

CHAPTER 5

DISCUSSION & CONCLUSION

5.1 Cytotoxicity of *H.erinaceus* Aqueous Extract

The cytotoxicity assessment of *H.erinaceus* aqueous extract was done using MTT colorimetric assay. The MTT assay is a rapid and sensitive spectrophotometric assay for determining viability and proliferation in monolayer cultured cell lines (Edmondson et al., 1988). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is a yellow tetrazolium salt reduced to insoluble purple formazan crystals by the mitochondria (mitochondrial succinic dehydrogenases) of viable cells. The crystals are solubilized by organic solvents to produce a coloured solution that can be quantified at the measurement endpoint using a spectrophotometer. As the production of formazan crystals greatly relies on the presence of metabolically active cells, there is a linear relationship between the absorbance value and number of viable cells present (Burton, 2005). As shown in **Figure 4.1** and **Figure 4.2**, the absorbance value is directly proportional to the cell density for both MRC-5 and NG108-15 cell lines. Further, a close correlation between MTT assay and the measurement of changes in cell resting membrane potential (RMP) – a sensitive indicator of the health of nerve cells, was reported by Xie & Harvey (1993). This denotes the importance and suitability of the MTT assay in evaluating cytotoxicity in neuronal cells *in vitro*.

The MRC-5 is a cell line derived from normal human lung tissue which is commonly used for *in vitro* cytotoxicity testing. As such, this lung fibroblast was chosen to test for the toxicity of *H.erinaceus* aqueous extract towards a cell line of human origin. There was a significant increase in cell viability when MRC-5 cells were incubated with 10

– 1000 µg/mL extract for 24 hr. This reflects an increase in cell number which indicates cell proliferation.

Cell proliferation was not evident in the neural cell NG108-15 within the concentration range tested (0 µg/mL – 50 000 µg/mL). The absence of cell proliferation in extract-treated NG108-15 can be attributed to the neuronal differentiation process. Neuronal differentiation has been closely linked to cessation of cell proliferation. The induction of nitric oxide synthase (NOS) plays an important role in cellular growth arrest, initiating the switch to cytostasis during differentiation (Peunova & Enikolopov, 1995). In the differentiation of PC12 cells, NGF blocks cell proliferation through a signal transduction pathway involving NOS, p53 tumour suppressor and p21^{WAF1} cyclin-dependent kinase inhibitor (Poluha et al., 1997). Overexpression of neuronal NOS in neuroblastoma cells promoted cell differentiation accompanied by a reduced cell proliferation rate (Ciani et al., 2004). In NG108-15 cells, the rate of cell proliferation decreased strongly when the cells were incubated with differentiation medium containing dibutyryl cAMP (Hamprecht, 1977). Differentiated NG108-15 cells were reported to have a lower growth rate, requiring 8 - 9 days to achieve cell confluence, compared to undifferentiated cells (Kawahara et al., 2002).

The *H.erinaceus* aqueous extract did not have significant cytotoxicity on both NG108-15 and MRC-5 cell lines tested. The IC₅₀ value, which is the 50% inhibitory concentration for the endpoint measured, of *H.erinaceus* aqueous extract is 34 094.57 ± 1199.80 µg/mL on MRC-5, and 14 446.07 ± 1548.34 µg/mL on NG108-15. Both IC₅₀ values are relatively high in comparison to the IC₅₀ value of known cytotoxic agents, which is usually less than 1000 µg/ml (1 mg/ml). The IC₅₀ values even exceed the maximum top concentration recommended for *in vitro* toxicity assays which is 1 mM or 0.5 mg/mL (International Conference on Harmonisation, 2008). A water extract mixture of Panax

ginseng and *H.erinaceus* (MUNOPHIL) was reported to be safe and non-toxic in an oral subchronic 28-day dose toxicity studies in rats (Park et al., 2008). Oral administration of hot water extract of *H.erinaceus* in mice showed no adverse toxicological effects on internal organs including heart, lung, liver and spleen (Mizuno, 1999). The extract of *H.erinaceus* promoted normal development of cultivated cerebellar cells and demonstrated a regulatory effect on the process of myelin genesis process *in vitro*. Also, there was no pathologic or toxic action observed in the extract-treated cells where the cellular ultrastructure remained intact and similar to that observed *in vivo* (Kolotushkina et al., 2003).

5.2 The Neurite Outgrowth Stimulation Activity of *H.erinaceus* Aqueous Extract

NG108-15 is a neuroblastoma-glioma hybrid cell line capable of expressing neuronal characteristics such as the synthesis of neurotransmitters and formation of functional synapses, when induced to differentiate (Smalheiser & Schwartz, 1987). Under normal growth conditions, the neuroblastoma-glioma hybrid cells are round, flat, and adhere loosely on cell culture surface. The cells tend to grow in clusters and pile up in nature, with short processes being formed occasionally. Poly-D-lysine coating has been employed in neurite outgrowth stimulation assays where the coating served as a neutral substratum for cells to attach on cell culture surface within the first hour of plating and thus, minimizing the clustering of cells. The survival and growth development of neuronal cell in culture greatly relies on substratum adhesion. Polycationic proteins such as polylysine (including levo and dextro enantiomers) and polyornithine are commonly used to promote attachment of neuronal cells on culture substrate (Yavin & Yavin, 1974). Extracellular matrix (ECM) molecules such as laminin, fibronectin, collagen and proteoglycans not only provide cell anchorage but also promote neural differentiation and axonal growth (Yu et al., 2008). The cell adhesion strength of NG108-15 was maximized on surfaces covalently

