitors for one hour. All the cells were then stimulated with mushroom extracts and uridine for three days prior to scoring neurite bearing cells and ELISA tests were carried out for signaling pathway elucidation and protein synthesis.

5.2.6 Preparation of cell lysate

To harvest the cells in a non-denaturing condition, the cells were rinsed with ice-cold PBS followed by addition of 0.5 mL of ice-cold cell lysis buffer (Cell Signaling Technology, Inc) with 1 mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma). The cell lysis buffer (1X) consists of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM b-lycerophosphate, 1 mM Na_3VO_4, and 1 µg/ml leupeptin. The plate or flask containing the cells was incubated on ice for 5 mins. The cells were scraped off, sonicated on ice, and microcentrifuged for 10 mins at 4 ℃. The supernatant is the cell lysate and stored at −80°C in single-use aliquots.

5.2.7 Protein quantification using Coomassie (Bradford) protein assay

A set of protein standards (0-2000 µg/mL) was prepared by diluting the contents of one albumin standard (BSA) ampoule into several vials. A total of 150 µL of standard or cell lysate was added into microplate wells. Coomassie reagent (150 µL) was added to each well and mixed with plate shaker for 30 seconds. The plate was incubated for 10 minutes at room temperature to obtain the most consistent results. The absorbance was measured at 595 nm on a plate reader. A standard curve was prepared by plotting the 595 nm measurement for each BSA standard versus its concentration in µg/mL. Using the standard curve, the protein concentration estimate was determined for each protein lysates.

5.2.8 Measurement of phosphorylated extracellular-signal regulated kinase (p-ERK), phosphorylated protein kinase B (p-Akt), phosphorylated mitogen-activated protein kinase (p-MEK), phosphorylated mammalian target of rapamycin (p-mTOR), and phosphorylated cAMP-response element binding protein (p-CREB)

Mushroom extracts and uridine were added to N2a cells with or without pretreatment with specific inhibitors. At indicated time points, the cells were harvested and cell lysates were prepared. The cell lysates were then incubated in microwells pre-coated individually with phospho-ERK (p-ERK; Thr202/Tyr204), p-Akt (Thr308), p-MEK1 (Ser217/221), p-mTOR (Ser2448), and p-CREB (Ser133) antibodies. To increase sensitivity, the plate was incubated overnight at 4^{o}C. The wells were then washed four times with washing buffer (10 mM phosphate buffer pH 7.4, 150 mM NaCl, and 0.05% Tween 20). Following washing, detection antibody was added (Cell Signaling Technology, Inc). Anti-rabbit IgG, HRP-linked antibody was then added to recognise the bound detection antibody. HRP substrate, which is TMB, was then added for colour development. Absorbance at 450 nm was recorded and the magnitude of absorbance is proportional to the quantity of the p-ERK, p-Akt, p-MEK1, p-mTOR, and p-CREB antibodies.

5.2.9 Measurement of growth associated protein 43 (GAP-43), tubulin alpha 4A (TUBA4A), and tubulin beta 1 (TUBb1)

Each dilution of standard (GAP-43, TUBA4A, and TUBb1; 100µL) and cell lysate samples (100µL) were added in appropriate wells (Cloud-Clone Corp., USA). The plate was incubated for two hours at 37^{o}C. After that, the liquid of each well was removed carefully and washing was not required. A total of 100 µL Detection Reagent A working solution (Cloud-Clone Corp., USA) was added to each well and was incubated for one hour at 37^{o}C after covering. The solution was aspirated and the wells were washed with 350 µL of 1× washing buffer using a multi-channel pipette; and was left to sit for two minutes. The remaining washing buffer was removed from all wells completely by snapping the plate onto an absorbent paper. The total number of wash was three times. Detection reagent B (100 µL) was added and incubated for 30 minutes

at 37^{o}C. The wash process was repeated for 5 times. Substrate solution (90 µL) was added to each well and incubated for 15-25 minutes. Stop solution (50µL) was added and measurement was conducted at 450nm. The levels of GAP-43, TUBA4A, and TUBb1were determined from a standard curve plotted with known concentrations of the respective proteins.

5.3 Results

5.3.1 Neurite outgrowth assays (Quantification of neurite bearing cells and chromogenic method)

The mean value of neurite-bearing cells in NGF treated cells (positive control) was 22.67 ± 0.74% as shown in Figure 5.3. The aqueous and ethanol extracts of the *P. giganteus* (20 µg/mL) caused a significant ($p < 0.05$) increase in neurite-bearing cells by 4.0- and 4.7-times when compared to the control cells with medium only. *P*-coumaric, cinnamic, and oleic acids (100 µM) caused no neuritogenic effects on differentiating N2a cells, i.e. 8.56 ± 1.51%, 10.17 ± 1.88%, 10.06 ± 1.32%; respectively. Linoleic acid caused 20.78 ± 2.43% of N2a cells to bear neurites. Uridine stimulated the highest ($p < 0.05$) percentage of neurite-bearing cell (43.09 ± 4.88%), which was 1.9-times higher than that of the positive control.

Indirect measurement (chromogenic method) was performed on N2a treated with mushroom extracts and compounds by using the neurite outgrowth quantification assay kit (Merck Millipore). The results were used to confirm the neurite outgrowth assay which records the neurite-bearing cells. Briefly, N2a cells after treatment were allowed to extend neurites for 48 hours, and then stained with Neurite Stain Solution for 30 minutes. A total of 100 µL of Neurite Stain Extraction Buffer was used for stain extraction and OD562 values were recorded for each of the treatments. As shown in Figure 5.3, uridine had the highest neurite outgrowth score as indicated by the highest

OD which was 0.53 ± 0.01 absorbance units (AU). This is in agreement with the counting method of neurite outgrowth, whereby the percentage of uridine-treated neurite bearing cell was also the highest i.e. 43.09 ± 4.88%. Overall, the neurite outgrowth score by using the chromogenic method in a descending order was uridine, NGF control, ethanol extract, aqueous extract, linoleic acid, benzoic acid, succinic acid, caffeic acid, cinnamic acid, *p*-coumaric acid, and oleic acid. The neurite bearing score also followed the similar descending trend: uridine, ethanol extract, aqueous extract, NGF control, linoleic acid, succinic acid, benzoic acid, caffeic acid, cinnamic acid, oleic acid, and *p*-coumaric acid. Therefore, it was concluded that the two methods can be used at the same time, in the condition that neurite bearing score (%) must be obtained and that the chromogenic method might be used as a confirmatory measurement to compliment the neurite bearing score results.

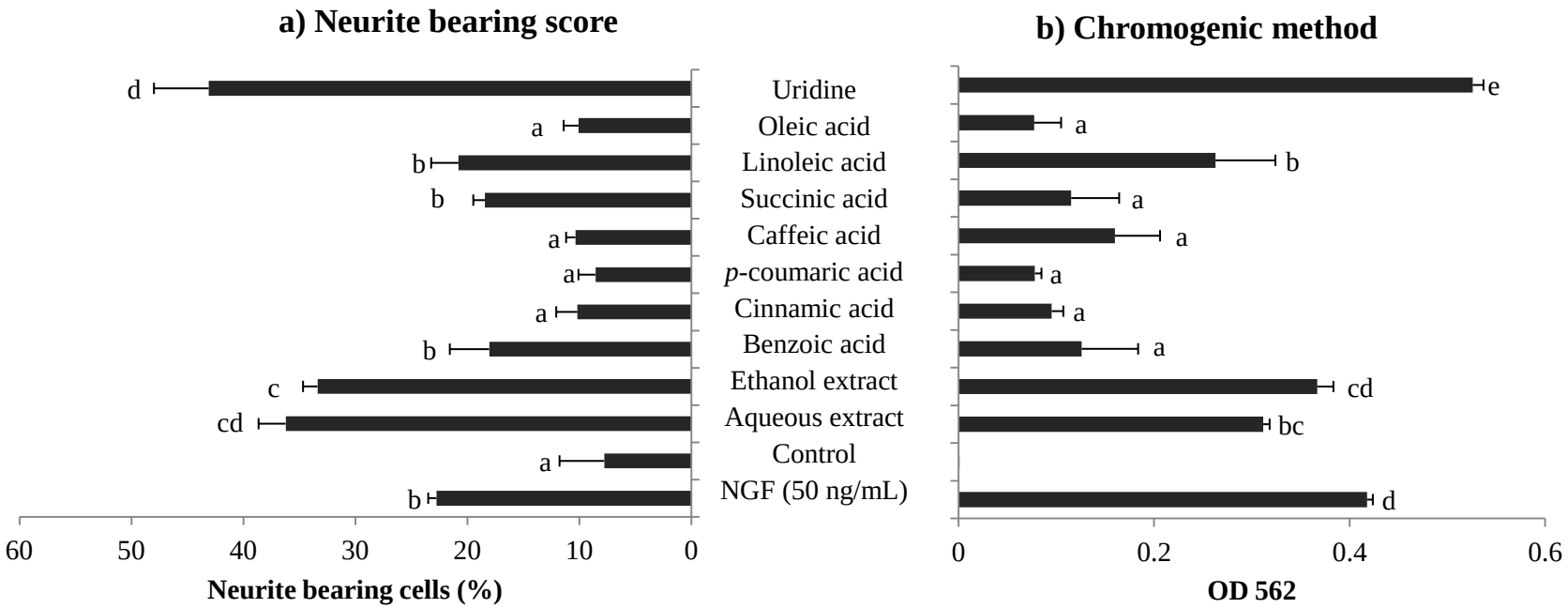


Figure 5.3: The effects of mushroom extracts and compounds on neurite outgrowth of differentiating N2a cells by using (a) quantification of neurite bearing cells, and (b) chromogenic method at optical density 562. NGF (50 ng/mL) was used as a positive control. Values are mean ± SD from three independent experiments. Different letters represent significant differences between samples ($p < 0.05$).

5.3.2 Quantification of uridine in *P. giganteus* extracts

Uridine (100 µM) caused the highest ($p < 0.05$) neurite bearing score of 43.09 ± 4.88%. As evidenced by the neurite outgrowth assay using chromogenic method, the neurite outgrowth was the highest (0.42 ± 0.01 AU) at OD 526. Therefore, the concentration of uridine was determined in the extracts of *P. giganteus*. On the basis of the calculations of the external reference uridine (Sigma), aqueous extracts contained 1.80 ± 0.03% (g of compound per 100 g extract) of uridine (Table 5.1). On the other hand, ethanol extract yielded a lower ($p < 0.05$) amount of uridine, i.e. 1.66 ± 0.03 g/100g. The HPLC chromatograms of the mushroom extracts showed a clear peak representing uridine (Figure 5. 4).

Table 5.1: Uridine (%, w/w) in the aqueous and ethanol extracts of *P. giganteus*

Compounds	**Content (%, w/w) in extract**	
	Aqueous	**Ethanol**
Uridine	1.80 ± 0.03[a]	1.66 ± 0.03[b]

Note: (w/w) = g of compound per 100 g of mushroom extract. In each line different letters mean significant differences at p =0.05.

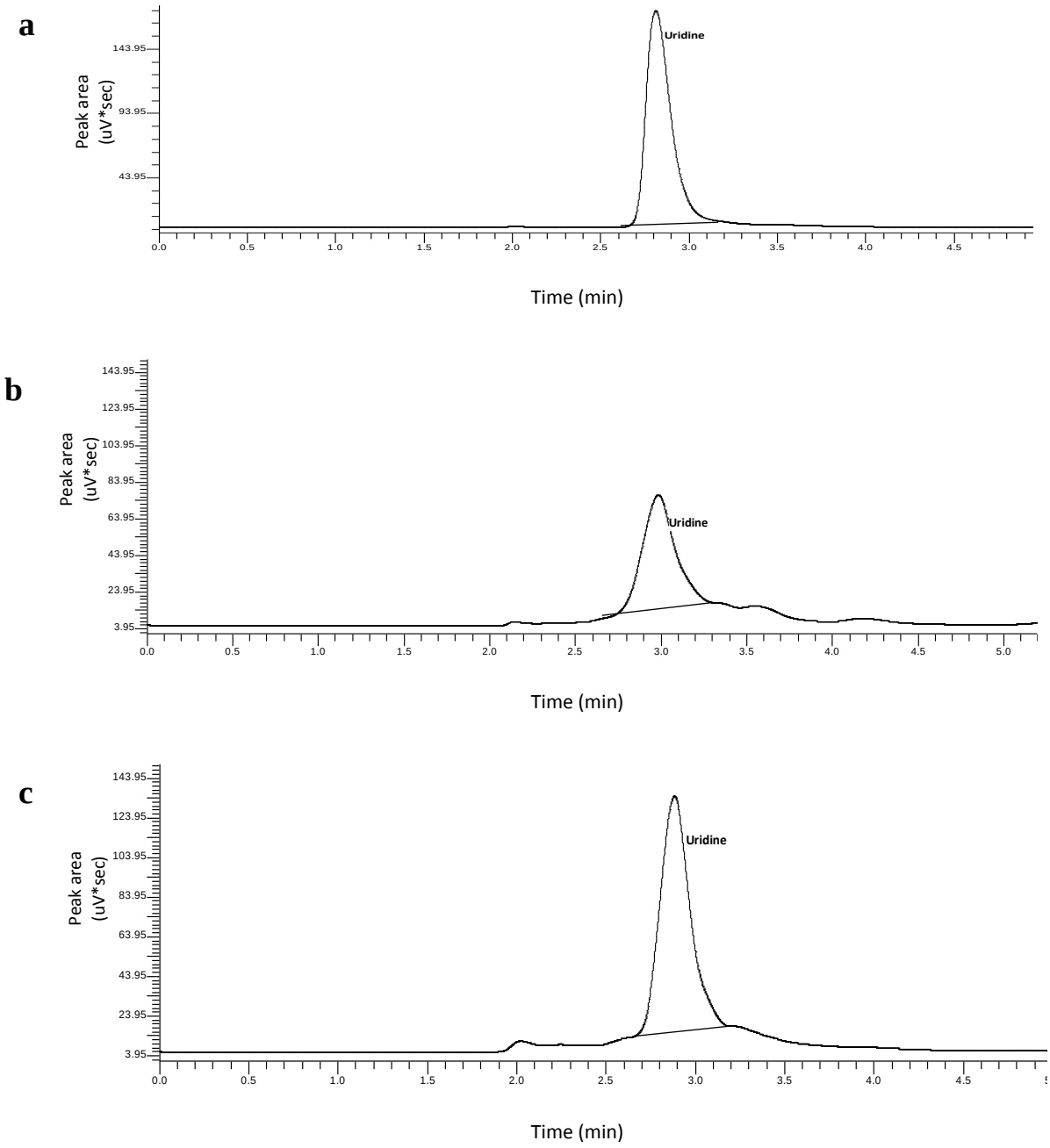


Figure 5.4: HPLC chromatogram of (a) Uridine reference (Lot no.:#030M1518V), (b) ethanol extract of *P. giganteus*, and (c) aqueous extract of *P. giganteus.*

5.3.3 The effects of uridine on neurite outgrowth activity of N2a cells

Treatment with uridine significantly ($p < 0.01$) increased neurite outgrowth in the N2a cells in a dose dependent manner up to 100 µM (Figure 5.5). The percentage of neurite bearing cells maintained at 45-50% upon increasing the concentration of uridine from 100 µM to 500 µM. Uridine at 100 µM also exhibited a significantly ($p < 0.01$) higher percentage in neurite-bearing cells when compared to NGF- and extract-treated cells. Figure 5.6 shows the morphology of the N2a cells with neurite outgrowth at 48 h of treatment with uridine (100 µM) and vehicle (serum-free medium).

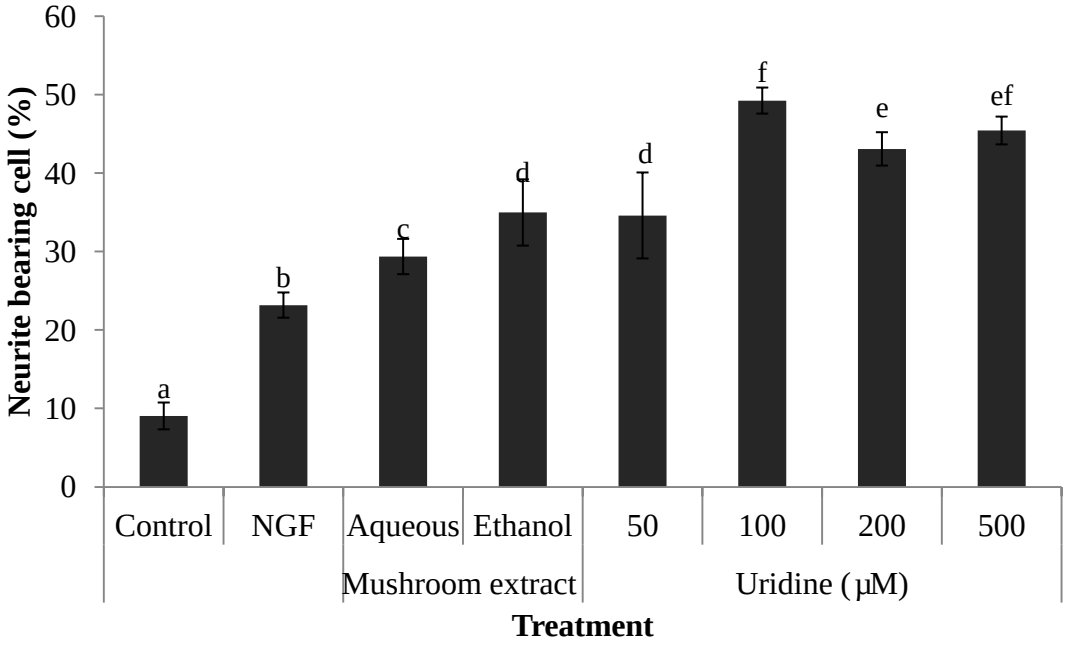


Figure 5.5: The effects of different concentrations of uridine (50-500 µM) on neurite outgrowth in differentiating N2a cells. NGF (50 ng/mL) was used as positive control and medium only (vechicle) with no treatment was used as control. Aqueous and ethanol extracts (20 µg/mL) were compared to uridine. Values are mean ± SD from three independent experiments. Different letters represent significant differences between samples ($p < 0.05$).

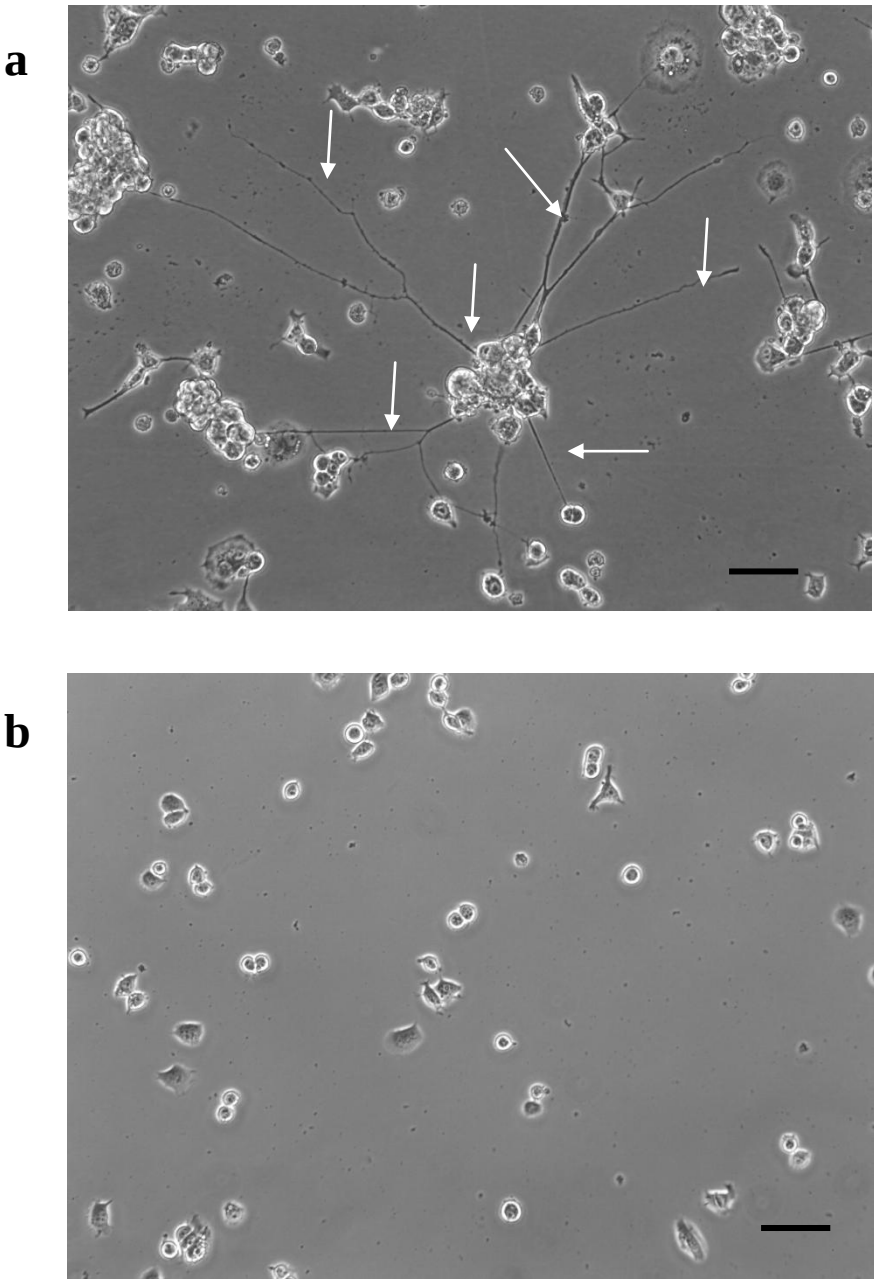
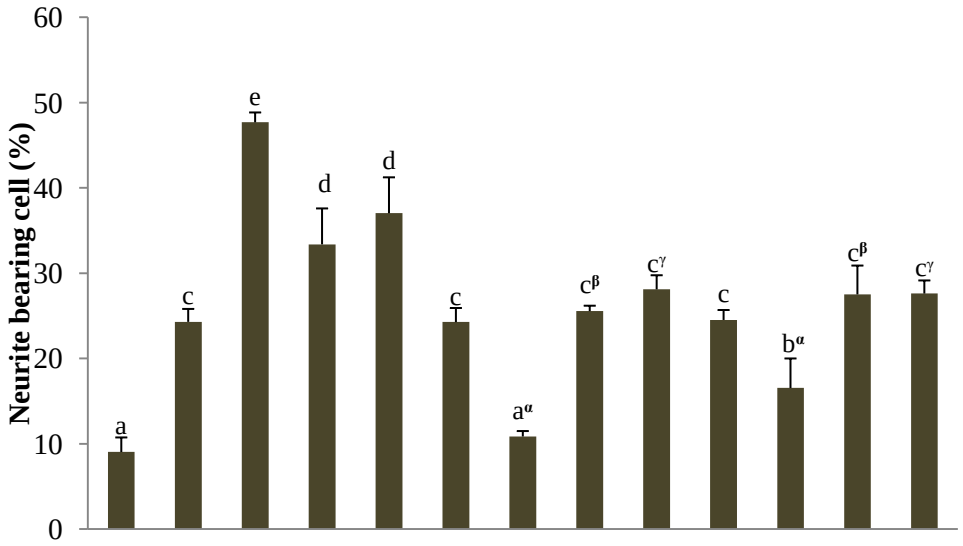


Figure 5.6: Phase-contrast photomicrographs of (a) uridine (100µM) induced neurites as indicated by arrows and (b) negative control cells treated with only vehicle (serum-free medium).

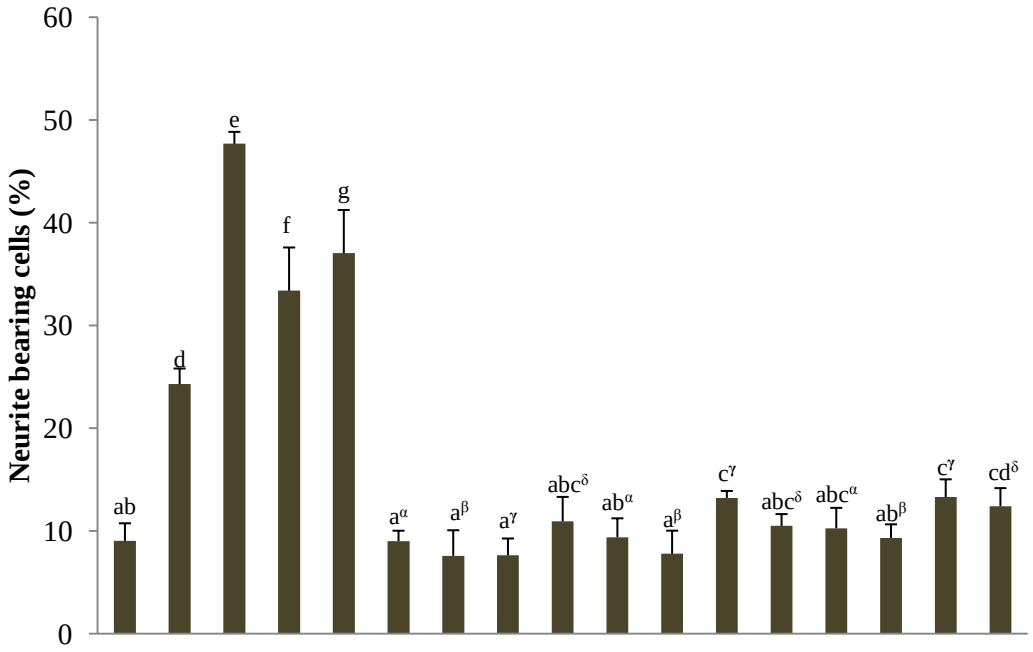
5.3.4 The effects of P2Y receptor, MAPK/ERK1/2 and PI3K/Akt signaling pathway inhibitors on neurite outgrowth activity of N2a cells

Specific inhibitors of key intermediates involved in neurite outgrowth signaling pathways were used to explore the mechanism of neuritogenesis in differentiating N2a cells potentiated by uridine and mushroom extracts. It was shown that neurite outgrowth induced by uridine was markedly inhibited ($p < 0.05$) by P2Y inhibitors suramin (30 μM) and PPADS (30 μM) (Figure 5.7). In N2a cells which were treated with aqueous and ethanol extracts combined with either suramin or PPADS, the decrease in the number of neuritic processes was significant ($p < 0.05$). On the contrary, both the inhibitors did not inhibit NGF-induced neurite outgrowth (Figure 5.7). This could be due to a different receptor, for example the Trk family that is responsible for binding of NGF, but not P2Y receptor. MAPK/ERK1/2 inhibitors U0126 and PD98059 at the concentrations of 10 μM and 40 μM, respectively, caused inhibition effects on N2a cells. The number of differentiating N2a cells with neurite lengths double the cell diameter decreased significantly for NGF-, extracts-, and uridine-treated cells (Figure 5.8). Cells pretreated with the PI3K/Akt inhibitor, LY294002 showed no difference ($p > 0.05$) to the negative controls, with differentiated cells bearing neurites ranging only from 8.9 – 10.3% (Figure 5.8). From these results, it is proposed that the *P. giganteus* extract and uridine induced neurite outgrowth on N2a cells *via* the activation of P2Y receptor. Activation of the P2Y receptor then triggered the activation of ERK1/2 and PI3K/AKt phosphorylation cascade.



NGF	-	+	-	-	-	+	-	-	-	+	-	-	-	50 ng/mL
Uridine	-	-	+	-	-	-	+	-	-	-	+	-	-	100 µM
AE	-	-	-	+	-	-	-	+	-	-	-	+	-	20 µg/mL
EE	-	-	-	-	+	-	-	-	+	-	-	-	+	20 µg/mL
Suramin	-	-	-	-	-	+	+	+	+	-	-	-	-	30 µM
PPADS	-	-	-	-	-	-	-	-	-	+	+	+	+	30 µM

Figure 5.7: The effects of two specific inhibitors of P2Y receptors (suramin and PPADS) on neurite outgrowth of differentiating N2a cells. NGF (50 ng/mL) was used as positive control and medium only with no treatment was used as control. Aqueous (AE) and ethanol extracts (AE; 20 µg/mL) were tested to compare with uridine treatment. Values are mean ± SD from three independent experiments. Different letters represent significant differences between samples ($p < 0.05$). $^{\alpha}p < 0.05$ vs. uridine, $^{\beta}p < 0.05$ vs. AE, and $^{\gamma}p < 0.05$ vs. EE.

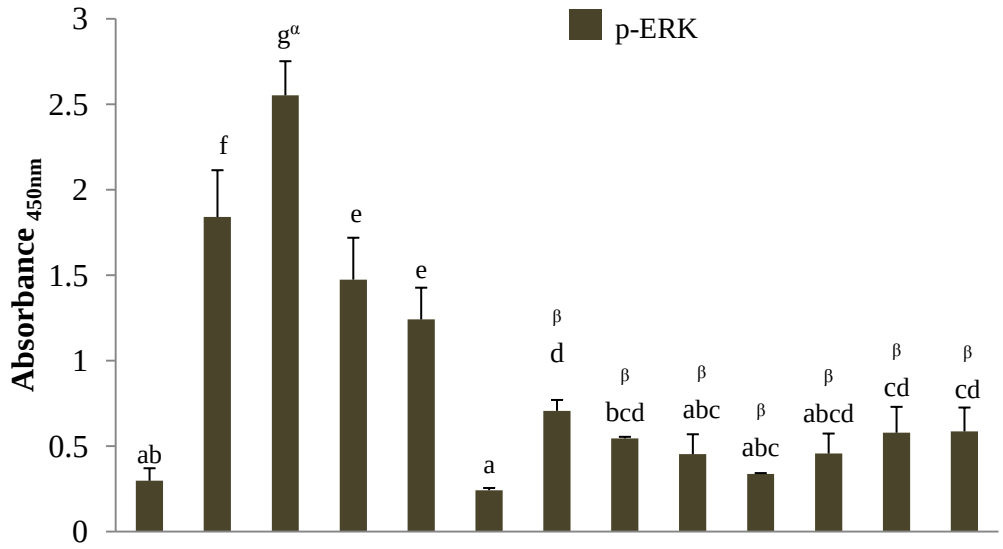


NGF	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	50 ng/mL
Uridine	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	100 µM
AE	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	20 µg/mL
EE	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	20 µg/mL
U0126	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	10 µM
PD98059	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	40 µM
LY294002	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	30 µM

Figure 5.8: The effects of specific inhibitors of MEK/ERK and PI3K/Akt pathways (U0126 and PD98059; and LY294002, respectively) on neurite outgrowth of differentiating N2a cells. NGF (50 ng/mL) was used as a positive control and medium only with no treatment was used as control. Aqueous (AE) and ethanol extracts (AE; 20 µg/mL) were tested to compare with uridine treatment. Values are mean ± SD from three independent experiments. Different letters represent significant differences between samples ($p < 0.05$). $^{\alpha}p < 0.05$ vs. NGF, $^{\beta}p < 0.05$ vs. uridine, $^{\gamma}p < 0.05$ vs. AE, and $^{\delta}p < 0.05$ vs. EE.

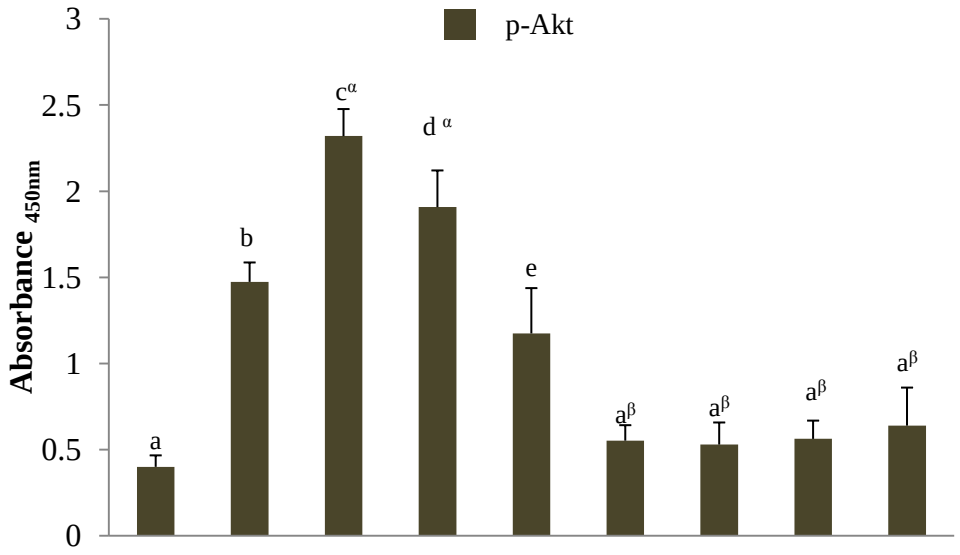
5.3.5 The effects of uridine on the expression of phosphorylated-(p-)ERK, p-Akt, p-MEK, p-mTOR, and p-CREB

Since the uridine-induced neurite outgrowth activity was found to be mediated by P2Y, MAPK/ERK1/2, and PI3K/Akt pathways, the ability of uridine to activate the specific protein kinases responsible for the pathways was tested. The phosphorylation of the downstream target proteins was measured. P-ERK activity (Figure 5.9) was significantly ($p < 0.05$) enhanced by uridine when compared to the NGF-treated cells. The presence of the aqueous and ethanol extracts also triggered significantly ($p < 0.05$) higher phospho-p44/42 (Thr202/Tyr204) levels when compared to cells with medium only. Besides, uridine resulted in the highest expression of p-ERK. As expected, ERK phosphorylation was significantly ($p < 0.05$) suppressed in the presence of inhibitors U0126 and PD98059. N2a cells treated with uridine also exhibited a significantly higher ($p < 0.05$) Akt (Thr308) phosphorylation when compared to the NGF control (50 ng mL−1) (Figure 5.10). Similarly, AKT inhibitor LY294002-treated cells added with mushroom extracts, uridine, or NGF did not result in phosphorylation of Akt. Considering the above results showing that uridine significantly ($p < 0.05$) enhanced the expression of p-ERK and p-Akt, it will be crucial to evaluate whether uridine could also increase p-MEK and p-mTOR. Treatment of N2a cells with uridine caused a significant ($p < 0.05$) and sustained increase of phosphorylation of MEK and mTOR in dose dependent manner (Figure 5.11). The highest phosphorylation levels of MEK and mTOR (2.65±0.2 and 3.33±0.2, respectively) were observed with 500 μM uridine. CREB is critical for activating the transcription of genes controlled by the cAMP-response element, and many of these genes may be involved in neuronal outgrowth and plasticity. Therefore, the phosphorylation of CREB was investigated. As shown in Figure 5.12, treatment of N2a cells with 500 μM of uridine caused approximately 2.8 fold increase of CREB phosphorylation when compared with the vehicle group.



NGF	-	+	-	-	-	+	-	-	-	+	-	-	-	50 ng/mL
Uridine	-	-	+	-	-	-	+	-	-	-	+	-	-	100 µM
AE	-	-	-	+	-	-	-	+	-	-	-	+	-	20 µg/mL
EE	-	-	-	-	+	-	-	-	+	-	-	-	+	20 µg/mL
U0126	-	-	-	-	-	+	+	+	+	-	-	-	-	10 µM
PD98059	-	-	-	-	-	-	-	-	-	+	+	+	+	40 µM

Figure 5.9: Enhancement of uridine-induced neurite outgrowth is ERK-dependent as evidenced by the expression of phospho-ERK1/2 (p44/p42) as detected using antibody specific for phospho-ERK1/2. U0126 and PD98059 are the inhibitors for ERK. Data are expressed as mean ± S.D. for n=3. $^{\alpha}p < 0.05$ represents a significantly higher value from the negative and NGF controls. $^{\beta}p < 0.05$ represents a significant difference from the non-inhibitors treated cells.



NGF	-	+	-	-	-	+	-	-	-	50 ng/mL
Uridine	-	-	+	-	-	-	+	-	-	100 µM
AE	-	-	-	+	-	-	-	+	-	20 µg/mL
EE	-	-	-	-	+	-	-	-	+	20 µg/mL
LY294002	-	-	-	-	-	+	+	+	+	30 µM

Figure 5.10: Enhancement of uridine-induced neurite outgrowth is PI3K/Akt-dependent as evidenced by the expression of phospho-Akt (Thr308) detected by the antibody specific for phospho-Akt. LY294002 is the inhibitor for PI3K/Akt. Data are expressed as mean ± S.D. for n=3. $^{\alpha}p < 0.05$ represents a significantly higher value from the negative and NGF controls. $^{\beta}p < 0.05$ represents a significant difference from the non-inhibitors treated cells.

