NEUROPROTECTIVE EFFECT OF *Centella asiatica* (L.) Urb. AGAINST TOXICITY INDUCED BY DIFFERENT NEUROTOXIC AGENTS IN NEUROBLASTOMA SH-SY5Y CELLS

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NEUROPROTECTIVE EFFECT OF *Centella asiatica* (L.) Urb. AGAINST TOXICITY INDUCED BY DIFFERENT NEUROTOXIC AGENTS IN NEUROBLASTOMA SH-SY5Y CELLS

ABSTRACT

Neurodegenerative diseases are characterized by the progressive dysfunction and death of neuronal cells and oxidative stress has been identified as one of the major cause of a range of neurodegenerative disorders. Most of the current therapies of neurodegenerative diseases involve usage of synthetic drugs which are believed to have some inadequacies. Thus, there has been a great interest towards using plant derived natural products as a potential neuroprotective agent. The present study aimed to investigate the neuroprotective effects of methanolic and water extracts of *Centella asiatica* against neurotoxic agents (hydrogen peroxide (H_2O_2) and acrylamide)-induced toxicity in human neuroblastoma SH-SY5Y cells. Prior to the neuroprotective assay, a preliminary screening was conducted to assess the toxicity of dimethyl sulfoxide (DMSO), neurotoxic agents and extracts of *C. asiatica* towards SH-SY5Y cells. The results demonstrated that DMSO and both extracts had no significant toxicity effect towards the cells at concentration range of 0.05 to 1.25% (v/v) and 1 to 100 µg/ml, respectively. The half-maximal inhibitory concentration (IC_{50}) of H_2O_2 and acrylamide was found to be 100 µM and 5 mM, respectively and these concentrations were used to induce toxicity in the cells during neuroprotective assays. The neuroprotective effect was assessed through cell viability using MTT assay whereby the cells were pre-treated with extracts of *C. asiatica* and then exposed to H_2O_2 and acrylamide separately. The results revealed that both methanolic and water extracts exhibited a mild neuroprotective activity against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells. The neuroprotective activity of methanolic extract was observed to

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be higher than water extract and both extracts conferred a better neuroprotection towards the cells against toxicity caused by H_2O_2 than acrylamide. In addition, the combined neuroprotective effects of extracts of *C. asiatica* and curcumin against H_2O_2 and acrylamide-induced toxicity in the cells was also investigated. Before that, the neuroprotective potential of curcumin against the neurotoxic agents-induced toxicity was investigated. The results revealed that curcumin had a considerable neuroprotective effect against both neurotoxic agents. For combination study, the results indicated that the combination of the extracts with curcumin slightly improved the neuroprotective activity against toxicity-induced by H_2O_2 while no improvement was observed in the neuroprotection against toxicity-induced by acrylamide. Besides that, antioxidant activity of curcumin and extracts of *C. asiatica* was determined through *in vitro* antioxidant assays such as DPPH radical scavenging assay, ABTS radical scavenging assay and iron chelating assay. The results showed that curcumin had an effective DPPH and ABTS radical scavenging activity than methanolic and water extracts. Among the extracts, water extract exhibited a slightly higher antioxidant activity compared to methanolic extract. The iron chelating activity of curcumin and methanolic extract was not able to be determined. In conclusion, methanolic extract of *C. asiatica* is a potential neuroprotectant and both the extracts and its combination with curcumin demonstrated a better neuroprotective activity against toxicity-induced by H_2O_2 compared to acrylamide. The mild neuroprotective effects of extracts of *C. asiatica* and its combination with curcumin might be due to the moderate antioxidant activity of the extracts. Further recommendations for future study were also suggested in this study.

Keywords: neuroprotective, *Centella asiatica*, hydrogen peroxide (H_2O_2), acrylamide, SH-SY5Y cells.

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KESAN NEUROPROTEKTIF *Centella asiatica* (L.) Urb. TERHADAP KETOKSIKAN YANG DISEBABKAN OLEH EJEN NEUROTOKSIK YANG BERBEZA DALAM SEL NEUROBLASTOMA SH-SY5Y

ABSTRAK

Penyakit neurodegenerasi dikenalpasti melalui disfungsi progresif dan kematian sel-sel neuron dan tekanan oksidatif merupakan salah satu punca utama pelbagai jenis penyakit tersebut. Kebanyakan rawatan semasa melibatkan penggunaan ubat sintetik yang dipercayai mempunyai beberapa kelemahan. Oleh itu, fokus ditumpukan terhadap penggunaan produk semula jadi berasaskan tumbuhan sebagai ejen berpotensi neuroprotektif. Kajian ini dijalankan untuk menyiasat kesan neuroprotektif ekstrak metanol dan air *Centella asiatica* terhadap ketoksikan yang disebabkan oleh ejen-ejen neurotoksik [hidrogen peroksida (H_2O_2) dan akrilamida] ke atas sel neuroblastoma SH-SY5Y. Sebelum kajian neuroprotektif, pemeriksaan awal mengenai ketoksikan DMSO, ekstrak-ekstrak *C. asiatica* dan ejen neurotoksik terhadap sel SH-SY5Y telah dilakukan. Keputusan menunjukkan bahawa DMSO dan kedua-dua ekstrak tidak mempunyai kesan ketoksikan yang signifikan terhadap sel-sel pada lingkungan kepekatan 0.05 hingga 1.25% (v/v) dan 1 hingga 100 µg/ml masing-masing. Separuh maksimum kepekatan perencatan (IC_{50}) ejen neurotoksik didapati 100 µM bagi H_2O_2 dan 5 mM bagi akrilamida dan kepekatan ini telah digunakan untuk menyebabkan ketoksikan dalam sel SH-SY5Y semasa kajian neuroprotektif. Kesan neuroprotektif telah dinilai melalui daya maju sel menggunakan kaedah MTT dimana sel SH-SY5Y telah diprarawat dengan ekstrak-ekstrak *C. asiatica* dan seterusnya didedahkan dengan H_2O_2 dan akrilamida secara berasingan. Hasil kajian menunjukkan bahawa kedua-dua ekstrak metanol dan air mempunyai aktiviti neuroprotektif yang serderhana terhadap ketoksikan yang disebabkan oleh H_2O_2 dan akrilamida dalam sel-sel SH-SY5Y. Aktiviti

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neuroprotektif esktrak metanol didapati lebih tinggi daripada esktrak air dan kedua-dua ekstrak tersebut memiliki aktiviti neuroprotektif yang lebih baik terhadap ketoksikan yang disebabkan oleh H_2O_2 berbanding akrilamida. Tambahan pula, kesan neuroprotektif gabungan ekstrak-ekstrak *C. asiatica* dengan kurkumin terhadap ketoksikan yang disebabkan oleh H_2O_2 dan akrilamida dalam sel-sel SH-SY5Y juga dikaji. Sebelum itu, potensi neuroprotektif kurkumin terhadap ketoksikan yang disebabkan oleh ejen-ejen neurotoksik telah dianalisa. Hasilnya menunjukkan bahawa kurkumin mempunyai kesan neuroprotektif yang memberangsangkan terhadap kedua-dua ejen neurotoksik. Bagi kajian gabungan, hasil kajian menunjukan bahawa kombinasi ekstrak metanol dan air dengan kurkumin meningkatkan aktiviti neuroprotektif terhadap ketoksikan yang disebabkan oleh H_2O_2 sementara tiada peningkatan yang diperhatikan dalam kesan neuroprotektif terhadap ketoksikan disebabkan oleh akrilamida. Di samping itu, aktiviti antioksidan kurkumin dan ekstrak-ekstrak *C. asiatica* telah dinilai melalui ujian antioksidan *in vitro* seperti ujian penghapusan radikal DPPH, ujian penghapusan radikal ABTS dan ujian 'chelating' besi. Hasilnya menunjukkan bahawa kurkumin mempunyai aktiviti penghapusan radikal DPPH dan ABTS yang efektif berbanding ekstrak metanol dan air. Antara kedua-dua ekstrak, ekstrak air menunjukkan aktiviti antioksidan yang lebih baik berbanding ekstrak metanol. Aktiviti 'chelating' besi kurkumin dan ekstrak metanol tidak dapat ditentukan. Kesimpulannya, ekstrak metanol *C. asiatica* merupakan ejen berpotensi neuroprotektif dan kedua-dua ekstrak dan kombinasinya dengan kurkumin menujukkan aktiviti neuroprotektif yang lebih baik terhadap ketoksikan yang disebabkan oleh H_2O_2 berbanding akrilamida. Kesan neuroprotektif ekstrak-ekstrak *C. asiatica* yang serderhana mungkin disebabkan oleh aktiviti antioksidan ekstrak yang sederhana. Beberapa cadangan bagi penambaikan kajian telah juga dicadangkan dalam kajian ini.

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Kata kunci: neuroprotektif, *Centella asiatica*, hidrogen peroksida (H_2O_2), akrilamida, sel SH-SY5Y.

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LIST OF SYMBOLS AND ABBREVIATIONS

C_3H_5NO : Acrylamide

α : Alpha

β : Beta

Ca^{2+} : Calcium ion

CO_2 : Carbon dioxide

Cu^{2+} : Copper(II) ion

cm^3 : Cubic centimeter

˚C : Degree celsius

N_2O_3 : Dinitrogen trioxide

IC_{50} : Half-maximal inhibitory concentration

H_2O_2 : Hydrogen peroxide

·OH : Hydroxyl radical

Fe^{2+} : Iron(II) ion

Fe^{3+} : Iron(III) ion

$FeCI_2$: Iron(II) chloride

Mg^{2+} : Magnesium ion

µg/ml : Microgram per milliliter

µl : Microliter

µM : Micromolar

mg/ml : Milligram per milliliter

ml : Milliliter

mM : Millimolar

nm : Nanometer

Ni^{2+} : Nickel(II) ion

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$NO^{\cdot}$: Nitric oxide

NO_2 : Nitrogen dioxide

HNO_2 : Nitrous acid

% : Percentage

$ONOO^-$: Peroxynitrite

KH_2PO_4 : Potassium dihydrogen phosphate

K^+ : Potassium ion

$K_2S_2O_8$: Potassium persulfate

$^1O_2$: Singlet oxygen

$NaHCO_3$: Sodium bicarbonate

NaCI : Sodium chloride

NaH_2PO_4 : Sodium dihydrogen phosphate

Na^{2+} : Sodium ion

$NaSO_4$: Sodium sulphate

$O_2^{\cdot -}$: Superoxide

v/v : Volume per volume

w/v : Weight per volume

6-OHDA : 6-hydroxydopamine

ABTS : 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AD : Alzheimer's disease

ADP : Adenosine diphosphate

AIDS : Acquired immune deficiency syndrome

ALS : Amyotrophic lateral sclerosis

ANOVA : Analysis of variance

ATP : Adenosine triphosphate

BHA : Butylated hydroxyanisole

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BHK : Baby hamster kidney

BHT : Butylated hydroxytoluene

BNDF : Brain-derived neurotrophic factor

CAT : Catalase

CBD : Corticobasal degeneration

CNS : Central nervous system

COX-2 : Cyclooxygenase-2

CUR : Curcumin

DMEM/F12 : Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

DPPH : 2,2-diphenyl-1-picrylhydrazyl

EDTA : Ethylenediaminetetraacetic acid

eNOS : Endothelial nitric oxide synthase

FBS : Fetal bovine serum

FTP : Frontotemporal dementia

GPx : Glutathione peroxidase

GSH : Glutathione

HD : Huntington's disease

HEPES : Hydroxyethyl-piperazineethane-sulfonic acid buffer

HNE : 4-hydroxyl-2-nonenal

iNOS : Inducible nitric oxide synthase

ME : Methanolic extract

MPO : Myeloperoxidase

MPTP : 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS : Multiple sclerosis

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MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	: Nicotinamide adenine dinucleotide phosphate hydrogen
nNOS	: Neuronal nitric oxide synthase
NOS	: Nitric oxide synthase
NPC	: Neural progenitor cells
PARP	: Poly (ADP-ribose) polymerase
PBS	: Phosphate-buffered saline
PD	: Parkinson's disease
RK	: Rabbit kidney
RNS	: Reactive nitrogen species
ROS	: Reactive oxygen species
SOD	: Superoxide dismutase
SPSS	: Statistical Package for the Social Sciences
TH	: Tyrosine hydroxylase
Trolox	: 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
WE	: Water extract
WHO	: World Health Organization

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LIST OF APPENDICES

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CHAPTER 1: INTRODUCTION

1.1 Background of Study

Neurodegeneration is defined as a process which involves the progressive loss of structure or function of neurons in the central nervous system (CNS) (Rasool *et al*., 2014). There are many types of diseases associated with neurodegeneration known as neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), corticobasal degeneration (CBD), frontotemporal dementia (FTP) and multiple sclerosis (MS) (Chen *et al*., 2012).

Oxidative stress has been identified as one of the key factors of neurodegeneration. Oxidative stress occurs because of excess production of reactive oxygen species (ROS) (oxidants) which can lead to the disability of the biological system to detoxify the reactive intermediates through antioxidant defence mechanism (Pham-Huy *et al*., 2008; Thanan *et al*., 2015). The generation of ROS can be caused by the internal factors such as the electron transport chain in the mitochondria, activation of oxidant producing enzymes and interaction between the ROS and metals through Fenton and Haber-Weiss reactions. External factors include exposure towards radiation and chemicals (e.g. lead, cadmium, arsenic, acrylamide) and consumption of drugs (e.g. cyclosporine, gentamicin, bleomycin, sodium nitroprusside), cooking oil and alcohol (Pham-Huy *et al*., 2008). The highly reactive properties of ROS causes damages to the biomolecules such as lipid, protein and DNA which leads to alteration in the neurons and glial cells function and eventually lead to the programmed cell death (apoptosis) (Thanan *et al*., 2015).

At present, the neurodegenerative diseases are becoming more prevalent and it is estimated to rise rapidly in the future (Melo *et al*., 2011). As claimed by World Health Organization (WHO), neurodegenerative disorders will exceed cancer as the second

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main cause of death in the world by 2040 (Andrade & Naus, 2016). The rapid growth in the number of people with neurodegenerative diseases triggers the development of various synthetic drugs and surgical procedures for treatment. Examples of drugs that are generally used for the treatment are tacrine, donepezil, rivastigmine and levodopa. Most of these drugs are expensive and thus creates immense burden for the society particularly those in poor and undeveloped countries (Casey *et al*., 2010). According to Lunn *et al*. (2011), stem cell therapy is emerging as one the effective treatments of neurodegenerative diseases, however its high cost may create a challenge for its application in the management of neurodegenerative diseases.

Besides that, synthetic drugs can also impose side effects such as nausea, muscular weakness, sleep disorder, gastrointestinal upset and weight loss (Kumar *et al*., 2015). Some of these drugs can cause severe side effects that can be harmful to health. For example, tacrine and anti-inflammatory drugs can cause liver and kidney toxicity (Wollen, 2010). In addition, Noble and Burns (2010) had claimed that most of these drugs are not effective as it only provides short term relief of the symptoms rather than fully curing the diseases.

Plant-based natural products in the form of fresh plants, herbs, extracts or their phytochemicals generally have wide range of pharmacological effects since ancient times. Its potential as neuroprotective agents were also evident from many previous literatures (More *et al*., 2013). One of such plant is *Centella asiatica* or locally known as “pegaga” in Malaysia. It is an important medicinal plant that is widely utilized in Traditional Chinese Medicine and Ayurveda. First medicinal use of *C. asiatica* was reported in approximately 1700 A.D. (Hamidpour *et al*., 2015). *C. asiatica* has various medicinal properties and its main component triterpene is believed to be responsible for those properties (Gohil *et al*., 2010). The traditional use of *C. asiatica* is mainly related to CNS management such as improvement of memory and learning, treat mental illness

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and epilepsy and as sedative besides being used to treat skin disease, fever, jaundice, diarrhoea, ulcer and asthma (Gohil *et al*., 2010; Roy *et al*., 2013). *C. asiatica* is commonly consumed as a cooked vegetable, fresh salad or as a drink (Hashim *et al*., 2011).

In addition, curcumin is a polyphenolic compound derived from the rhizome of *Curcuma longa* L. (commonly known as tumeric) which is native to Southeast Asia particularly India (Farkhondeh *et al*., 2016). The main bioactive component of tumeric is curcuminoids, a group of polyphenols which include curcumin, demethoxycurcumin and bisdemethoxycurcumin. Among these constituents, curcumin is the main bioactive constituent of tumeric (Iriti *et al*., 2010). Curcumin also have wide application in Ayurveda and Traditional Chinese Medicine for its antioxidant, anti-inflammatory, antimicrobial and anticancer properties (Mishra & Palanivelu, 2008). The daily consumption of curcumin as dietary supplement (tumeric is used as spice in most of Indian's culinary) have been hypothesized as the main reason for reduced prevalence of AD in India compared to US (Iriti *et al*., 2010). Moreover, some studies have suggested that the improved cognitive function in elder people is related to their dietary consumption of curry (Mazzanti & Giacomo, 2016).

Thus, in the present study, the *in vitro* neuroprotective effects of extracts of *C. asiatica* and combination of extracts of *C. asiatica* with curcumin against neurotoxic agents [hydrogen peroxide (H_2O_2) and acylamide]-induced toxicity in human neuroblastoma SH-SY5Y cells were investigated. A preliminary study was conducted to assess the toxicity of dimethyl sulfoxide (DMSO), neurotoxic agents and extracts of *C. asiatica* towards SH-SY5Y cells. In addition, the antioxidant activity of extracts of *C. asiatica* and curcumin was also determined in this study.

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1.2 Research Objectives

1.2.1 General Objective

The aim of the present study is to evaluate the neuroprotective potential of extracts of *Centella asiatica* towards neurotoxic agents-induced toxicity in human neuroblastoma SH-SY5Y cells.

1.2.2 Specific Objectives

The following are the specific objectives of the study:

i. to investigate the neuroprotective effects of extracts of *C. asiatica* towards neurotoxic agents-induced toxicity in human neuroblastoma SH-SY5Y cells.
ii. to assess the improvement of neuroprotective effects of extracts of *C. asiatica* towards neurotoxic agents-induced toxicity in SH-SY5Y cells after combining with curcumin.
iii. to evaluate the antioxidant activity of extracts of *C. asiatica* and curcumin.

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CHAPTER 2: LITERATURE REVIEW

2.1 Neurodegeneration

Neurodegeneration is composed of the word "neuro-", which indicates the nerve cells and "degeneration", which indicates the action of losing structure and function (Przedborski *et al*., 2003). Therefore, neurodegeneration is defined as a process which involves the progressive loss of structure or function of nerve cells in the central nervous system (CNS). The common effects of neurodegeneration are cognitive disorders such as memory loss, impaired reasoning, difficulties in reading, writing and speaking and personality changes and ataxia like tremor, muscular rigidity and postural imbalance (Massano & Bhatia, 2012; Sepand *et al*., 2013).

Neurodegeneration is generally caused by both internal and external factors. The internal factors include genetic, oxidative stress, mitochondrial dysfunction, inflammation, protein aggregation, depletion and degradation of neurotransmitter, abnormal ubiquitination, excitotoxicity and damage of blood-brain barrier (Perez-Hernandez *et al*., 2016); while external factor is related with the environmental factors such as chemicals and radiation exposure and food (Thanan *et al*., 2015).

2.1.1 Neurodegenerative Diseases

Neurodegenerative diseases are diseases associated with neurodegeneration. Examples of neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). Extracellular and intracellular deposition of aggregated protein in neuronal cells is the hallmark of many neurodegenerative diseases (Li *et al*., 2013).

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AD and PD are two most prevalent types of neurodegenerative diseases with higher occurrences with an average of 35.6 million people in the world are affected by AD and this amount is estimated to double by 2030 (Huang *et al*., 2012). Meanwhile, approximately 1.5 million people in the United State are affected with PD and nearly 50,000 new cases reported each year (Melo *et al*., 2011). AD is associated with deposition of extracellular beta-amyloid plaques and intracellular tau protein tangles which leads to neuronal loss. PD involves the progressive loss of dopaminergic neurons in the substantia nigra and aggregation of the protein alpha-synuclein (Huang *et al*., 2012; Fu *et al*., 2015).

To date, there is no cure found for neurodegenerative diseases although many significant funding and research have been dedicated to discover the therapeutic of the diseases. Most of the treatments of neurodegenerative diseases are symptomatic treatments that temporarily alleviate the symptoms related to the disease without affecting the progression of the disease (Kiaei, 2013). Examples of symptomatic treatment are the use of anticholinesterase inhibitor drugs for AD such as donepezil, galantamine and rivastigmine while levodopa and selegiline for PD (Casey *et al*., 2010; Kartika *et al*., 2010).

Therefore, many of current researches have been focused on disease modifying treatments which could impede the development of disease by inhibiting the critical processes involved in the progression of the disease (Kiaei, 2013). One of such emerging treatment is natural product based treatment. Many *in vitro* and *in vivo* studies have demonstrated that variety of bioactive components from natural products could interfere with the pathophysiological mechanisms related to neurodegeneration mainly via their antioxidant and anti-inflammatory properties (Rocha *et al*., 2011).

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2.2 Oxidative Stress

Oxygen is an important element of life whereby it is generally used as a substrate for energy metabolism for normal function and survival of most of the living organisms. During the normal cellular metabolism, oxygen can be converted into reactive oxygen species (ROS) and nitrogen into reactive nitrogen species (RNS) (Li *et al*., 2013). At low concentration, they regulate the physiological functions such as they involve in cellular signalling pathways that are responsible for cell growth, survival and differentiation, gene expression, mitosis, migration and apoptosis.

ROS and RNS also involve in the defence system against infectious agents and regulate the cellular homeostasis (Birben *et al*., 2012; Dhawan, 2014). In contrast, high concentration of ROS and RNS exert oxidative stress in which they cause damages to the cell's biomolecules such as protein, lipid and DNA. Oxidative stress occurs due to imbalance between the level of ROS or RNS and the antioxidant defences in the body (Gandhi & Abramov, 2012). This oxidative damages lead to the development of diseases such as neurodegenerative, cancer, atherosclerosis, hypertension, diabetes, and asthma (Birben *et al*., 2012).

2.2.1 Types of ROS and RNS

ROS is a group of reactive molecules derived from oxygen. The serial reduction of oxygen which has two unpaired electrons leads to the formation of ROS (Dhawan, 2014). Examples of ROS are superoxide ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). $O_2^{\cdot -}$ which is the precursor of many other ROS is formed by the reduction of molecular oxygen, H_2O_2 is produced by dismutation of $O_2^{\cdot -}$ and partial reduction of H_2O_2 forms $^{\cdot}OH$ (Turrens, 2003).

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RNS is primarily produced by reaction of nitric oxide ($NO^{\cdot}$) with other free radicals (Patel *et al*., 1999). $NO^{\cdot}$ has one unpaired electron and it is formed by nitric oxide synthases (NOS). Examples of RNS are $NO^{\cdot}$, nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3) and nitrous acid (HNO_2) (Dhawan, 2014). $ONOO^-$ is produced by the reaction of $NO^{\cdot}$ with $O_2^{\cdot -}$ and autoxidation of $NO^{\cdot}$ forms N_2O_3 (Dedon & Tannenbaum, 2004).

2.2.2 Mechanism of ROS and RNS Generation

The generation of ROS can be caused by the internal factors such as (i) the electron transport chain in the mitochondria, endoplasmic reticulum and peroxisomes, (ii) activation of oxidant producing enzymes [NADPH oxidase, xanthine oxidase, myeloperoxidase (MPO), cyclooxygenase-2 (COX-2) and NOS], and (iii) interaction between the ROS and metals through Fenton and Haber-Weiss reactions. External factors include (i) radiation, (ii) chemicals (iron, lead, cadmium, arsenic, benzene, acrylamide), (iii) drugs (cyclosporine, gentamicin, bleomycin, sodium nitroprusside), (iv) infectious agents, and (v) alcohol (Pham-Huy *et al*., 2008).

(i) Electron Transport Chain in Mitochondria

Mitochondrion is one of the major endogenous contributor of ROS/RNS. It is the site of energy production that comprises electron transport chain with a series of membrane bound respiratory complexes (Dasuri *et al*., 2013). During the process of oxidative phosphorylation, electrons are transferred across the electron transport chain for the reduction of oxygen into water. However, around 1–3% of electrons might leak

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from the system and thus reduce molecular oxygen into $O_2^{\cdot -}$ which also results in the formation of H_2O_2 and $^{\cdot}OH$ (Birben *et al*., 2012).

(ii) Activation of NADPH Oxidase

NADPH oxidase is a transmembrane enzyme complex which is an important source of endogenous ROS/RNS. It produces ROS by catalysing the electron transfer from NADPH to oxygen (Gandhi & Abramov, 2012). The generation of ROS by NADPH oxidase is dependent on cell type whereby more ROS is produced by phagocytes (monocytes, macrophages and neutrophils) compared to other cells like endothelial and smooth muscle cells. Aging also contribute to the increasing level of NADPH activity (Dasuri *et al*., 2013).

(iii) Activation of Nitric Oxide Synthase (NOS)

NOS plays an important role in $NO^{\cdot}$ production by catalysing the oxidation of L-arginine and L-citrulline. Neuronal (nNOS), endothelial (eNOS) and inducible isozyme (iNOS) are the three main isoforms of NOS involved in the production of $NO^{\cdot}$ (Dasuri *et al*., 2013). These isoforms also can produce $O_2^{\cdot -}$ when appropriate substrate and cofactor are available (Sun *et al*., 2010).

(iv) Chemicals Exposure

Environmental and occupational chemical exposure is the major example of exogenous source of ROS/RNS. Exposure to transition metals such as iron and copper can cause ROS generation by Haber-Weiss and Fenton reactions whereby $O_2^{\cdot -}$, and

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H_2O_2 can interact to produce ·OH (Birben *et al*., 2012). Exposure to benzene can cause the production of reactive molecules such as hydroquinone and benzoquinone which can produce ROS by redox cycling (Lodovici & Bigagli, 2011). In addition, arsenic is another type of chemical that can cause the formation of wide range of ROS/RNS such as $O_2^{\cdot -}$, H_2O_2, $^1O_2$ and NO·.

(v) Drugs

Drug is another example of external source of ROS/RNS in which the metabolisms of many drugs produce reactive intermediates that can reduce molecular oxygen to directly produce ROS and indirectly RNS. Doxorubicin is a drug that is used to treat different types of cancer. Reduction of doxorubicin by mitochondria reductases may produce free radicals which are unstable and readily reduce oxygen to generate $O_2^{\cdot -}$ and H_2O_2 (Deavall *et al*., 2012).

2.3 Oxidative Stress in Neurodegeneration

As one of the component of CNS, brain is particularly vulnerable to oxidative stress due to several reasons. First, brain which consists of neurons and astrocytes has high oxygen demand as it is one of the most metabolically active organs in the body. Although the brain represents only 5% of the body weight, it utilizes approximately 20% of the total oxygen consumption in which considerable amount of that oxygen can be converted into ROS. Second, abundant polyunsaturated fatty acids that are highly susceptible to lipid peroxidation are found in brain cell membranes. In addition, brain also has a weak endogenous antioxidative system (Uttara *et al*., 2009). Fourth, brain has high level of metal ions such as copper and iron that act as a catalyst for ROS formation

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and the metabolism of excitatory amino acids and neurotransmitters is the source of ROS production. Lastly, brain cells have large reliance on oxidative phosphorylation as a source of energy compared to other cells (Chen *et al*., 2012).

