NEUROPROTECTIVE EFFECT OF SELECTED HERBS ON H_2O_2-INDUCED NEUROBLASTOMA CELL DAMAGE IN SH-SY5Y

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NEUROPROTECTIVE EFFECT OF SELECTED HERBS ON H_2O_2-INDUCED NEUROBLASTOMA CELL DAMAGE IN SH-SY5Y

ABSTRACT

Neurodegenerative diseases occur as a result of the chronic breakdown and deterioration of neurons. Oxidative stress resulting from free radical formation by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is known to be the main causative factor in the initiation of neurodegeneration. A number of current therapeutic approaches lead to the upsurge of undesirable side effects. Hence, a great deal of attention has been focused on the use of natural antioxidant from plants to protect neuronal cells from oxidative damage or death which can lead to a number of neurodegenerative diseases. The present work was designed to establish the neuroprotective effect of selected herbs; *Piper betle* L. and *Clitoria ternatea* L., on hydrogen peroxide (H_2O_2) -induced neuronal cell damage on human neuroblastoma SH-SY5Y cells. The leaves of *P. betle* and *C. ternatea* were subjected to extraction method using methanol and water to yield methanolic and water extract. In prior to the neuroprotective assay, toxicity test on dimethyl sulfoxide (DMSO) as solvent, H_2O_2 as oxidative stress agent and, the plant extracts were analysed. The results were presented as percentage of cell viability which was assessed using MTT assay after each of the experiment. Total phenolic (TPC) and flavonoid contents (TFC) of the herbal extracts were quantified by colorimetric assay. Antioxidant capacity of the herbal extracts were assessed through 1, 1-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2‟-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] free radical scavenging assay. Relationship between neuroprotective activity, TPC, TFC, DPPH and ABTS scavenging activity was analysed through correlation coefficient analysis. The neurotoxicity test of the extracts revealed that the extracts of *P. betle* were toxic towards SH-SY5Y cells when the concentration used was >10 µg/mL, meanwhile the extracts of *C. ternatea* were not toxic to the cells at the range of concentration tested (1–100 µg/mL). Matching with the toxicity results, *P. betle* extracts show significant neuroprotective activity at lower concentrations (0.001–10 µg/mL). *C. ternatea* extracts also show good neuroprotective activity at the respective range of concentration tested (1–100 µg/mL). The present work also found that the methanolic extract of *P. betle* show better neuroprotective effect in pre-treatment group and its water extract in post-treatment

group, suggesting that this plant might be suitable for prevention and treatment respectively in neurodegenerative disease management. Meanwhile for *C. ternatea* extracts, the results revealed that this plant might be suitable to be used as prevention in managing neurodegenerative diseases. The extracts of *P. betle* were also shown to record greater TPC, DPPH and ABTS free radical scavenging activity, while the extracts of *C. ternatea* was found to have higher TFC. Furthermore, correlation analysis between neuroprotective activity, TPC, TFC, DPPH and ABTS radical scavenging capacity for the extracts of *P. betle* demonstrated negative correlations, meanwhile *C. ternatea* provided positive correlations. Correlation analysis between TPC, TFC, DPPH and ABTS showed positive correlation. In conclusion, current findings suggest that *P. betle* and *C. ternatea* possess good phenolic, flavonoid and antioxidant capacity, and ameliorated H_2O_2-induced neuronal cell death in dose-dependent manner. Therefore, these herbs have potential for development as neuroprotective agent to be used as alternative or complementary therapy in neurodegenerative diseases management.

Keywords: oxidative stress, neuroprotective, phenolic, flavonoid, antioxidant capacity

KESAN NEUROPROTEKTIF HERBA YANG TERPILIH TERHADAP SEL NEUROBLASTOMA SH-SY5Y YANG TELAH DIARUHROSAKKAN OLEH H_2O_2

ABSTRAK

Penyakit neurodegeneratif terhasil akibat kerosakan dan kemerosotan kronik yang dialami oleh sel neuron. Tekanan oksidatif kesan daripada pembentukan radikal bebas oleh spesies oksigen reaktif (ROS) dan spesies nitrogen reaktif (RNS) telah diketahui sebagai faktor penyebab utama permulaan neurodegenerasi. Beberapa pilihan pendekatan terapeutik semasa membawa kepada kemunculan kesan sampingan yang tidak dingini. Oleh itu, perhatian hebat telah tertumpu pada penggunaan antioksida semulajadi daripada tumbuh-tumbuhan untuk mengelakkan atau untuk melindungi sel neuron daripada kerosakan oksidatif atau kematian di mana boleh membawa kepada beberapa penyakit neurodegeneratif. Kajian ini telah direkabentuk untuk menentukan aktiviti neuroprotektif herba yang terpilih; *Piper betle* L. dan *Clitoria ternatea* L., terhadap sel SH-SY5Y neuroblastoma manusia yang telah diaruhrosakkan oleh hidrogen peroksida (H_2O_2). Bahagian daun *P. betle* dan *C. ternatea* telah menjalani kaedah pengekstrakan dengan menggunakan metanol dan air untuk menghasilkan ekstrak metanolik dan air. Sebelum ujian neuroprotektif dijalankan, ujian ketoksikan dimetil sulfoksida (DMSO) sebagai pelarut dan H_2O_2 sebagai agen tekanan oksidatif telah dianalisis. Keputusan telah dibentangkan dalam bentuk peratus daya maju sel yang telah dinilai melalui ujian MTT selepas setiap eksperimen. Jumlah kandungan fenolik (TPC) dan flavonoid (TFC) bagi ekstrak-ekstrak telah ditentukan kuantitinya menggunakan kaedah kolorimetrik. Kapasiyi antioksida bagi ekstrak-ekstrak herba telah dinilai melalui ujian penyingkiran radikal bebas 1, 1-diphenyl-1-picrylhydrazyl (DPPH) dan 2, 2‟-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS]. Hubungan antara aktiviti neuroprotektif, TPC, TFC, ujian penyingkiran radikal bebas DPPH dan ABTS telah diuji melalui analisis pekali korelasi. Ujian neurotoksik ke atas ekstrak mendedahkan kedua-dua herba berkelakuan dalam cara yang bergantung kepada kepekatan. Ekstrak metanol dan air bagi *P. betle* telah ditemui toksik terhadap sel SH-SY5Y apabila kepekatan yang digunakan adalah >10µg/mL. Kedua-kedua ekstrak methanol dan air bagi *C. ternatea* adalah tidak toksik pada julat kepekatan yang telah diuji (1–100 µg/mL). Seiring dengan keputusan ujian ketoksikan, ekstrak *P. betle* menunjukkan aktiviti neuroprotektif ketara pada konsentrasi yang lebih rendah (0.001–