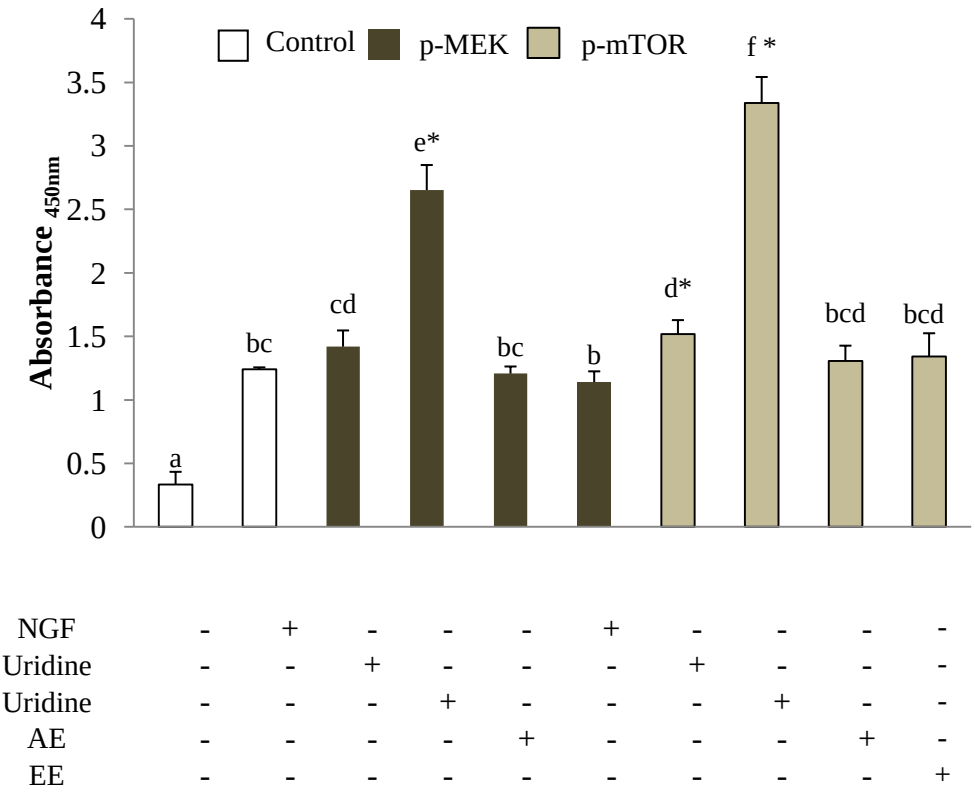
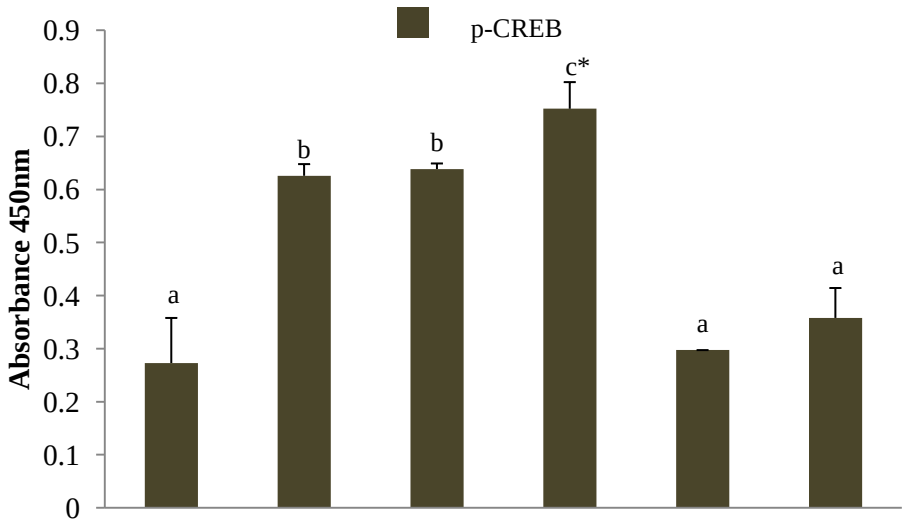


Figure 5.11: Enhancement of uridine-induced neurite outgrowth is MEK- and mTOR-dependent as evidenced by the activation of phospho-MEK (Ser217/221) and phospho- mTOR (Ser2448) as detected by using specific antibodies. Data are expressed as mean ± S.D. for n=3. *$p < 0.05$ represents a significantly higher value from the negative and NGF controls.



NGF	-	+	-	-	-	-	50 ng/mL
Uridine	-	-	+	-	-	-	100 µM
Uridine	-	-	-	+	-	-	500 µM
AE	-	-	-	-	+	-	20 µg/mL
EE	-	-	-	-	-	+	20 µg/mL

Figure 5.12: Enhancement of uridine-induced neurite outgrowth is MEK/ERK-CREB and PI3 K/Akt-mTOR-CREB dependent. Activation of phospho-CREB (Ser133) was detected using antibody specific for phospho-CREB. Data are expressed as mean ± S.D. for n=3. *$p < 0.05$ represents a significantly higher value from the negative and NGF controls.

5.3.6 The effects of uridine on the expression of GAP-43, TUBA4A, and TUBb1

As shown in Figure 5.13a, uridine (100 and 500 μM) significantly ($p < 0.05$) increased total GAP-43 (0.63±0.1 and 1.07±0.1 ng/mL of protein, respectively) level in a dose-dependent manner in N2a cells. The cytoskeleton requirement in uridine-induced neurite outgrowth was then investigated. Tubulin a4a, the neurite outgrowth marker was evaluated in the presence of uridine. The up-regulation in tubulin a4a expression was significant ($p < 0.05$) when treated with 100 and 500 μM of uridine when compared to NGF (Figure 5.13b). Notably, concentration of 500 μM uridine resulted in a significantly ($p < 0.05$) higher tubulin a4a expression when compared to the NGF-treated cells by 2.18-fold. Moreover, aqueous extract-treated N2a cells expressed 0.79±0.1 ng/mL of tubulin a4a, which was 1.6-fold higher than that of NGF-treated cells. Tubulin beta represents another important cytoskeleton component in neurite outgrowth. As shown in Figure 5.13c, the expression of tubulin beta b1 was significantly ($p < 0.05$) up-regulated by the aqueous extract of *P. giganteus* (1.56±0.3 ng/mL of protein). Treatment with uridine (500 μM) led to an up-regulation of tubulin beta b1 level by 1.8-fold (2.16±0.06 ng/mL of protein) when compared to NGF-treated cells.

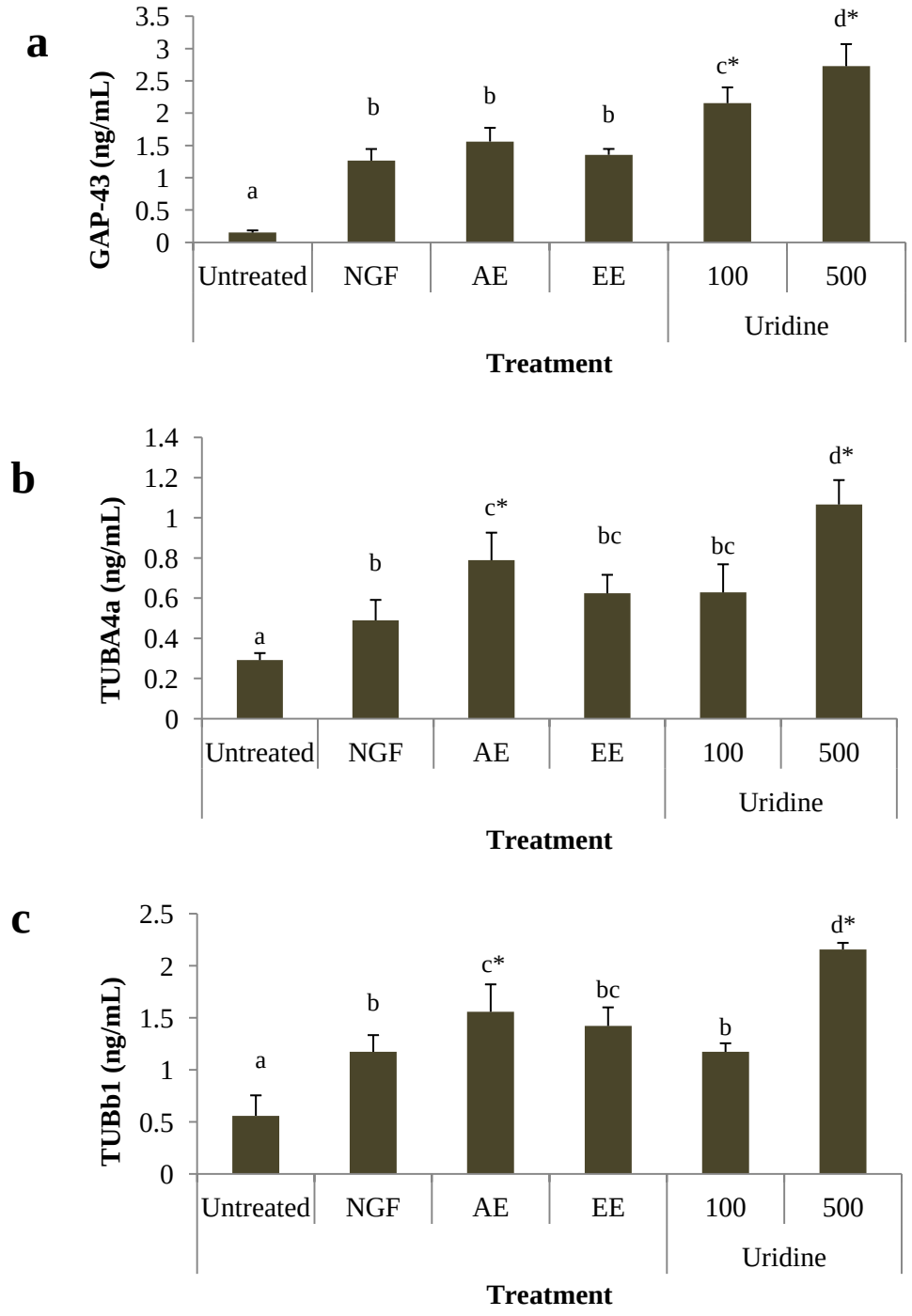


Figure 5.13: Uridine and mushroom extracts increased the activity of GAP-43 (a), Tubulin aipha 4a (b), and tubulin beta b1 (c) in N2a cells. Data are expressed as mean ± S.D. for n=3. *$p < 0.05$ represents a significantly higher value from the untreated and NGF-treated cells.

5.4 Discussion

In this study, linoleic acid promoted neurite outgrowth of N2a cells by 20.78 ± 2.43% (Figure 5.3). On the other hand, oleic acid caused little or no neurite outgrowth in N2a cells. Extension of neurites from neuronal cell body is an important step in neuronal development and requires the generation of additional plasma membrane (Kamata, Shiraga, Tai, Kawamoto, & Gohda, 2007). Polyunsaturated fatty acids like linoleic, linolenic, docosahexanoic, and arachidonic acids are known to promote neurite extension (Darios & Davletov, 2006). This suggests that linoleic acid which was present in abundance in the extracts of *P. giganteus* may play a key role in neuritogenesis. Similar to our results, other studies also showed that monounsaturated fatty acids and saturated long-chain fatty acids like oleic, stearic and palmitic acids caused little or no effects in neurite outgrowth (Darios & Davletov, 2006).

Long-chain polyunsaturated fatty acids (LCPUFA) are essential nutrients in the development and functioning of the central nervous system. The most abundant LCPUFA in the brain are docosahexaenoic acid (DHA) which is mainly derived from fish, and arachidonic acid (ARA) from meat and eggs (Janssen & Kiliaan, 2014). The desaturation and elongation of linoleic acids and alpha-linolenic acids to ARA and DHA; respectively, are very crucial for the infant's brain development. Since the percentage of ARA decreases in the brain during prenatal development, the balance in the dietary ratio of linoleic acid is very crucial to maintain a healthy brain development in preterm infant (Enke et al., 2011). Apart from that, many studies have demonstrated the importance of LCPUFA as a potent neuroprotectant. Linoleic acid was found to protect mouse cortical neurons against glutamate excitotoxicity (Hunt, Kamboj, Anderson, & Anderson, 2010). Linoleic acid and its derivatives also prevented sodium nitroprusside-induced cell death of cultured rat cerebral cortical neurons (Yaguchi, Fujikawa, & Nishizaki, 2010).

Nucleosides (adenosine, guanosine, uridine, and inosine) bind purinergic and/or pyrimidine receptors and their functions include the regulation and modulation of various physiological processes in the human body. Beside acting as precursors in nucleic acid synthesis, nucleotides were reported to enhance immune response, control metabolism of fatty acids, contribute to iron absorption in the gut, and improve gastrointestinal tract repair after damage (Yang, Lv, Zhang, & Xia, 2012). In this study, uridine was found to be present in a considerably high amount (1.66-1.80 g/100g extract). To date, uridine has been recognised as one of the main bioactive compounds in *Cordyceps militaris*. The mycelia extract of *C. militaris* NBRC 9787 and *C. militaris* G81-3 were reported to contain 106.8 and 45.4 mg/kg extract of uridine (Das, Masuda, Sakurai, & Sakakibara, 2010). Most recently, a total of 0.20, 0.79, 1.50, 1.40, and 0.80 mg/g extract of uridine was detected in *Agrocybe aegerita*, *Boletus nigricans*, *Boletus fulvus*, *Tricholoma matsutake*, and *Auricularia auricular-judae*; respectively (Yang et al., 2012). Besides, fractions F-K of the methanol extract of *Gomphus clavatus* demonstrated a high scavenging activity (63.8 –0.1 70.3%) against 1,1-diphenyl-2-picrylhy-drazyl (DPPH) radicals. Uridine and several other compounds like nicotinic acid and inosine were isolated from the fractions (Makropoulou et al., 2012).

Neurons form synapses through outgrowth of neurite throughout life. The numbers of neurite outgrowth and membrane synapses formed depend on the levels of three key nutrients in the brain, i.e. uridine, omega-3 fatty acid DHA, and choline (Wurtman, Cansev, Sakamoto, & Ulus, 2010). Therefore, it is thought that giving these compounds to patients with AD, a disease characterised by loss of neurite outgrowth and brain synapses, could be beneficial (Wurtman et al., 2010). Uridine is present as such in breast milk (Thorell, Sjöberg, & Hernell, 1996), but also as constituents of RNA; nucleotides (5'-UMP); and nucleotide adducts (UDP-glucose or UDP-galactose) (Leach, Baxter, Molitor, Ramstack, & Masor, 1995). Synthetic infant formulas are also

routinely fortified with uridine and uridine monophosphates. Cytidine (as cytidine triphosphate, CTP) and uridine (which is converted to UTP and then CTP) contribute to brain phosphatidylcholine and phosphatidylethanolamine synthesis *via* the Kennedy pathway (Cansev, 2006) (Figure 5.14). Uridine and cytidine circulating in our body can serve as the substrates to synthesise the respective nucleotides. They act as the precursors of the cytidine triphosphate (CTP) needed in the phosphatidylcholine (PC) biosynthetic pathway (Kennedy & Weiss, 1956). The principal constituents of mammalian cell membranes are phosphatides, the most abundant of which is phosphatidylcholine (PC) (Cansev, 2006). PC biosynthesis is initiated by the phosphorylation of choline to form phosphocholine, which then combines with cytidine triphosphate (CTP) to form 5'-cytidine diphosphocholine (CDP-choline); this compound then reacts with diacylglycerol (DAG) to produce PC (Richardson, Watkins, Pierre, Ulus, & Wurtman, 2003) (Figure 5.14). Uridine or cytidine increases CTP levels, hence, in turn can be rate-limiting in the syntheses of PC.

In gerbils (*Meriones unguiculatus*) and humans, the primary circulating pyrimidine is uridine. Uridine readily penetrates the blood-brain barriers (BBB) and enters the brain *via* a high-affinity transporter yielding UTP which is then converted to CTP by CTP synthase (Figure 5.14). Intracellular levels of uridine triphosphate (UTP) depend on the availability of free uridine. Since it seems possible that uridine would also enhance the production and extension of neurites, it is hypothesised that increasing the availability of uridine may further promote neurite outgrowth in N2a cells.

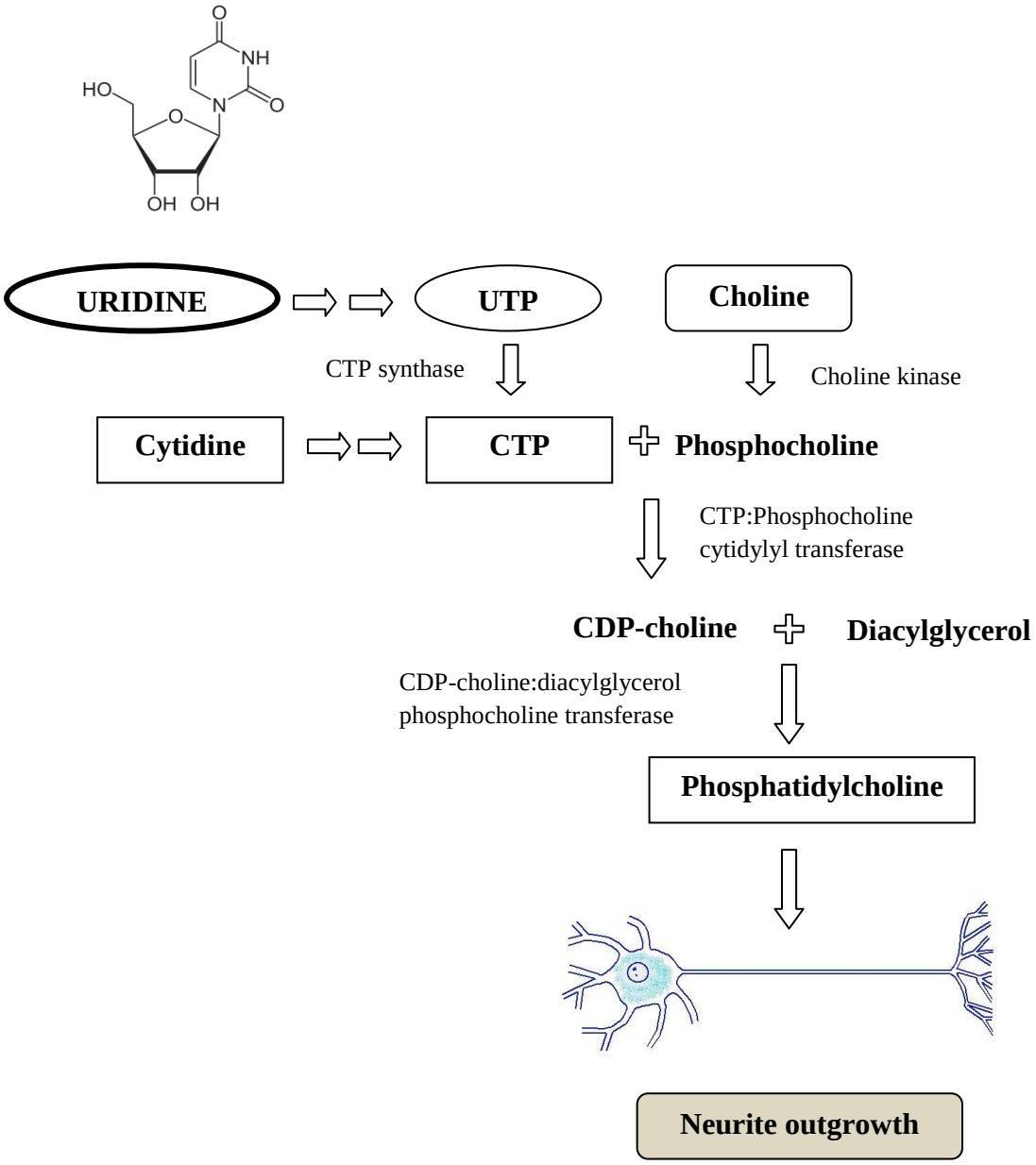


Figure 5.14: Phosphatidylcholine (PC) biosynthesis *via* the Kennedy pathway (Kennedy & Weiss, 1956; Richardson et al., 2003).

In a study using PC12 cells, uridine increased the number of neurites per cell significantly and in a dose-dependent manner after 4 days (Pooler, Guez, Benedictus, & Wurtman, 2005). This increase was accompanied by an increase in neurite branching and the neurofilament M and neurofilament 70. Uridine treatment also increased intracellular levels of CTP which suggests that uridine may affect neurite outgrowth by enhancing phosphatidylcholine synthesis. In an in vivo study, gerbils were given

supplemental uridine as its monophosphate (UMP, 0.5%), DHA (300 mg/kg/day), and/or choline (0.1%) via diet and by gavage for 4 weeks (Holguin, Martinez, Chow, & Wurtman, 2008). When uridine was co-administered with choline, phosphatides were increased. Uridine also further enhanced the animals' performance on the neurobehavioral tests, i.e. the four-arm radial maze, T-maze, and Y-maze tests. The effects of dietary supplementation with uridine (as in UMP-2Na+, an additive in infant milk formulas) on striatal dopamine (DA) release in aged rats were also tested (L. Wang, Pooler, Albrecht, & Wurtman, 2005). As a result, DA release was significantly greater among UMP-treated rats. Besides, the levels of neurofilament-70 and neurofilament-M proteins (biomarkers of neurite outgrowth) were also increased significantly with UMP consumption. Co-supplementation of uridine with DHA also increased the number of dendritic spines in adult gerbil hippocampus by more than 30% (Sakamoto, Cansev, & Wurtman, 2007).

Another hypothesis for uridine-induced neurite outgrowth is the involvement of P2Y receptor, a family of purinergic G protein-coupled receptors (Holguin et al., 2008). Exogenous uridine and its phosphorylated products, such as UMP, UDP, and UTP act as ligands for P2Y receptors which then can activate downstream protein synthesis related to neuronal differentiation and the promotion of brain glycogen synthesis *via* UDP- glucose (Brown, 2004). While P2X receptors recognise adenine nucleotides, P2Y receptors can recognise both adenine and uridine nucleotides. Members of the P2Y family are widely distributed throughout the body, including the brain (Wurtman et al., 2010). To date, eight P2Y receptors of human origin (P2Y1, 2, 4, 6, 11, 12, 13, and 14) have been cloned and characterised (Haas, Ginsburg-Shmuel, Fischer, & Reiser, 2014; Wurtman et al., 2010).

In gerbils, a single oral dose of a uridine source, i.e., UMP (which was approximately 300 mg/kg of uridine), yielded a two-fold increase in brain uridine levels (Cansev, Watkins, van der Beek, & Wurtman, 2005). Besides, uridine can also have effects on the brain by activating the P2Y receptors. There are eight different mammalian P2Y receptor subtypes (P2Y1, 2, 4, 6, 11, 12, 13, and 14) and only P2Y2, P2Y4 and P2Y6 accept uridine nucleotides as ligands (Dobolyi, Juhasz, Kovacs, & Kardos, 2011). In this study, uridine has been shown to mediate neurite outgrowth. This effect was accompanied by an increase in tubulin alpha and beta synthesis. Further, uridine's effect was blocked by P2Y receptor antagonists, suggesting that uridine may promote neurite outgrowth by uridine-mediated stimulation of a P2Y receptor-coupled signaling pathway. This observation is in agreement with previously reported neurotrophic effects of P2Y receptors. UDP and UTP have been reported previously to modulate noradrenaline release from cultured rat superior cervical ganglia (Boehm, Huck, & Illes, 1995). In addition to that, the pathway involved in UTP-evoked noradrenaline release was then shown to be mediated by P2Y6 receptors *via* activation of protein kinase C (Vartian et al., 2001). Uridine has been shown to excite sensory neurons *via* P2Y2 receptors (Molliver, Cook, Carlsten, Wright, & McCleskey, 2002) and most recently, extracellular UDP-glucose has been reported to stimulate neurite outgrowth *via* the purinergic P2Y14 receptor (Haanes & Edvinsson, 2014).

All eukaryotic cells possess multiple mitogen-activated protein kinases (MAPKs) pathways, which co-ordinate to regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation (Seger & Krebs, 1995). The conventional MAPKs comprise the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms (α, β, γ, and δ), and ERK5. By far the most extensively studied MAPKs are the ERK1/2, JNKs, and p38 isoforms. The Erk1/2 (p44/42) signaling pathway can be activated in response

to many extracellular stimuli, for example mitogens and growth factors (W. Zhang & Liu, 2002). Upon stimulation, a chronological protein kinase cascade is initiated. The three-part kinase consists of a MAP kinase kinase kinase (MAPKKK or MAP3 K), a MAP kinase kinase (MAPKK or MAP2 K), and a MAP kinase (MAPK). MEK1 and MEK2, which are MAPKKs, activate p44 and p42 through phosphorylation of activation loop residues Thr202/Tyr204 and Thr185/Tyr187, respectively. Here, it was demonstrated that 100 μM of uridine activated the phosphorylation of ERK1/2. Previous studies have shown that induction of ERK activation by some medicinal mushrooms was consistent with their ability to stimulate neurite outgrowth and that treatment with specific inhibitors resulted in inhibition of neuritogenesis by *G. lucidum* (Cheung et al., 2000), *G. neo-japonicum* (Seow et al., 2013), and *G. frondosa* (Nishina et al., 2006).

Activation of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of the downstream signaling effector, the Akt has been implicated in the neuronal survival and differentiation (H. S. Kim, Hong, Kim, & Han, 2011). Akt plays a critical role in controlling cell survival and apoptosis; and is activated by phospholipid binding and activation loop phosphorylation at Thr308 by pyruvate dehydrogenase lipoamide kinase 1 (PDK1) (Bozulic & Hemmings, 2009). PI3K/Akt has been proposed as a potential therapeutic target in neurodegenerative diseases since activation of Akt inhibits stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) that causes oxidative stress during neuronal degeneration (Burke, 2007). LY294002, a specific inhibitor of Akt has been widely used to elucidate PI3K/Akt pathway. In this study, based on the results it was hypothesised that neuritogenesis potentiated by uridine was regulated by PI3K/Akt pathway. As a result, uridine-treated N2a cells exhibited a significantly higher (p<0.05) Akt (Thr308) phosphorylation when compared to NGF control (50 ng/mL). This is consistent with the other reported studies. Dilong extracts (Chinese medicinal

preparation from the earthworm species *Lumbricus rubellus*) were found to promote neuron regeneration (Chang et al., 2011). Treatment with extract of Dilong induced the phosphorylation of the insulin-like growth factor-I (IGF-I)-mediated PI3K/Akt pathway, resulting in cell proliferation and survival of RSC96 Schwann cells. Besides, sargaquinoic acid isolated from a marine brown alga *Sargassum macrocarpum*, was found to promote neurite outgrowth in PC12 cells and inhibition of PI3K by wortmannin significantly suppressed the neuritogenic activity of sargaquinoic acid (Tsang & Kamei, 2004). More recently, luteolin (3′,4′,5,7-tetrahydroxyflavone) isolated from rosemary, *Rosmarinus officinalis* (Lamiacea), has been reported to induce PC12 cell differentiation (El Omri, Han, Kawada, Ben Abdrabbah, & Isoda, 2012). Luteolin treatment significantly enhanced acetylcholinesterase (AChE) activity and increased the level of total choline and acetylcholine in PC12 cells. In addition, treatment with U0126 and LY294002 also attenuated luteolin-induced AChE activity and neurite outgrowth in PC12 cells, suggesting that the neuritogenic properties of luteolin was regulated by activation of ERK1/2 and PI3K/Akt signalings.

The cAMP responsive element binding protein, CREB is a bZIP transcription factor that activates target genes through cAMP response elements. CREB is able to mediate signals from numerous physiological stimuli, resulting in regulation of a wide array of cellular responses. CREB plays a dominant regulatory role in the nervous system (Scott Bitner, 2012). CREB is believed to play a key role in promoting neuronal survival, precursor proliferation, neurite outgrowth and neuronal differentiation in certain neuronal populations (Yamashima, 2012). Some of the kinases involved in phosphorylating CREB at Ser133 are the MAPK and PI3K/Akt. Therefore, it is hypothesised that uridine present in the *P. giganteus* extracts could be metabolised in the N2a cells, and uridine along with its phosphates derivatives bind P2Y receptors and activates the MAPK and PI3K/Akt pathways, leading to the phosphorylation of the

transcription factor CREB that is able to selectively activate numerous downstream genes such as the growth associated protein 43 (GAP-43) and microtubules.

There is mounting evidence supporting the fact that growing neurons express high levels of GAP-43 and that the up-regulation of GAP-43 mRNA and protein is associated with neurite outgrowth (Benowitz & Routtenberg, 1997). The results in this study showed that after exposure to uridine, N2a cells exhibited morphological changes and neurite formation along with up-regulation of GAP-43. This is consistent with previous findings which demonstrated that DHA significantly increased the cellular GAP-43 immunoactivity and GAP-43 content in N2a cells (Wu et al., 2009). Dishevelled (Dvl), a cytoplasmic protein involved in the Wnt-Frizzled signaling cascade, has also been shown to interact with the cytoskeleton through modulation of GAP-43, and caused neurite outgrowth in N2a cells (Fan, Ramirez, Garcia, & Dewhurst, 2004). Claulansine F (Clau F) is a carbazole alkaloid isolated from the stem of wampee, *Clausena lansium* (Lour) Skeels. Clau F was found to have a critical role in elevating GAP-43 expression, which in turn triggered neuritogenesis in PC12 cells promoted by Clau F. Besides, 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (5-OH-HxMF), which is found exclusively in the Citrus genus (particularly in the peels of sweet orange) was found to promote neurite outgrowth in PC12 cells. Accordingly, it was reported that there was a strong positive correlation of elevated GAP-43 expression with the neuronal outgrowth states (Lai et al., 2011). Further, an increase in GAP-43 protein is associated with neuritogenesis in NGF-treated PC12 cells, potentiated by green tea polyphenols (Gundimeda et al., 2010). Finally, Figure 5.15 shows the hypothetic mechanism of uridine in promoting neurite outgrowth in differentiating N2a cells.

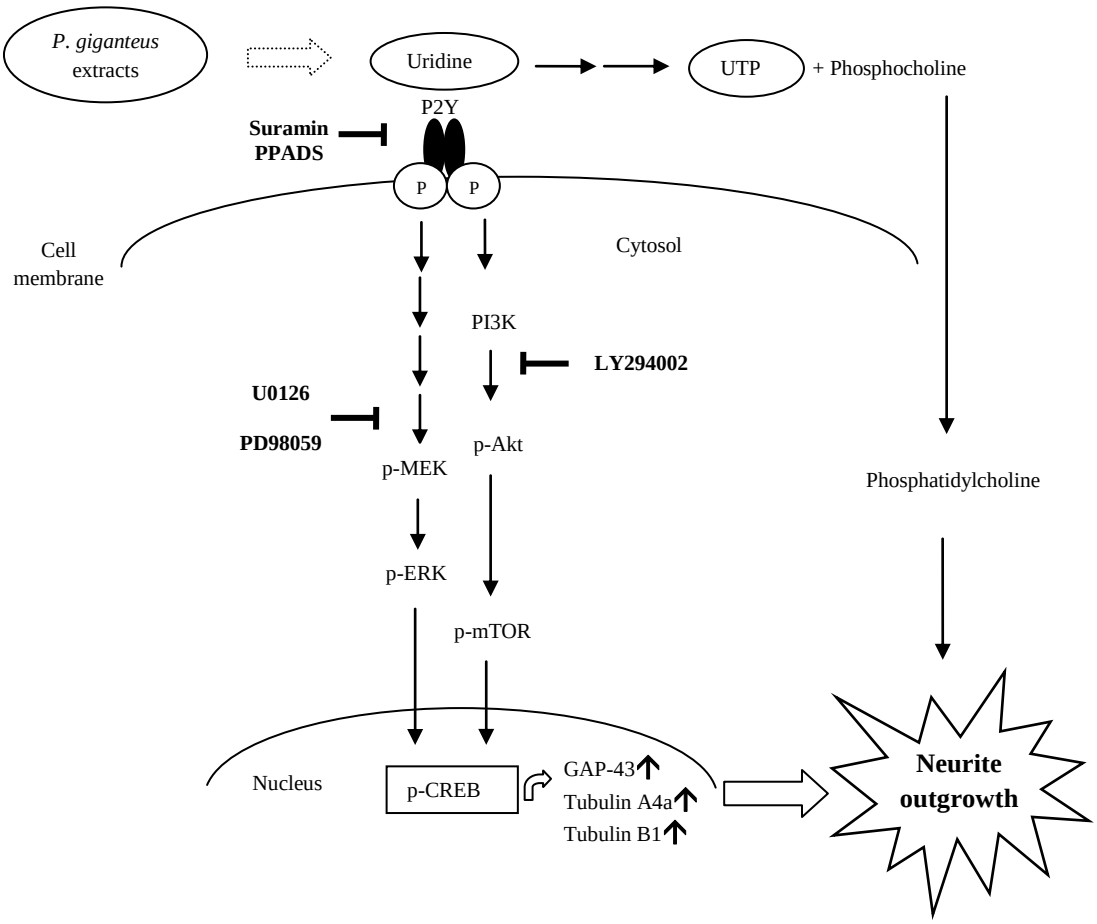


Figure 5.15: Hypothetic mechanism of uridine in promoting neurite outgrowth in N2a cells. Uridine could induce neurite outgrowth associated with expression of neuronal differentiation marker (GAP-43, tubA4a, and tubB1). Uridine stimulated CREB phosphorylation and neurite outgrowth mainly through activation of P2Y-dependent pathway. MEK/ERK1/2 and PI3K/Akt/mTOR were also partly involved in the uridine-induced neurite outgrowth. In addition, uridine conversation to UTP which is further combined with phosphocholine yielding phosphatidylcholine, may also partially contribute to the uridine-mediated neurite outgrowth. ⊣ = inhibit. ↑ increase.

5.5 Conclusion

The neurite outgrowth stimulatory effect of the chemical compounds and *P. giganteus* extracts in descending order was: uridine > aqueous extract > ethanol extract > NGF > linoleic acid > succinic acid > benzoic acid > cinnamic acid > caffeic acid > oleic acid > *p*-coumaric acid. The findings in this study clearly demonstrate that uridine (1.66-1.80 g/100g extract) may have potential in promoting neurite outgrowth as evidenced by the neurite bearing cell scores (43.09 ± 4.88%) and the chromogenic method (0.53 ± 0.02 AU). This finding supports *P. giganteus* as a potential mushroom that may be used as a functional food for reducing the impact of neurodegenerative diseases which involve the decrease or absence of neurite outgrowth. Moreover, uridine may serve as a marker component for the standardisation of *P. giganteus* as a functional food. Uridine promoted neurite outgrowth in differentiating N2a cells in a dose-dependent manner. The results indicated that uridine- and *P. giganteus* extracts-induced neuritogenesis was regulated, at least in part, by cross-talk between the MEK/ERKs and PI3K/Akt/mTOR pathways and required the activation of the transcription factor CREB. The neuronal biomarkers (GAP-43, tubulin alpha 4a, and beta) in N2a cells were also significantly ($p < 0.05$) increased when treated with uridine.

CHAPTER VI

PROPOSED FUTURE STUDIES AND CONCLUSIONS

The following is recommended as important and promising research directions for developing functional food of *P. giganteus* and other edible mushrooms with neurite outgrowth activity:

a) Additional effort is needed to determine the uridine level in different mushroom species as this nucleoside is clearly shown to promote neurite outgrowth.

b) In view of the neurite outgrowth activity of *P. giganteus* observed in this study, this mushroom can become a popular functional food. It is therefore necessary to examine the (subacute) toxicity of the mushroom extracts in compliance with the guidelines from the Organization of Economic Cooperation and Development (OECD, 1995).

c) Animal model studies with a dose-response design are needed to clearly establish the efficacy and mechanisms of action of uridine in *P. giganteus* in stimulating neurite outgrowth *in vivo*. Further, if *P. giganteus* is proposed to be a functional food, the animal model study should reflect this hypothesis by using the oral route of administration. Most importantly, the bioavailabity of uridine in *P. giganteus* extracts must be determined.

Therapeutic strategy to stimulate neuronal cell events including neurite outgrowth and to some extent, neuroprotection are needed for several neurodegenerative disorders like dementia. Many mushrooms have been known to have health benefits, but little is

known about their neurite outgrowth stimulatory effects. The main findings of this study are:

a. *Pleurotus giganteus* was identified as a potential neuritogenic mushroom species based on its high score in percentage of neurite-bearing N2a cells (33.4 ± 4.6%) which was significantly ($p < 0.05$) higher than the NGF control. The mushroom extract also recorded mean neurite length of 116.72 ± 29.5 µm which was about 6-time longer than cell body. The neuritic process was further confirmed by neurofilaments staining.
b. It was revealed that *P. giganteus* extracts contained considerably high amount of potassium, vitamins B1, B2, and B3, as well as linoleic acid; all of which may contribute to the neurite outgrowth stimulatory effects. The extracts also exhibited antioxidant activity which was believed to potentiate neuritogenesis by scavenging reactive species and influent intracellular redox status.
c. The uridine constituent (1.66-1.80 g/100g extract) present in *P. giganteus* was the most promising compound in promoting neurite outgrowth as evidenced by the neurite bearing cell score (43.09 ± 4.88%). Nucleoside, such as uridine, can cross blood-brain barrier. Uridine may work as therapeutic agent that possessed high neurotrophic potency and involved in numerous effects within the brain.
d. Uridine from *P. giganteus* was able to stimulate neuritogenesis *via* its interactions with critical neuronal intracellular signaling pathways. The uridine-induced neurite outgrowth involved the activation of MEK/ERK1/2, PI3k/Akt, and to some extends the mTOR pathways. Since uridine was the major bioactive constituent present in the extract, the involvement of specific P2Y receptor is suggested as the addition of P2Y inhibitor blocked the uridine-induced neurite outgrowth activity. Further, the phosphorylation of CREB may be associated with the neuronal function of uridine on differentiating N2a cells. Additionally,

uridine and *P. giganteus* extracts induced the accumulation of intracellular GAP-43, tubulin A4a and tubulin B1 activities.