Oxidative stress generally causes damages to the biomolecules such as lipid, protein and DNA which leads to alteration in the neurons and glial cells function and eventually lead to the programmed cell death (apoptosis) (Ghandhi & Abromov, 2012). Lipid peroxidation involves the oxidation of the polyunsaturated fatty acids that are abundant in the brain which produces end products such as malondialdehyde, 4-hydroxy-2-nonenal (HNE), acrolein and isoprostanes that are toxic to the neurons (Chen *et al*., 2012). These reactive end products also react with other cellular macromolecules such as protein and DNA that can indirectly induces cellular apoptosis. According to Chen *et al*. (2012), HNE inhibits the function of membrane proteins such as neuronal glucose and glutamate transport protein and this phenomenon has been recognized as one of the cause for the degeneration of the dopaminergic neurons in substantia nigra. Lipid peroxidation also affects the stability of the cell membrane (Chen *et al*., 2012). In addition, Gandhi and Abramov (2012) reported that an increased level of HNE and malondialdehyde was detected in brain and cerebrospinal fluid of AD patients which revealed that lipid peroxidation is evident in neurodegeneration disorders.

Besides that, oxidation of protein is an irreversible process which also contributes to the development of neurodegeneration. Protein misfolding and aggregation is one of the effects of protein oxidation whereby the functionality of the protein will be affected and it also leads to further formation of oxidants. Protein oxidation also causes the formation of protein carbonyls, inhibition of proteosomal activity, impairment of neurotransmitter and abnormal energy metabolism in the neurons (Thanan *et al*., 2015). According to Li *et al*. (2013), aggregation of protein

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subjected to oxidative damage is the hallmark in neurodegenerative diseases such as AD and PD. Moreover, an increased level of carbonyl proteins compared to the control observed in brain of PD patients indicates the association of protein oxidative damage in the neurodegenerative diseases (Thanan *et al*., 2015).

DNA damage by oxidative stress also plays an important role in neurodegeneration based on molecular mechanism whereby the ROS can cause DNA double or single strands breaks, DNA-protein crosslinks and modification of purine and pyrimidine bases which results in gene mutation and thus interferes with the gene transcription and translation in the neurons (Chen *et al*., 2012). Shukla *et al*. (2011) reported that elevated levels of DNA oxidation products such as 8-hydroxy-2-deoxyguanosine and 8-hydroxyguanosine was exhibited by patients with AD.

2.4 Antioxidant

Antioxidant is a molecule that can counteract the damages caused by oxidative stress and thus preventing the deleterious diseases associated with it. The mechanism of antioxidant action includes the scavenging of the ROS, molecular repairing of ROS damages, activating internal antioxidant enzymes, inhibiting ROS generating enzymes and by chelating metals involved in ROS production (Lu *et al*., 2010). There are two different types of antioxidant which are endogenous and exogenous antioxidants.

Endogenous antioxidant (enzymatic and non-enzymatic) is naturally produced in the body while exogenous antioxidant is derived from external source mainly form diet. Endogenous antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) while exogenous antioxidants include vitamin C, vitamin E, carotenoids and polyphenols (Uttara *et al*., 2009). At present, exogenous antioxidant which is mainly plant-based antioxidant has drawn

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considerable attention. Plants are rich with many bioactive phytochemicals which act as an antioxidant agent and found to be less expensive, easily available, have less side effects compared to synthetic antioxidants (Lu *et al*., 2010).

2.5 Medicinal Plants

Medicinal plants are an inevitable source of alternative medicine to treat various types of diseases as they generally have a wide range of biological activities. At present, many scientific studies have reported the role of medicinal plants as a therapeutic for neurodegenerative diseases. Examples of such plants are *Ginkgo biloba, Bacopa monnieri, Withania somnifera, Terminalia chebula, Centella asiatica, Panax ginseng, Ocimum sanctum, Melissa officinalis, Hypericum perforatum, Curcuma longa,* and *Nardostachys jatamansi* (Roy & Awasthi, 2017).

The mechanism in which these plants exert neuroprotective role includes direct uptake of free radicals, regulation of enzymes related with oxidative stress and chelation of metals involved in Fenton reactions (Hernandez *et al*., 2015). Kumar and Mondal (2016) reported the neuroprotective function of *B. monnieri* in which it increased the endogenous antioxidant levels and reduced lipid peroxidation and α-synuclein protein accumulation in brain. Besides that, the antioxidant activity was demonstrated by *H. perforatum* which is capable of chelating iron ions and scavenging hydroxyl radical while *P. ginseng* inhibited the nitric oxide production (Altun *et al*., 2013; Jang *et al*., 2016). In addition, *W. somnifera* is a nervine tonic whereby it is able to rejuvenate the neuronal cells (Roy & Awasthi, 2017).

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2.5.1 *Centella asiatica* (L.) Urban

Centella asiatica (L.) Urban (Figure 2.1), is a perennial herbaceous creeper plant which is native to Southeast Asia and belongs to the family Apiaceae (Meena *et al*., 2012). It has small fan shaped green leaves with long green-reddish stem and can grow approximately up to 15 cm (Figure 2.2). The plant can be found abundantly in moist areas (Arora *et al*., 2002). *C. asiatica* is also known as Gotu kola, Indian pennywort, Indian water navelwort, Asiatic pennywort, wild violet, and tiger herb (Orhan, 2012).

The major bioactive component reported in *C. asiatica* is triterpene which include both triterpene glycoside and triterpene acid such as asiaticoside, brahmoside, brahminoside, madecassoside, asiatic acid and madecassic acid. It also consists of flavonoids such as quercetin and kaempferol, phenolic acid, polysaccharides, polyacetylenes and essential oil (Orhan, 2012; Marques *et al*., 2015).

Figure 2.1: *Centella asiatica* (L.) Urban.

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Figure 2.2: The aerial parts of *C. asiatica*.

2.5.2 Biological Activities of *C. asiatica*

C. asiatica is well known for its wound healing, anti-inflammatory, antioxidant, cytotoxic and antitumor, immunostimulant, antidiabetic, antifungal, antimicrobial and antiviral effects (Orhan, 2012). These different types of therapeutics potential of *C. asiatica* are studied in the form of extracts or purified single compound in various *in vivo* and *in vitro* models. A summary of the biological activities of *C. asiatica* from several pharmacological studies is presented in Table 2.1.

Aqueous extract of *C. asiatica* demonstrated wound healing activity by increasing the collagen synthesis and cellular proliferation at wound site in rats (Sunilkumar *et al*., 1998) while asiaticoside derived from *C. asiatica* exhibited good antioxidant activity which was a contributing factor in the wound healing process in

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wounded rats (Shukla *et al*., 1999). According to George *et al*. (2009), aqueous and alcoholic extracts of *C. asiatica* reported to have *in vivo* anti-inflammatory activity which is similar to the standard Ibuprofen against induced carrageenan paw oedema rats.

Besides that, *C. asiatica* also possess anticancer activity whereby methanolic extract of *C. asiatica* induced apoptosis in human breast MCF-7 cancer cells (Babykutty *et al*., 2009). A study conducted by Jayashress *et al*. (2003) reported the antioxidant property *C. asiatica* in which oral treatment of methanolic extract of *C. asiatica* increased the level of antioxidants and antioxidant enzymes in mice with lymphoma. Its antidiabetic potential was reported by Emran *et al*. (2015) and the results revealed that *C. asiatica* extract decreased blood glucose level in rats with diabetic caused by alloxan. *C. asiatica* is also a potent antimicrobial agent whereby a recent study conducted by Idriz and Nadzir (2017) showed that the extracts of *C. asiatica* significantly inhibit the growth of fungus *Aspergillus niger* and gram-positive bacteria *Bacillus subtilis*. In addition, methanolic and water extracts of *C. asiatica* demonstrated antiviral activity against alpha-herpesvirus in *in vitro* model (Hanisa *et al*., 2014).

Besides these various therapeutics potential, *C. asiatica* is also widely known in traditional medicine as brain tonic or brain food in which it acts as neuroprotectant, memory enhancer, antidepressant, sedative, rejuvenant and anticonvulsant (Chong *et al*., 2009). The memory enhancing ability of *C. asiatica* was also detected in a study conducted by Sari *et al*. (2014) which revealed that ethanolic extract of *C. asiatica* improved memory performance after an induced chronic stress in rats. In addition, extracts of *C. asiatica* exhibited antidepressant (Goola & Tirupathi, 2016) and antiepileptic activity in *in vivo* model (Visweswari *et al*., 2010). Mukherjee *et al*. (2007) reported that hydroalcoholic extract of *C. asiatica* inhibit enzyme acetylcholinesterase which is responsible for the development of AD. Asiatic acid extracted from *C. asiatica*

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also exert neuroprotective effect against rotenone in *in vitro* model by reducing the production of ROS, increasing mitochondrial membrane potential and thus inhibiting apoptosis (Nataraj *et al*., 2017).

Table 2.1: Summarized biological activities of *C. asiatica.*

Biological Activities	Descriptions	References
Wound Healing	Increased collagen content, tensile strength and rapid epithelialisation.	Sunilkumar *et al*. (1998)
	Increased enzymatic and non-enzymatic antioxidants (CAT, SOD, vitamin E) in newly formed tissues and rate of wound contraction.	Shukla *et al*. (1999)
Anti-inflammatory	Increased percentage of inhibition of oedema in rat model.	George *et al*. (2009)
Anticancer	Induced apoptosis in human breast MCF-7 cancer cells which revealed by nuclear condensation and loss of mitochondrial membrane potential.	Babykutty *et al*. (2009)
Antioxidant	Increased antioxidant enzymes (SOD, CAT) and antioxidant level in mice with lymphoma.	Jayashress *et al*. (2003)
Antidiabetic	Significantly reduced blood glucose and cholesterol level in alloxan-induced diabetic rats.	Emran *et al*. (2015)
Antimicrobial	Significantly inhibit the growth of *A. niger* and *B. subtilis.*	Idriz and Nadzir (2017)
Antiviral	Demonstrated virucidal and antiviral attachment activity against alpha-herpesvirus in African green monkey kidney (Vero), baby hamster kidney (BHK) and rabbit kidney (RK) cells.	Hanisa *et al*. (2014)
Memory Enhancer	Improved memory which evident by increased level of serum brain-derived neurotrophic factor (BDNF) in rat model.	Sari *et al*. (2014)

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Table 2.1, continued.

Antidepressant	Significantly reduced the immobility time in forced swimming test in mice model.	Goola and Tirupathi (2016)
Antiepileptic	Increased the activity level of Na^+/K^+, Mg^{2+} and Ca^{2+}-ATPase in rat brain during pentylenetetrazol-induced epilepsy.	Visweswari *et al*. (2010)
Neuroprotective	Showed acetylcholinesterase inhibitory activity.	Mukherjee *et al*. (2007)
	Reduced the production of ROS, increased mitochondrial membrane potential and inhibited apoptosis in rotenone-induced SH-SY5Y cells.	Nataraj *et al*. (2017)

2.6 Phytochemicals

Phytochemicals which is also known as "plant chemicals" are naturally occurring bioactive compounds that are found in plants, fruits, vegetables and herbs (Kumar & Khanum, 2012). Common classes of phytochemicals are (i) phenols (quercetin, curcumin, resveratrol, rosmarinic acid), (ii) flavonoids (anthocyannis), (iii) alkaloids (huperzine A, berberine, withanolides), (iv) saponins (bacoside A), (v) terpenes (asiatic acid, madecassic acid, ginkgolide, ginsenoside), (vi) sterols (diosgenin, spicatoside A), (vii) fatty acids and (viii) tannins are examples of phytochemicals (Kumar *et al*., 2015). Significant medicinal properties of phytochemicals make these phytochemicals as potential therapeutics for a wide range of diseases.

Many scientific studies reported on the role of phytochemicals as an alternative natural therapeutic for neurodegenerative diseases due to their significant antioxidant activity (Kumar & Khanum, 2012). Guo *et al*. (2007) demonstrated that catechins protected dopaminergic neurons from 6-hydroxydopamine (6-OHDA)-induced oxidative stress in rats while resveratrol which is a polyphenol found mainly in red grapes protected the neurons from amyloid beta peptide toxicity and also reduced the

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intracellular amyloid beta peptide formation (Marabaud *et al*., 2005). In addition, huperzine A is another well-known phytochemical that can act as acetylcholinesterase inhibitor and improves learning and memory in *in vivo* model (Zhang & Tang, 2006).

2.6.1 Curcumin

Curcumin (diferuoyl methane) was isolated from the rhizome of *Curcuma longa* L. *C. longa* is a short-stemmed, perennial plant naturally growing throughout the Indian subcontinent and in tropical Asia, particularly in Southeast Asia and it belongs to the family of Zingiberaceae. The dried ground rhizome of *C. longa* is known turmeric and is well known as flavouring, colouring and preservative agent in Asian cooking and in cosmetics. Curcumin (a polyphenol) is the major bioactive component of turmeric which is also responsible for turmeric bright yellow colour (Iriti *et al*., 2010). Figure 2.3 shows the chemical structure of curcumin.

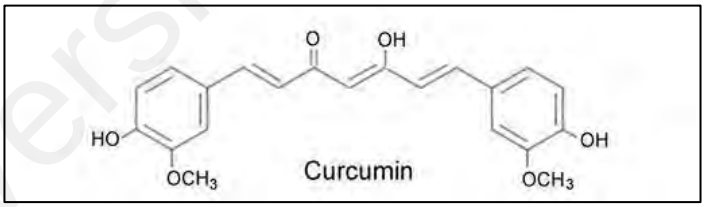

Figure 2.3: Chemical structure of curcumin.

2.6.2 Biological Activities of Curcumin

Curcumin has been widely utilized in traditional Indian and Chinese medicine as treatment for eye infection, jaundice, skin diseases, dental diseases, cough and respiratory problem, digestive problem and also to treat bites, burns, acne and to dress wound (Hatcher *et al*., 2015). Due to its significant role in traditional medicine, a large number of studies have been carried to explore the different types of therapeutics

potential of curcumin in various *in vivo* and *in vitro* models. Its biological activities include anti-inflammatory, antioxidant, anticancer, radioprotection and radiosensitization, antiviral, antimicrobial and neuroprotective. A summary on the pharmacological activities of curcumin from several literatures is presented in Table 2.2.

Curcumin was shown to exhibit an inhibitory effect on inflammation in various *in vivo* and *in vitro* models (Abe *et al*., 1999). A study conducted by Kim *et al*. (2012) reported that curcumin have strong antioxidant activity whereby it is able to inhibit the formation of ROS/RNS, significantly reduce lipid peroxidation and mitochondrial dysfunction in *in vitro* model. Curcumin also exert its anticancer activity mainly by regulating the processes involved in cell growth and apoptosis (Alok *et al*., 2015). A study conducted by Goel *et al*. (2001) reported the anticancer activity of curcumin against human colon HT-29 cancer cells.

In addition, curcumin also demonstrated both radioprotection and radiosensitization effects against normal and cancer cells respectively (Chendil *et al*., 2004; Lopez-Jornet *et al*., 2016). A study carried out by Chen *et al*. (2010) revealed that curcumin is a potent antiviral agent against human influenza (H1N1) and avian influenza (H6N1) virus. Curcumin also demonstrated antimicrobial activity against many bacteria and fungus (Alok *et al*., 2015).

Apart from that, curcumin is also a potent neuroprotectant. According to Mishra and Palanivelu (2008), curcumin reduced the formation of beta amyloid peptide and α-synuclein protein in the neuronal cells which is responsible for AD and PD respectively. Curcumin also showed neuroprotective effects against the sodium nitroprusside-induced neurotoxicity in rat (Nazari *et al*., 2013). Its neuroprotective effect against pentylenetetrazole-induced epilepsy in mice was showed by Agarwal *et al*. (2011).

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Table 2.2: Pharmacological activities of curcumin.

Biological Activities	Descriptions	References
Anti-inflammatory	Reduced the production of inflammatory cytokine such as interleukin-1β, interleukin-8, monocyte inflammatory protein-1 and tumour necrosis factor-α in lipo-polysaccharides induced monocytes and macrophages.	Abe *et al*. (1999)
	Oral administration of curcumin inhibits inflammation in carrageenan-induced mice.	Hatcher *et al*. (2008)
Antioxidant	Inhibited the formation of ROS/RNS, significantly reduce lipid peroxidation and mitochondrial dysfunction *in vitro* model.	Kim *et al*. (2012)
Anticancer	Significantly reduced the expression of COX-2 and thus inhibited the growth of human colon HT-29 cancer cells.	Goel *et al*. (2001)
Radioprotection	Reduced the structural damage to the salivary glands in rats exposed to radiation.	Lopez-Jornet *et al*. (2016)
Radiosensitization	Enhanced the radiation induced apoptosis and clonogenic inhibition in prostate PC-3 cancer cell.	Chendil *et al*. (2004)
Antivirus	Showed anti-influenza activity by inhibiting the virus propagation by interrupting the virus-cell attachment in *in vitro* model.	Chen *et al*. (2010)
Antimicrobial	Inhibited the growth several bacteria such as *Streptococci, Lactobacillus* and *Staphylococci* and showed antifungal effect against *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium digitatum*.	Alok *et al*. (2015)
Neuroprotective	Reduced the formation of beta amyloid peptide and α-synuclein protein in the neuronal cells.	Mishra and Palanivelu (2008)
	Significantly inhibited sodium nitroprusside-induced cytotoxicity and increased intracellular antioxidant level.	Nazari *et al*. (2013)
	Reduced the progression of epilepsy in mice.	Agarwal *et al*. (2011)

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2.7 Neurotoxic Agent

Neurotoxic agent is defined as a substance that causes reversible or permanent structural or functional change in the nervous system and most of the effects are in the form of oxidative stress (Farkhondeh *et al*., 2016). Types of neurotoxic agents include (i) endogenous neurotoxic agents such as ROS, RNS, beta amyloid protein and neurotransmitter (glutamate and dopamine) which exert neurotoxicity when present in excess and (ii) exogenous neurotoxic agents such as rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), sodium nitroprusside, vincristine, arsenic, mercury, aluminium and acrylamide (Gandhi & Abramov, 2012). The effect of various neurotoxic agents either in *in vivo* or *in vitro* model from several studies is summarized in Table 2.3.

Table 2.3: The effect of various neurotoxic agents either in *in vivo* or *in vitro* model.

Neurotoxic Agents	Functions	Descriptions	References
Glutamate	Endogenous excitatory neurotransmitter.	Increased the generation of ROS and intracellular calcium (Ca^{2+}) and caused mitochondrial dysfunction and depletion of intracellular antioxidants in HT-22 mouse hippocampal neuronal cells.	Lee *et al*. (2017)
Beta amyloid protein	Pathological characteristic of AD.	Reduced cell viability, increased level of lactate dehydrogenase, induced caspase-3 activity, DNA condensation and morphological changes in SH-SY5Y cells.	Zhang *et al*. (2010)
Sodium nitroprusside	Drug to reduce blood pressure (vasodilation agent).	Caused mitochondrial and plasma membrane damage, increased apoptotic protein biomarkers, stress marker of neuronal cells and reduced BDNF level in rat	Pandareesh and Anand (2014)

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Table 2.3, continued.

		pheochromocytoma PC-12 cells.	
Aluminium	Food additives, flocculating agents, used in medicine and cosmetics.	Reduced cognitive and motor function, increased lipid and protein oxidation and altered the structure of cerebral cortex due to neurodegeneration and, gliosis in rat model. Reduced the level of intracellular antioxidants (CAT, GSH and reductase).	Lakshmi *et al*. (2014)
Monosodium glutamate	Flavour enhancers.	Significantly elevated DNA and lipid oxidation, nitric oxide, beta-amyloid protein, neurotransmitters (dopamine and glutamate) levels, excitotoxicity and neurodegeneration in rat brain tissue.	Hussein *et al*. (2017)
Rotenone	Pesticides and insecticides.	Increased the behavioural and postural characteristics linked to PD, lipid peroxidation and ROS level. Reduced the tyrosine hydroxylase (TH) activity and GSH level in substantia nigra pars compactal of mice.	Cui *et al*. (2015)
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP)	Environmental toxin.	Reduced motor activity, level of dopamine and its metabolites, loss of dopaminergic neurons.	Cheng *et al*. (2008)

2.7.1 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a type of ROS that is naturally produced in the body by dismutation of superoxide radical, activity of oxidase enzyme and exposed externally through consumption of beverages (Haliwell *et al*., 2000). This highly diffusible molecule can be converted into highly reactive hydroxyl radical in the

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presence of transition metal such as iron(II) ion (Fe^{2+}) through the process known as Fenton reaction. Studies have revealed that H_2O_2 mainly cause lipid peroxidation and DNA damages to the neuronal cells which leads to apoptotic cell death (Uttara *et al*., 2009).

According to Park *et al*. (2009), H_2O_2 at concentration range from of 100–250 μM results in increment of intracellular beta amyloid protein levels in human neuroblastoma SH-SY5Y cells. In addition, exposure to H_2O_2 have also decreased the intracellular antioxidant level and increased the level of lactate dehydrogenase (a cytoplasmic enzyme) which indicates that there is an increase in plasma membrane damaged or dead cells (Wang *et al*., 2009).

2.7.2 Acrylamide

Acrylamide (C_3H_5NO) is water soluble monomer that has various industrial applications such as in dye synthesis, food packaging, gel electrophoresis, water and wastewater management. Diet is recognized as the main source of acrylamide exposure in humans as acrylamide formation occurs in carbohydrate rich food cooked at high temperature (Song *et al*., 2013). Studies have revealed that acrylamide associated with different types of toxicity such as neurotoxicity, carcinogenicity, development and reproductive toxicity and genotoxicity.

Acrylamide-induced oxidative stress occurs when acrylamide affect the cellular redox status which eventually lead to the production of ROS (Chen *et al*., 2014). According to Mehri *et al*. (2012), acrylamide can increase lipid peroxidation, decreases neurotransmitter release and reduces the neuronal cell antioxidant capacity. In addition, acrylamide also reported to induce apoptosis in SH-SY5Y cells via the activation of caspase-3 (Chen *et al*., 2014). A study conducted by Pennisi *et al*. (2013) revealed that

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neurotoxic effects had been observed in animal model exposed to certain concentration of acrylamide in daily basis.

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CHAPTER 3: METHODOLOGY

3.1 Chemicals and Reagents

Methanol was purchased from Fisher Scientific, US. Sodium sulphate ($NaSO_4$) and hydrogen peroxide (H_2O_2) were purchased from Systerm, Malaysia. Dimethyl sulfoxide (DMSO), sodium bicarbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCI) and iron(II) chloride ($FeCI_2$) were purchased from R&M Chemicals, UK. Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (1:1 DMEM/F12), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), hydroxyethyl-piperazineethane-sulfonic acid buffer (HEPES), trypan blue, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, ethylenediaminetetraacetic acid (EDTA), ferrozine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, US. Fetal Bovine Serum (FBS) was purchased from TIC, Europe, Accutase solution from Innovative Cell Technologies, US, acrylamide from Bio-Rad, US and curcumin from Nacalai Tesque, Japan.

3.2 Preparation of Extracts of *Centella asiatica*

The aerial parts of *Centella asiatica* were purchased from the local market at Pantai Dalam, Kuala Lumpur. The aerial parts of *C. asiatica* were rinsed with tap water, dried at 50˚C using hot air oven (Memmert, Germany) for three days, grounded into fine powder and stored in an air tight container until further use. The extraction process was

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carried out using two different types of solvents which were methanol and water according to the method described by Auddy *et al*. (2003) with slight modification.

3.2.1 Methanol Extract

The extraction was performed using maceration method whereby the powdered material of *C. asiatica* was soaked in methanol 90% (v/v) in a ratio of 1:10 for three days at room temperature. Then, solvent containing extract was filtered and the extraction step was repeated thrice using methanol. The filtrate from each extraction was combined and excess solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland).

The dark-greenish methanol extract of *C. asiatica* was stored in dark at 4˚C until further use. DMSO was used to dissolve the methanolic extract to yield a stock solution with concentration of 20 mg/ml and stock solution was further diluted in 10% DMSO to obtain the desired test concentration shortly before the experiments.

3.2.2 Water Extract

The sliced fresh *C. asiatica* was boiled in distilled water at a ratio of 1:25 for an hour at 80˚C using water bath (Memmert, Germany). Then, the mixture was filtered and the water filtrate was freeze dried. The resulting brown solid extract of *C. asiatica* was kept at 4˚C until further use. Distilled water was used to dissolve the water extract to yield a stock solution with concentration of 20 mg/ml and stock solution was further diluted in distilled water to obtain the desired test concentration shortly before the experiments.

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3.3 Neuroprotective Assays

3.3.1 Cell Culture

Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured in 1:1 DMEM/F12 supplemented with 10% (v/v) FBS. The SH-SY5Y cells were maintained in 25 cm^3 flasks (Nunc, Denmark) at 37˚C in a humidified incubator containing 5% of carbon dioxide (CO_2) (ESCO, Singapore). All experiments were carried out 24 hours after the cells were seeded. The cells were routinely checked under phase contrast inverted microscope (Leica, Germany) for any contamination and medium was replaced every two to three days.

Once the cells reached nearly 80% of confluency, SH-SY5Y cells were sub-cultured. Prior to the neuroprotective assay, the medium was discarded and the cells were rinsed with phosphate-buffered saline (PBS) at pH 7.4. The SH-SY5Y cells were detached from the tissue culture flask by using accutase solution and harvested by centrifugation (Kubota, Japan) at 100 × g for five minutes. The number of viable cells were counted by trypan blue (0.4% w/v) exclusion method using a hemocytometer. Then, the cells were seeded in 96-well culture plate (Orange Scientific, Belgium) according to the desired concentration.

3.3.2 Assessment of Toxicity of Dimethyl Sulfoxide towards SH-SY5Y Cells

A preliminary experiment was carried out to determine the appropriate concentration of DMSO that is not toxic towards SH-SY5Y cells. This assay was carried out according to the method described by Piah *et al*. (2010). The SH-SY5Y cells were cultured in 96-well culture plates at a density of 3×10^4 cells/ml (200 µl) and

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incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, the cells were incubated with different concentrations of DMSO [final exposure concentrations of 0.05, 0.25, 0.50, 1.25, 2.50, 5.00% (v/v)] for 24 hours. Cells without addition of DMSO served as negative control. At the end of the incubation, MTT assay was utilized to determine the cell viability (refer to section 3.5).

3.3.3 Assessment of Toxicity of Neurotoxic Agents towards SH-SY5Y Cells

The toxicity of H_2O_2 and acrylamide towards the viability of SH-SY5Y cells were assessed according to the method described by Piah *et al*. (2010). This assay was carried out to determine the half-maximal inhibitory concentration (IC_{50}) of the neurotoxic agents that was used to induce toxicity in SH-SY5Y cells during the neuroprotective assay.

