NEUROPROTECTIVE PROPERTIES OF *Sanguinoderma rugosum* EXTRACTS AGAINST GLUTAMATE-INDUCED HIPPOCAMPAL CELLS

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FACULTY OF SCIENCE
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NEUROPROTECTIVE PROPERTIES OF *Sanguinoderma rugosum* EXTRACTS AGAINST GLUTAMATE-INDUCED HIPPOCAMPAL CELLS

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NEUROPROTECTIVE PROPERTIES OF *Sanguinoderma rugosum* EXTRACTS AGAINST GLUTAMATE-INDUCED HIPPOCAMPAL CELLS

ABSTRACT

Oxidative stress and neuroinflammation are two intertwined pathologic factors in a wide range of neurological diseases. With heightening prevalence of neurological diseases due to rising life expectancy around the globe, searching for novel and alternative therapeutic candidates is urgently needed. *Sanguinoderma rugosum* (synonym: *Amauroderma rugosum*) has been traditionally used as a natural remedy to reduce inflammation, prevent unceasing crying, cancer and epileptic fits in China and Malaysia. However, the potential neuroprotective and neurorescue effects of *S. rugosum* against glutamate-induced neurotoxicity remain largely unexplored. In the present study, extracts from *S. rugosum* were evaluated for their neuroprotective and neurorescue properties using *in vitro* HT-22 mouse hippocampal neuronal cells. The mycelia of *S. rugosum* were cultivated in submerged liquid culture and freeze-dried prior to solvent extraction. MTT cell viability assay was performed to determine the neurotoxicity, neuroprotective and neurorescue activities of *S. rugosum* extracts at 24 and 48 h. Flow cytometric analysis was conducted to investigate the effects of *S. rugosum* extracts on the intracellular reactive oxygen species (ROS) production and cell death induced by glutamate. The constituents present in SR-HF fraction were identified through GC/MS analysis. The preliminary findings showed that all extracts did not exhibit cytotoxic effect on HT-22 cells. Upon 24 h incubation, pre-treatment with both SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) markedly (P<0.05) restored the loss of cell viability to 65.80 ± 1.85% and 89.76 ± 9.50%, respectively. The glutamate-induced ROS generation was effectively declined by SR-EE and SR-HF to 13.8% and 8.0%, respectively. The population of late apoptotic/early

necrotic cells showed reduction after pre-treatment with SR-HF, with value of 13.3%. However, an increase in the percentage of late apoptotic/early necrotic cells were shown in the glutamate-induced cells after pre-treatment with SR-EE, with value of 26.6%. In general, all extracts demonstrated neurorescue effect against glutamate-induced cells at 24 and 48 h. Eleven compounds were identified in SR-HF by GC/MS analysis, of which the linoleic acid, ergosterol and ethyl linoleate are the major chemical compounds. In short, these results demonstrated that SR-HF may be considered as a potent therapeutic agent to be used in treating neurological disorders related to oxidative stress and neuroinflammation.

Keywords: Medicinal mushroom, reactive oxygen species, neurological disorder, glutamate, mouse hippocampal cells.

SIFAT NEUROPROTEKTIF EKSTRAK *Sanguinoderma rugosum* TERHADAP SEL-SEL HIPOKAMPAL DIARUH GLUTAMAT

ABSTRAK

Tekanan oksidatif dan radang saraf adalah dua faktor patologi yang terjalin dalam pelbagai penyakit neurologi. Memandangkan peningkatan kelaziman penyakit neurologi disebabkan jangka hayat yang meningkat di seluruh dunia, pencarian calon perubatan yang baru dan alternatif amat diperlukan. *Sanguinoderma rugosum* (sinonim: *Amauroderma rugosum*) telah digunakan secara tradisional sebagai penawar semula jadi untuk mengurangkan keradangan, mencegah tangisan yang tidak berhenti, kanser dan penyakit epilepsi di China dan Malaysia. Untuk pengetahuan kami, kesan berpotensi neuroprotektif dan neuroselamat *S. rugosum* terhadap neurotoksisiti yang diaruh oleh glutamat masih belum diterokai. Dalam kajian ini, ekstrak daripada *S. rugosum* dinilai untuk sifat neuroprotektif dan neuroselamat mereka dengan menggunakan sel-sel saraf hipokampal tikus HT-22 sebagai model *in vitro*. Miselia *S. rugosum* dikultur dalam kultur cairan terendam dan dibeku-keringkan sebelum pengekstrakan pelarut. Ujian viabiliti sel MTT dijalankan untuk menentukan aktiviti neurotoksisiti, neuroprotektif dan neuroselamat ekstrak *S. rugosum* pada 24 dan 48 jam. Analisis sitometrik aliran dijalankan untuk menyiasat kesan ekstrak *S. rugosum* terhadap pengeluaran spesies oksigen reaktif (ROS) intraselular dan kematian sel yang diaruh oleh glutamat. Konstituen dalam fraksi SR-HF dikenalpasti melalui analisis GC/MS. Hasil awalan menunjukkan bahawa semua ekstrak tidak mempamerkan kesan sitotoksik terhadap sel HT-22. Setelah inkubasi 24 jam, pra-rawatan dengan SR-EE (12.5 µg/mL) dan SR-HF (100 µg/mL) mengembalikan kehilangan viabiliti sel secara signifikan (P<0.05) sebanyak 65.80 ± 1.85% dan 89.76 ± 9.50%, masing-masing. Generasi spesies oksigen reaktif yang

diaruh oleh glutamat ditolak secara berkesan oleh SR-EE dan SR-HF kepada 13.8% dan 8.0%, masing-masing. Populasi sel-sel apoptotik lewat/nekrotik awal menunjukkan pengurangan selepas pra-rawatan dengan SR-HF, dengan nilai 13.3%. Namun, peningkatan peratusan sel-sel apoptotik lewat/nekrotik awal ditunjukkan dalam sel-sel yang diaruh glutamat setelah pra-rawatan dengan SR-EE, dengan nilai 26.6%. Secara umum, semua ekstrak menunjukkan kesan neuroselamat terhadap sel-sel yang diaruh glutamat pada 24 dan 48 jam. Sebelas sebatian dalam SR-HF dikenalpasti oleh analisis GC/MS, di mana asid linoleat, ergosterol dan etil linoleat adalah sebatian kimia utama. Secara ringkas, keputusan tersebut menunjukkan bahawa SR-HF boleh dianggap sebagai agen terapeutik berpotensi untuk digunakan dalam merawat gangguan neurologi yang berkaitan dengan tekanan oksidatif dan radang saraf.

Kata kunci: Cendawan perubatan, spesies oksigen reaktif, gangguan neurologi, glutamat, sel hipokampal tikus.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
°C	:	Degree Celsius
Aβ	:	Amyloid-beta peptide
AD	:	Alzheimer's disease
AIF	:	Apoptosis-inducing factor
ALS	:	Amyotrophic lateral sclerosis
AMPAR	:	α-amino-3-hydroxy-5-methylisoxazole-4-propionate acid receptor
ANOVA	:	Analysis of variance
ATCC	:	American Type Culture Collection
ATP	:	Adenosine triphosphate
BAX	:	BCL2-associated X protein
BBB	:	Blood brain barrier
Bcl-2	:	B-cell lymphoma 2 gene
Ca^{2+}	:	Calcium ion
Cl^-	:	Chloride ion
CNS	:	Central nervous system
CO_2	:	Carbon dioxide
DCF	:	2',7'-dichlorofluorescein
DMEM	:	Dulbecco's modified Eagle's medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	1,1-diphenyl-2-picrylhydrazyl
e.g.	:	For example

EGCG	:	(-)-epigallocatechin-3-gallate
eV	:	Electric vehicle
FBS	:	Fetal bovine serum
FITC	:	Fluorescein isothiocyanate
g	:	Gram
GC/MS	:	Gas chromatography/mass spectroscopy
GPCR	:	G-protein-coupled receptor
GSH	:	Glutathione
h	:	Hour
H_2DCF	:	2',7'-dichlorodihydrofluorescein
H_2DCF-DA	:	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	:	Hydrogen peroxide
IL	:	Interleukin
iNOS	:	Inducible NO synthase
JNK	:	Jun nuclear kinase
KAR	:	Kainate receptor
L	:	Litre
LC3		Microtubule-associated protein 1A/1B-light chain 3
LPS	:	Lipopolysaccharide
m	:	Metre
M	:	Molarity
µg	:	Microgram
µm	:	Micrometre
µL	:	Microlitre
µM	:	Micromolar
MPTP	:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mtDNA	:	Mitochondrial DNA
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na^+	:	Sodium ion
NAC	:	N-acetyl-L-cysteine
NADPH	:	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	:	Sodium bicarbonate
NCDs	:	Non-communicable diseases
Nec-1	:	Necrostatin-1
NF-κB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDAR	:	N-methyl-D-aspartate receptor
NOX	:	Nicotinamide adenine dinucleotide phosphate oxidase
nm	:	Nanometre
No	:	Number
NO	:	Nitric oxide
NO•	:	Nitric monoxide
O_2	:	Molecular oxygen
O_2^-	:	Superoxide anion
OD	:	Optical density
OH•	:	Hydroxyl radical
OHDA	:	Hydroxydopamine
PD	:	Parkinson’s disease
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
pH	:	Power of hydrogen
PBS	:	Phosphate buffered saline
PI	:	Propidium iodide

PS	:	Phosphatidylserine
Q	:	Quadrant
Rac	:	Rho-related C3 botulinum toxin substrate
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
rpm	:	Revolutions per minute
RT	:	Retention time
SD	:	Standard deviation
SR-AF	:	*Sanguinoderma rugosum*-aqueous fraction
SR-EAF	:	*Sanguinoderma rugosum*-ethyl acetate fraction
SR-EE	:	*Sanguinoderma rugosum*-ethanolic extract
SR-HF	:	*Sanguinoderma rugosum*-hexane fraction
× g	:	Times gravity
TNF	:	Tumour necrosis factor
USD	:	United States Dollar
UV	:	Ultraviolet
v/v	:	Volume per volume
w/v	:	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Non-communicable diseases (NCDs) are the priority of global public health and development challenges. Among the NCDs, neurological diseases such as Alzheimer's disease (AD) and neuro-inflammatory disease (*e.g.*, multiple sclerosis) represent the leading causes of disability and death (Mentis et al., 2021). The direct economic burden of neurological diseases has been of colossal nature. According to World Alzheimer Report, it was estimated that the global cost for dementia was USD 1 trillion in 2018 and forecast to double by 2030 (Fleming et al., 2020).

The inextricable relationship between oxidative stress and neuroinflammation has been suggested to play significant role in neurological diseases. Disruption in the redox balance would alter the ROS-induced intracellular signalling, thereby creating the vicious cycle that promote the constant release of pro-inflammatory mediators (Solleiro-Villavicencio & Rivas-Arancibia, 2018). Reactive oxygen species (ROS) and redox balance have been thought to play pivotal role in the modulation of inflammatory cytokine production (Rada et al., 2011; Schieber & Chandel, 2014). Oxidative stress can be the cause or consequence of neuroinflammation. For instance, cytokine-stimulated hippocampus is prone to produce high amount of ROS which leads to oxidative stress in neurons. On the other hand, pro-oxidants can promote the expression of pro-inflammatory genes in hippocampus, further giving rise to numerous inflammatory responses (Mhatre et al., 2004).

Glutamate-induced neurotoxicity is known as one of the key players that initiate and perpetuate the brain injury, further triggering oxidative stress and neuroinflammation. Furthermore, excessive accumulation of extracellular glutamate is primarily implicated in unleashing a cascade of cellular events which results in the uncontrolled neuronal depolarization and cell damage or demise (Kritis et al., 2015). In the present study, glutamate was used as a pathologically relevant inducer due to its association with the pathological role in various neurological diseases.

Considering the harmful adverse effects of current drug therapy for neurological disorders, better therapeutic option such as natural products has been prospective in the development of new effective therapeutic agent owing to their little or no toxic side effects. Mushrooms have long been constituted as an essential part of normal human diet over the past decades. Apart from that, mushrooms have decade-old tradition of use as source of nutraceuticals due to the abundance of bioactive compounds (Reis et al., 2017; Martinez-Medina et al., 2021). The significant role of mushrooms as nutritionally functional food has attracted considerable attention from researchers to discover novel therapeutic strategies capable to suppress both oxidative stress and neuroinflammation.

Based on these premises, *Sanguinoderma rugosum*, also known as *Amauroderma rugosum* has been proclaimed to exert antioxidative, anti-inflammatory (Chan et al., 2013; Chan et al., 2015) and neuroprotective activities (Li et al., 2021). Nevertheless, there is limited empirical evidence on the neuroprotective and neurorescue potentials of *S. rugosum* extracts against glutamate-induced HT-22 mouse hippocampal cells. Therefore, it is of importance that the claims on the role of *S. rugosum* to serve as potent neuroprotective and neurorescue agent in treating neurological diseases be validated. The experimental design of the present study is outlined in Figure 1.1.

Objectives

This study aimed to investigate the pharmacological action of *S. rugosum* extracts against glutamate-induced neurotoxicity. This may be achieved through the following objectives:

i. To determine the neurotoxicity induced by *S. rugosum* extracts in hippocampal neuronal cells;
ii. To evaluate the neuroprotective and neurorescue effects of *S. rugosum* extracts against glutamate-induced hippocampal neuronal cells;
iii. To elucidate the underlying molecular mechanisms responsible for the neuroprotection on glutamate-induced oxidative stress and cell death by *S. rugosum* extracts;
iv. To identify the constituents present in the active fraction of *S. rugosum* that has shown neuroprotective effect.