10 µg/mL). Ekstrak *C. trenatea* juga menunjukkan aktiviti neuroprotektif yang bagus pada julat kepekatan yang telah diuji (1–100 µg/mL). Kajian ini juga telah menemui bahawa ekstrak metanolik *P. betle* menunjukkan kesan neuroprotektif yang lebih baik dalam kumpulan rawatan sebelum dan ektrak air dalam rawatan selepas, mencadangkan bahawa tumbuhan ini berkemungkinan lebih sesuai untuk pencegahan dan rawatan masing-masing dalam pengurusan penyakit neurodegeneratif. Manakala untuk ekstrak *C. ternatea*, keputusan mendedahkan bahawa tumbuhan ini berkemungkinan sesuai digunakan untuk pencegahan dalam menguruskan penyakit neurodegeneratif. Ekstrak *P. betle* juga menunjukkan nilai yang lebih tinggi untuk TPC, ujian penyingkiran radikal bebas DPPH dan ABTS, manakala *C. ternatea* menunjukkan nilai yang lebih tinggi untuk TFC. Tambahan lagi, analisis korelasi di antara activiti neuroprotektif, TPC, TFC, kapasiti penyingkiran radikal bebas DPPH dan ABTS untuk ekstrak-ekstrak *P. betle* menunjukkan korelasi negatif, manakala *C. ternatea* menunjukkan korelasi positif. Analisis korelasi di antara TPC, TFC, DPPH dan ABTS menunjukkan korelasi positif. Kesimpulannya, penemuan ini mencadangkan bahawa *P. betle* dan *C. ternatea* mempunyai kandungan fenolik dan flavonoid, dan kapasiti antioksida yang baik serta mengatasi sel neuron yang telah diaruhrosakkan oleh H_2O_2 bergantung kepada dos kepekatan. Oleh itu, herba-herba ini mempunyai potensi untuk penubuhan sebagai agen neuroprotektif untuk digunakan sebagai terapi alernatif atau pelengkap dalam pengurusan penyakit neurodegeneratif.

Kata kunci: tekanan oksidatif, neuroprotektif, fenolik, flavonoid, kapasiti antioksida

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Farah Azirah Mohd Yusuf
Univerity of Malaya, 2018

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LIST OF SYMBOLS AND ABBREVIATIONS

List of Symbols;

A	: Absorbance
Aβ	: Amyloid beta
ACh	: Acetylcholine
AChE	: Acetylcholinesterase
$AlCl_3$	: Aluminium chloride
CH_3COOK	: Potassium acetate
GPx	: Glutathione peroxidaxe
H_2O_2	: Hydrogen peroxide
IC	: Inhibitory concentration
$K_2S_2O_8$	: Potassium persulfate
Na_2CO_3	: Sodium carbonate
O^{-2}	: Superoxide anion
OH^-	: Hydroxyl anion
R	: Correlation coefficient

List of Abbreviations;

APP	: Amyloid beta precursor protein
ABTS	: 2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BHA	: Butylated hydroxyanisole
BHT	: Butylated hydroxytoluene
CAT	: Catalase
CNS	: Central nervous system
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DPPH	: 1, 1-diphenyl-1-picrylhydrazyl
FBS	: Foetal bovine serum
GAE	: Gallic Acid equivalent
MTT	: 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NSAID	: Non-steroidal antiinflammatory drug
PBS	: Phosphate buffered saline
PG	: Propyl gallate
QE	: Quercetin
RNS	: Reactive nitrogen species
ROS	: Reactive oxygen species
SEM	: Standard error mean
SOD	: Superoxide dismutase
TBHQ	: Tert-butylhydroquinone
TFC	: Total flavonoid content
TPC	: Total phenolic content
WHO	: World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Neurodegenerative diseases such as Alzheimer‟s disease, Parkinson‟s disease, and Huntington‟s disease are a major health concern and a leading cause of disability in the aging population worldwide (Pal *et al*., 2015). These disorders are expected to rise dramatically in the next few decades, and the World Health Organization (WHO) estimates that neurodegenerative diseases will transcend cancer by 2040 in industrialized countries (Ip *et al*., 2012; Yacoubian, 2017).

Neurological disorders are referred to as hereditary and sporadic conditions which are attributed as progressive loss and degeneration of structure or function of neurons in sensory, motor and cognitive systems. Despite various advances in the understanding of these diseases, current therapeutic approaches by conventional synthetic medicine have not obtained satisfactory results (Ip *et al.*, 2012). Medications for this group of these disorders are also claimed to be limited, aimed to treat the symptoms only, and caused several undesirable side effects (Yacoubian, 2017).

As an outcome, a great deal of attention has been focused on the use of herbal remedies to protect neuronal cells from damage or death that lead to neurodegenerative diseases and aging-related cognitive decline (Park *et al.*, 2015). Despite being more accessible and affordable, herbal medicine have been used traditionally and always been a favoured source of medicine for improving cognitive function and treating mental disease in decades (Fennel *et al.*, 2004; Ip *et al.*, 2014; Vats, 2014). Researchers identified several major functional groups of phytochemicals that can be obtained from nervine herbs that exert neuroprotective action and also able to regulate varieties of enzymes as well as cell receptors in neuroprotection regulation. Examples of these phytochemicals include polyphenols, isoprenoids, alkaloids, flavonoids and fatty acids,

in which play important roles in maintaining neuronal cell viability, modulating neuronal function and provide protection against neurodegeneration (Phani *et al.*, 2015).

For many years, a large number of modern drugs have been isolated from herbs that are used in traditional medicine (Ghorbani *et al.*, 2006). Studies on medicinal herbs have become more crucial in promoting and enhancing community"s acceptance towards the importance of traditional and indigenous remedies, its therapeutic value, and the integration of derivatives from natural resources in pharmaceutical products (Alsarhan *et al.*, 2014). Moreover, the importance and demand on medicinal herbs has elevated rapidly because of the increasing need to make health care affordable as well as the belief that natural remedies are safe and effective.