The SH-SY5Y cells were cultured in 96-well plate at a density of 3×10^4 cells/ml and incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, the cells were exposed to either H_2O_2 (final exposure concentrations of 25, 50, 75, 100, 200, 300, 400 and 500 μM) or acrylamide (final exposure concentrations of 1, 3, 5, 7, 10 and 20 mM) for 24 hours. Cells without addition of any of these neurotoxic agents served as negative control. At the end of the incubation period, the viability of SH-SY5Y cells was assessed by MTT assay (refer to section 3.5).

3.3.4 Assessment of Neurotoxicity Effects of Extracts of *C. asiatica* towards SH-SY5Y Cells

The neurotoxicity effects of the methanolic and water extracts of *C. asiatica* on SH-SY5Y cells were assessed according to the method described by Piah *et al*. (2010).

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This assay was carried out to determine the range of concentration of extracts that was not toxic towards SH-SY5Y cells. The SH-SY5Y cells were seeded in 96-well plate at a density of 3×10^4 cells/ml and incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, cells were incubated with several concentrations of the extracts (final exposure concentrations of 1, 10, 25, 75, 50 and 100 µg/ml) for 24 hours. Cells treated with vehicles (DMSO or distilled water) served as vehicle control. At the end of the incubation period, the viability of SH-SY5Y cells was assessed by the MTT assay (refer to section 3.5).

3.3.5 Evaluation of Neuroprotective Effects of Extracts of *C. asiatica* towards Neurotoxic Agents-Induced Toxicity in SH-SY5Y Cells

The analysis of neuroprotective effects of methanolic and water extracts of *C. asiatica* against toxicity-induced by H_2O_2 or acrylamide was carried out according to the method described by Piah *et al*. (2010) and Custodio *et al*. (2013) with some modifications. In this assay, the IC_{50} value of H_2O_2 of 100 µM and acrylamide of 5 mM determined from section 3.3.3 was used to induce toxicity in the SH-SY5Y cells and a range of non-toxic concentration of extracts of 1–100 µg/ml determined from section 3.3.4 was used to evaluate its neuroprotective efficacy. The SH-SY5Y cells were seeded in 96-well plate at a density of 3×10^4 cells/ml and incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, the cells were pre-treated with different concentrations of extracts of *C. asiatica* (1, 10, 25, 50, 75 and 100 µg/ml) for 24 hours and followed by treatment with either H_2O_2 (100 µM) or acrylamide (5 mM) for another 24 hours. Cells treated with vehicles (DMSO or distilled water) served as vehicle control. Cells treated with neurotoxic agents without addition of extracts were the negative control. Trolox was used as a positive control. At the end of the incubation

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period, the viability of SH-SY5Y cells was assessed by the MTT assay (refer to section 3.5).

3.4 Neuroprotective Effects in Combination Model

3.4.1 Assessment of Neurotoxicity Effect of Curcumin towards SH-SY5Y Cells

The neurotoxicity effect of curcumin on SH-SY5Y cells were assessed to determine the range of concentration of curcumin that was not toxic towards SH-SY5Y cells. The SH-SY5Y cells were seeded in 96-well plate at a density of 3×10^4 cells/ml and incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, cells were incubated with several concentrations of curcumin (final exposure concentrations of 0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 μg/ml) for 24 hours. Cells treated with vehicle (DMSO) served as vehicle control. At the end of the incubation period, the viability of SH-SY5Y cells was assessed by the MTT assay (refer to section 3.5).

3.4.2 Evaluation of Neuroprotective Effects of Curcumin towards Neurotoxic Agents-Induced Toxicity in SH-SY5Y Cells

In this assay, the neuroprotective effects of curcumin against toxicity-induced by H_2O_2 or acrylamide was evaluated. The SH-SY5Y cells were seeded in 96-well plate at a density of 3×10^4 cells/ml and incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, the cells were pre-treated with different concentrations of curcumin (0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 μg/ml) for 24 hours and followed by treatment with either H_2O_2 (100 μM) or acrylamide (5 mM) for another 24 hours. Cells treated with vehicle (DMSO) served as vehicle control. Cells treated with neurotoxic

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agents without addition of curcumin served as negative control. At the end of the incubation period, the viability of SH-SY5Y cells was assessed by the MTT assay (refer to section 3.5).

3.4.3 Evaluation of Neuroprotective Effects of the Combined extract-compound of *C. asiatica* and Curcumin

In this assay, the neuroprotective effects of the combined *C. asiatica* extracts and curcumin against toxicity-induced by H_2O_2 and acrylamide was evaluated. The concentration of methanolic and water extracts of *C. asiatica* and curcumin that provided the highest neuroprotective effect determined from section 3.3.5 and 3.4.2 were combined. The SH-SY5Y cells were seeded in 96-well plate at a density of 3×10^4 cells/ml and incubated for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. Then, the cells were pre-treated with extracts alone, curcumin alone and combination of extracts with curcumin in 1:1 ratio according to Table 3.1 for 24 hours. This is followed by treatment with either H_2O_2 (100 μM) or acrylamide (5 mM) for another 24 hours. Cells treated with vehicles (DMSO or distilled water) served as vehicle control. Cells treated with neurotoxic agents without extracts and curcumin were the negative control. At the end of incubation period, the viability of SH-SY5Y cells was assessed by the MTT assay (refer to section 3.5).

Table 3.1: Concentration of extracts of *C. asiatica* and curcumin used in combination study.

Neurotoxic Agents	Concentration of Extracts and Curcumin with the Highest Neuroprotective Activity (μg/ml)		
	Methanolic Extract	Water Extract	Curcumin
H_2O_2	75	10	0.25
Acrylamide	75	25	0.50

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3.5 Cell Viability Analysis Using MTT Assay

In MTT assay, 20 µl of 5 mg/ml MTT solution in PBS was added into each well of 96-wells plate after the cells were subjected to the treatment of interest and incubated at 37˚C for 4 hours. Then, the culture medium was carefully removed and 200 µl of DMSO was added to dissolve the insoluble purple formazan crystals from the MTT solution. The amount of formazan produced which is directly proportional to the number of viable cells was measured at wavelength of 570 nm with a reference wavelength at 690 nm using a microplate reader (Thermo Fisher Scientific, US). The cell viability which was expressed as a percentage of control value was calculated using the following equation (Adewusi *et al*., 2013):

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of cell subjected to treatment}}{\text{Absorbance of control cells}} \times 100$$

3.6 Assessment of Antioxidant Activity

The antioxidant activity of methanolic and water extracts of *C. asiatica* and curcumin was determined by using *in vitro* antioxidant assays; (i) DPPH radical scavenging, (ii) ABTS radical scavenging, and (iii) iron chelating activity based on method described by Venuprasad *et al*. (2013). Methanol was used as the solvent to prepare the desired test concentrations for methanol extract and curcumin, while distilled water was used as dilution solvent for water extract.

3.6.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

Briefly, 150 µl of 0.3 mM methanolic solution of DPPH was added to the 96-wells plate containing 50 µl of different concentrations of either extracts of *C. asiatica*

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(200, 400, 600, 800, 1000, 1200, 1500 and 2000 µg/ml) or curcumin (10, 20, 40, 60, 80 and 100 µg/ml). The mixture was shaken and incubated in dark for 30 minutes at room temperature.

Then, the absorbance of the solution was measured at 517 nm. Ascorbic acid was used as positive control while negative control was prepared based on type of solvent either by mixing DPPH solution with methanol or distilled water. DPPH radical scavenging activity of the extracts of *C. asiatica* and curcumin was compared with ascorbic acid and percentage of DPPH radical scavenging activity of the samples was calculated by using the following equation whereby A_0 is absorbance of negative control and As is absorbance of sample/positive control.

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_o - A_s}{A_o} \times 100$$

3.6.2 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity

The working solution (ABTS radical cation) was prepared by mixing equal volume of stock solutions of 7 mM ABTS and 2.45 mM $K_2S_2O_8$ and the reaction was allowed to occur in dark for 12 hours at room temperature. Prior to the assay, the ABTS radical cation solution was diluted with distilled water to obtain an absorbance of 0.706 ± 0.02 at 734 nm. Then, 20 µl of different concentrations of either extracts of *C. asiatica* (200, 400, 600, 800, 1000, 1200, 1500 and 2000 µg/ml) or curcumin (10, 20, 40, 60, 80, 120 and 150 µg/ml) were allowed to react with 200 µl of diluted ABTS cation solution for 15 minutes at room temperature and then the absorbance was measured at 734 nm. A mixture of ABTS radical cation and methanol or distilled water served as the negative control and ascorbic acid was used as positive control. The percentage of ABTS radical scavenging activity of extracts of *C. asiatica* and curcumin

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was calculated by using the following equation whereby A_0 is absorbance of negative control and As is absorbance of sample/positive control.

$$\text{ABTS radical scavenging activity (\%)} = \frac{A_o - A_s}{A_o} \times 100$$

3.6.3 Iron Chelating Activity

In this method, 5 µl of 2 mM $FeCI_2$ solution and 130 µl of distilled water were added into different concentrations of either extracts of *C. asiatica* (500, 1000, 1500, 2500, 3500 and 4500 µg/ml) or curcumin (10, 20, 40, 60, 80, 120 and 150 µg/ml). The reaction was initiated by the addition of 15 µl of 5 mM ferrozine solution. The mixture was shaken, incubated for 10 minutes at room temperature and then the absorbance of the solution was measured at 562 nm. EDTA was used as positive control while negative control contained all the reagents except the extracts and curcumin which were replaced by methanol or distilled water. The percentage of iron chelating potential of the samples was measured using the following equation whereby A_0 is absorbance of negative control and As is absorbance of sample/positive control.

$$\text{Iron chelating activity (\%)} = \frac{A_o - A_s}{A_o} \times 100$$

3.7 Statistical Analysis

Results are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by a Tukey's HSD-post hoc test using SPSS software (SPSS Statistics, Version 22, IBM, USA) in order to determine the

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significance of the data whereby p-value less that 0.05 ($p<0.05$) were regarded as statistically significant.

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CHAPTER 4: RESULTS

4.1 Extraction Yield

In the present study, methanolic and water extracts were prepared from the aerial part of *Centella asiatica*. The extraction yield of methanolic and water extracts of *C. asiatica* is presented in Table 4.1.

Table 4.1: Extraction yield (%) of methanolic and water extracts of *C. asiatica.*

Types of Solvent	Extraction Yield (%)
Methanol	11.67
Water	1.73

This result demonstrated that the extraction yield of methanol extract was higher than water extract with yield of 11.67% and 1.73%, respectively.

4.2 Neuroprotective Assays

4.2.1 Assessment of Toxicity of Dimethyl Sulfoxide towards SH-SY5Y Cells

The toxicity of dimethyl sulfoxide (DMSO) towards human neuroblastoma SH-SY5Y cells was investigated to determine whether DMSO causes any toxicity towards the cells. For that purpose, SH-SY5Y cells were exposed to DMSO with concentration range from 0.05 to 5.00% (v/v) for 24 hours and then the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of control. The cell viability (%) at different concentrations of DMSO is presented in Table 4.2. The toxicity effect of DMSO towards the SH-SY5Y cells is illustrated in Figure 4.1.

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Table 4.2: SH-SY5Y cells viability (%) at different concentrations of DMSO.

Concentrations of DMSO (%)	Cell Viability (%)
0.00	100^{ab}
0.05	105.99 ± 6.62^{a}
0.25	97.92 ± 8.25^{ab}
0.50	97.45 ± 4.21^{ab}
1.25	79.01 ± 4.55^{bc}
2.50	57.38 ± 2.85^{c}
5.00	12.13 ± 1.25^{d}

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ($^{a-d}$) in the same column were significantly different ($p<0.05$).

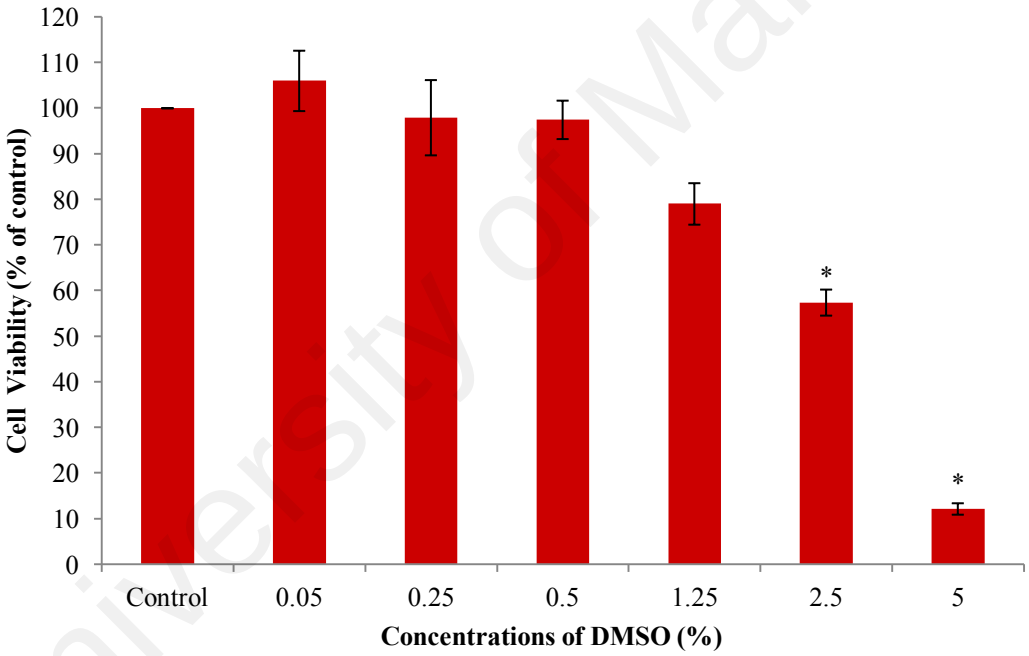

Figure 4.1: Toxicity effect of DMSO towards SH-SY5Y cells. Cells were incubated with different concentrations of DMSO [0.05, 0.25, 0.50, 1.25, 2.50 and 5.00% (v/v)] for 24 hours. Cell viability was determined using MTT assay and expressed as percentage of control. Control comprised of cells without DMSO treatment. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$ versus control group.

DMSO was used as a solvent to dissolve methanolic extract of *C. asiatica* during biological assay. Result in Table 4.2 and Figure 4.1 indicates that as the concentration of DMSO increased from 0.05 to 5.00%, the cell viability decreased from

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105.99 to 12.13%. At concentrations of 0.05, 0.25, 0.50 and 1.25%, the solvent had no significant toxicity effect towards SH-SY5Y cells compared to the control cells without DMSO exposure. The untreated control cells were considered with 100% cell viability. Thus, DMSO concentration of 0.50% was chosen to dissolve the methanolic extract for the neuroprotective analysis.

4.2.2 Assessment of Toxicity of Neurotoxic Agents towards SH-SY5Y Cells

Hydrogen peroxide (H_2O_2) and acrylamide were selected in the present study as the neurotoxic agents to induce toxicity in SH-SY5Y cells. The toxicity effect of H_2O_2 and acrylamide towards SH-SY5Y cells was investigated to determine the half-maximal inhibitory concentration (depicted as IC_{50}) of both the neurotoxic agents against SH-SY5Y cells.

For that, the cells were incubated with different concentration of H_2O_2 (25–500 μM) and acrylamide (1–20 mM) for 24 hours and then the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of control. The cell viability (%) at different concentrations of H_2O_2 and acrylamide is presented in Table 4.3 and Table 4.4, respectively. The toxicity effects of H_2O_2 and acrylamide towards SH-SY5Y cells is illustrated in Figure 4.2 and Figure 4.4, respectively.

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Table 4.3: SH-SY5Y cells viability (%) at different concentrations of H_2O_2.

Concentrations of H_2O_2 (µM)	Cell Viability (%)
0	100[a]
25	97.92±0.95[a]
50	77.85±5.00[b]
75	68.34±6.27[b]
100	44.14±0.26[c]
200	20.98±1.66[d]
300	17.76±2.83[d]
400	12.65±2.43[d]
500	12.27±2.70[d]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-d]) in the same column were significantly different ($p<0.05$). H_2O_2: hydrogen peroxide.

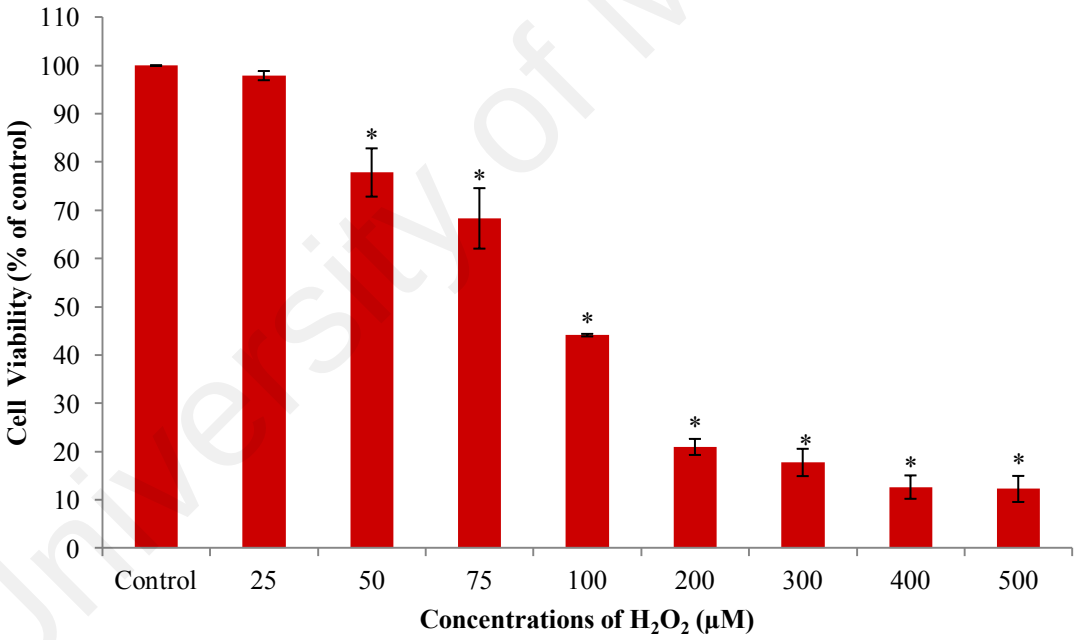

Figure 4.2: Toxicity effect of H_2O_2 towards SH-SY5Y cells. Cells were incubated with different concentrations of H_2O_2 (25, 50, 75, 100, 200, 300, 400 and 500 µM) for 24 hours. Cell viability was determined using MTT assay and expressed as percentage of control. Control comprised of cells without H_2O_2 treatment. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$ versus control group. H_2O_2: hydrogen peroxide.

Based on the result in Table 4.3 and Figure 4.2, the viability of SH-SY5Y cells decreased from 97.92 to 12.27% as concentration of H_2O_2 increased from 25 to 500 µM.

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The morphological changes of SH-SY5Y cells exposed to H_2O_2 observed using phase contrast inverted microscope illustrated in Figure 4.3 indicated that increasing concentration of H_2O_2 caused shrinkage and clumps of the cells, reduction in cell number, loss of cell processes (neurites) compared to the untreated SH-SY5Y cells which showed neuronal-like phenotypes such as less clustered cells with pyramidal shaped cell body and branching neurites. H_2O_2 highly influenced the cell viability at concentration of 50 μM and above compared to the untreated control cells. The untreated control SH-SY5Y cells were considered with 100% cell viability. The concentration of H_2O_2 that inhibit nearly 50% of viability of SH-SY5Y cells was recorded as 100 μM. Therefore, concentration of 100 μM of H_2O_2 was selected as the optimum concentration to induce toxicity in SH-SY5Y cells during the analysis of the neuroprotective effect of the extracts of *C. asiatica*.

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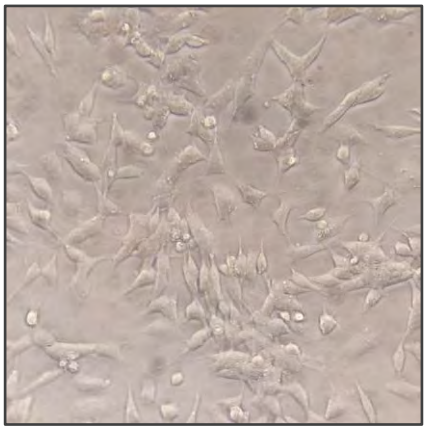
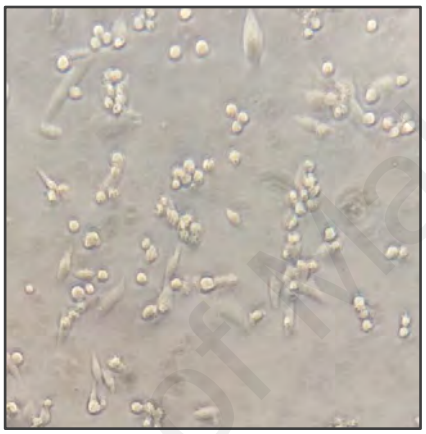
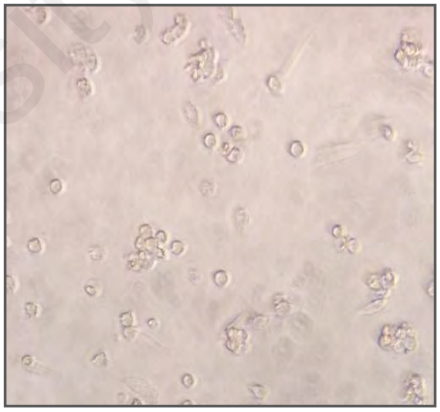


(a) Untreated SH-SY5Y cells



(b) 100 µM of H_2O_2



(c) 500 µM of H_2O_2

Figure 4.3: Toxicity effects of H_2O_2 towards SH-SY5Y cells after 24 hours' exposure. (a) Untreated SH-SY5Y cells without addition of any test agents, (b) SH-SY5Y cells exposed with 100 µM of H_2O_2 which caused 55.86% of cell death, and (c) SH-SY5Y cells exposed with 500 µM of H_2O_2 which caused 87.73% of cell death.

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Table 4.4: SH-SY5Y cells viability (%) at different concentrations of acrylamide.

Concentrations of Acrylamide (mM)	Cell Viability (%)
0	100[a]
1	94.77±2.43[a]
3	60.81±2.02[b]
5	46.93±3.04[b]
7	27.79±3.16[c]
10	22.58±5.20[c]
20	18.91±4.59[c]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-c]) in the same column were significantly different ($p<0.05$).

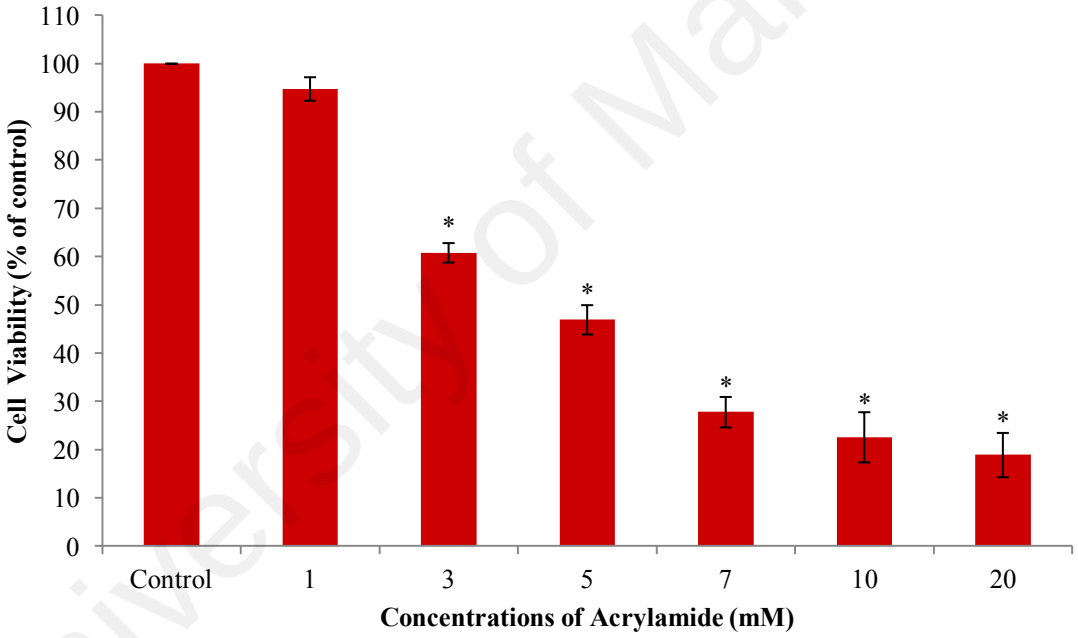

Figure 4.4: Toxicity effect of acrylamide towards SH-SY5Y cells. Cells were incubated with different concentrations of acrylamide (1, 3, 5, 7, 10 and 20 mM) for 24 hours. Cell viability was determined using MTT assay and expressed as percentage of control. Control comprised of cells without acrylamide treatment. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$ versus control group.

Based on the result presented in Table 4.4 and Figure 4.4, as the concentration of acrylamide increased from 1 to 20 mM, the cell viability decreased from 94.77 to 18.91%. A similar morphological changes as observed in H_2O_2-induced toxicity in SH-SY5Y cells were detected in the cells that had been exposed to increasing

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concentration of acrylamide as illustrated in Figure 4.5. Exposure of the SH-SY5Y cells to acrylamide with concentration of 3 mM and above significantly reduced the cell viability compared to the untreated control cells. The untreated control cells were considered with 100% cell viability. The concentration of acrylamide that inhibit approximately 50% viability of SH-SY5Y cells was recorded as 5 mM. Therefore, concentration of 5 mM of acrylamide was selected as the optimum concentration to induce cell toxicity in SH-SY5Y cells during the analysis of the neuroprotective effect of the extracts of *C. asiatica*.