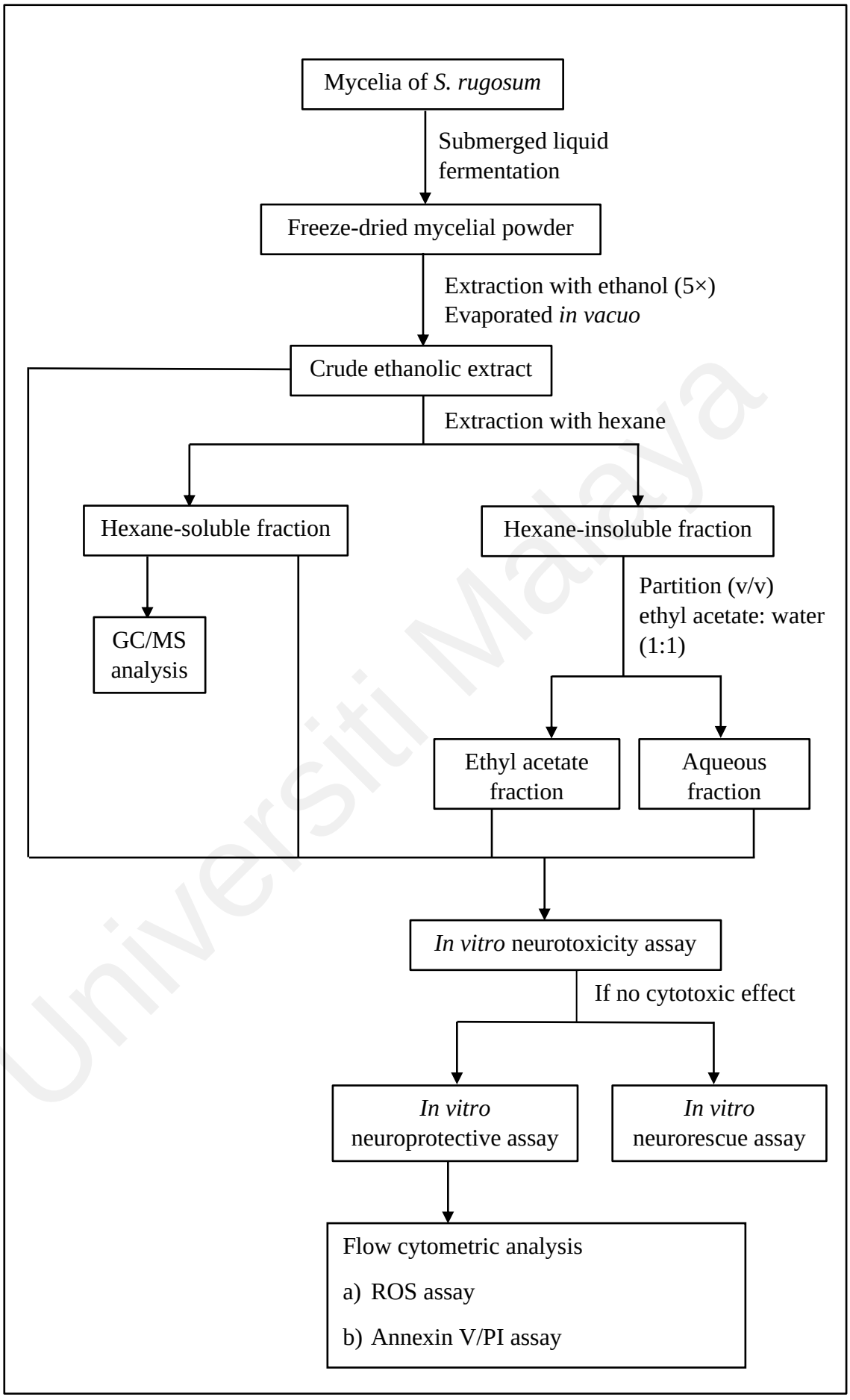


Figure 1.1: Outline of experimental design.

CHAPTER 2: LITERATURE REVIEW

2.1 Mushrooms

For centuries, mushrooms have been cultivated and consumed extensively, owing to their unique taste and texture (Kalac, 2016), plentiful nutritional value (Mingyi et al., 2019) and immense therapeutic properties (Rathore et al., 2017). Mushrooms are commonly eaten in their natural form or as a dietary supplement (Reis et al., 2017; Khan et al., 2019). Mushroom species are considered as an "inherent functional food" (Reis et al., 2017). Their chemical composition includes several bioactive compounds which enable them to perform diverse biological activities (Smith & Charter, 2011; Roupas et al., 2012).

Out of 140,000 mushroom species that are present on the Earth, only 10% of them are known to science (Miletić et al., 2020). There are at least 2,000 edible species and about 650 species exhibit various medicinal properties (Chang, 1999; Thatoi & Singdevsachan, 2014). It is also reported that 7,000 undiscovered mushroom species in which 5% of them bring potential health benefits to humankind (Atri et al., 2019). The most cultivated edible mushrooms are *Pleurotus ostreatus*, *Lentinula edodes*, *Agaricus bisporus*, and medicinal mushrooms with representatives of *Ganoderma lucidum*, *Poria cocos* and *Cordyceps sinensis* (Ma et al., 2018). Edible mushrooms are also known as "superfoods" because they exhibit numerous health-promoting properties such as antioxidant, immunomodulating, anti-inflammatory, antibacterial, antiviral and antidiabetic effects (Wasser, 2014; Bederska-Łojewska et al., 2017). Medicinal mushrooms are fungi that are particularly used to prevent diseases and maintain a balanced diet (Wasser, 2010). Beta-glucans in mushrooms have drawn the attention of researchers by anti-tumour (Liu et al., 2015a), anti-inflammatory (Du et al., 2018) and

immunostimulant properties (Zhu et al., 2014). In Malaysia, mushrooms are utilised by the local communities or aborigines not only for consumption, but also as medicinal or related to myths and beliefs (Fui et al., 2018; Lee & Mohammad, 2020).

2.1.1 Medicinal mushrooms and their biological functions

Currently, about 80 to 85% of medicinal mushroom products are derived from fruiting bodies, which are either available in the market or collected from the wild. Only 15% of all products are produced *via* extraction from mycelia (Antunes et al., 2020). Medicinal mushrooms are characterised by having more fungal cell wall materials and bioactive secondary metabolites which possess more pharmacological activities compared with edible mushrooms (Dumlupinar, 2021). The fungal cell wall is mainly made up of β-glucan-chitin complexes and mannoproteins (Usman et al., 2021), which are not digested in human gastrointestinal tract, thus makes them to resist gastric acidity and remain non-digestible by digestive enzymes (Cerletti et al., 2021). Due to this non-digestible property, mushrooms are considered as potential candidate for prebiotics as it meets part of the prebiotics criteria (Mitsou et al., 2020). For example, the polysaccharides derived from fruiting bodies and mycelia of *G. lucidum* and *P. cocos* act as prebiotics to modulate gut microbiota composition (Khan et al., 2018). Furthermore, an exo-polysaccharide released from mycelia of *Tramates versicolor*, namely tramesan was proved to display antioxidant properties through the manipulation of gene expression (Scarpari et al., 2017).

Apart from cell wall polysaccharides, medicinal mushrooms contain high molecular weight compound such as lignin and low molecular weight compounds such as triterpenes and phenolics (Shaffique et al., 2021). Lignin derivatives from chaga mushroom, *Inonotus obliquus* was reported to induce cell apoptosis in A549 human lung adenocarcinoma (Wang et al., 2015). Presently, more than 100 different types of triterpenes have been isolated from *G. lucidum*, 50 of them are mainly acids, ganodermic and lucidenic, as well as ganoderols and ganoderiols (Muszyńska et al., 2018). Triterpenes from methanol extracts of *G. lucidum* was reported to exhibit antibacterial activity against *Escherichia coli, Salmonella typhimurium* and *Bacillus subtilis* (Sheena et al., 2003).

Besides, mycelium, fruiting bodies and culture broth of various medicinal mushrooms have been widely reported on their antioxidant properties. Phenolic compounds are one of the most vital groups of bioactive secondary metabolites found in mushrooms with antioxidant activities (Cheung, 2010; Muszyńska et al., 2018). They are also able to chelate elements such as iron and copper that release free radicals (Muszyńska et al., 2018). It was found that methanolic extracts of *G. lucidum* and *G. tsugae* possessed high antioxidant activity and excellent scavenging effect of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals (Mau et al., 2002). The low reducing power ability observed in medicinal mushrooms could be attributed to the high abundance of reductone that potentially react with radicals to halt radical formations (Mau et al., 2002; Abdullah et al., 2012). Additionally, a comparative investigation on the chemical composition and antioxidant activities demonstrated that the ethanol extract from *I. obliquus* showed better antioxidant activities than *G. lucidum*, as evident from its higher phenolic and flavonoid content. This could be ascribed to the presence of p-hydroxybenzoic acid, quercetin and kaempferol (Kim et al., 2008; Zhang et al., 2015). The antioxidative potential of *I. obliquus* was proven by Nakajima et al. (2009) in which

the ROS accumulation and apoptosis were effectively inhibited by the phenolic ingredients isolated from *I. obliquus* on H_2O_2-induced PC-12 rat pheochromocytoma cells.

Furthermore, numerous studies have attributed the antioxidant and anti-inflammatory effect of *Hericium erinaceus*. In an investigation performed by Wang et al. (2017a), the crude mycelial polysaccharide extract of *H. erinaceus* was reported to play significant role in the suppression of oxidative stress by showing high oxygen radical absorbance capacity and strong radical scavenging activity. The report also demonstrated that the antioxidant activity of purified polysaccharide from *H. erinaceus* was responsible for the prevention of H_2O_2-induced apoptosis by inhibiting the overexpression of apoptosis-inducing signals such as Bax, caspase 3 and cytochrome c (Wang et al., 2017a). Apart from that, polysaccharides of *H. erinaceus* were tested *in vivo* on the anti-inflammatory activity against dextran sulphate sodium-injured colitis in C57BL/6 mice. Downregulation of several inflammatory mediators including nitric oxide (NO) and interleukin (IL) were observed, further suggesting the amelioration of *H. erinaceus* polysaccharides to regulate the signalling pathways related to oxidative stress and inflammation (Ren et al., 2018). Besides, it was claimed that erinacine A-enriched *H. erinaceus* mycelia reduced the level of proinflammatory cytokines and inducible NO synthase (iNOS) as well as attenuated the neuronal apoptotic cell death in an ischemic rat model (Li et al., 2018).

Cordyceps sinensis or caterpillar fungus, is well known for its wide variety of medicinal properties such as anti-inflammatory, antioxidant and immuno-modulatory properties (Wang et al., 2005; Kim et al., 2006; Li et al., 2006; Yang et al., 2011). For instance, the water and ethanol extracts of fruiting bodies of *C. sinesis* were found to elicit potent antioxidant effects *via* suppression of lipid peroxidation (Yamaguchi et al., 2000). Further *in vitro* study performed by Wang et al. (2009b) showed that the

polysaccharide isolated from fruiting bodies of *C. sinensis* could serve as potential antioxidant responsible for the treatment of renal failure.

2.1.2 Genus *Sanguinoderma* and medicinal properties of *Sanguinoderma* spp.

In the taxonomic and phylogenetic analyses conducted by Sun et al. (2020), species of *Amauroderma* sensu lato (*Ganodermataceae*) has been divided into four clades which are *Amauroderma* sensu stricto., *Foraminispora*, *Furtadoa* and a new genus *Sanguinoderma*. The *Sanguinoderma* sp. is characterized by corky to woody hard basidiocarp, dark-coloured pileus and pore surface which changes to blood red when bruised (Sun et al., 2020). To date, *Sanguinoderma* species group comprises of *S. flavovirens*, *S. laceratum*, *S. microporum*, *S. reniforme* and *S. sinuosum*, *S. bataanense*, *S. elmerianum*, *S. perplexum*, *S. rude* and *S. rugosum* (Sun et al., 2020).

Up to the present, the chemical and biological activities of most of the *Sanguinoderma* sp. have yet to be explored. Mounting evidence have revealed *Sanguinoderma rude* (synonym: *Amauroderma rude*) with various medicinal potentials. The *S. rude* which is known as "Xuezhi" in China was shown to induce apoptosis in human breast cancer cell lines, suggesting its potential to serve as a functional medicinal mushroom with anti-cancer effects (Jiao et al., 2013). Li et al. (2015) have identified ergosterol from *S. rude* as the main anti-cancer molecule. They have proved that ergosterol inhibited almost every stages of tumour growth *in vitro* and *in vivo* through activation of apoptotic signal pathway (Li et al., 2015). In another investigation carried out by Pan et al. (2015), it has been reported that polysaccharides purified from *S. rude* played an important role in immunopotentiation *in vitro* and *in vivo*. Crude polysaccharides from the water extract of *S. rude* were found to improve immunomodulatory activity in molecular and cellular level (Pan et al., 2015).

The *Sanguinoderma rugosum* (synonym: *Amauroderma rugosum*) (Figure 2.1) was classified under *Sanguinoderma* clade and distributed in warm temperate to subtropical or tropical areas (Sun et al., 2020). *S. rugosum* was first described by Blume and Nees (1920) and classified under the class of Basidiomycetes. The *S. rugosum*, known as black lingzhi is able to alleviate inflammation, to heal diuresis and upset stomach as well as to prevent cancer (Dai & Yang, 2008). Besides, *S. rugosum* is named as "epileptic child mushroom" or "cendawan budak sawan" in Malay language. In Malaysia, the fresh hollow stipe is diced, strung and worn as a necklace by indigenous Malaysian Temuan community to prevent epileptic fits and unremitting crying by babies (Chang & Lee, 2004; Azliza et al., 2012).

Several reports have attributed that freeze-dried mycelial extracts of *S. rugosum* possessed significant antioxidant activity, alluding to the presence of high phenolic content (Chan et al., 2013; Zhang et al., 2017). Besides, it has been reported that *S. rugosum* extracts inhibited the production of inflammatory mediators including TNF-α, NO and IL-10 in lipopolysaccharide (LPS)-stimulated murine macrophage RAW264.7 cells, suggesting the potent *in vitro* anti-inflammatory effects of *S. rugosum* (Chan et al., 2015; Zhang et al., 2017). Moreover, *S. rugosum* extracts demonstrated anti-cancer activity against MCF-7 cell line, leading to its potential to treat breast cancer (Zhang et al., 2017). The significant anti-cancer effect was found to be highly correlated with the quantity of polyphenols in *S. rugosum* extracts (Zhang et al., 2017). Particularly, polysaccharides isolated from the water-soluble *S. rugosum* extract displayed immunostimulatory activities. This property supported the interaction of β-glucans with the immune cell receptors to stimulate immune response (Zhang et al., 2017). In addition, it was claimed that the fruiting bodies of *S. rugosum* exhibited strong antioxidative capacity, antihyperlipidemic, anti-platelet aggregation and antithrombotic activities which are responsible for cardiovascular benefits (Chan et al., 2017). Recent studies

suggested that aqueous extract of *S. rugosum* could possess neuroprotective activity which involves both antioxidant and antiapoptotic properties, further providing rationalization that *S. rugosum* could serve as therapeutic agent to counteract neurodegenerative diseases related to oxidative stress (Li et al., 2021). Additionally, Chen et al. (2021) indicated anti-inflammatory activities against LPS-induced RAW264.7 cells on the sterols and unsaturated fatty acids isolated from *S. rugosum*. The same studies also demonstrated that *S. rugosum* exerted antiproliferative activities against HepG2 and MDA-MB-231 cancer cell lines (Chen et al., 2021).