Two herbs; *Piper betle* L. and *Clitoria ternatea* L., were selected in this present study due to their known ethno medicinal importance in the management of neurological disorders since time immemorial (Norfaizatul *et al.*, 2010; Lijon *et al.*, 2017). The neuroprotective effect of these herbs will be assessed on H_2O_2-induced damage SH-SY5Y neuroblastoma cells, a well known *in vitro* model used in neuropharmacological studies (Ruffels *et al.*, 2004). Eventhough reports on the neuroprotective effect of *P. betle* and *C. ternatea* on induced damage SH-SY5Y cell line is barely available, a number of researchers documented significant benefits of these herbs in managing neurological disorders since ancient times. *P. betle* has not only been known for its traditional and routine use on the social, cultural and religious occasions, but this herb has been well utilized in enhancing memory and learning function by many traditional medicine system (Vyawahare & Bodhankar, 2007; Roy & Vijayalaxmi, 2013). Meanwhile *C. ternatea* has been traditionally used as brain tonic, sedative agent and antidepressant in decades (Lijon *et al.*, 2017).

In vitro tests using animal cell culture has been a popular method in current neuropharmacological studies to investigate such neuroprotective potential of herbal

medicine that can scavenge free radicals and protect cells from oxidative damage. Human neuroblastoma SH-SY5Y cells, specifically, were reported to be commonly used as an *in vitro* model in neuroscience research including neuroprotective assessment (Park *et al.*, 2015). In this study, exogenous H_2O_2 treatment was used to induce toxicity or cell damage in SH-SY5Y cell line and the neuroprotective capability of the herbs were observed through the analysis of percentage of cell viability. H_2O_2-induced cytotoxicity has been claimed as one of the most common methods employed for the measurement of potential neuroprotective agents (Chow *et al.*, 2005; Garcia-Alonso *et al.*, 2006).

Besides neuroprotective analysis, this present study also determined the phytochemical contents and antioxidant capacity of the herbs, in which, could be responsible for the neuroprotection mechanism. The interrelation between the variables was then evaluated through a correlation coefficient analysis. Thus, it is believed that the findings of this study could provide useful information on the medicinal importance of *P. betle* and *C. ternatea* as they might have a potential to be used in alternative or complementary therapy in the management of neurodegenerative diseases.

1.2 Research Objectives

1.2.1 General Objective

Therefore, the present study was carried out to determine the neuroprotective effects of selected herbs against hydrogen peroxide (H_2O_2)-induced neuronal cell damage in human neuroblastoma SH-SY5Y cell line.

1.2.2 Specific Objectives

The specific objectives of present study are as follows:

i. to investigate the neuroprotective effect of methanolic and water extracts of *Piper betle* L. and *Clitoria ternatea* L. on H_2O_2-induced cell damage in human neuroblastoma SH-SY5Y cell lines.

ii. to determine the phenolic and flavanoid content of methanolic and water extract of *P. betle* and *C. ternatea*.

iii. to determine the antioxidant activity of methanolic and water extract of *P. betle* and *C. ternatea*; and

iv. to determine the correlation between phenolic and flavonoid content, antioxidant potential and neuroprotective activity of methanolic and water extract of *P. betle* and *C. ternatea*.

CHAPTER 2: LITERATURE REVIEW

2.1 Neurodegenerative Diseases

Neurodegenerative diseases are progressive disorders which result from a chronic breakdown and deterioration of neurons, particularly those of the central nervous system (CNS). Gradual and progressive loss of these neurons may leads to the accumulation of proteins which cause dysfunction on the central and peripheral nervous systems (Adewusi, 2012; Kumar *et al.,* 2015).

National Institute of Neurological Disorders and Stroke has reported more than 600 neurological disorders across the globe (Meek *et al.,* 1998). Some of the common forms are Alzheimer‟s disease, Parkinson‟s disease, multiple sclerosis, amyotrophic lateral sclerosis and spongiform encephalopathy (Chiba *et al.,* 2007). These diseases are usually found in elderly people as they are characterized by a gradual onset of progressive symptoms including loss of memory, difficulty in learning or retaining information, inability to handle complex tasks, impaired spatial orientation and abilities, language deficits and behavioural changes, attributed to neuronal cell death (Houghton & Howes, 2005; Coppede *et al.*, 2006).

Furthermore, neurodegenerative disorders are commonly related to a number of factors that lead to the progressive degeneration of neurons. Neurodegeneration triggers the dysfunction of neuronal system and ultimately causes the emerging of neurodegenerative disorders. Factors causing neurodegeneration has been well documented in earlier studies and further discussed in Section 2.1.1.

2.1.1 Causes of Neurodegeneration

Aging is highly relatable to mental deterioration as most of the neurodegenerative diseases occur sporadically in age-dependant manner. An example of an age-related neurodegenerative disorder is Alzheimer's disease, observed the elderly above 65 (Shah *et al.,* 2008). Most theories on aging progression are on the idea that cumulative oxidative stress leads to mitochondrial mutations, mitochondrial dysfunction and oxidative damage. Apart from that, genetics, stressful lifestyle and bad eating habits have been ponded as some of the considerable risk factors in contributing to the upsurge of neurodegenerative diseases (Lin & Beal, 2006).

According to Small and Mayeux (2005), the pathological hallmarks of many neurodegenerative diseases can be categorized into three factors: (i) cholinergic deficits, (ii) amyloid-β (Aβ) depositions and (iii) oxidative stress. Cholinergic deficit can be defined as a loss of cholinergic function in the central nervous system significantly caused cognitive declination associated with advanced age (Heinrich & Teoh, 2004). Acetylcholine (ACh) is a neurotransmitter that plays important role for an adequately functioning memory. Deficit in ACh is highly associated with the presence of acetyl cholinesterase (AChE), an enzyme that hydrolyses ACh, resulting to loss of stimulatory activity (Adewusi *et al.*, 2010). AChE has also been reported for their involvement in the pathogenesis of plaques. The enzyme appears to enhance the aggregation of Aβ peptide, a major event in the process of plague formation (Inestrosa *et al.*, 1996). It also increases the toxicity of Aβ peptides (Reyes *et al.*, 2004). On top of that, the aggregation of Aβ peptides may also arise prior to improper metabolism of amyloid-β precursor protein (APP) (Shah *et al*., 2008). A number of study proposed that Aβ peptide deposits are responsible for triggering a neurotoxic cascade of events which ultimately results in neurodegeneration (Castro *et al.*, 2002; Dastmalchi *et al.*, 2007). Thus, to overcome such factors, most therapeutic strategies are focused on inhibiting

AChE, in order to boost the endogenous level of ACh in brain and also to reduce the toxicity of Aβ leading to cognitive function improvement.