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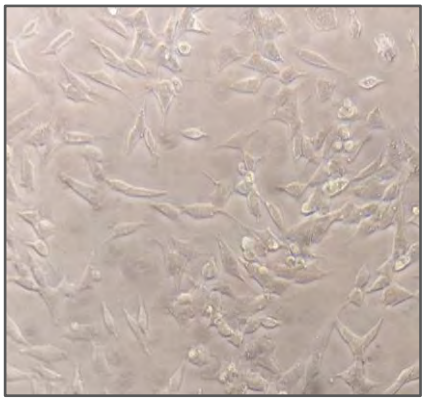
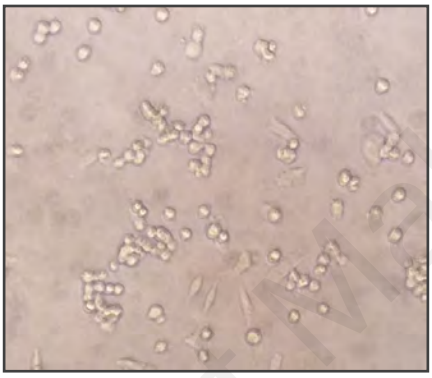
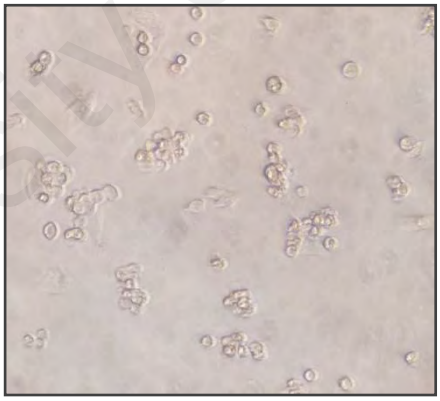


(a) Untreated SH-SY5Y cells



(b) 5 mM of acrylamide



(c) 20 mM of acrylamide

Figure 4.5: Toxicity effects of acrylamide towards SH-SY5Y cells after 24 hours' exposure. (a) Untreated SH-SY5Y cells without addition of any test agents, (b) SH-SY5Y cells exposed with 5 mM of acrylamide which caused 53.07% of cell death, and (c) SH-SY5Y cells exposed with 20 mM of acrylamide which caused 81.09% of cell death.

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4.2.3 Assessment of Neurotoxicity Effects of Extracts of *C. asiatica* towards SH-SY5Y Cells

The neurotoxicity effects of methanolic and water extracts of *C. asiatica* towards SH-SY5Y cells was analysed to determine whether both extracts exhibit any toxicity towards the cells. For that purpose, SH-SY5Y cells were incubated with different concentration of extracts (1–100 µg/ml) for 24 hours and then the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of control. Cell viability (%) at different concentrations of methanolic and water extracts of *C. asiatica* is presented in Table 4.5. Neurotoxicity effect of extracts of *C. asiatica* towards SH-SY5Y cells is illustrated in Figure 4.6.

Table 4.5: SH-SY5Y cells viability (%) at different concentrations of methanolic and water extracts of *C. asiatica.*

Concentration of Extracts (µg/ml)	Cell Viability (%)	
	Methanolic Extract	Water Extract
0	100^{b}	100^{b}
1	103.41 ± 1.51^{ab}	101.63 ± 1.06^{ab}
10	114.75 ± 6.07^{a}	109.37 ± 1.29^{a}
25	102.11 ± 2.48^{ab}	110.51 ± 2.04^{a}
50	98.39 ± 2.06^{b}	108.31 ± 1.21^{ab}
75	94.13 ± 1.71^{b}	108.18 ± 3.39^{ab}
100	92.31 ± 0.86^{b}	108.38 ± 2.25^{ab}

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ($^{a-b}$) in the same column were significantly different ($p<0.05$).

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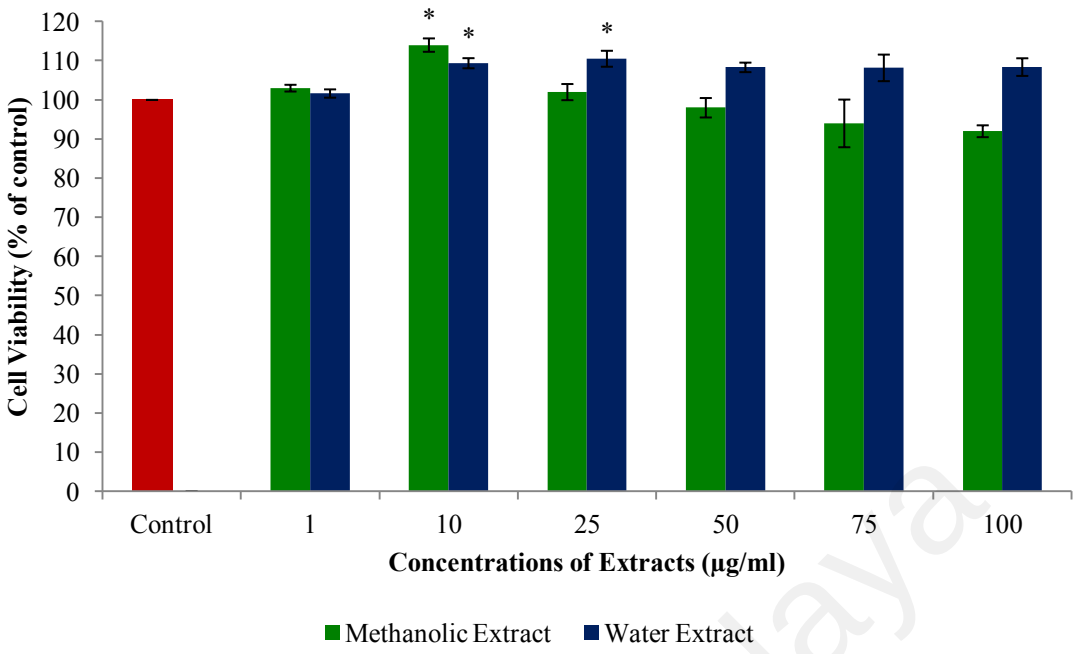

Figure 4.6: Neurotoxicity effects of methanolic and water extracts of *C. asiatica* towards SH-SY5Y cells. Cells were pre-treated with different concentrations of extracts (1, 10, 25, 50, 75 and 100 µg/ml) for 24 hours. The cell viability was determined using MTT assay and expressed as percentage of control. Control comprised of cells treated with vehicle (DMSO or distilled water). Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate.* denotes $p<0.05$ versus control group.

Based on the result presented in Table 4.5 and Figure 4.6, it can be seen that different concentrations (1, 10, 25, 50, 75 and 100 µg/ml) of methanolic and water extracts of *C. asiatica* have different effect towards the SH-SY5Y cell viability. For methanolic extract, the increasing concentration of extracts from 1 to 10 µg/ml increased the cell viability from 103.41 to 114.75% while further increment in the extract concentration from 10 to 100 µg/ml decreased the cell viability slightly from 114.75 to 92.31%.

For water extract, all the concentrations of the extract promoted the SH-SY5Y cells growth with a peak value of 110.51% of cell viability at concentration of 25 µg/ml. The untreated control cells were considered with 100% cell viability. Since both of the extracts concentrations did not have significant toxicity effect on the cell viability, this

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range of concentration was used for the analysis of its neuroprotective effects against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells.

4.2.4 Evaluation of Neuroprotective Effects of Extracts of *C. asiatica* towards Neurotoxic Agents-Induced Toxicity in SH-SY5Y Cells

The neuroprotective effects of methanolic and water extracts of *C. asiatica* towards H_2O_2 or acrylamide-induced toxicity in SH-SY5Y cells was evaluated by subjecting the cells to the extracts pre-treatment for 24 hours followed by exposure to neurotoxic agents for another 24 hours and finally the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of vehicle control. Generally, this assay consisted of four groups: Group 1: vehicle control (cell treated with vehicle only without addition neurotoxic agents or extracts); Group 2: negative control (cell treated with neurotoxic agents without addition of extracts); Group 3: cell treated with neurotoxic agents and extracts; Group 4: positive control (cell treated with neurotoxic agents and Trolox).

Cell viability (%) at different concentrations of methanolic and water extracts of *C. asiatica* in H_2O_2-induced toxicity in SH-SY5Y cells is presented in Table 4.6. The neuroprotective effects of methanolic and water extracts of *C. asiatica* in H_2O_2-induced toxicity in SH-SY5Y cells is illustrated in Figure 4.7.

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Table 4.6: Cell viability (%) at different concentrations of methanolic and water extracts of *C. asiatica* in H_2O_2-induced toxicity in SH-SY5Y cells.

Concentrations (µg/ml)	Cell Viability (%)		
	Methanolic Extract	Water Extract	Trolox
Vehicle Control	100[a]	100[a]	100[a]
0	46.87±6.56[c]	40.73±1.93[b]	46.87±6.56[c]
1	53.40±5.32[bc]	47.57±6.60[b]	-
10	53.57±6.62[bc]	48.73±2.06[b]	-
25	55.42±7.32[bc]	45.86±3.99[b]	75.89±3.26[b]
50	59.99±5.07[bc]	42.29±1.88[b]	-
75	60.94±7.93[bc]	40.98±2.50[b]	-
100	49.24±4.64[bc]	35.92±3.45[b]	-

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-c]) in the same column were significantly different ($p<0.05$). H_2O_2: hydrogen peroxide; Trolox: positive control.

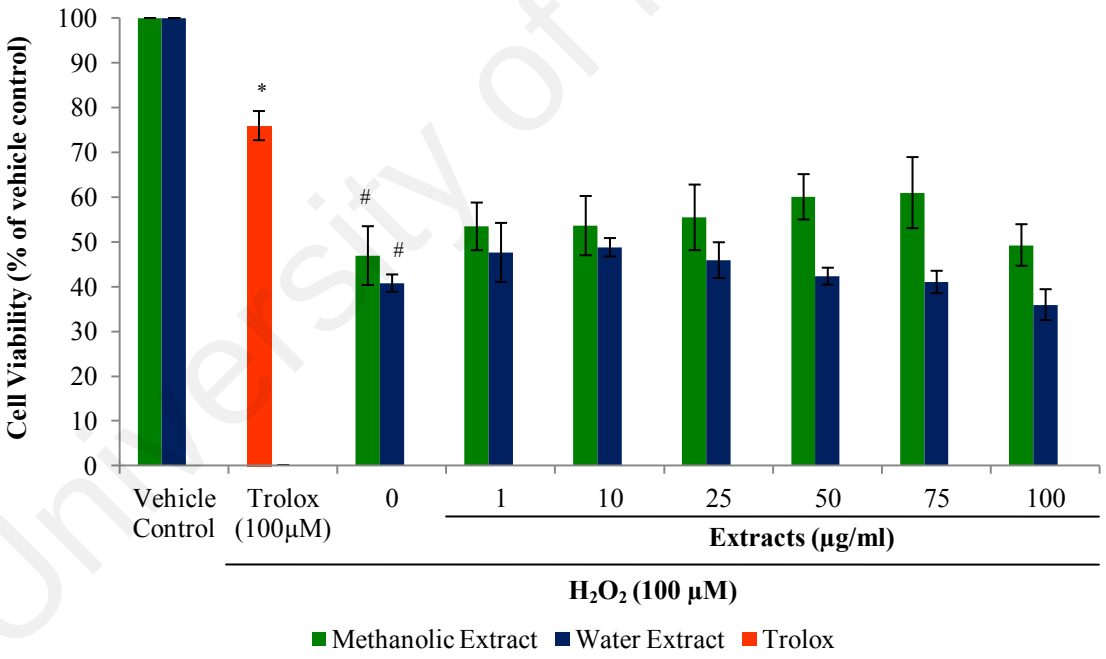

Figure 4.7: Neuroprotective effects of methanolic and water extracts of *C. asiatica* towards H_2O_2-induced toxicity in SH-SY5Y cells. Cells were pre-treated with different concentrations of extracts (1, 10, 25, 50, 75 and 100 µg/ml) for 24 hours and then treated with 100 µM of H_2O_2 for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Vehicle control comprised of cells treated with vehicle (DMSO or distilled water). Trolox was used as positive control. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. # denotes $p<0.05$, H_2O_2 alone treated group versus vehicle control group, * denotes $p<0.05$, extracts/trolox treated group versus H_2O_2 alone treated group. H_2O_2: hydrogen peroxide.

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Based on data obtained in section 4.2.2, the IC_{50} value of 100 μM of H_2O_2 was used to induce toxicity in SH-SY5Y cells and the non-toxic concentrations of the extracts ranging from 1 to 100 μg/ml obtained from section 4.2.3 were used to evaluate their neuroprotective efficacy. Based on the results obtained, it can be seen that the neuroprotective activity of both methanolic and water extracts of *C. asiatica* was not in concentration dependent manner whereby the cell viability generally increased and reached a peak before decreasing as the concentration of the extracts increased.

For methanolic extract, pre-treatment with extract provided protection to the cells against H_2O_2-induced toxicity at all concentrations examined compared to the cells treated with H_2O_2 alone. As the concentration of methanolic extract increased from 1 to 75 μg/ml, the cell viability steadily increased from 53.40 to 60.94%. Further increment in the concentration of the extract from 75 to 100 μg/ml had a reverse effect on the cell viability whereby the cell viability declined from 60.94 to 49.24%. A maximum protection towards the SH-SY5Y cells against H_2O_2-induced toxicity was observed in the presence of 75 μg/ml of extract with cell viability of 60.94% compared to the cells with H_2O_2 alone with cell viability of 46.87%, in which the cell death was reduced by nearly 14%.

In contrast, water extract of *C. asiatica* slightly inhibited toxicity caused by H_2O_2 at all concentrations of extract tested except at 100 μg/ml. As the concentration of water extract increased from 1 to 10 μg/ml, the cell viability increased from 47.57 to 48.73%. However, there was a decline in the cell viability from 48.73 to 35.92% as the concentration of extract was raised from 10 to 100 μg/ml. The highest neuroprotective effect was observed in SH-SY5Y cells treated with 10 μg/ml of extract with cell viability of 48.73% compared to the untreated cells with cell viability of 40.73%.

This finding also showed that methanolic extract provided a better neuroprotection compared to the water extract although a higher concentration of

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methanolic extract (75 µg/ml) is required in comparison with water extract (10 µg/ml). Trolox was used as a positive control and at a non-toxic concentration of 25 µg/ml, trolox significantly inhibited H_2O_2-induced toxicity in SH-SY5Y cells compared to the cells treated with H_2O_2 only which improved the cells survival by 29%.

The cell viability (%) at different concentrations of methanolic and water extracts of *C. asiatica* in acrylamide-induced toxicity in SH-SY5Y cells is presented in Table 4.7. The neuroprotective effects of methanolic and water extracts of *C. asiatica* in acrylamide-induced toxicity in SH-SY5Y cells is illustrated in Figure 4.8.

Table 4.7: Cell viability (%) at different concentrations of methanolic and water extracts of *C. asiatica* in acrylamide-induced toxicity in SH-SY5Y cells.

Concentrations	**Cell Viability (%)**		
(µg/ml)	**Methanolic Extract**	**Water Extract**	**Trolox**
Vehicle Control	100^{a}	100^{a}	100^{a}
0	43.00 ± 6.02^{c}	48.79 ± 5.90^{b}	43.00 ± 6.02^{c}
1	43.11 ± 5.46^{c}	50.85 ± 6.89^{b}	-
10	45.93 ± 4.88^{ab}	50.65 ± 6.21^{b}	-
25	45.04 ± 5.46^{ab}	51.98 ± 5.59^{b}	65.96 ± 2.69^{b}
50	46.91 ± 4.41^{ab}	49.24 ± 5.60^{b}	-
75	48.68 ± 5.49^{ab}	45.39 ± 2.39^{b}	-
100	47.53 ± 2.90^{ab}	44.26 ± 2.16^{b}	-

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ($^{a-c}$) in the same column were significantly different ($p<0.05$). Trolox: positive control.

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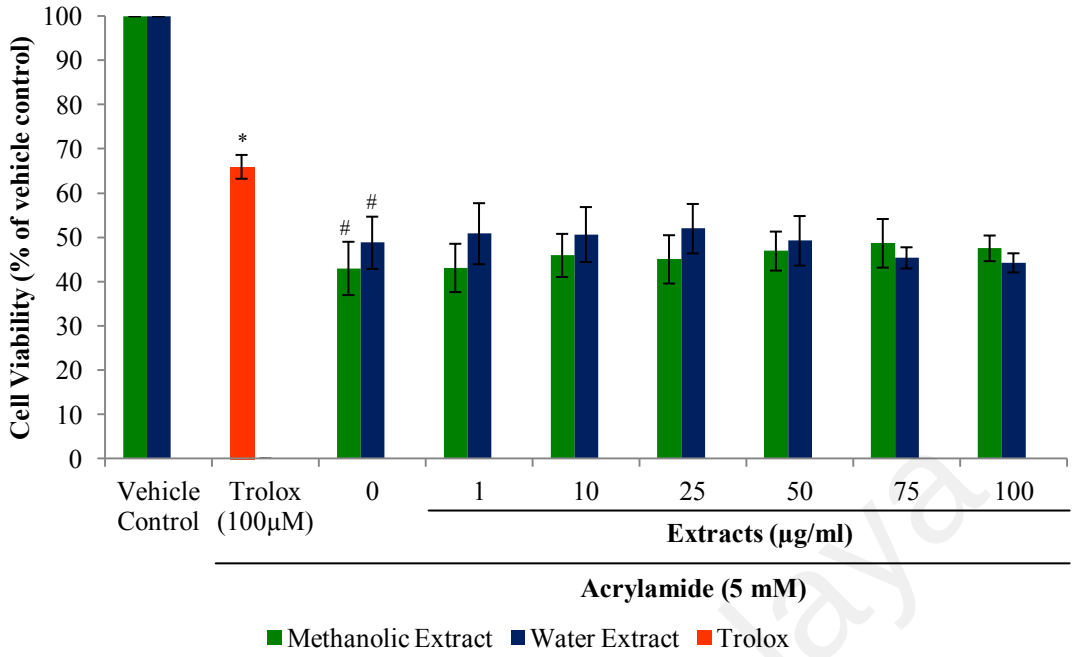

Figure 4.8: Neuroprotective effects of methanolic and water extracts of *C. asiatica* towards acrylamide-induced toxicity in SH-SY5Y cells. Cells were pre-treated with different concentrations of extracts (1, 10, 25, 50, 75 and 100 µg/ml) for 24 hours and then treated with 5 mM of acrylamide for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Vehicle control comprised of cells treated with vehicle (DMSO or distilled water). Trolox was used as positive control. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. # denotes $p<0.05$, acrylamide alone treated group versus vehicle control group, * denotes $p<0.05$, extracts/trolox treated group versus acrylamide alone treated group.

In this assay, the IC_{50} value of 5 mM of acrylamide was used to induce toxicity in SH-SY5Y cells and the non-toxic concentrations of extracts ranging from 1 to 100 µg/ml was used to evaluate its neuroprotective efficacy as reported in section 4.2.2 and 4.2.3. The result indicated that both methanolic and water extracts of *C. asiatica* did not exert a dose dependent neuroprotective effect against acrylamide-induced loss in cell viability.

A fluctuation in the cell viability over the concentrations of both extracts was observed. Pre-treatment with methanolic extract conferred a mild protection to cells against acrylamide caused toxicity at all the concentrations of the extract tested. The highest neuroprotective effect exhibited by methanolic extract pre-treatment was noted at 75 µg/ml which prevented loss of cell viability approximately by 5%.

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In contrast, water extract of *C. asiatica* slightly inhibited toxicity caused by acrylamide at all concentrations tested except at 75 and 100 µg/ml. The observed results also indicate that pre-treatment with water extract notably reversed the effect of acrylamide caused neurotoxicity in cells at 25 µg/ml with cell viability of 51.98% compared with untreated cells with cell viability of 48.79% which nearly protected 3% of cells.

This finding revealed that acrylamide-induced neurotoxicity was better blunted by methanolic extract compared to water extract although a higher concentration of methanolic extract (75 µg/ml) is required in comparison with water extract (25 µg/ml). Trolox was used as a positive control and at a non-toxic concentration of 25 µg/ml, trolox was able to significantly counteract the toxicity-induced by acrylamide in which the cell death was declined by nearly 23%. Overall, extracts of *C. asiatica* provided a stronger neuroprotection to SH-SY5Y cells against H_2O_2-induced toxicity compared to acrylamide-induced toxicity.

4.3 Neuroprotective Effects in Combination Model

4.3.1 Assessment of Neurotoxicity Effect of Curcumin towards SH-SY5Y Cells

The neurotoxicity effect of curcumin towards SH-SY5Y cells was assessed to determine the range of concentration of curcumin that is not toxic to the cells. For that, SH-SY5Y cells were incubated with several concentrations of curcumin (0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 µg/ml) for 24 hours and then the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of control. Cell viability (%) at different concentrations of curcumin is presented in Table 4.8. Neurotoxicity effect of curcumin towards SH-SY5Y cells is illustrated in Figure 4.9.

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Table 4.8: SH-SY5Y cells viability (%) at different concentrations of curcumin.

Concentrations of Curcumin (µg/ml)	Cell Viability (%)
0.00	100[a]
0.01	88.82±3.77[a]
0.10	89.02±5.67[a]
0.25	92.52±1.60[a]
0.50	93.18±5.42[a]
0.75	90.90±6.98[a]
1.00	88.71±1.12[a]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with same superscripts ([a]) in the same column were not significantly different ($p<0.05$).

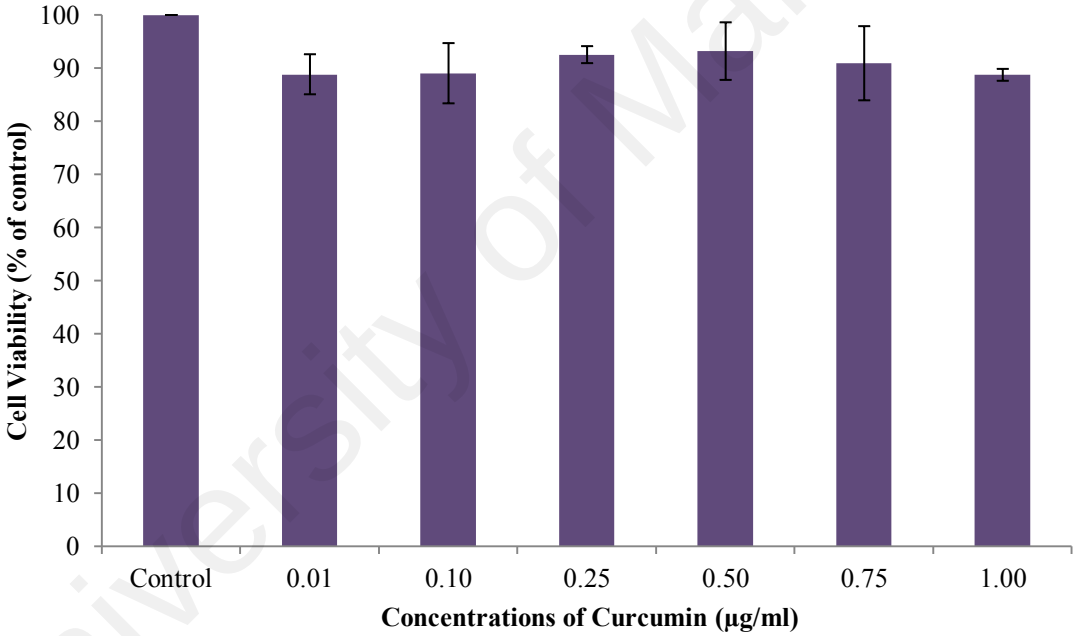

Figure 4.9: Neurotoxicity effect of curcumin towards SH-SY5Y cells. Cells were pre-treated with different concentrations of curcumin (0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 µg/ml) for 24 hours. The cell viability was determined using MTT assay and expressed as percentage of control. Control comprised of cells treated with vehicle (DMSO). Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate.

It can be seen from the results that the increasing concentration of curcumin from 0.01 to 0.50 µg/ml increased the cell viability from 88.82 to 93.18% while further increment in curcumin concentration from 0.50 to 1.00 µg/ml decreased the cell viability slightly from 93.18 to 88.71%. The untreated control cells were considered

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with 100% cell viability. Since all the tested curcumin concentrations did not have significant toxicity effect on the cell viability, this range of concentration was used for the analysis of its neuroprotective effects against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells.

4.3.2 Evaluation of Neuroprotective Effects of Curcumin towards Neurotoxic Agents-Induced Toxicity in SH-SY5Y Cells

In this assay, the neuroprotective effect of curcumin against toxicity induced by H_2O_2 or acrylamide was evaluated. For that, SH-SY5Y cells were pre-treated with different concentrations of curcumin (0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 μg/ml) for 24 hours prior to the exposure of neurotoxic agents for another 24 hours. Then, the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of vehicle control. Generally, this assay consisted of three groups: Group 1: vehicle control (cell treated with vehicle without addition of neurotoxic agents and curcumin); Group 2: negative control (cell treated with neurotoxic agents without addition of curcumin); Group 3: cell treated with neurotoxic agents and curcumin. The cell viability (%) at different concentrations of curcumin in H_2O_2-induced toxicity in SH-SY5Y cells is presented in Table 4.9. The neuroprotective effect of curcumin in H_2O_2-induced toxicity in SH-SY5Y cells is illustrated in Figure 4.10.

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Table 4.9: Cell viability (%) at different concentrations of curcumin in H_2O_2-induced toxicity in SH-SY5Y cells.

Concentrations of Curcumin (µg/ml)	Cell Viability (%)
Vehicle Control	100[a]
0.00	59.15±1.67[b]
0.01	68.44±6.18[b]
0.10	76.87±6.35[b]
0.25	79.33±2.48[ab]
0.50	73.27±5.41[b]
0.75	68.94±5.21[b]
1.00	63.65±4.81[b]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-b]) in the same column were significantly different ($p<0.05$). H_2O_2: hydrogen peroxide.

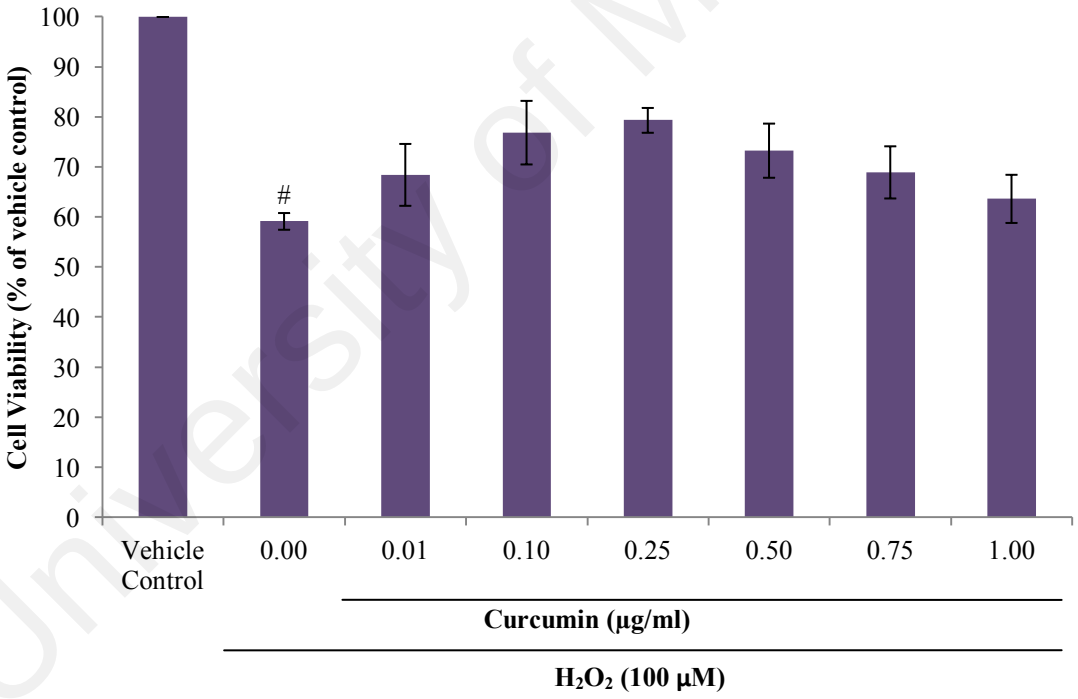

Figure 4.10: Neuroprotective effect of curcumin towards H_2O_2-induced toxicity in SH-SY5Y cells. Cells were pre-treated with different concentrations of curcumin (0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 µg/ml) for 24 hours and then treated with 100 µM of H_2O_2 for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Vehicle control comprised of cells treated with vehicle (DMSO). Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. # denotes $p<0.05$, H_2O_2 alone treated group versus vehicle control group. H_2O_2: hydrogen peroxide.