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APPENDICES

A. DATA AND STATISTICAL TABLES

Table 1: Multiple range test: The effects of different NGF concentrations (5-100 ng/mL) on stimulation of neurite outgrowth

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD

Col_1	Count	Mean	Homogeneous Groups
11	3	3.3	X
10	3	5.23333	XX
12	3	6.16667	XX
1	3	7.6	XXX
9	3	8.73333	XX
5	3	9.86667	XX
4	3	11.5667	X
2	3	11.6	X
8	3	12.1667	X
3	3	12.3667	X
6	3	15.8667	X
7	3	26.1667	X

Table 2: Multiple range test: The mean neurite length of N2a cells treated with different mushroom extracts

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD

Col_1	Count	Mean	Homogeneous Groups
8	5	7.486	X
7	5	78.578	X
3	5	82.39	X
1	5	90.576	XX
4	5	95.564	XXX
5	5	116.722	XX
2	5	121.51	X

Table 3: Multiple range test: TPC results

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
1	3	9.57653	X
2	3	12.1436	X
3	3	21.6116	X
4	3	24.0762	X

Table 4: Multiple range test: Total polysaccharides

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
4	3	2.462	X
3	3	2.972	X
2	3	3.22967	X
1	3	3.862	X

Table 5: Multiple range test: DPPH scavenging effects between the commercial and cultivated wild *P. giganteus*

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
2	3	8.10132	X
1	3	11.2823	XX
3	3	16.1761	XX
4	3	21.4631	X

Table 6: Multiple range test: FRAP results between the commercial and cultivated wild *P. giganteus*

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
1	3	2.03596	X
3	3	2.25992	XX
2	3	2.69474	XX
4	3	2.9864	X

Table 7: Multiple range test: Lipid peroxidation inhibition results between the commercial and cultivated wild *P. giganteus*

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
2	3	44.406	X
1	3	44.6145	X
4	3	49.5831	X
3	3	49.8018	X

Table 8: Correlation table of the antioxidant activities

Correlations

		DPPH	TPC	Polysaccharides	TFC	FRAP	LIPID
DPPH	Pearson Correlation	1	.827	.133	-.970	.820	.321
	Sig. (2-tailed)		.380	.915	.157	.388	.792
	N	3	3	3	3	3	3
TPC	Pearson Correlation	.827	1	.668	-.939	.355	-.267
	Sig. (2-tailed)	.380		.535	.223	.769	.828
	N	3	3	3	3	3	3
Polysaccharides	Pearson Correlation	.133	.668	1	-.371	-.459	-.896
	Sig. (2-tailed)	.915	.535		.758	.696	.293
	N	3	3	3	3	3	3
TFC	Pearson Correlation	-.970	-.939	-.371	1	-.655	-.080
	Sig. (2-tailed)	.157	.223	.758		.546	.949
	N	3	3	3	3	3	3
FRAP	Pearson Correlation	.820	.355	-.459	-.655	1	.806
	Sig. (2-tailed)	.388	.769	.696	.546		.403
	N	3	3	3	3	3	3
LIPID	Pearson Correlation	.321	-.267	-.896	-.080	.806	1
	Sig. (2-tailed)	.792	.828	.293	.949	.403	
	N	3	3	3	3	3	3

Table 9: Multiple range test: Percentage of neurite bearing cells of different chemical compounds from *P. giganteus*

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD

Col_1	Count	Mean	Homogeneous Groups
1	3	9.03333	X
7	3	10.8527	X
11	3	16.535	X
6	3	24.2633	X
2	3	24.2967	X
10	3	24.5167	X
8	3	25.55	X
12	3	27.5133	X
13	3	27.6133	X
9	3	28.1067	X
4	3	33.3833	X
5	3	37.03	X
3	3	47.6873	X

Table 10: Multiple range test: Quantification of neurite using chromogenic methods

```
Multiple Range Tests for Col_2 by Col_1

------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
2              3         0.0419            X
11             3         0.119133           X
7              3         0.12               X
6              3         0.1371             X
9              3         0.157133           X
8              3         0.16               X
5              3         0.167833           X
10             3         0.304833            X
3              3         0.353367            XX
4              3         0.4088               XX
1              3         0.459667              X
12             3         0.567767                X
```

Table 11: Multiple range test: The effects of different concentration of uridine on neurite outgrowth

```
Multiple Range Tests for Col_2 by Col_1

------------------------------------------------------------------------------
Method: 95.0 percent LSD
Col_1          Count     Mean              Homogeneous Groups
------------------------------------------------------------------------------
1              3         9.03333           X
5              3         21.9403            X
2              3         23.1667            X
3              3         29.3567              X
4              3         34.9733                X
7              3         43.0785                 X
8              3         45.4244                 XX
6              3         49.2406                   X
------------------------------------------------------------------------------
```

Table 12: Multiple range test: The effects of two specific inhibitors of P2Y receptors (suramin and PPADS) on neurite outgrowth

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
1	3	9.03333	X
7	3	10.8527	X
11	3	16.535	X
6	3	24.2633	X
2	3	24.2967	X
10	3	24.5167	X
8	3	25.55	X
12	3	27.5133	X
13	3	27.6133	X
9	3	28.1067	X
4	3	33.3833	X
5	3	37.03	X
3	3	47.6873	X

Table 13: Multiple range test: GAP-43 expression

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
1	3	0.151555	X
2	3	1.2664	X
4	3	1.35624	X
3	3	1.56145	X
5	3	2.15596	X
6	3	2.72776	X

Table 14: Multiple range test: Tubulin alpha 4a expression

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
1	3	0.291242	X
2	3	0.489628	X
4	3	0.623897	XX
5	3	0.628423	XX
3	3	0.789093	X
6	3	1.06668	X

Table 15: Multiple range test: Tubulin beta b1 expression

Multiple Range Tests for Col_2 by Col_1

Method: 95.0 percent LSD			
Col_1	Count	Mean	Homogeneous Groups
5	3	0.297333	X
1	3	0.329333	X
6	3	0.358	X
2	3	0.682333	X
3	3	0.695	X
4	3	0.808667	X

Figure 1: Calibration plot for TPC

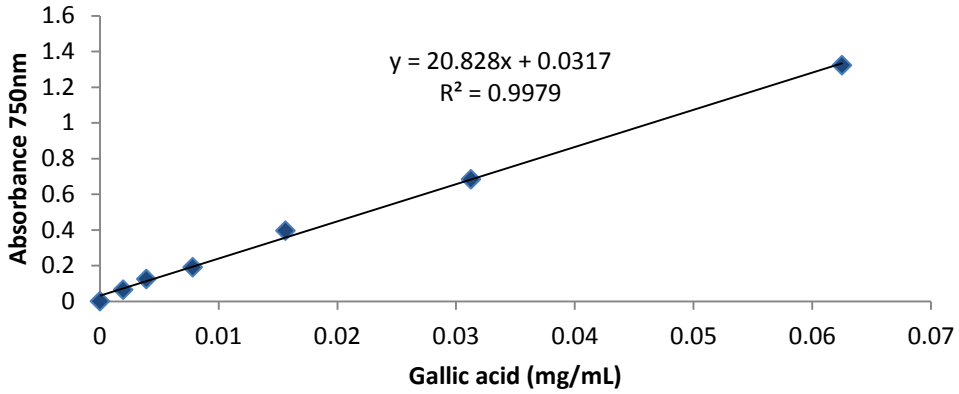


Figure 2: Calibration plot for total polysaccharides

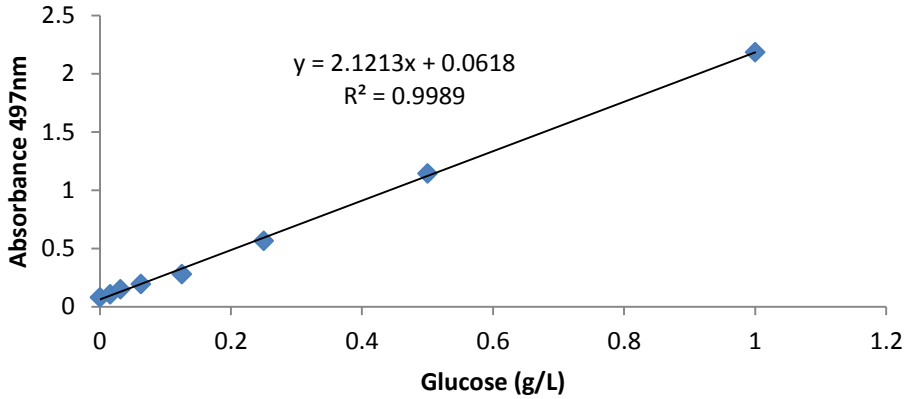


Figure 3: Calibration plot for total flavonoids

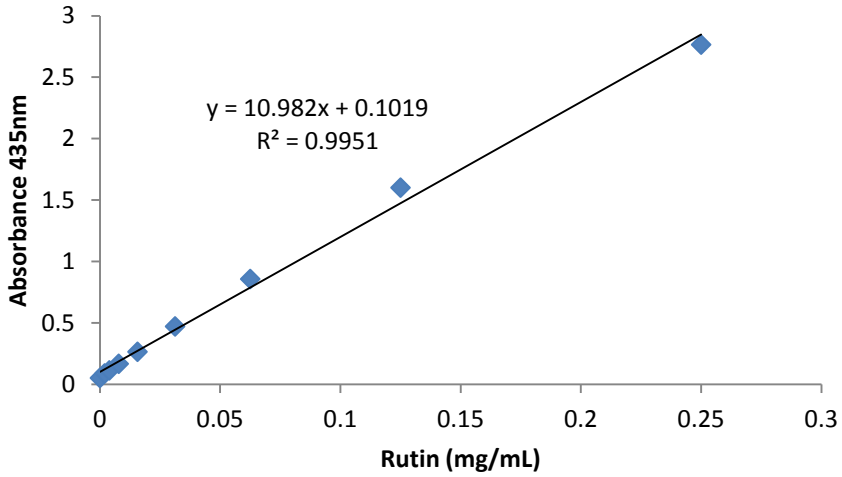


Figure 4: Calibration plot for FRAP

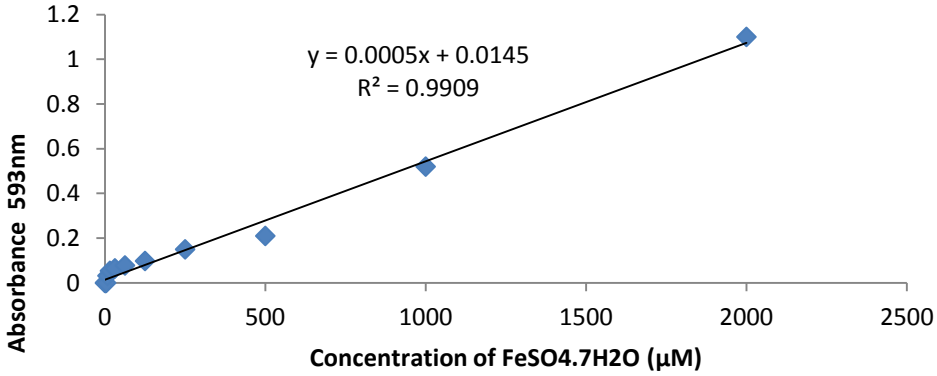


Figure 5: Calibration plot for uridine

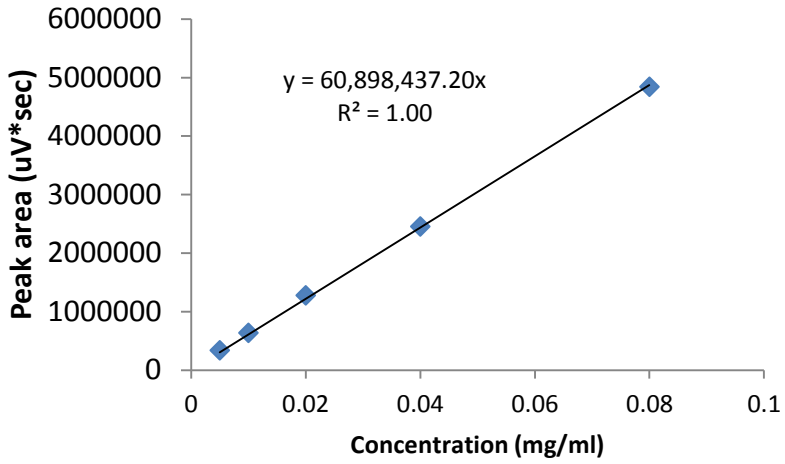


Figure 6: Calibration plot for GAP43

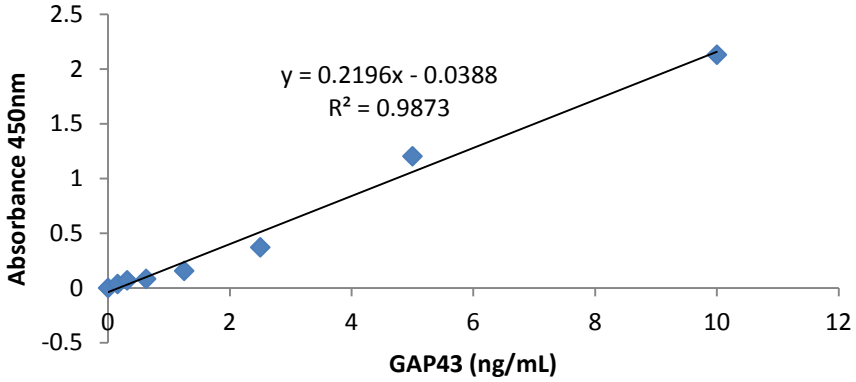


Figure 7: Calibration plot for Tubulin alpha A4a

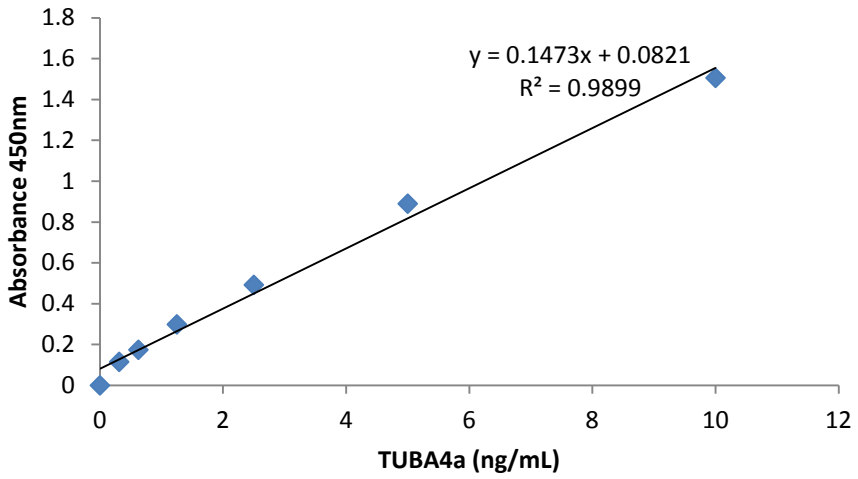
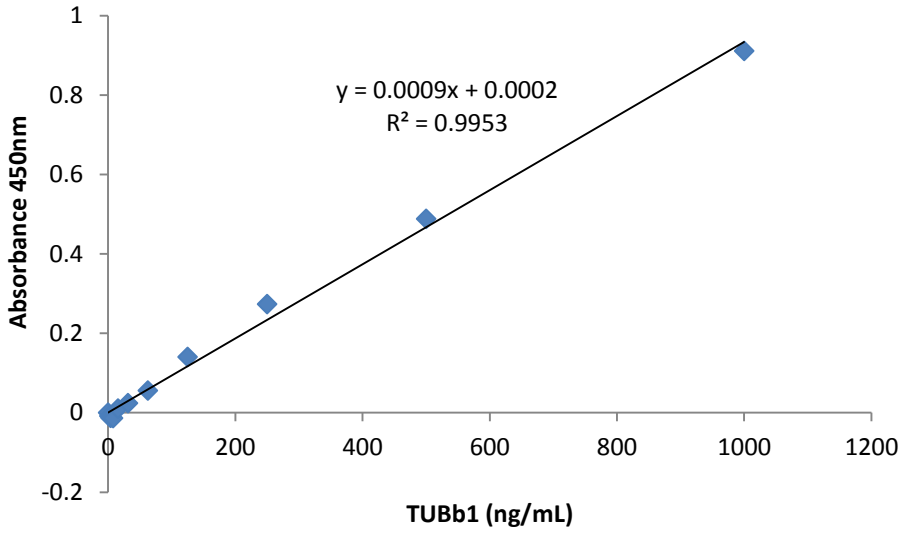


Figure 8: Calibration plot for Tubulin beta b1



B. PUBLICATIONS AND PRESENTATIONS

Journal articles

1. **Phan, C.-W**., David, P., Naidu, M., Wong, K.-H., & Sabaratnam, V. (2014). Therapeutic potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases: Diversity, metabolite, and mechanism. *Critical Reviews in Biotechnology*. In press doi:10.3109/07388551.2014.887649 **(I.F. 7.837)**
2. **Phan, C. -W.**, Lee, G. -H., Macreadie, I. G., Malek, S., Pamela, D., & Sabaratnam, V. (2013). Lipid constituents of the edible mushroom, *Pleurotus giganteus* demonstrate anti-*Candida* activity. *Natural Product Communications*, *8*, 1763–1765. **(I.F 0.924)**
3. **Phan, C.-W**., David, P., Naidu, M., Wong, K.-H., & Sabaratnam, V. (2013). Neurite outgrowth stimulatory effects of culinary-medicinal mushrooms and their toxicity assessment using differentiating Neuro-2a and embryonic fibroblast BALB/3T3. *BMC Complementary and Alternative Medicine*, *13*(1), 261. (**I.F 1.877)**
4. **Phan, C. -W.**, Wong, W. -L., David, P., Naidu, M., & Sabaratnam, V. (2012). *Pleurotus giganteus* (Berk.) Karunarathna & K. D. Hyde : Nutritional value and in vitro neurite outgrowth activity in rat pheochromocytoma cells. *BMC Complementary & Alternative Medicine*, *12*,102. **(I.F 1.877)**
5. **Phan, C. -W.**, David, P., Tan, Y-S., Naidu, M., Wong, K.-H., Kuppusamy, U.R., & Sabaratnam, V. (2014). Intrastrain comparison of the chemical composition and antioxidant activity of an edible mushroom, *Pleurotus giganteus*, and its potent neuritogenic properties. *Scientific World Journal*, Article ID 378651. **(I.F 1.219)**

Oral Presentations

1. **Phan, C. -W.**, David, P., Naidu, M., Wong, K.-H., & Sabaratnam, V. (2013). *Investigation of the role of culinary and medicinal mushrooms in neurodevelopment by using differentiating neuroblastoma-2a cells.* Paper presented at the 7th International Medicinal Mushroom Conference, Beijing, 26-29 August (pp. 76-77).
2. **Phan, C. -W.**, Moroney, S., Wong, W.-L., Sabaratnam, V., David, P., Naidu, M., Wong, K.-H., & Tan, Y.-S. (2013). *Pharmagological potential of an edible mushroom, Pleurotus giganteus (Berk) Karunarathna & K.D. Hyde.* Paper presented at the International Functional Food Conference, Cyberjaya, Malaysia, 18-20 August (pp. 5).
3. **Phan, C. -W.**, Moroney, S., David, P., Naidu, M., Sri Nurestri, M., & Sabaratnam, V. (2012). *In vitro neuronal differentiation and neurite outgrowth of rat pheochromocytoma cells evoked by Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde*. Paper presented at the 18th Congress of International Society of Mushroom Science, Beijing, China, 26-30 August (pp. 122-123).

Poster Presentations

1. **Phan, C. -W.**, Tan, Y.-S., & Sabaratnam, V. (2014). *A snapshot of the medicinal properties of Pleurotus giganteus.* Paper presented at the Mushroom Day Workshop, Klang, Malaysia, 24-26 August (pp. 34).
2. **Phan, C. -W.**, David, P., Naidu, M., & Sabaratnam, V. (2011). *In vitro neuronal differentiation of rat pheochromocytoma cells by aqueous extract of Panus*

giganteus (Berk. Corner. Paper presented at the International Congress Malaysian Society of Microbiology 2011- "Leveraging on Microbial Diversity for a Sustainable Future", Bayview Beach Resort, Penang, Malaysia, 8-11 December (pp. 347-349).

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REVIEW ARTICLE

Therapeutic potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases: diversity, metabolite, and mechanism

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Abstract

Mushrooms have long been used not only as food but also for the treatment of various ailments. Although at its infancy, accumulated evidence suggested that culinary-medicinal mushrooms may play an important role in the prevention of many age-associated neurological dysfunctions, including Alzheimer's and Parkinson's diseases. Therefore, efforts have been devoted to a search for more mushroom species that may improve memory and cognition functions. Such mushrooms include *Hericium erinaceus*, *Ganoderma lucidum*, *Sarcodon* spp., *Antrodia camphorata, Pleurotus giganteus, Lignosus rhinocerotis, Grifola frondosa*, and many more. Here, we review over 20 different brain-improving culinary-medicinal mushrooms and at least 80 different bioactive secondary metabolites isolated from them. The mushrooms (either extracts from basidiocarps/mycelia or isolated compounds) reduced beta amyloid-induced neurotoxicity and had anti-acetylcholinesterase, neurite outgrowth stimulation, nerve growth factor (NGF) synthesis, neuroprotective, antioxidant, and anti-(neuro)inflammatory effects. The *in vitro* and *in vivo* studies on the molecular mechanisms responsible for the bioactive effects of mushrooms are also discussed. Mushrooms can be considered as useful therapeutic agents in the management and/or treatment of neurodegeneration diseases. However, this review focuses on *in vitro* evidence and clinical trials with humans are needed.

Keywords

Alzheimer's disease, antioxidant, culinary-medicinal mushroom, neurite outgrowth, nerve regeneration, neurodegeneration, neuroprotection, secondary metabolite

History

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Introduction

Life expectancy of humankind had increased to 50–60 years at the beginning of the twentieth century due to improved medicinal, dietary, and sanitation conditions. It is, however, foreseen that society will witness an elevated life expectancy of 80–90 years by the twenty-first century (Candore et al., 2006). Nevertheless, ageing is inexorable with an age-associated decline in immune competence and the onset of chronic inflammation leading to neurodegenerative diseases including dementia, Alzheimer's disease (AD) and Parkinson's disease (PD); atherosclerosis and stroke; diabetes; sarcopenia; and cancer (Martorana et al., 2012). With the increased lifespan of the world's population, it is estimated that about 80 million people will suffer from dementia by 2040 whereby AD accounted for almost 60% of dementia cases (Bharadwaj et al., 2010).

The pathological hallmarks of AD are characterised by amyloidogenic processing of amyloid precursor protein (APP) and a subsequent β-amyloid cascade and tau hyperphosphorylation (Claeysen et al., 2012). Other hypotheses of AD pathogenesis include microglial activation associated with neuroinflammation, increased level of acetyl cholinesterase (AChE) activity, and free radical generation (Martorana et al., 2012). Drug therapies for AD include nicotine, melatonin, estrogens (Côté et al., 2012) cholinesterase inhibitors, and an *N*-methyl-D-aspartate receptor antagonist named memantine (Hong-Qi et al., 2012). However, the current AD drug therapy is ineffective and only provides a short-term delay progression of AD. Moreover, although there was a close association of the use of non-steroidal anti-inflammatory drugs (NSAIDs) and a lower incidence of AD, patients suffered from withdrawal syndrome as a result of gastrointestinal toxicity (Hong-Qi et al., 2012).

There has been a recent upsurge of interest in complementary and alternative medicine, especially dietary supplements and functional foods in delaying the onset of age-associated neurodegenerative diseases. As recently reviewed by Perry & Howes (2011), phyto-chemical approach for dementia and AD treatment includes galantamine from *Narcissus* sp., lemon balm (*Melissa officinalis*), and periwinkle (*Vinca minor*). Other edible ''brain food'' consists primarily of blueberry, grape seed, pomegranate, and walnut. The polyphenol entities found in the vegetables,

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fruits and nuts, inhibited neuro-inflammation by preventing APP signaling, and Aβ aggregation (Essa et al., 2012).

Mushrooms offer great potential as a polypharmaceutic drug because of the complexity of their chemical contents and different varieties of bioactivities. Available evidence suggests that mushrooms exhibit anti-oxidants, anti-tumor, anti-virus, anti-cancer, anti-inflammatory, immune modulating, anti-microbial, and anti-diabetic activities (Roupas et al., 2012). In contrast to plant herbal medicines, which are widely explored and relatively more advanced, the brain and cognition health effects of mushrooms are in the early stages of research. Palmitic, oleic, and linoleic acids dominate fatty acid profiles in mushrooms (Doğan & Akbaş, 2013). These fatty acids are important nutritionally, as oleic acid (C18:1 n-9) has been shown to promote axon generation in the striatum during brain development (Guest et al., 2013). Furthermore, *in vitro* toxicology assessment across different mushroom extracts on embryonic fibroblast and neuroblastoma cell lines suggested that the extracts are safe to be consumed even at high doses and they may be developed as a dietary supplement to improve brain and cognitive health. An elaborated discussion on the toxicity of various mushroom metabolites can be found in Phan et al. (2013).

Disease prevention is better than cure especially in neurodegenerative diseases as degeneration process is nearly impossible to be arrested or delayed once the process has commenced. In the present review, brain and cognition health effects of higher Basidiomycetes are analyzed with emphasis on dementia, AD, and PD. The review summarizes the biodiversity of brain-health promoting mushrooms, the chemical structure of the responsible bioactive metabolites, their biological actions, and molecular mechanisms, i.e. neurite outgrowth, cholinesterase inhibition, BACE1 inhibition, anti-neuroinflammation and neuroprotection. The positive, as well as negative, results of experimental testing (*in vitro* and *in vivo*) are also included.

Diversity of mushrooms with brain health promoting effects

In mushroom biology, species boundaries are always indistinct and many mushrooms are subsumed under erroneous names (Hallenberg et al., 2012). Therefore, common names and taxonomic descriptions of different culinary-medicinal mushrooms, which were found to promote brain and cognitive health, are included in Supplemental Table 1. Common names and a morphological description of the mushroom basidiocarps can also be found in Supplemental Table 1.

Inhibition of beta-amyloid, p-tau, and acetylcholinesterase

Beta-amyloid 1–42 (Aβ1–42), a 42-amino acid-length polypeptide, is a cleavage product of amyloid precursor protein (APP) by two secretase enzymes: beta (β-) and gamma (γ-) secretases. Aβ peptides self assemble into soluble oligomers and deposit as insoluble senile plaque in the hippocampus; causing impaired memory and cholinergic dysfunctions in the brains of Alzheimer's patients. Therefore, AD may be prevented by inhibiting the production of Aβ or preventing the aggregation of Aβ into amyloid plaques. Following this hypothesis, the potential of β-secretase (β-site APP cleaving enzyme, BACE1) inhibitor is promising (Sabotič & Kos, 2012). Some BACE1 (EC3.4.23.46) inhibitors such as KMI-429, GSK188909, and PMS777 have provided new insights for clinical application in the near future (Sathya et al., 2012). Apart from that, the level of acetylcholine, a neurotransmitter involved in the regulation of learning and memory functions, decreased dramatically in the neocortex and hippocampus in AD. Therefore, AChE inhibitors can be used to restore acetylcholine levels and therefore cholinergic brain activity.

Aβ 1–40 causes oxidative stress and inflammation in the brain leading to the secretion of *p*-tau protein which is involved in neuron damage (Bharadwaj et al., 2010). In a study by Wang et al. (2012), the mycelium and/or fruiting body of *Antrodia camphorata* were able to reverse the damaging effects of *in vivo* Aβ-40 infusion and *in vitro* Aβ-40 treatment. A working memory test to evaluate short-term memory and learning abilities of Aβ brain infusion rats was carried out. The mushroom-supplemented group displayed better improvement in memory and learning abilities. Also, the expression of *p*-tau protein in rat pheochromocytoma (PC-12) cells was significantly decreased by the treatment of *A. camphorata.* However, *A. camphorata* did not have significant inhibitory effects on BACE expression. This result was interpreted to indicate that *p*-tau inhibition, rather than BACE modulation, played a vital role in AD prevention by *A. camphorata*.

The effects of *Hericium erinaceus* on Aβ25–35 peptide-induced cognitive dysfunction in mice was investigated by Mori et al. (2011). The powder of *H. erinaceus* was mixed with a normal powdered diet and the Aβ25–35 peptide was administered by intracerebroventricular injection. The results revealed that *H. erinaceus* prevented impairments of spatial short-term and visual recognition memory induced by Aβ25–35 in mice. Human trials with *H. erinaceum* derivatives also showed promising results in patients with dementia based on Revised Hasegawa Dementia Scale (HDS-R) (Mori et al., 2009).

Aqueous extract of *Ganoderma lucidum* significantly attenuated Aβ-induced synaptotoxicity and apoptosis by preserving the synaptic density protein called synaptophysin (Lai et al., 2008). Further, a study by Wang et al. (2004) concluded that senescence-accelerated mice (strain SAMP8) given a diet supplemented with *Ganoderma* extract exhibited significantly lower brain amyloid and higher antioxidation activities such as superoxide dismutase, glutathione peroxidase (GPx), and glutathione reductase when compared with the control mice. Moreover, a study by Pinweha et al. (2008) suggested that *G. lucidum* mycelium extract might possess nerve growth factor (NGF)-like properties for the processing of APP *via* an enhanced NGF signaling pathway. As a result, the increased APP expression promoted non-amyloidogenic protein secretion (sAPP).

The mushroom *Cortinarius infractus* has a strong bitter taste and an unpleasant odor due to the presence of indole alkaloids infractine, 6-hydroxyinfractine, and infractopicrine (Brondz, et al., 2007). Infractopicrin (**1**) and 10-hydroxy-infractopicrin (**2**) (Supplemental Figure 1) showed AChE-inhibiting activity with non-detectable cytotoxicity (Geissler et al., 2010). Topological polar surface area (TPSA) of below

70 Å^2 suggested that the compounds could pass through the blood–brain barrier. Aggregation of Aβ1–40 (fibril formation) was also inhibited by the two alkaloids as revealed by the thioflavin T fluorescence assay. In addition, *in vitro* AChE and butyrylcholinesterase-inhibiting activities of extracts of *Tricholoma* species (*T. fracticum*, *T. imbricatum,* and *T. terreum*) were tested. As a result, only the hexane extract of *T. imbricatum* (0.2 mg/mL) was confirmed to inhibit AChE and butyrylcholinesterase by 71.8 ± 0.3% and 52.6 ± 1.0%, respectively (Tel et al., 2011).

According to Dai et al. (2010), hispidin (**3**), a class of polyphenols is an important medicinal metabolite from *Phellinus* spp. Hispidin (Supplemental Figure 1) were isolated from the culture broth of *P. linteus*, and it has been shown to be a non-competitive inhibitor of BACE1 with an IC_{50} value of 4.9×10^{-6} M and a K_i value of 8.4×10^{-6} M (Park et al., 2004a). In addition, hispidin was shown to be an efficient reactive oxygen species (ROS) scavenger (Park et al., 2004b). *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (enoki), and *Lentinula edodes* (shiitake) neither inhibited nor activated BACE1. The major polysaccharide of button mushroom, β-D-glucan, in contrast, did not cause BACE1 activation (Sheean et al., 2012). The results indicated that BACE1 activity behaved differently with different compound features. Nevertheless, the effects of button mushrooms, enoki, and shiitake together with β-D-glucan need to be tested further. Most recently, BACE1 activity was shown to be inhibited by extracts of fresh basidiocarps of *Auricularia polytricha* (wood ear mushroom). The BACE1 inhibitory activity was most likely due to the hispidin-derived polyphenolics (Bennett et al., 2013b).

Stimulation of neurite outgrowth and NGF synthesis

Neurotrophic factors (neurotrophins) such as NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and glia-derived neurotrophic factor (GDNF) play an important role in differentiation, survival, and maintenance of the neuronal cells. Insufficient neurotrophins is believed to result in dysfunction of the nervous system, causing dementia, AD, and PD. However, polypeptides such as NGF in therapy are unfavorable as they are unable to cross the blood–brain barrier. Therefore, finding small molecules that show neurotrophic properties and/or enhancing the action of endogenous neurotrophic factors is important (Shi et al., 2011).

Sarcodon spp., also called ''bitter tooth'', are widely distributed in Europe, North America, and Asia. *Sarcodon* mushrooms are considered inedible due to their bitter taste. On one hand, cyrneines A (**4**) and B (**5**) (Figure 1) isolated from *Sarcodon cyrneus* stimulated neurite outgrowth in PC12 at 100 μM with no cytotoxicity as indicated by lactate dehydrogenase (LDH) analysis (Marcotullio et al., 2006a) (Table 1). Later, it was shown that both cyrneines A and B promoted NGF production in 1321N1 cells (Marcotullio et al., 2007). Neurite outgrowth activity was also observed in NG108-15 cells, a hybrid neuronal cell line derived from mouse neuroblastoma and rat glioma (Obara et al., 2007). On the other hand, cyrneines C (**6**) and D (**7**) failed to induce neurite outgrowth. In addition, glaucopine C (**8**), isolated from the hexane extract of *Sarcodon glaucopus* (Marcotullio et al., 2006b), did not significantly promote neurite outgrowth in PC12 cells but induced NGF gene expression to a lesser extent when compared with cyrneines A and B. It seemed that the presence of the hydroxyl cycloheptadienyl carbaldehyde system in cyrneines could be important for neuritogenesis (Marcotullio et al., 2007). In other words, minor differences in functional groups on cyathane structures in cyrneines A, B, C, and D can influence the responses in neuronal cells. Figure 1 shows the chemical structure of different cyrneines.

Scabronine A (**9**) (Table 2), isolated from *Sarcodon scabrosus,* showed potent inductive activity of NGF synthesis in 1321N1 human astrocytoma cells (Ohta et al., 1998). Further investigation led to the isolation of novel cyathane diterpenoids named scabronines B–F (**10**–**14**) (Kita et al., 1998), G (**15**) (Obara et al., 1999), K (**16**), and L (**17**) (Shi et al., 2011). However, only scabronines B, C, E, and G showed NGF-synthesis stimulating activity. It appeared that the presence of the α,β-unsaturated aldehyde system in the seven-membered ring could be crucial for the bioactivity. Recently, the first synthesis of scabronine G in optically pure form has been reported, and the neurite outgrowth activity was comparable with NGF and natural scabronine G (Waters et al., 2005). Meanwhile, scabronine G-methyl-ester (**18**) synthesized from scabronine G also potently promoted the secretion of NGF and interleukin-6 (IL-6), another major neurotrophic factor released from astrocytes. Most recently, secoscabronine M (**19**), a hemiacetal cyathane diterpenoid was isolated from *S. scabrosus* but no neuritogenesis has been reported for this compound. Figure 1 shows the structures of scabronine A-G, K, L, scabronine G-methyl-ester, and secoscabronine M isolated from *S. scabrosus*.

There is a possible use of *Hericium erinaceus* (Bull.: Fr.) Pers. in the treatment of neurological disorders and dementia as reported by Kawagishi & Zhuang (2008). In a study by Wong et al. (2007), the extracts of *H. erinaceus* fruiting body and mycelium induced neurite outgrowth of neuronal cells NG108-15 *in vitro* (Supplemental Figure 2). Also, ethanol extract of *H. erinaceus* promoted the neurite outgrowth of PC12 cells, enhanced NGF mRNA expression, and the secretion of NGF from 1321N1 human astrocytoma cells (Mori et al., 2008). Further, *in vivo* functional recovery of axonotmetic peroneal nerve injury in Sprague–Dawley rats was assessed by walking-track analysis and toe-spreading reflex (Wong et al., 2009) (Supplemental Figure 3). The peroneal functional index (PFI) and toe-spreading reflex improved more rapidly in the group treated with daily administration of *H. erinaceus* extract. These data suggested that *H. erinaceus* could promote the regeneration of nerve injury in the early stage of recovery (Wong et al., 2010). Although preliminary, it was demonstrated that the *H. erinaceus* extract exerted neurotrophic action and improved the myelination process in the rat brain without affecting nerve cell growth and toxicity (Moldavan et al., 2007). There was an attempt to isolate a polysaccharide from the mycelium of *H. erinaceus* and the polysaccharide (1 000 000 dalton; molar ratio of 1.5:1.7:1.2:0.6:0.9; glucose:galactose:xylose:mannose:fructose) promoted neurite outgrowth in PC12 cells *in vitro* (Park et al., 2002).