In addition, oxidative stress is implicated and has been established to be the main causative factor in the neuronal damage associated with most of the common neurodegenerative diseases. It is thought to be mediated by free radical formation by the reactive oxygen species (ROS) and reactive nitrogen species (RNS), in which, the balance between production of ROS and level of antioxidants is significantly disturbed (Gandhi & Abramov, 2012; Phani *et al.*, 2015). This results in cellular damage and subsequent cell death especially in organs such as brain. The brain is highly vulnerable and more susceptible to oxidative damage as it consumes about 20% of the body"s total oxygen with high content of polyunsaturated fatty acids and lower levels of endogenous antioxidant activity as compared to other tissues (Halliwell & Gutteridge, 1985; Floyd & Hensley, 2002; Shulman *et al.*, 2004). Some active oxygen and free radicals, such as superoxide anion (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl anion (OH^-) are spontaneously generated and formed continuously in our body through natural metabolic actions, while some of them can also be generated due to exogenous factors, such as radiation or drug exposure (Alsarhan *et al.*, 2014; Park *et al.*, 2015). Their actions can be diverged through balanced organization of antioxidant defences, including antioxidant synthesis and enzymes. A breach in this equilibrium causes oxidative strain, which may lead to DNA and protein damage, lipid peroxidation, neuroinflammatory activity and ultimately cell death (Saxena & Saxena, 2013; Alsarhan *et al.*, 2014). Hence, neurodegeneration caused by oxidative stress could be prevented by the potential use of antioxidants as radical scavengers and their natural biological counterparts for protecting cells and tissues from oxidative damage (Deng *et al.*, 2004).

Figure 2.1 illustrates the relationship and role of AChE and beta amyloid plaque in the chain reaction that leads to neurodegeneration.

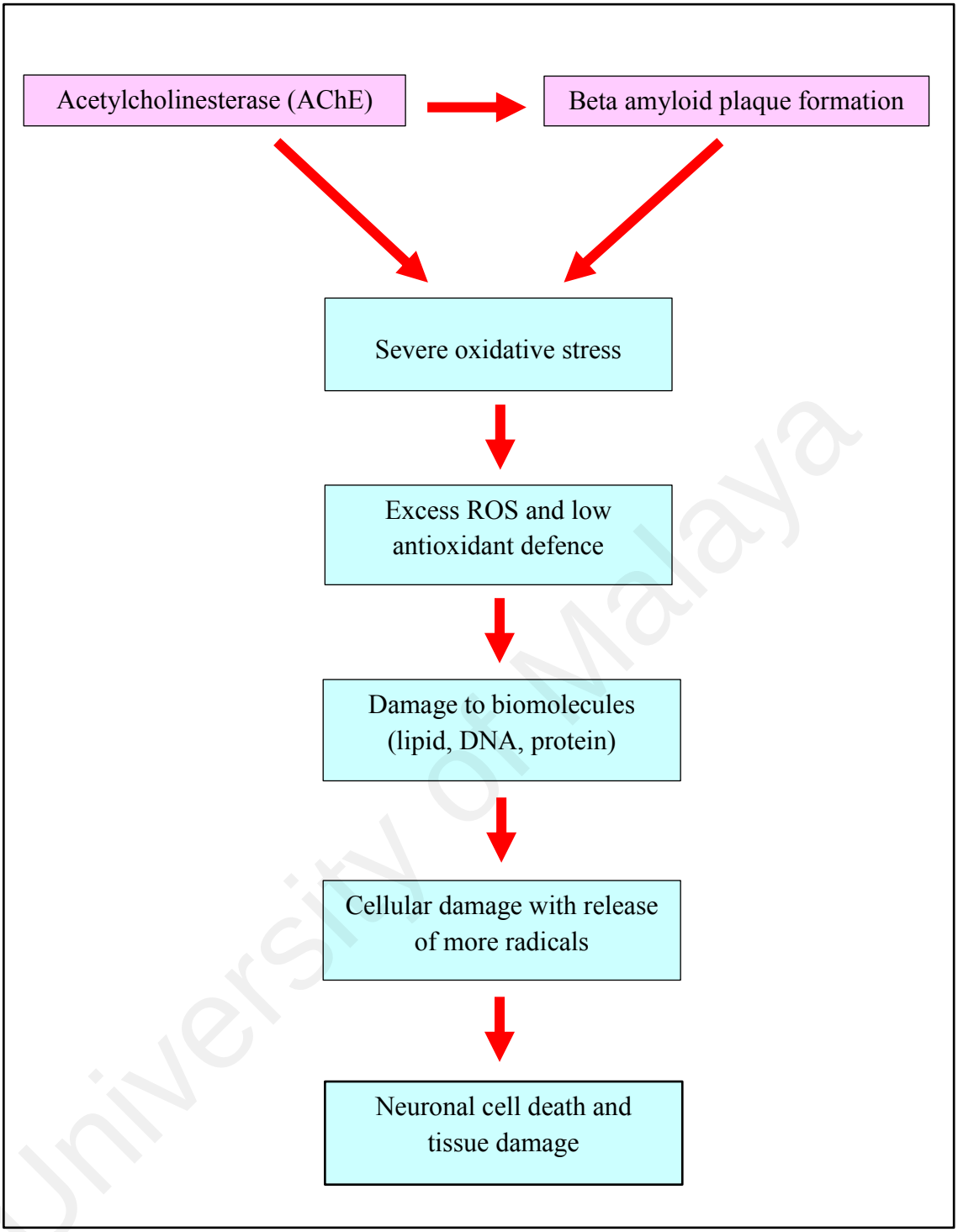

Figure 2.1: Acetylcholinesterase, beta amyloid plaque formation and oxidative stress as part of a chain reaction leading to neurodegeneration (Adewusi, 2012).