Figure 2.1: *Sanguinoderma rugosum* fruiting body.

2.2 Oxidative stress and neuronal cell death in neurological diseases

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical (OH•) and nitric monoxide (NO•) are highly reactive oxygen-containing molecules being produced through stepwise reduction of molecular oxygen (O_2) (Nita & Grzybowski, 2016). The brain's oxygen demand is enormous; although the brain represents only 2% of body mass, it accounts for 20% of total oxygen metabolism in the body (Jain et al., 2010). The discrepancy in per weight oxygen uptake is believed to induce the formation of ROS, which are commonly known as the toxic by-products of oxygen in aerobic metabolism (Patel, 2016). Besides, the brain is highly vulnerable to lipid peroxidation due to the simultaneous abundance of polyunsaturated fatty acids and lipids that serve as the hot-spots of ROS attack (Liu et al., 2008). Lipid peroxidation can cause damage of membrane components and results in cellular degeneration, thereby disrupting the cell integrity and viability (Salim, 2017).

ROS are generated endogenously in moderate or low level for neuronal development and function. When ROS production exceeds scavenging capacity of antioxidant defence system, oxidative stress is generated (Ray et al., 2012). Oxidative stress refers to the self-propagating phenomenon where there is an imbalance between ROS production and biological system's ability to mitigate reactive oxygen intermediates (Burton & Jauniaux, 2011; Nita & Grzybowski, 2016). The disequilibrium between antioxidants and prooxidants can directly or indirectly incur structural damage of deoxyribonucleic acid (DNA), protein oxidation and lipid denaturation (Bhat et al., 2015). DNA damage induced by oxidative stress may result in the mutation of mitochondrial DNA (mtDNA) and thus affect the oxidative phosphorylation and neuronal function due to impaired ATP generation in mitochondria (Kroemer & Reed, 2000; Bhat et al., 2015). Furthermore, excessive amount of carbonylated proteins are used as a biomarker for protein oxidation (Møller & Kristensen, 2004). It has been reported that elevated

carbonylated proteins which causes oxidative damage are found in patients with AD (Fedorova et al., 2014).

Among the brain regions, hippocampus is highly sensitive to oxidative stress (Santos et al., 2009). To date, burgeoning experimental and clinical evidence demonstrate that oxidative stress is the causative factor of neurological diseases (Jenner & Olanow, 2006; Zhou et al., 2018; Jayaraj et al., 2019). Signs of oxidative stress such as lipid peroxidation and reduced glutathione (GSH) levels have been revealed in the patients with AD, Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Barnham et al., 2004; Zhou et al., 2008; Blesa et al., 2015; Butterfield & Halliwell, 2019). In the absence of efficient protective antioxidant defence mechanisms, loss of dopaminergic neurons in PD is believed to be resulted from low GSH levels as monitored in adult dopaminergic neurons (Martin & Teismann, 2009; Smeyne & Smeyne, 2013). Besides, an elevated level of lipid peroxidation due to increased ROS production was observed in the pilocarpine-induced seizures in rats' hippocampus homogenate (Santos et al., 2009).

Due to high reactivity and availability of ROS in the brain, it was reported that ROS can chemically interact with nucleic acid, protein or lipid and induce radical-mediated injury in cells (Salim, 2017). Although the mechanisms by which ROS leads to neuronal damage are not well elucidated, ROS has been proclaimed to trigger cascades of events that destructs the blood-brain barrier (BBB), eventually leading to the progression of neuroinflammation and neuronal death (Uttara et al., 2009; Salim, 2017). Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is known as one of the major sources of ROS production in variety of neurological diseases (He et al., 2020). In traumatic brain injury, the activation of NOX associated with oxidative stress increases the permeability of BBB, ultimately results in secondary neuronal damage that enhances neuroinflammation and neurodegeneration in hippocampus (Maas et al., 2008; Niesman et al., 2014). Moreover, overexpression of NOX has been implicated in the development

of AD and PD. Elevated NOX2 due to activated microglia by β-amyloid (Aβ) which induce ATP release could give rise to NOX2 activation (Angeloni et al., 2015). Thus, increased ROS formation plays critical roles to upregulate the proinflammatory mediators and further lead to neuronal death (Kim et al., 2007; Angeloni et al., 2015).

2.3 Role of glutamate in the brain

Glutamate is regarded as the predominant excitatory neurotransmitter and the most abundant free amino acid in the brain (Brosnan & Brosnan, 2013). In addition to its role in protein synthesis, it plays essential role in normal neural transmission, development, learning and memory (Mcentee & Crook, 1993; Kritis et al., 2015). Within the central nervous system, glutamate has evolved into a molecule with multiple metabolic pathways and major signalling receptors or transporters (Shirlee et al., 2001; Zhou & Danbolt, 2014).

Glutamate exerts as a neurotransmitter *via* interaction with ionotropic and metabotropic glutamate receptors (Kew & Kemp, 2005; Olloquequi et al., 2018). Ionotropic receptors are associated with ligand-gated cation channels that are activated upon glutamate binding (Kew & Kemp, 2005). They are subdivided into three major receptors that belong to same superfamily, namely α-amino-3-hydroxy-5-methylisoxazole-4-propionate acid receptor (AMPAR), kainate receptor (KAR) and N-methyl-D-aspartate receptor (NMDAR) (Nedergaard et al., 2002; Fu et al., 2019). On the other hand, metabotropic receptors are members of the class C G-protein-coupled receptor (GPCR) superfamily, the most abundant receptor gene family in the human genome (Spampinato et al., 2018). Compared to ionotropic receptors that mediate fast synaptic transmission (Hayashi, 2021), metabotropic receptors mediate the slow responses *via* coupling to G-protein signalling systems (Roth, 2019).

2.3.1 Glutamate-induced neurotoxicity

High concentration of glutamate is neurotoxic and kill central neurons. Hence, the level of extracellular glutamate is maintained at low level by a family of sodium-dependent glutamate transporters found on the surface of both nerve and glial cells (Nedergaard et al., 2002; Mahmoud et al., 2019). Glutamate-induced toxicity occurs through two pathways which are receptor-mediated excitotoxicity and non-receptor-mediated oxidative stress (Kritis et al., 2015).

Olney and co-workers established such neurotoxicity as excitotoxicity which is a common feature of the brain activity or synaptic transmission mediated by glutamate (Olney & Sharpe, 1969; Olney et al., 1986). It was believed that glutamate is the major causal factor of neuronal cell loss that is implicated in several neurological diseases such as stroke (Li & Wang, 2016), epilepsy (Choi, 1988; Ambrogini et al., 2019) and certain neurodegenerative diseases (Simões et al., 2018). Excitotoxicity is characterised by the accumulation of glutamate in central nervous system (CNS) due to the malfunction or aberrant expression of glutamate transporters (Spampinato et al., 2018). Excessive activation of ionotropic glutamate receptors (NMDA receptors) leads to influx of extracellular Ca^{2+} into neurons through the ion channel which triggers a cascade of events resulting in the loss of nerve function, cell damage and apoptotic or necrotic death (Lau & Tymianski, 2010; Pina-Crespo et al., 2014; Viviani et al., 2014). Although activation of glutamate receptors involves different ions, there is an evidence which supported the paramount role of Ca^{2+} in excitotoxicity. Choi (1988) proposed two components involved in excitotoxicity: Na^{+} and Cl^{-} influx through ion channels due to acute cell swelling, and disrupted neuronal degeneration resulted from excessive Ca^{2+} influx being the major cause of neuronal death. In contrast to the ionotropic receptors, the intervention of metabotropic receptors is not significant in excitotoxicity (Casson, 2006; Działo et al., 2013).

Apart from those neuronal cells in which the glutamate-induced toxicity is exerted through overstimulation of ionotropic receptors while there are other cells that do not express active receptors, the toxicity is caused by glutamate-induced oxidative stress (Murphy et al., 1989). In year 1989, glutamate-induced Ca^{2+} dependent cell death has been observed by Murphy and colleagues in the N18-RE-105 neuroblastoma X retina cell line. The study showed that glutamate-induced neurotoxicity is mediated *via* the inhibition of cystine uptake through the cystine/glutamate antiporter (Murphy et al., 1989; Sato et al., 1999). The inhibition of cystine import to enter the cell gives rise to the depletion of GSH and further leads to oxidative stress and cell death (Shih et al., 2006; Maher et al., 2018). As such, it can be concluded that glutamate acts as a neurotoxicant at high concentration to initiate a series of events that elevates the intracellular level of ROS and reactive nitrogen species (RNS) (Xin et al., 2019). This type of cytotoxicity has been termed as oxidative glutamate toxicity or oxytosis (Shirlee et al., 2001).

2.3.2 *In vitro* model for glutamate-induced neurotoxicity

The use of animals in researching neurotoxicity has been ethically controversial but also high-cost, labour-intensive and inappropriate for high-throughput investigations (Heusinkveld & Westerink, 2017). On the contrary, *in vitro* approaches permit relatively more convenient and higher speed in testing chemicals of interest for potential neurotoxic effects with added ethical benefits (Fritsche et al., 2015). Cell culture is generally described as a technique that the cells are removed from the tissues of animal or plant and grown under artificial controlled conditions (Verma, 2014). Primary cells are derived directly from the tissue of origin; and hence, they have similar biological response with the parental tissue (Bhatia et al., 2019). An ideal model of primary cells used to study glutamate-induced neurotoxicity is the cerebellar granule cells isolated postnatally from the cerebellum of rats (Büyükokuroğlu et al., 2003; Pereira et al., 2017). A pitfall of using

primary cells, however, is that the presence of astrocytes could render the cells to exhibit different functional and metabolic characteristics compared to neurons (Krämer & Minichiello, 2010). On the other hand, cell lines are culture of cells arose from primary cultures. One of the benefits of cell lines is that they can be propagated continuously and grown indefinitely compared to the primary cells that have limited life span (Bols et al., 2011). Further, the protocol to culture the cell line is readily available and the maintenance of cells can be performed conveniently using the culture flasks and media.

Various cell models have been reported to be suitable for *in vitro* studies on glutamate-induced neurotoxicity. One of the widely used cell lines is rat pheochromocytoma nerve PC-12 cells, originally developed from adrenal tumour (Wang et al., 2009a; Ma et al., 2013; Zhang et al., 2020). As there is a lack of NMDA receptors in PC-12 cells, the mechanism of cytotoxicity is mainly caused by the oxidative stress from inhibition of cystine uptake and depletion of GSH (Lv et al., 2017). Innumerable experimental findings also supported that glutamate-induced apoptosis is associated with the increased intracellular ROS and calcium (Zhao et al., 2017; Chang et al., 2020; Zhang et al., 2020). Morphological study showed that the apoptotic PC-12 cells are shrunken and become thin with pyknotic nuclei after glutamate exposure (Chen et al., 2017). Besides, increased glutamate level was reported to upregulate the ratio of proapoptotic and anti-apoptotic protein (Bax/Bcl-2) as well as to activate both c-jun N-terminal kinase (JNK) and p38 signalling cascades through a series of apoptotic signals (Chang et al., 2014; Yu et al., 2014). It was also noteworthy that low glutamate concentration (10 μM) could lead to induction of microtubule-associated protein 1A/1B-light chain 3 (LC3) that further indicate the occurrence of autophagy in PC-12 cells (Stamoula et al., 2015).

Apart from that, human neuroblastoma cell line SH-SY5Y has been found to respond to glutamate-induced neurotoxicity through the increased level of ROS and reduction in ATP level due to mitochondrial dysfunction (Cunha et al., 2016; Shah et al., 2016; TerziOğlu BebiToğlu et al., 2020). Experimental evidence suggested that glutamate-induced elevation of cytoplasmic calcium was independent to both ionotropic and metabotropic receptors in SH-SY5Y cells (Sun et al., 2010). In addition, glutamate-induced oxidative stress has been proclaimed to induce competitive inhibition of cystine uptake through cystine/glutamate antiporter, decreased GSH level and downregulation of antioxidant proteins such as superoxide dismutase and glutathione peroxidase, resulting in apoptotic cell death (Lee et al., 2019). Members of Bcl-2 family such as Bax and Bcl-2 proteins have been implicated to play important roles to regulate mitochondrial apoptotic pathway in glutamate-treated SH-SY5Y cells (Hu et al., 2012). Nikolova et al. (2005) also showed that Rac-NADPH oxidase-mediated ROS production could be the main underlying mechanism of glutamate-induced apoptosis in SH-SY5Y cells.

Last but not least, HT-22 murine hippocampal neuronal cells (Figure 2.2) originally immortalized from primary murine neuronal culture, a subclone of the hippocampal cell line HT-4 cell line was the most glutamate-sensitive among the 25 clones tested (Maher & Davis, 1996). Despite HT-22 cells are deficient in ionotropic glutamate receptors, the cells are still susceptible to high concentrations of glutamate and hence have been commonly used as an *in vitro* cellular model to study the non-receptor-mediated oxidative toxicity (Maher & Davis, 1996; Kritis et al., 2015). Impaired mitochondrial function and increased intracellular ROS are the initial events leading to glutamate-induced cell death (Tobaben et al., 2011). It was indicated that glutamate shows sign of necrosis at early time point (before 12 h) whereas apoptosis is the main cell death program at late time point (after 12 h) after glutamate induction (Fukui et al., 2009). Accumulating evidence has suggested apoptotic cell death induced by glutamate was

mediated *via* calpain pathway, implying that glutamate kills HT-22 cells in a caspase-independent mechanism (Zhang et al., 2003; Zhang & Bhavnani, 2006). Calpain-dependent apoptosis was further supported by the expression of both apoptosis-inducing factor (AIF) and p53 proteins (Cao et al., 2007). Moreover, translocation of mitochondrial AIF resulted in chromatin condensation and DNA fragmentation (Cregan et al., 2002). Xu et al. (2007) suggested that glutamate-induced oxytosis may link to necroptosis (regulated necrosis) which was observed from the role of necrostatin-1 (Nec-1) to protect the glutamate-induced HT-22 cells against necroptotic cell death.