modified with amine groups, where the net surface charge of the substrate was increased (Cargill et al., 1999). Undifferentiated NG108-15 cells were reported to express an exuberant, multipolar outgrowth of diverse neurites over first 4 hours post-plating when plated upon laminin-coated substrate; however, this was not observed in cells plated on polylysine-coated substrate (Smalheiser & Schwartz, 1987). This is in accordance with the results in this study where poly-D-lysine served as a neutral substratum in providing cell attachment.

Two distinct neurite patterns, 'rapid-onset' and 'slow-onset' neurites, were observed in the differentiated NG108-15 cells treated with *H.erinaceus* aqueous extract and/or NGF. Rapid-onset neurites are easily spotted with profuse multipolar outgrowth and extensive branching with filopodia and lamellipodia arising at various sites along the neurite and not restricted to the growing tips. On the polylysine substratum, methylation inhibitors, kinase antagonists and Mn^{2+} ions were reported to stimulate rapid-onset neurites in NG108-15 cells by affecting the manner in which the cells respond to the substratum (Smalheiser, 1990). Laminin, a complex multi-domain glycoprotein which interacts with a variety of cell-surface receptors, stimulates rapid-onset neurites in NG108-15 cells by stimulating active motility responses (Smalheiser, 1991). Neurite plasticity was well observed in rapid-onset neurites where the cells engaged in extension and resorption, with lifetime of any single neurite ranging from minutes to hours (Smalheiser, 1989b). In contrast, slow-onset neurites formed long, smooth, cable-like neurites with motility restricted to the growing tips. It was reported that rapid-onset neurites in actively translocating cells became fused and gradually formed smooth, unipolar, axon-like neurites over several days of plating (Smalheiser, 1989a). This explains the presence of two distinct neurite patterns observed in the treated cell populations with slow-onset mode gradually dominating the expression of the rapid-onset mode.

Neurofilaments are one of the major neuronal protein building blocks in the cytoskeleton of neurites. Neurofilaments are useful immunocytochemical markers for axons due to the abundance of neurofilaments present in mature axons (Audesirk & Audesirk, 1998). Neurite extensions of cultured cells may be related to outgrowth of axonal or dendritic processes. The NG108-15 cell line has been reported to be able to extend axon-like and dendrite-like processes when induced with different neuritogenic agents. Neuritogenic agents including neuraminidase, ionomycin, KCl + dibutyryl cAMP and cholera toxin B subunit induce axon-like neurites that stained positive for phosphorylated high molecular weight neurofilament protein (NF-H) and synaptic vesicle protein-2 (SV2) but stained negative for microtubule-associated protein-2 (MAP-2). Conversely, agents such as retinoic acid, dibutyryl cAMP and GM1 ganglioside induce dendrite-like processes which stained positive for MAP-2 and negative for NF-H and SV-2 (Wu et al., 1998). NF-200, also known as high molecular weight neurofilament protein (NF-H), is a useful immunocytochemical marker for neurons whereas MAP-2 is a specific marker for dendrites. However, it was found that NG108-15 cells differentiated with dibutyryl cAMP stained positive for NF-200 and MAP-2 which contradicts with earlier findings (Tojima et al., 2000). Upregulation of GM1 ganglioside in the nuclear envelope along with elevation of intracellular Ca^{2+} was found to occur in axon-like outgrowth in NG108-15 cells (Kozireski-Chuback et al., 1999). The positive staining of NF-200 in NG108-15 cells treated with *H.erinaceus* extract and/or NGF may indicate the formation of axon-like outgrowth. Yet, it is worthwhile to study the expression of other neuronal proteins and GM1 ganglioside to further confirm the type of neurite formation.

The *H.erinaceus* aqueous extract, applied individually or in combination with NGF, induced visible neurite extensions in NG108-15 cells compared to untreated control cells. When the *H.erinaceus* extract and NGF was applied individually, the neurite outgrowth

stimulation activity was dependent on the application dose in a bell-shaped manner. The cells appeared to be more sensitive and responsive to the mushroom extract and in having maximal increment of 64.8% as compared to 43.4% in NGF. This may be due to the difference in neurite outgrowth signalling pathway employed by both agents. The extracellular NGF level in the cell supernatant was maximally increased by 85.5% when treated with the optimal concentration of *H.erinaceus* extract (50 µg/mL) in neurite outgrowth activity. This reflects the association between secreted NGF levels in culture and neurite outgrowth stimulation activity in NG108-15 cells treated with *H.erinaceus* aqueous extract. The neuroactive compounds in *H.erinaceus* mushroom act as inducers of NGF-synthesis in cells of neural origin rather than having neuritogenic activity *per se*.