2.1.2 Current Therapeutic Approaches for Neurodegenerative Diseases

Several current therapeutic approaches adopted for most of the neurological disorders include the implication of enzyme inhibitors, artificial antioxidants, statins, non-steroidal anti-inflammatory drugs and diet (Adewusi, 2012). However, synthetic drug development is high cost and a challenging task. Selective drugs with free dose-limiting side effects are not currently available, and current available drugs may not elicit the full therapeutic response (Felder *et al.*, 2000). On top of that, there are also claims made upon consumptions of synthetic medicine causing gastrointestinal disturbances and problems associated with bioavailability (Melzer, 1998; Schulz, 2003). Phani *et al.* (2015) also reported that synthetic neuroprotective agents are believed to contribute certain side effects such as dry mouth, tiredness, drowsiness, sleepiness, anxiety or nervousness and difficulty with balance. Table 2.1 summarizes several therapeutic approaches in neurological disorders with their side effects.

Table 2.1: Current therapeutic approaches for neurodegenerative diseases and their side effects.

Class	Description	Drugs	Side effects	References
AChE inhibitors	Suppress the levels of Aβ protein.	Tacrine, donepezil, galanthamine, rivastigmine	Facial flushing, dyspepsia, nausea, vomiting and diarrhoea.	Mulugeta *et al.* (2003); Shah *et al.* (2008)
Synthetic antioxidants	Act as radical scavengers and prevent cognitive decline.	Butylhydroxyanisole (BHA), butylhydroxytoluene (BHT)	Potential carcinogenic at high dosage and long term use.	Kahl & Kappus (1993); Sano *et al.* (1997); Shah *et al.* (2008)
Statins	Reduce cholesterol level, prevent cardiovascular disease and neuroprotective.	Simvastatin, rosuvastatin, lovastatin	Muscle pain, weakness, memory loss, confusion, digestive problem and dizziness.	Fassbender *et al*. (2001)
Non-steroidal antiinflammatory drugs (NSAIDs)	Employed as anti-inflammatory, analgesic, and antipyretic agents.	Aspirin, ibuprofen	Aseptic meningitis, seizure, ataxia, vertigo, dizziness, recurrent falls, nystagmus, headache, encephalopathy, and disorientation.	Auriel *et al.* (2014)

2.2 Neuroprotection

The term neuroprotection refers to the homeostatic strategies and mechanisms within the CNS that protect neurons from apoptosis or degeneration following an acute brain injury or as consequences of chronic neurodegeneration. Neuroprotection through neuroprotective agents has been the primary tactic in targeting acute and chronic neurological diseases such as ischemia and Alzheimer"s disease respectively (Kim, 2010). The term „neuroprotection" had been widely used in the late 1980s in review articles, and there were numerous studies on the potential and role of some neuroprotective agents on acute brain damage and injury. Later, the research interest started to change its lane to include various other types of chronic neurodegenerative diseases.

Generally, the role of neuroprotection involves a number of effects including a potential capability against injury induced by neurotoxic species, a capability to defeat neuroinflammation and to stimulate effective cognitive function (Phani *et al.*, 2015). Even so, scientists also suggested that the action mechanism of neuroprotection can be categorized into several groups including (i) free radical scavengers, (ii) antiexcitotoxic agents, (iii) apoptosis inhibitors, (iv) neurotrophic agents; and (v) ion channel modulators (Fiszman, 2003; Peng *et al*., 2008; Hardeland, 2009; Xia *et al.*, 2009).

2.3 Herbs for Management of Neurodegenerative Diseases

In human history, herbs have been used since antiquity in traditional medicinal systems for the management of several neurological disorders worldwide. In this era, a sudden rise in neurological disorders and recognition of acute side effects associated with long term administration of synthetic drugs has alarmed the attention of researchers towards natural resources (Phani *et al.*, 2015). It is approximated that there are about more than 100 traditional herbs are being used for the therapy of neurological disorders in Asian countries (Kumar & Khanum, 2012).

Herbs that has been used in the management of neurodegenerative diseases are commonly refered as nerve tonics or calming herbs that are mildly relaxing without the overtly suppressant effects of sedatives. They are also commonly used to restore emotional balance and to nourish the nerves and nervous system. Furthermore, consumption or applications of these herbs help in coping with stress, anxiety, insomnia, irritability and hypertension (Bhattacharjee & Manna, 2016).

Herbs like *Bacopa monniera*, *Gingko biloba*, *Panax ginseng*, *Nardostachys jatamansi*, *Centella asiatica*, *Valeriana officinalis* and *Withania somnifera* have been used extensively in various traditional systems because of their adaptogenic, psychotropic and neuroprotective properties. In Indian system of traditional medicine, several herbs that have shown promising activity in neuropsychopharmacology including *Acorus calmus*, *Allium sativum*, *Angelica sinensis*, *Bacopa monniera*, *Centella asiatica*, *Celastrus paniculatus*, *Crocus sativus*, *Curcuma longa*, *Enhydra fluctuans*, *Ginko biloba*, *Glycyrrhiza glabra*, *Huperriza serata*, *Hypericum perforatum*, *Nicotiana tabaccum*, *Physostigma venosum*, *Salvia officinalis*, *Terminalia chebula*, *Uncaria tomentosa*, *Valeriana wallichii* and *Withania somnifera*. As for Chinese traditional medicine system, some of the nervine herbs being used are *Angelica pubescens*, *Ledebouriella divaricate*, *Ligusticum chuanxiong*, *Morus alba*, *Salvia*

miltiorrhiza, *Scutella baicalensis* and *Uncaria rhynchophylla* (Phani *et al.*, 2015). Table 2.2 summarizes the traditional use of some herbs in neurological disorders management.

Table 2.2: Herbs used in neurological disorders management.

