COMPARATIVE STUDY ON NEUROPROTECTION OF AQUEOUS AND ETHANOLIC EXTRACTS OF *Calocybe indica* (PURKAYASTHA & CHANDRA) IN NEURO-2A-BLASTOMA CELL

MUHAMMAD TAHA BENNETT

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MUHAMMAD TAHA BENNETT

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COMPARATIVE STUDY ON NEUROPROTECTION OF AQUEOUS AND ETHANOLIC EXTRACTS OF *Calocybe indica* (PURKAYASTHA & CHANDRA) IN NEURO-2A-BLASTOMA CELL

ABSTRACT

Mushrooms have gained worldwide popularity for both their nutritional and medicinal properties. Despite thousands of edible mushrooms worldwide, only a handful of mushrooms are available on a commercial scale or have been extensively studied for their therapeutic properties. A lot of research on health properties of mushrooms focus on chronic diseases such as cancer, heart disease and diabetes. In particular, a lot of research is currently focusing on neurodegenerative diseases such as Alzheimer's disease. The current research focuses on *Calocybe indica* Purkayastha & Chandra (milky white mushroom), a relatively unknown mushroom. Fresh fruiting bodies of mushrooms were sliced then either freeze-dried or oven-dried before extracted using aqueous and ethanolic (99.8%) solvents. Cultures of mouse Neuro-2a-blastoma (N2a) cells were seeded into a 96-well plate and assessed by using a colorimetric 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay with varying concentrations of the mushroom extracts (0-500 µg/mL) in order to determine potential neuroprotective properties of the mushroom. A negative control of N2a cells was used to determine cell viability and a positive control of H_2O_2 was used to determine cell toxicity. Oven-dried aqueous extract of *Calocybe indica* showed the highest cell viability (121%), followed by oven-dried ethanol extract (113%), freeze-dried aqueous extract (112%), and freeze-dried ethanol extract (105%). Neuroprotection against H_2O_2 followed a similar trend. Lower concentrations of *Calocbye indica* correlated with higher cell viability and neuroprotection. The total phenolic content and antioxidant activity of the mushroom were evaluated using a Folin-Ciocalteu reagent and 2,2-diphenyl-1- picrylhydrazyl (DPPH) dye.

Oven-dried ethanol extracts showed the highest neuroprotective effects, the second highest total phenolic content (5.04 mg GAE/50 g dried mushroom weight), and the third highest antioxidant activity (12.89 mg/mL). Freeze-dried ethanol extracts showed the highest total phenolic content (7.2 mg GAE/50 g dried mushroom weight) and the highest antioxidant activity (EC_{50}=8.63 mg/mL) despite having the lowest neuroprotective effects. A moderate positive correlation (R^2 = 0.61) was found between total phenolic content and antioxidant activity. While phenolic content plays a considerable role in antioxidant activity, there is reasonable evidence that non-phenolic compounds contribute greatly to antioxidant activity and neuroprotection.

Keywords: milky white mushroom, mouse neuroblastoma, neuroprotection, hydrogen peroxide

KAJIAN PERBANDINGAN NEUROPROTECTION EKSTRAK AQUEOUS DAN ETANOLIK *Calocybe indica* (PURKAYASTHA CHANDRA) DALAM SEL NEURO-2A-BLASTOMA

ABSTRAK

Cendawan telah mendapat populariti di seluruh dunia kerana mempunyai kedua-dua sifat yang berkhasiat dan ciri-ciri perubatan. Walaupun beribu-ribu cendawan boleh dimakan di seluruh dunia, hanya segelintir cendawan boleh disediakan pada skala komersil ataupun telah banyak dikaji untuk sifat-sifat terapeutik mereka. Kebanyakan kajian menunjukkan bahawa sifat perubatan cendawan hanya tertumpu kepada penyakit-penyakit kronik seperti kanser, penyakit jantung dan kencing manis. Terdapat sejumlah penyelidikan kini sedang tertumpu kepada penyakit neurodegeneratif seperti penyakit Alzheimer secara khusus. Kajian yang dijalankan ini memberi tumpuan kepada *Calocybe indica* Purkayastha & Chandra (cendawan melati), iaitu sejenis cendawan yang agak tidak diketahui. Cendawan yang segar telah dihiris kemudian dibeku kering atau dikeringkan dengan ketuhar sebelum diekstrak dengan pelarut air dan etanol (99.8%). Budaya garis sel tikus Neuro-2a-blastoma (N2a) dinilai dengan menggunakan kolorimetri ujian MTT (*3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide*), dengan menggunakan konsentrasi ekstrak cendawan yang berbeza (0-500 μg/mL) untuk mengkaji potensi neuroproteksi dalam cendawan. Kawalan negatif sel N2a digunakan untuk menentukan daya maju sel dan kawalan positif H_2O_2 digunakan untuk menentukan ketoksikan sel. Akueus ekstrak *Calocybe indica* yang telah dikeringkan menggunakan ketuhar menunjukkan viabiliti sel yang tertinggi (121%), diikuti dengan etanol ekstrak yang dikeringkan dengan ketuhar (113%), akueus ekstrak yang dikering beku (112%), dan etanol ekstrak yang dikering beku (105%). Neuroproteksi terhadap H_2O_2 juga menunjukkan aliran yang sama. Konsentrasi *Calocybe indica* yang rendah menunjukkan korelasi yang tinggi dengan viabiliti sel dan neuroproteksi.

Jumlah kandungan fenolik dan aktiviti antioksida juga telah dikaji dengan mengaplikasikan kaedah Folin-Ciocalteu dan 2,2-difenil-1-pikrilhidrazil (DPPH). Etanol ekstrak yang dikering menggunakan ketuhar menunjukkan kesan neuroproteksi yang tertinggi, kedua tertinggi ialah kandungan fenolik total (5.04 mg GAE/50 g cedawan kering) dan ketiga tertinggi ialah aktiviti antioksidan (EC_{50}=12.89 mg/mL). Ekstrak etanol yang dikering beku menunjukkan jumlah kandungan fenolik tertinggi (7.2 mg GAE/50 g berat cendawan kering) dan aktiviti antioksidan tertinggi (EC_{50}=8.63 mg/mL) biarpun mempunyai kesan neuroprotektif terendah. Terdapat korelasi positif sederhana (R^2=0.61) diantara kandungan fenolik dengan aktiviti antioksidan. Walaupun kandungan fenolik memainkan peranan yang cukup besar dalam aktiviti antioksidan,
ada bukti yang munasabah bahawa sebatian bukan fenolik sangat menyumbang kepada aktiviti antioksidan dan neuroproteksi.

Kata kunci: cendawan melati, neuroblastoma tikus, neuroproteksi, hidrogen peroksid

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Muhammad Taha Bennett

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α	:	Alpha
β	:	Beta
μ	:	Micro
%	:	Percent
ABTS	:	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AD	:	Alzheimer's disease
Bax	:	Apoptosis regulator BAX
Bcl-2	:	Bcl-2 family protein
BHA	:	Beta hydroxy acid
BHT	:	Butylated hydroxytoluene
C.I.	:	*Calocybe indica*
R^2	:	Coefficient of determination
CAT	:	Catalase
COX-2	:	Cyclooxygenase-2
CUPRAC	:	Cupric ion reducing antioxidant capacity
DLPE	:	Dilinoleoyl-phosphatidylethanolamine
DMEM	:	Dulbecco's modified eagle medium
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	:	50% effective concentration
ERK ½	:	Extracellular signal-regulated kinase ½
FBS	:	Fetal bovine serum
FC	:	Folin ciocalteu
FD	:	Freeze-dried

FDA	:	Food and Drug Administration
FRAP	:	Ferric reducing ability of plasma
g	:	gram
GAE	:	Gallic acid equivalent
Gpx	:	Glutathione peroxidase
GSH	:	Reduced glutathione
H_2O_2	:	Hydrogen peroxide
HD	:	Huntington's disease
HIV	:	Human immunodeficiency virus
HOCl	:	Hypochlorous acid
IL-1β	:	Interleukin 1β
iNOS	:	Nitric oxide synthase
JNK	:	c-Jun N-terminal kinase
MAPK	:	p38 mitogen-activated protein kinase
MCA	:	Middle cerebral artery occlusion
MDH	:	Malate dehydrogenase
mg	:	milligram
mL	:	millilitre
MRP	:	Maillard reaction products
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	:	Monounsaturated fatty acids
N2a	:	Neuro-2a blastoma cells
NCDs	:	Non-communicable diseases
NIH	:	National Institutes of Health
$NO^{\bullet}$	:	Nitric oxide

NO_2	:	Nitrogen dioxide
$O2^{\bullet}$	:	Superoxide
OD	:	Oven-dried
$OH^{\bullet}$	:	Hydroxyl
PC-12	:	Pheochromocytoma-12 cell line
PD	:	Parkinson's disease
PDH	:	Pyruvate dehydrogenase
PKC	:	Protein kinase C pathway
PUFA	:	Polyunsaturated fatty acids
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
SDH	:	Succinate dehydrogenase
SOD	:	Superoxide dismutase
TBHQ	:	tert-Butylhydroquinone
TNF-α	:	Tumour necrosis factor-α

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CHAPTER 1: INTRODUCTION

'Mushrooms are macro-fungi with distinctive and visible fruiting bodies that may grow above or below ground, formed from spacious underground myella (hyphae) by the process of frutification' (Zeng et al., 2012). Mushrooms are taxonomically classified in two different groups: Basidiomycetes, which include many known genera, and Ascomycetes. When scientists refer to fungi for medicinal purposes, they frequently refer to the phylum Basidiomycetes or Ascomycetes. Together, they are often referred to as the "higher fungi" (Mattos-Shipley et al., 2016). Higher fungi are of therapeutic interest for their pharmacological action in promoting human health for over a millennium. In particular, Asia has traditionally been and is still exploiting mushrooms for medicinal purposes and treating major diseases (Poucheret et al., 2006). Today, mushrooms are officially designated as 'healthy food' by the Food and Drug Administration (FDA) and the National Institutes of Health (NIH) is currently conducting clinical studies using mushrooms to treat patients suffering from chronic diseases such as cancer, HIV, obesity and neurological disorders (Stamets, 2005).