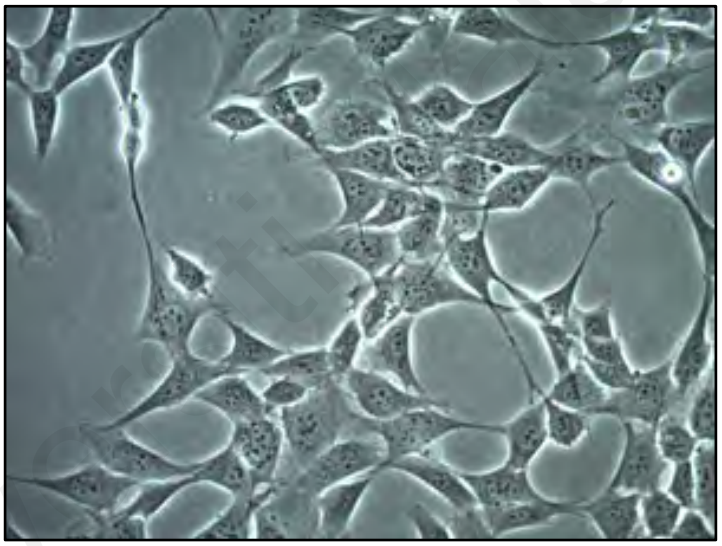


Figure 2.2: HT-22 murine hippocampal neuronal cells (Photo adapted from www.merckmillipore.com).

2.4 Neuroprotective and neurorescue properties of natural products

Neuroprotection covers all the pharmacological and physical strategies which are important to recover neuronal functionality and to suppress cell death through the attenuation of pathophysiological cascade triggered by acute injury (Hilton et al., 2017; Miras-Portugal et al., 2019). Neuroprotection is known to be useful to prevent the adverse effects after brain insult or injury (Stafstrom & Sutula, 2005). Generally, neuroprotection is a therapeutic mechanism which the neurons are protected against damage, eventually avoiding the neuronal dysfunction or death (Schapira, 2010).

Research has focused on developing neuroprotective agents for the ailments of both acute (trauma and stroke) (Vosler et al., 2014; Abu Deiab & Croatt, 2019) and chronic degenerative diseases (Alzheimer's disease, Parkinson's disease and epilepsy) (Stafstrom & Sutula, 2005; Wang et al., 2017b; Zheng et al., 2017; Liu et al., 2018). The neuroprotectants cannot reverse the existing damage, instead they protect against further nerve damage and slow down degeneration of the CNS (Schapira, 2010).

In recent years, the use of natural products has greatly contributed to neuroprotection. Tetrahydrocurcumin isolated from *Curcuma longa* (turmeric) has been claimed to demonstrate excellent anti-inflammatory and neuroprotective activities against glutamate-induced oxidative stress and cell death in HT-22 cells (Park et al., 2020a). Additionally, Park et al. (2021) has shown that polyphenolic compounds such as catechins from green tea extract of *Camellia* (*Camellia sinensis* L. arachnid) exhibited neuroprotective effect by reducing the glutamate-induced intracellular Ca^{2+} accumulation and free radicals in HT-22 cells. Furthermore, it has been proved that co-treatment with the rhizome extract of *Kaempferia parviflora* Wall. ex Baker exerted both neuroprotective and longevity-inducing properties against glutamate-induced HT-22 cells through the suppression of apoptosis and production of intracellular ROS (Tonsomboon et al., 2021).

Neurorescue can be viewed as a corrective treatment to repair and rehabilitate the damaged cells that have undergone progressive insult (Thomas Tayra et al., 2013). The relevance of neurorescue as potential therapeutic strategy was established due to the delay of diagnosis in certain neurological diseases such as AD. For instance, AD was often diagnosed after the initiation of synaptic loss or neuronal cell death while some neurons are undergoing damage (Soll et al., 2016). Post-treatment is considered as a suitable approach to study the neurorescue effects of a particular therapeutic agent (Singh et al., 2021).

Various evidence demonstrated neurorescue potential of natural products in the treatment of neurological diseases, especially PD both *in vitro* and *in vivo*. Singh and colleagues reported that *Bacopa monnieri (*L.*) Wettst* extract exhibited neurorescue properties against MPTP-induced mice model of PD (Singh et al., 2021). Besides, the progression of PD has been shown to slow down after the administration of black tea extract using 6-hydroxydopamine (6-OHDA)-injured rat model, indicating the neurorescue effect of black tea (Chaturvedi et al., 2006; Xu et al., 2017). Moreover, it was suggested that antioxidant flavonoids and phenolics from the eggplant (*Solanum melongena* L) extract exhibited neurorescue activity in 6-OHDA-lesioned rodent model (Li et al., 2017).

Furthermore, a polyphenol constituent ((-)-epigallocatechin-3-gallate, EGCG) isolated from green tea was able to reverse the loss of cell viability in Aβ-induced PC-12 cells, suggesting the neurorescue effect exerted by EGCG (Levites et al., 2003). The neurorescue activity of EGCG was further demonstrated in the long-term serum deprived PC12 cells by promoting the neurite outgrowth activity (Reznichenko et al., 2005). Additionally, an investigation carried out by Yael et al. (2007) demonstrated that novel iron chelators and EGCG possessed neurorescue properties through the attenuation of apoptosis in serum-starved human SH-SY5Y neuroblastoma cells.

2.4.1 Neuroprotective properties of mushrooms

Mushrooms have long been regarded as one of the functional foods worldwide not only for their nutritional properties, but also for their pharmacological potential as well as source of essential bioactive compounds to treat diseases. Numerous investigations have been conducted to show that mushrooms exerted potent neuroprotective activities. Biological evaluation performed by Zhang and co-workers have revealed that meroterpenoids isolated from *Ganoderma austral* prevent neural cells against glutamate-induced cellular toxicity in human neuroblastoma SH-SY5Y cells (Zhang et al., 2019b). Besides, Kondeva-Burdina et al. (2019) found that the muscimol isolated from the ethanol extract of *Amanita muscaria* possessed remarkable neuroprotective effect on the neurotoxicity models, thereby proving that *A. muscaria* could serve as promising candidate for the neuroprotective potential in the treatment of Parkinson's disease.

Moreover, bioactive evaluation of the known isolated aromatic compounds from *G. lucidum* presented that the compounds displayed significant neuroprotective activities against corticosterone-induced PC-12 cell damage and demonstrated anti-inflammatory activities against LPS-induced NO production in RAW264.7 macrophages (Lu et al., 2019). Additionally, Lemieszek et al. (2018) investigated the neuroprotective effect of *Cantharellus cibarius* extract in different *in vitro* models of neurodegeneration. They further suggested that polysaccharide fraction from *C. cibarius* could be developed as effective source of neuroprotective agent against neurodegenerative diseases (Lemieszek et al., 2018).

Besides, *Morchella importuna* can be considered as a dietary supplement or traditional medicine to prevent oxidative stress, owing to its profound neuroprotective property (Xiong et al., 2016). Polysaccharides extracted from the fruiting bodies of *M. importuna* has been shown to ameliorate the viability of PC-12 cells against H_2O_2-induced toxicity and suppress the programmed cell death *via* down-regulation of the NF-κB pathway and the p38-JNK pathway (Xiong et al., 2016). Moreover, erinacines isolated from *H. erinaceus* have been reported to possess neuroprotective effects by ameliorating the amyloid-β deposition in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated PD model (Li et al., 2018). Neuroprotective properties against seizure activity were also demonstrated using *H. erinaceus* which further highlight the potential application of *H. erinaceus* to prevent neuronal demise after seizures (Jang et al., 2019).

Neuroprotective effects of *Lignosus rhinoceros* sclerotial methanol extract was found to ameliorate the dexamethasone-induced apoptosis in human embryonic stem cells, (Yeo et al., 2019). Apart from that, Liu and co-workers suggested that the cordycepin from *C. militaris* possessed neuroprotective action in the ischemic brain which could be owed to the anti-inflammatory and antioxidant properties (Liu et al., 2015b). Kim et al. (2019b) investigated the effect of *C. militaris* in improvement of cognitive dysfunction and found out that *C. militaris* could be developed into a potent neuroprotective agent to improve the cognitive improvement on ischemic-induced WISTAR rats.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and reagents

Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Difco (BD, New Jersey, USA). Ethanol, hexane and ethyl acetate were of analytical grade purity and procured from Merck KGaA (Darmstadt, Germany). All other cell culture reagents were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA) unless stated otherwise.

3.2 Production of *Sanguinoderma rugosum* mycelia

Mycelia of *S. rugosum* (KUM61131) were acquired from Mushroom Research Centre, Universiti Malaya, and cultured on PDA (Appendix A (a)) plates according to the protocol outlined by Chan et al. (2013). After one week, ten plugs of the actively growing mycelia were inoculated into 500 mL baffled Erlenmeyer flasks filled with 100 mL of PDB medium (Appendix A (b)) and grown with an agitation rate of 100 rpm, 27°C for two weeks. The entire mycelium broth was freeze-dried and kept at 4°C prior to extraction.

3.3 Extraction and fractionation

The preparation of crude ethanolic extract and its fractions was carried out according to the method previously described by Chan et al. (2013). Briefly, the freeze-dried mycelia (119.0 g) were soaked in ethanol for ten days at a ratio of 1:10 (w/v) at room temperature. The solvent-containing extracts were decanted, filtered and the culture filtrate was re-dissolved in equal volume of ethanol. The extraction was repeated at two days interval. The resulting supernatant from five extractions were collected and

concentrated *in vacuo* by means of a rotary evaporator (Buchi, Switzerland) at 40°C to attain a dark brown viscous crude ethanolic extract (SR-EE, 59.4 g; 49.9% yield from the freeze-dried mycelia). Subsequent fractionation of the SR-EE (58.4 g) was consecutively performed with hexane at a ratio of 1:10 (w/v) to generate a yellow brown gummy hexane-soluble fraction (SR-HF, 0.4 g; 0.7% yield from the SR-EE) and hexane-insoluble residue. The hexane-insoluble residue was subjected to further partition between ethyl acetate and water (1:1; v/v). The top ethyl acetate layer was separated using separatory funnel and vaporized under reduced pressure in a rotary vacuum evaporator to obtain a dark brown-coloured ethyl acetate fraction (SR-EAF, 1.0 g; 1.7% yield from the SR-EE). The partition process was repeated five times or until the colour of ethyl acetate became pale. The bottom aqueous layer was sequentially submitted to lyophilization (Christ, Germany) to produce a light brown-coloured aqueous fraction (SR-AF, 26.6 g; 45.5% yield from the SR-EE). The crude ethanolic extract and partitioned fractions were stored at 4°C before further analysis.

3.4 Cell culture

HT-22 mouse hippocampal neuronal cells (Merck Millipore, Darmstadt, Germany) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Appendix B (a, ii)) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.5% amphotericin B at 37°C in the humidified 5% CO_2 incubator (Esco, Singapore) to reach 70–80% confluency. The cells were washed with phosphate buffer saline (PBS) (Appendix B (b, i)) and detached with accutase (passaged) every two to three days (Appendix B (d)).

3.5 Cell viability assessment

The HT-22 cell viability treated with *S. rugosum* extracts were measured by the quantitative colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). The SR-EE, SR-HF and SR-EAF were dissolved in sterile dimethyl sulfoxide (DMSO) with the exception of SR-AF which was solubilised in sterile PBS to form stock solutions of 80 mg/mL. In brief, a total of 1 × 10^4 cells/well were seeded in the single well of 96-well plate and following 24 h incubation, the medium was replaced with or without *S. rugosum* extracts (concentrations ranging from 12.5 to 400 µg/mL), not exceeding 0.5% (v/v) DMSO in content. Untreated cells and N-acetyl-L-cysteine (NAC) were served as negative control and positive control, respectively. After treatment for 24 h, MTT reagent (20 µL) at concentration of 5 mg/mL (Appendix B (b, ii)) was added into the tested well followed by an additional 3 h incubation at 37°C. The culture supernatant was thoroughly removed from the well and replaced with DMSO solution (200 µL/well) to terminate the reaction. Absorbance of dissolved formazan salt at 570 nm with a reference wavelength of 650 nm was measured using the Multiskan GO micro plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). The same protocol was repeated for 48 h with different cell density (4 × 10^3 cells/well) such that the optical density (OD) was between 0.75–1.25 according to the instruction guide provided by American Type Culture Collection (ATCC, USA). The cell viability was calculated using the formula: percentage of cell viability (%) = (OD value of treated cells/OD value of control without treatment) × 100%.

3.6 Optimization of glutamate concentration

HT-22 cells (1×10^4 cells/well) cultured in a 96-well microtiter plate were incubated overnight for adherence. The culture medium was then aspirated from the wells and replaced with different concentrations of glutamate (2.5, 5, 10, 15, 20 and 25 mM). The plates were left in the dark for 24 h at 37°C. Thereafter, the density of metabolically active cells was assessed by MTT assay with respect to Section 3.5.

3.7 *In vitro* neuroprotective and neurorescue assays

The neuroprotective and neurorescue properties of *S. rugosum* extracts were assessed by the pre-treatment and post-treatment of *S. rugosum* extracts against glutamate-induced HT-22 cells, respectively using MTT assay as mentioned in Section 3.5. For neuroprotective assay, HT-22 cells were cultivated at a density of 1×10^4 cells/well in 96-well plates one day before the pre-incubation with or without various concentrations of *S. rugosum* extracts for 3 h followed by the exposure to 5 mM glutamate. For neurorescue assay, the post-treatment was performed with or without the presence of 5 mM glutamate 3 h prior to treatment with different concentrations of *S. rugosum* extracts. After incubation for an additional 24 h, the cell viability was assessed by MTT assay, which reflects the mitochondrial succinate dehydrogenase activity. The exact procedure was repeated for 48 h with different cell density (4×10^3 cells/well) for both neuroprotective and neurorescue assays.

3.8 Flow cytometric analysis

The possible underlying molecular mechanisms responsible for the neuroprotection on glutamate-induced oxidative stress and cell death by SR-EE and SR-HF were investigated through flow cytometry.