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Based on the results obtained, the neuroprotective activity of curcumin against H_2O_2 caused toxicity in SH-SY5Y cells was not in concentration dependent manner. The cell viability generally increased and reached a peak before declining as the concentration of curcumin was increased. As the concentration curcumin increased from 0.01 to 0.25 µg/ml, the number of viable cell steadily increased from 68.44 to 79.33%. However, there was a decline in the cell viability from 79.33 to 63.65% as the concentration of curcumin increased from 0.25 to 1.00 µg/ml. Generally, curcumin pre-treatment provided protection to the cells against H_2O_2-induced toxicity at all the concentrations examined compared to cells treated with H_2O_2 alone. Pre-treatment with curcumin provided a maximum protection towards SH-SY5Y cells against H_2O_2-induced toxicity at 0.25 µg/ml with cell viability of 79.33% compared to H_2O_2 alone treated group with cell viability of 59.15% in which the cell death was reduced by nearly 20%.

The cell viability (%) at different concentrations of curcumin in acrylamide-induced toxicity in SH-SY5Y cells is presented in Table 4.10. The neuroprotective effect of curcumin in acrylamide-induced toxicity in SH-SY5Y cells is illustrated in Figure 4.11.

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Table 4.10: Cell viability (%) at different concentrations of curcumin in acrylamide-induced toxicity in SH-SY5Y cells.

Concentrations of Curcumin (µg/ml)	Cell Viability (%)
Vehicle Control	100[a]
0.00	58.20±2.22[b]
0.01	62.88±1.16[b]
0.10	63.66±0.79[b]
0.25	64.51±1.53[b]
0.50	65.14±3.82[b]
0.75	63.39±3.01[b]
1.00	58.65±1.02[b]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-b]) in the same column were significantly different ($p<0.05$).

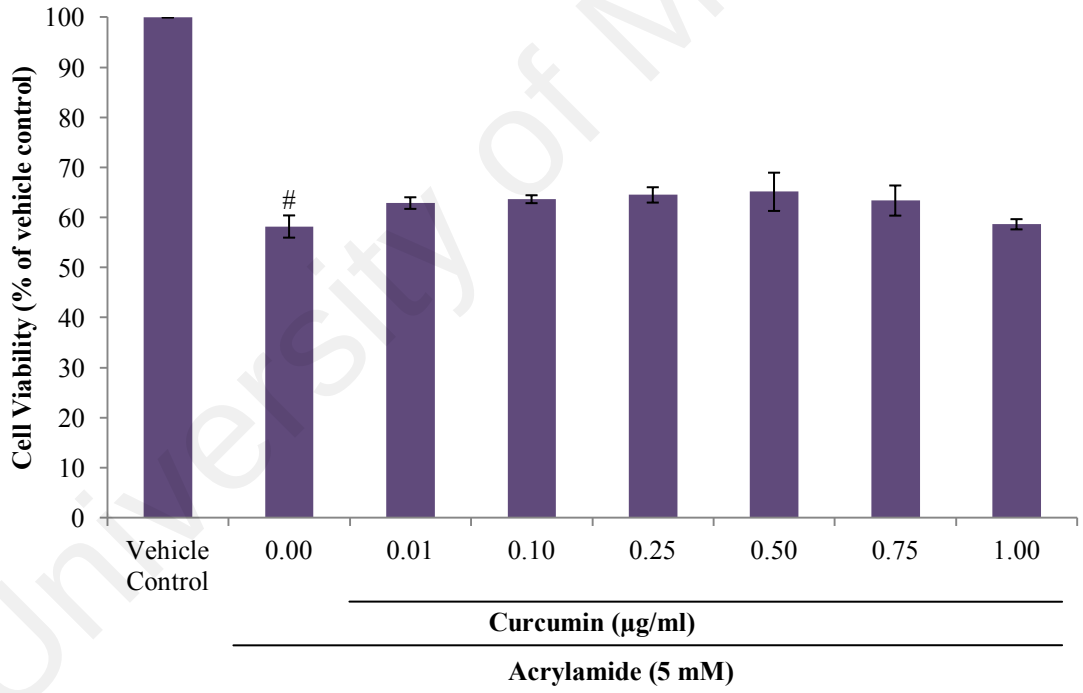

Figure 4.11: Neuroprotective effect of curcumin towards acrylamide-induced toxicity in SH-SY5Y cells. Cells were pre-treated with different concentrations of curcumin (0.01, 0.10, 0.25, 0.50, 0.75 and 1.00 µg/ml) for 24 hours and then treated with 5 mM of acrylamide for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Vehicle control comprised of cells treated with vehicle (DMSO). Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. # denotes $p<0.05$, acrylamide alone treated group versus vehicle control group.

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As it can be seen from the results obtained, curcumin pre-treatment did not provide a dose dependent neuroprotection towards the SH-SY5Y cells against toxicity caused by acrylamide. A similar trend was observed whereby the cell viability increased from 62.88 to 65.14% when the concentration of curcumin increased from 0.01 to 0.50 µg/ml whereas the cell viability decreased from 65.14 to 58.65% as the concentration was raised from 0.50 to 1.00 µg/ml. Generally, curcumin pre-treatment provided protection to SH-SY5Y cells against acrylamide-induced toxicity at all the concentrations examined compared to the cells treated with acrylamide alone. The highest neuroprotective effect was observed at 0.50 µg/ml with cell viability of 65.14% compared to untreated cells with cell viability of 58.20% which improved the cell viability by 7%. Overall, the findings indicate that curcumin provide a better neuroprotection towards the cell against H_2O_2 compared to acrylamide.

4.3.3 Evaluation of Neuroprotective Effects of the Combined extract-compound of *C. asiatica* and Curcumin

In this assay, neuroprotective effects of combined extracts of *C. asiatica* and curcumin against toxicity-induced by H_2O_2 and acrylamide was evaluated. For that, SH-SY5Y cells were pre-treated either with curcumin, methanolic or water extract of *C. asiatica* alone; and combination of curcumin and extracts at the concentration that provided highest neuroprotective effect. This is followed by addition of H_2O_2 (with concentration of 100 µM) and acrylamide (with concentration of 5 mM) respectively for another 24 hours. Then, the cell viability was assessed using MTT assay. The cell viability is expressed as percentage of vehicle control.

Generally, this assay consisted of four groups: Group 1: vehicle control (cells treated with vehicle only); Group 2: negative control (cells treated with neurotoxic

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agents only); Group 3: cells treated with either curcumin or extracts alone with neurotoxic agents; Group 4: cell treated with combination of curcumin and extracts with neurotoxic agents. Viability (%) of SH-SY5Y cells pre-treated with the extracts of *C. asiatica*, curcumin and combination of the extracts and curcumin in H_2O_2 and acrylamide-induce toxicity is presented in Table 4.11 – 4.14. Neuroprotective effects of extracts of *C. asiatica,* curcumin and combination of the extracts with curcumin in H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells is illustrated in Figure 4.12 – 4.15.

Table 4.11: Viability (%) of SH-SY5Y cells pre-treated with methanolic extract, curcumin and methanolic extract-curcumin combination in H_2O_2-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%)
Vehicle Control	NA	100^{a}
Negative Control	NA	55.80 ± 2.74^{b}
Methanolic Extract	75	64.86 ± 3.26^{b}
Curcumin	0.25	66.00 ± 5.31^{b}
Methanolic Extract-Curcumin Combination	75+0.25	67.88 ± 5.75^{b}

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ($^{a-b}$) in the same column were significantly different ($p<0.05$). H_2O_2: hydrogen peroxide; NA: not applicable.

Table 4.12: Viability (%) of SH-SY5Y cells pre-treated with water extract, curcumin and water extract-curcumin combination in H_2O_2-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%)
Vehicle Control	NA	100^{a}
Negative Control	NA	51.59 ± 2.58^{b}
Water Extract	10	57.80 ± 3.91^{b}
Curcumin	0.25	62.44 ± 6.13^{b}
Water Extract-Curcumin Combination	10+0.25	62.83 ± 7.05^{b}

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ($^{a-b}$) in the same column were significantly different ($p<0.05$). H_2O_2: hydrogen peroxide; NA: not applicable.

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Table 4.13: Viability (%) of SH-SY5Y cells pre-treated with methanolic extract, curcumin and methanolic extract-curcumin combination in acrylamide-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%)
Vehicle Control	NA	100[a]
Negative Control	NA	51.32±7.40[b]
Methanolic Extract	75	59.19±6.67[b]
Curcumin	0.50	60.43±9.47[b]
Methanolic Extract-Curcumin Combination	75+0.50	52.08±8.66[b]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-b]) in the same column were significantly different ($p<0.05$). NA: not applicable.

Table 4.14: Viability (%) of SH-SY5Y cells pre-treated with water extract, curcumin and water extract-curcumin combination in acrylamide-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%)
Vehicle Control	NA	100[a]
Negative Control	NA	49.80±5.87[b]
Water Extract	25	51.94±6.22[b]
Curcumin	0.50	54.75±5.00[b]
Water Extract-Curcumin Combination	25+0.50	45.54±6.44[b]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-b]) in the same column were significantly different ($p<0.05$). NA: not applicable.

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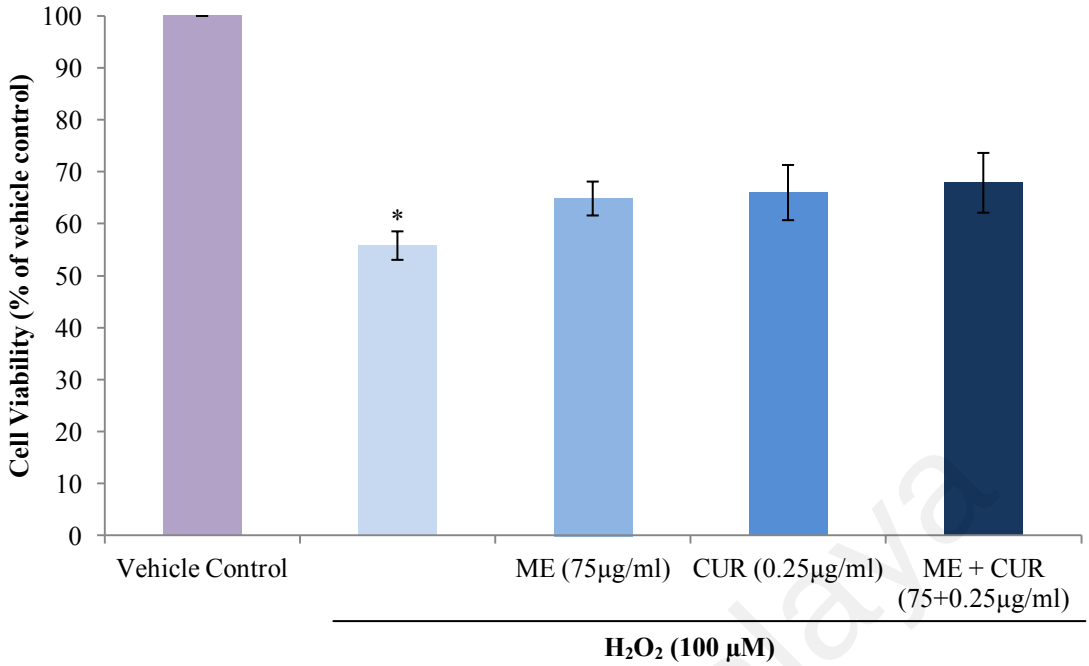

Figure 4.12: Neuroprotective effects of methanolic extract of *C. asiatica,* curcumin and methanolic extract of *C. asiatica*-curcumin combination towards H_2O_2-induced toxicity in SH-SY5Y cells. Cells were pre-treated with methanolic extract (75 µg/ml), curcumin (0.25 µg/ml) and combination of the extract (75 µg/ml) and curcumin (0.25 µg/ml) for 24 hours. Then, the cells were treated with 100 µM of H_2O_2 for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$, H_2O_2 alone treated group versus vehicle control group. ME: methanolic extract; CUR: curcumin; ME+CUR: methanolic extract-curcumin combination; H_2O_2: hydrogen peroxide.

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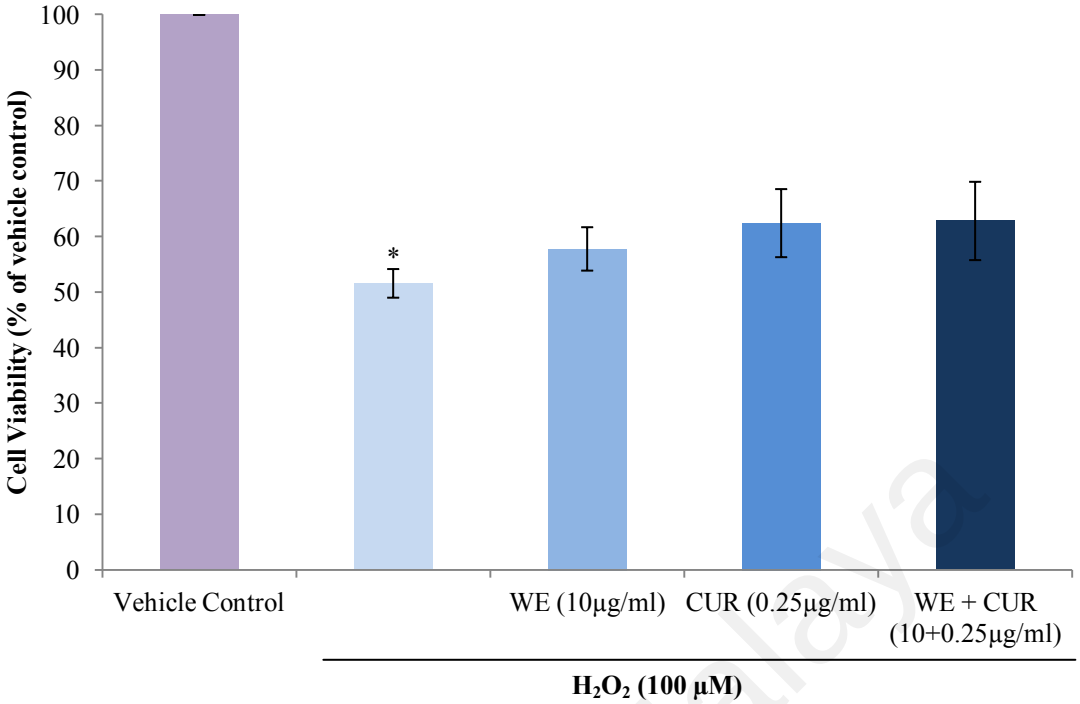

Figure 4.13: Neuroprotective effects of water extract of *C. asiatica,* curcumin and water extract of *C. asiatica*-curcumin combination towards H_2O_2-induced toxicity in SH-SY5Y cells. Cells were pre-treated with water extract (10 µg/ml), curcumin (0.25 µg/ml) and combination of the extract (10 µg/ml) and curcumin (0.25 µg/ml) for 24 hours. Then, the cells were treated with 100 µM of H_2O_2 for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$, H_2O_2 alone treated group versus vehicle control group. WE: water extract; CUR: curcumin; WE+CUR: water extract-curcumin combination; H_2O_2: hydrogen peroxide.

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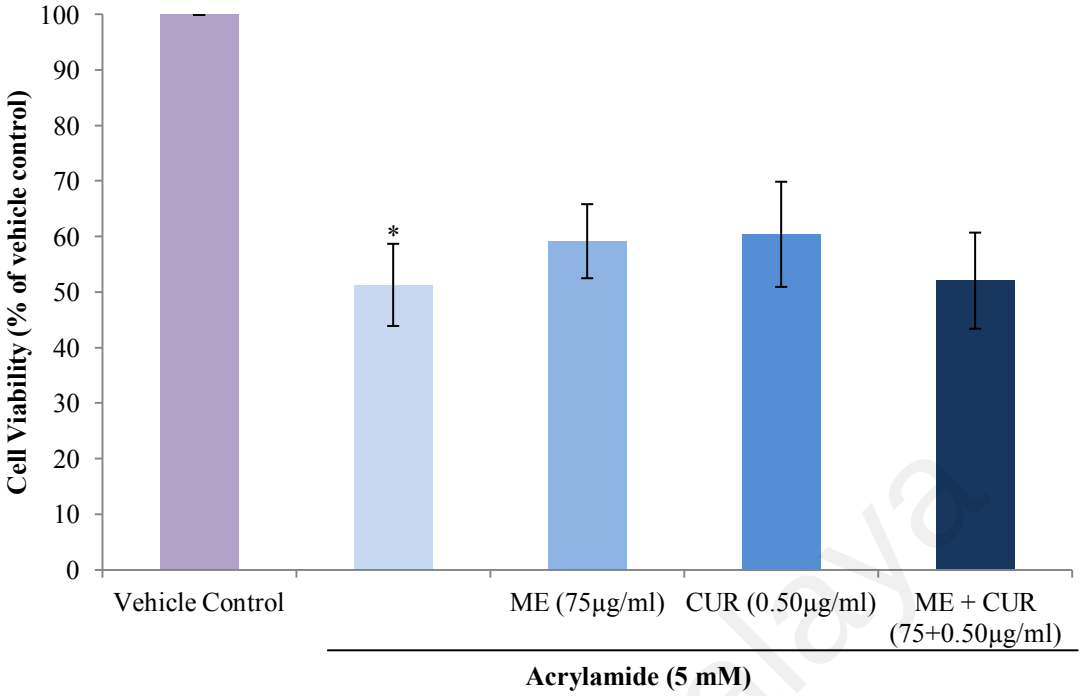

Figure 4.14: Neuroprotective effects of methanolic extracts of *C. asiatica,* curcumin and methanolic extract of *C. asiatica*-curcumin combination towards acrylamide-induced toxicity in SH-SY5Y cells. Cells were pre-treated with methanolic extract (75 µg/ml), curcumin (0.50 µg/ml) and combination of the extract (75 µg/ml) and curcumin (0.50 µg/ml) for 24 hours. Then, the cells were treated with 5 mM of acrylamide for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$, acrylamide alone treated group versus vehicle control group. ME: methanolic extract; CUR: curcumin; ME+CUR: methanolic extract-curcumin combination.

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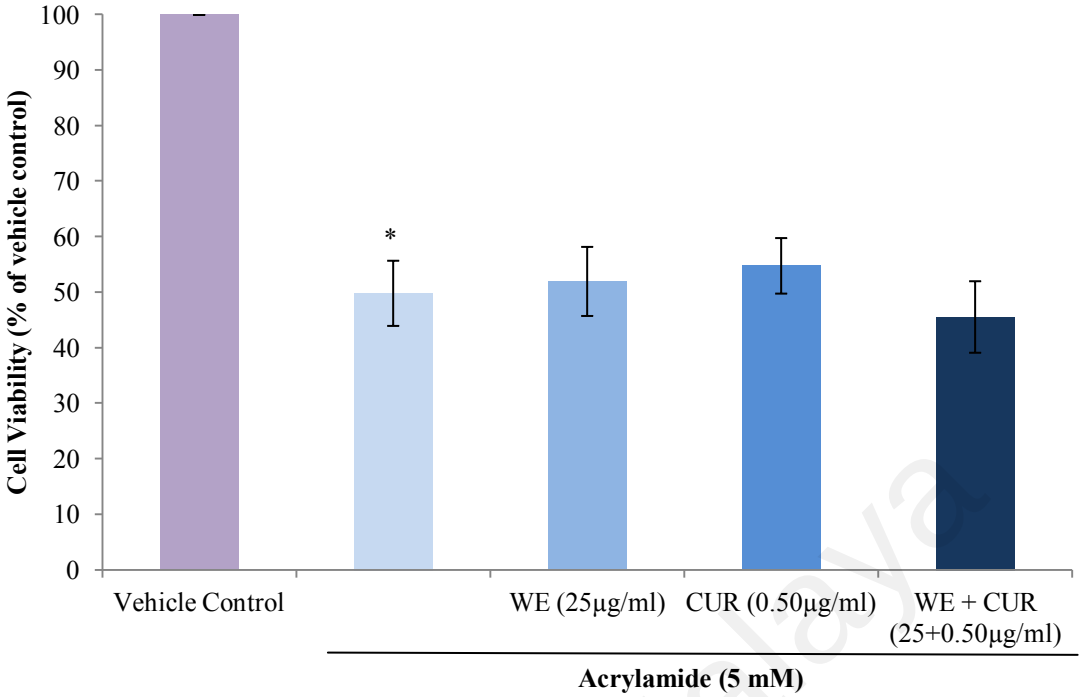

Figure 4.15: Neuroprotective effects of water extract of *C. asiatica,* curcumin and water extract of *C. asiatica*-curcumin combination towards acrylamide-induced toxicity in SH-SY5Y cells. Cells were pre-treated with water extract (25 µg/ml), curcumin (0.50 µg/ml) and combination of the extract (25 µg/ml) and curcumin (0.50 µg/ml) for 24 hours. Then, the cells were treated with 5 mM of acrylamide for another 24 hours. Cell viability was determined using MTT assay and expressed as percentage of vehicle control. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. * denotes $p<0.05$, acrylamide alone treated group versus vehicle control group. WE: water extract; CUR: curcumin; WE+CUR: water extract-curcumin combination.

Based on the results obtained, combination of both methanolic and water extract with curcumin provided a slightly higher neuroprotective effect against toxicity-induced by H_2O_2 than the neuroprotective effect achieved with curcumin and extracts alone. Pre-treatment with methanolic extract (75 µg/ml) and water extract (10 µg/ml) alone improved the cell viability by 9 and 6%, respectively. Curcumin (0.25 µg/ml) pre-treatment reduced the cell death by nearly 11%. Combination of methanolic extract with curcumin recorded a cell viability of 67.88% compared to H_2O_2 alone treated group with cell viability of 55.80% in which the cell death was reduced by nearly 12%.

Besides that, combination of water extract with curcumin recorded a cell viability of 62.83% compared to H_2O_2 alone treated group with cell viability of 51.59%

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in which the cell survival was improved by approximately 11%. Thus, combination of methanolic extract with curcumin demonstrated a better improvement in cell viability against toxicity-induced by H_2O_2 compared to combination of water extract and curcumin.

In contrast, combination of both methanolic and water extract with curcumin did not improve the neuroprotective activity against toxicity-induced by acrylamide compared the neuroprotective effect achieved with curcumin and extracts alone. Pre-treatment with methanolic extract (75 µg/ml) and water extract (25 µg/ml) alone improved the cell viability by 8 and 2%, respectively. Curcumin (0.50 µg/ml) pre-treatment reduced the cell death by nearly 9%. Combination of methanolic extract with curcumin recorded a cell viability of 52.08% compared to acrylamide alone treated group with cell viability of 51.32% in which the cell death was reduced by nearly 1%.

Besides that, combination of water extract with curcumin did not show any improvement in the cell viability compared to acrylamide alone treated group. Overall, combination of the extracts with curcumin demonstrated a better improvement in the neuroprotective activity against toxicity induced by H_2O_2 compared to toxicity induced by acrylamide.

4.4 Assessment of Antioxidant Activity

4.4.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

In DPPH assay, different concentrations of curcumin (concentrations range from 10–100 µg/ml), methanolic and water extracts of *C. asiatica* (concentrations range from 200–2000 µg/ml) were tested for their DPPH radical scavenging potential. The radical scavenging activity of curcumin and both extracts is reflected through the IC_{50} value.

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IC_{50} value depicted the concentration of test agents that able to scavenge 50% of the free radicals and the value is obtained by extrapolating from the graph of percentage of free radical inhibition versus concentration of the test agents. DPPH radical scavenging activity of curcumin, methanolic and water extracts of *C. asiatica* and ascorbic acid (positive control) are illustrated in Figure 4.16 and the IC_{50} values of the DPPH radical scavenging activity are presented in Table 4.15.

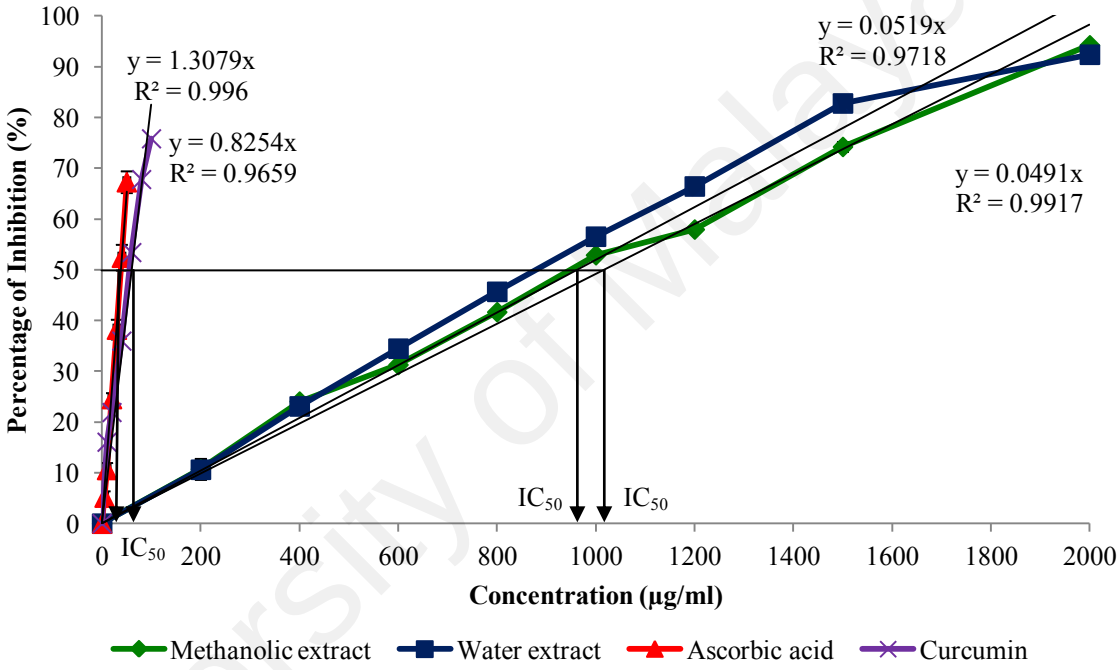

Figure 4.16: DPPH radical scavenging activity of curcumin, methanolic and water extracts of *C. asiatica* and ascorbic acid. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. DPPH: 2,2-diphenyl-1-picrylhydrazyl; ascorbic acid: positive control.