The *H.erinaceus* aqueous extract was further studied to investigate whether the extract would enhance neurite outgrowth stimulation activity in exogenous NGF-treated cells. There was a small magnitude of increase in the percentage of neurite-bearing cells when NG108-15 cells were treated with *H.erinaceus* extract in combination with 5 ng/mL NGF. Yet, the enhancement effect was negligible. However, the combined treatments at 10 ng/mL NGF did have a positive effect with respect to neurite outgrowth. Addition of *H.erinaceus* extract at the concentration range of 1 – 10 µg/mL increased the neurite outgrowth activity by 20% compared to 10 ng/mL NGF treatment only. The combined application of 1 µg/mL *H.erinaceus* extract (29.8%) and 10 ng/mL NGF (33.3%) resulted in an additive response (60.6%) in neurite outgrowth stimulation activity of NG108-15. Nevertheless, the additive effect was not observed with increasing concentrations of *H.erinaceus* extract and appeared to be saturated at 1 µg/mL. When the NGF concentration was increased to 20 ng/mL in the combined treatments, the enhancement of neurite outgrowth activity was absent. Further, increasing concentrations of *H.erinaceus* extract combined with 20 ng/mL NGF reduced the percentage of neurite-bearing cells.

As the combined treatments of *H.erinaceus* aqueous extract and 10 ng/mL NGF significantly enhanced neurite outgrowth activity in morphological studies, the extracellular NGF level in cell supernatant was measured to determine whether the neurite-inducing enhancement was related to the secreted NGF levels as in *H.erinaceus* extract-treated cells. There was a stepwise increase in the extracellular NGF level in NG108-15 cells treated with combined treatment of 10 ng/mL exogenous NGF and *H.erinaceus* aqueous extract where a 4-fold (1 – 10 µg/mL extract) and 5-fold (50 – 100 µg/mL extract) increase was observed compared to individual application of 10 ng/mL exogenous NGF. The elevated levels of extracellular NGF may have contributed to the enhancement of neurite outgrowth activity in NG108-15 cells in the combined treatments of 10 ng/mL NGF and 1 – 10 µg/mL mushroom extract. However, the percentage of neurite outgrowth declined upon application of 10 ng/mL exogenous NGF combined with high concentrations of mushroom extract (50 – 100 µg/mL). This suggests that excessive levels of NGF may have inhibited neurite outgrowth stimulation activity in NG108-15. It was reported that high concentrations of NGF arrest axonal growth in dorsal root ganglia by inducing cytoplasmic relocation of the receptor TrkA independent of functional p75^{NTR} receptors (Conti et al., 2004). Hence, the application of *H.erinaceus* extract should fall within the range of 1 - 10 µg/mL when combined with 10 ng/mL NGF to achieve optimal neurite outgrowth activity.

Neurite outgrowth, a critical process in neuronal differentiation, is modulated by a number of factors. Extrinsic cues such as trophic factors, extracellular matrix molecules and activity-dependent depolarization activate intracellular signalling cascades that promote neurite initiation and extension (Radio & Mundy, 2008). Extracellular NGF binds to TrkA receptors found on the neuronal membrane and activates phosphatidylinositol-3-kinase (PI3K) and Akt pathway which promotes neuronal survival (Lau & Huganir, 2006). The activation of PI3K is essential for neuronal survival as signalling of the protein kinase Akt

controls biological functions of several proteins responsible in modulating cell survival. In most neurons, neurotrophins convey survival-promoting signals via Ras-dependent activation of PI3K pathway (Huang & Reichardt, 2001). The activation of extracellular signal regulated kinases (ERKs) is a common effect of cellular stimulation by all growth factors of which its activation is required for the initiation and extension of neuritic processes and morphological differentiation of neurons (Landreth, 2006). The ERKs are the best characterized family of mitogen-activated protein kinases (MAPKs) in the brain (Bibb & Nestler, 2006). MAPKs comprise of a family of protein kinases which regulates extensive cellular functions including gene expression, mitosis, movement, metabolism and programmed death (Johnson & Lapadat, 2002).

Hericium erinaceus extract has been reported to promote NGF gene expression via phosphorylation of c-jun N-terminal kinase (JNK) and its downstream substrate c-Jun, as well as enhance c-fos gene expression (Mori et al., 2008). JNKs, a subfamily of MAPKs, is referred to as stress-activated protein kinases (SAPKs) due to their activation in response to cellular stress (Bibb & Nestler, 2006). JNKs are activated in response to inflammatory cytokines, growth factors, environmental stress (heat shock, ionizing radiation, oxidative stress and DNA damage) and inhibition of DNA and protein synthesis (Raman et al., 2007). Conversely, recent studies have shown that JNKs mediate physiological functions and restorative actions such as neurite outgrowth and axonal regeneration. In response to NGF, a concerted activity of PI3K, ERK and JNK pathways is involved in neuronal cells where PI3K induces neurite sprouting followed by ERK and JNK signalling, resulting in the induction of genes such as c-Jun and neurofilament proteins (Waetzig et al., 2006). The activation of JNK in a biphasic manner was essential for neuritogenesis induced by NGF in a variant PC12 cell line (PC12-N1) (Xiao et al., 2006). JNKs were found to support neuronal recovery in NGF-treated PC12 cells by turning apoptotic Fas ligation into

sprouting signals (Waetzig et al., 2008). Also, activated JNK was required in the formation of axon in hippocampal neurons but not for the development of dendrites (Oliva et al., 2006).