3.8.1 Measurement of intracellular ROS level

The relative level of ROS generation in HT-22 cells was measured with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) using the ROS detection kit (PromoCell GmbH, Germany). In brief, HT-22 cells were seeded onto 6-well plates with a density of 1.5×10^5 cells in 2 mL culture media per well. After 24 h incubation, the cells were pre-treated with SR-EE (12.5 μg/mL) and SR-HF (100 μg/mL) for 3 h and then exposed to 5 mM glutamate. Following 24 h treatment, the cells were pooled and washed twice with PBS. A volume of 1 mL PBS containing 1×10^6 cells was centrifuged for 5 min at 400 × g at room temperature followed by the resuspension of cell pellet in ROS assay buffer loaded with H_2DCF-DA working solution. The fluorescence intensity of 2',7'-dichlorofluorescein (DCF) was determined after 30 min of incubation in the dark by using FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). A minimum of 10,000 events were collected and recorded.

3.8.2 Annexin V-FITC/Propidium iodide apoptosis assay

HT-22 cells (1.5×10^5 cells/well) were plated onto 6-well plates and incubated overnight for attachment. After pre-treatment with SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) for 3 h followed by an overnight glutamate exposure, the cells were harvested and stained with Annexin V-FITC and PI according to the protocol supplied in Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA). The stained cells were resuspended in the binding buffer and a total of 10,000 cells were analysed using a BD FACS Canto II flow cytometer within one hour. The population (%) of viable, early apoptotic, late apoptotic/secondary necrotic and early necrotic/dead cells were determined on the respective quadrants.

3.9 Gas chromatography/mass spectrometry (GC/MS) analysis

The GC/MS analysis was conducted with an Agilent Technologies 6890N (Palo Alto, CA, USA) GC System equipped with a 5979-mass selective detector (70 eV direct inlet) on a HP-5ms (5% phenyl-methylpolysiloxane) capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness) with helium as the carrier gas at a flow rate of 1 mL min^{-1} (split ratio 1:20). The column temperature was initially set at 60°C (isothermal for 1 min), with an increase of 5°C/min, to 280°C and held for 10 min. The obtained total ion chromatogram was autointegrated by ChemStation. The identification of constituents present in SR-HF was performed by comparing the spectrum of the constituents with spectrum of known compounds stored in the data base (NIST Library, 2011) and with those spectroscopic data reported in the literature (Chan et al., 2013). The matching quality index was set at 90% and any compound with matching quality index below 90% was not considered.

3.10 Data analysis

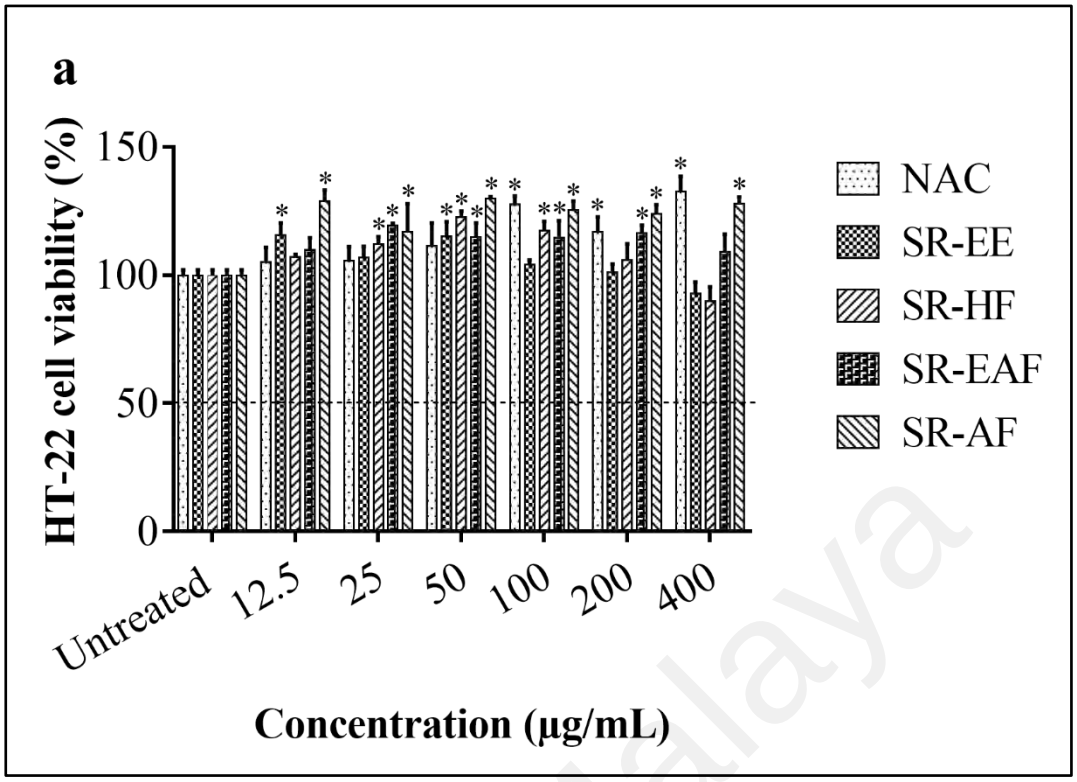
The results were shown as the mean ± standard deviation (SD). Each experiment was repeated at least three times. Quantitative data were evaluated for statistical difference by two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using GraphPad Prism 7.0 software (San Diego, CA, USA). Statistical difference was accepted at *P* value less than 0.05.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Effect of *S. rugosum* extracts on the viability of HT-22 cells

The neuroprotective and neurorescue activities of *S. rugosum* extracts were investigated using cell-based assays. The percentage of viable HT-22 cells after treatment with different concentrations of *S. rugosum* extracts was determined using MTT assay to exclude the possible occurrence of cytotoxicity caused by the extracts. Upon 24 h incubation, no cytotoxic effect was exerted on HT-22 cells after exposure with *S. rugosum* extracts, as the percentage of viable cells was found to be higher than 80% (Figure 4.1a). Therefore, *S. rugosum* extracts at various concentrations were selected for subsequent experiments.

As reported by Rossiana et al. (2018), the extract or compound was shown to exhibit cytotoxicity towards the cells if the percentage of viable cells was reduced by half (50% cell viability). After 48 h incubation, *S. rugosum* extracts showed low cytotoxicity on the HT-22 cells at any evaluated concentrations, except for a reduction in cell viability with SR-HF treated at 200 and 400 µg/mL and NAC treated at 25 and 50 µg/mL (Figure 4.1b). It was found that SR-HF remarkably (P<0.05) decreased the cell viability at concentrations of 200 and 400 µg/mL to 41.96 ± 1.59% and 7.79 ± 1.02%, respectively. NAC at concentrations of 25 and 50 µg/mL demonstrated significant (P<0.05) cytotoxicity as evident from the decrement of cell viability to 48.13 ± 2.52% and 43.96 ± 3.79%, respectively. According to Nemudzivhadi and Masoko (2014), a potential therapeutic agent with minimal toxic effect is considered as a good candidate in the development of new drug. Hence, *S. rugosum* extracts at various concentrations were selected for subsequent 48 h assays except SF-HF (200 and 400 µg/mL) and NAC (25 and 50 µg/mL) which showed appreciable cytotoxic effect.



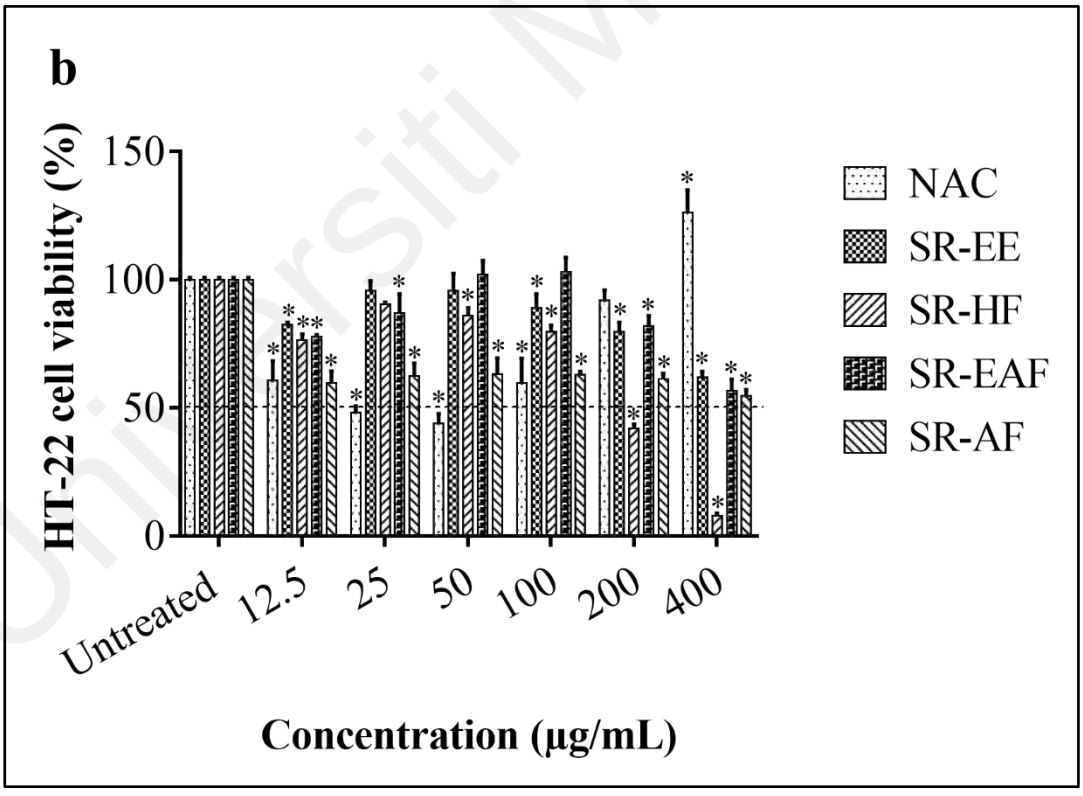


Figure 4.1: Effect of *S. rugosum* extracts on HT-22 cells after (a) 24 h and (b) 48 h incubation. The dashed line (50%) represents the threshold level of cell viability with cytotoxic effect. All data were expressed as mean ± SD (n=3). *significantly different from untreated control (P<0.05).

4.2 Cytotoxic effect of glutamate on HT-22 cells

Exposure of glutamate on HT-22 cells for 24 h gave rise to the reduction of cell viability in a concentration-dependent manner (Figure 4.2). At the lowest concentration (2.5 mM), the percentage of viable cells decreased significantly (P<0.05) by 26.67 ± 4.47% compared to the non-treated cells. Furthermore, consistent cell viability was shown when HT-22 cells were treated with glutamate with concentrations ranging from 10 to 25 mM. On the other hand, the data demonstrated that 5 mM glutamate remarkably (P<0.05) reduced the cell viability by 84.68 ± 3.44%. Therefore, it was reasonable to select 5 mM glutamate to induce cell injury in the following assays due to the substantial decline in the cell viability. This selection was further supported by the earlier literatures on neuroprotection studies (Yang et al., 2013; Park et al., 2019).

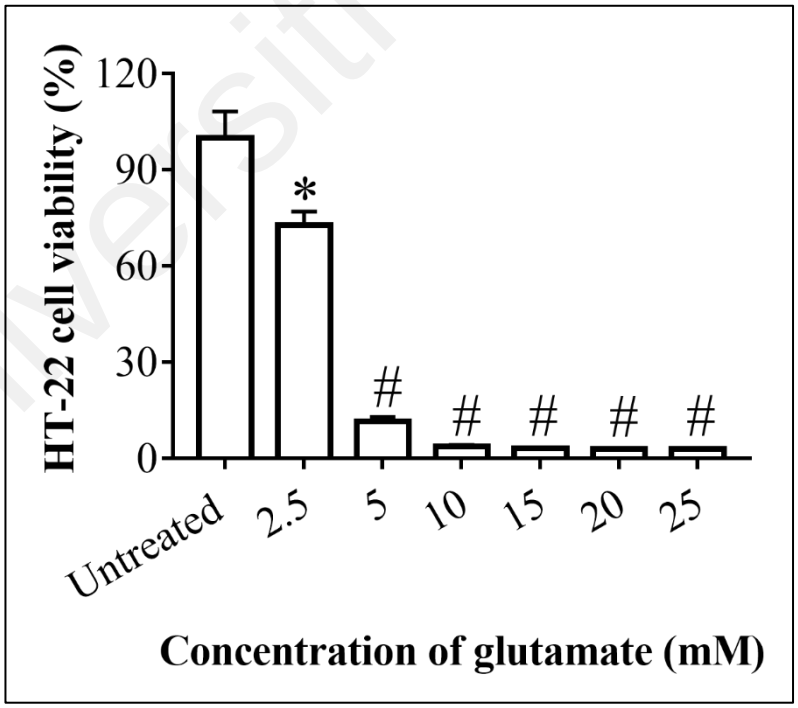


Figure 4.2: Cytotoxic effect of glutamate on HT-22 cells. HT-22 cells were exposed to various glutamate concentrations for 24 h and the cell viability was examined using MTT assay. Each bar was presented as mean ± SD (n=3). *2.5 mM glutamate is significantly different from untreated control (P<0.05); #5–25 mM glutamate is significantly different versus 2.5 mM glutamate and untreated control (P<0.05).

4.3 Neuroprotective effect of *S. rugosum* extracts against glutamate-induced HT-22 cells

Immortalized hippocampal cell line HT-22 has been extensively employed to unravel the glutamate-induced oxidative toxicity because it does not express functional NMDA-type glutamate receptors in its undifferentiated state. This eliminates the possibility of glutamate-induced excitotoxicity and, thus suggests HT-22 neuronal cell as a well-established *in vitro* system for studying glutamate-induced cell death through the stimulation of oxidative stress (Maher & Davis, 1996; Albrecht et al., 2010; Lee et al., 2013). Over the years, mounting literatures have demonstrated the potent neuroprotective property of different medicinal mushrooms such as *G. lucidum*, *C. militaris*, *H. erinaceus* and *L. rhinocerotis* (Sun et al., 2017; Kim et al., 2019b; Kushairi et al., 2019; Kittimongkolsuk et al., 2021). The ability to protect the oxidative stress-induced cells is depending on the type of extracts and presence of bioactive compounds in extracts (Geng et al., 2017; Lee et al., 2018).