Scientific name	Ethno-medicinal use	References
Allium sativum	Antioxidant, antimicrobial, antitumour and reduces cardiovascular risk and dementia.	Mathew and Biju (2008); Bayan *et al*. (2014)
Alstonia macrophylla	Antiinflammatory, antidepressant, sedative, relieves depression and antipsychotic to treat madness and epilepsy.	Chattopadhyay *et al.* (2004) ; Elisabetsky and Costa-Campos (2006)
Amaranthus spinosus	Antiinflammatory, treat hallucinations, nausea, diabetes and epileptic seizures.	Gul *et al*. (2011); Chaudary *et al*. (2012)
Bacopa monniera	Brain tonic for learning, memory enhancement and antistress agent in anxiety	Anand *et al*. (2012)
Celastrus paniculatus	Antioxidant, antiinflammatory, stimulating intellect and sharpening memory	Ahmad *et al*. (1994); Kumar and Gupta (2002)
Centella asiatica	Antioxidant, improve cognitive function, revitalizing and strengthening nervous function and memory	Kumar and Gupta (2002); Adewusi (2012)
Coleus amboinicus	Antiepileptic, antioxidants, antiinflammatory and antitumour.	Franca *et al.* (1996); Taraphdar *et al.* (2001)

Table 2.2 continued,

Curcuma longa	Antioxidant, antiinflammatory, anticancer, anticardiovascular, enhances immune system and memory impairment.	Araujo (2001); Cole *et al*. (2007); Gupta *et al.* (2012)
Gingko biloba	Antioxidant, improve cognitive function and memory loss associated with abnormalities in blood circulation	Rigney *et al*. (1999)
Huperzia serrata	Antioxidant and improve memory impairment	Hostettman *et al*., (2006)
Hypericum perforatum	Antidepressant, improve memory and learning dysfunction	Widy- Tyszkiewicz *et al.* (2002); Trofimiuk *et al.* (2005)
Panax ginseng	Increase body‟s resistance to various stresses like anxiety, trauma and fatigue by modulating the immune function	Phani *et al.* (2015)
Salvia officinalis	Improve memory and cognitive function, treatment for seizure, dementia, dizziness, paralysis and age-related cognitive disorders	Ahmad and Mahdi (2017); Adrian (2017)
Scutellaria baicalensis	Antioxidant, antitumour, antiinflammatory, treat hypertension and insomnia.	Zhao *et al.* (2016)
Uncaria rhynchophylla	Treat insomnia, sleep tremors epilepsy, seizure and dementia	Fujiwara *et al.* (2006); Liu *et al.* (2012)
Uncaria tomentosa	Antioxidant, anticancer, improves memory and cognitive impairment.	Hardin (2007)

Table 2.2 continued,

Valeriana wallichii	Sleep remedy, sedative in hysteria, epilepsy and nervous anxiety.	Nandhini *et al*. (2018)
Withania somnifera	Sedative, antistress, antiinflammatory, epilepsy, insomnia, dementia, leprosy and nervous disorders.	Umadevi *et al.* (2012)

2.3.1 *Piper betle* L.

Piper betle L., commonly known as betel leaf, is the leaf of a vine belonging to the Piperaceae family. *P. betle* has long been recognised as one of the medicinal plants that has tremendous health benefits. This plant is originated from Malaysia but is distributed and cultivated extensively in India, Sri Lanka, Thailand, Taiwan and other South-east Asian countries. *P. betle* is called by different names in different languages; *Paan* in Hindi, *Vettilai* in Tamil, *Tanbol* in Arabic and *Sirih* in Malay (Suryasnata *et al.*, 2016).

In general, *P. betle* is a dioeciously tropical shade loving perennial root climber and it may climb as high as 10–15 feet in length. *P. betle* prefers a warm and humid climate to live. The leaves of this plant are simple, alternate and heart shaped with 4–7 inch long and 2–4 inch broad in size (Figure 2.2). *P. betle* has male spikes which are dense and cylindrical, while female spikes are pendulum in shape. Roots arise from each node aid in fixing the plant to the host tree. The colour of the leaves is yellowish green to dark green with glossy upper surface (Bhaleraol *et al.*, 2010; Lakshmi *et al.*, 2010).

P. betle also has a pleasant odour, in which it is one of the most noticeable characteristics of the plant. *P. betle* leaves are aromatic with varied taste, ranging from sweet to pungent which are believed due to the presence of essential oils (Suryasnata *et al.*, 2016).

Figure 2.2: Leaves of *P. betle*.

Traditionally, *P. betle* has gained valuable attention at ceremonial events for various religious rituals and customs in Asia. Among Indians, it is the most favourite herbs used for chewing purpose according to the native people (Jaiswal *et al.*, 2014). *P. betle* is eaten raw with various condiments inside the edible leaves such as sliced areca nut, slaked lime, coriander, aniseed, clove, cardamom, sweetener, coconut scrapings, pepper mint, fruit pulp and many more (Aishwarya *et al.*, 2016). It is generally taken after meals to cleanse palate or as mouth freshener and masticatory (Norfaizatul *et al.*, 2010).

The economical, medicinal and traditional importance of this herb has been very well understood worldwide. Every parts of the plant are well-off in macro and micro nutrients, antioxidants and a wide variety of biologically active compounds which makes this herb one of the most famous medicinal plants that has been used since

ancient times. Table 2.3 summarizes the ethno-botanical uses of *P. betel* respective to the usable parts of the plant.

In Ayurveda medicinal system, *P. betle* is known by its Vedic name *Saptasira* and used as adjuvant with different medicines for voice, purifying blood, laxative and appetizer (Jaiswal *et al.*, 2014). *P. betle* leaves are known for its richness in moisture, protein, fats, minerals, vitamins (Table 2.4). Indian traditional medicine systems claimed that it helps in curing communicable and non-communicable diseases such as, cough, cold, asthma and used to treat other diseases like bad breath, boils and abscesses, conjunctivitis, constipation, swelling of gums, cut and injuries (Gundala *et al.*, 2014).