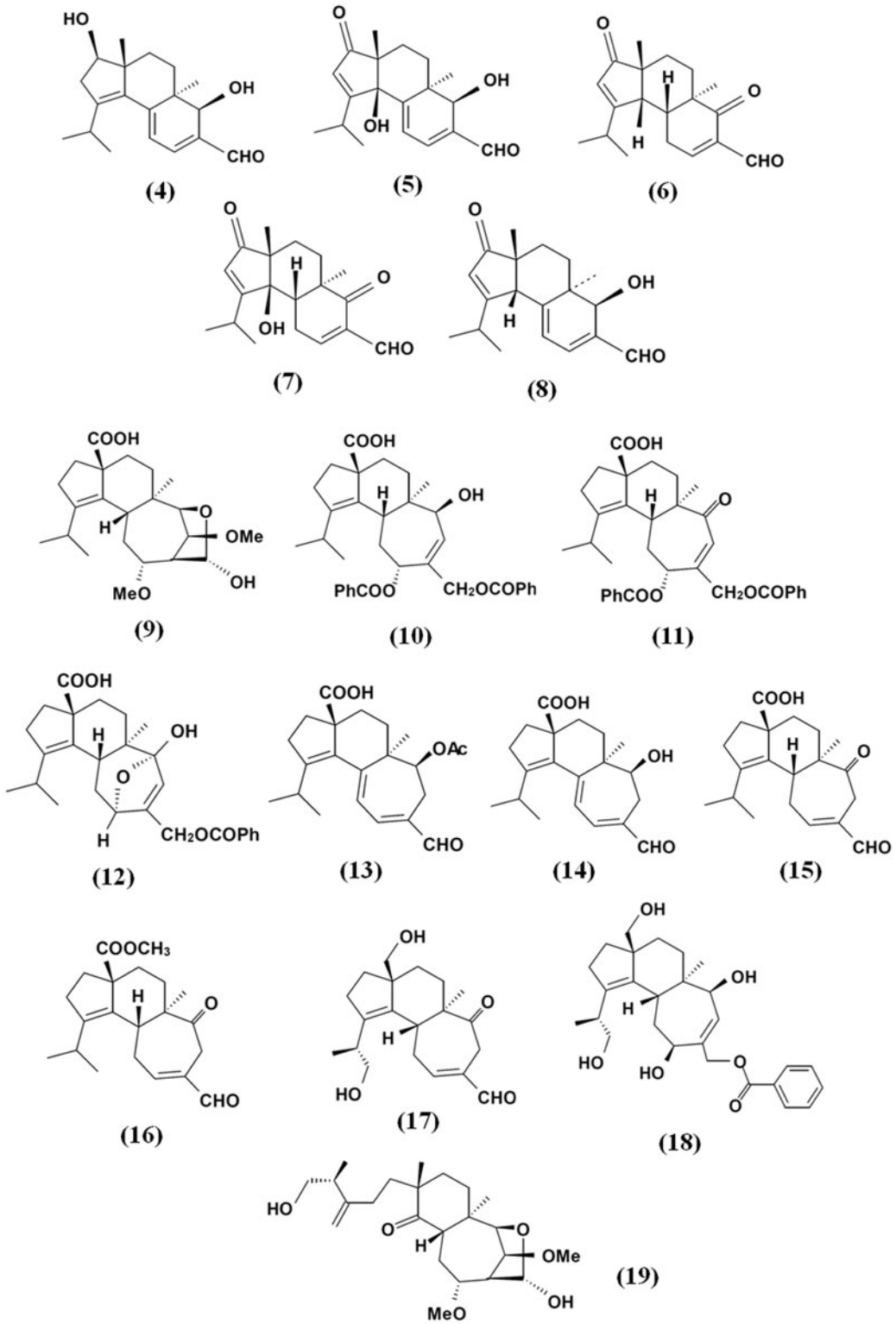


Figure 1. Cyrneines A (**4**), B (**5**), C (**6**), and D (**7**) from *Sarcodon cyrneus*; and glaucopine C (**8**), isolated from the hexane extract of *Sarcodon glaucopus*. Scabronine A–G (**9**–**15**), K (**16**), L (**17**), scabronine G-methyl-ester (**18**), and secoscabronine M (**19**), isolated from *Sarcodon scabrosus*.

However, it needs to be clarified that this *in vitro* evidence cannot be assumed to occur in *vivo* and that the *in vitro* activity of polysaccharides cannot be extrapolated to explain *in vivo* observations.

On one hand, hericenones (benzyl alcohol derivatives) were isolated from the fruiting bodies of *H. erinaceus* (Table 2). Hericenones A (**20**) and B (**21**) were first reported in 1990 but no neurite outgrowth activity was reported (Kawagishi et al., 1990). Hericenones C (**22**), D (**23**), E (**24**), F (**25**), G (**26**), and H (**27**) exhibited stimulating activity for the biosynthesis of NGF *in vitro* (Kawagishi & Ando, 1993; Kawagishi et al., 1991). On the other hand, diterpenoid derivatives (named erinacines) were isolated from the mycelium of *H. erinaceus*. Erinacines A–I (**28**–**36**) significantly induced the synthesis of NGF *in vitro* (Kawagishi et al., 1994, 1996a,b; Lee et al., 2000) and *in vivo* (Shimbo et al., 2005).

Table 1. Compounds isolated from mushroom *Sarcodon* spp. that were screened for neurite outgrowth activity.

No.	Compound	*Sarcodon spp.*	*In vitro* study	Neurite outgrowth activity	References
4	Cyrneine A	SC	PC12; NG108–15; 1321N1	Neurite outgrowth ↑, NGF ↑	Marcotullio et al. (2006a) and Obara et al. (2007)
5	Cyrneine B	SC	PC12	Neurite outgrowth ↑, NGF ↑	Marcotullio et al. (2006b, 2007)
6	Cyrneine C	SC	PC12	–	Marcotullio et al. (2007)
7	Cyrneine D	SC	PC12	–	Marcotullio et al. (2007)
8	Glaucopine C	SG	PC12	NGF gene expression ↑	Marcotullio et al. (2006a) and Marcotullio et al. (2007)
9	Scabronine A	SS	1321N1	Neurite outgrowth ↑	Ohta et al. (1998)
10	Scabronine B	SS	Rat astroglial cells	NGF ↑	Kita et al. (1998)
11	Scabronine C	SS	Rat astroglial cells	NGF ↑	Kita et al. (1998)
12	Scabronine D	SS	Rat astroglial cells	–	Kita et al. (1998)
13	Scabronine E	SS	Rat astroglial cells	NGF ↑	Kita et al. (1998)
14	Scabronine F	SS	Rat astroglial cells	–	Kita et al. (1998)
15	Scabronine G	SS	1321N1	Neurite outgrowth ↑	Obara et al. (1999) and Waters et al. (2005)
16	Scabronine G-Methyl ester	SS	PC12	NGF and IL-6 ↑	Obara et al. (2001)
17	Scabronine K	SS	PC12	–	Shi et al. (2011)
18	Scabronine L	SS	PC12	–	Shi et al. (2011)
19	Secoscabronine M	SS	–	–	Shi et al. (2012)

SC, *S. cyrneus*; SG, *S. glaucopus*; SS, *S. scabrosus*; –, no effect on neurite outgrowth; NGF, nerve growth factor; ↑, promoted/increased.

Table 2. List of hericenones and erinacines in *Hericium erinaceus*, some of which showed neurite outgrowth activity.

No.	Compound	Mushroom component	*In vitro* study	Neurite outgrowth activity	References
20	Hericenone A	F	–	–	Kawagishi et al. (1990)
21	Hericenone B	F	–	–	Kawagishi et al. (1990)
22	Hericenone C	F	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1991)
23	Hericenone D	F	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1991)
24	Hericenone E	F	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1991)
25	Hericenone F	F	Mouse astroglial cells	NGF ↑	Kawagishi & Ando (1993)
26	Hericenone G	F	Mouse astroglial cells	NGF ↑	Kawagishi & Ando, (1993)
27	Hericenone H	F	Mouse astroglial cells	NGF ↑	Kawagishi & Ando, (1993)
28	Erinacine A	M	Mouse astroglial cells Rat (*in vivo*)	NGF ↑; catecholamine ↑ in the CNS of rats	Kawagishi et al. (1994) Shimbo et al. (2005)
29	Erinacine B	M	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1994)
30	Erinacine C	M	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1994)
31	Erinacine D	M	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1996b)
32	Erinacine E	M	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1996a)
33	Erinacine F	M	Mouse astroglial cells	NGF↑	Kawagishi et al. (1996a)
34	Erinacine G	M	Mouse astroglial cells	NGF ↑	Kawagishi et al. (1996a)
35	Erinacine H	M	Rat astroglial cells	NGF ↑	Lee et al. (2000)
36	Erinacine I	M	Rat astroglial cells	NGF ↑	Lee et al. (2000)
37	Erinacine J	M	MRSA	–	Kawagishi et al. (2006)
38	Erinacine K	M	MRSA	–	Kawagishi et al. (2006)
39	Erinacine P	M	–	Biosynthesis of erinacines	Kenmoku et al. (2000)
40	Erinacine Q	M	–	Biosynthesis of erinacine C	Kenmoku et al. (2002)
41	Erinacine R	M	–	–	Ma et al. (2010, 2008)
42	Erinacol	M	–	Biosynthesis of erinacine Q	Kenmoku et al. (2004)

F, fruiting body; M, mycelium; –, none; NGF, nerve growth factor; CNS, central nervous system; MRSA, methicillin-resistant *Staphylococcus aureus*.

Isolation of new compounds from this mushroom continued with the discovery of erinacines J (**37**), K (**38**), P–R (**39**–**41**), as well as erinacol (**42**), a novel cyathadien-14β-ol (Kawagishi et al., 2006; Kenmoku et al., 2000; Kenmoku et al., 2002, 2004; Ma et al., 2010, 2008). Structures of hericenones and erinacines are given in Figure 2.

Cheung et al. (2000) reported that *G. lucidum* extract reduced PC12 cell proliferation and induced neuronal differentiation and neurite outgrowth *via* the activation of MAP kinases and cAMP-response element binding protein (CREB) signaling pathways. In addition, a lipophilic fraction of *G. lucidum* (125 and 500 mg/L) was also shown to induce neurite outgrowth of PC12 cells (Zhang et al., 2005).

Mycoleptodonoides aitchisonii is a rare mushroom that improves brain function in rats. The mycelium-containing cultivation medium was found to bear fragrant compounds of phenylpentane, which consists of 1-phenyl-3-pentanol and 1-phenyl-3-pentanone. The compounds improved dopamine liberation in the brains of rats fed with the mushroom powder or aqueous extracts (Okuyama et al., 2004a). Further, NGF synthesis in the cerebral cortex and hippocampus of newborn rats was also enhanced after the pregnant rats were fed

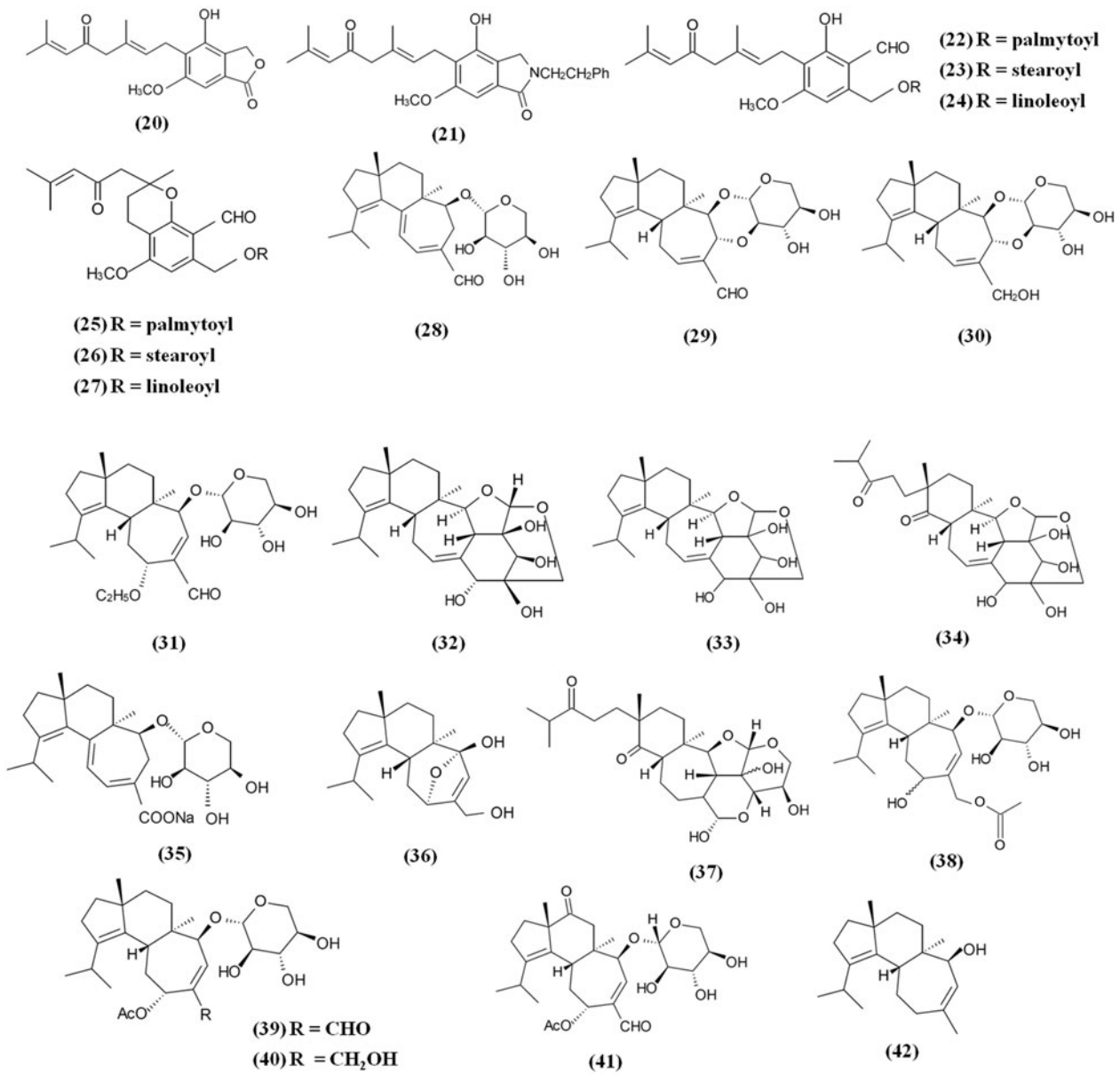


Figure 2. Hericenones A–H (**20**–**27**)**;** erinacine A–K (**28**–**38**), P–R (**39**–**41**), and erinacol (**42**).

with either *M. aitchisonii* powder or its aqueous extract for 7 d before delivery (Okuyama et al., 2004b). A recent study concluded that *M. aitchisonii* aqueous extract prevented the reduction of dopaminergic and serotoninergic neuronal activities following brain ischemia damage in the cerebral cortex (Okuyama et al. 2012). The concentrations of the neurotransmitters, dopamine, and its metabolites were increased after treatment with this mushroom. Moreover, *M. aitchisonii* was shown to activate NF-E2-related factor 2 (Nrf2) and might contribute to the prevention of oxidative stress-related diseases (e.g. Alzheimer's) by inducing anti-oxidative and phase II detoxifying enzyme series (Kokubo et al., 2011).

Dictyophora indusiata is a famous edible mushroom used in Chinese cuisine and medicine. Two eudesmane-type sesquiterpenes, dictyophorines A (**43**) and B (**44**) (Supplemental Figure 4), were isolated from the mushroom and were found to promote NGF synthesis by astroglial cells (Kawagishi et al., 1997). It was shown that NGF secreted into the medium in the presence of 3.3 mM of dictyophorines A was four times higher than the negative control. Meanwhile, lysophosphatidylethanolamine (LPE) isolated from *G. frondosa* (GLPE) was found to induce neurite outgrowth and it upregulated the neurofilament M expression in cultured PC12 cells (Nishina et al., 2006). This study also showed the suppressive effect of *G. frondosa* on serum deprivation-induced apoptosis of the PC12 cells.

The aqueous extract of *Tremella fuciformis* not only promoted neurite outgrowth of the PC12 cells but also significantly reversed the scopolamine- and trimethyltin-induced memory deficit in rats, as revealed by the Morris water maze test and choline acetyltransferase (ChAT) immunohistochemistry (Kim et al., 2007; Park et al., 2012). Besides, neuritogenic compounds named tricholomalides A–C (**45**–**47**) (Supplemental Figure 4) were also isolated from *Tricholoma* sp. and neurite outgrowth in PC-12 cells was significantly induced at concentrations of 100 μM (Tsukamoto et al., 2003). Whereas for *Termitomyces*

albuminosus, the cerebrosides named termitomycesphins A–D (**48**–**51**) (Qi et al., 2000), E–F (**52**–**53**) (Qi et al., 2001), and G–H (**54**–**55**) (Qu et al., 2012) (Supplemental Figure 4) were identified to potentiate neuritogenesis in PC12 cells. It is interesting that termitomycesphin with a 16-carbon-chain fatty acid (A, C, and G) showed higher neuritogenic activity than that of termitomycesphin with an 18-carbon-chain fatty acid (B, D, and H), suggesting that the chain length of the fatty acyl moiety played a determining role in neuritogenesis. A number of new mushrooms have been reported to possess neuritogenic effects (Sabaratnam et al., 2013). Aqueous extract of *L. rhinocerotis* sclerotium (Eik et al., 2012), *L. rhinocerotis* mycelium (John et al., 2013), *Ganoderma neo-japonicum* (Seow et al., 2013), and *Pleurotus giganteus* (Phan et al., 2012) were shown to induce neuronal differentiation and stimulate neurite outgrowth of PC12 and N2a cells. Meanwhile, a methanol extract of *Cordyceps militaris* (5–20 μg/mL) was able to increase primary neurite sprouting and ChAT expression in differentiated N2a cells (Lee et al., 2011). Administration of *C. militaris* also restored the scopolamine-induced memory deficit *in vivo*.

Neuroprotection, anti-inflammatory, and anti-oxidant activities

Accumulating evidence have indicated that oxidative stress and ROS play an important role in the progression of many chronic diseases including cardiovascular diseases, diabetes, and neurodegenerative disorders (Chu et al., 2012). Imbalance between ROS generation and antioxidant enzyme activities will cause lipid peroxidation, nuclear mitochondrial DNA damage and protein oxidation, resulting in brain damage and amnesia (Biasibetti et al., 2013). Therefore, a drug with antioxidant and anti-inflammatory activities may prevent neuronal degeneration in AD. Mushrooms, known for their potent antioxidant property, have attracted interest due to their potential in neuroprotection, antioxidant, and anti-inflammatory effects, in a variety of experimental models (Gunawardena et al., 2014).

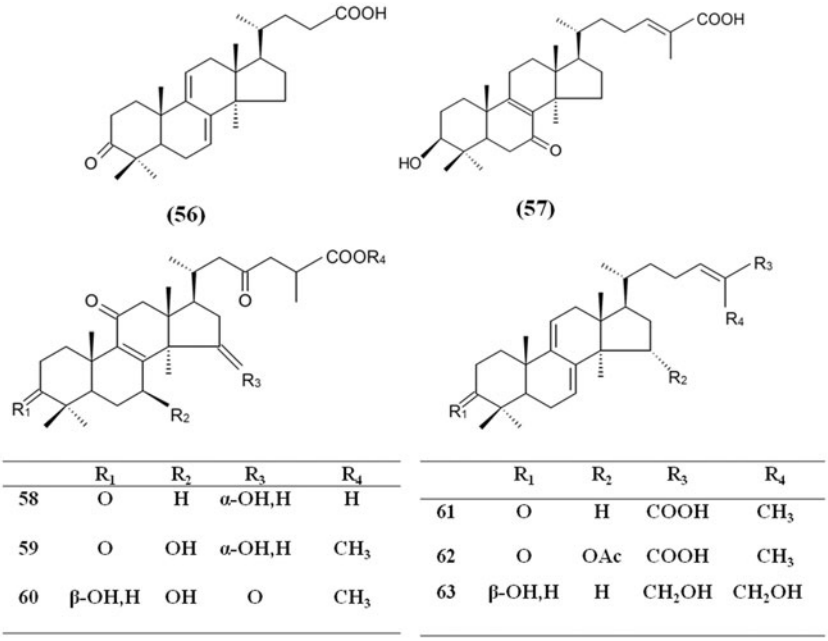
At least 140 different triterpenes have been identified in *G. lucidum* and they include ganoderic, lucidenic, ganodermic, ganoderenic, ganolucidic and applanoxidic acids, lucidones, ganoderals, and ganoderols (Connolly & Hill, 2003; Smina et al., 2011; Wu et al., 2001). The total triterpenes from *G. lucidum* scavenged 2,2-diphenyl-1-picryl hydrazyl ($DPPH^+$), 2,2′-azinobis-(3-ethylbenzothiazolin-6-sulphonic acid ($ABTS^+$) and superoxide radicals (Smina et al., 2011). Also, the administration of total triterpenes to mice enhanced the superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT), and GPx in the blood and liver tissues. Further, the aqueous extracts of *G. lucidum* fruiting bodies were shown to prevent H_2O_2-induced oxidative damage to cellular DNA (Shi et al., 2002). Dietary intake of natural or synthetic products with a putative antioxidant effect has been shown to delay the onset AD (Praticò, 2008). Therefore, the ability of the triterpenes of *G. lucidum* to scavenge free radicals may suppress reactive oxygen damage that leads to AD pathology.

For PD, a neuroprotective approach to salvage dopamine neurons from progressive death in the brain (substantia nigra region) is currently being explored. A study by Zhu et al. (2005) has shown that rats fed with *G. lucidum* spores oil ameliorated Parkinsonism induced by neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The number of surviving dopamine neurons in the substantia nigra and the level of dopamine in the striatum of MPTP-induced mice has increased after treatment with the oil of *Ganoderma* spores. Furthermore, involuntary movement of mice was also significantly reduced. Microglia is the resident innate immune cells of central nervous system (CNS) and it plays a major role in the neuroinflammatory process. Activation of microglia can trigger neurotoxicity *via* the production of pro-inflammatory and cytotoxic factors including tumor necrosis factor-(TNF)-α, nitric oxide (NO), superoxide radicals, interleukin- β (IL-β), and cyclooxygenase 2 (COX2) (Liu et al., 2006). The over-activation of microglia in the CNS contributes to neurodegenerative processes (Brown & Neher, 2010). To test for the potential neuroprotective effect of *G. lucidum*, co-cultures of 1-methyl-4-phenylpyridinium-(MPP^+)-treated dopaminergic neuronal cell line MES23.5 and LPS-activated microglia were used (Zhang et al., 2011a). MPP^+ is a metabolite of the neurotoxin MPTP. The *G. lucidum* extracts significantly inhibited the production of microglia-derived proinflammatory and cytotoxic factors (NO, TNF-α, and IL-1β) suggesting that *G. lucidum* is a promising agent in deterring inflammation-induced Parkinson's disease.

Ganoderic acid is a member of highly oxygenated C_{30} lanostane-type triterpenoids. However, its biological activity on the nervous system is still unknown. Recently, a new lanostanoid, 4,4,14-trimethyl-5-chol-7,9(11)-dien-3-oxo-24-oic acid (**56**) was isolated from an ethyl acetate extract of the dried fruiting bodies of *G. lucidum*. The triterpenoids, together with seven other known triterpenoids, i.e. 7-oxo-ganoderic acid Z (**57**), ganolucidic acid A (**58**), methyl ganoderic acid A (**59**), methyl ganoderic acid B (**60**), ganoderic acid S1 (**61**), ganodermic acid TQ (**62**) and ganodermatriol (**63**) (Figure 3), have shown NGF- and brain-derived neurotrophic factor-like neuronal survival-promoting effects (Table 3).

The role of vitamin D2-enriched button mushrooms (*Agaricus bisporus*) was studied especially for their memory improving effects in rats (Bennett et al., 2013a). Fungi, especially the members of Basidiomycetes, are rich in ergosterol. The ergosterol in mushrooms can be converted to vitamin D2 following exposure to ultra violet (UV) light. Recent research suggested that higher vitamin D dietary intake was associated with a lower risk of developing AD among older women (Annweiler et al., 2012). Compound (**56**) has a steroidal feature resembling cholesterol that can be converted to vitamin D by enzymatic pathways, in response to UV irradiation. Therefore, there is a potential for this class of compounds to interact with vitamin D receptors and exert bioactivity *via* vitamin D mimicry.

The endoplasmic reticulum (ER) is an organelle within fungal cells in which protein folding, lipid biosynthesis, and calcium storage takes place (Brown & Naidoo, 2012). The ER, by serving as quality control machinery, suppresses protein aggregation in the cells under normal physiological



	R_1	R_2	R_3	R_4
58	O	H	α-OH,H	H
59	O	OH	α-OH,H	CH_3
60	β-OH,H	OH	O	CH_3

	R_1	R_2	R_3	R_4
61	O	H	COOH	CH_3
62	O	OAc	COOH	CH_3
63	β-OH,H	H	CH_2OH	CH_2OH

Figure 3. New lanostanoid (**56**), 7-oxo-ganoderic acid Z (**57**), ganolucidic acid A (**58**), methyl ganoderic acid A (**59**), methyl ganoderic acid B (**60**), ganoderic acid S1 (**61**), ganodermic acid TQ (**62**), and ganodermatriol (**63**), isolated from *Ganoderma lucidum*.

Table 3. List of ganoderic acids in *Ganoderma lucidum* which showed neuroprotection effects in *in vitro* studies using NIH-3T3/TrkA and NIH-3T3/TrkB cells.

No.	Compound	Neuronal surviving effect	References
56	4,4,14-Trimethyl-5-chol-7,9(11)-dien-3-oxo-24-oic acid	NGF ↑	Zhang et al. (2011b)
57	7-Oxo-ganoderic acid Z	BDNF ↑	Li et al. (2006) and Zhang et al. (2011b)
58	Ganolucidic acid A	BDNF ↑	Zhang et al. (2011b)
59	Methyl ganoderic acid A	BDNF ↑	Zhang et al. (2011b)
60	Methyl ganoderic acid B	NGF ↑	Zhang et al. (2011b)
61	Ganoderic acid S1	BDNF ↑	Zhang et al. (2011b)
62	Ganodermic acid TQ	BDNF ↑	Zhang et al. (2011b)
63	Ganodermatriol	BDNF ↑	Zhang et al. (2011b)

NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor.

Table 4. Protective effects of mushrooms from endoplasmic reticulum stress-induced neuronal cell death.

No.	Mushroom	Compound	References
64	*Hericium erinaceum*	3-Hydroxyhericenone F	Ueda et al. (2008)
		Dilinoleoyl-phosphatidylethanolamine	Nagai et al. (2006)
65	*Stropharia rugosoannulata*	Strophasterol	Wu et al. (2011, 2012)
66	*Leccinum extremiorientale*	Ethyl 2-(*N*-phene-thylformamido)acetate; also Leccinine A	Choi et al. (2011)
67–71	*Termitomyces titanicus*	Termitomycamides A–E	Choi et al. (2010)
72	*Mycoleptodonoides aitchisonii*	3-(Hydroxymethyl)-4-methylfuran-2(5*H*)-one	Choi et al. (2009)
73		3-(10-Hydroxyethyl)-4-methyldihydrofuran-2(3*H*)-one	
74		3-Hydroxyethyl-4-methyldihydrofuran-2(3*H*)-one	
75		1-Hydroxy-3-pentanone	

conditions. However, with age and under stress, ER homeostasis will be interrupted and brings about the ER-stress response or the activation of the unfolded protein response, followed by programmed cell death (apoptosis) in the brain and/or insoluble protein fibrils formation. ER stress accompanies and contributes to several neurological disorders including PD. Due to that, the demand for new protective substances against the ER-stress-dependent cell death is high. In this review, the protective effects of medicinal mushrooms, namely *H. erinaceus*, *Stropharia rugosoannulata*, *Leccinum extremiorientale*, *Termitomyces titanicus*, and *Mycoleptodonoides aitchisonii* against age-implicated ER stress are discussed (Table 4).

In the protection assay against ER stress-dependent cell death, a popular cell line Neuro2a (N2a) cell is widely used. In general, the ER stress was either induced by addition of tunicamycin or thapsigargin. Tunicamycin is a protein glycosylation inhibitor that induces accumulation of misfolded protein in the ER and ultimately causes cell death. Thapsigargin is a non-competitive inhibitor of Ca^{2+} ATPase in ER that causes Ca^{2+} reduction. 3-hydroxyhericenone F (**64**) (Supplemental Figure 5), which was isolated from the fresh fruiting bodies of *H. erinaceus,* was found to protect N2a cells against both tunicamycin and thapsigargin toxicities (Ueda et al., 2008). Another ER stress attenuating compound, dilinoleoyl-phosphatidylethanolamine, was also isolated and

identified from the dried fruiting bodies of *H. erinaceum* (Nagai et al., 2006). A compound from *S. rugosoannulata* attenuated the ER stress caused by thapsigargin, but not by tunicamycin (Wu et al., 2011). The compound was later found to be strophasterol (**65**) with a new steroid skeleton not previously reported (Wu et al., 2012) (Supplemental Figure 5). Similarly, leccinine A (**66**) (Supplemental Figure 5) from *L. extremiorientale* also showed significant protective activity against thapsigargin toxicity but not tunicamycin (Choi et al., 2011). Meanwhile, five fatty acid amides, termitomycamides A–E (**67–71**) isolated from *T. titanicus* (Supplemental Figure 5), were screened for their protective effects against tunicamycin toxicity. Only termitomycamides B and E showed significant protective effects, suggesting that these compounds blocked the inhibitory action of tunicamycin and N-linked glycosylation in ER was not repressed. Another four compounds (**72–75**) (Supplemental Figure 5) were also successfully isolated from the mushroom *M. aitchisonii* and they have shown attenuating effects on ER stress-dependant neuronal cell death (Choi et al., 2009).

Inonotus obliquus is another mushroom popular for its antioxidative effects in neuronal cells (Jung et al., 2008). An acid protein-bound polysaccharide from *I. obliquus* exhibited notable quenching of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals (Chen et al., 2010). Enzymatically hydrolyzed *I. obliquus* by carbohydrase (Celluclast) and protease (Protamex) showed the highest DPPH radical-scavenging activities (Kim et al., 2011). Pepsin extracts of *I. obliquus* managed to decrease generation of ROS and cell death in PC12 cells against H_2O_2-induced oxidative damage. In another study using the peroxide-treated human fibroblasts, *I. obliquus* showed cytoprotective effects by scavenging intracellular ROS and preventing lipid peroxidation and ultimately stopping premature senescence. Most recently, a significant cognitive enhancement was observed in amnesic mice after orally administration of methanolic extracts of *I. obliquus* (Giridharan et al., 2011). The critical metabolites responsible for the neuroprotection were thought to be the phenolic ingredients namely 3,4-dihydroxybenzalacetone (**76**) and caffeic acid (**77**) (Nakajima et al., 2009) (Supplemental Figure 6).

Armillaria mellea produces an array of different metabolites, including carbohydrates, sterols, sphingolipids, fatty acids, sesquiterpenes, non-hallucinogenic indole compounds, peptides, enzymes, and adenosine derivatives (Muszyńska et al., 2011). N6-(5-hydroxy-2-pyridyl)-methyl-adenosine (**78**) (Supplemental Figure 6) from the mycelia of *A. mellea* displayed an adenosine-like cerebral protecting activity (Gao et al., 2009; Watanabe et al., 1990). Compounds of *Daldinia concentric*, 1-(3,4,5-trimethoxyphenyl) ethanol (**79**) and caruilignan C (**80**) (Supplemental Figure 6) showed neuroprotective effects against iron-induced neurotoxicity in mouse cortical cell cultures (Lee et al., 2002).

Five compounds were isolated from the fruiting bodies of *Antrodia camphorata* (Chen et al., 2006) (Supplemental Figure 6). The compounds, 19-hydroxylabda-8(17)-en-16,15-olide (**81**), 3β,19-dihydroxylabda-8(17),11*E*-dien-16,15-olide (**82**), 13-*epi*-3β,19-dihydroxylabda-8(17),11*E*-dien-16,15-olide (**83**), 19-hydroxylabda-8(17),13-dien-16,15-olide (**84**), and 14-deoxy-11,12-didehydroandrographolide (**85**), were shown to protect neurons from $A\beta_{25–35}$ damage. In the study, primary cultures of neonatal cortical neurons from the cerebral cortex of Harlan Sprague–Dawley rat pups at postnatal day 1 were used. The cell stress model for this particular study was serum-deprived PC12 cells (Huang et al., 2005; Lu et al., 2008). Lu et al. (2006) unraveled that the protective effect of *A. camphorata* was due to adenosine (**86**) (Supplemental Figure 6). The protective effect of adenosine was found to be mediated through Adenosin-$_{2A}$ receptor (A_{2A}-R) activation on PC12 cells. A_{2A}-R has been regarded as a potential therapeutic target in protecting against neuronal injury and it has been reported that A_{2A}-R activation delayed apoptosis in human neutrophils (Lu et al., 2006).

Mechanisms and signaling pathways of bioactivity of mushrooms secondary metabolites in neurodegenerative diseases

Signal transduction cascades like the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase-Akt (PI3K-Akt), and protein kinase C (PKC) pathways play important roles in neurons downstream of multiple signals including neurotrophins and neurotransmitters (Martin & Arthur, 2012). Certain mushrooms have shown NGF-like neuritogenic effects. Therefore, it is of utmost importance to elucidate the molecular mechanism responsible for the activity. Essentially, the process where a cell translates an external signal into cellular response is ''signal transduction'' (Martin & Arthur, 2012). Signal transduction begins with the binding of an external ligand (NGF, neurotransmitter, or mushroom compound in this case) to a specific receptor on a cell. This ultimately causes a systematic signalling cascade that initiates a response in a cell, for instance cell differentiation and extension of neurite.

The MAPK signal cascade is known to regulate cell growth and differentiation (Zhang & Liu, 2002). Three MAPK families have been characterized namely extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 kinase. Small and selective molecule protein kinase inhibitor is a powerful tool to study kinase function. Since NGF induces the activation of MEK and phosphorylation of ERK1/2, MEK inhibitors (U0126 and PD98059) were widely used as one of the checkpoints to assess the MAPK cascade. As reported by Phan et al. (2012), the induction of activation of ERK1/2 by both NGF and *P. giganteus* extracts was inhibited by U0126 and PD98059. Therefore, the mushroom extracts (as well as NGF) induced the activation of MEK1/2, resulting in neurite outgrowth. Similar observations were reported by Cheung et al. (2000) for *G. lucidium* extracts and Nishina et al. (2006), for lysophosphatidylethanolamine from *Grifola frondosa*. Interestingly, there was no direct involvement of the Trk family of receptor tyrosine kinase, (TrkA) for the above mushroom-potentiated neuritogenesis, as opposed by the classical NGF. It is thus predicted that activation of TrKA may not be necessary for NGF-independent neuritogenic effects by mushrooms. It is widely accepted that PI3K/Akt regulates neuritogenesis (Kimura et al., 1994). Akt is a serine/threonine kinase essential for neurotrophin-induced cell survival and the activation of Akt by neurotrophins is

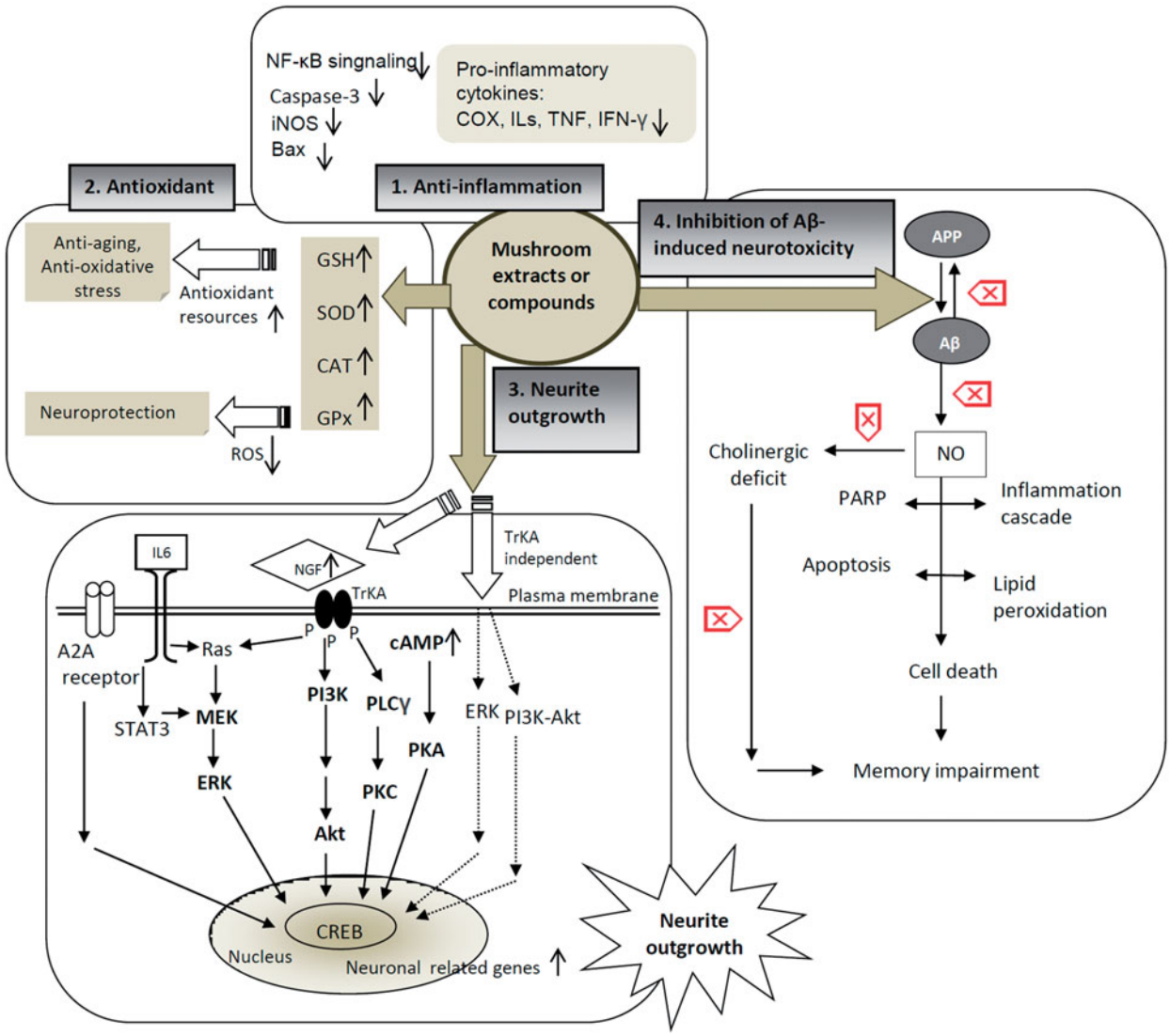


Figure 4. Schematic model of the antioxidative, anti-inflammatory, neurite outgrowth, and neuroprotective effects of mushroom extracts/compounds. ↑, increased; ↓, decreased; ⊠, inhibited; COX, cyclooxygenase; ILs, interleukins; TNF, tumor necrosis factor; IFN-γ, interferon-γ; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; Bax, BCL2-associated X; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; ROS, reactive oxygen species; cAMP, cyclic adenosine monophosphate; STAT3, signal transducers and activators of transcription 3: CREB, cAMP response element-binding; PLC-γ, phosphoinositide phospholipase C-γ; NO, nitric oxide; APP, amyloid precursor protein; PARP, poly (ADP-ribose) polymerase.

mediated by phosphatidylinositol-3 kinase (PI3K). Inhibition of PI3K/Akt by inhibitor LY294002 negatively affected neurite outgrowth of PC12 potentiated by *P. giganteus*. This finding suggested that *P. giganteus* induced-neurite outgrowth is also regulated by PI3K/Akt cascade.