Neuroprotective activity of *S. rugosum* extracts was assessed by pre-treating the HT-22 cells with various concentrations of extracts prior to the glutamate exposure and the results are presented in Figure 4.3a. The cell viability was significantly (P<0.05) reduced to 16.09 ± 2.46% after incubation with 5 mM glutamate for 24 h (shown as horizontal dotted line in Figure 4.3a). Interestingly, the glutamate-induced neurotoxicity was remarkably (P<0.05) abolished by the pre-treatment with extracts at all tested concentrations except SR-AF at 12.5 µg/mL. In addition, the percentage of viable cells was higher than NAC when HT-22 cells were pre-incubated with SR-EE and SR-HF at concentrations of 12.5–200 µg/mL, suggesting that they exhibited stronger neuroprotective activity against glutamate-induced oxidative stress over the positive control. The result also showed that the cell viability achieved 89.76 ± 9.50% and 90.21

± 6.30% when SR-HF at concentrations of 100 and 200 µg/mL were treated to the glutamate-induced HT-22 cells, respectively.

The pronounced neuroprotective activity demonstrated in SR-EE could be attributed to their richness of bioactive constituents. The selection of ethanol as the primary solvent used in the extraction was known to extract a wide range of agents with different polarities (Chan et al., 2011). It is plausible that the synergism may take place between bioactive components in SR-EE whereby the compounds appear in SR-HF could also appear in SR-EE (Olszowy-Tomczyk, 2020). On the other hand, hexane as the second solvent was consecutively employed for the extraction of non-polar compounds (Chan et al., 2011; Muhamad et al., 2019). Hence, the conspicuous neuroprotective effect exerted by SR-HF might be ascribed to the abundance of fatty acids and sterols.

In addition, the neuroprotective effect elicited by SR-EAF was less potent compared to SR-EE and SR-HF, as evident from its lower effectiveness to restore the loss of glutamate-induced cell viability. Furthermore, SR-AF exhibited the weakest protective effect against glutamate-induced neurotoxicity. NAC, a GSH precursor was used as the positive control because it exhibits potent antioxidant, anti-inflammatory and neuroprotective properties in counteracting age-related neurological diseases (Garg et al., 2018; Tardiolo et al., 2018). The present study indicated that NAC successfully improve the loss of glutamate-induced cell viability, which is in agreement with the recent studies (Song et al., 2018; Park et al., 2020b).

As shown in Figure 4.3b, treatment with 5 mM glutamate markedly (P<0.05) reduced the cell viability by 90.08 ± 0.60% of the cultured cells after 48 h (shown as horizontal dotted line). In general, pre-treatment with SR-HF showed neuroprotective activity at all tested concentrations except 12.5 µg/mL, further providing evidence that SR-HF could be developed into a potent neuroprotective agent. Besides, pre-treatment of HT-22 cells with the highest concentration of SR-EE and SR-EAF (400 µg/mL)

remarkably (P<0.05) protected the cells against glutamate-induced oxidative toxicity, preserving the cellular viability up to 30% with value of 31.47 ± 5.15% and 27.86 ± 1.51%, respectively. Furthermore, SR-AF showed insignificant neuroprotective effect to attenuate the glutamate-induced cells at all tested concentrations except 400 µg/mL after 48 h incubation. This data was found to be similar with the results for 24 h treatment (Figure 4.3a) which further suggested that SR-AF exhibited the weakest neuroprotective activity compared to other extracts.

Collectively, the result implied that the neuroprotective activity could be dependent on the polarity of solvent used in extraction and the respective bioactive compounds present in each extract. Hexane (non-polar solvent) has been shown to have strongest neuroprotection which could be attributed to the rich content in fatty acids and sterols (Sulmartiwi et al., 2018). Water with the highest polarity appeared to have least potent neuroprotection, indicating that the abundance of polar agents elicit weakest antioxidant and anti-inflammatory activities (Abu et al., 2017).

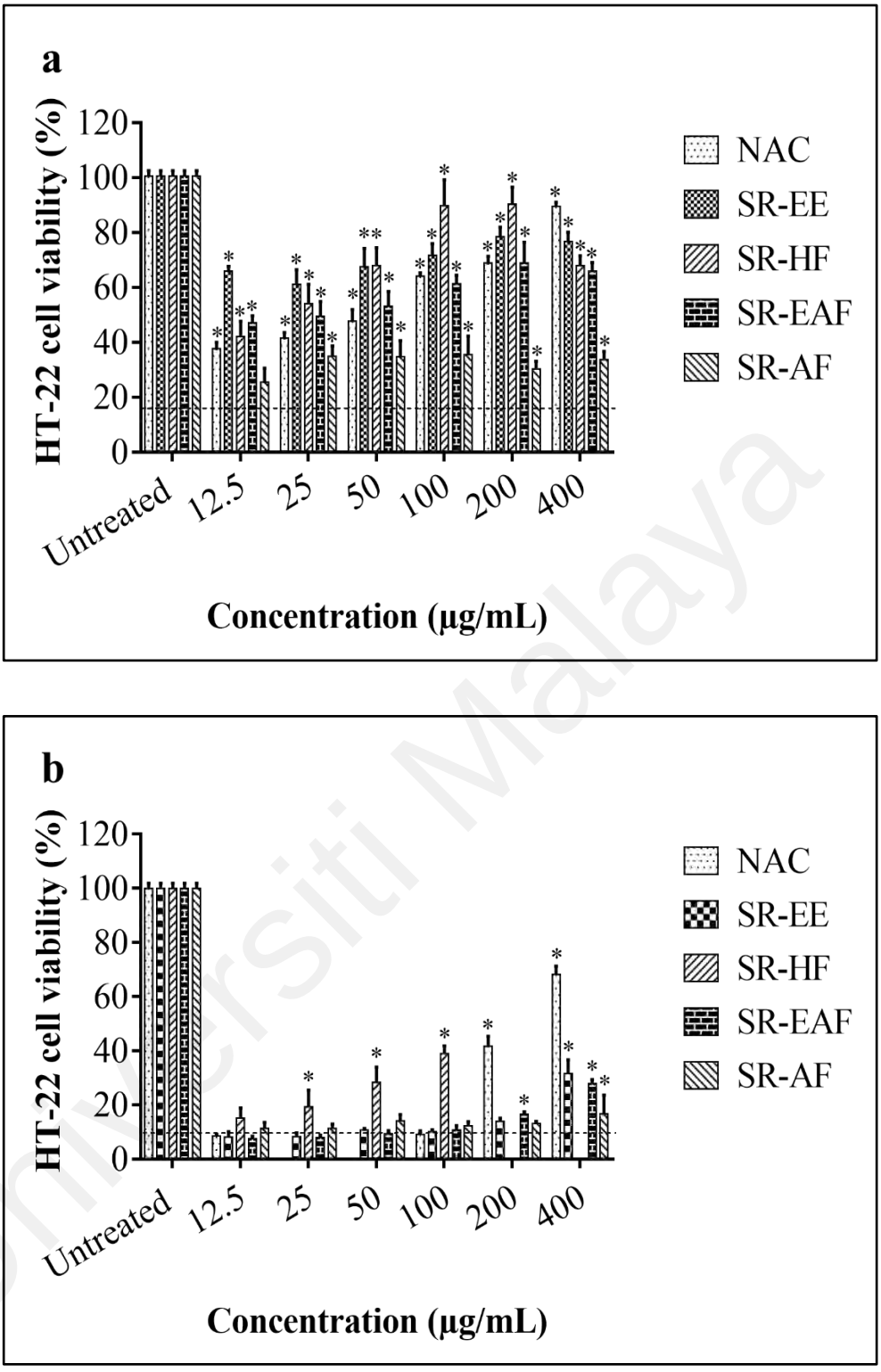


Figure 4.3: Neuroprotective effect of *S. rugosum* extracts against glutamate-induced HT-22 cells. HT-22 cells were treated in the presence or absence of *S. rugosum* extracts prior to the incubation with glutamate for (a) 24 h and (b) 48 h. MTT assay was performed to investigate the neuroprotective effect of *S. rugosum* extracts against glutamate-induced neurotoxicity. The dotted lines (16.09 ± 2.46% and 9.92 ± 0.60%) represent the percentage of cell viability after glutamate exposure for (a) 24 h and (b) 48 h, respectively. The readings of each tested well were expressed as percentage of untreated cells (100%). *P<0.05 was considered significantly different compared to the glutamate-treated cells.

4.4 Neurorescue effect of *S. rugosum* extracts against glutamate-induced HT-22 cells

Neurorescue potential of different natural compounds such as extracts of green tea, black tea, eggplant and medicinal herb have been extensively studied both *in vitro* and *in vivo* to search for the potential pharmacotherapies that prevent and treat Parkinson's disease (Reznichenko et al., 2005; Chaturvedi et al., 2006; Li et al., 2017; Singh et al., 2021). To the best of our knowledge, this is the first study to demonstrate the neurorescue potential of medicinal mushroom in the glutamate-induced HT-22 cells. The neurorescue effect of *S. rugosum* extracts were examined by pre-treating the HT-22 cells with glutamate followed by the incubation with extracts at various concentrations (post-treatment with extracts). The results are presented in Figure 4.4a (24 h incubation) and 4.4b (48 h incubation).

Whilst incubation with 5 mM glutamate for 24 h led to substantial (P<0.05) reduction of cell viability to 16.87 ± 2.96% (shown as horizontal dotted line in Figure 4.4a), the post-treatment with SR-HF at concentrations evaluated from 12.5 µg/mL until 400 µg/mL generally rescued the cells from the ongoing injury due to glutamate exposure, and the similar neurorescue activity was shown in the post-treatment with NAC. Additionally, the results revealed that SR-EAF at the highest concentration (400 µg/mL) ameliorated the reduction in cell viability to the greatest extent among the extracts, with recorded value of 73.84 ± 9.02%. The neurorescue effect of SR-EAF (400 µg/mL) was found to be comparable with NAC (84.69 ± 4.70%).

Upon 48 h treatment, glutamate at concentration of 5 mM significantly (P<0.05) decreased the cell viability by 95.16 ± 1.00% (shown as horizontal dotted line in Figure 4.4b). Generally, *S. rugosum* extracts were able to rescue HT-22 cells from glutamate-induced cell damage at all concentrations, compared to NAC which failed to rescue the cells at concentration of 100 µg/mL. Post-treatment with SR-EE and SR-EAF exhibited

strongest neurorescue effect from glutamate exposure at the highest concentration (400 µg/mL) with cell viability of 21.15 ± 3.09% and 23.69 ± 1.00%, respectively. Furthermore, SR-AF markedly (P<0.05) rescued the glutamate-induced HT-22 cells at all concentrations. This could be ascribed to the presence of polysaccharides in SR-AF that contributed to the remarkable neurorescue activity (Sun et al., 2017).

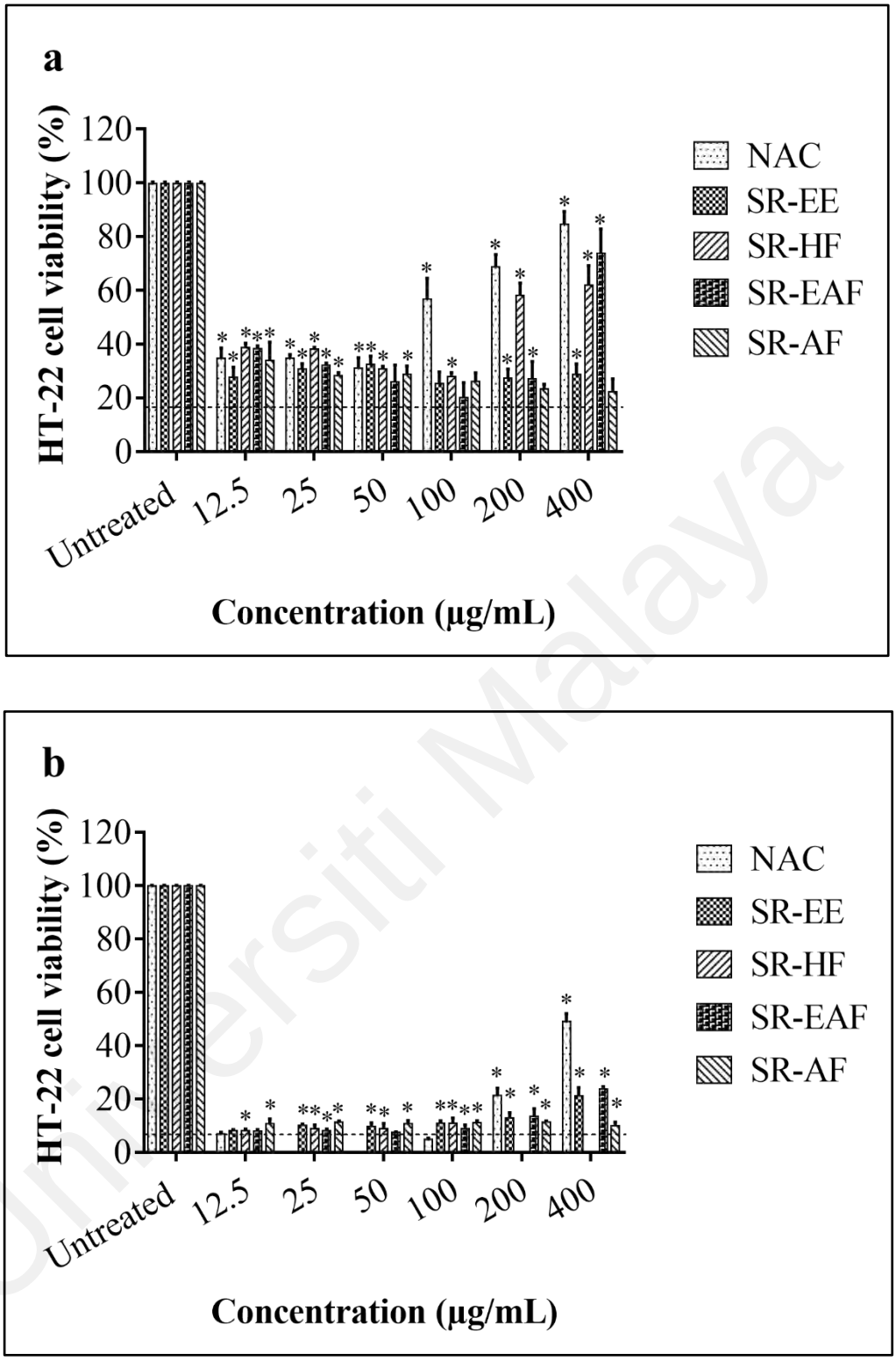


Figure 4.4: Neurorescue effect of *S. rugosum* extracts against glutamate-induced HT-22 cells. Pre-incubation with or without 5 mM glutamate prior to the treatment with *S. rugosum* extracts for (a) 24 h and (b) 48 h were carried out to assess the neurorescue activity of *S. rugosum* extracts on the glutamate toxicity in HT-22 cells using MTT assay. The dotted lines (16.87 ± 2.96% and 4.84 ± 1.00%) represent the percentage of cell viability after glutamate exposure for (a) 24 h and (b) 48 h, respectively. Each column represents the mean ± SD (n=3). *Significant difference from the glutamate-induced cells (*P*<0.05).