Among the recognized 14,000 species of mushrooms, at least a couple thousand in 30 genera have been identified as fit for human consumption (Sabaratnam et al., 2013). Despite the 270 potential species of mushrooms considered for their therapeutic or preventive remedies, only about 25 species are commercially available with the top three being *Agaricus bisporus*, *Lentinula edodes*, and *Pleurotus ostreatus* (Corrêa et al., 2016).

Apart from being praised for their texture and flavour, edible mushrooms are also very nutritional. In addition to being a low-calorie food, they are low in fat yet rich in polyunsaturated fatty acids (PUFA), have all the 9 essential amino acids, are high in fibre and a rich source of vitamins B to E (Rathee et al., 2012; Valverde et al., 2015). Thus, mushrooms are an excellent functional food that can be used directly for its overall effects in the human diet to promote health.

Despite living in the age of modern medicine, increasing aging population and unhealthy lifestyle practices have given rise to a variety of noncommunicable diseases (NCDs), more commonly known as chronic diseases. A high proportion of senior citizens (projected around 80%) will have at least one long-term health-related disease (Millington et al., 2014) In particular, neurodegenerative diseases such as Alzheimer's (AD), Huntington's (HD) and Parkinson's (PD) diseases cause losses of motor and cognitive function which can be very distressing for the elderly (Sabaratnam et al., 2013). One of the key features in the development of many human's diseases is oxidative stress. In particular, it is believed that the main factors behind neurodegenerative diseases are the result of oxidative stress-mediated cell injury via apoptosis or necrosis.

When reactive oxygen species (ROS) levels go beyond the threshold capacity of a cell's natural defence; the ROS scavenging antioxidant defence system is said to be stressed (Xiong et al., 2016). In other words, foods rich in antioxidants may be able to retard neurodegenerative disease progression as a result of their neuroprotective effects. A great deal of current *in vitro* and *in vivo* studies have focused on anti-inflammatory, antioxidant, and neuroprotective properties of either crude mushroom extracts or their isolated bioactive compounds (Zhang et al., 2016).

Calocybe indica, or milky mushroom, was first reported and cultivated in India (Phutela & Phutela, 2012) and is gaining popularity in tropical countries like Malaysia due to its tolerance for high temperature and humidity (Subramanian & Shanmugasundaram, 2015). The name is derived from the ancient Greek terms kalos "pretty" and cubos "head". In Orissa, it is known as *dudha chhatu* and in some places they are called *kuduk* (as cited in Anju, 2013).

Optimizing cultivation of *Calocybe indica* using different substrates have been reported in Bangladesh (Alam et al., 2010) and India (Jadhav et al., 2014) and reported to have higher biological efficiency than oyster mushrooms (Navathe et al., 2014). It has been studied for antioxidant properties (Mirunalini et al., 2012; Mowsumi et al., 2015), anti-hyperglycemic activity (Krishnakumari et al., 2013), anti-cancer potential (Gurunathan et al., 2015; Selvi et al., 2011), immunoenhancing polysaccharides (Maity et al., 2011; Mandal et al., 2012), and anti-lipid peroxidative potential (Selvi et al., 2010; Selvi et al., 2006).

In the present study, *Calocybe indica* was extracted using aqueous and ethanol solvents. The most common solvents used in research of crude mushroom extracts are either hot water, miscible solvents such as ethanol and methanol, or a combination of an aqueous and miscible solvent (Abugria & McElhenny, 2013; Mau et al., 2002; Tsai et al., 2007).

Numerous major bioactive compounds in mushrooms such as polysaccharides, triterpenes, phenols, sterols, and proteins have been identified and characterized in mushrooms with known medicinal properties (Taofiq et al., 2016). In particular, a lot of neuroprotective studies focus on the antioxidant effects of bioactive phenolic compounds found in mushrooms (Cui et al., 2002; Lee et al., 2003a; Lee et al., 2003b; Nukata et al., 2002; Phan et al., 2016). Several antioxidant assays can be done to evaluate the scavenging activity of mushrooms based on electron transfer including 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Cupric Ion Reducing Antioxidant Capacity (CUPRAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu (FC) and ferric reducing antioxidant power (FRAP) methods, each using different chromogenic redox reagents with different standard capabilities (Apak et al., 2007). Hydrogen peroxide (H_2O_2) is a common reactive oxygen species used to evaluate oxidative damage in neuronal cells (Hardaway et al., 2012; Hazekawa et al., 2010; Heo et al., 2012; Hu et al., 2014).

In addition to studies neuroprotective properties of bioactive compounds, an abundance of research has focused on optimizing the extraction of mushrooms through a combination of various drying methods and solvents. Although some research has concluded freeze-dried extracts are more suitable for preserving the bioactive compounds in mushrooms (Ma et al., 2013; Zhang et al., 2009), other findings suggest that heat treatments such as oven-drying or microwave enhance the antioxidant activity (Choi et al., 2006; Tian et al., 2016).

Objectives:

The objectives of the current study were to:

a) evaluate the effects of drying methods on moisture content of dried *Calocybe indica* extracts.

b) evaluate the toxicity and neuroprotective properties of *Calocybe indica* on neuro-2a- blastoma cell.

c) determine the total phenolic content and antioxidant activity of *Calocybe indica* extracts.

CHAPTER 2: LITERATURE REVIEW

2.1 Mushrooms in neurodegenerative research

The economic cost of neurodegenerative disease is enormous and is projected to grow considerably with increased ageing populations worldwide. In the US, at least 100 million Americans suffer from some neurological disease, with 7 million being dementia-related cases. Dementia-related chronic diseases alone cost Americans USD 243 billion annually, with Alzheimer's disease being the most prevalent (Gooch et al., 2017). Mushrooms are not only considered a functional food because in addition to being highly nutritious they contain diverse yet exclusive bioactive compounds that are not found in plants or animals. Hence, they might have beneficial effects on human health that go beyond basic nutrition (Phan et al., 2017). Common neuronal research of mushrooms includes studies on their antioxidant and neuroprotective effects.

2.1.1 Antioxidants

Oxidation is the production of free radicals in food and chemicals and is also a necessary part of the biological processes in living organisms. Reactive oxygen species (ROS), commonly referred to as free radicals, contribute to various human ailments such as aging, cancer, inflammatory and cardiovascular diseases (Barku et al., 2013). ROS can be generated internally during exercise and inflammation as well as in certain organelles such as mitochondria and peroxisomes. In addition, ROS can be produced from external sources such as ultraviolet light, ionizing radiation, industrial chemicals, and air pollutants (Lobo et al., 2010).

ROS include free radicals such as superoxide (O2•-) and hydroxyl (OH•), and non-free radicals such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Others are reactive nitrogen species (RNS) including nitric oxide (NO•) and nitrogen dioxide (NO_2) (Diaz et al., 2012). These deleterious radicals are normally kept in check by a well-developed antioxidant defence system within our bodies but can sometimes be overwhelmed (Chakrabarti et al., 2011). However, overproduction of ROS results in oxidative stress which can damage cells, cell organelles, and DNA. In particular, the mitochondrial lipid membrane is easily attacked by ROS and often referred as lipid peroxidation (Khatua et al., 2013).

Antioxidants are generally divided into two categories; natural and synthetic. Natural antioxidants are categorized into plant and fungal extracts. These include phenolics, flavonoids, organic acids, monoterpenoids, diterpenoids, trace elements such as zinc (Zn) and selenium (Se), vitamins A, B, C and E, synthetic antioxidants including compounds like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate, *tert*-butylhydroquinone (TBHQ) can effectively inhibit oxidation (Khatua et al., 2013; Kozarski et al., 2015). Even though some synthetic antioxidants are authorized for use as food additives, they may cause adverse toxic effects under certain conditions (Aksoy et al., 2013). As a result, there is a rapid growing interest in naturally derived antioxidants.

According to Ferreira et al. (2009) the phenolic compounds are the main source of antioxidants found in mushrooms which are reported to have protective role against chronic diseases related to oxidative stress. In contrast, a review by Zhang et al. (2016) considers the polysaccharide content in mushrooms as the main contributor to antioxidant activity. Unfortunately, there is no single, universally accurate antioxidant profiling test due to several underlying mechanisms such as termination, donation, chelation and elimination (Abdullah et al., 2012).

Mushrooms have two main advantages over plants when considering antioxidant sources. Mushroom can be grown more quickly and the growth requirements can be optimized more economically. Barros et al. (2007) measured the antioxidant profile of *Lactarius piperatus* fruiting bodies in four different stages of maturity. Mature mushrooms with closed caps and immature spores (stage 2) showed the highest antioxidant profile with significantly higher phenolic, flavonoid, β-carotene, and lycopene than any other growth stages. However, Soares et al. (2009) showed little difference in phenolic content between young and mature *Agaricus brasiliensis*. While both measured similar levels of scavenging activity, reducing power, and lipid peroxide inhibition, mature *Agaricus brasiliensis* had significantly higher chelating ability. Five mg/mL of mature *Agaricus brasiliensis* was able to chelate 50% of ferrous ions compared to 13 mg/mL of young *Agaricus brasiliensis*.