The application of *H.erinaceus* aqueous extract enhanced NGF secretion in NG108-15 cells both in the presence and absence of exogenous NGF. This indicates that the neuroactive compounds present in the mushroom extract stimulate neurite outgrowth by inducing NGF synthesis in the neuronal cells. Soluble peptide growth factors synthesized in endoplasmic reticulum are stored in secretory vesicles and secreted via the regulated pathway, where the vesicles are actively transported for extracellular delivery in response to extracellular stimuli (Lessmann et al., 2003). NGF-promoting compounds may trigger regulated exocytosis via vesicle membrane fusion, which results in a transient local rise in intracellular Ca^{2+} and fusion of the vesicle membrane with plasma membrane. These series of events eventually lead to the release of mature and biologically active NGF to the extracellular space (Landreth, 2006). Hericenones isolated from the fruiting bodies of *H.erinaceus* are the active compounds responsible for promoting NGF biosynthesis (Ma et al., 2010). Several NGF-inducing agents have been found to engage different signaling pathways in the biosynthesis of NGF including signalling of cyclic AMP (cAMP), protein kinase C (PKC), intracellular Ca^{2+} , nuclear factor- κ B (NF- κ B) and MAPK (Obara & Nakahata, 2002). Yet, the mechanism of hericenones in NGF biosynthesis remains elusive. Also, it was reported that hericenones C, D and E did not increase NGF mRNA expression in 1321N1 cells and there may be unknown NGF-promoting compounds present in the fruiting body of *H.erinaceus* (Mori et al., 2008). This warrants further investigation on the identification of neuroactive compounds, especially water-soluble components, present in the mushroom along with their mechanism in promoting neurite outgrowth in neuronal cells.

Neuropeptide Y (NPY) is a 36-amino acid peptide that acts as an important transmitter-like mediator in the hypothalamic feeding circuitry and participates in several mechanisms in the development of nervous system (Hökfelt et al., 2008). Vasoactive intestinal polypeptide (VIP) and NPY indirectly increase neurite outgrowth in nerve injury-induced sensory neurons via neurotrophic factors derived from the spinal cord (White & Mansfield, 1996). Further studies have shown that NPY acts via neurotrophin-3 to mediate increased expression of neurite outgrowth in dorsal root ganglion cells (White, 1998). The exposure of NG108-15 cells to dexamethasone (a synthetic glucocorticoid with anti-inflammatory and immune suppressive effects) or NGF was found to increase cellular concentrations of NPY. The changes in cellular concentration of NPY appear to accompany increased neuronal differentiation in NG108-15. Further, a synergistic effect between both agents (dexamethasone and NGF) was observed in the enhancement of NPY concentration in NG108-15 cells (Yeats et al., 1983). Upon differentiation, increased expression of NPY and laminin was observed in NG108-15 cells transfected with synapsin. Synapsin-transfected NG108-15 exhibit stronger neuronal phenotype upon differentiation with higher numbers of neuritic varicosities and NPY-containing large dense-cored vesicles (LDVs) (Fried et al., 1995). It is not known whether NPY is expressed in the neuronal differentiation process of NG108-15 treated with *H.erinaceus* extract. As NPY are known to exert trophic actions similar to growth factors, it may be involved in the additive effect of *H.erinaceus* extract and NGF in the neurite outgrowth stimulation activity of NG108-15.

Several phytochemicals were reported to enhance neurite outgrowth activity of NGF. The ethanolic extracts of *Centella asiatica* at 100 µg/mL elicited a marked increase in the neurite elongation of human neuroblastoma SH-SY5Y cells in the presence of NGF. Asiatic acid, a triterpenoid compound found in non-polar fractions, was responsible for the neuritogenic activity in *C.asiatica*. Conversely, the neurite inducing effect was absent in the

water extract of *C.asiatica* and the extract significantly inhibit the activity of NGF (Soumyanath et al., 2005). In this study, the aqueous extract of *H.erinaceus* not only induced neurite outgrowth independent of the presence of NGF but enhanced the activity of NGF in the stimulation of neurite outgrowth. The crude water extract of *Tremella fuciformis* induced neurite outgrowth of PC12h cells at fairly low concentrations of 0.1 µg/mL and 1 µg/mL (Kim et al., 2007). The neurite length of the treated cells was measured as an indicator of neurite outgrowth instead of the percentage of neurite-bearing cells. Hence, it is difficult to compare the potency of neurite outgrowth activity in the aqueous extract of *H.erinaceus* and *T.fuciformis*.

The aqueous extract of locally grown *H.erinaceus* was shown to enhance the activity of NGF in promoting neuronal differentiation in NG108-15 cell line in this study. The extract promoted neurite outgrowth activity by inducing the synthesis of extracellular NGF. These findings hold out promising prospects for this mushroom to be a dietary supplement for brain health in aging individuals. Age-dependent degeneration of cholinergic neurons resulting in progressing memory deficits is caused by a decrease in trophic support. However, extended trophic support to degenerating neurons may reverse aging-related neuronal atrophy (Schliebs & Arendt, 2011). This study has shown that the aqueous extract of *H.erinaceus* enhanced the neurite outgrowth stimulation activity of NGF when applied in combination with suboptimal concentrations of NGF. Hence, the consumption of *H.erinaceus* may provide the necessary trophic support in an aging brain by increasing the concentration of NGF to normal healthy levels. Regular intake of *H.erinaceus* may promote healthy brain functions and prevent age-related learning and memory decline.