As for the inhibitory mechanism of *G. lucidum* on Aβ25–35 neurotoxicity, the levels of stress kinases, namely phosphorylated JNK, phosphorylated c-Jun, and phosphorylated p38 were markedly attenuated (Lai et al., 2008). Meanwhile, the phosphorylation levels of ERK, JNK, and p38 were found to increase in microglia after lipopolysaccharide (LPS) and/or interferon gamma treatment. The methanol extracts of *A. camphorata* significantly inhibited the phosphorylation of ERK and JNK, slightly inhibited the activator of transcription (STAT-1) phosphorylation, in the course of anti-inflammatory activity in microglia. Another study also agreed that *A. camphorata* prevented serum deprivation-induced PC12 cell apoptosis through a PKA-dependent pathway and by suppression of JNK and p38 activities (Lu et al., 2008). 3,4-Dihydroxybenzalacetone (DBL) isolated from *I. obliquus*, inhibited H_2O_2-induced apoptosis of neurons by suppressing the intracellular ROS levels and inhibited Bax and caspase-3 activation. Treatment of DBL significantly inhibited the H_2O_2-dependent phosphorylation of p38-MAPK, but not the ERK and JNK, since p38 was responsible for phosphorylate p53, which ultimately lead to apoptosis.

IL-6 is an important interleukin to promote neuronal survival and neuronal differentiation. Scabronine G-ME-induced neuritogenesis was mediated by PKC cascades, since a selective inhibitor of PKC, GF109203X inhibited the process (Obara et al., 2001). In contrast, GF109203X, as well as the wortmannin (another inhibitor of PI3K), did not inhibit neurite outgrowth of PC12 in response to cyrneine A from the mushroom *Sarcodon cyrneus*. This indicated that PKC and PI3K/Akt were not

involved. However, the neurite outgrowth process was blocked by PD98059, indicating that ERK1/2 is required for cyrneine A-induced neuritogenesis. The activity of Rac1, which is a GTPase protein that regulates actin, was also increased by cyrneine A. Both scabronine G-methylester and cyrneine A enhanced the activation of nuclear factor-κB, but not phospho-cAMP-response element-binding protein (CREB). In contrast to this, *Tremella fuciformis* (Park et al., 2012) and *G. lucidum* (Cheung et al., 2000) enhanced the neurite outgrowth of PC12 cells *via* activation of CREB transcription. *A. camphorata* was also found to prevent serum deprivation-induced PC12 cell apoptosis through CREB-dependent protein kinase A (PKA) pathway (Huang et al., 2005). The coordinated events involved in the mechanisms of antioxidant, anti-inflammation, neurite outgrowth, and inhibition of neurotoxicity are presented in Figure 4.

Conclusions

In this review, we have summarized mushrooms that have been reported to show beneficial effects in neuronal health, with particular emphasis on either crude extracts or isolated metabolites. Taken as a whole, these medicinal mushrooms have shown neurological properties such as neuronal survival and neurite outgrowth activities, including improvement in recovery and function in both *in vitro* and *in vivo* mammalian nervous systems. Therefore, based on the studies discussed in this review, including our own research over the last decade, we propose that these medicinal mushrooms may have therapeutic values to treat human neurological diseases. However, any such endeavor, involving human models, must be carried out with great care and caution as the pharmacological and negative effects of these mushrooms are not well established even though many of these mushrooms are edible. We hope this review will promote interest in medicinal mushroom research in the experimental clinical neurology area with a long-term objective of developing effective therapies for neurological diseases.

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Declaration of interest

The authors report no conflicts of interest.

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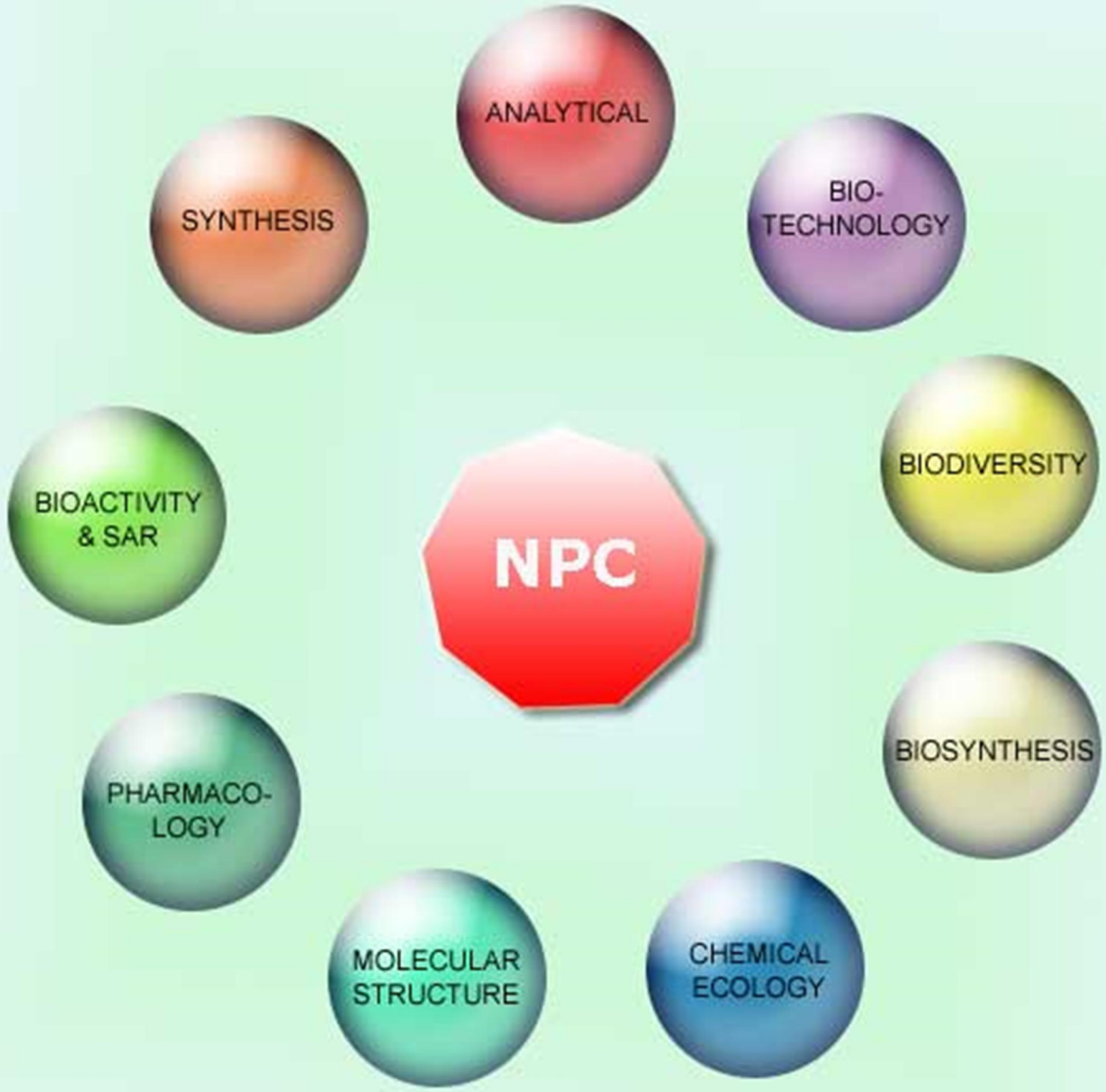

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Lipid Constituents of the Edible Mushroom, *Pleurotus giganteus* Demonstrate Anti-Candida Activity

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Different solvent extracts of *Pleurotus giganteus* fruiting bodies were tested for antifungal activities against *Candida* species responsible for human infections. The lipids extracted from the ethyl acetate fraction significantly inhibited the growth of all the *Candida* species tested. Analysis by GC/MS revealed lipid components such as fatty acids, fatty acid methyl esters, ergosterol, and ergosterol derivatives. The sample with high amounts of fatty acid methyl esters was the most effective antifungal agent. The samples were not cytotoxic to a mammalian cell line, mouse embryonic fibroblasts BALB/c 3T3 clone A31. To our knowledge, this is the first report of antifungal activity of the lipid components of *Pleurotus giganteus* against *Candida* species.

Keywords: *Pleurotus giganteus*, *Candida*, yeast, Antifungal, Medicinal mushroom, Fatty acid, Fatty ester methyl ester, Ergosterol.

Fungal infections are problematic for human health and are responsible for high rates of morbidity and mortality worldwide. Species of *Candida* are the dominant cause of opportunistic mycoses and among them, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* account for 95–97% of all Candida infections [1,2]. *C. albicans* and *C. tropicalis* are susceptible to polyenes, flucytosine, azoles and echinocandins, while *C. glabrata* is either susceptible or resistant to fluconazole[3]. Furthermore, *C. krusei* displays decreased susceptibility to amphotericin B, as well as fluconazole. Considering the increasing incidence of drug-resistant *Candida* infections, the search for more effective anti-Candida agents as an alternative to synthetic ones is needed. The interest in natural products from medicinal plants as a source of anti-Candida agents has grown dramatically. A wide variety of plant extracts have been reported to have anti-*C. albicans* activity. Examples include propolis, *Punica granatum* (pomegranate), *Streblus asper* (Siamese rough bush), *Vitis vinifera* (common grape vine), and tea tree oil from *Melaleuca alternifolia* [4].

Medicinal mushrooms are relatively less researched for their antifungal properties. However, in the last five years, there has been a renewed interest in using mushrooms as antimicrobial agents. *Lentinula edodes* (shiitake), *Boletus edulis* (Penny bun), *Pleurotus ostreatus* (oyster mushroom), *Coprinus comatus* (shaggy mane), *Astraeus hygrometricus* (earthstar mushroom), and *Cordyceps militaris* were shown to exhibit antifungal activity against *C. albicans* [5–8]. *Pleurotus giganteus* (Zhudugu, Dabeijun, morning glory mushroom), a saprobic mushroom, is one of the largest mushroom which grows on soil either as solitary or gregarious fruiting bodies [9]. This mushroom has gained popularity in China for its culinary properties. The medicinal properties of this mushroom are less known. We have previously reported the hepatoprotective and neuronal stimulating effects of *P. giganteus* [10,11]. In this study the antifungal activities of different solvent extracts of this mushroom were evaluated. The extracts prepared with different solvents had different profiles of fatty acids, and fatty acids have been shown to demonstrate antimicrobial activities [12].The main metabolites / components in the extracts were analysed by GC-MS. As a preliminary *in vitro* toxicity assessment, the *P. giganteus* extracts were also investigated for cytotoxicity to mouse embryonic 3T3 fibroblast cells.

Table 1: Activity of different extracts of *Pleurotus giganteus* against *Candida* species.

Candida strains	Untreated	Solvent extracts (µg/mL)								
		Methanol			Ethyl acetate			Aqueous		
		25	50	100	25	50	100	25	50	100
Candida albicans WM1172	++++	++++	+++	++	+	-	-	++++	++++	++++
Candida albicans ATCC90028	++++	+++	+++	+	+	-	-	++++	++++	+++
Candida dubliniensis	++++	++++	+++	-	+	-	-	++++	++++	+++
Candida glabrata CBS138	++++	++++	++	-	+	-	-	++++	++++	++++
Candida glabrata ATCC90030	++++	++++	++++	-	+	-	-	++	+++	+++
Candida krusei ATCC6258	++++	+++	+++	+	-	-	-	++++	+++	+++
Candida pseudotropicalis	++++	+++	+++	++	-	-	-	++++	++++	++++
Candida tropicalis WM30	++++	+++	++++	++	+	-	-	++++	++++	++++

Strains were grown with different mushroom extracts at the concentrations shown for two days on YEPD media. Growth was scored from "-" to "++++", indicating no growth to strong growth.

The anti-Candida activity of methanol, ethyl acetate and aqueous extracts of *P. giganteus* against all yeast species tested are summarised in Table 1. *Candida* species showed strong growth (denoted as "++++") when extracts were not added to the medium. The aqueous extract had minimum or no inhibitory activity against all *Candida* spp. The ethyl acetate extract completely inhibited the growth of all *Candida* spp. when tested at 50 and 100 µg/mL. Thus, the ethyl acetate extract was fractionated to identify the active component/s responsible for the antifungal activity. Sub-fractions A to H were obtained and the minimum inhibitory concentration (MIC) values against all the tested yeasts are given in Table 2. The MIC values for all the *Candida* spp. tested, ranged from 2.0 ± 1.0 to 10.3 ± 2.5 µg/mL for sub-fraction A; and 9.3 ± 2.3 to 34.3 ± 10.8 µg/mL for sub-fraction B; respectively. The MIC values of

Table 2: Activity of the sub-fractions of ethyl acetate extracts against Candida species.

Candida strains	Sub-fractions from ethyl acetate extract (MIC[a])								IC_{50} (mM)	
	A	B	C	D	E	F	G	H	Fluconazole	Amphotericin B
Candida albicans WM1172	7.3 ± 1.5	16.0 ± 6.1	26.6 ± 11.6	*	*	*	*	*	1.0	0.6
Candida albicans ATCC90028	7.0 ± 1.0	22.6 ± 11.5	40.6 ± 16.6	*	*	*	*	*	2.0	0.7
Candida dubliniensis	8.2 ± 3.7	31.6 ± 7.8	37.0 ± 7.0	*	*	*	*	*	24.0	2.1
Candida glabrata CBS138	8.1 ± 1.5	12.5 ± 2.4	28.3 ± 6.6	*	*	*	*	*	>10.0	1.3
Candida glabrata ATCC90030	9.2 ± 1.6	29.4 ± 7.0	41.3 ± 7.6	*	*	*	*	*	>10.0	1.2
Candida krusei ATCC6258	10.3 ± 2.5	37.2 ± 5.0	*	*	*	*	*	*	8.0	0.4
Candida pseudotropicalis	3.8± 1.4	9. 3 ± 2.3	23.0 ± 11.0	*	*	*	*	*	9.0	0.6
Candida tropicalis WM30	2.0 ± 1.0	34.3 ± 10.8	*	*	*	*	*	*	<1.0	1.2

Results were from three independent experiments performed in triplicate. [a]MIC is expressed in μg/mL. * : >50 μg/mL

sub- fraction C varied from 23.0 ± 11.0 to >50 μg/mL; whereas the MIC values for sub-fractions D-H were all >50 μg/mL. Overall, sub-fraction A showed the lowest MIC value for all *Candida* spp. tested.

Sub-fractions A and B were further analysed by GC-MS. Both samples were pale yellow-colored oils with a distinct odor. Constituents of sub-fractions A and B are listed in Table 3. Twelve compounds were identified in sub-fractions A and B. Sample A was characterized by high amounts of fatty acid methyl esters, namely: methyl palmitate, ethyl palmitate, methyl linoleate, methyl oleate, methyl stearate, and ethyl oleate. Sample B contained fatty acids (palmitic acid and oleic acid), fatty acid methyl esters (methyl linoleate and methyl oleate), ergosterol, ergosta-5,7,9 (11),22-tetraen-3β-ol, ergost-5,8(14)-dien-3-ol, and γ-ergostenol.

Table 3: Chemical composition of lipids in sub-fractions A and B of .*P. giganteus*.

Constituents	RT (min)	Percentage (%)	Quality
Sub-fraction A			
Methyl palmitate	20.50	14.8	99
Ethyl palmitate	21.81	1.2	98
Methyl linoleate	23.70	19.8	99
Methyl oleate	23.80	39.3	99
Methyl stearate	24.26	3.3	99
Ethyl oleate	24.99	12.3	99
Sub-fraction B			
Methyl palmitate	20.49	0.2	95
Palmitic acid	21.28	14.4	99
Methyl linoleate	23.68	0.4	93
Methyl oleate	23.79	1.0	93
Oleic acid	24.61	31.7	99
Ergosta-5,7,9(11),22-tetraen-3β-ol	39.83	2.2	90
Ergosterol	40.33	24.4	98
Ergost-5,8(14)-dien-3-ol	40.51	10.2	87
γ-Ergostenol	41.32	3.7	94

The methanol, ethyl acetate, and aqueous extracts were not toxic to 3T3 fibroblasts cells and the IC_{50} values were more than 2 mg/mL (Fig. 1). Meanwhile, cell viability (%) decreased steadily with increasing concentrations of sub-fractions A and B at levels up to 500 μg/mL. The IC_{50} value of sub-fraction A was 352 μg/mL and the R^2 value was 0.9609. For sub-fraction B, the IC_{50} was 362 μg/mL with the R^2 value recorded at 0.9552.

To our knowledge, this is the first report on the antifungal activity of the lipid components of *P. giganteus*. It has been reported that crude extracts of *P. ostreatus* and *C. comatus* inhibited the growth of *C. albicans* [6]. However, the MICs were much higher (up to 1 mg/mL) when compared with this study, which recorded an MIC of

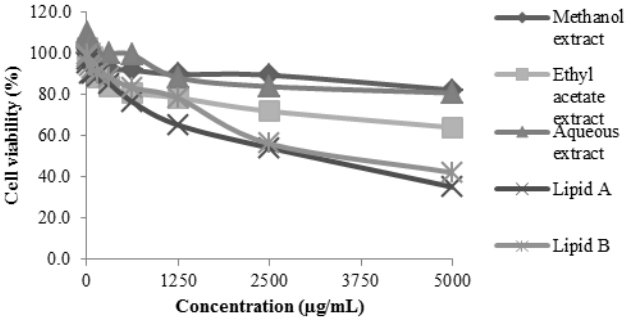

Figure 1: Cell viability of embryonic fibroblast cells after treatment with various extracts of *P. giganteus*.

100 μg/mL. The sub-fractions A and B were shown to contain several bioactive components. Since they are blends of fatty acids and fatty acid methyl esters, they do not act on specific targets in the fungal cells, and fungal resistance may be unlikely to occur. Furthermore, fatty acids and their methyl esters were reported to have fungicidal activity to *C. albicans, C. krusei*, *C. tropicalis* and *C. parapsilosis* [13]. The entities might play crucial roles in lipophilic or hydrophilic effects on the cell wall and membrane, hence affecting the distribution of the lipids in the cells [14]. Moreover, ergosterol present in the sample could disrupt the ergosterol biosynthesis pathway in the yeast, causing growth inhibition or cell death. This was further supported by a study of Irshad *et al.* [15], who reported that ergosterol-rich *Cassia fistula* oil significantly decreased the *in vivo* ergosterol content in the *Candida* cell wall.

In this study, the sub-fractions A and B were not cytotoxic to mouse fibroblasts at the concentrations tested (Fig. 1). Animal testing is becoming less popular and is gradually being replaced by *in vitro* methods for toxicity assessment of pharmaceutical products. In conclusion, *P. giganteus* lipids are promising natural products to be further explored as antifungal agents against *Candida* species.

Experimental

Mushroom: The fruiting bodies of *Pleurotus giganteus* (Berk) Karunarathna & K.D. Hyde were obtained from Nas Agro Farm, Selangor, Malaysia. A voucher specimen (KLU-M 1227) was deposited in the Herbarium in the University of Malaya.

Chemicals: Fluconazole and amphotericin B were purchased from Sigma Co. (St. Louis, MO, USA). The stocks were prepared in dimethyl sulfoxide (DMSO) prior to bioassays. [3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), was also obtained from Sigma. Methanol (MeOH), ethyl acetate (EtOAc), *n*-hexane and acetone were from Merck (Darmstadt, Germany).

Extracts preparation: The fresh fruiting bodies of *P. giganteus* were sliced, freeze-dried and ground to a fine powder (500 g). The mushroom powder was extracted with 80% MeOH to yield a MeOH extract (115 g, 23.0%). This (125 g) was further partitioned in EtOAc-H_2O (100 mL: 100 mL) to give an EtOAc-soluble extract (6.96 g, 6.05%) and a H_2O extract (74.2 g, 64.52%).

Fractionation of extract: The EtOAc extract (5.00 g) was further fractionated by CC over silica gel. The extract was eluted with *n*-hexane containing increasing concentrations of acetone to obtain 8 fractions (A to H) based on similarity of spots on TLC.

Cell culture: Mouse embryonic fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%, v/v, heat-inactivated fetal bovine serum (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were routinely passaged every 2-3 days and incubated at 37°C and 5%, v/v, CO_2 in a humidified atmosphere.

Cytotoxicity: The crude MeOH and fractionated EtOAc extracts were dissolved in DMSO (10 mg/mL) as stock solutions. The H_2O extract (10 mg/mL) was stocked in sterilised distilled water. The cytotoxic effects of varying concentrations of MeOH, EtOAc and H_2O extracts, as well as the fractions A-H in DMSO to 3T3 fibroblast cells were tested by the established colorimetric MTT assay [16]. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of extract or fraction that reduced fibroblast cell growth by 50%.

Anti-yeast activity: *Candida albicans* WM1172, *C. albicans* ATCC90028, *C. dubliniensis*, *C. glabrata* CBS138, *C. glabrata* ATCC90030, *C. krusei* ATCC6258, *C. pseudotropicalis*, and *C. tropicalis* WM30 were used in this study. The yeast inhibition assay was performed according to the method of Macreadie *et al.* [17]. The yeast strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). If required, media were solidified by the addition of 1.5% agar. Yeast inocula (100 µL) with a starting optical density at A_{595} of 0.02-0.04 were added to each well of a 96-well microplate (Orange Scientific, Braine-l'Alleud, Belgium). Mushroom extracts were then added as two-fold serial dilutions commencing with a 100 µg/mL concentration. Fluconazole (0.1 mM) and amphotericin B (1.0 mM) were used as positive controls. A growth control DMSO solvent alone was also included. The microplate was incubated in a microplate shaker at 35ºC. After 2 h and 4 h incubation, the A_{595} was recorded using a microplate reader (Sunrise™, Tecan, Austria). Each sample was assayed in triplicate. The lowest concentration of extracts that inhibited growth of *Candida* spp. is the minimum inhibitory concentration (MIC).

Gas chromatography-mass spectrometry (GCMS): GCMS analysis was performed on sub-fractions A and B using Network Gas Chromatography system (Agilent Technologies 6890N) equipped with an Inert Mass Selective Detector (Agilent Technologies 5975) (70eV direct inlet) on a HP-5ms (5% phenyl methyl siloxane) capillary column (30 m × 250 µm × 0.25 µm) initially set at 150ºC, then increased at 5ºC per min to 300ºC and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL per min. The total ion chromatogram obtained was autointegrated by chemstation and the constituents were identified by comparison with the accompanying mass-spectra database (Wiley 9th edition with NIST 11 Mass Spectral Library, USA) wherever possible.

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Review/Account

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Neurite outgrowth stimulatory effects of culinary-medicinal mushrooms and their toxicity assessment using differentiating Neuro-2a and embryonic fibroblast BALB/3T3

Chia-Wei Phan[1,2], Pamela David[1,3*], Murali Naidu[1,3], Kah-Hui Wong[1,3] and Vikineswary Sabaratnam[1,2]

Abstract

Background: Mushrooms are not only regarded as gourmet cuisine but also as therapeutic agent to promote cognition health. However, little toxicological information is available regarding their safety. Therefore, the aim of this study was to screen selected ethno-pharmacologically important mushrooms for stimulatory effects on neurite outgrowth and to test for any cytotoxicity.

Methods: The stimulatory effect of mushrooms on neurite outgrowth was assessed in differentiating mouse neuroblastoma (N2a) cells. Neurite length was measured using Image-Pro Insight processor system. Neuritogenesis activity was further validated by fluorescence immunocytochemical staining of neurofilaments. *In vitro* cytotoxicity was investigated by using mouse embryonic fibroblast (BALB/3T3) and N2a cells for any embryo- and neuro-toxic effects; respectively.

Results: Aqueous extracts of *Ganoderma lucidum*, *Lignosus rhinocerotis*, *Pleurotus giganteus* and *Grifola frondosa*; as well as an ethanol extract of *Cordyceps militaris* significantly ($p < 0.05$) promoted the neurite outgrowth in N2a cells by 38.4 ± 4.2%, 38.1 ± 2.6%, 33.4 ± 4.6%, 33.7 ± 1.5%, and 35.8 ± 3.4%; respectively. The IC_{50} values obtained from tetrazolium (MTT), neutral red uptake (NRU) and lactate dehydrogenase (LDH) release assays showed no toxic effects following 24 h exposure of N2a and 3T3 cells to mushroom extracts.

Conclusion: Our results indicate that *G. lucidum*, *L. rhinocerotis*, *P. giganteus*, *G. frondosa* and *C. militaris* may be developed as safe and healthy dietary supplements for brain and cognitive health.

Keywords: Culinary-medicinal mushrooms, Neurite outgrowth, Cytotoxicity, Mouse neuroblastoma N2a cell, Mouse 3T3 embryonic fibroblast, Neurofilament

Background

Neurite outgrowth is an important event in neuronal path finding and the establishment of synaptic connections during development [1,2]. It is also essential in neuronal plasticity, neuronal regeneration after injury [3,4] and neurodegenerative conditions such as Alzheimer's and Parkinson's diseases [5]. Therefore, treatments aiming at promoting neurite outgrowth and preserving the neurite network and synaptic connections are needed.

The potential use of culinary-medicinal mushrooms in neurodegenerative diseases is being explored [6]. On-going research in our laboratory shows that *Hericium erinaceus* (Bull.: Fr) Pers. (monkey's head mushroom, lion's mane mushroom and Yamabushitake) [7], *Lignosus rhinocerotis* (Cooke) Ryvarden (tiger milk mushroom) [8,9], and *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde (morning glory mushroom, cow's stomach mushroom) [10] exhibit neurite outgrowth stimulatory effects in NG108-15 and PC12 cell lines. This observation raised a question with respect to the neurodevelopmental effects,

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if any, of culinary-medicinal mushrooms. Birth defects have been identified as a growing social and healthcare issue. Congenital diseases are present in 2–3% of human newborns [11]. About 20% of the birth defects are due to genetic anomaly and 10% are caused by environmental factors during pregnancy [12]. Therefore, toxicological safety assessments of food, chemicals and drugs to evaluate the effects on reproductive health and for embryotoxicity have become an important requirement. Thus, the aims of the present study were (a) to evaluate neurite outgrowth stimulatory effects of selected culinary-medicinal mushrooms using neuroblastoma-2a (N2a) cells and (b) to assess the neuro- and embryotoxicity of the mushroom extracts using N2a and 3T3 fibroblasts. The results will enable us to select potential mushrooms for further in depth *in vivo* developmental toxicity evaluation.

Methods

Mushroom and plant samples

The mushrooms were authenticated by experts in the Mushroom Research Centre, University of Malaya and voucher specimens were deposited in the University of Malaya herbarium at Rimba Ilmu (Table 1). Fresh fruiting bodies of *Ganoderma lucidum* (Fr) P. Karst (KLU-M 1233) and *H. erinaceus* (KLU-M 1232) were obtained from Ganofarm Sdn Bhd. *Pleurotus giganteus* (KLU-M 1227) was provided by Nas Agro Farm and Dong Foong Biotech. Freeze dried powder of *Cordyceps militaris* (L.:Fr.) Link and *L. rhinocerotis* were purchased from BioFact Life Sdn Bhd and Ligno Biotek Sdn Bhd, respectively. *Pleurotus pulmonarius* (Fr.) Quél. (KLU-M 1309) and *Gingko biloba* extracts were obtained from Reishilab Sdn Bhd, Selangor. Wild *Ganoderma neo-japonicum* Imazeki 1939 (KLU-M 1231) was collected from forest in Ulu Grik, Perak, Malaysia. *Ganoderma neo-japonicum* was used as traditional medicine by the indigenous people in Malaysia. *Grifola frondosa* (Dicks.: Fr.) S.F. Gray (KLU-M 1229) imported from Japan was obtained from supermarkets in Selangor, Malaysia. *Lycium barbarum (*wolfberry), a traditional Chinese medicine was purchased from a Chinese medicine shop in Selangor, Malaysia and deposited in University of Malaya herbarium. Curcumin was purchased from NatXtra, Synthite Co., India.

Chemicals

Phosphate buffered saline (PBS), [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), dimethyl sulfoxide (DMSO), nerve growth factor (NGF) from murine submaxillary gland, 3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride (neutral red), and foetal bovine serum (FBS) were obtained from Sigma Co. (St. Louis, MO, USA).

Cell culture

Mouse neuroblastoma cells (N2a, ATCC CCL-131) and mouse embryonic fibroblast cells (BALB/c 3T3, ATCC clone A31) were purchased from American Type Culture Collection (ATCC; MD, USA). N2a cells were cultured in Eagle's minimum essential medium (MEM) with L-glutamine (PAA) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (PAA), 100 U/ml penicillin, and 100 μg/ml streptomycin. 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (high glucose at 4.5 g/l) supplemented with 10% FBS. All the cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere. N2a cells were subcultured at 3 – 4 days intervals while 3T3 cells were routinely passaged every 2 – 3 days. For preservation, the cells were frozen at −70°C liquid nitrogen in complete medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO; Sigma) as a cryoprotecting agent.

Preparation of mushroom extracts

The fresh fruiting bodies of *P. giganteus*, *P. pulmonarius*, *H. erinaceus*, and *G. frondosa* were sliced, frozen and then freeze-dried for two days. The freeze-dried fruiting bodies were then ground to powder and kept at 4 - 8°C. For aqueous extraction, the freeze dried powder was soaked in distilled water (1:20, w/v) at room temperature and 200 rpm in a shaker for 24 h. The mixture was then double boiled in water bath for 30 min, cooled and then filtered (Whatman No. 4). The resulting aqueous extracts were freeze-dried and kept at −20°C prior to use. The process was repeated for the freeze-dried powder of *C. militaris* and *L. rhinocerotis* sclerotia. For ethanol extraction, the freeze dried powder was soaked in 95% ethanol at room temperature for three days and the process was repeated three times. The solvent was then evaporated using a rotary evaporator (Eyela N-1000, USA) to give a brownish viscous extract.

Neurite outgrowth assay

N2a cells were seeded in 24-well culture plate at an initial density of 5,000 cells per well containing complete growth medium (1 ml/well) and incubated overnight. Concentrations of NGF ranging from 5–100 ng/ml (w/v) were tested to determine the optimum concentration that stimulates maximum neurite outgrowth. The optimum concentration was then used as a positive control throughout the subsequent assays. Aqueous and ethanol mushroom extracts were stocked at 10 mg/ml and were subsequently dissolved in sterile distilled water or DMSO, to the appropriate concentrations. The final concentration of DMSO in the assays was 0.1 - 0.25%. To induce cell differentiation, the complete medium was carefully replaced with 5% serum medium before exposure to mushroom extracts at 10–50 μg/ml. Cells with medium only served as a

Table 1 Medicinal mushrooms used in this study, their common names, and culinary nature

Mushroom species	Voucher number	Common names	Local names (in Malay)	Part used	Edible/ culinary	Wild/ cultivated	Medicinal properties	References
Pleurotus giganteus	KLU -M 1227	Zhudugu, cow's stomach mushroom	*Cendawan seri pagi* (morning glory), *perut lembu* (cow's stomach)	Fruiting body	Culinary	Cultivated	Antioxidant, neurite outgrowth simulation	[10,13]
Pleurotus pulmonarius	KLU-M 1309	Grey oyster mushroom	*Cendawan tiram kelabu* (grey oyster)	Fruiting body	Culinary	Cultivated	Antioxidant, anti-diabetic	[14]
Lignosus rhinocerotis	Purchased from Ligno Biotek Sdn Bhd	Tiger milk mushroom,	*Cendawan susu rimau* (tiger's milk)	Sclerotium and mycelium	Non-culinary but edible	Cultivated	Anticancer, anti-inflammatory, neurite outgrowth stimulation	[8,15]
Hericium erinaceus	KLU-M 1232	Monkey's head mushroom, lion's mane mushroom, Yamabushitake	*Cendawan bunga kubis* (cauliflower)	Fruiting body	Culinary	Cultivated	Anti-ulcer, neurite outgrowth stimulation	[7,16-18]
Ganoderma lucidum	KLU-M 1233	Lingzhi, reishi	*Cendawan merah* (red mushroom)	Fruiting body	Non-culinary but edible	Cultivated	Anticancer, neuroprotection	[19]
Ganoderma neo-japonicum	KLU-M 1231	Purple reishi	*Cendawan senduk* (cobra mushroom)	Fruiting body	Non-culinary but edible	Wild	Antioxidant, antihepatoxic, neurite outgrowth stimulation	[9,20]
Cordyceps militaris	Purchased from BioFact Life Sdn Bhd	Winter worm summer grass, caterpillar fungus	-	Fruiting body (ascocarp)	Non-culinary but edible	Cultivated	Anti-inflammatory, anticancer, relief respiratory disorders	[21]
Grifola frondosa	KLU- M 1229	Maitake, hen of the woods	*Cendawan maitake*	Fruiting body	Culinary	Cultivated	Anti-inflammatory, anti-cholesterol	[22]

negative control. All the cells were incubated for 48 h at 37°C, 95% air and 5% CO_2 to observe neuritogenesis activity, if any. Curcumin, *G. biloba* and *L. barbarum* extracts were also tested to compare the neurite outgrowth activities with those of mushroom extracts.

Quantification of neurite bearing cells

Five random fields (100 – 200 cells/well) were examined in each well by using a phase contrast microscope (20× magnifications) equipped with QImaging Go-3 camera (QImaging, Canada). Neurite length was measured in at least 30 cells in randomly chosen fields by using image processor system Image-Pro Insight (MediaCybernetics, MD). The number of neurite outgrowths, defined as axon-like extensions that were double or more than the length of the cell body diameter was recorded. The percentage of neurite bearing cells (%) is the number of neurite bearing cells divided by the total number of cells in a field and then multiplied by 100%. At least three independent experiments were conducted and results were expressed as mean ± standard deviation (S.D).

Fluorescence immunocytochemistry study

The axon-like extensions were confirmed as neurite outgrowth by immunofluorescence study. N2a cells were seeded in 12-well μ-dishes (ibidi, Martinsried, Germany) and were exposed to treatments for 48 h. The cells were fixed with 4% paraformaldehyde in PBS (pH 7) for 20 min. After two washes with PBS, the cells were incubated with rabbit anti-neurofilament 200 polyclonal antibody (1:80 in 10% sheep serum as blocking buffer) for 1 h. The cells were washed and then incubated in a mixture of fluorescein isothiocyanate (FITC)-conjugated secondary antibody and sheep anti-rabbit IgG (1:160 in blocking buffer) for 2 h at room temperature in the dark. The cells were then washed three times. 4'-6-diamidino-2- phenylindole (DAPI) was used to counter stain the nuclei. Images were observed under a fluorescent microscope (Nikon Eclipse 80i microscope).

Evaluation of embryo- and/or neurotoxic effects of mushroom extracts

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

N2a and 3T3 cells (1×10^4) per well were seeded in 96-well plates. After incubation for 24 h, different concentrations of mushroom extracts (0 – 5 mg/ml) dissolved in phenol red free culture medium were added to each well. The samples were incubated for 24 h at 37°C under humidified atmosphere of 5% CO_2 and 20 μl MTT (5 mg/ml) was added to each well. The crystal dyes which were up-taken by the cells were then dissolved with DMSO. Absorbance was measured at 570 nm in a microplate reader (Tecan, Austria) using 630 nm as a reference wavelength. All measurements were done in triplicates, and at least three independent experiments were carried out. To calculate IC_{50} values which estimated the concentration of mushroom extract that caused 50% inhibition of proliferation (viability) in N2a and 3T3 cells, Probit analysis was conducted using SPSS 17.0 (SPSS Science Inc., Chicago, IL).