Jayakumar et al. (2011) did an extensive study on the *in vitro* and *in vivo* antioxidant activity of *Pleurotus ostreatus*. Methanolic extracts from a number of commercial mushrooms including *Lentinula edodes*, *Volvariella volvacea*, *Flammulina velutipes*, and *Pleurotus ostreatus* have been shown to inhibit lipid peroxidation very effectively, which can be correlated to their phenolic contents (Zeng et al., 2012).

Likewise, Vamanu & Nita (2013) found that ethanol and methanol solvents showed higher scavenging and reducing activity compared to hot and cold-water extracts of *Boletus edulis*. Ethanol extracts recorded the highest level of tested antioxidant components including phenols, flavonoids, anthocyanins, ascorbic acid, rosmarinic acid, β- carotene, lycopene, and tocopherol.

Similarly, Tsai et al. (2007) reported higher antioxidant activities in ethanolic extracts than hot water extracts of *Agaricus blazei*, *Agrocybe cylindracea*, and *Boletus edulis*. Sudha et al. (2016) found that methanol extracts showed a higher phenolic content than aqueous extracts for *Pleurotus djamor*. A positive correlation between phenolic content and antioxidant activity showed that compounds contained in methanol extracts were active free radical scavengers, reducing agents, and metal chelators.

In contrast, Gan et al. (2013) found that water was a more suitable solvent than 60% ethanol for total phenolic content recovery for both *Agaricus bisporous* and *A. brasiliensis* due to its polarity. The lower water content in *A*garicus *brasiliensis* attributed to drying was used to partially explain the higher phenolic content. Similarly, Pal et al. (2010) found that hot water extracts of *Pleurotus squarrosulus* were higher in phenolic, flavonoid, β-carotene, and lycopene content compared to cold water and methanol extracts. Shi et al. (2002) found that cold aqueous extracts of *Agaricus bisporus* and hot aqueous extracts of *G. lucidum* significantly protected DNA of human lymphocyte cells from oxidative stress.

Jayakumar et al. (2007) found that extracts of *Pleurotus ostreatus* showed therapeutic potential in the brain, heart, kidney, and liver of aged rats against oxidative stress. Furthermore, the extracts showed elevated activity of major enzyme antioxidant defence systems such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), and reduced glutathione (GSH). Jeong et al. (2010) found that extracts of *Agaricus bisporus* improved levels of unhealthy blood glucose, cholesterol, triglycerides, and lipoproteins in rats. Liu et al. (2010) showed that crude extracts of *Cordyceps sinensis* improved recovery of rat brains suffering from cerebral artery occlusion while Wang et al. (2012) attributed the isolated peptide cordymin as a neuroprotective agent because of its anti-inflammatory and antioxidant properties.

2.1.2 Neuroprotective properties of mushrooms

Unlike animals and plants, fungi do not ingest their food or make their own food through photosynthesis but rely on decaying material as an energy source. Fungi contain chitin rather than cellulose found in plants as well as sterols and ergosterol rather than cholesterol found in animal cells (Feeney et al., 2014). Hence, fungi play an important role as natural products, most notably for their secondary metabolites that fight off infection, improve immunity, and lower cholesterol levels (Alam et al., 2008). Many edible species, however, are also regarded for their specific protective effects relating to the nerves and brain.

Due to advances of medicine and technology, society is experiencing an increased ageing population. Ageing is associated with a deterioration in immune capability and the beginning of chronic inflammation and oxidative stress leading to neurodegenerative diseases (Zhang et al., 2016). It has been estimated that close to 80 million people will suffer from some form of dementia in the next two decades (Phan et al., 2015b). Alzheimer's disease is mainly an aging disorder which results in the loss of cognitive function such as memory. It is characterized biologically by the death of neurons in various parts of the brain along with the presence of amyloid deposition (Sabaratnam et al., 2013). Like numerous other chronic diseases, oxidative stress plays a key role in the progression of neurodegenerative diseases such as Alzheimer's which is why a lot of neuroprotective studies on mushrooms focuses on their antioxidant properties. Neuroprotective effect against excitotoxicity and oxidative stress have been reported on crude and fractionated mushroom extracts including bioactive compounds as summarized in Table 2.1.

Lee et al. (2002) showed neuroprotective effects of dictyoquinazols extracted from *Dictyophora indusiata* protected primary cultured mouse cortical neurons from glutamate-induced excitotoxicity. Similarly, Lee et al. (2003a) also showed *p*-ter-phenyl curtisians isolated from *Paxillus curtisii* protected against glutamate-induced excitotoxicity in mouse N18-RE-105 cortical cells. Lee et al. (2003b) found that the neuroprotective mechanism of *p*-terphenyl leucomentis isolated from the mushroom *Paxillus panuoides* demonstrated neuroprotective properties against glutamate and H_2O_2 toxicity in mouse cortical neurons. The findings also reported that H_2O_2-induced neurotoxicity are iron-mediated, resulting from the iron-catalysed production of hydroxyl radicals. Thus, leucomentis is considered a neuroprotectant because of its ability to chelate iron rather than as a free radical scavenger.

Table 2.1 Neuroprotective properties of mushrooms (*in vitro* and *in vivo*)

Extract	Target organ/cells	Antioxidants	Reference
Dictyophora. indusiate, *Paxillus curtisii*, *Paxillus panuoides*	Mouse cortical cells	Dictyoquinazols, *p*-ter-phenyl curtisians, and *p*-terphenyl protect against glutamate-induced excitotoxicity and H_2O_2	Lee et al., 2002; 2003a; 2003b
Ganoderma lucidium	Rats	Oligosaccharides show neuroprotective anticonvulsant protection against seizures.	Aguirre-Moreno et al., 2013
Ganoderma lucidum	Rat brain	Neuroprotective properties upregulate mitochondrial enzyme complexes.	Ajith et al., 2009
Ganoderma lucidium	Rat spine	Crude polysaccharides show significant neuroprotective in spinal injuries.	Gokce et al., 2015
Hericium erinaceus	Mice brain	Cerebro-protective effects against middle cerebral artery occlusion (MCA).	Hazekawa et al., 2010
Hericium erinaceus	PC-12	Protection against H_2O_2	Lee et al., 2010
Hericium erinaceus	Rat brain	Erinacine A prevents neural death in stroke-induced brain injury.	Lee et al., 2014
Hericium erinaceus	N2a	Dilinoleoyl-phosphatidylethanolamine (DLPE) extracts protect endoplasmic reticulum (ER)	Nagai et al., 2006
Hericium erinaceus	Rat peroneal nerves	Early-stage axon regeneration.	Wong et al., 2011
Inonotus obliquus	PC-12	Crude water-soluble and alkali-soluble extracts polysaccharides showed protection against H_2O_2	Mu et al., 2012
Morchella importuna	PC-12	Crude polysaccharides showed protection against H_2O_2	Xiong et al., 2016

In a study on a human skin cell line, Cui et al. (2005) reported that polyphenolic extracts of *Inonotus obliquus* showed strong protective effects against H_2O_2-induced oxidative stress in human keratinocyte cell line (HaCaT) while triterpenoids, steroids and water-soluble polysaccharides were not found to show any protection. In contrast, a study on a mouse nerve cell line by Mu et al. (2012) found that both water-soluble and alkali-soluble crude polysaccharides in *Inonotus obliquus* showed neuroprotection against H_2O_2 toxicity in pheochromocytoma (PC-12) cells.

A few neuroprotective studies have focused on cell organelles and signalling pathways involving protein kinases and apoptotic factors. For example, Lee et al. (2010) found that pepsin-treated extracts of *Hericium erinaceus* showed neuroprotection against H_2O_2 toxicity in PC-12 cells through the mitochondrial apoptotic pathway by decreasing the expression of proapoptotic protein (Bcl-2) and increasing the expression of antiapoptotic protein (Bax). Nagai et al. (2006) found that dilinoleoyl-phosphatidylethanolamine (DLPE) extracted from *Hericium erinaceus* protected the endoplasmic reticulum (ER) in neuro-2A-blastma cell (N2a) against cell death possibly via the protein kinase C (PKC) pathway. Lee et al. (2014) found that erinacine A, extracted from *Hericium erinaceus*, prevented neural death in rats subjected to stroke-induced brain injury by suppressing reactive nitrogen species and down-regulating the iNOS and p38 MAPK pathways.

Wong et al. (2011) found that daily oral administration of aqueous extracts of *Hericium erinaceus* was capable to regenerate early-stage axon of injured rat peroneal nerves. In addition, Hazekawa et al. (2010) reported the cerebro-protective effects of *Hericium erinaceus* against cerebral infarctions induced by middle cerebral artery occlusion (MCA) in mice.