The study of neurotrophic factors as therapeutic agents in neurodegenerative diseases has long been contemplated. It is known that the balance of NGF trophic system is

altered in the brains of Alzheimer's disease patients. Thus, with NGF acting as a therapeutic agent, it is deduced that this neurotrophic factor will impede further death of cholinergic neurons and restore functions of degenerating cells (Williams et al., 2006). Current drug development studies have focused on the therapeutic potential of CERE-110 (AAV2-NGF), an adeno-associated virus-based gene delivery vector that encodes human NGF (Bishop et al., 2008). As the *H.erinaceus* aqueous extract was shown to enhance neurite differentiation when used in combination with NGF, the supplementation of this mushroom extract in NGF therapeutic treatments may be beneficial. However, further studies have to be conducted to investigate whether the enhancement effect of neurite outgrowth activity by NGF combined with *H.erinaceus* aqueous extract is effective *in vivo*.

5.3 The Neuroprotective Effect of *H.erinaceus* Aqueous Extract

Hydrogen peroxide (H₂O₂) is a reactive oxygen species (ROS) which induces oxidative stress in cellular constituents. In this experimental setting, cell viability of NG108-15 was maintained above 90% when treated with low levels (0.5 – 10 µM) of H₂O₂. Cell viability was not adversely affected at this inferior level of H₂O₂ even when the duration of incubation was prolonged from 2 to 24 hr. When the cells were treated with moderate levels (50 – 100 µM) of H₂O₂, the cell viability of NG108-15 started to reduce as the incubation period was prolonged. At 50 µM H₂O₂, the cell viability reduced 2-fold (84.27% to 36.98%) when the duration of incubation was extended from 2 to 24 hr. Similarly, the cell viability reduced 4-fold (54.89% to 13.04%) when treated with 100 µM H₂O₂. The cytotoxicity of H₂O₂ towards NG108-15 cells was apparent with prolonged incubation periods. Also, the cytotoxic potency of H₂O₂ was similarly observed with increased concentrations of H₂O₂. The treatment of moderately high levels (250 – 500 µM) of H₂O₂ was lethal to NG108-15 cells even with minimal exposure (2 hr).

The concentration-dependent effects of H₂O₂ in mammalian cells was reported in Wiese et al. (1995) in which extremely low concentrations (3 – 15 µM) stimulated cell growth and proliferation; low concentrations (120 – 150 µM) induced a transient adaptive response; intermediate concentrations (250 – 400 µM) caused permanent loss of replicative or divisional competence and high concentrations (> 1 mM) caused destructive necrotic cell death. This suggested that low concentrations of H₂O₂ may play an important role as a physiological growth and proliferative agent in mammalian systems. Temporary adaptation to the oxidative stress of H₂O₂ may be a mechanism for mammalian cells to cope with fluctuating levels of oxidants generated in biochemical processes such as respiration, autooxidation and oxidative burst of neighbouring phagocytes (Wiese et al., 1995). The cytotoxic potency of H₂O₂ in cell culture can be influenced by various factors including the type of cell used, adaptation to oxidative stress, treatment media, cell density and duration of H₂O₂ exposure (Gülden et al., 2010). In this experimental setting, the cell viability of NG108-15 has shown that the cytotoxic effect of H₂O₂ is enhanced with increased concentrations of H₂O₂ and longer duration of incubation. As such, the treatment condition of 100 µM H₂O₂ for 2 hr (cell viability at 54.89 ± 4.40 %) was used to evaluate the neuroprotective activity of *H.erinaceus* aqueous extract.

Pre-treatment and co-treatment of NG108-15 cells with *H.erinaceus* aqueous extract was studied to assess the neuroprotective effect of the mushroom extract against oxidative stress. The pre-treatment mode allowed the neuronal cells to be “primed” with the mushroom extract before the introduction of oxidative stress; conversely, the co-treatment mode evaluated the protective effect of the mushroom extract in the presence of ROS. The pre-treatment of *H.erinaceus* extract was carried out for different duration – 2 hr and 24 hr, to investigate whether extended “priming” hours would confer better protection effects towards the neuronal cells. It was observed that there was no significant improvement in

the cell viability of NG108-15 treated with *H.erinaceus* aqueous extract in both treatment modes. This indicates that both the pre-treatment and co-treatment of *H.erinaceus* aqueous extract did not confer protective effects on NG108-15 under oxidative assault when evaluated using MTT assay. The neuroprotective activity of *H.erinaceus* extract was also evaluated using trypan blue exclusion assay which evaluated cellular viability based on the ability of a cell with an intact membrane to exclude the dye trypan blue. The loss of membrane integrity in late apoptosis and necrosis can be detected by cellular uptake of the vital dye. It was observed that cell viability of NG108-15 was improved when the cells were pre-treated with mushroom extract followed by oxidative assault when evaluated using trypan blue assay. The treatment of 100 µg/mL *H.erinaceus* extract (24 hr) prior to the introduction of H₂O₂ improved the cell viability up to 30.9% compared to untreated cells. However, the neuroprotective activity of *H.erinaceus* extract was not observed in the co-treatment mode using trypan blue exclusion assay.