Neutral red uptake assay

Neutral red medium (40 μg/ml) was prepared fresh before use by diluting the neutral red stock (4 mg/ml) with phenol red free culture medium. Neutral red medium was centrifuged at 1800 rpm for 10 min to remove any precipitated dye crystals before use. After cell seeding and treatment (0 – 5 mg/ml), the medium was discarded and replaced with equal amount of neutral red medium to each well of the plate. The plate was then incubated for 2 h. The neutral red medium was then removed and the cells were washed quickly with adequate amount of PBS. A total of 100 μl of neutral red solubilising solution (1% acetic acid in 50% ethanol) was added to each well and allowed to stand for 10 minutes at room temperature until the neutral red extracted from the cells reached a homogeneous solution. The absorbance at a wavelength of 540 nm with 690 nm of background absorbance was spectrophotometrically measured (Tecan, Austria). The experiment was repeated at least three different times.

Lactate dehydrogenase (LDH) release assay

After cell seeding and treatment with mushroom extracts (0 – 5 mg/ml), the culture plates were centrifuged at 1500 rpm for 5 minutes and 50 μl of supernatant was then transferred to a new plate for LDH analysis according to manufacturer instruction (Sigma). To each well, 100 μl of LDH mixture solution comprising of LDH assay substrate, dye and cofactor was added and incubated at room temperature for 30 min. The reaction was stopped by adding 10 μl of 1 N HCl to each well. Absorbance was spectrophotometrically measured at 490 nm with background absorbance at 690 nm. Triton X-100 (0.5%, Scharlau) was used as a positive control and was thus set to 0% viability representing a 100% cell death.

Statistical analysis

All the experimental data are expressed in mean ± standard deviation (S.D). Statistical differences between groups were analysed and calculated by one-way analysis of variance (ANOVA) from at least three independent experiments. This was followed by Duncan's multiple range tests. $P < 0.05$ was considered to be significant between groups.

Results

The effects of NGF on neurite outgrowth activity in N2a cells

Nerve growth factor induced neurite outgrowth of N2a in a dose-dependent manner (Figure 1). After 48 h of NGF stimulation, the percentage of neurite bearing cells increased significantly ($p < 0.05$) to 26.1 ± 1.8% in N2a cells treated with 50 ng/ml NGF when compared to negative control (7.6 ± 2.5 %). At 60 ng/ml of NGF, the percentage of neurite bearing cells significantly decreased to 12.2 ± 2.1% ($p < 0.05$). Based on these findings, the optimised concentration of NGF (50 ng/ml) was selected for the following experiments as a positive control.

The effects of different mushroom extracts on neurite outgrowth activity in N2a cells

The positive control (NGF) recorded 26.4 ± 3.6% of neurite-bearing cells (Figure 2). The extraction yield of extracts from the mushrooms are summarised in Table 2. The percentage of neurite bearing cells after treatment with aqueous extracts of *G. lucidum* (38.4 ± 4.2%), *L. rhinocerotis* (38.1 ± 2.6%), and ethanol extract of *C. militaris* (35.8 ± 3.4%) were significantly higher ($p < 0.01$) than NGF control by approximately 1.45-, 1.44- and 1.35-fold, respectively. Aqueous extracts of *G. frondosa* (33.7 ± 1.5%) and *P. giganteus* (33.4 ± 4.6%) were also shown to induce significantly ($p < 0.05$) higher neurite bearing cells compared to the NGF control. Meanwhile, the aqueous extracts of *L. rhinocerotis* mycelium, *H. erinaceus, G. neo- japonicum*, *P. pulmonarius*, as well as ethanol extracts of *H. erinaceus*, *P. pulmonarius* and *P. giganteus* showed varied neurite outgrowth stimulatory effects with average neurite bearing cells ranging from 26.4 ± 5.4% to 29.6 ± 2.2%. Further, these extracts showed no significant difference when compared to NGF control ($p > 0.05$). Among the plant extracts tested, wolfberry extract did not show any neurite outgrowth activity. The percentage of neurite bearing cells obtained after treatment with 20 μg/ml of ethanol extract of *G. biloba* (30.3 ± 2.5%) was better than curcumin which gave 26.4 ± 5.4% at 20 μg/ml. The five different mushroom extracts (*G. lucidum*, *L. rhinocerotis*, *P. giganteus, G. frondosa* and *C. militaris*) each at 20 μg/ml were selected for neurofilament staining.

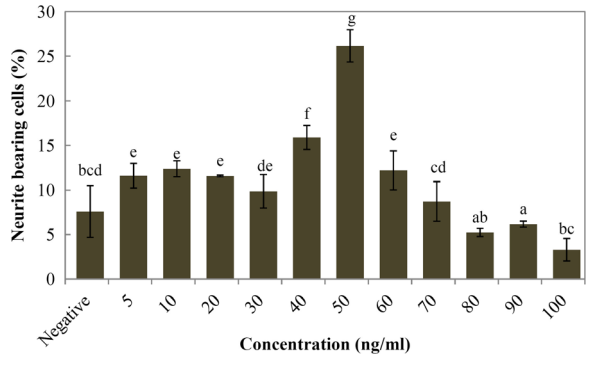

Figure 1 Effects of different NGF concentrations (5 – 100 ng/ml) on stimulation of neurite outgrowth using differentiating N2a cells as an *in vitro* model. The results shown represent the mean ± SD; n = 3. Means not sharing a common letter were significantly different at $p < 0.05$.

The mean diameter of N2a cell body was found to be 19.45 ± 0.72 μm (Figure 3). To qualify as a "neurite", the axon-like extension needs to be double or more than the cell body length of N2a. The average neurite length of NGF-stimulated cells was 78.58 ± 18.6 μm, which is approximately 4-time longer than the cell body. Cells treated with aqueous extract of *G. lucidum* were found to develop the longest mean neurite length i.e. 121.51 ± 28.6 μm (6.25-time longer than cell body), followed by aqueous extract of *P. giganteus* which recorded mean neurite length of 116.72 ± 29.5 μm (5.99-time longer than cell body). Figure 4 shows the morphology of differentiating N2a cells with neurites after 48 h of treatment with 50 ng/ml NGF (a) and 20 μg/ml of aqueous extracts of *G. lucidum* (b), *L. rhinocerotis* (c), *P. giganteus* (d) and *G. frondosa* (e); as well as ethanol extract of *C. militaris* (f).

Immunofluorescence staining of neurofilament

Neurofilament belongs to a class of intermediate filament found in neuronal cells that provides specific support for axons. There is a direct relationship between neurite outgrowth and neurofilament expression as neurofilament protein levels increase with differentiation of cell lines [23]. Figure 5 shows the immunocytochemical labeling of neurons. The expression of neurofilament protein during neurite outgrowth was stained green while nuclei were stained blue.

The cytotoxic effects of mushroom extracts on 3T3 and N2a by using MTT, NRU and LDH release assay

Table 3 shows the results of cytotoxicity screening of mushroom and plant extracts to N2a cells and 3T3 fibroblasts. The cytotoxicity determination are expressed as IC_{50} values, which is the concentration resulting in 50% inhibition of cell growth and proliferation after 24 h exposure. All the extracts of mushrooms and plants did not show cytotoxic effects to the two tested cell lines ($IC_{50} \geq 1$ mg/ml in all cases). Three cytotoxicity endpoints were used in this experiment, which were MTT, NR uptake assay and LDH release assay. Interestingly, IC_{50} determined by NRU test and LDH test was higher than that of MTT. In general term, the ethanol extracts showed lower IC_{50} too.

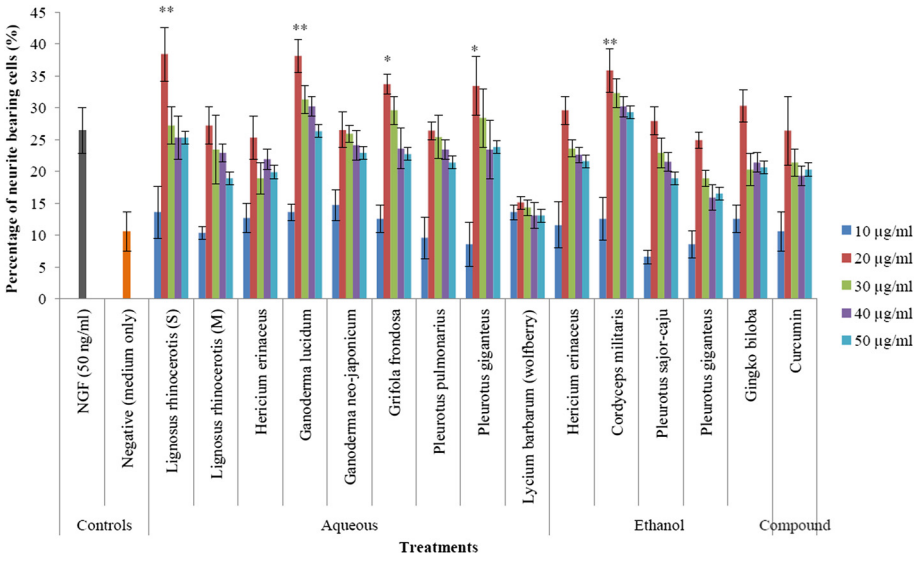

Figure 2 Percentage of neurite bearing cells after treatment of different mushroom and plant extracts. * $p < 0.05$, ** $p < 0.01$ versus the positive control (NGF). (S) = sclerotium, (M) = mycelium.

Discussions

Eight species of medicinal mushrooms were investigated and categorised into two groups: culinary and non-culinary. The former group represents mushrooms that can be used for culinary purposes like preparing meal and cooking, especially from the fruiting bodies. This group (culinary-medicinal mushrooms) comprised of *P. giganteus*, *P. pulmonarius*, *H. erinaceus*, and *G. frondosa*. Medicinal mushrooms of no culinary properties are appreciated for their pharmacological merits and are not cooked as a meal. The fruiting bodies or sclerotia are often handpicked, ground to powder and subjected to various extraction methods before being used as a traditional medication [24]. *L. rhinocerotis, G. lucidum, G. neo-japonicum,* and *C. militaris* are non-culinary medicinal mushrooms.

Ganoderma lucidum (also known as Lingzhi in Chinese or Reishi in Japanese) has been widely investigated for its potential therapeutic benefits and longevity. Our results showed that aqueous extract of *G. lucidum* promoted neuritogenesis in N2a cells with 38.4 ± 4.2% of neurite

Table 2 Extraction yield of aqueous and ethanol extracts from the studied mushrooms

Mushrooms	Extract	Yield (%, w/w)
Lignosus rhinocerotis (S)	Aqueous	3.56
Lignosus rhinocerotis (M)		4.65
Hericium erinaceus		8.56
Ganoderma lucidum		3.67
Ganoderma neo-japonicum		5.75
Grifola frondosa		3.54
Pleurotus pulmonarius		3.60
Pleurotus giganteus		6.70
Hericium erinaceus	Ethanol	4.50
Cordyceps militaris		3.45
Pleurotus pulmonarius		6.70
Pleurotus giganteus		5.30
Lycium barbarum (wolfberry)	Aqueous	9.76
Gingko biloba	Ethanol	n.d

(S) = sclerotium, (M) = mycelium. n.d. = not determined.

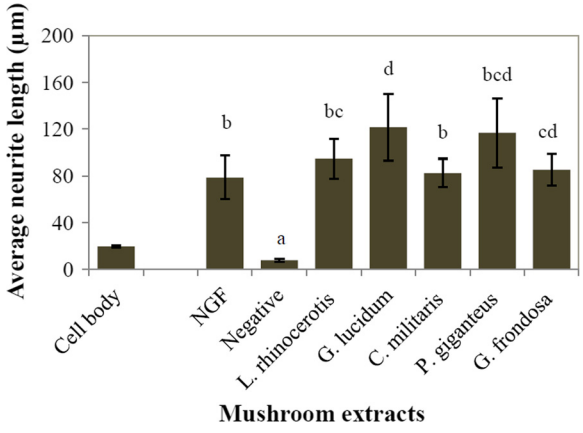

Figure 3 The mean neurite length of N2a treated with different mushroom extracts at 20 µg/ml. The results shown represent the mean ± SD; n = 3. Means not sharing a common letter were significantly different at $p < 0.05$. S = sclerotium, aq = aqueous, EtOH = ethanol.

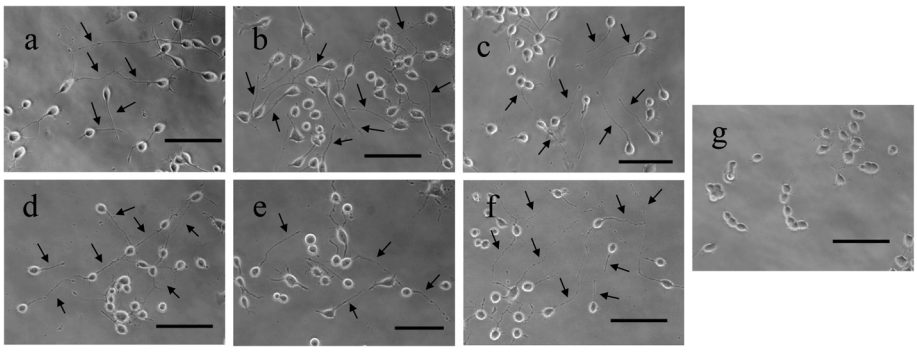

Figure 4 Phase-contrast photomicrographs showing the effects of (a) NGF, (b) *G. lucidum*, (c) *L. rhinocerotis*, (d) *P. giganteus*, (e) *G. frondosa*, and (f) *C. militaris* on the morphology of differentiating N2a cells after 48 h. Untreated cells serve as control (g) and only contained 5% FBS as vehicle. Arrows indicate typical neurites of N2a. Scale bar represents 20 μm. Photomicrographs of representative microscope fields were taken with a 20× objective.

bearing cells. This agrees with the finding of Cheung et al. [25] who showed that *Ganoderma* extract contained NGF-like compounds that mediated the neuronal differentiation and elongation of rat pheochromocytoma (PC12) cells. The *Ganoderma* neuroactive constituents that accounted for neurite outgrowth activity are triterpenoids, such as lucidenic acid [26], 7-oxo-ganoderic acid Z, ganolucidic acid A, methyl ganoderic acid A, ganoderic acid S1, and 4,4,14α-trimethyl-5α-chol-7,9(11)-dien-3-oxo-24-oic acid [27]. Further, the water-soluble polysaccharides of *G. lucidum* were shown to significantly ($p < 0.05$) reduce neuronal cell death and apoptosis of rat primary cortical neurons (model of brain cerebral ischemia) induced by oxygen/glucose deprivation treatment [19]. *Ganoderma* extracts may also provide mitigation to Parkinson's disease as it was shown to prevent dopaminergic neuron degeneration by attenuating the pro-inflammatory response of microglial cells [28].

The tiger milk mushroom, *L. rhinocerotis* has been described as the national treasure of Malaysia as this macrofungus is rare and is often used as folk remedy to treat a variety of diseases. Consistent with our previous study [8], the sclerotia of *L. rhinocerotis* improved neurite outgrowth in N2a. Interestingly, in the present study, the sclerotial extract (38.4 ± 4.2% of neurite bearing cells) performed better than the mycelial extract (27.2 ± 2.9%). However, the active components in *L. rhinocerotis* that play the role in neurite outgrowth activity need further investigation. Lee et al. [15] highlighted that the protein or carbohydrate/protein complex of the cold water scerotial extract (4°C) is responsible for the anti-proliferative activity against human breast and lung carcinoma. In this study, a hot water extraction approach was employed and we hypothesise that polysaccharides or triterpenoids rather than peptides are involved in the neurite outgrowth stimulatory activity of *L. rhinocerotis.*

Wild *Pleurotus giganteus* has been reported to be consumed by the indigenous tribes of Semai, Temuan, and Jakun in Malaysia [29]. This wild mushroom has been successfully domesticated for large scale production and is

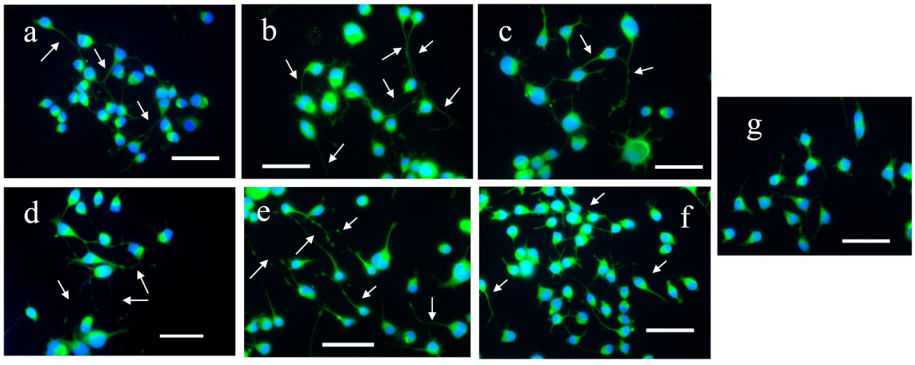

Figure 5 Immunocytochemical staining of neurofilament in N2a cells treated with (a) NGF, (b) *G. lucidum*, (c) *L. rhinocerotis*, (d) *P. giganteus*, (e) *G. frondosa*, and (f) *C. militaris*, and untreated cells as control (g). DAPI stains for nuclei blue, while anti-neurofilament 200 kD labeled with FITC stains neuronal cells green. Scale bar represents 20 μm. Arrows indicate neurite outgrowth. Photomicrographs of representative microscope fields were taken with a 20× objective.

Table 3 IC_{50} values obtained by using different cytotoxicity assays- MTT, NRU and LDH release

Mushroom/Plant	Species	Extracts	Neuroblastoma 2a cells			3T3 embryonic fibroblast		
			IC_{50} (mg/ml) after 24 h			IC_{50} (mg/ml) after 24 h		
			MTT	NRU	LDH	MTT	NRU	LDH
Culinary/medicinal mushroom	*Pleurotus giganteus*	Aqueous	4.07 ± 0.67^{e}	6.95 ± 1.00^{g}	-	2.16 ± 0.05^{c}	2.67 ± 0.47^{d}	-
	Pleurotus pulmonarius		2.85 ± 0.06^{cd}	2.13 ± 0.87^{cdef}	-	1.75 ± 0.14^{bc}	2.60 ± 0.55^{d}	-
	Lignosus rhinocerotis (sclerotium)		3.27 ± 0.77^{d}	2.72 ± 0.65^{ef}	-	5.63 ± 0.06^{e}	5.93 ± 0.05^{f}	-
	Lignosus rhinocerotis (mycelium)		2.43 ± 0.12^{c}	1.75 ± 0.18^{abc}	-	5.23 ± 0.17^{e}	5.60 ± 0.1^{f}	-
	Hericium erinaceus		2.60 ± 0.52^{cd}	2.85 ± 0.56^{e}	-	3.43 ± 0.29^{d}	3.53 ± 0.06^{e}	-
	Ganoderma lucidum		1.35 ± 0.03^{a}	1.52 ± 0.17^{ab}	2.20 ± 0.52^{ab}	1.19 ± 0.06^{ab}	1.35 ± 0.02^{ab}	1.50 ± 0.08^{a}
	Ganoderma neo-japonicum		1.17 ± 0.006^{a}	1.11 ± 0.03^{a}	2.00 ± 0.53^{ab}	1.47 ± 0.37^{ab}	1.78 ± 0.56^{bc}	1.58 ± 0.44^{a}
	Grifola frondosa		2.72 ± 0.08^{cd}	4.60 ± 1.10^{f}	-	6.67 ± 0.87^{f}	7.60 ± 0.1^{g}	-
	Pleurotus giganteus	Ethanol	2.43 ± 0.10^{c}	2.81 ± 0.15^{e}	5.8 ± 0.10^{c}	1.66 ± 0.56^{bc}	1.96 ± 0.42^{c}	1.73 ± 0.48^{a}
	Pleurotus pulmonarius		2.30 ± 0.72^{bc}	2.64 ± 0.16^{def}	2.53 ± 0.96^{bc}	1.27 ± 0.06^{ab}	1.48 ± 0.05^{abc}	1.67 ± 0.12^{a}
	Hericium erinaceus		2.47 ± 0.50^{c}	1.61 ± 0.51^{ab}	2.10 ± 0.72^{ab}	1.31 ± 0.03^{ab}	1.84 ± 0.57^{bc}	1.47 ± 0.31^{a}
	Cordyceps militaris		1.65 ± 0.015^{ab}	1.81 ± 0.10^{abcd}	2.37 ± 0.21^{b}	1.02 ± 0.08^{a}	1.10 ± 0.02^{a}	1.40 ± 0.29^{a}
Medicinal plant	*Lycium barbarum*	Aqueous	4.37 ± 1.00^{e}	6.96 ± 0.73^{g}	-	8.40 ± 0.72^{g}	7.86 ± 0.06^{g}	-
	Gingko biloba	Ethanol	1.56 ± 0.08^{ab}	1.66 ± 0.11^{ab}	1.43 ± 0.39^{a}	1.56 ± 0.58^{abc}	1.37 ± 0.07^{ab}	1.78 ± 0.07^{a}
	Curcumin	Compound	1.13 ± 0.01^{a}	1.15 ± 0.01^{a}	1.30 ± 0.16^{a}	1.16 ± 0.06^{ab}	1.23 ± 0.03^{ab}	1.60 ± 0.22^{a}

The data represent the mean ± SD of three determinations. Means not sharing a common letter were significantly different at $p < 0.05$.

gaining popularity for culinary uses. The medicinal properties of *P. giganteus* are comparatively little as compared to *P. pulmonarius* (grey oyster mushroom). Recently *P. giganteus* (synonyms: *Lentinus giganteus* and *Panus giganteus*) was shown to have *in vivo* hepatoprotective effect in rat [13] and *in vitro* neuritogenic effects in PC12 cells [10]. On-going studies aimed at isolating and identifying the chemical constituents of the *P. giganteus* fruiting bodies indicate the presence of phenolics (caffeic acid and cinnamic acid), organic acid (succinic acid) and triterpenoids (unpublished data). The compounds may work synergistically and accounted for neuritogenesis.

Cordyceps militaris is a parasitic fungus that colonises moth larvae (Lepidoptera) and has been valued in Traditional Chinese Medicine for more than 2000 years. The major bioactive components in *C. militaris* include adenosine, cordycepin and polysaccharides [30]. Our finding is in agreement with the work by Lee et al. [21] where methanol extract of *C. militaris* was shown to significantly reverse the scopolamine-induced deficit in memory of rat and improve neurite outgrowth in N2a. Similar to our results, lysophosphatidylethanolamine isolated from *G. frondosa* (Maitake) was also reported to induce neuronal differentiation in PC12 cells, causing up-regulation of neurofilament M expression of PC12 cells [22].

It is well known that N2a cells, upon the withdrawal of serum, differentiate and elaborate neurites [31,32]. This well-defined neuronal model is often employed for studies relating to neuronal differentiation [33]. It is also a popular cell line in studying neurotoxicity as the brain is a first target in situations such as ageing and neurodegenerative diseases [34]. NGF is the most appropriate positive control in neurite outgrowth assays as its role in neural development have been characterised extensively as supported by Sofroniew et al. [35]. The therapeutic application of neurotrophins like nerve growth factor (NGF) is not possible as NGF cannot penetrate the blood–brain barrier. Studies indicate that lower-molecular-weight molecules may be a promising alternative for therapeutic intervention, for example, α-phenyl-N-tert-butylnitron [36]. However, most of the experiments testing natural products have been conducted *in vitro*, and few studies evaluated these compounds in the brain *in vivo*. Our previous study showed that aqueous extract of *P. giganteus* induced neuronal differentiation of PC12 cells *via* the activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase-Akt (PI3K/Akt) signaling pathways [10]. In fact, the most studied pathway controlling the consolidation of neurites involves signaling through neurotrophin receptors to a Ras-dependent, mitogen-activated protein kinase (MAPK) cascade [33]. At present, the precise neuritogenic signal transduction pathway involved in the actions of NGF, serum withdrawal, and the mushroom extracts is yet to be

elucidated. It is anticipated that mushroom extracts (comprising of neuroactive polysaccharides or triterpenoids) under certain condition (serum deprivation) participate in triggering NGF signals, hence activating the downstream neuronal responses to axonal growth [37].

It is important that functional and health food remedies recommended for the prevention or treatment of diseases undergo safety assessment. Today, a number of natural products with potential biomedical application are being launched, although some could be potentially toxic when ingested at high doses or in combination with other medications [38]. Our results indicated that no cytotoxicity was detected for the concentrations tested. Notably, ethanol extract of *C. militaris* showed the lowest IC_{50} ($p < 0.05$) value against 3T3 fibroblast. Similarly, curcumin although was reported beneficial in neuroprotection [39], the IC_{50} value detected against N2a was the lowest by means of MTT, NRU and LDH assays. Elsewhere, *in vivo* toxicity evaluation of *Ganoderma boninense* (Pat.) was carried out [40] and a significant toxicity (IC_{50} = 640 μg/ml) against *Artemia salina* (brine shrimp) was demonstrated after 24 h. However, *Ganoderma* extract is granted safe on short-term exposure. Conversely, *in vivo* toxicity profiling of total triterpene fraction from *G. lucidum* against Swiss albino mice showed that ganoderma triterpenes did not possess significant toxicity [41] and administration of *G. lucidum* β−glucan (2000 mg/kg body weight/day) to Sprague Dawley rats did not cause toxicological abnormality [42]. Mutagenicity studies by means of *Salmonella typhimurium* also did not reveal any genotoxicity. Meanwhile, sub-acute toxicity study of the sclerotial powder of *L. rhinocerotis* by using rat model showed no treatment-related toxicity at 1000 mg/kg [43]. Taken together, beneficial mushroom extracts hardly exert any significant toxicity.

We have chosen more than one cytotoxicity assay namely MTT, NRU and LDH release assay to determine *in vitro* cell viability in order to increase the reliability of the results obtained and also to avoid over- or underestimation of the mushroom or plant toxicity. The mechanisms of the chosen assays are different. While MTT is based on the enzymatic conversion of MTT in the mitochondria, NRU assay is based on the dye uptake capability by lysosomes [44]. Both served as colorimetric assays, whereby viable and uninjured cells stain blue and red, for MTT and NRU assays, respectively. LDH release assay, on the other hand is based on the release of the enzyme into the culture medium after the disruption of cell membrane [45]. It is noteworthy that the toxicity profiles detected by the three different assays generally followed a similar trend although some results were not in agreement. For instance, no IC_{50} values were recorded for some mushroom extracts by using LDH assays, suggesting that LDH may be the least sensitive method among the three.

Conclusions

The extracts of *G. lucidum*, *L. rhinocerotis*, *P. giganteus*, *G. frondosa* and *C. militaris* showed potential in promoting neurite outgrowth of differentiating N2a cells. The synergism of the various active entities in these mushroom extracts may be responsible for the neurite outgrowth activity and further experiments are warranted to isolate and identify the compounds. The signaling pathways involved is yet to be elucidated but based on our previous results, among other possibilities, phosphorylation and activation of the ERK and Akt may be involved. This study also showed the absence of embryotoxic and neurototoxic effects of the various mushroom extracts in 3T3 and N2a cells, respectively.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

CWP carried out the study, performed the data collection, data management, statistical analysis, data interpretation, and manuscript writing. PD and VS conceived the study, participated in its design and coordination. MN participated in the design of the study. KHW took part in data interpretation. MN and VS contributed to conception of the design and execution of the study. All authors read and approved the final manuscript.

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RESEARCH ARTICLE Open Access

Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde: Nutritional value and *in vitro* neurite outgrowth activity in rat pheochromocytoma cells

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Abstract

Background: Drugs dedicated to alleviate neurodegenerative diseases like Parkinson's and Alzheimer's have always been associated with debilitating side effects. Medicinal mushrooms which harness neuropharmacological compounds offer a potential possibility for protection against such diseases. *Pleurotus giganteus* (formerly known as *Panus giganteus*) has been consumed by the indigenous people in Peninsular Malaysia for many years. Domestication of this wild mushroom is gaining popularity but to our knowledge, medicinal properties reported for this culinary mushroom are minimal.

Methods: The fruiting bodies *P. giganteus* were analysed for its nutritional values. Cytotoxicity of the mushroom's aqueous and ethanolic extracts towards PC12, a rat pheochromocytoma cell line was assessed by using 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Neurite outgrowth stimulation assay was carried out with nerve growth factor (NGF) as control. To elucidate signaling mechanisms involved by mushroom extract-induced neurite outgrowth, treatment of specific inhibitor for MEK/ERK and PI3K signalling pathway was carried out.

Results: The fruiting bodies of *P. giganteus* were found to have high carbohydrate, dietary fibre, potassium, phenolic compounds and triterpenoids. Both aqueous and ethanolic extracts induced neurite outgrowth of PC12 cells in a dose- and time-dependant manner with no detectable cytotoxic effect. At day 3, 25 μg/ml of aqueous extract and 15 μg/ml of ethanolic extract showed the highest percentage of neurite-bearing cells, i.e. 31.7 ± 1.1% and 33.3 ± 0.9%; respectively. Inhibition treatment results suggested that MEK/ERK and PI3K/Akt are responsible for neurite outgrowth of PC12 cells stimulated by *P. giganteus* extract. The high potassium content (1345.7 mg/100 g) may be responsible for promoting neurite extension, too.

Conclusions: *P. giganteus* contains bioactive compounds that mimic NGF and are responsible for neurite stimulation. Hence, this mushroom may be developed as a nutraceutical for the mitigation of neurodegenerative diseases.

Keywords: *Pleurotus giganteus*, Medicinal mushroom, Edible mushroom, Neurite outgrowth, Neurodegenerative disease, MEK/ERK signalling pathway, PI3K/Akt signalling pathway

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Background

Neurodegenerative diseases are on the rise. The most common form of neurodegenerative disease is Alzheimer's disease, which causes thinking and memory to become seriously impaired due to neuronal loss in brain [1]. The second most common neurodegenerative syndrome, Parkinson's disease is characterised by the classic symptoms of tremors, rigidity and gait impairment [2]. Medications to alleviate these neurodegenerative diseases can only provide benefits for several years but are not effective as the diseases progress [3]. Some undesired side effects associated with these drugs include hallucinations, dyskinesia, nausea and constipation [2,3]. In this regard, complementary and alternative medicine which is now gaining momentum may be a promising way for prevention and protection against such neurodegenerative diseases [4].

Mushrooms are largely consumed not only in Asian countries but across Western countries. Mushrooms are of considerable interest because of their organoleptic merit, medicinal properties and economic significance. We have documented the effects of an edible mushroom *Hericium erinaceus* (Bull.:Fr.) Pers. (also known as monkey's head, lion's mane, and yamabushitake) on neurite outgrowth and peripheral nerve regeneration both *in vitro* and *in vivo* [5-7]. More recently, the aqueous extracts of *Lignosus rhinoceros* (Cooke) Ryvarden (tiger's milk mushroom) was reported to induce neurite outgrowth with or without the trigger of nerve growth factor (NGF) [8]. The cocktail of bioactive compounds present in these mushroom extracts exhibits NGF-like properties and play important roles in the growth, differentiation and survival of neuronal cells [9-11].

Formerly known as *Panus giganteus* (Berk) Corner, *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde is a culinary mushroom that is gaining popularity for its organoleptic properties and commercial prospects. In fact, consumption of this used-to-be wild mushroom has long been a tradition in the indigenous villages in Peninsular Malaysia [12]. A variety of *P. giganteus* from China is now being cultivated in Malaysia and the common commercial name in Malay language for *P. giganteus* is "*Seri Pagi*" (morning glory). In China, *P. giganteus* is widely referred as "Zhudugu" (swine's stomach) [13]. It is noteworthy to mention that the "*Panus-Pleurotus-Lentinus*" complex has long existed and has resulted in the confusion of nomenclature and taxonomy of these three species. Briefly, *Panus giganteus* (Polyporaceae, Polyporales) is characterised by its unbranched skeletal hyphae that usually grow on buried woody substrates [14]. While Pegler [15] has merged *Panus* as a subgenus within *Lentinus*, Corner [14], has grouped the genus *Panus* to species with skeletal hyphae and separated those species with ligative hyphae in *Lentinus*. Hence, *Lentinus giganteus* is regarded as synonym for *Panus giganteus* and *Lentinus giganteus* should be used if recommendation of Pegler is ever adopted [13,16]. However, Karunarathna and colleagues have revisited this issue and concluded that *Panus*/*Lentinus giganteus* should be unified and positioned in *Pleurotus* as supported by molecular evidences [17].

Cell cultures derived from nervous system tissue have proven to be powerful tools for elucidating cellular mechanisms of nervous system function [18]. The effect of chemicals, drugs, natural products or even growth factors on neurite outgrowth can be quantified by enumerating the number of cells that bear neurites using *in vitro* cell line model [19]. Neurite refers collectively to "axons and dendrites extended by primary cells growing in culture, or processes extended by neuronal cell lines, which are neither definitive axons, nor dendrites" [19,20]. Pheochromocytoma (PC12) cells, originated from a rat adrenal medullary tumour (pheochromocytoma) have been widely employed as a model of neuronal differentiation and neurite outgrowth [21]. PC12 cells respond to NGF and when triggered, cease proliferation, extend neurites, and become electrically excitable [22].

There is, however, minimal information on the medicinal properties of *P. giganteus*. The aqueous and ethanolic extracts of *P. giganteus* have shown antioxidant, genoprotection (unpublished data) and liver protection properties [23]. To our knowledge, there are no reports on the nutritional composition of *P. giganteus* and its benefits on neurite outgrowth stimulation, if any. In the present study, aqueous and ethanolic extracts of *P. giganteus* fruiting bodies were investigated for their effects in neurite outgrowth of rat pheochromocytoma (PC12) cells. Prior to this, the cytotoxicity of the extracts was determined by using [3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide] (MTT) assay. The hypothesis that MEK/ERK and PI3K/Akt are required for the neuronal differentiation and neurite outgrowth of PC12 cells was also tested using specific inhibitors.

Methods

Materials and chemicals

The fruiting bodies of *P. giganteus* were obtained from Nas Agro Farm, Sepang, Selangor, Malaysia. Rat pheochromocytoma (PC-12) cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA; Catalogue Number: CRL-1721.1TM). [3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide] (MTT), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), F-12 K medium (Kaighn's Modification of Ham's F-12 Medium), NGF-7 S from murine submaxillary gland, MEK inhibitor (U0126, PD98059), and PI3K inhibitor (LY294002) were obtained from Sigma Co. (St.

Louis, MO, USA). Fetal bovine serum (FBS) and horse serum (HS) were purchased from PAA Laboratories (Cölbe, Germany).

Cultivation condition of mushrooms

Pleurotus giganteus (KUM61102) was maintained on potato dextrose agar (PDA) at 4 - 10 °C and regularly subcultured. The substrate formulation for the cultivation of *P. giganteus* is similar to that for oyster mushroom cultivation, i.e. 89 - 94% (w/w) rubber wood sawdust, 5 - 10% (w/w) rice bran and 1% (w/w) calcium carbonate. Polypropylene bags are used for substrate bagging and the moisture content in the substrate was kept at 60% - 65%. The temperature for mycelia growth, spawn run, and fruiting body formation is 26 - 32 °C. Relative humidity of 70% and 80 - 90% during mycelia growth and fruiting; respectively, should be maintained. Direct illumination should be avoided as it has been reported to inhibit the fruiting body formation. A 20-day cycle after complete colonization of the artificial log is needed for each harvest and about four harvests (a total yield of 280 g) can be obtained from each bag of 900 g (Nas Agro Farm, personal communication).

Cell culture

The PC12 cells (adherent variant, PC-12Adh) from ATCC were maintained in F-12 K medium (Sigma) supplemented with 2.5% (v/v) heat-inactivated fetal bovine serum (PAA) and 15% (v/v) horse serum (PAA) with final pH 6.8 - 7.2. All incubations were performed at 37 °C in a humidified environment of 5% CO_2 and 95% air. The cells were maintained in the logarithmic phase of growth and were subcultured at 2–3 day intervals. For storage, the cells were frozen at −70 °C liquid nitrogen in complete medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO) (Sigma) as a cryoprotectant.

Extraction of *P. giganteus* fruiting bodies

The fresh fruiting bodies were sliced, weighed and freeze-dried (Christ, Germany) for 1–2 days. The freeze-dried fruiting bodies were then ground using a blender. The resulting dried powder was weighed and kept in 4 - 8 °C. Aqueous extraction method was according to Eik et al. [8]. Briefly, the freeze dried powder was soaked in distilled water (1:20 ratio, w/v) and was left overnight at room temperature and 200 rpm in a shaker. The mixture was double boiled in water bath for 30 min and filtered (Whatman Grade 4) after cooling. The resulting aqueous extract was freeze-dried and kept at −40 °C prior to use. For ethanol extraction, the freeze dried powder was soaked in 95% ethanol at room temperature for three days and the process was repeated three times. The ethanol solvent was evaporated using a rotary evaporator (Eyela N-1000, USA) to give a brownish viscous extract.

Nutritional composition of freeze dried fruiting bodies of *P. giganteus*

Fifty grams sample of *P. giganteus* fruiting bodies was sent to Consolidated Laboratory (M) Sdn. Bhd. for nutritional analysis.