Xiong et al. (2016) reported that crude polysaccharides from *Morchella importuna* showed protection against H_2O_2 in PC-12 cells by improving ERK expression while downregulating JNK1/2. Ajith et al. (2009) also found that extracts of *Ganoderma lucidum* showed neuroprotective properties by upregulating complex I and II, succinate dehydrogenase (SDH), malate dehydrogenase, (MDH), a-ketoglutarate dehydrogenase (a-KGDH), and pyruvate dehydrogenase (PDH) in the mitochondria of rat brains. Gokce et al. (2015) showed that polysaccharides from *Ganoderma lucidium* had significant neuroprotective effects against spinal injuries in rats. Aguirre-Moreno et al. (2013) discovered that oligosaccharides from *Ganoderma lucidium* demonstrated neuroprotective anticonvulsant properties against kainic acid-induced seizures in rats.

Some neuroprotective research involving mushrooms focused on the involvement of inflammatory and cytotoxic factors in neurodegenerative diseases (Chang et al., 2011; Hu et al., 2014). Microglial cells, the primary form of active immune defence in the central nervous system (CNS), are chronically activated in patients with Alzheimer's and Parkinson's, releasing pro-inflammatory cytokines which disrupt normal CNS activity (Wang et al., 2015). Zhang et al. (2011) found that *G. lucidum* extracts protected dopaminergic neurons against inflammatory damage induced by microglia-derived toxic factors such as nitric oxide (NO), tumour necrosis factor-α (TNF-α), interleukin 1β (IL-1β) and superoxide.

Similarly, Peng et al. (2015) found that cordycepin extracted from *Cordyceps militaris* protected MES 23.5 cells by reducing release of TNF-α and IL-1β from rat microglial cells. The anti-inflammatory properties of cordycepin downregulate nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2); two enzymes involved in microglia mediated inflammation.

2.2 Extraction of bioactive compounds from mushrooms

Numerous studies have also tried to optimize recovery of bioactive compounds and improve antioxidant activity in mushrooms using a variety of techniques; primarily solvents coupled with heat, electromagnetic radiation or high pressure (Mendiola et al., 2007; Tian et al., 2012). Barros et al. (2007) found low heating temperatures (40˚C) of dried samples improved the content of bioactive compounds in *Lactarius deliciosus*, *Macrolepiota mastoidea*, *Macrolepiota procera*, and *Sarcodon imbricatus* while higher temperatures of cooked samples in olive oil showed deleterious effects. Cooking was found to increase the monounsaturated fatty acid (MUFA) content while decreasing the polyunsaturated fatty acid (PUFA) and sugar content. It was concluded that cooking possibly leads to heat transformation of fatty acids into glucose. For example, linoleic acid might be converted into 1-octen-3-ol or trehalose, a glucose disaccharide, might be converted into glucose.

Ju et al. (2010) tested the effect of steam treatment (120˚C, 3 h) on the antioxidant profile of *Inonotus obliquus*. Steam treatment proved to be an effective method for improving the radical scavenging activity by liberating low molecular weight free phenolic acids. The concentrations of vanillic acid, protocatechuic acid, syringic acid, and 2,5-dihydroxyterephthalic acid were significantly improved by steam treatment.

Ma et al. (2013) found that both polysaccharides yield and antioxidant activity were higher in freeze-dried extracts of *Inonotus obliquus* compared to oven-dried and vacuum-dried extracts. Zhang et al. (2009) found that antioxidant recovery for freeze-drying was higher than oven, vacuum or sun-drying methods in *Lentinula edodes*. Zhang et al. (2013) found that freeze-drying was beneficial for the stability of eritadenine and protein in *Lentinula edodes* while hot air drying (50 ˚C) improved the total phenolics, amino acid, uronic acid and neutral sugar content.

Results reported by Choi et al. (2006) showed that a prolonged heating time (30 min) and higher heating temperature (121˚C) significantly enhanced the overall antioxidant profile of *Lentinula edodes*. It was concluded that high heating temperatures improved the antioxidant profile of mushrooms either by releasing free polyphenolic and flavonoids from the insoluble portions of mushrooms such as the cell wall or by the formation of novel compounds having antioxidant activities.

Li & Shah (2013) measured the free, bound and total phenolic content of freeze-dried, oven-dried, and boiled extracts of *Pleurotus eryngii*. The total phenolic content was highest in freeze-dried extracts followed by oven-dried and boiled extracts, respectively. Freeze-dried extracts showed higher recovery of bound phenolics while oven-dried extracts were higher for free phenolic recovery. Boiling significantly reduced the total phenolic content indicating thermal or oxidative degradation of phenolics.

Abugria & McElhenney (2013) reported the total phenolic and flavonoid content of five different mushrooms using methanol, ethanol and water solvents. No single solvent was ideal. Ethanol and methanol extracts yielded higher phenolic and flavonoid content in *Fomes fomentarius* and *Ganoderma applanatum*. In contrast, water extracts yielded higher phenolic and flavonoid content in white and brown *Agaricus bisporus* and *Trametes versicolor*. The moisture content of dried mushroom fruiting bodies, solvent polarity and the formation of protein complexes with phenolics or flavonoids may all play a role in the extractability of antioxidant compounds.

A couple of studies on *Agaricus bisporus* found that vacuum oven-dried extract showed comparable or higher total phenolic content and antioxidant activity than freeze-dried extracts (Giri & Prasad, 2009; Ji et al., 2012). Wong et al. (2009) found that the total phenolic content of oven-dried extracts was 3-fold higher than freeze-dried extracts in *Hericium erinaceus* and also showed higher scavenging activity.

The result of heat treatment of fruit bodies may not only help release phenolic compounds but also increase the generation of Maillard's reaction products (MRPs). The protein-polysaccharide conjugates formed through the Maillard reaction are often cited in literature as the cause of the antioxidant and antimicrobial properties of mushrooms (Ju et al., 2010; Wong et al., 2009). The extent of the Maillard reaction can be measured by several colorimetric assays which measure the average number of lysine amino acids that react with polysaccharides (Oliveira et al., 2016).

2.3 *Calocybe indica* and its medicinal properties

There are numerous mushrooms which are cultivated in Malaysia, but only eight are currently grown on an industrial scale; *Pleurotus pulmonarius* (grey oyster), *Pleurotus flabelletus* (red oyster), *Pleurotus floridanus* (white oyster), *Ganoderma applanatum*, *Lentinula edodes* (shiitake), *Pleurotus cystidiosus* (abalone), *Volvariella volvacea* (paddy straw mushroom) and *Auricularia polytricha* (jelly mushroom). While these mushrooms have been utilized for a long time in Malaysia, *Calocybe indica* is relatively unknown despite its biological efficiency as a native tropical mushroom (Amin et al., 2014).

Calocybe indica, a summer-grown tropical edible mushroom, of the class Basidiomycetes, belonging to the family Tricholomataceae of the order Agaricales. More commonly known as milky white mushroom, it is commonly cultivated in countries such as India and Bangladesh (Figure 2.1).

Figure 2.1 *Calocybe indica* (Photo taken by Dr. Tan Yee Shin)

Apart from being rich source of carbohydrates, amino acids, vitamins and fibres, *Calocybe indica* is becoming popular due to its appearance, organoleptic properties, sustainable yield, and long shelf-life (Alam et al., 2010; Amin et al., 2010). It can be considered as a high calorific value food among other mushrooms; higher protein content than that of *Auricularia* and *Lentinus* species while its soluble sugars are almost double that of *Pleurotus sajor-caju* (Kaur, 2016). Sorghum and wheat straw have been found to be the most suitable substrates for *Calocybe indica* spawn production (Subbiah & Balan, 2015).

Apart from nutritional and optimization studies, a growing number of studies have been conducted on the antioxidant, anti-inflammatory, anti-cancer and immunoenhancing properties of *Calocybe indica*. Mirunalini et al. (2012) found that antioxidant activity of *Calocybe indica* ethanol extracts increased in a dose-dependent manner. EC_{50} scavenging of DPPH radicals was recorded at 20 µg/ml. In contrast, Selvi et al. (2010) recorded an EC_{50} of 43 µg/mL for *Calocybe indica* ethanol extracts. Mowsumi et al. (2015) found that methanol extracts of *Calocybe indica* showed moderate antioxidant activity (3.9 mg/ml) most likely due to the loss of bioactive compounds following heat treatment.

Prabu et al. (2016) found that *Calocybe indica* was comparable to *Pleurotus florida* in terms of antioxidant activity and anti-inflammatory properties. Maity et al. (2011) found that water-soluble polysaccharides isolated from *Calocybe indica* showed an EC_{50} of 6.2 mg/mL against DPPH radicals.

A preliminary *in vitro* experiment by Selvi et al. (2006) found that aqueous *Calocybe indica* extracts were effective in inhibiting lipid peroxidation in goat liver and red blood cell membrane model. Ghosh (2015) found that oven-dried aqueous and methanol extracts of *Calocybe indica* were effective against cancer cell lines of Human Ewing's sarcoma MHH–ES-1 and breast cancer MCF-7.

Ethanolic extract of *Calocybe indica* showed significant inhibitory effect against carrageenan-induced inflammation in paw edema experimental rat models by nearly 50% (Prabu & Kumuthakalavalli, 2014). Govindan et al. (2016) treated ageing rats with D-galactose which induced oxidative damage as well as cognitive and mitochondrial dysfunction. It was found that 6 weeks of treatment with *Calocbye indica* crude polysaccharides significantly improved the learning and memory ability of rats in Morris water maze test.

Despite the growing popularity of cultivating *Calocybe indica* and a growing number of therapeutic studies on *Calocybe indica*, there remains a gap in research for its neuroprotective properties. To the best of our knowledge, there is no literature reported on the neuroprotective effects of *Calocybe* spp. A brief summary of *Calocybe indica* research is shown in Table 2.2. Therefore, the present study into the neuroprotective properties of *Calocybe indica* laid the basis to encourage the study of neurodegenerative diseases.