Both the MTT and trypan blue exclusion assays are regular cell viability assays whereby the former evaluates cellular metabolic activity and the latter evaluates membrane structural integrity. The MTT assay determines the ability of viable cells to convert the yellow tetrazolium salt (MTT) into insoluble purple formazan precipitate. The tetrazolium salt is reduced at the ubiquinone and cytochrome b and c sites of the mitochondrial electron transport system via the activity of succinate dehydrogenase (Supino, 1995). The trypan blue exclusion assay determines cellular viability by assessing functional integrity in which viable cells have intact membranes and exclude the dye trypan blue; non-viable cells are stained with the dye and are visible under bright field optics (Coder, 1997). Although the pre-treatment of *H.erinaceus* aqueous extract demonstrated protective effects against oxidative stress when trypan blue exclusion assay was employed, the disruption of cellular metabolic activity in NG108-15 cells was clearly revealed in MTT assay. During oxidative

stress, mitochondrial membrane transition pore permeability is increased, followed by loss of mitochondrial NAD^+ and further generation of superoxide radicals lead to cell injury. The presence of free radicals impairs mitochondrial electron transport and leads to mitochondrial dysfunction (Maiese et al., 2010). On the other hand, cell membrane of cells undergoing apoptosis remains intact for a relatively long time, thus, only cells in late stages of apoptosis and necrotic cells take up the dye trypan blue and appear as blue cells (Zhivotovsky & Orrenius, 2001). It was observed that when mammalian cells are subjected to very high concentrations of H_2O_2 (5.0 - 10.0 mM), the cell membrane disintegrates, proteins and nucleic acids denature, and lastly necrosis ensues (Davies, 1999).

As the results in trypan blue exclusion assay showed possible protective activity against oxidative stress, the terminal deoxynucleotide transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay was further employed to determine the protective effect of *H.erinaceus* extract in NG108-15 cells undergoing apoptosis. The TUNEL method is based on direct, specific, *in situ* labelling of DNA fragmentation sites in the nuclei of fixed cells. Apoptotic cells exhibit distinct morphological and biochemical hallmarks including phosphatidylserine exposure, cell shrinkage, chromatin cleavage, nuclear condensation and formation of pyknotic bodies of condensed chromatin (Zhivotovsky & Orrenius, 2001). With TUNEL, apoptotic cells are identified using TdT to transfer biotin-dUTP to free 3'-OH ends of cleaved DNA. The biotin-labeled cleavage sites are then visualized by the reaction with fluorescein conjugated avidin (avidin-FITC) (Millipore Corporation, 2007). It was observed that there was no significant difference between the percentages of apoptotic cells present in extract treated and non-extract treated cells. This denotes that the *H.erinaceus* extract was unable to protect NG108-15 cells against H_2O_2 -induced oxidative stress.

Apoptotic cells are not easily distinguishable in the trypan blue exclusion assay due to the intact cellular membrane that excludes the dye trypan blue as in viable cells. Hence, the increased cell viability observed in NG108-15 cells pre-treated with *H.erinaceus* aqueous extract using trypan blue exclusion assay may have included apoptotic cells. The TUNEL assay further clarifies the obscure protective effect of the mushroom extract seen in the trypan blue exclusion assay. The combination of assays employed - MTT assay, trypan blue exclusion assay and TUNEL assay, showed that the *H.erinaceus* aqueous extract failed to protect NG108-15 cells against oxidative stress. This indicates that the aqueous preparation of *H.erinaceus* does not possess neuroprotective properties.

Clinical findings revealed that the oxidation of lipid, protein and DNA levels are higher than normal levels in the brains of Alzheimer's disease patients. Oxidative stress plays an important role in the neurotoxic effect of amyloid- β peptide in Alzheimer's disease (Matteo et al., 2007). The aqueous extract of locally grown *H.erinaceus* was reported to possess antioxidant properties with the presence of high phenolic content (Abdullah et al., 2012). However, the extract did not confer protection to NG108-15 cells subjected to oxidative stress. It was reported that the phenolic content and antioxidant activity of oven-dried fruitbody of *H.erinaceus* was higher compared to extracts of freeze-dried fruitbody (Wong et al., 2009b). Thus, the aqueous extract of oven-dried fruitbody may demonstrate better protective effect against oxidative assault. The enzymatic extracts of *H.erinaceus* were found to possess more effective antioxidative activity than water or organic solvent extracts of the mushroom. Furthermore, pepsin-treated extracts of *H.erinaceus* exhibited neuroprotective effects against H₂O₂-induced oxidative stress in PC12 cells by regulating the anti-apoptotic protein Bcl-2 (Lee et al., 2010). Hence, different extract preparation of locally grown *H.erinaceus* can be further studied for their neuroprotective effects. The aqueous extract of *Auricularia auricular-judae* was reported to have high antioxidant

capacity with high phenolic content, β -carotene bleaching activity, plasma lipid peroxidation inhibitory and DPPH radical scavenging activities. The pre-treatment of baby hamster kidney fibroblast (BHK 21) cells with the extract showed potent protective effects against H_2O_2 -induced oxidative stress (Oke & Aslim, 2011). However, it is not known whether the extract would confer protection in neuronal cells.