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Table 4.15: DPPH radical scavenging activity (IC_{50}, µg/ml) of extracts of *C. asiatica*, curcumin and ascorbic acid.

Test Agents	IC_{50} (µg/ml)[e]
Methanolic extract of *C. asiatica*	1018.37±4.33[a]
Water extract of *C. asiatica*	962.23±5.89[b]
Curcumin	61.10±3.96[c]
Ascorbic acid	38.41±1.50[d]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-d]) in the same column were significantly different ($p<0.05$). [e]IC_{50} value: concentration of test agents that able to scavenge 50% of the free radicals and the value was obtained by extrapolating from the graph of percentage of free radical inhibition versus concentration of the test agents; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ascorbic acid: positive control.

As shown in Figure 4.16, curcumin and both extracts of *C. asiatica* exhibited concentration-dependent radical scavenging activity. The radical scavenging activity of curcumin with an IC_{50} value of 61.10±3.96 µg/ml was observed to be higher than both the methanolic and water extracts with an IC_{50} value of 1018.37±4.33 µg/ml and 962.23±5.89 µg/ml, respectively. However, curcumin and both of the extracts had lower radical scavenging potential than the positive control, ascorbic acid with IC_{50} value of 38.41±1.50 µg/ml.

4.4.2 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity

In ABTS assay, different concentrations of curcumin (concentrations range from 10–150 µg/ml), methanolic and water extracts of *C. asiatica* (concentrations range from 200–2000 µg/ml) were tested for its ABTS radical scavenging activity. The ABTS radical scavenging activity of curcumin, methanolic and water extracts of *C. asiatica* and ascorbic acid (positive control) are illustrated in Figure 4.17 and the IC_{50} values of the ABTS radical scavenging activity are summarized in Table 4.16.

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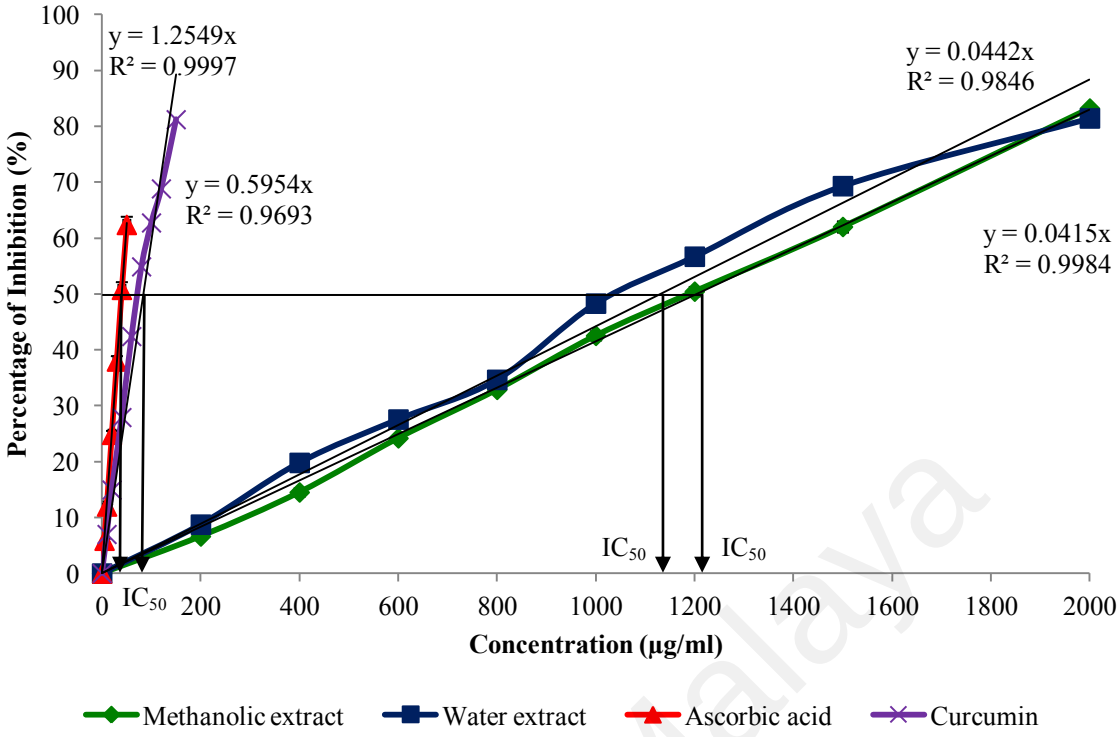

Figure 4.17: ABTS radical scavenging activity of curcumin, methanolic and water extracts of *C. asiatica* and ascorbic acid. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ascorbic acid: positive control.

Table 4.16: ABTS radical scavenging activity (IC_{50}, µg/ml) of extracts of *C. asiatica*, curcumin and ascorbic acid.

Test Agents	IC_{50} (µg/ml)[e]
Methanolic extract of *C. asiatica*	1205.93±9.26[a]
Water extract of *C. asiatica*	1123.27±16.88[b]
Curcumin	83.98±0.33[c]
Ascorbic acid	40.86±0.42[d]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-d]) in the column were significantly different ($p<0.05$). [e]IC_{50} value: concentration of test agents that able to scavenge 50% of the free radicals and the value was obtained by extrapolating from the graph of percentage of free radical inhibition versus concentration of the test agents; ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ascorbic acid: positive control.

As depicted in Figure 4.17, ABTS radical scavenging activity was directly proportional to the concentration of curcumin and both extracts whereby curcumin and extracts exhibited scavenging activity at all the concentrations that were examined. The

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ABTS radical scavenging activity of curcumin with IC_{50} value of 83.98±0.33 µg/ml was higher than methanolic and water extracts with an IC_{50} value of 1205.93±9.26 µg/ml and 1123.27±16.88 µg/ml, respectively. Nevertheless, the IC_{50} value of ascorbic acid, 40.86±0.42 µg/ml was relatively more pronounced than that of curcumin and extracts.

4.4.3 Iron Chelating Activity

The antioxidant activity of curcumin, methanolic and water extracts of *C. asiatica* extracts was also evaluated by its ability to chelate or deactivate transition metals through the iron chelating assay. The iron chelating activity of water extract of *C. asiatica* and EDTA (positive control) are illustrated in Figure 4.18. The IC_{50} values of the iron chelating activity are presented in Table 4.17.

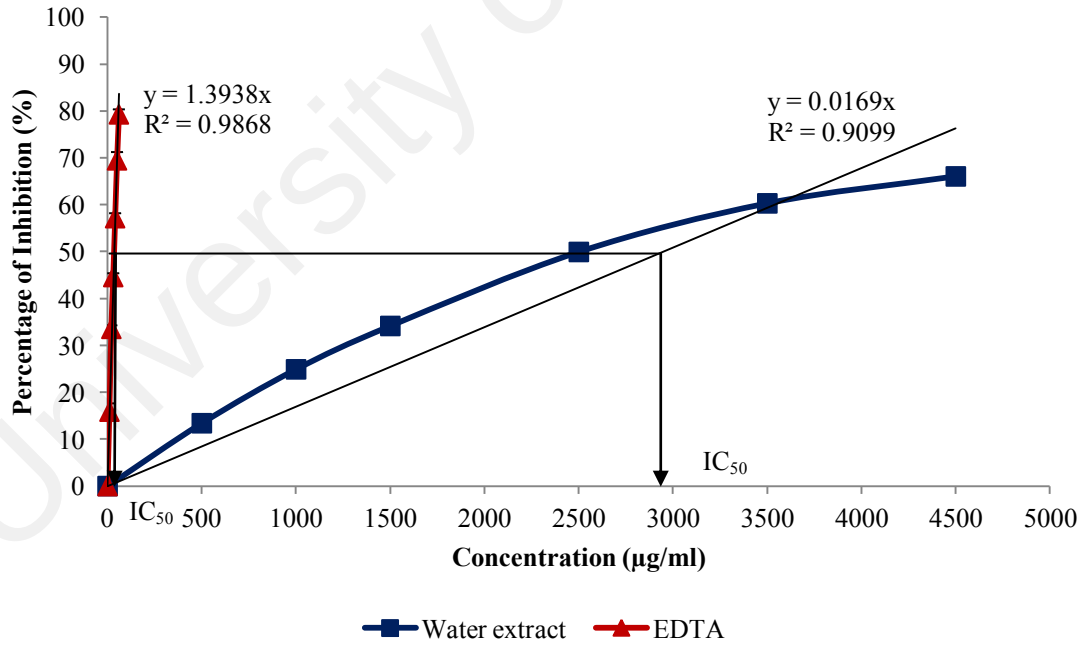

Figure 4.18: Iron chelating activity of water extract of *C. asiatica* and EDTA. Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. EDTA: ethylenediaminetetraacetic acid (positive control).

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Table 4.17: Iron chelating activity (IC_{50}, µg/ml) of extracts of *C. asiatica*, curcumin and EDTA.

Test Agents	IC_{50} (µg/ml)[c]
Methanolic extract of *C. asiatica*	ND
Water extract of *C. asiatica*	2952.80±5.80[a]
Curcumin	ND
EDTA[d]	35.46±0.72[b]

Data are presented as mean ± standard error of three independent experiments with each experiment performed in triplicate. Means with different superscripts ([a-b]) in the column were significantly different ($p<0.05$). [c]IC_{50} value: concentration of test agents that able to scavenge 50% of the free radicals and the value was obtained by extrapolating from the graph of percentage of free radical inhibition versus concentration of the test agents; [d]EDTA: ethylenediaminetetraacetic acid (positive control); ND: not determined.

The result showed that methanolic extract of *C. asiatica* and curcumin possess no iron chelating activity while the water extract of *C. asiatica* exhibits iron chelating activity with an IC_{50} value of 2952.80±5.80 µg/ml. However, the iron chelating activity of water extract of *C. asiatica* was lower than the standard EDTA with an IC_{50} of 35.46±0.72 µg/ml.

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CHAPTER 5: DISCUSSION

5.1 Preparation of Extracts of *Centella asiatica*

In this present study, methanolic and water extracts of *C. asiatica* were prepared. The preparation of methanolic extract involved collection of the fresh aerial parts of the plant, drying, grinding into fine powder and finally extraction of the dried powder using methanol. The aerial parts of *C. asiatica* were dried in oven for three days at low temperature (50˚C) prior to the extraction process in order to remove moisture and thus preserves the phytochemicals of the plant from deterioration by microorganisms. Low temperature drying was used to prevent the heat labile components of the plant from degradation (Castro *et al*., 2010; Azwanida, 2015).

Extraction is the most crucial step in the analysis of constituents of plants that are responsible for plant's various medicinal properties. Extraction step allows the separation of medicinally active components of plants by using suitable solvent system. The method of extraction chosen in this study was maceration. Maceration involves the soaking of the powdered plant in a solvent in a closed container at room temperature for a minimum of three days. This method is simple, easiest and does not require the boiling of the solvent which can cause thermal decomposition of bioactive compounds of the plant (Castro *et al*., 2010; Azwanida, 2015). Disadvantages of this method are it is time consuming and require large volume of solvent (Azwanida, 2015).

Selection of solvent is the most important factor in maceration method. Methanol is chosen as solvent in this study as it is most commonly used polar solvent that has been reported to effectively extract many different types of plant's bioactive compounds (Widyawati *et al*., 2014; Dhawan & Gupta, 2016). It is also low cost and has lower boiling point. In contrast, decoction method was used to prepare the water

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extract by boiling the fresh plant sample in distilled water for a shorter period of time. Water extraction in general is a most commonly known method of herbal remedies preparation in ancient times and many traditional healers mainly use water as a solvent (Kaneria *et al*., 2012).

Based on the results obtained for extraction yield, the yield of methanolic extract was higher than the yield of water extract. This result indicates that although water is more polar than methanol, many of the plant constituents are more readily soluble in methanol compared to water. A similar result was reported by Do *et al*. (2014), which showed that the extraction yield of methanolic extract of *Limnophila aromatica* was higher than water extract and this result was supported with identification of high phenolics and flavanoids content in methanolic than water extract. Another study done by Iloki-Assanga *et al*. (2015) also showed that a lower yield of phenolic components of *Bucida buceras* was obtained when water was used as solvent compared to methanol and this study suggest that it might due to the thermal decomposition of certain phenolics during the preparation of water extract which require the boiling of plant material in distilled water. There are also studies demonstrated that extraction yield of water was higher than other organic solvents (Mohd Farhan *et al*., 2012). Hence, extraction efficiency can also be influenced by polarity, chemical characteristics and structure of components of plant besides the polarity of solvent (Sultana *et al*., 2009; Do *et al*., 2014).

5.2 Neuroprotective Assays

Neuroprotection is known as strategies and mechanisms involved to preserve the structure and function of neurons from neuronal injury caused by various agents (Elufioye *et al*., 2017). It has been well documented that medicinal plants have a long

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history in the treatment of various diseases. An approximately 5,000 years old of written evidence found on Sumerian clay slab revealed that medicinal plants were used to prepare drugs (Petrovska, 2012) and until now 35,000 to 70,000 plant species were evaluated for their medicinal properties (Veeresham, 2012). Thus, there has been a great interest towards using medicinal plants as neuroprotective agents since there are many of the plants have been reported to have neuroprotective potential (Kumar *et al*., 2015) and one of such plant is *C. asiatica*. The *C. asiatica* is traditionally known as nerve tonic and memory enhancer (Hashim, 2011).

In this study, neuroprotective effects of methanolic and water extracts of *C. asiatica* against different neurotoxic agents [hydrogen peroxide (H_2O_2) and acrylamide]-induced toxicity in human neuroblastoma SH-SY5Y cells was investigated. An *in vitro* model is generally required as it provides a platform to understand the pathology of diseases and infections, to identify protein and molecular mechanism of specific phenomena and to conduct preliminary drug testing (Shipley *et al*., 2016).

The SH-SY5Y cells are long-established *in vitro* model in neuroscience research as they have neuronal cell phenotype such as neuritic processes with functional synapses, produce neuron related enzymes and neurotransmitters and can be easily maintained. In addition, the cells also play an important role in researches that require an accurately reproduced human nervous system as they produce some human specific proteins since they are derived from human compared to traditional use of primary neuronal cultures of rodents (Shipley *et al*., 2016). The cells were originated from three consecutive sub clones of parental SK-N-SH cell line which was derived from a metastatic bone tumour biopsy of a 4-year old female with neuroblastoma (Kovalevich & Langford, 2013). Thus, SH-SY5Y cells were selected in the current study as the *in vitro* model to investigate the neuroprotective activity of extracts of *C. asiatica* against toxicity-induced by neurotoxic agents.

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The neuroprotective potential of the extracts was evaluated using cell viability assay. The cell viability assay selected in this study was MTT assay as this assay is simple, reliable, sensitive and suitable for high-throughput assessment (Patravale *et al*., 2012; Riss *et al*., 2016). It had been also claimed by many researches as the gold standard to determine the cell viability since its invention in 1980's (Riss *et al*., 2016). There are also other well-known cell viability assays such as neutral red uptake, adenosine triphosphate (ATP) assay, lactate dehydrogenase assay and resazurin reduction assay.

MTT assay is a colorimetric assay. The yellow MTT is reduced by the mitochondrial dehydrogenase enzyme (present in only metabolically active and viable cells) into insoluble purple formazan which can be measured spectrophotometrically after dissolution in DMSO. Thus, the production of formazan is directly proportional to the number of viable cells and it indirectly indicates mitochondrial integrity and activity of the cells (Janhom & Dharmasaroja, 2015). The amount of formazan produced was measured at wavelength of 570 nm with a reference wavelength at 690 nm using a microplate reader.

The neuroprotective assays comprised of the preliminary assessment of the toxicity of the DMSO, neurotoxic agents and extracts of *C. asiatica* prior to the investigation of the neuroprotection potential of the extracts against the toxicity-induced by the neurotoxic agents in SH-SY5Y cells. In addition, the improvement of neuroprotective effect of extracts of *C. asiatica* on neurotoxic agents-induced toxicity in the cells after combining with curcumin was also assessed.

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5.2.1 Assessment of Toxicity of Dimethyl Sulfoxide towards SH-SY5Y Cells

Dimethyl sulfoxide (DMSO) [$(CH_3)_2SO$] is a colourless polar aprotic solvent classified as an organosulfur complex that has ability to dissolve both polar and nonpolar compounds (Hebling *et al*., 2015). DMSO has wide application in cell biology as an effective solvent, carrier of various drugs, cryoprotectant and free radical scavenger. As a pharmacological carrier, it helps to increase the cell permeability and enhance the penetration of pharmaceuticals through the cells (Hajighasemi & Tajik, 2017). DMSO has been classified under class III solvents according to Q_3C guidelines which indicate that DMSO is less toxic and thus reduces risk to human health (Jamalzadeh *et al*., 2016).

However, the application of DMSO in biological studies had demonstrated some cytotoxicity effect such as it can affect the structure and conformation changes of cell membrane and protein which can severely influence the metabolism and cell membrane function (Trivedi *et al*., 1990; Violante *et al*., 2002). Therefore, the toxicity of DMSO towards the SH-SY5Y cells was assessed in this study as DMSO was used as the solvent to dissolve the methanolic extract of *C. asiatica*. The findings showed that concentration of 2.5% (v/v) and above significantly affect the cell viability which can be due to damaging effect of DMSO as discussed above. Thus, concentration of DMSO of 0.5% (v/v) was chosen to dissolve the methanolic extract of *C. asiatica*.

Besides that, a study conducted by Hajighasemi and Tajik (2017) revealed that no significant cytotoxicity was observed at DMSO concentration less than 2% (v/v) on human leukemic cell lines. Concentration of DMSO less than 0.5% (v/v) did not significantly affect the cell viability of human breast MCF-7 cancer cell line (Jamalzadeh *et al*., 2016). These results indicate that different concentration of DMSO shows different cytotoxicity effect towards various cell lines of different origin. An

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ideal solvent used as vehicle of a pharmacological active compound should be non-toxic to the cells, optimize the solubility, avoid the loss of biological activities of that compound, compatible with the media and do not influence the result of the experiment (Jamalzadeh *et al*., 2016).

5.2.2 Assessment of Toxicity of Neurotoxic Agents towards SH-SY5Y Cells

In the present study, H_2O_2 and acrylamide were two neurotoxic agents used to induce toxicity in SH-SY5Y cells. H_2O_2 is a naturally produced reactive oxygen species (ROS) in the body during normal cell metabolism and it acts as an important cellular signalling molecule. However, when it is produced in excess it can cause oxidative stress. H_2O_2 is widely known as cytotoxic agent as many previous studies have reported the usage of H_2O_2 as cytotoxicity and oxidative stress inducer in order to screen the protective and antioxidant activity of plant extracts in various *in vivo* and *in vitro* models (Lee & Jeong, 2007; Piah *et al*., 2010; Custodio *et al*., 2013; Al-Sheddi *et al*., 2015; Luo *et al*., 2018).

H_2O_2 has high membrane permeability and it can be converted into highly reactive hydroxyl radical which can cause oxidative damage to the cellular biomolecules and eventually leads to cell death (Lee & Jeong, 2007). For the assessment of the toxicity of H_2O_2 towards the SH-SY5Y cells, the findings indicated that exposure to H_2O_2 in concentration range of 25 to 500 μM decreased the cell viability in dose dependent manner and the cell viability declined to nearly 50% in response to 100 μM of H_2O_2 exposure. This is because exogenous H_2O_2 treatment can cause the abnormal increase of intracellular ROS level which induce oxidation of lipid, protein and DNA and thus inhibit the cell growth (Han *et al*., 2017). Yang *et al*. (1999) reported that H_2O_2 is well regarded as genotoxic agent as it cause oxidative DNA

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damage which includes DNA strand breakage and base modification. The decrease in the cell viability can also due to the mitochondrial dysfunction due to loss mitochondria membrane potential and decrease in the level of intracellular antioxidant level (Luo *et al*., 2018).

Many researches revealed that exposure of neuronal cells to H_2O_2 cause the increase of caspase-3 activity which leads to cell death via apoptosis (Masilamani *et al*., 2017). Finding in this present study agreed well with study conducted by Xiong *et al*. (2009), which revealed that exposure of 100 μM of H_2O_2 for 24 hours to SH-SY5Y cells reduced the cell viability to 41.5%. In addition, Halliwell *et al*. (2000) indicated that concentration of H_2O_2 above 50 μM is toxic to many types of animal, plant and bacterial cells cultures.

Acrylamide is another type of neurotoxic agent used in this study to induce toxicity in SH-SY5Y cells. Acrylamide is a potent neurotoxic agent and it also has been classified as class 2A by the International Agency for Research on Cancer, which indicate that it is a probable human carcinogen (Cao *et al*., 2008). Consumption of foods cooked at high temperature especially carbohydrate based foods has been identified as one of the major source of human exposure to acrylamide (Pennisi *et al*., 2013). Many studies have claimed that cytotoxicity caused by acrylamide is associated with oxidative stress as acrylamide can be biotransformed in the body into glycidamide and eventually into ROS (Song *et al*., 2013). The possible neurotoxicity effects caused by acrylamide are interaction with cellular biomolecules (lipid peroxidation, DNA fragmentation) (Mehri *et al*., 2012), reduction of the intracellular antioxidative enzymes level (Chen *et al*., 2014), and further influence the receptors, neurotransmitter concentration and membrane integrity of neuronal cells (Pennisi *et al*., 2013).

For the assessment of the toxicity of acrylamide towards the SH-SY5Y cells, findings revealed that exposure to acrylamide in the concentration range of 1 to 20 mM

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decreased the cell viability in dose dependent manner and the cell viability reduced to nearly 50% in response to 5 mM of acrylamide exposure. This data indicates that increasing concentration of acrylamide causes neurotoxicity effect towards the cells and thus inhibits its growth. This result was in good agreement with study conducted by Mehri *et al*. (2012), which revealed that exposure of rat pheochromocytoma PC-12 cells to 5 mM of acrylamide for 24 hours reduced the cell viability to 50%. Another study conducted by Chen *et al*. (2014) also showed that the cell viability was nearly 50% when the human epithelial colorectal CaCo-2 carcinoma cells were treated with 5 mM of acrylamide for 24 hours. In addition, a study conducted by Park *et al*. (2010) demonstrated that high dose of acrylamide (5 mM) activated the cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in neural progenitor cells (NPC) which suggested that acrylamide induced both necrotic and apoptotic cell death in NPCs. In summary, the IC_{50} values obtained from this assay also indicate that SH-SY5Y cells were more sensitive towards H_2O_2 than acrylamide induced-toxicity.

5.2.3 Assessment of Neurotoxicity Effects of Extracts of *C. asiatica* towards SH-SY5Y Cells

Traditionally, the preparation of medicine using plants is in the form of methanolic/alcoholic extract or water extract (Kaneria *et al*., 2012). In this study, methanolic and water extracts of *C. asiatica* were prepared to evaluate its neuroprotective potential against different neurotoxic agents-induced toxicity in SH-SY5Y cells. Prior to the neuroprotective analysis, the neurotoxicity effect of the extracts towards the cells was determined in order to identify the suitable range of concentration of extracts that do not cause any toxicity effect to the cells. The results of

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the experiment will be affected if there is any toxicity effect of the extracts towards the cells.

Based on the results obtained, methanolic and water extracts of *C. asiatica* did not cause any significant toxicity towards SH-SY5Y cells at all the concentrations examined (ranges from 1–100 µg/ml) in which high percentage of cell viability was recorded. A study conducted by Chen *et al*. (2016) showed that ethanolic extract of *C. asiatica* was not significantly cytotoxic to rat pheochromocytoma PC-12 cells at concentration less than 100 µg/ml. Custodio *et al*. (2013) demonstrated that methanolic extracts of cork oak and holm oak did not affect the cell viability of SH-SY5Y cells at concentration range of 10 to 100 µg/ml.

In contrast, another study carried out by Masilamani *et al*. (2017) revealed that treatment of human neuroblastoma IMR cells with ethanolic extract of *Peltophorum pterocarpum* had no significant cytotoxicity effect towards the cells at concentration ranging from 8 to 250 µg/ml. Thus, it can be seen from above studies that cytotoxicity effect of plant extracts varies upon plant and cell types. The concentration ranges from 1 to 100 µg/ml of the extracts of *C. asiatica* was used for the analysis of its neuroprotective effects against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells.

5.2.4 Evaluation of Neuroprotective Effects of Extracts of *C. asiatica* towards Neurotoxic Agents-Induced Toxicity in SH-SY5Y Cells

Methanolic and water extracts of *C. asiatica* were evaluated for their neuroprotective activity against H_2O_2 or acrylamide-induced toxicity in SH-SY5Y cells. The results revealed that both of the extracts at their non-toxic concentration range had a mild neuroprotective activity against both of the neurotoxic agents. Pre-treatment of

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SH-SY5Y cells with the extracts did not significantly improve the cell viability compared to cells exposed to neurotoxic agents alone. In H_2O_2-induced toxicity model, methanolic and water extracts showed the highest neuroprotective activity at 75 and 10 μg/ml, respectively. Meanwhile, in acrylamide-induced toxicity model, methanolic and water extracts showed the highest neuroprotective activity at 75 and 25 μg/ml, respectively.

The exact mechanisms of the neuroprotective action of *C. asiatica* remain to be unknown though many hypotheses have been formulated (Lokanathan *et al*., 2015). The possible mechanisms underlying the neuroprotective activity of the extracts of *C. asiatica* are by (i) reduction of intracellular ROS-induced by the toxicity of H_2O_2 or acrylamide by scavenging or neutralizing the ROS, (ii) restoration of antioxidative defence system in cells (Chen *et al*., 2016), (iii) reduction of oxidative damage to biomolecules of cells such as decrease the lipid peroxidation and protein oxidation, (iv) prevention of mitochondrial dsyfunction and (v) reduction of apoptotic cell death (Xu *et al*., 2012). These neuroprotective properties of *C. asiatica* have been related with its major constituent, triterpenes such asiatic acid, asiaticoside, madecassic acid and madecassoside (Lokanathan *et al*., 2015).

Several previous studies have reported the neuroprotective activity of whole extract and individual components of *C. asiatica* in both *in vitro* and *in vivo* models. The neuroprotective roles of *C. asiatica* includes (i) memory and cognitive improvement, (ii) regeneration of nerve cells, (iii) reduction of oxidative stress-induced by neurotoxic agents, (iv) as antioxidant agent, (v) inhibition of cholinesterase's enzyme involved in Alzheimer's disease (AD) and (vi) prevention of apoptosis neuronal cell death (Lokanathan *et al*., 2015). Many of the neurotoxicity related studies were conducted in *in vivo* model while only a few done using *in vitro* model. The *C. asiatica* have demonstrated protective effect against glutamate (Xu *et al*., 2012), arsenic (Gupta

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& Flora, 2006), beta amyloid (Gray *et al*., 2014), rotenone (Teerapattarakan *et al*., 2018), sodium nitroprusside (Marques *et al*., 2015), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Haleagrahara & Ponnusamy, 2010) and aluminium-induced neurotoxicity (Amjad & Umesalma, 2015).