Table 2.2 Biological activity of *Calocybe indica* reported from the literature

Assay	Extract	Biological activity	Reference
In vitro	Ethanol extracts	Antioxidant activity	Mirunalini et al., 2012; Selvi et al., 2010
In vitro	Methanol extracts	Antioxidant activity	Mowsumi et al., 2015
In vitro and liver model	Aqueous extracts	Antioxidant, Anti-inflammatory	Prabu et al., 2016
Antioxidant and structural characterization	Water-soluble polysaccharides	Antioxidant	Maity et al., 2011
Liver and RBC model	Aqueous extracts	Antioxidant	Selvi et al., 2006
MHH – ES -1 and MCF -7	Aqueous and methanol extracts	Anti-cancer	Ghosh et al., 2015
Rat paw edema	Ethanol extracts	Anti-inflammatory	Prabu & Kumuthakalavalli, 2014
Rat memory in MWM test	Crude polysaccharides	Improved memory and learning	Govindan et al., 2016

CHAPTER 3: MATERIALS & METHODS

3.1 Preparation of mushroom extracts

The fruiting bodies of *Calocybe indica* were purchased from Nas Agro Farm, Sepang, Malaysia. Prior to extraction, fresh fruiting bodies were kept in the fridge (4°C) for 2 days prior to extraction. Two kilograms of fresh *Calocybe indica* fruiting bodies were sliced. After that, the fruiting bodies were either freeze-dried for two days at -50°C or oven-dried for 4 days between 40-45°C. All the processed mushrooms were powdered in a blender and kept in an airtight bottle and stored at 4-8°C prior extraction. The extraction method was based on Phan et al. (2012). For aqueous extraction, powdered freeze-dried or oven-dried aqueous fruiting bodies of *Calocybe indica* was soaked in distilled water (1:20, w/v) and was left in a Schott bottle overnight at room temperature. The aqueous mixture was then placed in a water bath and double boiled for 30 min. After cooling, the aqueous mixture was filtered (Whatman No 4). The filtered aqueous extract was freeze-dried and subsequently stored at −20°C before use. For ethanol extraction, powdered freeze-dried or oven-dried fruiting bodies of *Calocybe indica* was soaked in 99.8% ethanol (1:20, w/v) for three days at room temperature. The ethanol solvent was evaporated using a rotary evaporator (Eyela OSB-2000). All the extractions were done in triplicate.

3.2 Cell culture maintenance

Neuro-2a blastoma cells (N2a) (ATCC® CCL-131™) were maintained in 75 cm^2 flasks in DMEM containing 10% FBS, supplemented with glutamine (2 mM), streptomycin (100 μg/mL) and penicillin (100 U/mL). The cell lines were sustained in a humidified atmosphere (5% CO_2, 95% air) at 37°C and subcultured with 2 mL of trypsin every 3-5 days.

3.3 Moisture content of mushroom extracts

The moisture content of the mushroom extracts was calculated based on Alam et al. (2008). For freeze-dried sample, the initial fresh weight of *Calocybe indica* was weighed on a digital balance (Shimadzu AUW220D) after slicing and the final weight was recorded. Similarly, for oven-dried sample, the initial fresh weight of *Calocybe indica* was weighed after slicing and the final weight was noted. The moisture content was determined as a percentage using the following equation: Moisture (%) = (initial fresh weight – final dry weight) × 100 / final dry weight of sample

3.4 (4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT) Assay

MTT assay was conducted based on the method adopted from Baskaran et al. (2017). The N2a cells (1×10^4 cells per well) were seeded with DMEM into a 96-well flat-bottomed culture plate and incubated at 37°C overnight in a humidified atmosphere (5% CO_2, 95% air) to allow cell attachment. The supernatant was carefully discarded after which the cells were treated with different concentrations between 0-500 μg/mL of either aqueous or ethanolic extracts of *Calocybe indica* in fresh DMEM for another 24 h. Following this, 20 μL of MTT was added to each well for 2h. Subsequently, the supernatant was discarded again and 100 μL of DMSO was then added as a bleaching agent to dissolve the MTT formazan crystals.

The absorbance was recorded using a microplate reader (Sunrise Techan) at 570 nm with a reference wavelength at 630 nm. DMEM served as the blank, and cells incubated in DMEM only without *Calocybe Indica* extracts were used as a negative control.

Cell viability was expressed as a percentage of the cells in the extract sample relative to the control using the following formula:

Cell viability (%) = [(OD sample-OD medium) / (OD control -OD medium)] x 100%

3.5 Hydrogen Peroxide Assay

3.5.1 Determination of Half Maximal Effective Concentration (EC_{50})

An H_2O_2 pre-treatment assay was determined based on the method adopted from Hardaway et al. (2012) in order to calculate the EC_{50} of H_2O_2 in N2a cells. N2a cells (1 x 10^4 cells per well) were seeded with DMEM into a 96-well flat-bottomed culture plate and incubated in a humidified atmosphere (5% CO_2, 95% air) at 37˚C overnight to allow cell attachment. Cells were then replenished with fresh DMEM and co-incubated with different concentrations of H_2O_2 (0-200 μM) and plated for 24 h. Subsequently, 20 μL of MTT solution were added into each well and incubated for an additional 2 h to develop formazan crystals. A Pearson correlation shown in Appendix A (Figure A.1) of cell viability against hydrogen peroxide concentration was used to calculate the EC_{50}.

3.5.2 Toxicity Assay

Incubated N2a cells were seeded in a flat bottom 96-well plate with a total volume of 100 μL of fresh DMEM and a cell density 1x10^4 cells per well. The cells were incubated overnight to allow attachment. Following this, the supernatant was removed. N2a cells were then treated with five different concentrations for aqueous and ethanolic *Calocybe indica* (0-500 μM) and co-incubated with H_2O_2 (50 μM) in a final volume of 100 μL of DMEM. Cells plated with media only was used as a negative control and cells plated with media and H_2O_2 was used as a positive control.

3.6 Total Phenolic Content (TPC) Determination

In order to estimate the reducing capacity of phenolic content and other reducing agents in *Calocybe indica*, a Folin-Ciocalteu reagent (a mixture of phosphomolybdic and phosphotungstic acids) was used. The phenolic content concentration was measured using a method adapted by Yim et al. (2010).

Fifty microlitres of mushroom aqueous or ethanol extract (5 mg/mL) and 50 μL of 10% Folin-Ciocalteau reagent were mixed for 3 minutes. Then 100 μL of 10% aqueous sodium carbonate solution was supplemented to complete the mixture. After incubation for 1 h in the dark, the absorbance of the mixture was measured on a microplate reader at 700 nm. Gallic acid was selected as the standard (0-1 mg/mL) and the mixture without extract was used as a control. The final concentration of total phenolic content in *Calocybe indica* was calculated and expressed in mg gallic acid equivalent per 50 g of dried weight mushroom (mg GAE/ 50 g of dried weight).

3.7 2,2-Diphenyl-1- Picrylhydrazyl (DPPH) Radical Scavenging Activity

One method of measuring the free radical scavenging activity of food is using the diphenyl-1-picryl-hyrazyl (DPPH) assay. The scavenging activity is measured based on discolouration of the purple DPPH dye. The higher number of molecules of DPPH reduced by one molecule of the reductant, the more discolouration and higher scavenging activity present. Estimation of free radical scavenging activity of *Calocybe indica* was determined by a method adapted in Tan et al. (2015).

Five mg/mL of *Calocybe indica* extracts and a 100 μM methanolic solution of DPPH radicals were prepared. Ten microlitres of *Calocybe indica* extracts were mixed and shaken thoroughly with 90 μL of methanolic solution containing DPPH radicals. The mixture was wrapped in aluminium foil and left in the dark for 30 min. The absorbance was determined at 515 nm using a spectrophotometer. Five mg/mL of ascorbic acid (10 μL) was used as standard, methanolic solution of DPPH radicals was used as a control and water was used as a blank. The radical scavenging activity was calculated as percentage of DPPH reduced (%) using the following formula.

Scavenging activity (%) = [(Absorbance of control−Absorbance of sample) / Absorbance of control] x 100%

3.8 Statistical analysis

All analyses were carried out using at least 3 independent triplicate experiments (mean ± standard deviation). Cell viability, cell toxicity, total phenolic content and DPPH were presented in graphs using GraphPad Prism (ver. 8.0.2) and Excel (ver. 2016). Data table values were recorded using SPSS for Windows (ver. 22). One-way analysis of variance (ANOVA) and Tukey's post hoc tests were carried out to test any significant differences (p<0.05, p<0.01) in cell viability, cell toxicity and EC_{50} values in scavenging activity. A Pearson's correlation analysis was carried out to determine the correlation between the total phenolic content and DPPH scavenging activity.

CHAPTER 4: RESULTS

4.1 Moisture content of mushroom extracts

Fresh and dry weight of *Calocybe indica* were recorded on a digital balance. Results are shown in Table 4.1. Oven-dried method removed 837 g of moisture and freeze-dried method removed 952 g of moisture. The freeze-dried method was able to remove 5.8% more moisture from mushrooms compared to the oven-dried method. Although the moisture content removed was lower, oven-dried extracts yielded higher cell viability and neuroprotection than freeze-dried extracts for both solvents.