Clenbuterol, a lipophilic β -adrenergic receptor agonist, induces NGF synthesis in a concentration dependent manner in cultured rat cortical astrocytes. The neuroprotection of clenbuterol was observed *in vivo* with enhanced NGF expression in the rat brain (Semkova & Krieglstein, 1999). Although the application of *H.erinaceus* aqueous extract induced NGF synthesis in NG108-15 cells, the neuroprotective action was not observed when the cells were subjected to H_2O_2 -induced oxidative stress. This may be due to the difference in the level of NGF induced by both agents. The application of clenbuterol (1 – 100 μ M) significantly enhanced the level of NGF (100 - 200 pg/mL) in the medium of rat hippocampal cells (Semkova et al., 1996). However, the level of NGF detected in the medium of NG108-15 cells was comparably lower (30 – 45 pg/mL) when treated with *H.erinaceus* aqueous extract. The levels of NGF induced by the mushroom extract in this study may be inadequate to protect the neuronal cells against oxidative stress.

A patent on the method of extraction of an anti-dementia substance from the fruiting body of *H.erinaceus* was filed by Zhuang et al. (2009). The anti-dementia substance was extracted from a fat-soluble fraction that includes benzyl alcohol derivatives, chromane derivatives and phosphatidylethanolamine derivatives as main bioactive compounds. The bioactive fraction was reported to increase the synthesis of NGF and reduce toxicity of amyloid- β peptide. Neuroprotective compounds present in *H.erinaceus* mushroom including dilinoleoyl-phosphatidylethanolamine (DLPE) (Nagai et al., 2006) were of lower

polarity and mainly extracted with organic solvents. It is necessary to investigate whether these neuroprotective compounds are present in the locally grown *H.erinaceus* mushroom.

Several extracts were reported to demonstrate both neurotrophic and neuroprotective activity. The hot water extract of *Tremella fuciformis* showed potent neuritogenic activity by inducing neurite outgrowth in PC12h cells at comparably low concentrations (0.1 – 1 µg/mL). Also, the *T.fuciformis* extract demonstrated protective effect against amyloid-β peptide-induced cytotoxicity when PC12h cells were pre-treated with the extract for 24 hr. It was suggested that neuroprotective effect of *T.fuciformis* is attributed to its superoxide-scavenging ability (Park et al., 2007). The water extract of dried *Silybum marianum* (milk thistle) significantly enhanced differentiation of PC12 cells in the presence of NGF. The extract protected PC12 cells against apoptosis induced by NGF withdrawal for a period up to 7 days. The protective effect of *S.marianum* was similarly observed in primary hippocampal neurons against FeSO₄-induced apoptosis (Kittur et al., 2002). The aqueous extract of *Ganoderma lucidum* induced neuronal differentiation of PC12 cells and protected the neurons from NGF withdrawal apoptosis (Cheung et al., 2000). In this study, the aqueous extract of *H.erinaceus* exhibited neurotrophic activity but not neuroprotective activity in NG108-15 cells.

5.4 Properties of *H.erinaceus* Aqueous Extract

In traditional medicine, medicinal plants and herbs are usually prepared in the form of decoction or infusions. Mushrooms are usually consumed as decoction in medical prescription or cooked as soup/stew in culinary. Decoction refers to a preparation made by adding cold water to the required amount of the drug, boiled and simmered for 5 – 10 min. The mixture is strained afterwards. Infusions are prepared by steeping herbs in boiling water, as in the preparation of tea (Gurib-Fakim, 2006). Hence, the aqueous extract of *H.erinaceus* was prepared by soaking the freeze-dried powder of the mushroom fruiting

body in distilled water followed by boiling. This is to simulate the decoction preparation usually prescribed in traditional medicine. Furthermore, the decoction method breaks down the cell walls of mushroom, allowing the medicinal components to become available and thereby readily absorbed once consumed (Halpern, 2007). Hence, most mushroom supplements available commercially are hot water extract preparations.

The extracts of locally grown *H.erinaceus* have been studied for their antimicrobial and antioxidant activities. The fruiting body and mycelia extracts prepared in distilled water demonstrated weak inhibitory effect towards bacterial and fungal growth. Conversely, the antioxidant activity assessment was carried out using methanolic extract of locally grown *H.erinaceus*. The fresh fruiting body extract showed the most potent DPPH radical scavenging activity while oven-dried fruiting body extract was excellent in reducing β -carotene bleaching. The total phenolic content and total antioxidant activity was highest in oven-dried fruiting body extract compared to extracts of other processing preparations (Wong et al., 2009b). It was found that the phenolic content of *H.erinaceus* hot water extract [10.20 ± 2.25 mg gallic acid equivalents (GAE) per gram extract] was higher than that of methanolic extract of fresh, oven-dried and freeze-dried fruiting body of the same species. The hot water extract of *H.erinaceus* exhibited moderately high antioxidant potential with an antioxidant index of 17.7% (Abdullah et al., 2012).