However, extensive research over the years found that this herb‟s popularity is not only limited to its chewing purposes, as it also possesses anti-inflammatory, anti-apoptotic, anti-cancer and anti-microbial properties (Suryasnata *et al.*, 2016). Recent studies demonstrated that *P. betle* shows antimicrobial activity against a number of microbes including urinary tract pathogenic bacteria such as *Enterococcus faecalis* and *Klebsiella pneumonia* (Tripathi *et al.*, 2011; Argawal & Singh, 2012).There are also a number of studies reported on the potent cytotoxicity and probable anticancer property of this herb towards several type of cancer (Murakami *et al.*, 2010; Fathilah *et al.*, 2010; Dwivedi *et al.*, 2010; Abrahim *et al.*, 2012). *In vivo* studies on the antidiabetic properties of *P. betle* suggested that this herbal plant can be used as a potential pharmaceutical for diabetic patients (Arambawela *et al.*, 2005).

On the other hand, the use of *P. betle* as medicinal herb in neurological disorders has been known since decades ago, as Ayurveda medicine believed that this herb is useful in enhancing memory and learning function. Attention of researches on exploiting medicinal herbs to improve neurodegenerative diseases such as Alzheimer‟s and Parkinson‟s disease has not been exceptional for *P. betle*. Several *in vivo* studies have claimed for its antioxidant and neuropharmacological properties especially for its

potential nootropic effect (Vyawahare & Bodhankar, 2007). Thus, current *in vitro* study on the neuroprotective properties of *P. betle* could be beneficial to provide information on the potential nootropic effect of this nervine herb in improving neurological disorders.

Table 2.3: Traditional uses of *P. betle*.

Parts used	Ethno-botanical uses	References
Leaf	Treat cough and indigestion, bad breath, hysteria, itches, swelling of gum, cuts and injuries, also as antimalarial, antibacterial, antifungal, insecticidal, antiinflammatory, antioxidant, and antidiabetic.	Ramji *et al.* (2002); Majumdar *et al.* (2003)
Stem	Treat indigestion, bronchitis, constipation, coughs and asthma.	Rekha *et al.* (2014)
Root	Female contraceptive purpose.	Arawwala *et al.* (2014)
Whole plant	As food and spices, fish bait, insecticides, oil, perfumes and helps in improving digestive system.	Rekha *et al.* (2014)
Essential oil	As antibacterial, antiprotozoan, antifungal.	Punuri *et al.* (2012)

Table 2.4: Nutritional composition of *P. betle* leaf (Agarwal *et al*., 2012).

Constituents	Approximate composition
Energy	44 kcal/100g
Water	85–90%
Protein	3–3.5%
Fat	0.4–1.0%
Minerals	2.3–3.3%
Fiber	2.3%
Carbohydrate	0.5–6.1%
Nicotinic acid	0.63–0.89 mg/100g
Vitamin C	0.005–0.01%
Vitamin A	1.9–2.9 mg/100g
Thiamine	10–70 µg/100g
Riboflavin	1.9–30 µg/100g
Nitrogen	2.0–7.0%
Phosphorus	0.05–0.6%
Potassium	1.1–4.6%
Calcium	0.2–0.5%
Iron	0.005–0.007%
Iodine	3.4 µg/100g
Essential oil	0.08–0.2%

2.3.2 *Clitoria ternatea* L.

Clitoria ternatea L. is a species belongs to Fabaceae family, and commonly known as *butterfly pea*, and *kordofan pea* in Sudan, *cunha* in Brazil, *Aparajit* in Hindi and *Kokkatan* in Tamil. (Zingare *et al.*, 2013). In Malaysia, *C. ternatea* is commercially known as *bunga telang* by the locals and it is widely used as the food dyes in commercially known as *bunga telang* by the locals and it is widely used as the food dyes in "Nasi Kerabu" – a Malay rice dish to make it blue in colour, and a dessert of the ethnic Baba and Nyonya known as "kueh tekan" (Lijon *et al.*, 2017).

This herb has twining fine stems, 0.5–3.0 meters long (Figure 2.3). The leaves are pinnate, with 5–7 elliptic to lanceolate leaflets, 3–5 cm long and shortly pubescent underneath. Flowers are vivid blue in colour (Figure 2.4), 1 to 2 inches long, having wavy-rimmed standard and white centre, which is rather common in gardens of Hawaii (Lijon *et al.*, 2017). Pods are flat, linear, beaked, 6–12 cm long, 0.7–1.2 mm wide and slightly pubescent with up to 10 seeds. The seeds are olive, brown or black in colour, often mottled, 4.5–7.0 mm long and 3–4 mm wide (Hall, 1985).

Figure 2.3: Twiner of *C. ternatea*

Figure 2.4: Flower of *C. ternatea*.

The major phytochemical constituents found in *C. ternatea* are pentacyclic triterpnoids such as taraxerol and taraxerone (Lijon *et al.*, 2017). A preliminary phytochemical screening of this herb also showed that *C. ternatea* contained tannins, phlobatannin, carbohydrates, saponins, triterpenoids, phenols, flavonoids, flavanol glycosides, proteins, alkaloids, antaraquinone, anthocyanins, cardiac glycosides, volatile oils and steroids (Al-Snafi, 2016). The presence of these phytochemicals made all parts of this herb is very useful for medical treatments and has been used in folk medicines for curing different diseases (Neda *et al.*, 2013).

Traditionally, this herb is very well known for its use in the treatment of chronic bronchitis, dropsy, goitre, leprosy, mucous disorder, sight weakness, skin diseases, sore throat and tumours (Ramaswamy *et al.*, 2011). Table 2.5 summarized the traditional uses of different parts of *C. ternatea*.

Table 2.5: Traditional uses of *C. ternatea*.

Parts used	Traditional uses	References
Leaf	Brain tonic.	Mukherjee *et al.* (2007); Nawaz *et al.* (2009); Ragupathy and Newmaster (2009)
Flower	Food dye.	Jain *et al.* (2003)
Root	Muscular strength, treatment of ascetics, enlargement of abdominal viscera, sore throat and skin disease.	Mukherjee *et al.* (2007); Nawaz *et al.* (2009); Ragupathy and Newmaster (2009)
Seed	Brain tonic, antidote for snake bite, also used in swollen joints and urinary problems.	Mukherjee *et al.* (2007); Nawaz *et al.* (2009); Ragupathy and Newmaster (2009)
Whole plant	Heat stable function, treat sexual ailments such as infertility and gonorrhoea.	Fantz (1991); Nguyen *et al.* (2011)

Over the years, attention paid towards the pharmacological importance of *C. ternatea* and demonstrated that this herb possesses other biological activity such as antioxidant, hypolipidemic, anticancer, antiinflammatory, analgesic, antipyretic, antidiuretic, antimicrobial, gastrointestinal, antiparasitic, insecticidal and many others (Al-Snafi, 2016). Researchers also suggest that *C. ternatea* is a potential herbal medicine for cancer and tumour treatment as the chemical profile of its extract is well correlated with other reports from different herbs on cancer suppressing activity or anti-carcinogenic activity (Ramaswamy *et al.*, 2011).