With respect to total phenolic content and scavenging activity, different trends were observed and were more dependent on solvent choice than moisture content. Oven-dried aqueous extracts recorded higher total phenolic content and scavenging activity than freeze-dried aqueous extracts. In contrast, freeze-dried ethanol extracts showed higher total phenolic content and scavenging activity than oven-dried ethanol extracts.

Table 4.1 Fresh and dry weight of *Calocybe indica*

Sample	*C. indica* (oven-dried)	*C. indica* (freeze-dried)
Initial fresh weight	997 g	1060 g
Final dry weight	160 g	108 g
Moisture content	84%	89.8%

4.2 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

A major concern about bioactive compounds from mushrooms is that some of these compounds are toxic to our normal system and is an important consideration in the development of novel drugs. As a result, this study used concentrations below 1 mg/mL (0–500 μg/mL). Cell viability was determined using the 3 [4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. In the assay, yellow tetrazolium salt was reduced to insoluble purple formazan crystals by the mitochondrial dehydrogenases of viable cells. The results were recorded in Figure 4.1. MTT assay was performed to determine the degree of cytotoxicity of *Calocybe indica* extracts in N2a cells.

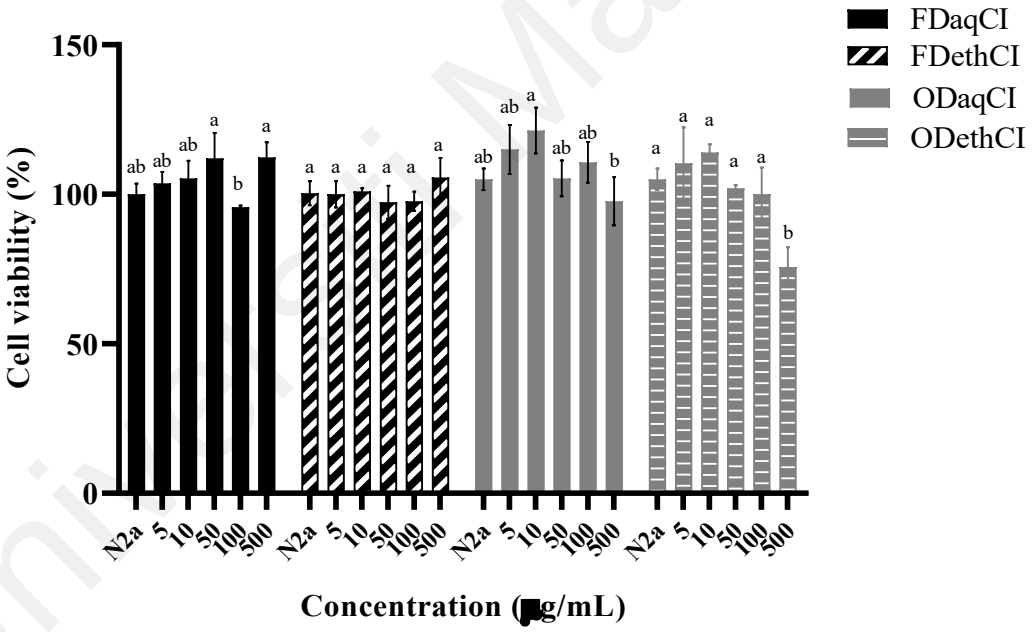

Figure 4.1 Cell viability of N2a upon treatment of different extracts of *Calocybe indica* (CI). Data marked with different letters were significantly different ($p< 0.05$) within a group. FDaqCI (freeze-dried aqueous extracts), FDeth CI (freeze-dried ethanol extracts), ODaqCI (oven-dried aqueous extracts), ODethCI (oven-dried ethanol extracts)

The cell viability and cell proliferation were denoted as 100% for the negative control (cells in complete growth medium without mushroom extracts). Medium without cells or mushroom extract served as the blank. The concentrations of 3 extracts demonstrated significant impact on cell viability between groups; freeze-dried aqueous extracts, $F(5,12)=4.69$ ($p=0.013$), oven-dried aqueous, $F(5,12)=4.39$ ($p=0.017$), and oven-dried ethanol extracts, $F(5,12)$ ($p=0.00$). However, different concentrations of freeze-dried ethanol extracts did not have a significant impact on cell viability $F(5,12)$ ($p=0.26$). In general, lower concentrations of all mushroom extracts showed higher cell viability but were not significantly different from the control. All extracts showed no significant difference from the control with one exception. At 500 μg/mL, oven-dried ethanol extracts showed significantly lower cell viability than the control ($p< 0.05$).

In regard to freeze-dried extracts (0-500 μg/mL), four out of five aqueous extracts exhibited higher viability than ethanol extracts although the difference was not significant. At 500 μg/mL, freeze-dried aqueous extracts showed the highest viability at 112% and freeze-dried ethanol extracts showed the highest viability at 105%. Looking at freeze-dried aqueous extracts alone, although there was no significant difference from control, 50 μg/mL and 500 μg/mL extracts were significantly higher than 100 μg/mL ($p< 0.05$). In contrast, none of the freeze-dried ethanol extract concentrations (0-500 μg/mL) were significantly different from the control.

Similarly, in relation to oven-dried aqueous and ethanol extracts, all concentrations were not significantly different than the control. In regard to oven-dried aqueous extracts alone, 10 μg/mL extracts were significantly higher than 500 μg/mL extracts ($p<0.05$). Five and ten µg/mL of oven-dried ethanol extracts improved viability, but the difference was not significant from control. Oven-dried aqueous *Calocybe indica* showed the highest viability at 10 μg/mL (121%) followed by 10 µg/mL of oven-dried ethanol extracts (113%).

4.3 Hydrogen Peroxide (H_2O_2) Assay

An H_2O_2 cell toxicity assay was carried out in order to determine the concentration at which the cell viability was reduced by half (EC_{50}). A preliminary H_2O_2 toxicity test (0-1000 µM) yielded 100% toxicity at 200 µM. A second H_2O_2 toxicity test was run and cells were treated with H_2O_2 (0-200 μM) and the results were recorded in Figure 4.2. The EC_{50} was determined to be 50 μM.

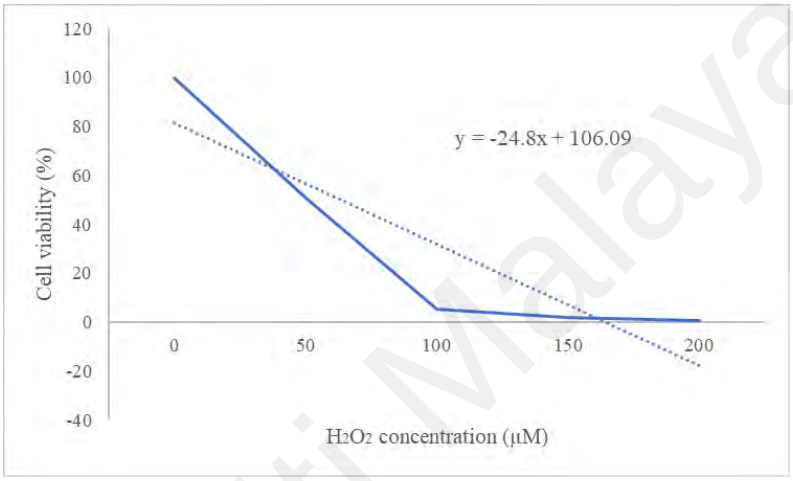

Figure 4.2 Cell viability of N2a upon co-incubation treatment with hydrogen peroxide

A hydrogen peroxide assay was carried out by co-incubating various concentrations of mushroom extract (0-500 μg/mL) using the determined EC_{50} of hydrogen peroxide (50 μM) as a negative control. The results were recorded in Figure 4.3. The concentrations of all 4 extracts demonstrated a significant impact on neuroprotection between groups; freeze-dried aqueous extracts, $F(6,14)=19.57$ ($p=0.00$), freeze-dried ethanol extracts $F(6,14)=15.54$ ($p=0.00$), oven-dried aqueous, $F(6,14)=12.03$ ($p=0.00$), and oven-dried ethanol extracts, $F(6,14)=17.57$ ($p=0.00$).

Freeze-dried aqueous *Calocybe indica* showed very significant neuroprotection (p<0.01) against the positive control (H_2O_2) at low concentrations (5-10 μg/mL) and were comparable to the negative control (N2a) but negligible neuroprotection at higher concentrations (50-500 μg/mL). Similarly, freeze-dried ethanol *Calocybe indica* showed moderate neuroprotection against H_2O_2 at low concentrations (5-50 μg/mL) but were not significantly different. In regard to oven-dried aqueous *Calocybe indica* showed very significant neuroprotection at 5-10 μg/mL (p<0.01) and significant neuroprotection at 50-100 μg/mL (p<0.05) than the negative control. Likewise, oven-dried ethanol *Calocybe indica* showed very significant neuroprotection (p<0.01) at 5, 10, and 100 µg/mL and significant neuroprotection at 50 μg/mL (p<0.05). Oven-dried ethanol, oven-dried aqueous and freeze-dried aqueous extracts showed a near 2-fold increase from the negative control (120%, 109%, 107%, respectively). Freeze-dried ethanol extracts had the lowest viability rate (78%) and were not significantly different from the negative control.