Cell viability and cytotoxicity assay

Cell viability and proliferation was determined by MTT assay [24]. Approximately 12,000 cells per well were seeded on a 96-well plate and incubated at 37 °C overnight in a humidified environment of 5% CO_2 and 95% air. Fresh medium were then replaced and the cells were exposed to 0 to 1000 μg/ml of aqueous or ethanolic extract of *P. giganteus* for 48 hours. Subsequently, 20 μl of sterilized MTT (5 mg/ml) in phosphate buffered saline (PBS) buffer (pH 7.4) was spiked into each well and incubated at 37 °C for 4 hours. The supernatant was then carefully removed, and 200 μl of dimethyl sulfoxide (DMSO) was added into each well to dissolve the MTT formazan (blue colour) at the bottom of the wells. After 15 min, the absorbance at 540 nm with 690 nm as background absorbance was measured with an ELISA microplate reader (Sunrise, Tecan, Austria). The complete growth medium was the blank, and cells incubated in medium only without mushroom extracts were denoted as positive control.

Neurite outgrowth stimulation activity

Neurite outgrowth stimulation assay was according to Eik et al. [8] with some modifications. The cells were seeded in a 6-well plate at an initial density of 5,000 cells per well in 2 ml complete growth medium with different concentrations of aqueous and ethanolic mushroom extracts. For freeze dried aqueous extract, a stock solution of 10 mg/ml was prepared freshly each time prior to assay. The stock solution was then diluted five times in sterile distilled water to final concentrations ranging from 5–100 μg/ml (w/v). For ethanolic extract, 10 mg/ml of stock solution in DMSO was prepared freshly. The solution was also diluted five times with sterile distilled water. In positive control experiments, cells were induced to differentiate by the addition of 50 ng/ml (w/v) NGF extracted from murine submaxillary gland (Sigma). Cells in complete growth medium only served as a negative control. All the cells were incubated for five days at 37 °C, 95% air and 5% CO_2 to observe any neuronal differentiation activity.

Quantification of neurite bearing cells

A cell was scored positive if it bears a thin neurite extension that was double or more the length of the cell body

diameter [20]. Ten fields per well were randomly examined under an inverted microscope (Nikon Eclipse TS100). The cells were photographed using a Nikon DS-Fi1 camera and processed with a Nikon's Imaging Software, NIS-Elements D. The percentage of neurite-bearing cells were quantified by scoring the number of neurite-bearing cells over the total number of viable cells in 10 microscopic fields with average of randomly chosen 200 to 300 cells per well.

Treatment with specific inhibitors of signaling pathways

Stock solution (10 mM) of MEK inhibitor (U0126, PD98059) and PI3K inhibitor (LY294002) were prepared in DMSO and stored in −20 °C in the dark. Each inhibitor i.e. 10 μM for U0126 [25], 10–50 μM of LY294002 [26]; and 40 μM for PD98059 [27] was then prepared by diluting in medium just before use. PC12 cells were either incubated with or without the treatment of inhibitors for 1 hour. All the cells were then stimulated with 25 μg/ml of *P. giganteus* aqueous extract for three days prior to scoring neurite bearing cells.

Statistical analysis

Results were expressed as the means ± standard deviation (SD). Data comparison between groups was performed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be significant between groups by using Duncan's multiple range tests (DMRT).

Results

Nutritional composition of freeze dried fruiting bodies of *P. giganteus*

The nutritional components of *P. giganteus* fruiting bodies are shown in Table 1. *Pleurotus giganteus* contains 67.2 g/100 g of carbohydrate, 15.4 g/100 g of protein and 33.3 g/100 g of dietary fibre. It is rich in minerals like magnesium (67.64 mg/100 g) and potassium (1345.7 mg/100 g).

The effects of aqueous and ethanolic extracts of *P. giganteus* on PC12 cell viability

MTT assay was performed to determine the degree of cytotoxicity of *P. giganteus* extracts in PC12 cell. The cell viability and cell proliferation was denoted as 100% for the positive control i.e. cells in complete growth medium without mushroom extracts. It was shown that the growth of PC12 cell decreased with the increasing concentrations of the mushroom extracts. Figure 1a and the negative region of Figure 1b and 1c indicates that treatment with 10–200 μg/ml of aqueous extract and 10 μg/ml of ethanolic extract induced cell proliferation significantly ($p < 0.05$) as compared to control after a 48 h incubation. Upon challenge with a threshold dosage (500 μg/ml for aqueous extract and 200 μg/ml for ethanolic extract), the number of viable cells decreased significantly ($p < 0.05$) to 13.9% and 37.1%, respectively. At a concentration of 1000 μg/ml, the different extracts inhibited the cell proliferation to 75.65 ± 5.8% for aqueous extract, and 85.67 ± 5.3 for ethanolic extract. The IC_{50} which is the concentration at which 50% of cell growth inhibition occurs for aqueous extract and ethanolic extract were 806.39 ± 48 μg/ml and 309.46 ± 46 μg/ml, respectively. Hence, ethanolic extract is more toxic compared to aqueous extract, as the IC_{50} of ethanolic extract was 2.6-fold higher than that of aqueous extract.

The effects of aqueous and ethanolic extracts of *P. giganteus* on neurite outgrowth of PC12 cells

All concentrations of mushroom extracts tested were non-cytotoxic to the cells, as determined by MTT assay. Aqueous extract of *P. giganteus* induced neurite outgrowth of PC12 cells in both a time- and dose-dependent manner (Figure 2a). On the second day, the percentage of neurite-bearing cells increased significantly ($p < 0.05$) to 18.8% after treatment with 25 μg/ml of aqueous extract when compared to time-matched negative control (9.5%). After stimulation with aqueous

Table 1 The breakdown of nutritional content of *Pleurotus giganteus* freeze-dried fruiting bodies

Test Parameter	Result *	Recommended daily allowance (RDA)
Total Fat	3.7	65 g
• Saturated fat	0.97	-
• Monosaturated fat	1.97	-
• Polyunsaturated fat	0.77	-
• Trans fat	N.D (<0.01 g/100 g)	-
Energy in Kilo Calorie	364 kcal/100 g	-
Protein	15.4	50 g
Cholesterol	N.D (<0.001 mg/100 g)	300 mg
Carbohydrate	67.2	300 g
Dietary fibre	33.3	25 g
Sodium (as Na)	5.7	2400 mg
Calcium (as Ca)	5.78	1000 mg
Magnesium (as Mg)	67.64	0.4 g
Iron (as Fe)	1.85	18 mg
Zinc (as Zn)	2.68	15 mg
Phosphorus (as P)	526.45	700 mg
Potassium (as K)	1345.7	3500 mg
Copper (as Cu)	0.59	2.0 mg
Manganese (as Mn)	0.41	2.0 mg
Selenium (as Se)	N.D (<0.02 mg/kg)	70 μg

* 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); ND: Not detectable.

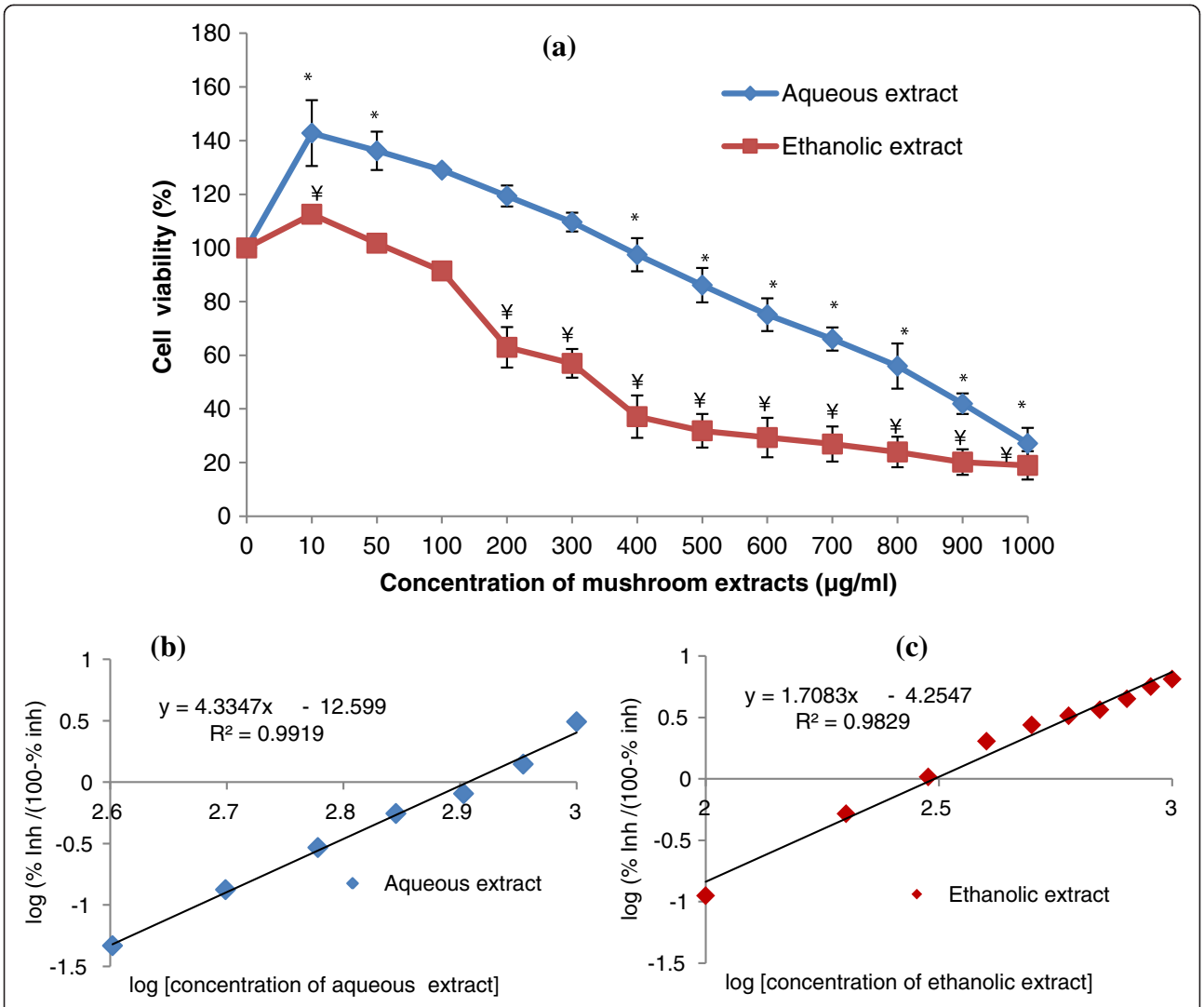

Figure 1 The effects of aqueous and ethanolic extracts of *P. giganteus* on PC12 cell viability. (**a**) Effect of aqueous extract and ethanolic extract on the cell proliferation of PC12 cells. The mean absorbance obtained using medium with cells only was designated 100%. Results shown represent the mean ± SD; $n = 3$. $^{*,¥}$ $p < 0.05$ for aqueous extract and ethanolic extract, respectively compared to the control 100%. (**b**) & (**c**): IC_{50} was obtained from the intercept on the x-axis (y = 0) of the regression line using the linear part of the percentage inhibition (% inh) curve (data not shown).

extract, the percentage of neurite-bearing cells significantly increased ($p < 0.05$) until the effect reached a plateau after day 3. Therefore, day 3 was selected for further studies as the neurite scoring for all concentrations were the highest. Similarly, ethanolic extract induced neurite outgrowth of PC12 cells in a time- and dose-dependent manner and the number of neurite-bearing cells remained constant after day 3, as shown in Figure 2(b).

Figure 2c and 2d give the percentage of neurite-bearing cells for aqueous extract and ethanolic extract, respectively, on day 3. As shown in Figure 2c, aqueous extract at 25 µg/ml had a significant ($p < 0.05$) effect (31.7 ± 1.1%) in stimulating neuronal differentiation compared to NGF (28.3 ± 0.4%). On day 3, 15 µg/ml of ethanolic extract induced 33.3 ± 0.9% of neurite-bearing cells (Figure 2d). There was no significant difference ($p > 0.05$) in the percentage of neurite-bearing cells at 25 µg/ml of aqueous extract and 15 µg/ml of ethanolic extract. However, both the extracts performed better than NGF ($p < 0.05$). It was obvious for ethanolic extract, that 50 µg/ml, 75 µg/ml and 100 µg/ml did not significantly ($p > 0.05$) trigger neuronal differentiation and neurite outgrowth of PC12 as compared to aqueous extract for the same concentrations. Figure 3 shows the morphology of PC12 cells with neurites at day-3 of treatment with 50 ng/ml NGF (a), 25 µg/ml of aqueous extract (b), and neither of them (c).

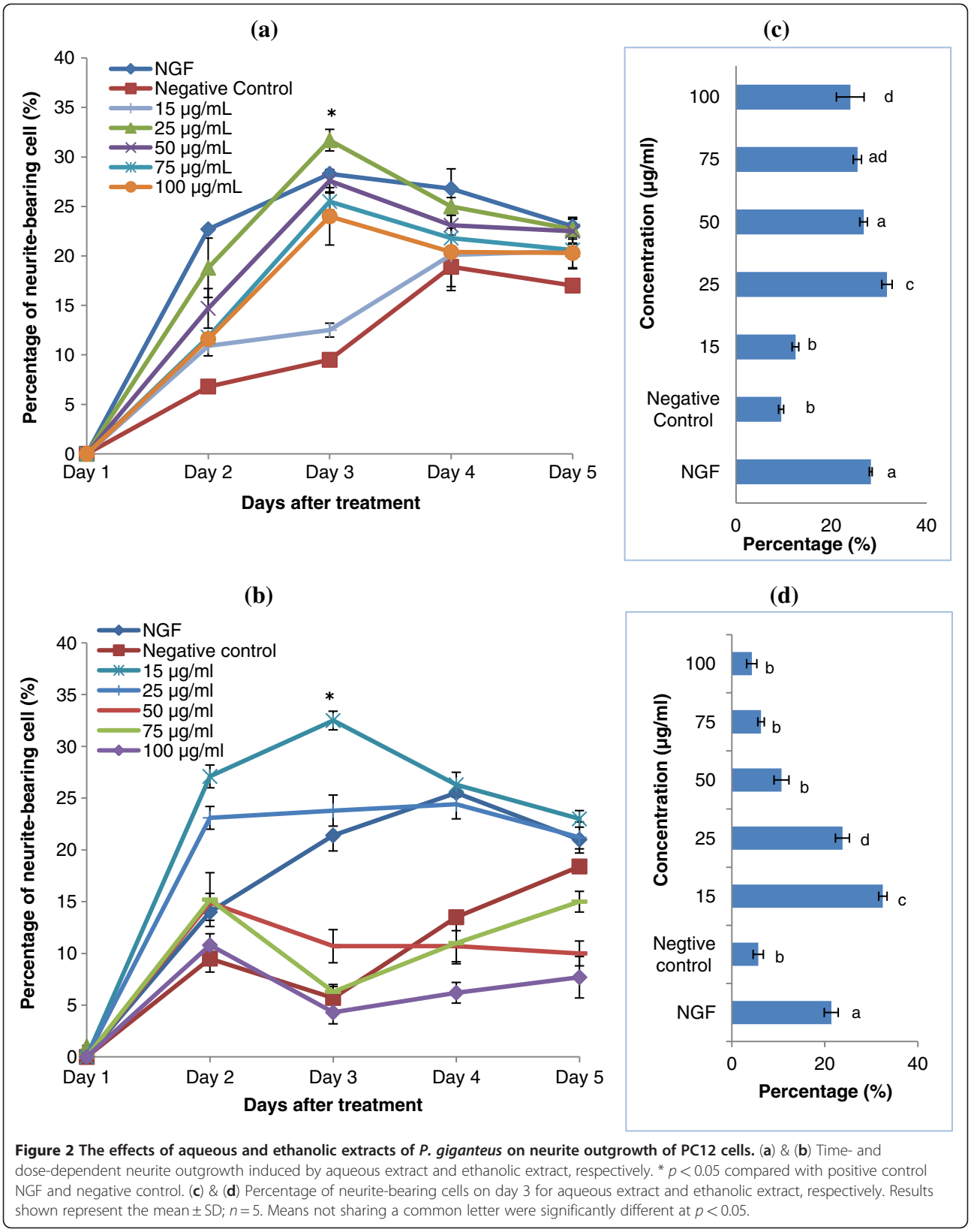

Figure 2 The effects of aqueous and ethanolic extracts of *P. giganteus* on neurite outgrowth of PC12 cells. (**a**) & (**b**) Time- and dose-dependent neurite outgrowth induced by aqueous extract and ethanolic extract, respectively. * $p < 0.05$ compared with positive control NGF and negative control. (**c**) & (**d**) Percentage of neurite-bearing cells on day 3 for aqueous extract and ethanolic extract, respectively. Results shown represent the mean ± SD; $n = 5$. Means not sharing a common letter were significantly different at $p < 0.05$.

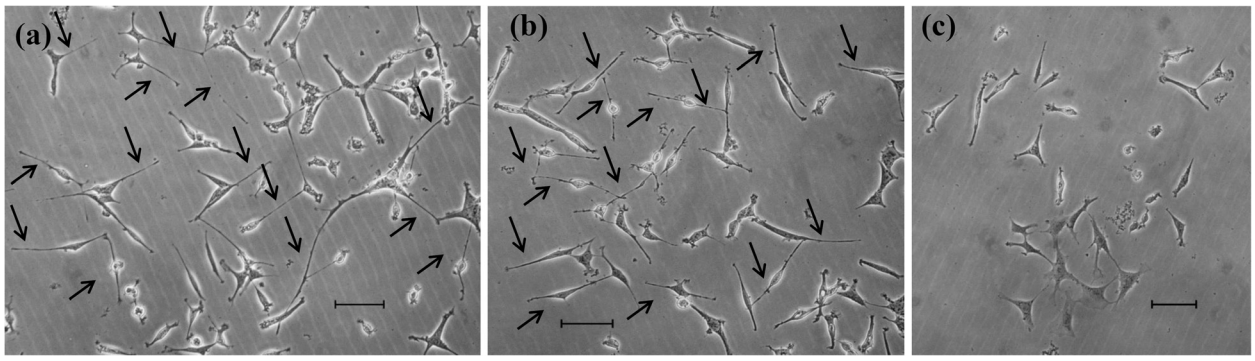

Figure 3 Phase-contrast photographs of PC12 neurites at day 3. (**a**) Treatment with 50 ng/ml NGF (**b**) Treatment with 25 μg/ml of aqueous extract, and (**c**) Negative control, treatment of nither of (**a**) and (**b**). Scale bar = 20 μm. Arrows indicate neurite extentions.

The mechanism of neurite outgrowth stimulation by the extracts of *P. giganteus*

It was shown that neurite outgrowth induced by NGF and aqueous extract of *P. giganteus* was markedly inhibited ($p < 0.05$) by MEK inhibitors U0126 and PD98059 (Figure 4a and 4b). In fact, in PC12 cell treated with aqueous extract combined with either 10 μM of U0126 or 40 μM of PD98059, the decrease in the number of neuritic processes was significant ($p < 0.05$). On the contrary, an inhibitor of PI3K/Akt pathway, LY294002, did not inhibit aqueous extract- and NGF-induced neurite outgrowth at the concentration of 10 μM and 20 μM ($p > 0.05$). LY294002 at the concentration of 30 μM started to cause inhibition effects on PC12 in a concentration-dependent manner. At 30 μM of LY294002, the number of elongated PC12 cells with neurites doubled the cell diameter decreased significantly, by 49.6% and 63.5%, for NGF- and aqueous extract-treated cells; respectively (Figure 4c). At 50 μM, all the cells pretreated with the inhibitor showed no difference ($p > 0.05$) to the negative controls, with differentiated cells bearing neurites ranging only from 3.2 – 5.3%. From this result, we proposed that aqueous extract induced neurite outgrowth on PC12 cells via the activation of ERK1/2 cascade and PI3K/AKt pathways.

Discussion

There is a vast amount of nutritional studies of wild and cultivated mushrooms across the world. However, relatively little data exist in the literature on the nutrient content of *Pleurotus giganteus*. Herein, it was intended to compare only the highly appreciated and most cultivated culinary-medicinal mushrooms, for example the *Pleurotus* genus and *Agaricus* genus. Generally, mushrooms have high (19 – 35%) protein contents [28]. In Brazil, it was reported that the fruiting bodies of *Pleurotus ostreatus* and *Pleurotus sajor-caju* presented protein content ranging from 13.1% to 18.4%, depending on the substrates used [29]. The present study showed that the protein level of *P. giganteus* is 5.3-time lower than that of *Agaricus bisporus* (white button mushroom) with reference to a study from Portugal [30]. On the other hand, the carbohydrate content in *P. giganteus* is 4-, 6-, 7.2-, 7.5-, 8-, 11-time higher than that of *Lentinula edodes*, shiitake (17.12 g/100 g), *Flammulina velutipes*, golden needle mushroom (10.57 g/100 g), *Pleurotus ostreatus,* oyster mushroom (9.30 g/100 g), *Pleurotus eryngii*, king oyster mushroom (8.95 g/100 g), *Agaricus bisporus* white button mushroom (8.25 g/100 g) (30), and *Agaricus bisporus* brown mushroom (5.98 g/100 g) [31]. This suggested that carbohydrates (glucose, mannitol, trehalose, oligosaccharide groups, and reserved polysaccharide like glycogen) account for the prevailing component of *P. giganteus* fruiting body. Reports related to the nutritional evaluation of *Pleurotus* genus carried out by other researchers from different regions (Japan, India, Bangladesh, Turkey, Finland, and Italy) can be retrieved from [32–37], respectively. Nevertheless, the differences between the nutrient values may be attributed to the type of mushroom, strain of mushroom, environmental factors, and composition of growth media [37].

MTT assay is by far the most convenient colorimetric assay based on the metabolic activity of a viable cell [24,38]. Basically, only viable cell has the mitochondrial dehydrogenase system that can cleave the yellow MTT tetrazolium salt and yield MTT formazan which is blue in colour. Thus, the optical density of the amount of solubilised MTT formazan is quantitatively correlated to the percentage of cell viability. The present study showed that cytotoxic effect of *P. giganteus* aqueous and ethanolic extracts towards PC12 cells were concentration dependant. This is consistent with the finding by Cheung et al. [39] whereby viability of PC12 cells was dose-dependently decreased by increasing *Ganoderma lucidum* extracts.

On-going studies show that the aqueous extract of *P. giganteus* contains bioactive secondary metabolites like

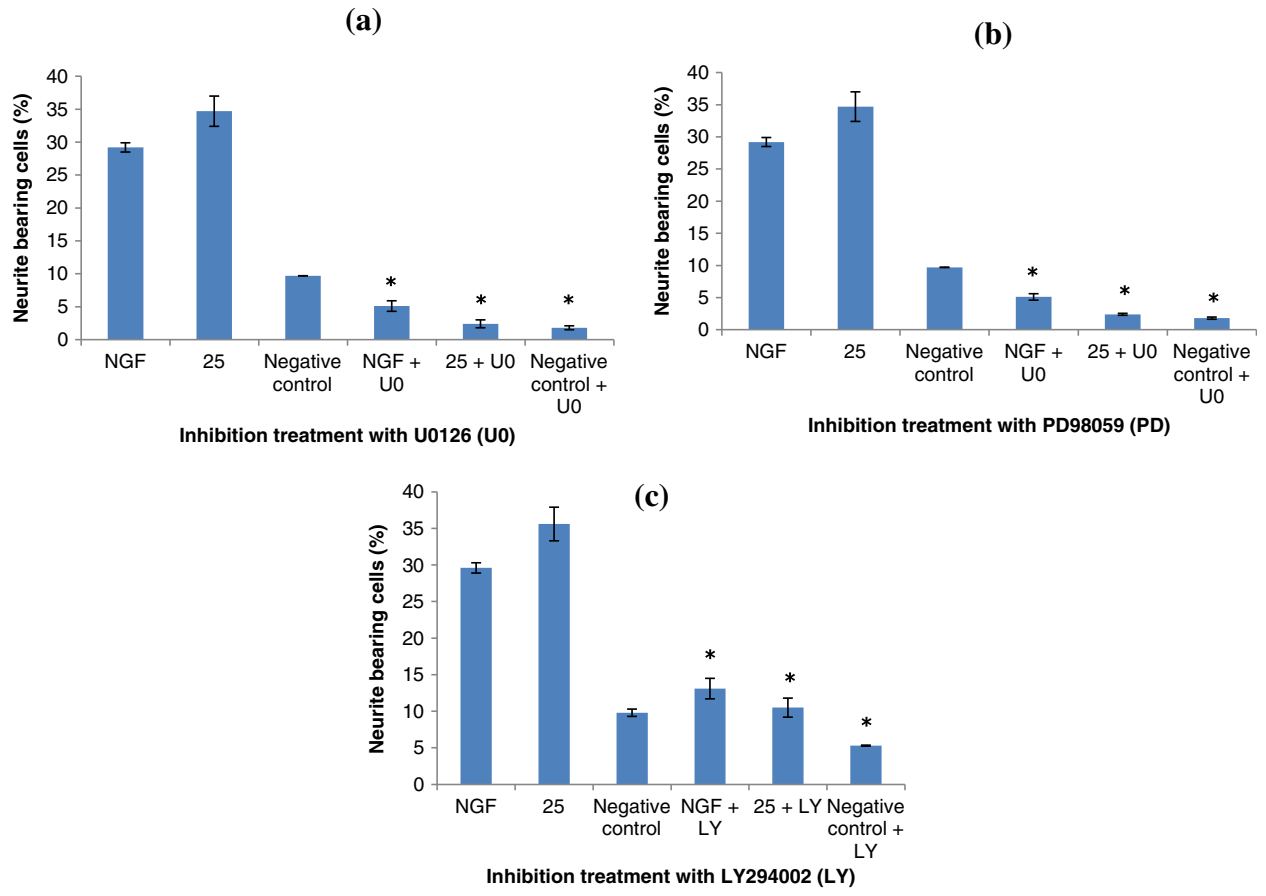

Figure 4 Comparison of the percentage of neurite-bearing cells with different specific inhibitors treatment. (**a**) U0126 (10 μM), (**b**) PD98059 (40 μM), and (**c**) LY294002 (30 μM). NGF, 25 μg/ml of aqueous extract (denoted simply by 25), and negative control were the control groups i.e. without the treatment with inhibitors. Results shown represent the mean ± SD; $n = 3$. * Significant difference at $p < 0.05$ versus control group.

sterols and triterpenes (unpublished data). These compounds are reported to have neutrophic NGF-like properties and caused neurite outgrowth activity in PC12 cells [40]. We have shown for the first time that *P. giganteus* extract can stimulate neurite outgrowth by using PC12 cell line model. It was shown that 25 μg/ml of aqueous extract and 15 μg/ml of ethanolic extract induced the highest percentage of neurite outgrowth in PC12 cells at day 3. The number of neurite bearing cells was significantly higher than that of NGF. The mushroom extracts may contain bioactive compounds either mimic NGF or trigger the production of NGF, hence resulting in neurite outgrowth. Further, the potassium level in *P. giganteus* was 1345.7 mg/100 g and according to Kalac [41], potassium level in fruiting bodies is between 20- and 40-fold higher than in the substrates used for mushroom cultivation. In the study by Cohen-Cory et al. [42], the cell number of Purkinje cells, the major efferent neurons of the brain cerebellum increased by 40% when treated with potassium. Besides, potassium alone or potassium coupled with NGF markedly increased the cell survival, cell differentiation and neurite outgrowth. In this study, the potassium present in *P. giganteus* extracts may be involved in the regulation of the morphological differentiation of PC12 cells by acting as a depolarising agent.

The present study extends recent findings that some mushroom extracts can have neuritogenesis effects. Prior studies by our group have shown that 0.2% (v/v) aqueous extract of freeze dried fruiting bodies from *Hericium erinaceus* caused maximal stimulation of neurite outgrowth (17.3% of neurite bearing cells and 88.2% increase compared to control) in NG108-15 cell line after 24 hours of incubation [5]. Besides, freeze drying was found to be the best approach to preserve the bioactive compounds in mushroom as compared to oven-dried method [43]. It had been reported that PC12 cells responded well to water extract of sclerotium of *Lignosus rhinocerus* (Cooke) Ryvarden [8]. It was found that synergistic effect, i.e. 42.12% of neurite bearing PC12 cells was

elicited when the cells were treated with 20 μg/ml of water extract combined with 30 ng/ml of NGF. Some other medicinal mushrooms that induced neurite outgrowth included *Grifola frondosa* (Maitake) [10], *Tricholoma* sp [44], *Termitomyces albuminosus* [45,46], *Dictyophora indusiata* [47], *Tremella fuciformis* [48], and *Ganoderma lucidium* (Lingzhi) [39].

The involvement of the MAPK/ERKs signaling pathway in neuronal differentiation by mushroom extracts has been reported. Neuroprotective and neuritogenesis effect of *Ganoderma lucidium* extracts on PC12 was stipulated to be mediated via the MAPK/ERK signalling pathway [39]. Besides, lysophosphatidylethanolamine from *Grifola frondosa* induced activation of ERK1/2 of PC12 cells thus stimulated neurite outgrowth and inhibited serum withdrawal-induced apoptosis [10]. Neurotrophins like NGF are mostly mediated by the Trk family of receptor tyrosine kinase, TrKA. However, discrepancy did occur in the case of *Ganoderma lucidium* extracts, whereby there was no direct involvement of TrkA [39]. Similarly, *α*-Phenyl-*N*-*tert*-butylnitron was also found to induce neurite outgrowth in PC12 independent of TrkA [49]. It is thus predicted, based on the ability of *P. giganteus* extract to stimulate neurite outgrowth of PC12 without NGF, that activation of TrKA receptor tyrosine kinase may not be necessary. According to Sweatt [50], the mitogen-activated protein kinase (MAP kinase, MAPK) cascade is a superfamily of signalling cascade and is a vital regulator of cell division and differentiation. Recently, MAPK was specified as the extracellular signal-regulated kinase (ERK) comprising ERK 1 and 2, or as ERK1/2. It has been demonstrated that ERK-cascade was necessary and sufficient enough for NGF-induced neuronal differentiation of PC12 cells. In the present study, upon inhibition by MEK-selective inhibitor U0126 and PD98059, the percentage of neurite outgrowth decreased significantly. This suggested that ERK1/2 phosphorylation was affected and this indirectly implied that activation of ERK1/2 is necessary for *P. giganteus*-mediated neuritogenesis. Inhibition of PI3K/Akt signalling by LY294002 also negatively affected neurite outgrowth of PC12. This finding suggested that neurite outgrowth potentiated by *P. giganteus* in PC12 cells is also regulated by PI3K/Akt signaling pathway. However, it was noted that PI3K/Akt inhibitor did not markedly affect the activities of ERK [51], therefore neurite extension of PC12 still could be observed at lower concentrations of LY294002. According to Naidu et al. [52], phospho-Akt and phospho-MAPK were expressed during neurodevelopment and nerve regeneration following sciatic nerve crush on rats. Collectively, these results demonstrated that *P. giganteus*-induced neurite extension is regulated at least by part between MEK/ERK and PI3K/Akt pathways. For the future work, confirmation by immunoblot analysis to detect the phosphorylation of TrKA, ERK, and Akt, is necessary.

Conclusions

To our knowledge, this is the first evidence on the effects of *Pleurotus giganteus* aqueous and ethanol extracts on neuronal differentiation and neurite outgrowth. The high potassium level in the fruiting bodies and the presence of bioactive compounds (mainly triterpenoids) could be responsible for the neuroactivity. Work is in progress to determine and identity the bioactive compound/s responsible for the activity. Our results suggested that neurite outgrowth stimulated by *P. giganteus* is mediated via the "cross-talk" between MEK/ERKs and PI3K/Akt pathways. However, further immunoblot analysis is required.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

CWP carried out the experiment, drafted the manuscript, and engaged in data acquisition and data interpretation. WLW carried out ethanol extraction, and preparation of samples for nutritional analysis. PD participated in the acquisition of funding and editing for manuscript. MN involved in the design of the study and manuscript editing. VS provided the grant, involved in coordinating and monitoring of research; and manuscript editing. All authors read and approved the final manuscript.

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Research Article

Intrastrain Comparison of the Chemical Composition and Antioxidant Activity of an Edible Mushroom, *Pleurotus giganteus*, and Its Potent Neuritogenic Properties

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Two strains of *Pleurotus giganteus* (commercial and wild) were tested for their ability to induce neurite outgrowth in rat pheochromocytoma (PC12) and mouse neuroblastoma-2a (N2a) cells. Treatment with the mushroom extracts resulted in neuronal differentiation and neuronal elongation, but not nerve growth factor (NGF) production. Linoleic acid (4.5–5.0%, w/w) which is a major fatty acid present in the ethanol extract promoted NGF biosynthesis when augmented with low concentration of NGF (5 ng/mL). The two strains of mushroom were found to be high in protein (154–192 g kg^{-1}), total polysaccharides, phenolics, and flavonoids as well as vitamins B1, B2, and B3. The total phenolics present in the mushroom extracts were positively correlated to the antioxidant activity (free radical scavenging, ferric reducing power, and lipid peroxidation inhibition). To conclude, *P. giganteus* could potentially be used in well-balanced diet and as a source of dietary antioxidant to promote neuronal health.

1. Introduction

Neurite outgrowth is a critical process in neuronal formation and development. Malfunction of this event will lead to demolition of synaptic connections and extended series of neuronal dysfunctions like Alzheimer's disease (AD) [1]. As AD progresses, programmed apoptotic neuronal death is triggered as a result of nerve growth factors (NGFs) depletion and oxidative stress exerted by reactive oxygen species (ROS) [2]. Therefore, strategies aimed at preserving and restoring the neurite network might be beneficial in treating AD. Dietary intake of antioxidants is also important to improve the intrinsic antioxidant mechanisms (superoxide dismutase, catalase, and glutathione peroxide) to avoid an environment where prooxidant species overwhelm antioxidant species.

Functional food plays a significant role in preventing or reducing severity of lifestyle diseases and improving physical and mental well-being of consumers. Edible and medicinal mushrooms are gaining recognition as preventative agents for age-related diseases including neurodegenerative diseases such as AD, Parkinson's disease, and dementia [3]. At the current stage, medications are prescribed for mild, moderate, to severe Alzheimer's disease to help delay or prevent behavioral symptoms, but that, too, is only for a limited time. Trials with NGF for Alzheimer's disease had gained some degree of success, but the high molecular weight of the NGF protein seems to suggest that it could not cross the blood-brain barrier [4]. Considering the limitation of the existing preventive methods, intervention strategy using mushrooms as functional food is of utmost importance. Edible and medicinal mushrooms are packed with a wide array of bioactive and nutritional components that could scavenge reactive oxygen species (ROS) and exert neuroprotective effects and promote neuritogenesis and neuroregeneration.

There are ample studies available in the literature regarding the chemical composition of different mushroom species from all over the world. However, such information on *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde, a popular edible mushroom in China, Thailand, and Sri Lanka is scanty. In Malaysia, the mushroom is also consumed as a delicacy by the indigenous communities. Studies on *P. giganteus* include domestication and cultivation [5], liver protection [6], and anti-*Candida* activities [7]. It has been shown that extracts of this mushroom exhibited neurite outgrowth activity in rat pheochromocytoma (PC12) and mouse neuroblastoma-2a (N2a) cells [8, 9]. In this study, the neuritogenic effects of the commercial strain of *P. giganteus* were compared to that of the wild domesticated strain. The nutritional components including vitamins, fatty acids, and amino acids as well as the phenolic, flavonoid contents, and antioxidant activities were also evaluated and compared.

2. Materials and Methods

2.1. Mushroom Species. Fresh basidiocarps of *P. giganteus* (commercial strain KLU-M 1227) were collected from Nas Agro Farm and Dong Foong Biotech. Wild *P. giganteus* (KLU-M 1228) was collected from Ayer Hitam Forest Reserve, Puchong, Malaysia. The mushroom identity (molecular fingerprinting) was authenticated by Dr. Yee-Shin Tan from Mushroom Research Centre, University of Malaya. Voucher specimens were deposited in the Herbarium of University of Malaya. Domestication of the wild strain was carried out as previously reported [8]. The substrate formulation for basidiocarp formation of both commercial and domesticated strains was similar and consisted of rubber wood sawdust, rice bran, and calcium carbonate.

2.2. Chemicals. Gallic acid, rutin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], phosphate buffered saline (PBS), nerve growth factor (NGF), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.3. Proximate Analysis of Basidiocarps. The freeze-dried powder of basidiocarps of *P. giganteus* was analyzed for the nutritional components using the standard American Oil Chemists' Society (AOCS) procedures [10]. Total fat, dietary fiber, and protein content were analyzed using AOAC 989.05, AOAC 985.25, and Kjeldahl method with boric acid modification, respectively. Carbohydrates were calculated using the formula carbohydrates (g) = 100 − (protein + fat + ash). Energy was calculated using the formula energy (kcal) = 4 × (protein + carbohydrate) + 9 × (fat) [10].

2.4. Determination of Free Sugars, Minerals, Vitamins, Fatty Acids, and Amino Acids. Free sugars and amino acids were analyzed by high performance liquid chromatography (HPLC). Minerals were determined using inductively coupled plasma optical emission spectrometry ICP-OES following the AOAC 985.01 and 922.02, as well as American Association of Cereal Chemists (AACC 40–70) procedures. Vitamin C was determined by the AOAC 967.21 method. Preparation of methyl esters of long-chain fatty acids was carried out based on AOCS Ce-2-66 test for further analysis by gas chromatography (GC). Omega-3 and Omega-6 fatty acids were analyzed using AOCS 1d-91 methods by capillary gas-liquid chromatography (GLC).