4.5 Effect of SR-EE and SR-HF on glutamate-induced ROS accumulation

Accumulation of exogenous glutamate induces oxidative stress through the inhibition of cystine uptake *via* the cystine/glutamate antiporter (system Xc−) (Murphy et al., 1989; Yang et al., 2014). Within the cell, reduction of cystine into cysteine promotes the imbalance of cystine homeostasis and rapid depletion of endogenous antioxidant GSH, ultimately renders the neuronal cells more susceptible to ROS accumulation and cell death (Shirlee et al., 2001; Fukui et al., 2010; Pfeiffer et al., 2014; Zhang et al., 2019a). Therefore, eliminating ROS production is a possible strategy to ameliorate the neuronal cell demise.

Based on the findings on neuroprotection in MTT cell viability assay (Figure 4.3a), SR-EE and SR-HF were shown to demonstrate more potent neuroprotective effect compared to SR-EAF and SR-AF as SR-EE and SR-HF were more effective in restoring the loss of glutamate-induced cell viability. Pre-treatment with SR-EE (12.5 µg/mL) was shown to restore the reduction of cell viability more effectively (4.96-fold) compared to NAC at concentration of 12.5 µg/mL (2.93-fold) against glutamate-induced cells. Besides, the loss of cell viabilities induced by glutamate was improved by the pre-treatment with SR-HF (100 µg/mL and 200 µg/mL) by 7.28-fold and 7.08-fold, respectively. Consequently, the higher cell viability shown in SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) were selected for subsequent flow cytometric analysis. To further investigate the effect of SR-EE and SR-HF on intracellular ROS production, an ROS-sensitive fluorescence indicator, H_2DCF-DA, was used. Upon the cell entry, the stable non-fluorescent H_2DCF-DA is deacetylated to form 2',7'-dichlorodihydrofluorescein (H_2DCF) which can be further oxidized to produce DCF by intracellular ROS (Christov et al., 2003). The representative dot plots of untreated cells, cells treated with glutamate only, cells pre-treated with SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) prior to glutamate exposure

are shown in Figure 4.5a, b, c and d, respectively. The population of cells that produce ROS (ROS positive) are indicated as blue dots in the right quadrant of each dot plot.

As shown in Figure 4.5b, incubation of HT-22 cells with glutamate at concentration of 5 mM led to increment of ROS level, with recorded value of 31.9% compared to the untreated cells (0.9%; Figure 4.5a). However, pre-treatment with SR-EE (12.5 µg/mL) successfully reduced the glutamate-induced ROS production to 13.8% (Figure 4.5c). This could be explained from the presence of phenolic compounds in the ethanol extract which function as antioxidants that attenuate the glutamate-induced intracellular ROS accumulation (Kittimongkolsuk et al., 2021). In contrast, pre-treatment with SR-HF (100 µg/mL) was able to suppress the ROS generation more effectively compared to SR-EE as it recorded ROS level of 8.0% (Figure 4.5d). This data was in line with the previous reports showing the antioxidant capabilities in hexane extracts of medicinal mushrooms such as *H. erinaceus, Metacordyceps neogunnii* and *Dictyophora indusiata* (Daba et al., 2020). Taken together, the results indicated that SR-HF served as better neuroprotective agent than SR-EE in terms of the ROS scavenging activity and inhibitory effect on intracellular ROS production.

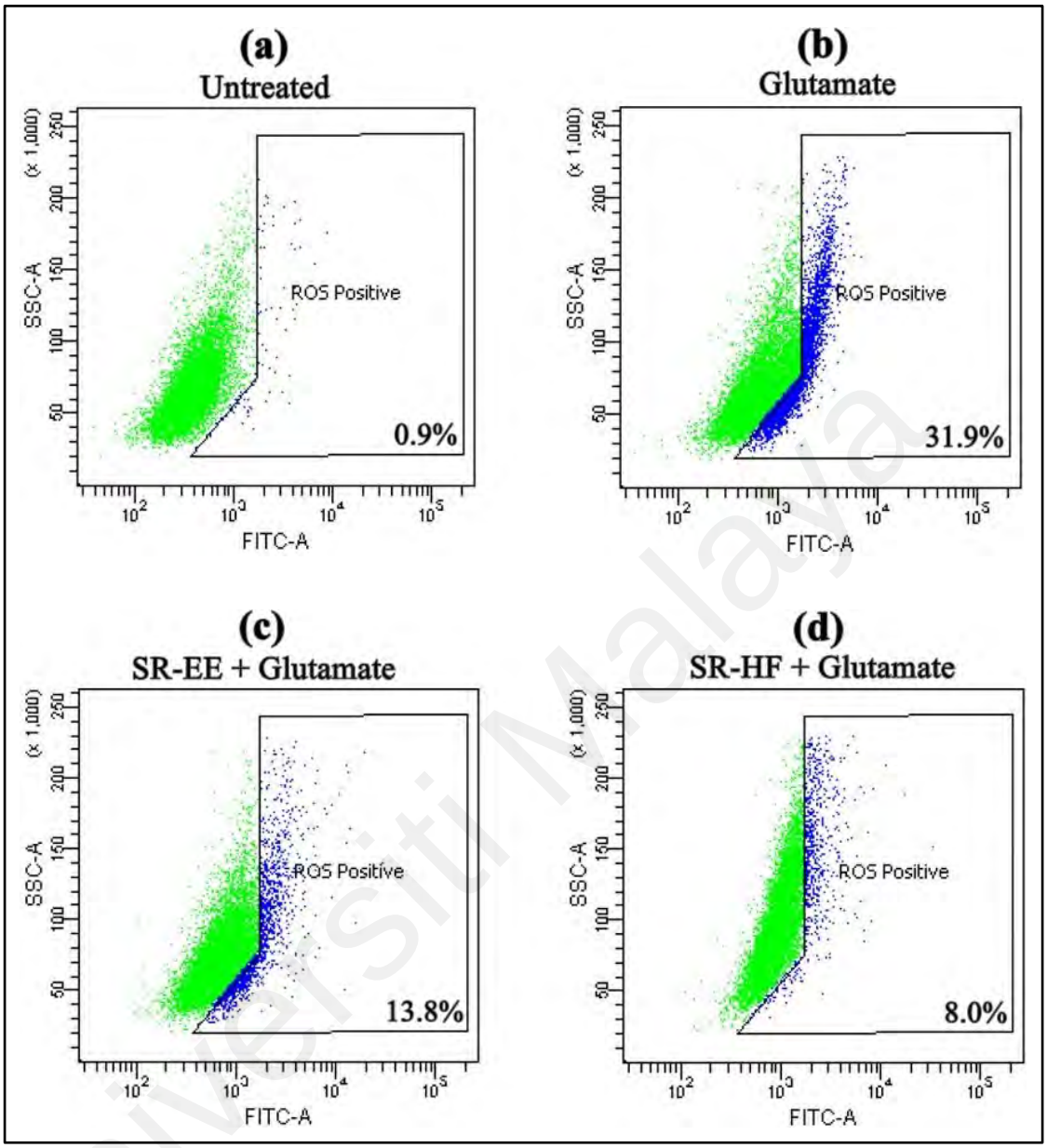


Figure 4.5: Effect of SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) on glutamate-induced ROS production. The level of intracellular ROS for (a) untreated cells, cells treated with (b) glutamate alone, cells pre-treated with both (c) SR-EE and (d) SR-HF followed by glutamate exposure for 24 h were determined using the fluorescent dye H_2DCF-DA followed by FACS analysis. The population of cells that releases ROS (ROS positive) are indicated as blue dots in the right quadrant of each dot plot.

4.6 Effect of SR-EE and SR-HF on glutamate-induced cell death

Glutamate-induced oxidative stress has been postulated to induce both apoptosis and necrosis in neuronal cells such as PC12 cells (Lu et al., 2011; Ma et al., 2012), HT-22 cells (Lee et al., 2017; Kim et al., 2019a) and SH-SY5Y cells (Nikolova et al., 2005; Yuksel et al., 2019). According to Fukui et al. (2009), glutamate-induced HT-22 cell death involved both necrotic and apoptotic cell death in a time-dependent manner. In the present study, Annexin V-FITC/PI double staining was conducted using flow cytometer to examine whether SR-EE and SR-HF could suppress glutamate-induced cell death as well as to characterize the cell death mechanisms involved. Annexin V has high affinity to phosphatidylserine (PS) which is normally located on the inner surface of plasma membrane. During early apoptosis, disruption of membrane asymmetry leads to the translocation of PS to the outer leaflet, which externalizes PS on the surface of apoptotic cell (Van Engeland et al., 1998). Hence, Annexin V which is often conjugated with the fluorochrome such as FITC can function as a sensitive probe to identify cells in the early stage of apoptosis. Whilst, PI is included in the incubation mix to identify the late apoptotic and necrotic cells that have lost their membrane integrity. As the membrane breaks down, these dead and damaged cells are particularly permeable to PI (Brauchle et al., 2014). Therefore, dual staining with fluorescent Annexin V and PI can be used to distinguish early apoptotic cells from necrotic cells.

Figure 4.6a and b show the representative dot plots of untreated HT-22 cells and glutamate-induced cells, respectively. The dot plots of cells pre-treated with SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) before incubation with glutamate for 24 h are demonstrated in Figure 4.6c and d. The population of viable cells with intact membrane and absence of PS exposure on the outer surface of membrane is shown in the lower left quadrant (Q3, Annexin-V−/PI−). Presence of early apoptotic cells are indicated in the lower right quadrant (Q4, Annexin-V+/PI−). The population of late apoptotic cells or

secondary necrotic cells are represented in the upper right quadrant (Q2, Annexin-V+/PI+). Cells in the upper left quadrant (Q1, Annexin-V−/PI+) are the early necrotic cells or dead cells.

In the present study, induction with 5 mM glutamate shifted the dot plot from Q3 to Q1 and that the percentage of late apoptotic and early necrotic cells was increased to 20.5% (Figure 4.6b) compared to the untreated cells (14.6%; Figure 4.6a). It is noticeable that the population of early apoptotic cells in Q4 was neither prominent nor significant in Figure 4.6b (0.2%) and majority of apoptotic cells were in late stage, hence this observation suggested that glutamate-induced cell death mainly underwent necrotic pathway (Vermes et al., 1995). As illustrated in Figure 4.6c, the cell death caused by the glutamate exposure was not reversed following the pre-treatment with SR-EE. This is because the population of late apoptotic and early necrotic cells was shown to increase with recorded percentage of 26.6% (Figure 4.6c) compared to the glutamate-treated group (20.5%; Figure 4.6b), implying the failure of complete activation of the antioxidant protection system which eventually exacerbate the cell death (Battistelli et al., 2016). On the other hand, pre-treatment with SR-HF (100 µg/mL) reduced the late apoptotic and early necrotic cells to 13.3% (Figure 4.6d) compared with that of the glutamate-treated group (20.5%; Figure 4.6b). The present findings showed that the protective effect of SR-HF against glutamate-induced neurotoxicity was associated with the attenuation of cell death.

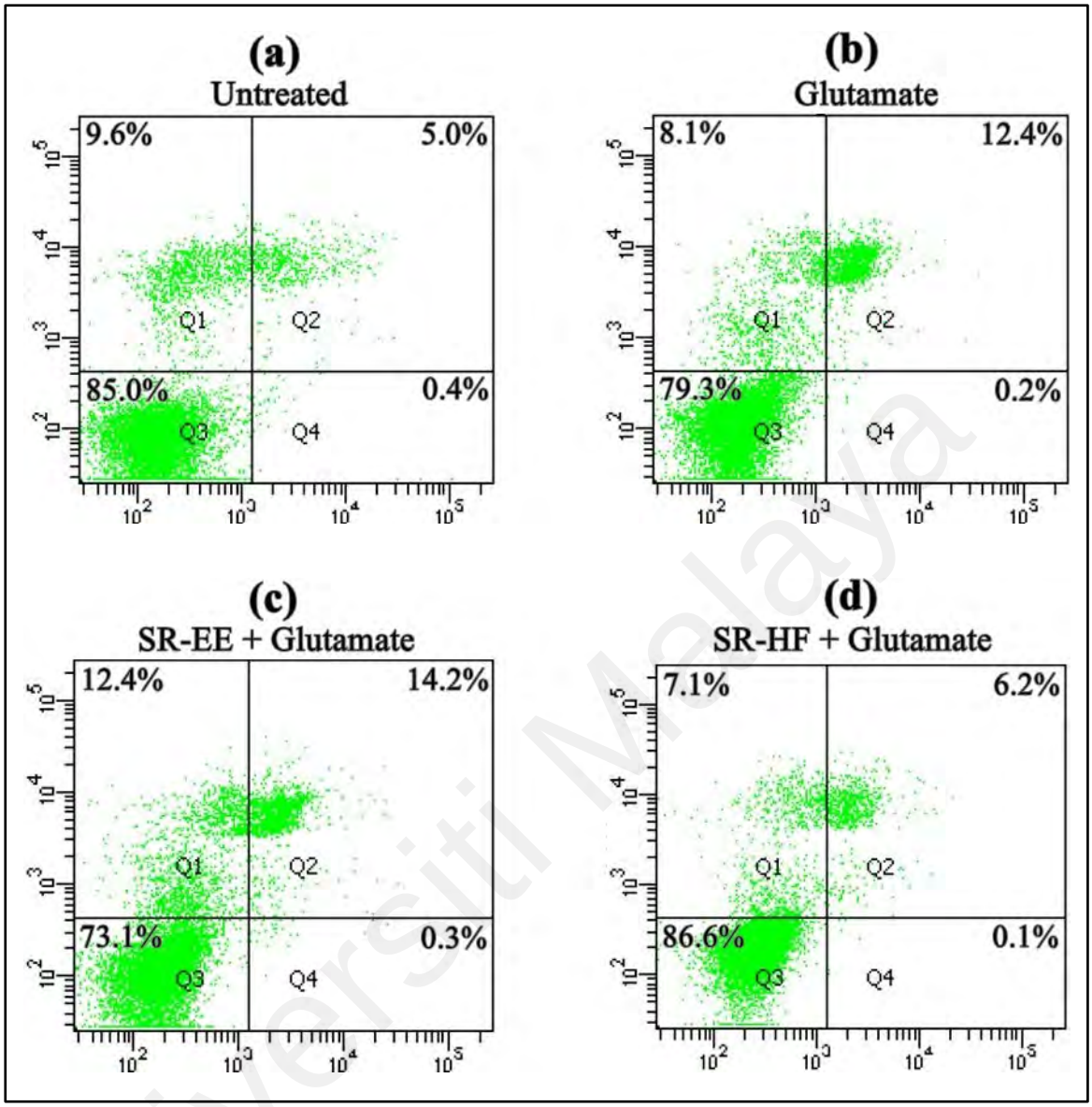


Figure 4.6: Effect of SR-EE (12.5 µg/mL) and SR-HF (100 µg/mL) on glutamate-induced cell death. The quantitative analysis was performed by flow cytometer using Annexin V/PI double staining in (a) untreated cells, (b) glutamate-treated cells, cells pre-exposed with both (c) SR-EE at concentration of 12.5 µg/mL and (d) SR-HF at concentration of 100 µg/mL following the administration of glutamate for 24 h. The quadrants were designated as Q1 (early necrotic/dead), Q2 (late apoptotic/secondary necrotic), Q3 (viable) and Q4 (early apoptotic) cells.