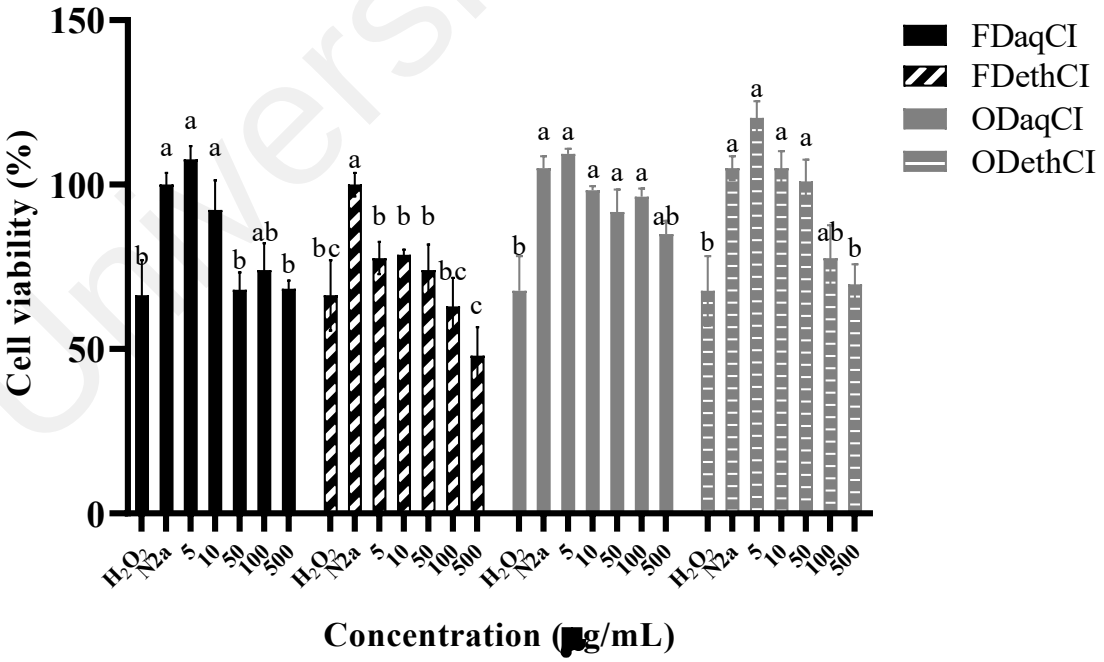

Figure 4.3 Cell viability of N2a after co-incubation of *Calocybe indica* and hydrogen peroxide. Data marked with different letters were significantly different from H_2O_2 (p<0.05) within a group. FDaqCI (freeze-dried aqueous extracts), FDeth CI (freeze-dried ethanol extracts), ODaqCI (oven-dried aqueous extracts), ODethCI (oven-dried ethanol extracts)

4.4 Total Phenolic Content (TPC) Determination

The total phenolic content of mushroom extracts ranged between 2.04 to 7.2 mg GAE per 50 g of dry weight mushroom. The gallic acid standard curve is shown in Appendix A (Figure A.2) and the gallic acid equivalent (GAE) is shown in Figure 4.4. Ethanol extracts had significantly higher phenolic content than all the aqueous extracts. Freeze-dried ethanol *Calocybe indica* recorded the highest total phenolic content (7.2 mg GAE/50 g dry weight) followed by oven-dried ethanol (5.04 mg GAE/50 g dry weight), oven-dried aqueous (2.52 mg GAE/ 50g dry weight), and freeze-dried aqueous *Calocybe indica* (2.04 mg GAE/ 50g dry weight). All the TPC extracts were significantly different from each other (p<0.05).

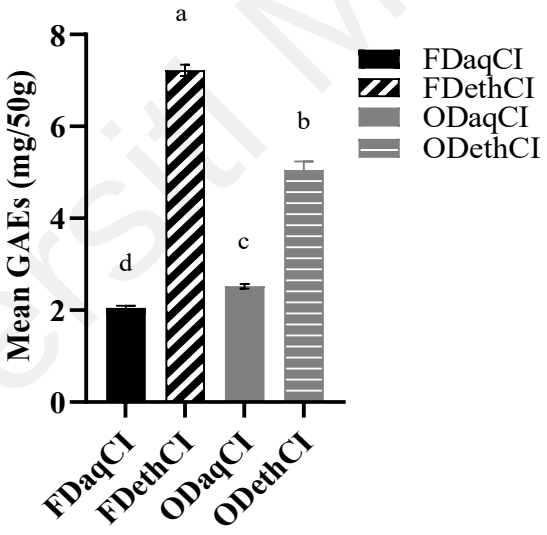

Figure 4.4 The total phenolic content of *Calocybe indica* extracts measured in gallic acid equivalent (GAE mg/50g dried weight) taken from the gallic acid standard curve. Data marked with different letters are significantly different at p< 0.05. FDaqCI (freeze-dried aqueous extracts), FDeth CI (freeze-dried ethanol extracts), ODaqCI (oven-dried aqueous extracts), ODethCI (oven-dried ethanol extracts)

Oven dried aqueous extracts were 23% higher in a phenolic content than freeze-dried aqueous extracts. Likewise, they both showed similar trends in viability and neuroprotective properties. Oven-dried ethanol extracts showed the second highest phenolic content and recorded the highest neuroprotection. In contrast, freeze-dried ethanol extracts had significantly highest phenolic content which was 43% higher than oven-dried ethanol extracts. Freeze-dried ethanol extracts also showed up to 3-fold higher phenolic content when compared to aqueous extracts but recorded the lowest cell viability and showed no significant neuroprotection most probably due to lack of any heat treatment.

4.5 2,2-Diphenyl-1- Picrylhydrazyl (DPPH) Radical Scavenging Activity

EC_{50} results were taken from the line graph in Figure 4.5 and recorded in Table 4.2. The scavenging activity of *Calocybe indica* extracts ranged from 8.63 to 53.4 mg/mL.

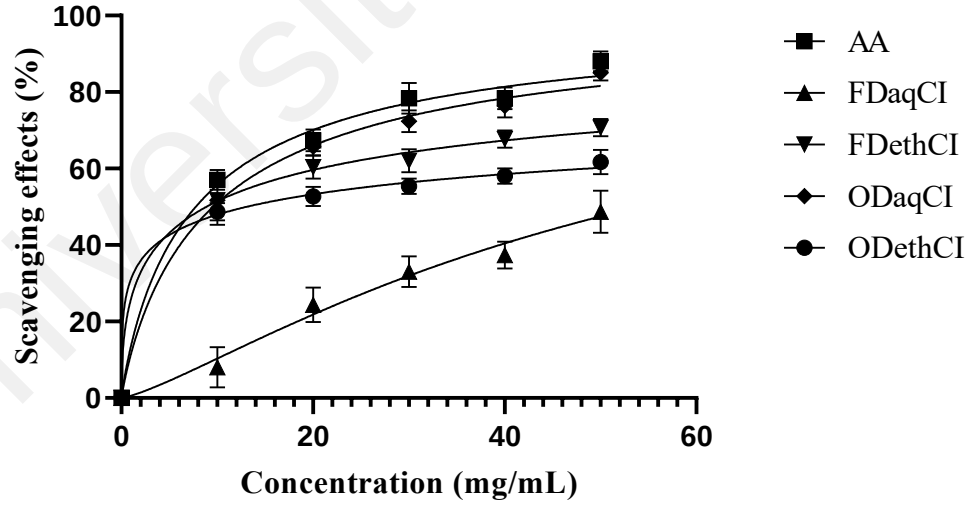

Figure 4.5 DPPH scavenging activity from *Calocybe indica* extracts compared to the positive control of ascorbic acid. AA (ascorbic acid), FDaqCI (freeze-dried aqueous extracts), FDeth CI (freeze-dried ethanol extracts), ODaqCI (oven-dried aqueous extracts), ODethCI (oven-dried ethanol extracts)

Table 4.2 EC_{50} of mushroom extracts obtained from the DPPH scavenging radical activity (mg/mL).

Mushroom extract	EC_{50} (mg/mL)
Freeze-dried eth *C. indica*	8.63 ± 2.32^{b}
Oven-dried aq *C. indica*	9.60 ± 3.63^{b}
Oven-dried eth *C. indica*	12.9 ± 2.43^{b}
Freeze-dried aq *C. indica*	53.43 ± 4.66^{a}
Ascorbic acid	7.72 ± 3.09^{b}

The results are expressed as mean ± SD (n = 3). Data marked with different letters are significantly different at $p<0.05$.

Freeze-dried ethanol extracts showed the highest scavenging activity followed by oven-dried aqueous, oven-dried ethanol and freeze-dried aqueous extracts, respectively. As with total phenolic content (Figure 4.4), freeze-dried ethanol extracts showed the highest scavenging activity. There was no significant difference in scavenging activity between freeze-dried and oven-dried ethanol extracts. In contrast, oven-dried aqueous extracts showed significantly higher scavenging activity (5-fold) compared to freeze-dried aqueous extracts.

4.6 Correlation between Total Phenolic Content (TPC) and Scavenging Activity (DPPH)

It is well-known that higher phenolic content in mushrooms is associated with higher scavenging activity. Figure 4.6 illustrates the correlation between total phenolic content (TPC) and radical scavenging activity (DPPH) of *Calocybe indica*. There was a positive but moderate correlation between TPC and DPPH radical scavenging activity (R^2=0.61).