Apart from that, the mild neuroprotective activity of extracts of *C. asiatica* might be due to the reason that the extraction process particularly the types of solvents used might not fully extract the phytochemicals that are responsible for neuroprotection against the toxicity-induced by the neurotoxic agents. Generally, the extraction process is affected by several factors such as solvent type, extraction method, solid to solvent ratio, extraction temperature, solvent concentration and particle size (Mohd Salim *et al*., 2013). Another reason could be due to the presence of low concentration of bioactive compound(s) that could be responsible for neuroprotection against the neurotoxic agents in the collected plant sample. Phytochemicals content of *C. asiatica* have been reported to vary according to its habitat as different habitat have different source of nutrients, climate, soil condition and water supply (Zainol *et al*., 2008; Devkota *et al*., 2010). Devkota *et al*. (2010) revealed that the concentration of asiaticoside in the leaves of *C. asiatica* obtained from Nepal was 4 to 10 times greater than those collected from India. Sharma and Gupta (2016) revealed that triterpene components in *C. asiatica* can be affected by diverse environmental conditions, location and accessions. This is justified by a study performed by Shinomol and Muralidhara (2008), which reported that water infusion of *C. asiatica* obtained from India conferred a marked neuroprotection against 3-nitropropionic acid-induced oxidative stress in both *in vivo* and *in vitro* models.

In addition, there are several studies reported on the neuroprotective activity of other different plants against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells (Mehri *et al*., 2012; Venuprasad *et al*., 2013; Esmaeelpanah *et al*., 2015; Park *et al*.,

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2015; Masilamani *et al*., 2017). Ability of these plants to provide a significant neuroprotection to the cells compared to *C. asiatica* might be attributed to extraction method and types and concentration of the phytochemicals that it contains. Most of the bioactive compounds with the neuroprotective property that have been identified in those plants reported to belong to the group of polyphenols [flavonoids (chrysin, epigenin, orientin, quercetin) and saponin (spicecatoside A)].

Furthermore, methanolic extract of *C. asiatica* demonstrated a better neuroprotective activity than water extract against H_2O_2 and acrylamide-induced toxicity. This finding indicates that methanol might be able to effectively extract the targeted endogenous bioactive compounds from *C. asiatica* than water. This is further supported by the results of extraction yield of extracts of *C. asiatica* which revealed that extraction yield of methanol was higher than water. According to Lui (2011), triterpenes easily dissolve in organic solvent but hardly dissolve in water. Since triterpenes in *C. asiatica* are known to play an important role in neuroprotection, its greater solubility in organic solvent compared to water could be the reason for the greater neuroprotective activity of methanolic extract than water extract against the neurotoxic agents. In addition, methanol is widely used solvent in many ethnopharmacological studies by traditional practitioners (Amid *et al*., 2010). Besides that, the results also revealed that both extracts of *C. asiatica* more effectively protected the cells against toxicity caused by H_2O_2 than acrylamide.

Trolox was used as the positive control in this study to compare its neuroprotective activity with the extracts of *C. asiatica*. Trolox is widely known antioxidant derived from Vitamin E that has been used as reference compound in many neuroprotective studies (De Pedro *et al*., 2014; Wean *et al*., 2014; Sarveazad *et al*., 2016; Supasuteekul *et al*., 2016). In this study, pre-treatment of SH-SY5Y cells with trolox at 25 μg/ml had significantly attenuated both H_2O_2 and acrylamide-induced

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toxicity in the cells. Yu *et al*. (2017) reported that pre-treatment with trolox had significantly protected rat pheochromocytoma PC-12 cells against H_2O_2-induced toxicity mainly by reducing the intracellular ROS level, lipid peroxidation and caspase-3 activity.

5.3 Neuroprotective Effects in Combination Model

Combination therapy is defined as a treatment of disease with two or more drugs (Karjalainen & Repasky, 2016). Drug combination generally aims to attain additive or synergistic therapeutics effect, reduce dose, toxicity, side effects and the chances in developing of drug resistance (Agrawal *et al*., 2005). The outcome of drug combination can be either synergistic or antagonistic. Synergism is obtained when the combined drugs provide a better effect than the individual effects of each drug. While antagonism is achieved when combined drugs provide undesirable effect than the individual effects of each drug (Yin *et al*., 2014). This multi-therapeutic system has proven its efficacy in treatment of cancers, diabetes, acquired immune deficiency syndrome (AIDS), infectious diseases and other complex human diseases (Agrawal *et al*., 2005; Zimmermann *et al*., 2007).

Evidently, neurodegenerative disease is a multifactorial disease and thus combination drug therapy would be an effective or the disease modifying therapy as it simultaneous targets multiple mechanisms and pathways that are responsible for neurodegenerative disease compared to the current conventional therapeutic approaches based on "one gene, one drug, one disease" philosophy (Schyf, 2014). There are several published works on neuroprotective potential of drugs combination. Combination of memoquin with several of its derivatives showed an effective anti-beta-amyloid and cholinesterase activity while combination of rivastigmine molecule with rasagiline

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molecule (Ladostigil) conferred protection against neurodegenerative disorders such as AD, Parkinson's disease (PD) and Lewy body diseases (Schyf, 2014).

At present significant attention has been paid to natural products from medicinal plants and herbs in the development of multi-component system as there are many previous studies reported on the various therapeutic potentials of plant extracts or phytochemicals (Chakraborty *et al*., 2017). In light with above knowledge, this study also evaluated the improvement in neuroprotective effects of the extracts of *C. asiatica* by combining it with curcumin on neurotoxic agents-induced toxicity in SH-SY5Y cells.

Curcumin is a polyphenolic compound obtained from the dried rhizome of *Curcuma longa* (turmeric). Many previous studies reported on therapeutic potential of curcumin against variety of diseases and one of such diseases is neurodegenerative diseases. Curcumin was selected in this study as it is known to exert at least 10 different neuroprotective actions (Cole *et al*., 2007). Many *in vitro* studies demostrated that curcumin is one of the powerful dietary phytochemical to prevent several neurological disorders (Kumar *et al*., 2015). Its neuroprotective properties are highly associated with its complex chemistry, ability to target multiple mechanisms related to the disease, ability to cause variety of important biological effects, high antioxidant, anti-inflammatory and anti-protein accumulation potentials (Cole *et al*., 2007; Menon & Sudheer, 2007; Hatcher *et al*., 2015). Velmurugan *et al*. (2008) revealed that among 214 compounds with antioxidant property, curcumin had the highest affinity towards aggregation of beta amyloid protein which is responsible for AD. Curcumin also improves cognition, reduces depression, prevents stroke and disruption of the blood-brain barrier (Nabiuni *et al*., 2011; Wang *et al*., 2016). Thus, the combination of extract of medicinal plant and phytochemical is expected to enhance the neuroprotective effect against the neurotoxic agents.

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5.3.1 Assessment of Neurotoxicity Effect of Curcumin towards SH-SY5Y Cells

Prior to the neuroprotective analysis of curcumin, the neurotoxicity effect of certain range of concentration of curcumin towards the cells was determined in order to identify the suitable range of concentration of curcumin that is not toxic towards SH-SY5Y cells. Based on the results obtained, curcumin did not cause any significant effect on the viability of SH-SY5Y cells at all the concentrations tested (ranges from 0.01–1.00 µg/ml). A study conducted by Mufti *et al*. (2015) demonstrated that treatment of SH-SY5Y cells with curcumin alone at a range of 0.3–7.0 µg/ml did not significantly affect the viability of the cells.

5.3.2 Evaluation of Neuroprotective Effects of Curcumin towards Neurotoxic Agents-Induced Toxicity in SH-SY5Y Cells

In this study, the neuroprotective effects of curcumin towards H_2O_2 or acrylamide-induced toxicity in SH-SY5Y cells was evaluated. The outcome of the analysis indicated that pre-treatment of the cells with curcumin at its non-toxic concentrations provided protection towards the cells against toxicity-induced by both of the neurotoxic agents. However, curcumin did not exhibit a significant neuroprotective activity as the cell viability was improved slightly compared to cells exposed to neurotoxic agents alone.

A similar result was reported by Zhao *et al*. (2011), which showed that pre-treatment with curcumin at concentration of 20 and 25 µg/ml restored the cell viability of murine neuroblastoma Neuro-2A cells by 7 and 8%, respectively in H_2O_2-induced cell damage. Perhaps the possible neuroprotective action of curcumin might be due to the membrane permeable property of curcumin (Zhao *et al*., 2011) which allows it to

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diffuse into the cells and scavenge or neutralize the intracellular ROS generated by the neurotoxic agents. This is further justified by a study carried out by Tuba and Gulcin (2008), which indicated that curcumin is a good antioxidant agent. The methoxyl, phenolic and β-diketone functional groups of curcumin were reported to have notable free radical scavenging properties. The detailed mechanism behind antioxidant property of curcumin that have been hypothesized is by the inhibition of oxidation of ferrous ion to ferric ion through Fenton reaction that causes the production of superoxide anion and hydroxyl radical (Hassani *et al*., 2014).

In addition, Prasad and Muralidhara (2013) have demonstrated the potential of curcumin to ameliorate the cytotoxicity caused by acrylamide in Drosophila model whereby curcumin effectively reduced the mortality rate by mainly increasing the intracellular antioxidant level and reduced the oxidative stress markers. It was also evident from previous studies that curcumin is a potential neuroprotective agent against neurotoxicity caused by different types of neurotoxic agents (beta amyloid, MPTP, rotenone, iodoacetate) in both *in vivo* and *in vitro* models (Park *et al*., 2008; Reyes-Fermin *et al*., 2012; Jayaraj *et al*., 2014; Cui *et al*., 2015).

Apart from that, according to Mufti *et al*. (2015), significant neuroprotective effect was observed upon curcumin post-treatment compared to pre-treatment in H_2O_2-induced SH-SY5Y cells. This study indicates that mode of curcumin treatment also influence the neuroprotective role exerted by curcumin. Findings of the present study also have indicated that curcumin provided a better neuroprotection to the SH-SY5Y cells against toxicity-induced by H_2O_2 compared to acrylamide.

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5.3.3 Evaluation of Neuroprotective Effects of the Combined extract-compound of *C. asiatica* and Curcumin

The improvement in the neuroprotective effects of methanolic and water extracts of *C. asiatica* after combining with curcumin against H_2O_2 or acrylamide-induced toxicity in SH-SY5Y cells was also evaluated in this study. The results showed that combination of methanolic or water extract with curcumin slightly improved the neuroprotective activity compared to the neuroprotection exerted by either the extracts or curcumin individually against H_2O_2-induced toxicity.

The modest additive effect in the neuroprotective activity could be due to the interaction between the active bioactive components of the extracts of *C. asiatica* and the polyphenol (from curcumin) that strengthen each other in this multi-component system. Besides *C. asiatica* major active compound which is triterpene, its polyphenol constituents such as phenolic acids (caffeic acid, gallic acid, vanillic acid), flavones (apigenin, luteolin), flavanone (naringin), flavonols (quercetin, kaempferol) and flavanols (catechin, epicatechin) (Ariffi *et al*., 2011) might be associated with the cooperative effect in combination model of neuroprotective activity against H_2O_2-induced toxicity.

To date, there are no studies have been reported on the neuroprotective action of combination model of extract of *C. asiatica* and curcumin against any types of neurotoxic agents. Jiang *et al*. (2016) reported that combination of asiatic acid with madecassic acid of *C. asiatica* showed significant synergism in promoting neuronal differentiation in rat pheochromocytoma PC-12 cells. Thoo *et al*. (2013) demonstrated that combination of ethanolic extract of *C. asiatica* and α-tocopherol exhibited synergistic antioxidant effects assessed by ABTS radical scavenging, β-carotene bleaching system and liposome peroxidation assays.

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Combination of curcumin with piperine improved motor performance, antioxidant enzyme levels, neurochemicals and reduced neuroinflammation and oxidants levels in quinolonic acid-induced neurodegeneration in rats (Singh & Kumar, 2016). According to Khatri and Juvekar (2018), in rotenone-induced mice, combined curcumin and ellagic acid markedly enhanced locomotor activity, mitochondrial enzyme complex activities and decreased oxidative damage.

Besides that, studies have been also reported on the neuroprotective activity of combination of other extracts of plants and phytochemicals (Mu *et al*., 2007; Lee *et al*., 2012; Liu *et al*., 2016; Sutalangka & Wattanathorn, 2017). A study conducted by Ramachandran *et al*. (2014) showed that combination of *Bacopa monnieri* extract with *Rosmarinus officinalis* antioxidant extract exhibited synergestic effects in antioxidant activity, anti-lipid peroxidation activity, and inhibition of amyloid precursor protein while no improvement was observed in inhibition of formation of phosphorylated tau protein in human glial and embryonic mouse hypothalamus cell lines. Combination of quercetin and resveratrol improved the antioxidant activity and reduction of anxiety in *in vivo* model. The synergism was due to the interaction between the combined polyphenols which enhanced the therapeutics properties and increased the bioavaibility of these compounds (Eddine *et al*., 2015).

Meanwhile, no additive effects were observed in the neuroprotective activity exhibited by the combined methanolic and water extracts of *C. asiatica* and curcumin in pre-treatment model against acrylamide caused toxicity in SH-SY5Y cells. However, the extracts and curcumin exhibited neuroprotective activity when treated individually. This can be due to the reason that in the extract-curcumin combination, the constituents present in the extracts of *C. asiatica* and polyphenol (from curcumin) may react antagonistically in the inhibition of neurotoxicity caused by acrylamide. Generally,

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factors such as types of compounds present and their concentrations and ratios at which they are mixed influence the multi-component system (Thoo *et al*., 2013).

Study conducted by Bhatnagar *et al*. (2017) revealed that no synergistic effect in behavioural and oxidative stress recovery in MPTP-induced Parkinson's model of mice was observed when *C. asiatica* was combined with *Withania somnifera* compared to individual administration of those two extracts. Another study carried out by Ruzicka *et al*. (2018) showed that combination of curcumin and epigallocatechin gallate did not exert any synergistic neuroprotective effect against spinal cord injury in rat model. Yin *et al*. (2014) indicated that drug combination may cause the drugs to interact in various unpredictable ways and demonstrate varieties of outcomes. Thus, in this work combination of methanolic or water extracts of *C. asiatica* with curcumin improved the neuroprotective activity against H_2O_2-induced toxicity while no improvement was observed against acrylamide-induced toxicity in SH-SY5Y cells.

5.4 Assessment of Antioxidant Activity

5.4.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that can be reduced into stable diamagnetic molecule, diphenylpicrylhydrazine when it accepts hydrogen atom from an antioxidant compound. The scavenging activity is indicated by the changes in the colour from purple to light yellow measured at absorbance of 517 nm. It is one of the most commonly used methods for evaluation of the antioxidant activity of extracts of plants and vegetables, beverages and foods (Fehli *et al*., 2017). This method allows an easy, rapid, sensitive, reproducible and convenient way to screen the antioxidant activity of compounds (Kaneria *et al*., 2012).

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In this study, the results showed that curcumin and extracts of *C. asiatica* exhibited DPPH scavenging capacity in concentration dependent manner at all the concentration tested with curcumin had a better DPPH radical scavenging activity compared to both methanolic and water extracts of *C. asiatica*. This could be due to the presence of curcumin in its pure form works better as DPPH free radical scavenger compared to the crude extracts of *C. asiatica* which is a mixture of many constituents. This result was similar with a study conducted by El-Beshbishy *et al*. (2009), which demonstrated that curcumin had higher DPPH radical scavenging activity than extracts of seed of *Vitis vinifera* (grape) (GSE) and *Nigella sativa* (blackseed) (NSE).

El-Beshbishy *et al*. (2009) also revealed that the antioxidant activity of a compound is affected by the number and position of phenolic groups in the structure of an antioxidant molecule and the presence of other functional groups. Compared to GSE and NSE, curcumin have two phenolic groups attached in a suitable position that allow two hydroxyl groups of it to scavenge free radical without any inhibition. Tuba and Gulcin (2008) reported that DPPH radicals easily accept an hydrogen atom from the hydroxyl group and this makes curcumin a powerful antioxidant.

Among the extracts of *C. asiatica*, the DPPH radical scavenging activity was higher in water extract compared to methanolic extract. This result was in good agreement with study conducted by Ahmed *et al*. (2015), which demonstrated that phytochemicals soluble in water possess a stronger potential to scavenge DPPH free radicals as the aqueous extract had a higher DPPH scavenging activity than methanolic extract. This finding is probably related to the polarity of the extraction solvents. As water is more polar than methanol, so it is able to extract more phytochemicals such as flavonoids and triterpenes that are responsible for the higher antioxidative action (Ahmed *et al*., 2015).

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In contrast, study conducted by Pittela *et al*. (2009) reported that water extract of *C. asiatica* had a higher DPPH radical scavenging activity with IC_{50} value of 31.25 μg/ml compared with the value obtained in this present study. The differences in DPPH radical scavenging activity in both similar species might be due to differences in the chemical constituents of *C. asiatica* that vary significantly between different locations (Chong & Aziz, 2011).

Overall, the DPPH radical scavenging activity of curcumin and extracts of *C. asiatica* were lower than the standard ascorbic acid. Ascorbic acid was used as positive control in DPPH and ABTS assays as it is a potent antioxidant with high reducing potential (Nenadis *et al*., 2007). Many previous studies have reported the use ascorbic acid as the reference compound in various antioxidant assays (Irshad *et al*., 2012; Kumarappan *et al*., 2012; Borra *et al*., 2013; Kumari *et al*., 2016). Besides ascorbic acid there are also other reference compounds used in antioxidant assays such as gallic acid, trolox, α-tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Nenadis *et al*., 2007; Gulcin *et al*., 2010; Venuprasad *et al*., 2013). The choice of the reference compound generally plays an important role in any antioxidant activity evaluation (Nenadis *et al*., 2007).

5.4.2 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay is another widely used spectrophotometric method for determination of antioxidant activity of compounds which is based on the degree of decolourization due to the presence of antioxidants that reverses the formation of ABTS radical. ABTS is radical cation formed in stable form by the reaction between ABTS and potassium

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persulfate with maximum absorbance at 734 nm. The degree of decolourization of this blue-green radical cation determines the antioxidant potential of a compound. ABTS radical cation is more reactive than DPPH. ABTS assay generally involves the transfer of electron while DPPH assay involves the transfer of hydrogen atom (Tuba & Gulcin, 2008).

From the results obtained, it can be seen that curcumin and extracts of *C. asiatica* demonstrated a dose-dependent ABTS radical scavenging activity at all the concentration examined with curcumin exhibited higher ABTS radical scavenging activity compared to methanolic and water extracts of *C. asiatica*. Tuba and Gulcin (2008) reported a lower IC_{50} value of curcumin of 18.07 µg/ml compared to the reading obtained in this study. This difference might because of the variation in the method used to conduct the ABTS assay and the difference in the source of curcumin.

Antioxidant mechanism of curcumin was described by the density functional theory which include five different mechanisms that are single electron transfer, radical adduct formation, transfer of hydrogen atom from neutral curcumin and deprotonated curcumin and serial proton loss electron transfer (Mosovska *et al*., 2016). This result also indicated that ABTS radical scavenging activity of water extract was higher than methanolic extract. Study conducted by Thoo *et al*. (2013) reported that ABTS radical scavenging activity of ethanolic extract of *C. asiatica* with nearly similar IC_{50} value of 1936 µg/ml while Arora *et al*. (2018) revealed that methanolic extract of *C. asiatica* had lower IC_{50} value of 50.86 µg/ml in ABTS assay. Overall, the ABTS radical scavenging activity of curcumin and extracts of *C. asiatica* were lower than the standard ascorbic acid. In addition, ABTS radical scavenging potential of curcumin and both extracts were lower than DPPH radical. This can be related to stereoselectivity of the radicals or the solubility of the extracts in different system (Adedapo *et al*., 2008).

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5.4.3 Iron Chelating Activity

Ferrozine is one of the examples of chelator that can form complexes with iron(II) ion (Fe^{2+}) and produces red coloured product that can be measured spectrophotometrically at 562 nm. In the presence of other chelator (antioxidant compounds) there will be reduction in the formation of the red coloured complex. Thus, the measurement of colour reduction will estimate the iron chelating activity of a compound.

In iron chelating assay, the result revealed that curcumin and methanolic extract of *C. asiatica* did not show any iron chelating activity. The iron chelating activity could not be determined although different ranges of concentration and methods have been tested. However, Tuba and Gulcin (2008) reported that curcumin is an effective iron chelator. Iron is naturally present in the body as it is one of the essential micronutrient and involved in many metabolic activities. When present in excess, iron can cause deleterious effect to the body mainly in the form oxidative stress as excess iron generally induce lipid peroxidation and involve in Fenton reaction to generate of ROS (Badria *et al*., 2015).

The iron chelating activity of curcumin is mainly due to its high Fe^{2+} binding affinity and capacity than ferrozine. The hydroxyl, methoxyl and carbonyl functional groups in curcumin are attributed to the iron chelating activity of curcumin (Tuba & Gulcin, 2008). Besides that, study conducted Mohd Salim *et al*. (2013) showed that methanolic extract of *C. asiatica* demonstrated iron chelating activity with an IC_{50} value of 930 µg/ml under an optimized extraction condition such as extraction temperature, agitation speed, extraction time and solvent to solid ratio. This finding suggests that extraction conditions might influence the iron chelation ability of a compound.

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In contrast, water extract of *C. asiatica* demonstrated iron chelating activity in the range of concentration examined. However, the iron chelating activity of water extract was lower than the standard ethylenediaminetetraacetic acid (EDTA). Amid *et al*. (2010) showed that aqueous extract of *C. asiatica* obtained by using conventional soxhlet extraction method exerted good iron chelating activity.

EDTA was used as positive control in this study as many of the iron chelating assays used EDTA as reference compound (Ebrahimzadeh *et al*., 2008; Raghavendra *et al*., 2011; Mohan *et al*., 2012; Sarkar *et al*., 2012). EDTA is a notable chelating agent as it has six potential metal cations binding sites and known to have chelate metal ions such as Fe^{2+}, copper(II) (Cu^{2+}), nickel(II) (Ni^{2+}) and iron(III) (Fe^{3+}) ions (Zaitoun & Lin 1997). The presence of transition metal ion in the body can cause the formation and propagation of many highly reactive free radicals that can cause oxidative damage. Thus, the formation of the radicals can be avoided through the metal chelating (Melo *et al*., 2011).

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CHAPTER 6: CONCLUSION

C. asiatica has a long history and use in traditional medicine due to its versatility and efficacy in treating various types of diseases. Above all, it is particularly well-known as brain tonic due to its memory, learning, intellect and cognitive disorders enhancement properties. Based on these traditional claims, this study evaluated the neuroprotective effects of methanolic and water extracts of *C. asiatica* against neurotoxic agents (H_2O_2 and acrylamide)-induced toxicity in neuroblastoma SH-SY5Y cells. Toxicity induced by these neurotoxic agents is claimed to be associated with oxidative stress and oxidative stress is one of the major known cause of neurodegenerative diseases. The findings of this study indicated that both methanolic and water extracts of *C. asiatica* exhibited a mild neuroprotective activity against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells. Among these extracts of *C. asiatica*, methanolic extract notably protected SH-SY5Y cells against toxicity-induced by both H_2O_2 and acrylamide compared to water extract. Furthermore, both extracts of *C. asiatica* exerted a better neuroprotection against toxicity-induced by H_2O_2 than acrylamide.

In this present study, the improvement in the neuroprotective effects of extracts of *C. asiatica* against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells after combining with curcumin was also evaluated. The result revealed that combination of both extracts with curcumin showed a slight amelioration in the neuroprotection against the H_2O_2-induced toxicity in the cells while no improvement was observed in the combination model against acrylamide-induced toxicity. This finding suggests that types of neurotoxic agents might influence the combined neuroprotective effect of extracts of *C. asiatica* and curcumin. In addition, the investigation of the neuroprotective potential of combination of *C. asiatica* with curcumin (combined

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effects of herb and phytochemical) against two different types of neurotoxic agents in an *in vitro* model would contribute new knowledge to the literature as most of the previous scientific studies evaluated the neuroprotective activity of individual extracts or bioactive compounds.

The antioxidant activity of methanolic and water extracts of *C. asiatica* and curcumin was evaluated using *in vitro* antioxidant assays such as DPPH and ABTS radical scavenging and iron chelating assays. Curcumin possessed a good DPPH and ABTS radical scavenging activity compared to the extracts. Among the extracts of *C. asiatica*, water extract demonstrated a better antioxidant activity than methanolic extract.

Overall, findings from this study revealed that the mild neuroprotective effects of extracts of *C. asiatica* against H_2O_2 and acrylamide-induced toxicity in SH-SY5Y cells might be attributed to the moderate antioxidant activity of the extracts of the plant. It is also evident from this study that the neuroprotective action of the extracts and curcumin might not only target the oxidative stress as the toxicity caused by the neurotoxic agents could be probably related to other consequences besides oxidative stress. Therefore, *C. asiatica* could be a potential dietary supplement or functional food which could help to reduce the toxicity effects of neurotoxic agents and thus minimizes the risk of neurodegenerative diseases.

Upon completion of the study, several recommendations were formulated in order to improve the current work in future. First, several methods involved in this study such as extraction method (parameters of extraction process) and neuroprotective assay [extracts pre-treatment duration, mode of extracts treatment (co-treatment or post-treatment), and method of cell viability analysis] need to be optimized. The quantitative and qualitative phytochemical analysis should be carried out in order to determine the different types and amount of phytochemicals present in the extracts of *C. asiatica*.

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Third, the mechanism of neuroprotection should be identified through assays such as determination of intracellular ROS and antioxidants level, lipid peroxidation, mitochondrial membrane potential, acetylcholinesterase and butyrylcholinesterase inhibitory and apoptosis assay so that the neuroprotective action of the extracts can be further validated. Besides that, for the combination study, different combination concentrations and ratios of extracts of *C. asiatica* and curcumin can be further evaluated. Lastly, the future study should also focus on the neuroprotective effects of extracts of *C. asiatica* against the neurotoxic agents in *in vivo* model.

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APPENDIX A- CELL CULTURE TECHNIQUES

1) Preparation of Growth Media

First basic DMEM/F12 media was prepared by adding 12 g of DMEM/F12, 2.44 g of sodium bicarbonate ($NaHCO_3$) and 0.5206 g of Hepes into sterile distilled water at a certain volume and the solution was stirred until completely dissolved. The pH of the solution was adjusted to approximately 7.0 and the media was brought to final volume of 1 liter using sterile distilled water. The basic media was sterilized by filtration using a syringe filter with pore size of 0.22 μm. Finally, the growth media was prepared by supplementing the basic media with 10% FBS and followed by filtration and refrigeration.

2) Preparation of Cryoprotectant Media

The freezing media contained basic DMEM/F12 media, FBS and DMSO in a ratio of 4.5:5:0.5. It was filtered and refrigerated until further use.