The most highly sought after property in *H.erinaceus* is its neuroactive components – hericenones and erinacines, which can stimulate the synthesis of NGF. An earlier study has shown that this bioactive attribute of this temperate mushroom is not affected by tropical cultivation conditions in Malaysia (Wong et al., 2007). Extracts prepared from fruiting bodies and mycelium of locally grown *H.erinaceus* was shown to stimulate neurite outgrowth in the neuroblastoma-glioma hybrid cell line NG108-15. Maximum stimulation of neurite outgrowth was recorded with extracts of freeze-dried fruiting body, followed by

fresh fruiting body and mycelium. The neurotrophic effect was absent in the extract of oven-dried fruiting body which may be due to inactivation via oxidation of the bioactive compound involved. Freeze-drying, also known as lyophilization, dries the mushrooms by freezing them under reduced pressure in a vacuum which allows a better preservation of flavor (Chang & Miles, 2004). Freeze-drying appears to be a feasible method for long term preservation of *H.erinaceus* mushroom to retain its neurotrophic properties. Thus, in this study, the filtrate of boiled freeze-dried powder of *H.erinaceus* was freeze-dried once again to allow longer storage periods.

The neuroactive components from *H.erinaceus* were mainly extracted using organic solvents such as acetone, ethanol, chloroform and ethyl acetate. Yet, the aqueous extract of *H.erinaceus* was shown to promote neurite outgrowth in neuronal cells by stimulating the synthesis of extracellular NGF. Water is a universal solvent of high polarity. It is able to extract small molecule and water-soluble components including sugars, amino acids and glycosides. Lipophilic and non-polar compounds are most likely absent in a water extract (Houghton & Raman, 1998). Sugars such as arabinose, glucose, rhamnose, deoxyribose and galactose were reported to be present in the hot water preparation of locally grown *H.erinaceus*. Arabinose, a neutral sugar, was the major sugar component in the locally grown *H.erinaceus* but was not present in *H.erinaceus* grown in China (Choong et al., 2007). It is not known whether these sugars contributed to the neurotrophic activity of locally grown *H.erinaceus*. An exo-biopolymer purified from the liquid culture broth of *H.erinaceus* mycelium which consists of glucose, galactose, xylose, mannose and fructose enhanced the growth and neurite extension of PC12 cells (Park et al., 2002). Thus, there may be bioactive polysaccharides in the aqueous extract of *H.erinaceus* responsible for the neurite outgrowth stimulation in NG108-15. The aqueous extract of *H.erinaceus* warrants

further investigation to determine the presence of bioactive polysaccharides with potential neurite-inducing properties in the mushroom.

The aqueous extract of *H.erinaceus* promoted neurite outgrowth activity by inducing the synthesis of extracellular NGF. The extract did not confer protection against oxidative stress in neuronal cells despite several neuroprotective compounds were reported to be extracted from *H.erinaceus*. This suggests that the neuroactive compounds responsible for neurotrophic activity may not necessarily possess neuroprotective activity. Previous studies of hericenones and erinacines primarily focused on their NGF-inducing property. Neurotrophic factors including NGF have been reported to play a role in neuroprotection; thus, it was assumed that hericenones and erinacines would similarly possess neuroprotective activities. Further research efforts are required to investigate whether these neurotrophic compounds possess neuroprotective activity.

Although *H.erinaceus* has been extensively studied over the past two decades, there is still a great deal of information about the mushroom remains unknown. The underlying mechanisms of the neuroactive compounds, i.e. hericenones and erinacines, in stimulating NGF-synthesis are yet to be fully understood. It is important to investigate whether these compounds are transported across the blood-brain barrier and acts on the targeted brain site when *H.erinaceus* extract is administered orally. At present, *H.erinaceus* is sold as a dietary supplement for brain and nerve health; most of the therapeutic properties of these products are based on data obtained from *in vitro* and animal-based experiments. Therefore, it is necessary to conduct long-term double-blinded, placebo-controlled clinical studies with large trial populations to validate the safety and efficacy of *H.erinaceus* in enhancing brain function and preventing memory impairment. Further studies should focus on the signalling pathways employed by *H.erinaceus* in expressing neurotrophic and neuroprotective activity. It is worthwhile to investigate the signalling pathway employed by the combined treatment

of *H.erinaceus* aqueous extract and NGF resulting in additive effect in neurite outgrowth activity of NG108-15 as seen in this study. Efforts to explore the therapeutic potential of *H.erinaceus* in the prevention and treatment of dementia remain imperative.

5.5 Conclusion

In this study, the aqueous extract of mushroom *H.erinaceus* grown in the tropical climate of Malaysia was studied for its cytotoxic, neurotrophic and neuroprotective actions.

This study concluded that the aqueous extract of *H.erinaceus*:

- a) was not cytotoxic to human lung fibroblast, MRC-5 and neural hybrid cell line, NG108-15,
- b) elicited neurite outgrowth stimulation in NG108-15 cells by inducing the synthesis of extracellular NGF,
- c) enhanced the neurite outgrowth stimulation activity of NGF in NG108-15 when combined with suboptimal concentrations of NGF, and
- d) did not exhibit neuroprotective activity in NG108-15 cells under oxidative stress.