2.5. Preparation of Mushroom Extracts. Crude aqueous and ethanol extracts were prepared as previously described [8]. For aqueous extract, the freeze-dried basidiocarps powder was soaked in distilled water (1 : 20, w/v) for 24 h at room temperature and 200 rpm in a shaker. After double-boiling in a water bath at 100°C, the mixture was filtered (Whatman No. 4). The filtrate was then freeze-dried and kept at −20°C prior to use. To obtain crude ethanol extract, the freeze-dried powder was soaked in 95% (v/v) ethanol at room temperature for three days. The solvent was then evaporated using a rotary evaporator (Eyela N-1000, USA) and a brownish viscous extract was obtained.

2.6. Determination of Total Polysaccharides. The total polysaccharide content of the aqueous and ethanol extracts was determined using the phenol-sulphuric acid method with D-glucose as a reference [11]. Briefly, 1 mL of 5% phenol was added to 1 mL of sample solution, followed by 5 mL of concentrated H_2SO_4. The absorbance was measured after 10 min at 497 nm.

2.7. Determination of Total Phenolic Contents (TPC) in Mushroom Extracts. The total phenolic contents in the mushroom extracts, expressed as gallic acid equivalents (GAEs), were determined by the Folin-Ciocalteu method [12]. Fifty microliters of sample was mixed with an equal volume of Folin-Ciocalteu phenol reagent. After 3 min, 100 μL of Na_2CO_3 (10%) was added to the mixture. The reaction was kept in the dark for 90 min, after which the absorbance was read at 750 nm using a spectrophotometer. A calibration curve was prepared with different concentrations of gallic acid (0–100 μg/mL) as standard. TPC was expressed as mg GAE/g of extract.

2.8. Determination of Total Flavonoids in Mushroom Extracts. Total flavonoids in the mushroom extract were estimated by using the aluminum calorimetric method as previously described [13]. Mushroom extract of 150 μL was mixed with an equal volume of $AlCl_3$ (2%). After 10 min, the absorbance of the supernatant was measured at 435 nm by using an ELISA microplate reader (Sunrise, Austria). The total flavonoid content of the mushroom extract was expressed as rutin equivalents in microgram per gram extract (mg RE/g extract).

2.9. Evaluation of Antioxidant Activity of Mushroom Extracts

2.9.1. DPPH Scavenging Activity Assay. The DPPH free radical scavenging activity was determined as previously

described [14]. Mushroom extracts of various concentrations (5 μL) were mixed with 195 μL of DPPH reagent. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm using a microtiterplate reader (Tecan, Austria). The radical scavenging activity was calculated using the following equation: % radical scavenging activity = $[(Abs_{Blank} - Abs_{Sample})/Abs_{Blank} \times 100\%]$, where Abs_{Sample} is the absorbance of the sample whereas Abs_{Blank} is the absorbance of the DPPH solution. The antioxidant property of the extracts was expressed in terms of IC_{50} value, that is, the concentration to quench 50% of available DPPH content. L-ascorbic acid (0–25 μM) was used as standard and butylated hydroxytoluene (BHT) was used as control.

2.9.2. FRAP (Ferric Reducing Antioxidant Power) Assay. The reducing power of mushrooms extracts was determined by the ferric reducing antioxidant potential (FRAP) assay as previously described [15]. To prepare FRAP reagent, 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM in 40 mM HCl) was mixed with 2.5 mL of 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) (10 mmol/L in 300 mmol/L acetate buffer). Then, 10 μL of mushroom extract was added to 300 μL of the FRAP reagent after which the absorbance of the product of the reaction between Fe^{2+} and TPTZ was measured at 593 nm against a blank for each sample. Ferrous sulfate ($FeSO_4$) of concentrations 0–20 mM was used as standard and BHT as control. The FRAP value was expressed as μM of $FeSO_4$ equivalents/g mushroom.

2.9.3. Inhibition of Lipid Peroxidation. The assay was based on the thiobarbituric acid reaction method [16]. Mushroom extracts of different concentrations (0–20 mg/mL) were mixed with 0.5 mL of egg yolk suspension and 0.5 mL of $FeSO_4$. The mixture was incubated at 37°C for an hour after which 0.5 mL of 20% trichloroacetic acid (TCA) and 1 mL of 0.8% thiobarbituric acid (TBA) were added. The mixture was then heated in boiling water for 15 min and centrifuged at 3500 rpm for 20 min. The absorbance of thiobarbituric acid reactive substances (TBARS) present in the supernatant was measured at 532 nm using a microtiterplate reader (Tecan, Austria). Result was expressed as percentage inhibition of lipid peroxidation at extract concentration of 10 mg/mL. BHT was used as a control in this assay.

2.10. Effect of Mushroom Extracts on Neuritogenesis

2.10.1. Cell Culture. Mouse neuroblastoma (N2a, CCL-131) and rat pheochromocytoma cells (adherent variant, PC-12Adh) were purchased from American Type Culture Collection (ATCC; MD, USA). N2a cells were cultured in Eagle's minimum essential medium (MEM) with L-glutamine (PAA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (PAA), 100 U/mL penicillin, and 100 μg/mL streptomycin. PC12 cells were maintained in F-12K medium (Sigma) supplemented with 2.5% (v/v) heat-inactivated fetal bovine serum (PAA) and 15% (v/v) horse serum (PAA). All plates were incubated at 37°C in a humidified environment of 5% CO_2 and 95% air. The cells were routinely passaged every 2-3 days.

2.10.2. Neurite Outgrowth Assay. N2a and PC12 cells were seeded at a density of 5×10^3 cells in growth medium per well in 24-well culture plates and incubated overnight. Mushroom extracts (0–50 μg/mL) were added to the cells and further incubated for 3 days. For N2a cells, the cells were induced to differentiate by replacing the growth medium with 5% serum medium. Nerve growth factor (50 ng/mL) was used as a positive control. After 3 days, the cells were then examined using an inverted light microscope (Nikon Eclipse TS100). Five random fields (200–300 cells/well) were examined in each well. The number of axon-like processes, defined as extensions longer than twice the cell body diameter, was recorded. The mean number of neurite-bearing cells was quantified by scoring the total number of neurite-bearing cells over the total number of viable cells per field. At least three independent experiments were conducted.

2.10.3. Measurement of NGF. The NGF level in the PC12 culture medium was performed following the ChemiKine NGF sandwich enzyme-linked immunosorbent assay (ELISA) kit procedure (Merck Millipore, Germany). After treatment, the culture medium was added into a microplate precoated with anti-mouse NGF polyclonal antibody. Anti-mouse NGF monoclonal antibody was then added. After 2 h, horseradish peroxidase- (HRP-) conjugated donkey anti-mouse IgG polyclonal antibody was added to react with TMB substrate solution. The color intensity of the sample was measured at 450 nm. The level of NGF was determined from a standard curve plotted with known concentrations of NGF.

2.11. Statistical Analysis. Results were expressed as mean ± standard deviation (SD) ($n = 3$). Analysis of variance (ANOVA) followed by Duncan's test was performed to test for differences between means by employing Statgraphics Plus (Statistical Graphics Corp., Herndon, VA). Correlations between total polysaccharides, TPC, and antioxidant activities were determined by Pearson's correlation coefficient (r) with the statistical program SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). The statistical significance of mean differences was based on P value of <0.05.

3. Results

3.1. Proximate Analysis of Mushroom Basidiocarps. The proximate nutritional components of commercial *P. giganteus* (KLU-M 1227) and domesticated wild *P. giganteus* (KLU-M 1228) are shown in Table 1. The commercial strain presented a significantly higher ($P < 0.05$) carbohydrate, dietary fiber, total fat, and monosaturated fat content compared to the domesticated wild strain by 3.7, 2.7, 16, and 2.1%, respectively. Both strains showed no difference in the gross energy value and saturated fat contents. However, the wild strain presented a significantly higher ($P < 0.05$) crude protein and polyunsaturated fat content.

Table 1: Chemical compositions, sugars, macro- and microelements, and vitamins of commercial *Pleurotus giganteus* (KLU-M 1227) and wild *P. giganteus* (KLU-M 1228).

Parameter	*Pleurotus giganteus* (commercial strain)	*Pleurotus giganteus* (wild strain)
Carbohydrate ($g\,kg^{-1}$)	672 ± 0.0^{a}	647 ± 0.0^{b}
Protein ($g\,kg^{-1}$)	154 ± 0.0^{c}	192 ± 0.0^{d}
Energy ($kcal\,kg^{-1}$)	3640 ± 0.00^{e}	3640 ± 0.0^{e}
Dietary fiber ($g\,kg^{-1}$)	333.5 ± 0.07^{f}	324.5 ± 0.07^{g}
Total fat ($g\,kg^{-1}$)	37.0 ± 0.0^{h}	31.0 ± 0.0^{i}
Saturated fat	9.7 ± 0.0^{j}	9.5 ± 0.0^{j}
Monosaturated fat	19.7 ± 0.0^{k}	13.2 ± 0.0^{l}
Polyunsaturated fat	7.8 ± 0.0^{m}	8.3 ± 0.0^{n}
Trans fat	ND	ND
Cholesterol ($g\,kg^{-1}$)	ND	ND
Free sugars ($g\,kg^{-1}$)		
Fructose	7.2 ± 0.2^{a}	10.6 ± 0.4^{b}
Glucose	31.4 ± 0.7^{c}	39.7 ± 0.2^{d}
Sucrose	ND	ND
Lactose	ND	ND
Maltose	ND	ND
Maltotriose	ND	ND
Macroelements ($g\,kg^{-1}$)		
Potassium (as K)	13.46 ± 0.0^{a}	11.71 ± 0.32^{b}
Phosphorus (as P)	5.27 ± 0.39^{c}	4.01 ± 0.04^{d}
Magnesium (as Mg)	0.67 ± 0.00^{e}	0.65 ± 0.00^{f}
Calcium (as Ca)	0.058 ± 0.00^{g}	0.087 ± 0.01^{h}
Sodium (as Na)	0.058 ± 0.07^{g}	0.047 ± 0.00^{i}
Microelements ($g\,kg^{-1}$)		
Iron (as Fe)	0.019 ± 0.04^{a}	0.014 ± 0.00^{b}
Zinc (as Zn)	0.027 ± 0.01^{c}	0.042 ± 0.00^{d}
Manganese (as Mn)	0.041 ± 0.01^{e}	0.043 ± 0.00^{e}
Copper (as Cu) ($mg\,kg^{-1}$)	0.60 ± 0.01^{f}	0.228 ± 0.00^{f}
Selenium (as Se) ($mg\,kg^{-1}$)	ND	ND
Vitamins ($g\,kg^{-1}$)		
Vitamin B1	0.004 ± 0.01^{a}	0.002 ± 0.00^{b}
Vitamin B2	0.009 ± 0.00^{c}	0.009 ± 0.00^{c}
Vitamin B3	0.09 ± 0.10^{d}	0.06 ± 0.02^{e}
Vitamin C	0.003 ± 0.00^{a}	0.001 ± 0.01^{f}

Each value is expressed as mean ± SD ($n = 3$). In each row, the different letters represent significant differences between samples ($P < 0.05$). ND: not detectable.

3.2. Determination of Sugars, Minerals, and Vitamins. The sugar composition of *P. giganteus* basidiocarps is given in Table 1. Glucose and fructose were detected in the basidiocarps of both strains. The glucose and fructose content of the domesticated wild strain was approximately 47% and 26% higher ($P < 0.05$) than that of the commercial strain. For macroelements composition, potassium in the basidiocarps of commercial strain ($13.46 \pm 0.0\,g\,kg^{-1}$) was significantly higher ($P < 0.05$) than the domesticated wild strain ($11.71 \pm 0.32\,g\,kg^{-1}$). On the other hand, the calcium level in the domesticated wild strain ($0.087 \pm 0.01\,g\,kg^{-1}$) was higher than that of the commercial strain ($0.058 \pm 0.0\,g\,kg^{-1}$). The vitamin profiles showed similarity, but the concentrations in the two strains were different. Vitamin C (ascorbic acid) in the wild strain was almost 3.9-fold higher than that of the commercial strain (Table 1). Vitamin B3 (niacin) is the most abundant vitamin found in this mushroom with 0.09 ± 0.10 and $0.06 \pm 0.02\,g\,kg^{-1}$ in the commercial and domesticated wild strain, respectively.

3.3. Determination of Amino Acids and Fatty Acids. All the essential amino acids (threonine, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan, lysine, histidine, and arginine) were detected in this mushroom with the exception of tryptophan (Table 2). Among the essential amino acids, leucine (commercial strain $20.4 \pm 0.10\,g\,kg^{-1}$; domesticated wild strain $19.4 \pm 0.0\,g\,kg^{-1}$) was found to be the largest constituent, followed by phenylalanine and histidine. The methionine, isoleucine, and valine were present in small amounts ranging from 0.31 to 0.80% for both the *P. giganteus* strains. In particular, *P. giganteus* had the highest concentration of glycine, which is categorized as the nonessential amino acid. Also, the wild strains exhibited higher amount of glutamine, alanine, and asparagine as compared to the commercial strains.

Table 3 presents the fatty acid profile of basidiocarps of *P. giganteus*. Among the saturated fatty acids, palmitic acid (C16:0) was predominant with 3.8 ± 0.01 and $3.3 \pm 0.0\,g\,kg^{-1}$ in the commercial strain and wild strain, respectively. Amongst the polyunsaturated fatty acids, oleic acid was the predominant fatty acid in this species. Oleic acid (C18: 1n9c) in the commercial strain ($10.3 \pm 0.06\,g\,kg^{-1}$) was significantly ($P < 0.05$) higher than that in the wild strain ($8.9 \pm 0.0\,g\,kg^{-1}$). Linoleic acid was also present in significant quantities (4.5–$5.0\,g\,kg^{-1}$) in *P. giganteus*. The medium chain fatty acid, caprylic acid (C8:0), and the long chain fatty acid, stearic acid (C18:0), were also present. Capric, undecanoic, palmitoleic, and eicosadienoic acids were detected in trace amounts in *P. giganteus*.

3.4. Determination of Total Polysaccharides, Total Phenolic, and Total Flavonoids Compound. The total polysaccharides of the commercial and wild *P. giganteus* were comparable. The aqueous extract had the highest amount of polysaccharides as shown in Table 4. Notably, the total polysaccharides in aqueous extract of the wild strains ($17.91 \pm 0.05\%$, w/w) were significantly ($P > 0.05$) higher than those of the commercial strain ($14.93 \pm 0.04\%$). On the other hand, the total phenolic content in the ethanol extracts was higher. The quantity of phenolics in the mushrooms extracts was in descending order: commercial strain ethanol extract > wild strain ethanol extract > commercial extract aqueous extract > wild strain aqueous extract. There was also a significant difference ($P < 0.05$) in the total flavonoids between the basidiocarps of the commercial and wild strains. The wild

Table 2: Amino acid content (g kg^{-1} protein) of *Pleurotus giganteus* KLU-M 1227and KLU-M 1228.

Amino acids	*Pleurotus giganteus* (commercial strain)	*Pleurotus giganteus* (wild strain)
Asp	6.9 ± 0.0^{c}	7.0 ± 0.0^{c}
Glu	15.7 ± 0.0^{jk}	17.2 ± 0.1^{l}
Ser	11.1 ± 0.01^{e}	11.0 ± 0.01^{e}
His*	15.4 ± 0.04^{ji}	15.1 ± 0.05^{j}
Gly	29.0 ± 0.11^{p}	29.5 ± 0.04^{p}
Thr*	5.3 ± 0.01^{b}	5.2 ± 0.00^{b}
Arg*	11.4 ± 0.04^{ef}	12.1 ± 0.00^{fg}
Ala	11.1 ± 0.01^{e}	13.5 ± 0.00^{h}
Tyr	5.8 ± 0.06^{b}	5.9 ± 0.00^{b}
Cys	ND	ND
Val*	8.0 ± 0.01^{d}	7.1 ± 0.00^{c}
Met*	3.1 ± 0.01^{a}	3.1 ± 0.00^{a}
Phe*	16.4 ± 0.09^{kl}	16.5 ± 0.00^{kl}
Ile*	7.7 ± 0.02^{cd}	7.3 ± 0.00^{cd}
Leu*	20.4 ± 0.10^{n}	19.4 ± 0.00^{m}
Lys*	14.5 ± 0.01^{i}	12.8 ± 0.00^{gh}
Asn	15.2 ± 0.07^{ij}	24.6 ± 0.00^{o}
Gln	ND	ND
Nva	ND	ND
Trp*	ND	ND

Asp: aspartate; Glu: glutamate; Ser: serine; His: histidine; Gly: glycine; Thr: threonine; Arg: arginine; Ala: alanine; Tyr: tyrosine; Cys: cystine; Val: valine; Met: methionine; Phe: phenylalanine; Ile: isoleucine; Leu: leucine; Lys: lysine; Asn: asparagine; Gln: glutamine; Nva: norvaline; Trp: tryptophan. Each value is expressed as mean ± SD ($n = 3$). In each row, the different letters represent significant differences between samples ($P < 0.05$). ND: not detected. *Essential amino acids.

Table 3: Fatty acids (g kg^{-1} dry weight) detected in the basidiocarps of *Pleurotus giganteus*.

	Fatty acid	*Pleurotus giganteus* (commercial strain)	*Pleurotus giganteus* (wild strain)
C8:0	Caprylic	1.3 ± 0.0^{a}	1.1 ± 0.0^{ab}
C10:0	Capric	0.4 ± 0.0^{c}	0.4 ± 0.0^{c}
C11:0	Undecanoic	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}
C16:0	Palmitic	3.8 ± 0.0^{d}	3.3 ± 0.0^{e}
C16:1	Palmitoleic	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}
C18:0	Strearic	1.1 ± 0.03^{ab}	0.8 ± 0.0^{b}
C18:1n9c	Oleic***	10.3 ± 0.06^{g}	8.9 ± 0.0^{h}
C18:2n6c	Linoleic**	5.0 ± 0.01^{f}	4.5 ± 0.0^{f}
C20:2	Eicosadienoic**	0.3 ± 0.01^{c}	0.3 ± 0.0^{c}
C22:0	Beheric	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}
C24:0	Lignoceric	0.4 ± 0.01^{c}	0.4 ± 0.0^{c}

**Omega-6 PUFAs; ***Omega-9 PUFAs. Each value is expressed as mean ± SD ($n = 3$). In each row, the different letters represent significant differences between samples ($P < 0.05$).

P. giganteus had approximately 2.09% higher flavonoids than the commercial strains.

3.5. Evaluation of Antioxidant Activity of Mushroom Extracts

3.5.1. DPPH Scavenging Activity Assay. The scavenging effect of the mushroom extracts on DPPH radicals increased with sample concentration, depending on the extraction solvent and strain type. In general, the ethanol extracts showed higher scavenging activity, hence lower IC_{50} (mg/mL) when compared to the aqueous extracts (Table 5). The scavenging activity obtained in descending order was wild strain ethanol extract > commercial strain ethanol extract > wild strain aqueous extract > commercial strain aqueous extract.

3.5.2. FRAP (Ferric Reducing Antioxidant Power) Assay. In the ferric reducing power assay, the reducers present in the mushroom extracts lead to the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The reducing capacity of the mushroom extract serves as an index of antioxidant activity. The reducing ability of the different extracts was in the range of 1.17–3.88 μM $FeSO_4 \cdot 7H_2O$/g mushroom (Table 5). The antioxidant activity obtained in descending order was commercial strain ethanol extract ≥ wild strain ethanol extract ≥ commercial strain aqueous extract ≥ commercial strain aqueous extract.

3.5.3. Inhibition of Lipid Peroxidation. The study of lipid peroxidation (LPO) inhibition is based on the measurement of malondialdehyde (MDA) generated by the polyunsaturated fatty acid peroxides upon decomposition. As a result of LPO, destruction of cellular components occurs and brings about oxidative stress in biological systems. As shown in Table 5, there was no significant difference ($P > 0.05$) in terms of lipid peroxidation inhibition between the commercial and domesticated wild mushroom extracts. However, the ethanol extracts of both the strains showed significantly higher ($P < 0.05$) lipid peroxidation inhibitory ability (49.58–49.80%) when compared to the aqueous extracts (44.41–44.61%). Therefore, the means of extractions instead of mushroom strains played a more prevailing role in lipid peroxidation inhibition.

3.6. Correlation between Total Polysaccharides, TPC, and Antioxidant Parameters.

The TPC in the mushroom extract was positively correlated to the DPPH scavenging capacity ($r = +.827$) and FRAP reducing power ($r = +.820$). This indicated that the antioxidant effects increased with increasing concentrations of the total phenolics present in the mushroom extracts. However, a weak correlation was found between the lipid peroxidation inhibition activity and the TPC ($r = +.321$). Nevertheless, the DPPH scavenging capacity, FRAP reducing power, and lipid peroxidation inhibition showed a strong positive correlation ($r = +.806$ to $+.820$) between each antioxidant activity.

3.7. Neurite Outgrowth Assay.

The mean value of neurite-bearing cells in NGF treated cells (positive control) was 22.67 ± 6.67% as shown in Figure 1(a). The ethanol extracts of the commercial and wild *P. giganteus* (20 μg/mL) caused a significant ($P < 0.05$) increase in neurite-bearing cells

Table 4: Total polysaccharides, phenolics, and flavonoids present in the crude aqueous and ethanol extracts of basidiocarps of *Pleurotus giganteus*.

	Pleurotus giganteus (commercial strain)		*Pleurotus giganteus* (wild strain)	
	Aqueous	Ethanol	Aqueous	Ethanol
Extraction yield (%, w/w)	15.60 ± 2.20^{a}	12.00 ± 1.00^{b}	13.77 ± 1.68^{ab}	6.67 ± 1.06^{c}
Total polysaccharides (%, w/w)	14.93 ± 0.04^{a}	11.31 ± 0.16^{b}	17.91 ± 0.05^{c}	13.72 ± 0.04^{d}
Total phenolic content (mg GAE/g)	12.14 ± 1.89^{a}	24.08 ± 1.04^{b}	9.58 ± 0.18^{c}	21.61 ± 1.47^{d}
Total flavonoids (mg RE/g)	ND	2.94 ± 0.00^{a}	ND	6.14 ± 0.01^{b}

In each row, the different letters represent significant differences between samples ($P < 0.05$). ND = not detected.

Table 5: Antioxidant activities of the aqueous and ethanol extracts from the commercial strain of *Pleurotus giganteus* (KLU-M 1227) and the wild strain (KLU-M 1228).

Antioxidant properties	Test method	Positive control (BHT)	*Pleurotus giganteus* (commercial strain)		*Pleurotus giganteus* (wild strain)	
			Aqueous	Ethanol	Aqueous	Ethanol
Free radical scavenging	DPPH (IC_{50}; mg/mL)	0.09 ± 0.01	21.46 ± 6.95^{a}	11.28 ± 3.54^{bc}	16.18 ± 1.76^{ab}	8.10 ± 2.15^{c}
Reducing power	FRAP (μM $FeSO_4 \cdot 7H_2O$/ g)	780.29 ± 13.4	2.26 ± 0.29^{ab}	2.99 ± 0.14^{b}	2.04 ± 0.32^{a}	2.69 ± 0.71^{b}
Lipid peroxidation inhibition	Inhibition of lipid peroxidation at extract concentration of 10 mg/mL (%)	79.07 ± 2.25	44.41 ± 1.00^{a}	49.58 ± 1.87^{b}	44.61 ± 1.42^{a}	49.80 ± 3.27^{b}

In each row, the different letters represent significant differences between samples ($P < 0.05$).

by 3.98- and 4-fold, respectively, when compared to the control cells with medium only. Additionally, in order to verify the neuritogenic activity of these mushroom extracts, PC12 cells, which only extend neurite upon NGF activation, were employed. Incubation of PC12 with 20 μg/mL of ethanol extracts resulted in a significant increase ($P < 0.05$) in neurite-bearing cells compared to cells treated with NGF alone. Phase-contrast micrographs of neurite-bearing cells were shown in Figure 1(b). The two most abundant fatty acids in the investigated extract were oleic acid and linoleic acid (Table 3). Therefore, the neuronal cells were treated with linoleic acid and oleic acid and the neurite outgrowth activity was examined. Linoleic acid enhanced the neuritogenic activity of PC12 and N2a cells significantly ($P < 0.05$) but not oleic acid (Figure 1(a)).

3.8. NGF Measurement. We measured the level of NGF in the culture medium after PC12 cells were cultivated in the presence of *P. giganteus* extracts and linoleic and oleic acids for 3 days. Linoleic acid significantly ($P < 0.05$) augmented NGF secretion by 1.4 times (Figure 2). On the other hand, oleic acid had a lower potency in promoting NGF secretion (255 pg/mL), as compared to linoleic acid (323 pg/mL). In contrast to the fatty acids, the extracts had a weak stimulatory effect on NGF secretion.

4. Discussion

The nutritional components found in the present work are in accordance with the literature. A study in Italy showed that the protein content in *Pleurotus ostreatus* (grey oyster mushroom) was 1.61 ± 0.02 g/100 g, which is lower than the protein content of *P. giganteus* in this study [17]. *Pleurotus giganteus* also showed a higher protein content when compared to *Pleurotus sajor-caju* (13.0–18.4 g/100 g) [18]. The protein content of mushrooms is dependent on the strain, substrate chemical composition, pileus size, and cultivation time [19, 20]. In this study, rice bran supplementation may have increased the soluble protein content present in both strains as the protein content in rice bran is about 10–15% of the total weight of the mushroom growth substrate [21].

Pleurotus ostreatus was composed mainly of glucose ($14.29\ g\ kg^{-1}$) and mannose ($10.55\ g\ kg^{-1}$) [22]. While fructose was not detected in *P. ostreatus*, $27.81\ g\ kg^{-1}$ of glucose was detected in *P. eryngii* with trace amounts of ribose and xylose. *Pleurotus* spp. in particular are rich in calcium, potassium, magnesium, iron, and phosphorus [23]. *Pleurotus sajor-caju*, *P. platypus*, and *P. citrinopileatus* were reported to contain 16.3 ± 0.22, 11.2 ± 0.3, and $10.3 \pm 0.2\ g\ kg^{-1}$ of potassium, respectively. The concentrations of vitamin B3 in mushrooms were highly species dependent and they vary from 34 to 109 mg/100 g dry weight for *P. ostreatus* [24]. Mushrooms contain higher vitamin B2 (riboflavin) as compared to vegetables. Some varieties of *Agaricus bisporus* (white button mushroom) have also been reported to contain vitamin B2 levels as high as those found in eggs and cheese [25]. In our study, vitamin B1 (thiamin) was higher in the commercial strain by 1.9-fold, while vitamin B2 level was not significantly different when compared to that of the wild strain.

The free amino acids in *P. giganteus* were comparable with reported literature values. Tanzanian wild mushrooms *Lactarius* sp. (milky cap), *Boletus pruinatus* (Matt Bolete mushroom), and *Boletinus cavipes* were reported to have leucine as high as 15.9%, 10.6%, and 8.40%, respectively

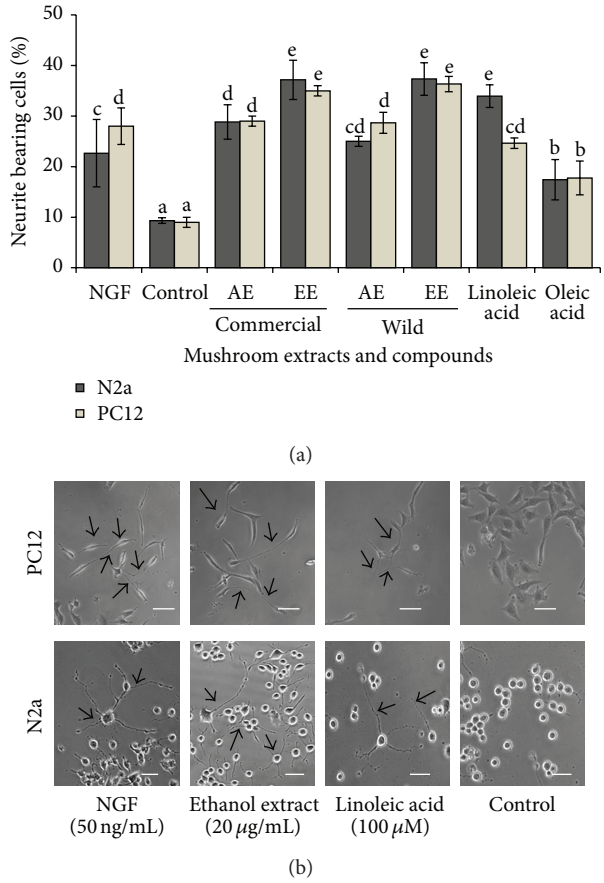

Figure 1: (a) Neurite-bearing cells (%) after 3-day incubation with NGF, mushroom extracts, linoleic acid, and oleic acid. AE: aqueous extract; EE: ethanol extract. Data are expressed as mean ± SD of three experiments. Different letters represent significant differences between samples ($P < 0.05$). (b) Phase-contrast photomicrographs showing the effects of NGF, ethanol extract, and linoleic acid on the morphology of PC12 and differentiating N2a cells after 3 days. Untreated cells serve as control and only contained 5% FBS as vehicle. Arrows indicate neurite extension and scale bar corresponds to 20 μm.

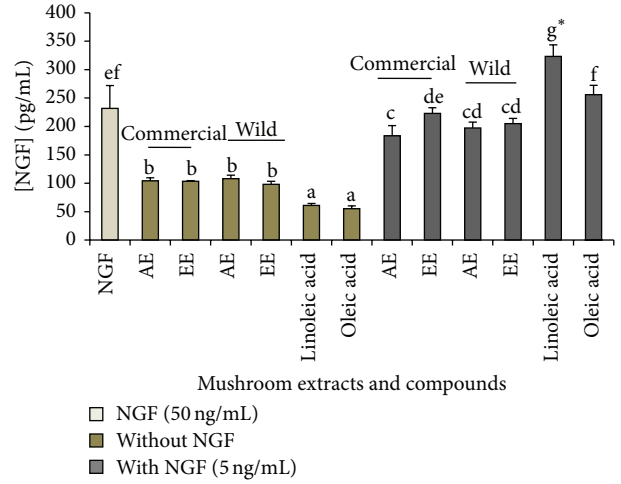

Figure 2: The effects of mushroom aqueous and ethanol extracts on the stimulation of NGF secretion by neurite-bearing PC12 cells in the presence or absence of NGF. NGF (50 ng/mL) was used as the positive control. Values are mean ± SD from three independent experiments. Different letters represent significant differences between samples ($P < 0.05$). $^*P < 0.05$ represents a significant difference from the control, that is, 50 ng/mL of NGF.

[26]. The fresh *P. ostreatus* was found to have 2.74, 1.76, and 1.43 g kg^{-1} of glutamine, asparagine, and arginine, respectively [27]. Similar to our findings, other edible mushrooms also contained high levels of oleic acid (% total fatty acid methyl esters): *Auricularia polytricha* (27.1), *Lentinus sajor-caju* (23.5), *Lentinus squarrosulus* (5.8), *Pleurotus djamor* (28.8), *P. sajor-caju* (16.4), and *Russula brevipes* (39.2) [28]. The presence of caprylic and capric acids in mushrooms is rare. *Calocybe gambosa* (St. George's mushroom) and *Clitocybe odora* (Aniseed funnel mushroom) were reported to have 0.25 ± 0.02 and 0.03 ± 0.00% of caprylic acid, respectively, while *Coprinus comatus* (shaggy mane mushroom) registered 0.09 ± 0.00% of capric acid [29].

Extraction with boiling water is used to obtain extracts with high molecular weight compounds, such as polysaccharides which play an important medicinal role in mushrooms [30]. Low molecular weight compounds, such as phenolic compounds, were usually from ethanol extraction [29]. Although flavonoid was not detected in *P. sajor-caju* [31], its concentration ranging from 0.24 to 0.32 mg/g was reported in *P. ostreatus* [32]. Flavonoids were also present in other mushroom species like *Clitocybe gibba* (3.56 mg chatequin/g extract) and *Boletus armeniacus* (8.59 mg chatequin/g extract) [33]. The rubber wood sawdust which is rich in lignin could have been depolymerized by the lignocellulosic enzymes of mushrooms into phenolic units and further dimerized or polymerized, creating flavonoids [34].

DPPH radical scavenging effects of the mycelia extracts of *Pleurotus* spp. (*P. citrinopileatus*, *P. djamor*, *P. eryngii*, *P. flabellatus*, *P. florida*, *P. ostreatus*, and *P. sajor-caju*) have been reported [32, 35]. The methanol and hot water extracts (10 mg/mL) of *P. eous* were found to scavenge DPPH radical by 85.19% and 70.21%, respectively [36]. The higher DPPH scavenging ability of ethanol extracts might be due to more hydrogen-donating components including phenolic compounds extracted from the mushroom. The higher phenolic content of ethanol extracts might account for the better results found in reducing power as compared to the aqueous extract [37]. When comparing the FRAP values of *P. giganteus* with *P. sajor-caju*, FRAP values of aqueous extract of *P. giganteus* were lower than those of *P. sajor-caju* (35.06 ± 0.86 μM $FeSO_4 \cdot 7H_2O$/g mushroom) [31]. The higher phenolic content in the ethanol extracts might contribute to a higher inhibitory effect on lipid peroxidation. *Pleurotus florida* was reported to display 57% of lipid peroxidation inhibition, while *P. flabellatus*, *P. cystidiosus*, *P. eryngii*, and *P. sajor-caju* showed 50%, 49.8%, 48%, and 43% of lipid peroxidation inhibition, respectively [38]. Meanwhile, *P. ostreatus* at a concentration of 10 mg/mL inhibited LPO activity in rat liver homogenate by 56.20% [39]. In accordance with the present results,

Dubost et al. [34] had demonstrated a positive correlation between the TPC in the mushroom extracts of *P. ostreatus* and *P. eryngii* and the antioxidant capacity.

It has been reported that tiger's milk mushroom, *Lignosus rhinocerotis*, demonstrated neuritogenic effects on PC12 cells [40]. The aqueous extract of *Ganoderma neo-japonicum* (50 μg/mL) was found to trigger a maximal stimulation of PC12 neurite outgrowth with 14.22 ± 0.43% of neurite-bearing cells [41]. Extension of neurites from neuronal cell body is an important step in neuronal development and requires the generation of additional plasma membrane [42]. Polyunsaturated fatty acids like linoleic, linolenic, docosahexanoic, and arachidonic acids promoted basal and nerve growth factor-(NGF-) induced neurite extension of the PC12. This suggested that linoleic acid which was present in abundance in the extracts of *P. giganteus* may play a key role in neuritogenesis. In contrast, studies showed that monounsaturated fatty acids and saturated long-chain fatty acids like oleic, stearic, and palmitic acids caused little or no effects in neurite outgrowth [43]. Studies have shown that cyathane diterpenes from the mushroom *Sarcodon cyrneus*, namely, cyrneines A, B, C, and D, and glaucopine C increased the NGF gene expression in 1321N1 astrocytoma cells [44]. Hericenones C, D, and E isolated from the basidiocarps of *Hericium erinaceus* exhibited stimulating activity for the biosynthesis of NGF *in vitro*. In the presence of hericenones C, D, and E at 33 μg/mL, mouse astroglial cells secreted 10.8 ± 0.8, 23.5 ± 1.0, 13.9 ± 2.1, and 45.1 ± 1.1 pg/mL of NGF into the culture medium, respectively [45].

Long-chain polyunsaturated fatty acids (LCPUFA) are essential nutrients in the development and functioning of the brain and central nervous system. The most abundant LCPUFA in the brain are docosahexaenoic acid (DHA) which is mainly derived from fish and arachidonic acid (ARA) from meat and eggs [46]. The desaturation and elongation of linoleic acids and alpha-linolenic acids to ARA and DHA, respectively, are very crucial for the infant's brain development. Since the percentage of ARA decreases in the brain during prenatal development, the balance in the dietary ratio of linoleic acid is very crucial to the brain development in preterm infant [47]. Apart from that, many studies have demonstrated the importance of LCPUFA as potent neuroprotectant. Linoleic acid was found to protect mouse cortical neurons against glutamate excitotoxicity [48]. Linoleic acid and its derivatives also prevented sodium nitroprusside-induced cell death of cultured rat cerebral cortical neurons [49]. However, to date, limited information is available on the role of polyunsaturated fatty acid as a stimulator of NGF synthesis in neuronal cells.

5. Conclusion

In conclusion, we report for the first time the chemical compositions of the commercial and domesticated wild strains of *P. giganteus*. The extracts of both the strains showed distinctive antioxidant activities due to the differential distributions of total phenolics and flavonoids. Aqueous extracts did not contain flavonoids and had a lower phenolic content, hence explaining its lower antioxidative capacity. Our study demonstrates that the ethanol extract and its major constituent, linoleic acid, induced neurite outgrowth and increased NGF biosynthesis. These findings provide support for the possible role of *P. giganteus* as a functional food to maintain neuronal differentiation and neuritogenesis, as well as a healthy NGF supply in central and peripheral nervous system.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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