3) Procedure of Handling Frozen Cells

a. The vials were quickly thawed in water bath.
b. The cell suspension was transferred into centrifuge tube containing 1 ml of 20% media and centrifuged at 100 × g for 5 minutes.
c. The supernatant was discarded and 1 ml of 20% media was added to the cell pellet and mixed gently.
d. The cell suspension was transferred into the cell culture flask containing 5 ml of 20% media and incubated in humidified incubator containing 5% of CO_2.

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4) Procedure of Subculture

a. Once the cells reached confluency, the cells were first checked for any contamination using phase contrast inverted microscope and then the spent media was discarded from the culture flask.

b. The cells were rinsed with 10 ml of PBS and were detached by using accutase (1 ml) solution.

c. The culture flask was incubated for 10 minutes and then the detachments of the cells were checked under the microscope. The bottom of the culture flask was tapped gently to increase the detachments of the cells from the flask.

d. Then, the cells were harvested by centrifugation at 100 × g for 5 minutes. The supernatant was discarded and the required amount of 10% media was added to the cell pellet and mixed gently.

e. Equal amount of the cell suspension or a specfic seeding density counted by trypan blue (0.4% w/v) exclusion method using a hemocytometer was transferred into the culture flasks containing 5 ml of 10% media and incubated.

5) Procedure of Cell Counting

a) The cells were detached from the flasks and harvested by centrifugation at 100 × g for 5 minutes.

b) The supernatant was discarded and 1 ml of 10% media was added to the cell pellet and mixed gently.

c) 100 µl of cell suspension was added into microcentrifuge tube containing 900 µl of trypan blue (1:10 dilution) and the mixture was mixed gently.

d) The hemocytometer and the cover slip were first cleaned with alcohol and the cover slip was placed on top of the hemocytometer.

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e) One side of the hemocytometer chamber was carefully filled with the tryphan blue-cell suspension (approximately 20 µl) at the edge of cover slip.

f) The hemocytometer was examined under microscope and number of cells in five squares (four corners and one middle square) was counted with proper counting techniques. Dead cells were stained blue while live cells were unstained (colourless).

g) The number of viable cells per ml in the original suspension was calculated using the formula below:

Number of viable cells per ml = $N \times 10^4 \times D$

N = average number of viable cells in five squares

10^4 = conversion factor

D = dilution factor, 10

h) The density of cells to be plated in the 96-well plate was calculated using the formula below:

$M_1 V_1 = M_2 V_2$

M_1 = original concentration of cells

M_2 = concentration of cells to be plated

V_1 = volume of cell suspension needed

V_2 = total volume needed for plating

Lets assume the $M_1 = 6 \times 10^6$ cells/ml

$M_2 = 3 \times 10^4$ cells/ml

V_2 = 10 ml

$(6 \times 10^6$ cells/ml) $(V_1) = (3 \times 10^4$ cells/ml) (10 ml)

V_1 = 0.05 ml

Therefore, 50 µl of cell suspension was added into 9950 µl of 10% media for plating.

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6) Procedure of Cryopreservation

a. The cells were given fresh 10% media for 24–48 hours before the cryopreservation.

b. The cells were first checked for any contamination and detached from the flask.

c. After centrifugation the supernatant was discarded and the cell pellet was suspended in freezing media.

d. The equal volume (1 ml) of cell suspension was transferred into the vials.

e. The vials were placed into a box containing liquid nitrogen overnight in the -80˚C freezer.

f. Then, the vials were transferred into the cryo canister in the liquid nitrogen tank.

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APPENDIX B- PREPARATION OF REAGENTS, SOLUTIONS AND TEST AGENTS

For cell culture,

1) Preparation of phosphate-buffered saline (PBS)

PBS was prepared by adding 0.76 g of sodium dihydrogen phosphate (NaH_2PO_4), 0.29 g of potassium dihydrogen phosphate (KH_2PO_4) and 4.25 g of sodium chloride (NaCl), 2.44 g of sodium bicarbonate ($NaHCO_3$) into distilled water at a certain volume and the solution was stirred until completely dissolved. The pH of the solution was adjusted to approximately 7.4 and the solution was brought to final volume of 500 ml using distilled water. The solution was filtered and autoclave at 121˚C, 15 psi for 15 minutes. PBS was stored at room temperature.

2) Preparation of Trypan Blue

0.4% (w/v) of tryphan blue was prepared by dissolving 200 mg of tryphan blue in 50 ml of sterile distilled water and stored at room temperature.

3) Preparation of Methanolic and Water Extracts

20 mg/ml of stock solutions of both extracts were made by dissolving 20 mg of the extracts in 1 ml of pure DMSO and stored in dark at -20˚C. Dilutions were made using 10% DMSO and the specific test concentrations were made as the final concentration in the well by using the formula $M_1\ V_1 = M_2\ V_2$.

4) Preparation of 1M of Hydrogen peroxide (H_2O_2)

Density = 1.11 g/ml

Original concentration, M_1 = 30% (v/v)

Molar mass = 34.01 g/mol

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First M_1 is converted to molarity

= (1.11 g/ml) (0.3 ml/ml) (1/34.01 mol/g)

= 9.79×10^{-3} mol/ml

= 9.79 M

Using $M_1 V_1 = M_2 V_2$

(9.79M) (V_1) = (1M) (1ml)

V_1 = 0.102 ml

Thus, 1M of H_2O_2 was prepared by dissolving 102 µl of H_2O_2 in 898 µl of basic DMEM/F12 media. The dilutions were made by using basic DMEM/F12 media from the stock 1M of H_2O_2 and the specific test concentrations were made as the final concentration in the well by using the formula $M_1 V_1 = M_2 V_2$.

5) Preparation of 1M of Acrylamide

7.108 g of acrylamide was dissolved in 100 ml of sterile distilled water. (MW: 71.08 g/mol). The dilutions were made by using basic DMEM/F12 media from the stock 1M of acrylamide and the specific test concentrations were made as the final concentration in the well by using the formula $M_1 V_1 = M_2 V_2$.

6) Preparation of 20 mg/ml of Trolox

20 mg of trolox was dissolved in 1 ml of pure DMSO. The dilutions were made using 10% DMSO and the specific test concentrations were made as the final concentration in the well by using the formula $M_1 V_1 = M_2 V_2$.

7) Preparation of MTT

5 mg/ml of MTT was prepared by dissolving 50 mg of MTT in 10 ml of PBS. The solution was filtered and stored in dark at 4˚C.

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For non-cell culture,

1) Preparation of 0.3 mM of DPPH solution

1.183 mg of DPPH was dissolved in 10 ml of methanol. MW: 394.32 g/mol.

2) Preparation of 2 mM of iron (II) chloride ($FeCl_2$) solution

3.976 mg of $FeCl_2$ was dissolved in 10 ml of distilled water. MW: 198.81 g/mol.

3) Preparation of 5 mM of ferrozine solution

24.623 mg of ferrozine was dissolved in 10 ml of distilled water. MW: 492.46 g/mol.

4) Preparation of 2.45 mM of potassium persulfate ($K_2S_2O_8$) solution

6.622 mg of $K_2S_2O_8$ was dissolved in 10 ml of distilled water. MW: 270.32 g/mol.

5) Preparation of 7 mM of ABTS solution

38.408 mg of ABTS was dissolved in 10 ml of distilled water. MW: 548.68 g/mol.

6) Preparation of 10 mg/ml of asorbic acid

10 mg of asorbic acid was dissolved in 10 ml of distilled water.

7) Prepartion of 10 mg/ml of EDTA

10 mg of EDTA was dissolved in 10 ml of distilled water.

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APPENDIX C-DATA OF RESULTS

1) Assessment of Toxicity of DMSO towards SH-SY5Y Cells

Table C1: SH-SY5Y cells viability (%) at different concentrations of DMSO.

Concentration of DMSO (%)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
0.00	100	100	100	0.00	100
0.05	93.85	107.49	116.63	6.62	105.99
0.25	87.67	91.84	114.24	8.25	97.92
0.50	89.31	99.65	103.39	4.21	97.45
1.25	72.90	76.23	87.91	4.55	79.01
2.50	54.40	54.66	63.08	2.85	57.38
5.00	13.14	9.64	13.60	1.25	12.13

2) Assessment of Toxicity of Hydrogen Peroxide (H_2O_2) towards SH-SY5Y Cells

Table C2: SH-SY5Y cells viability (%) at different concentrations of H_2O_2.

Concentration of H_2O_2 (μM)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
0	100	100	100	0.00	100
25	99.68	97.65	96.42	0.95	97.92
50	72.83	87.85	72.85	5.00	77.85
75	63.45	80.77	60.78	6.27	68.34
100	44.42	44.38	43.62	0.26	44.14
200	17.89	23.57	21.47	1.66	20.98
300	12.40	22.00	18.89	2.83	17.76
400	8.71	12.17	17.07	2.43	12.65
500	7.63	12.20	16.98	2.70	12.27

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3) Assessment of Toxicity of Acrylamide towards SH-SY5Y Cells

Table C3: SH-SY5Y cells viability (%) at different concentrations of acrylamide.

Concentration of Acrylamide (mM)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
0	100	100	100	0.00	100
1	99.48	91.37	93.48	2.43	94.77
3	61.71	63.77	56.96	2.02	60.81
5	45.07	42.85	52.88	3.04	46.93
7	24.35	24.93	34.10	3.16	27.79
10	18.06	16.72	32.96	5.20	22.58
20	16.62	12.35	27.75	4.59	18.91

4) Assessment of Neurotoxicity Effects of Extracts of *C. asiatica* towards SH-SY5Y Cells

Table C4: SH-SY5Y cells viability (%) at different concentrations of methanolic extract of *C. asiatica.*

Concentration of Methanolic Extract (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
0	100	100	100	0.00	100
1	101.55	90.89	106.40	1.51	103.41
10	126.21	96.58	105.47	6.07	114.75
25	99.73	102.31	99.53	2.48	102.11
50	95.34	107.08	97.53	2.06	98.39
75	94.97	112.57	90.84	1.71	94.13
100	92.21	102.27	93.85	0.86	92.31

Table C5: SH-SY5Y cells viability (%) at different concentrations of water extract of *C. asiatica.*

Concentration of Water Extract (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
0	100	100	100	0.00	100
1	100.16	101.03	103.68	1.06	101.63
10	107.10	111.56	109.45	1.29	109.37
25	114.46	109.39	107.67	2.04	110.51
50	106.08	110.24	108.60	1.21	108.31
75	105.79	103.87	114.87	3.39	108.18
100	105.33	107.05	112.76	2.25	108.38

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5) Evaluation of Neuroprotective Effects of Extracts of *C. asiatica* towards H_2O_2-Induced Toxicity in SH-SY5Y Cells.

Table C6: Cell viability (%) at different concentrations of methanolic extract in H_2O_2-induced toxicity in SH-SY5Y cells.

Concentration of Methanolic Extract (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0	41.43	39.25	59.94	6.56	46.87
1	43.68	54.49	62.02	5.32	53.40
10	47.14	46.76	66.80	6.62	53.57
25	51.30	45.30	69.64	7.32	55.42
50	53.61	56.36	70.01	5.07	59.99
75	48.25	59.03	75.53	7.93	60.94
100	41.62	48.47	57.63	4.64	49.24

Table C7: Cell viability (%) at different concentrations of water extract in H_2O_2-induced toxicity in SH-SY5Y cells.

Concentration of Water Extract (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0	37.80	44.38	40.01	1.93	40.73
1	44.63	60.19	37.90	6.60	47.57
10	46.46	52.84	46.88	2.06	48.73
25	41.51	53.83	42.24	3.99	45.86
50	39.44	45.83	41.60	1.88	42.29
75	36.40	41.55	45.00	2.50	40.98
100	39.34	39.40	29.02	3.45	35.92

Table C8: Viability (%) of SH-SY5Y cells pre-treated with trolox in H_2O_2-induced toxicity.

Concentration of Trolox (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0	41.43	39.25	59.94	6.56	46.87
25	71.42	82.23	74.02	3.26	75.89

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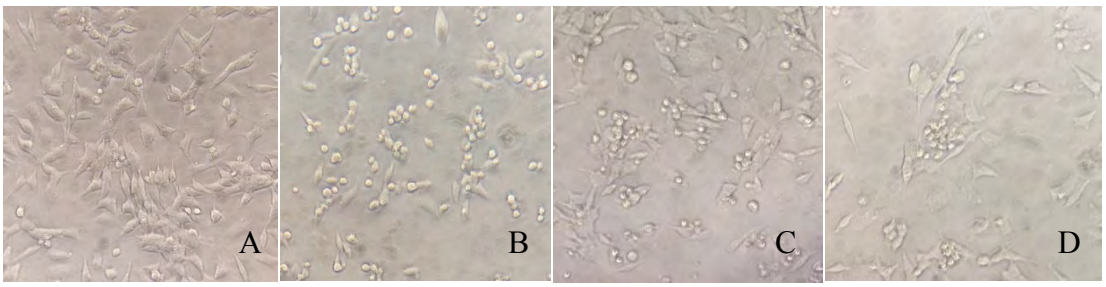

Figure C1: Neuroprotective effects of methanolic and water extract of *C. asiatica* on H_2O_2-induced toxicity in SH-SY5Y cells. A: Untreated SH-SY5Y cells; B: SH-SY5Y cells treated with 100 µM of H_2O_2; C: SH-SY5Y cells pre-treated with 75 µg/ml of methanolic extract and followed by the toxicity-induced by 100 µM of H_2O_2; D: SH-SY5Y cells pre-treated with 10 µg/ml of water extract and followed by the toxicity-induced by 100 µM of H_2O_2.

6) Evaluation of Neuroprotective Effects of Extracts of *C. asiatica* towards Acrylamide-Induced Toxicity in SH-SY5Y Cells.

Table C9: Cell viability (%) at different concentrations of methanolic extract in acrylamide-induced toxicity in SH-SY5Y cells.

Concentration of Methanolic Extract (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0	41.81	33.21	53.98	6.02	43.00
1	37.86	37.44	54.04	5.46	43.11
10	44.65	38.19	54.95	4.88	45.93
25	42.17	37.36	55.60	5.46	45.04
50	43.55	41.54	55.65	4.41	46.91
75	44.26	42.17	59.60	5.49	48.68
100	47.17	42.71	52.72	2.90	47.53

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Table C10: Cell viability (%) at different concentrations of water extract in acrylamide-induced toxicity in SH-SY5Y cells.

Concentration of Water Extract (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0	39.90	46.51	59.97	5.90	48.79
1	40.18	48.65	63.73	6.89	50.85
10	42.01	47.25	62.70	6.21	50.65
25	43.45	49.99	62.50	5.59	51.98
50	41.52	46.06	60.14	5.60	49.24
75	41.41	45.09	49.67	2.39	45.39
100	40.12	45.23	47.42	2.16	44.26

Table C11: Viability (%) of SH-SY5Y cells pre-treated with trolox in acrylamide-induced toxicity.

Concentration of Trolox (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0	41.81	33.21	53.98	6.02	43.00
25	62.14	64.59	71.14	2.69	65.96

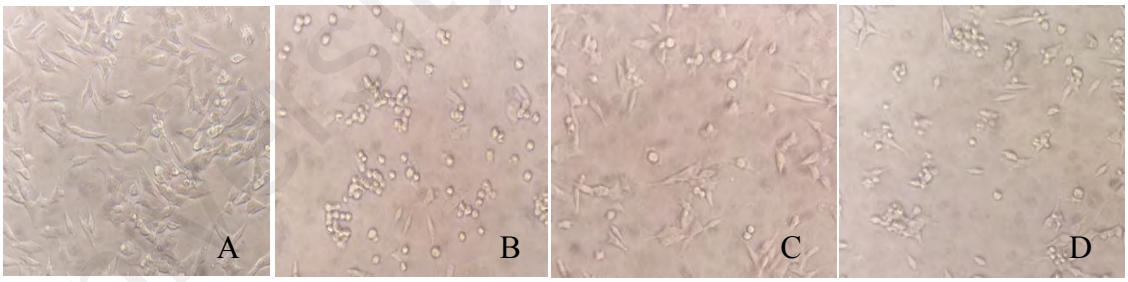

Figure C2: Neuroprotective effects of methanolic and water extract of *C. asiatica* on acrylamide-induced toxicity in SH-SY5Y cells. A: Untreated SH-SY5Y cells; B: SH-SY5Y cells treated with 5 mM of acrylamide; C: SH-SY5Y cells pre-treated with 75 µg/ml of methanolic extract and followed by the toxicity-induced by 5 mM of acrylamide; D: SH-SY5Y cells pre-treated with 25 µg/ml of water extract and followed by the toxicity-induced by 5 mM of acrylamide.

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7) Assessment of Neurotoxicity Effect of Curcumin towards SH-SY5Y Cells

Table C12: SH-SY5Y cells viability (%) at different concentrations of curcumin.

Concentration of Curcumin (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
0.00	100	100	100	0.00	100
0.01	90.00	81.78	94.68	3.77	88.82
0.10	100.33	84.00	82.74	5.67	89.02
0.25	95.70	90.65	91.20	1.60	92.52
0.50	100.44	82.58	96.52	5.42	93.18
0.75	103.95	80.09	88.65	6.98	90.90
1.00	90.85	87.05	88.25	1.12	88.71

8) Evaluation of Neuroprotective Effect of Curcumin towards H_2O_2-Induced Toxicity in SH-SY5Y Cells

Table C13: Cell viability (%) at different concentration of curcumin in H_2O_2-induced toxicity in SH-SY5Y cells.

Concentration of Curcumin (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0.00	59.88	55.97	61.60	1.67	59.15
0.01	66.04	59.14	80.15	6.18	68.44
0.10	71.63	69.47	89.52	6.35	76.87
0.25	80.46	74.59	82.94	2.48	79.33
0.50	70.27	65.77	83.77	5.41	73.27
0.75	66.33	61.50	78.97	5.21	68.94
1.00	61.39	56.67	72.88	4.81	63.65

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9) Evaluation of Neuroprotective Effect of Curcumin towards Acrylamide-Induced Toxicity in SH-SY5Y Cells

Table C14: Cell viability (%) at different concentration of curcumin in acrylamide-induced toxicity in SH-SY5Y cells.

Concentration of Curcumin (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
	1	2	3		
Control	100	100	100	0.00	100
0.00	61.05	59.73	53.82	2.22	58.20
0.01	65.09	62.42	61.15	1.16	62.88
0.10	65.22	62.62	63.14	0.79	63.66
0.25	67.44	63.84	62.26	1.53	64.51
0.50	70.97	66.51	57.93	3.82	65.14
0.75	68.08	64.30	57.78	3.01	63.39
1.00	60.67	57.89	57.40	1.02	58.65

10) Evaluation of Neuroprotective Effects of the Combined Methanolic Extract of *C. asiatica* and Curcumin towards H_2O_2-Induced Toxicity in SH-SY5Y Cells

Table C15: Viability (%) of SH-SY5Y cells pre-treated with methanolic extract, curcumin and methanolic extract-curcumin combination in H_2O_2-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
		1	2	3		
Control	NA	100	100	100	0.00	100
0	NA	55.28	51.35	60.79	2.74	55.80
Methanolic Extract	75	64.65	59.33	70.60	3.26	64.86
Curcumin	0.25	65.14	57.28	75.60	5.31	66.00
Methanolic Extract-Curcumin Combination	75+0.25	70.76	56.79	76.08	5.75	67.88

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11) Evaluation of Neuroprotective Effects of the Combined Water Extract of *C. asiatica* and Curcumin towards H_2O_2-Induced Toxicity in SH-SY5Y Cells

Table C16: Viability (%) of SH-SY5Y cells pre-treated with water extract, curcumin and water extract-curcumin combination in H_2O_2-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
		1	2	3		
Control	NA	100	100	100	0.00	100
0	NA	47.17	56.11	51.50	2.58	51.59
Water Extract	10	49.98	61.98	61.43	3.91	57.80
Curcumin	0.25	50.20	69.01	68.12	6.13	62.44
Water Extract-Curcumin Combination	10+0.25	50.43	63.25	74.83	7.05	62.83

12) Evaluation of Neuroprotective Effects of the Combined Methanolic Extract of *C. asiatica* and Curcumin towards Acrylamide-Induced Toxicity in SH-SY5Y Cells

Table C17: Viability (%) of SH-SY5Y cells pre-treated with methanolic extract, curcumin and methanolic extract-curcumin combination in acrylamide-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
		1	2	3		
Control	NA	100	100	100	0.00	100
0	NA	40.19	65.34	48.43	7.40	51.32
Methanolic Extract	75	46.32	68.66	62.60	6.67	59.19
Curcumin	0.50	42.07	73.61	65.60	9.47	60.43
Methanolic Extract-Curcumin Combination	75+0.50	38.01	67.86	50.36	8.66	52.08

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13) Evaluation of Neuroprotective Effects of the Combined Water Extract of *C. asiatica* and Curcumin towards Acrylamide-Induced Toxicity in SH-SY5Y Cells

Table C18: Viability (%) of SH-SY5Y cells pre-treated with water extract, curcumin and water extract-curcumin combination in acrylamide-induced toxicity.

Test Agents	Concentrations (µg/ml)	Cell Viability (%) Replicates			Standard Error	Average Cell Viability (%)
		1	2	3		
Control	NA	100	100	100	0.00	100
0	NA	49.32	60.20	39.87	5.87	49.80
Water Extract	25	52.15	62.61	41.06	6.22	51.94
Curcumin	0.50	54.87	63.34	46.03	5.00	54.75
Water Extract-Curcumin Combination	25+0.50	38.02	58.35	40.32	6.44	45.54

14) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity of Methanolic Extract

Table C19: DPPH radical scavenging activity of different concentrations of methanolic extract.

Concentration of Methanolic Extract (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
200	9.31	12.14	10.72	0.82	10.72
400	28.10	21.73	22.31	2.03	24.05
600	31.73	30.72	31.32	0.29	31.36
800	41.23	40.95	42.78	0.57	41.66
1000	52.75	52.39	53.47	0.32	52.87
1200	58.27	57.54	57.96	0.21	57.92
1500	75.38	74.15	73.07	0.67	74.20
2000	93.02	93.47	96.16	0.98	94.22

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15) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity of Water Extract

Table C20: DPPH radical scavenging activity of different concentrations of water extract.

Concentration of Water Extract (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
200	6.81	13.50	11.54	1.98	10.62
400	21.24	24.80	23.23	1.03	23.09
600	32.39	35.97	35.01	1.07	34.46
800	44.60	47.18	45.21	0.78	45.66
1000	56.52	58.11	54.93	0.92	56.52
1200	66.56	67.62	65.06	0.74	66.41
1500	85.24	81.87	81.18	1.25	82.76
2000	92.49	92.36	92.23	0.08	92.36

16) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity of Curcumin

Table C21: DPPH radical scavenging activity of different concentrations of curcumin.

Concentration of Curcumin (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
10	15.53	18.30	14.29	1.18	16.04
20	25.15	18.80	21.71	1.83	21.89
40	31.74	35.17	40.96	2.69	35.96
60	45.31	51.24	63.63	5.40	53.39
80	57.82	68.07	77.45	5.67	67.78
100	70.83	73.89	82.69	3.55	75.80

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17) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity of Ascorbic Acid

Table C22: DPPH radical scavenging activity of different concentrations of ascorbic acid.

Concentration of Ascorbic acid (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
5	3.18	4.83	7.40	1.23	5.14
10	8.37	10.25	13.10	1.37	10.57
20	22.89	23.93	26.79	1.17	24.54
30	35.76	36.83	42.05	1.94	38.21
40	50.51	49.24	57.40	2.54	52.38
50	65.99	64.33	71.42	2.14	67.25

18) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity of Methanolic Extract

Table C23: ABTS radical scavenging activity of different concentrations of methanolic extract.

Concentration of Methanolic Extract (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
200	7.36	6.20	6.33	0.37	6.63
400	15.13	15.37	13.02	0.75	14.51
600	24.66	24.41	23.50	0.35	24.19
800	33.07	32.17	33.35	0.35	32.86
1000	41.93	41.49	44.15	0.82	42.52
1200	52.12	49.78	49.39	0.85	50.43
1500	63.34	60.03	62.64	1.01	62.00
2000	83.68	82.80	83.09	0.26	83.19

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19) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity of Water Extract

Table C24: ABTS radical scavenging activity of different concentrations of water extract.

Concentration of Water Extract (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
200	9.84	8.82	7.57	0.66	8.75
400	21.01	18.04	20.33	0.90	19.79
600	29.99	27.46	25.20	1.38	27.55
800	36.02	33.35	34.59	0.77	34.65
1000	48.19	48.73	47.95	0.23	48.29
1200	56.80	56.78	56.46	0.11	56.68
1500	69.26	70.17	68.47	0.49	69.30
2000	82.66	82.06	79.55	0.95	81.42

20) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity of Curcumin

Table C25: ABTS radical scavenging activity of different concentrations of curcumin.

Concentration of Curcumin (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
10	6.15	6.75	8.08	0.57	6.99
20	15.11	15.01	14.69	0.13	14.94
40	27.33	28.88	27.51	0.49	27.91
60	42.14	43.62	41.43	0.65	42.39
80	55.21	54.64	54.76	0.17	54.87
100	64.68	63.70	59.79	1.49	62.72
120	67.86	69.60	69.00	0.51	68.82
150	82.59	78.69	82.32	1.26	81.20

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21) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity of Ascorbic Acid

Table C26: ABTS radical scavenging activity of different concentrations of ascorbic acid.

Concentration of Ascorbic acid (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
5	6.55	5.44	5.64	0.34	5.88
10	12.13	11.22	12.65	0.42	12.00
20	24.40	23.88	26.19	0.70	24.83
30	37.00	36.94	39.87	0.97	37.94
40	49.61	49.17	53.51	1.38	50.76
50	61.27	60.59	65.26	1.46	62.37

22) Iron Chelating Activity of Water Extract

Table C27: Iron chelating activity of different concentrations of water extract.

Concentration of Water Extract (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
500	13.13	14.23	12.87	0.42	13.41
1000	24.15	26.24	24.29	0.67	24.90
1500	36.06	32.78	33.60	0.99	34.15
2500	49.70	50.38	49.71	0.23	49.93
3500	60.89	60.38	59.61	0.37	60.30
4500	65.84	65.42	66.88	0.43	66.05

23) Iron Chelating Activity of EDTA

Table C28: Iron chelating activity of different concentrations of EDTA.

Concentration of EDTA (µg/ml)	% of Inhibition Replicates			Standard Error	Average % of Inhibition
	1	2	3		
0	0.00	0.00	0.00	0.00	0.00
10	19.08	13.01	15.57	1.76	15.88
20	34.61	33.89	32.11	0.75	33.54
30	42.98	45.52	45.25	0.81	44.58
40	56.23	59.24	55.95	1.05	57.14
50	66.78	68.86	72.79	1.76	69.48
60	77.21	80.80	79.83	1.07	79.28

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24) Extraction Yield

Table C29: Extraction yield of methanolic and water extracts.

Types of Solvent	Weight Before Extraction (g)	Weight After Extraction (g)	Extraction Yield (%)
Methanol	40	4.668	11.67
Water	20	0.346	1.73

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