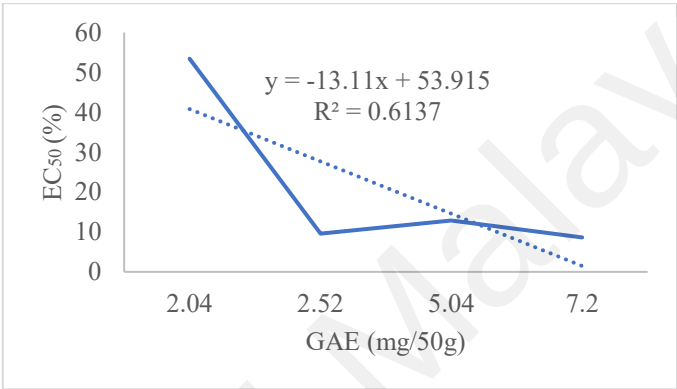

Figure 4.6 Correlation between total phenolic content and free-radical scavenging activity

CHAPTER 5: DISCUSSION

5.1 Moisture analysis and cell viability

Although oven-dried extracts had 5.8% more moisture than freeze-dried extracts, the oven-dried extracts showed higher cell viability, particularly at lower concentrations (5-10 µg/mL). This would indicate that small differences in moisture content between freeze-dried and oven-dried extracts have little effect on the viability of N2a cells. It is possible to reason that lower moisture content will provide higher phenolic content and scavenging activity and thus improve neuroprotection. Zhang et al. (2009) found that lower moisture content of freeze-dried *Lentinus edodes* significantly showed a higher total antioxidant capacity compared to sun-, oven- and microwave-dried extracts. Furthermore, an orthogonal array design matrix was able to demonstrate optimum extraction conditions using different ethanol concentrations (40-80%) and temperature (30-90 ˚C). It was found that 40% ethanol extracts showed the highest total phenolic content when heated at 90 ˚C for 1h. In contrast, Barros et al. (2007) found that lower temperatures (40 ˚C) increased mushroom bioactive compound content and higher temperatures destroyed them. However, there remains a gap in knowledge as the most suitable extraction method and temperature for optimizing bioactive compounds extraction. The results also agree with Phan (2015a) that aqueous and ethanol extracts do not show cytotoxicity at concentrations lower than 1 mg/mL. The overall trend agreed with the findings of Phan et al. (2012) and Baskaran et al. (2017) which showed lower concentrations of mushroom extract demonstrated higher viability in pheochromocytoma (PC-12) and macrophage (RAW 264.7) cells, respectively. At 500 µg/ml, over-dried ethanol extract was significantly lower than the positive control. This would indicate that heat treatment and browning, rather than moisture content or solvent, negatively affected cell viability.

Looking at freeze-dried aqueous extracts, although there was no significant difference from control, 50 μg/mL and 500 μg/mL extracts were significantly higher than 100 μg/mL ($p< 0.05$). This indicates a physiological stress limit of cells has been reached at 50 μg/mL; cell proliferation is not likely to improve with increasing mushroom extract dosage (Ben-Porath & Weinberg, 2004).

5.2 Neuroprotective effects of *Calocybe indica*

The trend showed that mushroom concentrations lower than 100 μg/mL demonstrate higher cell protection against H_2O_2. This agrees with previous research such as Shi et al. (2002) reported that 0.5 mg/mL of both hot water crude extracts of *G. lucidum* and cold-water crude extracts of *Agaricus bisporus* provided nearly complete (87%) protection against H_2O_2. Cui et al. (2005) reported that the polyphenolic extracts of *Inonotus obliquus* exhibited protective activity against hydrogen peroxide-induced cell damage at low concentrations (10–50 μg/ml). In the current study, N2a cells were sensitive to H_2O_2 stress which is very different from Hardaway et al. (2012) who found that N2a cells did not show significant decrease in viability with up to 300μM of H_2O_2 after 24 h, most likely as a result of higher seeding in well plate (19,000 cells per well).

Comparing aqueous extracts, freeze-dried extracts showed significant neuroprotection at 0-10 μg/mL whereas oven-dried extracts showed significant neuroprotection at higher concentrations (0-100 μg/mL). Comparing ethanol extracts, none of the freeze-dried extracts (0-500 μg/mL) showed significant neuroprotection against H_2O_2 despite recording the highest total phenolic content and scavenging activity. Oven-dried ethanol extracts, although lower in total phenolic content and antioxidant activity, demonstrated significant neuroprotection at lower concentrations (0-50 μg/mL).

Based on the results of the current study, oven-drying might be a more suitable method for recovering bound phenolic acids which show higher scavenging activity when compared to free phenolic acids as previous research has reported (Li & Shah, 2013). It is also possible that prolonged heating time and temperature could significantly enhance the formation of novel compounds such as Maillard reaction products (Choi et al., 2006). Similarly, comparing aqueous extracts, the oven-drying method showed significantly higher neuroprotection than the freeze-drying method at 50-500 μg/mL. It can be concluded that regardless of solvent choice, oven-drying extracts greatly improve neuroprotection. To sum up, as with cell viability, despite the higher moisture content, oven-dried extracts showed higher neuroprotection than freeze-dried extracts and played a more important role than solvent choice.

5.3 Antioxidant activity of *Calocybe indica*

The results agree with the findings of Vamanu & Nita (2013) who found that ethanol solvents showed higher phenolic content than aqueous extracts of *Boletus edulis* but disagreed with Wong et al. (2009) who found that oven-dried extracts showed a 3-fold higher phenolic content and higher scavenging activity than freeze-dried extracts of *Hericium erinaceus*. The results also disagreed with Pal et al. (2010) who found that hot water extracts were higher in phenolic, flavonoid, β- carotene, and lycopene content compared to cold water and methanol extracts of *Pleurotus squarrosulus*. The results agreed with Li & Shah (2013) who found that freeze-drying yielded higher scavenging activity than oven-drying ethanol extracts of *Pleurotus eryngii*. The lower moisture content in freeze-dried extracts can partially explain the higher total phenolic content in *Calocybe indica* due to a higher concentrating effect (Gan et al., 2013).

In contrast, Zhan et al. (2013) found that hot air-drying at 50°C, 100°C, and 120°C improved the total phenolic content and scavenging activity of ethanol extracts of *Lentinula edodes* when compared to freeze-drying. This can primarily be due to cell disruption releasing more antioxidants and possibly because of the formation of new antioxidant compounds.

With the exception of freeze-dried ethanol extracts, there appears to be a positive trend between total phenolic content and neuroprotection. While oven-dried ethanol extracts were lower in scavenging activity and lower in phenolic content than freeze-dried ethanol extracts, it recorded higher neuroprotection. This indicates that non-phenolic compounds could also play a synergistic role in neuroprotection (Phan et al., 2012).

As with total phenolic content, freeze-dried ethanol extracts showed higher scavenging activity when compared to oven-dried ethanol extracts which agrees with Zhang et al. (2009). Freeze-dried aqueous extracts were consistently the lowest in terms of phenolic content and scavenging activity but showed significantly higher neuroprotection compared to freeze-dried ethanol extracts. This further indicates that the neuroprotective properties of aqueous extracts are not dependent on the phenolic content of mushrooms. Ma et al. (2013) explained higher polysaccharide content in freeze-dried extracts may be responsible for the improved scavenging activity.

The higher total phenolic content in ethanol extracts suggests that less polar solvents are responsible for the bulk presence of total phenolic metabolites in *Calocybe indica*. Ethanol is especially a suitable solvent for extracting both polar and nonpolar compounds (Elbatrawy et al., 2015). Freeze-dried and oven-dried aqueous extracts were both potential neuroprotective agents despite having significantly different levels of total phenolic content and scavenging activity. This differed from Lai et al. 2013 who found that the moderate scavenging activity of aqueous extracts of *Hericium erinaceus* did not provide sufficient protective effect against H_2O_2 in NG108-15 cells. Interestingly, it was determined that enzymatic extracts of *Hericium erinaceus* provided more effective antioxidative and superoxide radical scavenging-activity compared to water and organic solvent extracts of the mushroom. From the current study, oven-drying improved neuroprotection, total phenolic content and scavenging activity in aqueous extracts. Furthermore, oven-drying improved neuroprotection in ethanol extracts but did not improve total phenolic content or scavenging activity. This partially agreed with the findings of Saad et al. (2014) who found that heat treatment of methanolic extracts of *Pleurotus sajor-caju* significantly increased the scavenging activity of heated mushrooms but did not change the phenolic content.

CHAPTER 6: CONCLUSION

The current study focused on the neuroprotective properties of four different *Calocybe indica* extracts; freeze-dried aqueous, freeze-dried ethanol, oven-dried aqueous and oven-dried ethanol. All four extracts at all tested concentrations (0-500 µg/ml) were not toxic to N2a cells. Lower concentrations of mushroom extracts showed improved cell viability and neuroprotection. Three out of four extracts- freeze-dried aqueous, oven-dried aqueous and oven-dried ethanol- showed a significant neuroprotection at 10 µg/mL or lower (p<0.05). The overall most suitable extraction method for neuroprotective properties was oven-dried ethanol extracts. Freeze-dried ethanol extracts recorded the highest total phenolic content and antioxidant activity but weak neuroprotective properties. Oven-dried aqueous extracts recorded the second highest antioxidant activity and neuroprotective properties. Oven-drying was found to be a suitable low-cost alternative to freeze-drying for neuroprotective and antioxidant research. It is recommended that future research focuses on oven-drying methods for optimum recovery of bioactive compounds, particularly oven vacuum-drying or steam-drying. In order to improve understanding of neuroprotective mechanisms of *Calocybe indica*, it is also recommended that oven-dried extracts be tested for possible Maillard reaction products and further identification of bioactive compounds in *Calocybe indica* responsible for its neuroprotective properties.

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