4.7 GC/MS analysis of SR-HF

Based on the above findings, SR-HF was selected for further identification of compounds which may contribute to the neuroprotective activity. As presented in Table 4.1, total eleven compounds including three fatty acids (50.67%), four fatty acid esters (13.14%) and one sterol (10.11%) have been detected in the composition of hexane fraction of *S. rugosum* (SR-HF) by GC/MS analysis. These compounds constituted 77.21% of the overall composition of SR-HF (Figure 4.7). Linoleic acid (41.70%) was found to be the most abundant fatty acid, followed by palmitic acid (8.13%) and margaric acid (0.84%). The fatty acid esters were dominated by ethyl linoleate (9.17%), ethyl oleate (1.65%), methyl linoleate (1.19%) and ethyl palmitate (1.13%). The ergosta-5,7,22-trien-3β-ol (ergosterol) contributed to 10.11% of the total fraction.

Linoleic acid, ergosterol and ethyl linoleate were the main compounds present among the eleven compounds identified in SR-HF. Previously, a study conducted by Chan et al. (2013) identified ergosterol as the main compound followed by ethyl linoleate. Therefore, the present findings provide additional data on the composition of SR-HF and offer the first analytical proof on the presence of eleven compounds in SR-HF. The difference in the compounds present in SR-HF could be due to the culture conditions in terms of temperature, humidity or light and the growth stage of mushrooms (Mizuno et al., 1995; Asgharpour et al., 2020). In the present study, most of the compounds were reported to exhibit antioxidant and anti-inflammatory activities such as palmitic acid (Guerrero et al., 2017), linoleic acid (Elagbar et al., 2016; Saiki et al., 2017), ethyl palmitate (Guerrero et al., 2017), methyl linoleate (Krishnamoorthy & Subramaniam, 2014), ethyl linoleate (Park et al., 2014), ethyl oleate (Asghar & Choudahry, 2011; Ponmathi et al., 2017) and ergosterol (Drori et al., 2016; Mei et al., 2019). The mass-spectral data and chemical structure of each compound identified were shown in Appendix C.

Ergosterol which is the most abundant sterol found in cell wall and membrane of mushroom can be easily converted into vitamin D_2 upon exposure of ultraviolet (UV-B) irradiation, which makes mushrooms to be the rich source of vitamin D (Duffy et al., 2018; Papoutsis et al., 2020). The technology to produce vitamin D_2 from ergosterol of commercial mushrooms has been widely used to develop dietary supplements and food fortification (Phillips et al., 2011). In the present study, it was revealed that ergosterol is the major sterol found in SR-HF, thus *S. rugosum* could serve as an excellent source of dietary vitamin D supplement for people with insufficiency of vitamin D. The variation in the ergosterol content is depending on the growing and storage temperature of mushrooms or UV intensity (Gąsecka et al., 2018). In this study, *S. rugosum* was grown in the absence of sunlight. Concerning the growing environment of *S. rugosum*, this may offer justification that ergosterol was not shown as the major compound in SR-HF.

Table 4.1: Phytochemical components in SR-HF.

No	Chemical constituents	RT (min)	MW	MF	*Percentage of peak area (%)	Reported biological activity
1	β-acetylacrylic acid	6.471	114	$C_5H_6O_3$	0.56	Anti-fertility (Lagervall, 1958)
2	1-Methylcyclohexane-carboxylic acid	14.591	142	$C_8H_{14}O_2$	2.73	Anticonvulsant (Sobol et al., 2004)
3	n-Hexadecanoic acid (Palmitic acid)	29.696	256	$C_{16}H_{32}O_2$	8.13	Antioxidant, anti-inflammatory (Guerrero et al., 2017), anticancer (Ravi & Krishnan, 2017)
4	Hexadecanoic acid, ethyl ester (Ethyl palmitate)	30.328	284	$C_{18}H_{36}O_2$	1.13	Antioxidant (Guerrero et al., 2017)
5	Heptadecanoic acid (Margaric acid)	31.549	270	$C_{17}H_{34}O_2$	0.84	Anticancer (Xu et al., 2019)
6	9,12-octadecadienoic acid (Z,Z)- methyl ester (Methyl linoleate)	32.196	294	$C_{19}H_{34}O_2$	1.19	Anti-inflammatory, anti-cancer (Krishnamoorthy & Subramaniam, 2014)
7	9,12-octadecadienoic acid (Z,Z)-, (Linoleic acid)	32.945	280	$C_{18}H_{32}O_2$	41.70	Antioxidant (Elagbar et al., 2016), anti-inflammatory (Saiki et al., 2017)
8	Linoleic acid ethyl ester (Ethyl linoleate)	33.405	308	$C_{20}H_{36}O_2$	9.17	Anti-inflammatory (Park et al., 2014)
9	Ethyl oleate	33.507	310	$C_{20}H_{38}O_2$	1.65	Antioxidant (Asghar & Choudahry, 2011), anti-inflammatory (Ponmathi et al., 2017)
10	Ergosta-5,7,22-trien-3β-ol (Ergosterol)	49.757	396	$C_{28}H_{44}O$	10.11	Anti-inflammatory (Drori et al., 2016; Mei et al., 2019)

RT: Retention time; MW: Molecular weight; MF: Molecular formula

* Percentage of peak area were calculated based on the results obtained from the total ion chromatogram.

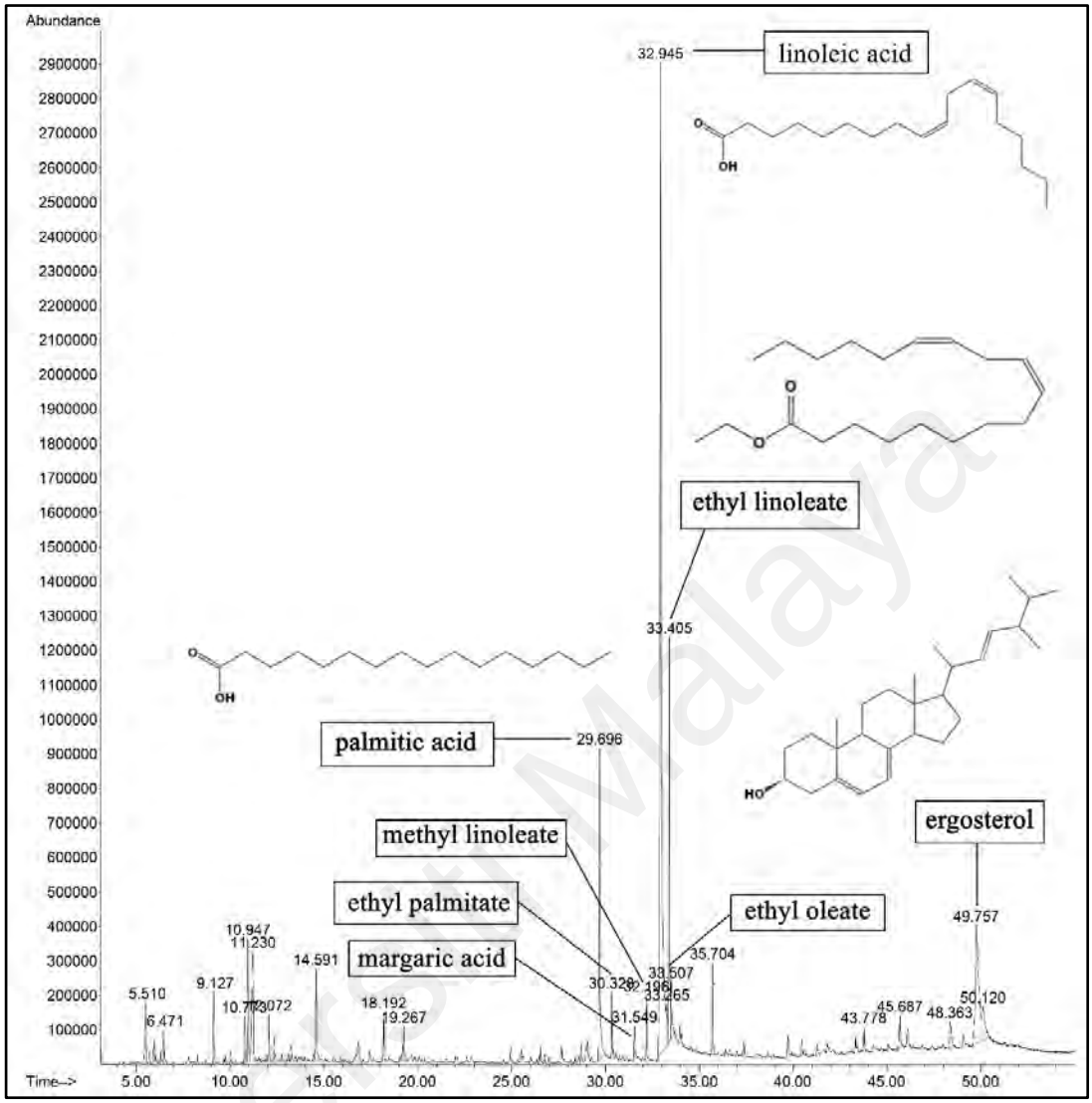


Figure 4.7: GC/MS total ion chromatogram of the SR-HF.

4.8 Overall discussion

Neurological diseases have become major public health concern around the world. These diseases including Parkinson's Disease, Alzheimer's Disease, stroke, epilepsy and multiple sclerosis, are among the most pervasive health issues in elder adults, chiefly those aged 55 and older. Neurological diseases are often accompanied with the impairment of cognitive or motor functions, muscle weakness, poor coordination and ache (Farooqui, 2018). Therefore, the diseases affecting the brain or nervous system are closely linked to the heightened risk of adverse health sequelae, such as fatality, disability and hospitalization (Callixte et al., 2015). Notwithstanding the pathogenesis of neurological disorders is complicated and not well understood, research resembling the fundamental characteristics of disease have manifested pivotal factors, including oxidative stress and neuroinflammation (Rehman et al., 2019). Thus, therapeutic strategies focusing on neuroprotection and neurorescue could be effective to prevent, or perhaps hamper the development of neurological diseases associated to oxidative stress and neuroinflammation.

Generally, neuroprotection (pre-treatment with *S. rugosum* extracts) represents the action taken to defend the brain against injury (Nzogang & Donkeng, 2020). Whilst, neurorescue (post-treatment with *S. rugosum* extracts) is viewed as the ability of neurons to recover from damage (Hjorth & Schultzberg, 2017). In brief, the cell viabilities of the pre- and post-treatment with two potential extracts at different concentration upon 24 h exposure were selected to make comparison. Interestingly, pre- and post-treatment with SR-EE (12.5 µg/mL) were shown to restore the reduction of cell viability by 4.96-fold and 2.26-fold, respectively compared to the glutamate-induced cells. In parallel, the loss of cell viabilities was improved by the pre- and post-treatment with SR-HF (100 µg/mL) by 7.28-fold and 2.11-fold, respectively compared to the glutamate-induced cells. Taken together, pre-treatment with SR-EE and SR-HF demonstrated better restoration in the loss

of cell viability than the post-treatment, implying that neuroprotection offered greater effectiveness than neurorescue to mitigate neurological diseases related to oxidative stress and neuroinflammation.

Both SR-EE and SR-HF were further selected for the evaluation of their ability to ameliorate oxidative stress and cell death. The present study indicates that SR-HF could serve as the potent neuroprotective agent among all extracts, as regards to its role in attenuating the ROS accumulation and cell death induced by glutamate. In view of these results obtained, SR-HF was subjected to GC/MS analysis whereby most of the identified compounds possessed antioxidant and anti-inflammatory activities, further suggesting that these activities are responsible for the excellent neuroprotective potential of SR-HF.

CHAPTER 5: CONCLUSION AND PROPOSED FUTURE DIRECTIONS

In conclusion, this study showed that SR-HF exhibited strongest neuroprotective activity against glutamate-induced cells among all the extracts. Taking into account that glutamate-induced oxidative stress is one of the main factors responsible for cell death, the experimental results demonstrated that SR-HF may develop into an alternative neuroprotective agent to treat neurological diseases related to oxidative stress and neuroinflammation.

This study evaluated the *S. rugosum* extracts as potent neuroprotective agent against glutamate-induced HT-22 mouse hippocampal cells. In this direction, further investigations to isolate and characterize the active compounds in SR-HF are warranted in an effort to create an avenue for the identification of an exact compound contributing to its remarkable neuroprotective activity. An extended investigation can also be carried out using the identified chemical constituents of SR-HF in order to ascertain the compounds that can act in a synergistic fashion. Moreover, additional studies on molecular basis are necessary to clearly establish the possible signalling pathways involved in the neuroprotective action of SR-HF against glutamate induction. Additionally, it is worthwhile to conduct *in vivo* studies and clinical trials so that the clinical relevance of SR-HF can be validated. Concerning the mild cytotoxic effect observed when higher concentrations of SR-HF were treated on the HT-22 cells for 48 h, it is imperative that the dose which will be administered in the future treatment regime be closely monitored.

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