The importance of *C. ternatea* in neurodegenerative diseases varies as it has been widely used for many centuries in the Ayurveda medicine as a memory enhancer, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative agent (Mukherjee *et al.*, 2008). It is also considered as a „Medhya" drug to improve intelligence and enhance memory function. A number of recent studies on animal models were evaluated for its effect on cognitive behaviour, anxiety, depression, stress and convulsions and *C. ternatea* was found to possess outstanding nootropic, anxiolytic, antidepressant, anticonvulsant and antistress activity (Taranalli & Cheeramkuzhy, 2003). A study also demonstrated that *C. ternatea* extract was beneficial in Alzheimers disease through many mechanisms (Shahnas & Akhila, 2014). Hence, this herb possesses significant potential to be used as alternative or complementary medicine in neurodegenerative diseases.

2.4 Herbs as Natural Antioxidant

Neuronal cell damage caused by oxidative stress is mainly due to the imbalance state exists between pro-oxidants and antioxidants level. Hence dietary intake of such phytochemical based antioxidants is one of the important strategies for inhibiting or delaying pathological conditions; dysfunction in mitochondrial and endoplasmic reticulum, which includes apoptosis and protein misfolding in neurons (Shankar *et al.*, 2013; Phani *et al.*, 2015). A number of *in vitro* studies also have demonstrated very promising neuroprotective activities of antioxidant agents and have shown potential benefit of treatment with radical scavengers in pathologies such as Alzheimer‟s and Parkinson‟s disease (Grundman, 2000; Shults, 2003; Nagayama *et al.*, 2004).

In general, antioxidants are molecules that play an important role in preventing or delaying degenerative diseases resulting from the oxidative damage in living cells caused by free radicals (Jaitak *et al.*, 2010). This means that they may have neuroprotective role by reducing or reversing cellular damage and by slowing the progression of neuronal cell loss (Moosmann & Behl, 2002).

In nature, antioxidants are categorized as endogenous or exogenous. The endogenous group includes enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and several proteins like albumin, ceruloplasmin and haptoglobin. Some important exogenous antioxidants are dietary phytochemicals including polyphenols, phenolic acids, flavonoids, terpenoids, saponins, and vitamins including ascorbic acid, alpha-tocopherol and beta-carotene (Berger, 2005).

For many years, artificial antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been tested for probable toxicity. However, there are also extensive studies on natural antioxidants from natural plant materials to replace artificial

antioxidants as they are much safer, more effective and cheaper alternative (Qader *et al.*, 2011).

Hence, in recent years, there has been an intense interest emphasized on the potential of phytochemicals in herbs to act as antioxidants, modulate neuronal function and protective mechanism against neurodegeneration (Phani *et al.*, 2015). Many studies reported that phytochemicals belong to medicinal herbs used in managing neurodegenerative diseases shown potential neuroprotective action in animal and cell culture model. They are also believed to provide neuroprotection by activating cellular stress-response pathways resulting in the upregulation of neuroprotective gene products. Polyphenols (phenolic acids, anthocyanins, proanthocyanidins, flavanols, tannin), isoprenoids (sesqiterpenes, diterpenes, triterpenes, steroids, saponins), alkaloids (indole alkaloids, lysergic acid diethylamide, tropane alkaloids, ergot group) and fatty acids are known to be the active components found in the herbs that regulate a variety of enzymes as well as receptors in neuroprotection (Facchini, 2001).

2.4.1 Phenolic Compounds

Recent studies suggested that the antioxidant effect of herbal plants is mainly attributed to phenolic compounds such as flavonoids, phenolic acids, tannins and others (Navagani & Rao, 2010; Cartea *et al.*, 2010). Table 2.6 summarizes several phenolic compounds and their neuroprotective activities.

Phenolics comprise the largest group of plant secondary metabolites characterized by the presence of more than one phenolic unit which is linked directly to the aromatic ring (Phani *et al.*, 2015). They are also known as the major contributor of antioxidant activity in plant extracts due to their higher value in total content, synergistic effectiveness as hydrogen donating capacity, reducing agent and free radical scavenger (Wong *et al.*, 2006; Hodzic *et al.*, 2009).

Table 2.6: Phenolic compounds and their benefits in neuroprotection.

Compound	Description	References
Anthocyanin	Improves brain cognitive function and defence mechanism on oxidative stress and lipid peroxidation.	Talav˜era *et al*. (2005)
Catechin	Provides protection against brain injuries, suppress neuroinflammation and delay onset of neurodegenerative disease through different mechanisms such as iron chelators, radical scavengers and modulators of prosurvival genes.	Wu *et al*. (2009)
Tannin	Reduces Aβ-induced neurotoxicity and attenuate stress-induced behaviour in depression.	Feng *et al*. (2009)
Quercetin	Protects neuronal cells against ischemic injury, attenuate Aβ-induced toxicity, protein oxidation and apoptosis.	Ansari *et al*. (2009)

2.4.2 Flavonoids

Among natural phytochemicals identified from herbs used in neurological disorders, flavonoids (Figure 2.5) represent one of the most important, and interesting and by far the best studied group of compounds for neuroprotective effects. Previous reports demonstrated that flavonoids are effective in the protection of numerous cell types from oxidative injury including neuronal cells (Zou *et al.*, 2010).

This group of compounds has a general shape of two phenyl rings and a heterocyclic ring (Figure 2.5). It has been known for its effectiveness in blocking oxidant induced neuronal damage by preventing the activation of enzyme involved in neuronal cells apoptosis (Phani *et al.*, 2015). Evidence also suggests that the antioxidant activities of flavonoids are stronger than antioxidants nutrient such as vitamin C, vitamin E and β-carotene (Hoe *et al*., 2004). Baicalein and Baicalin, compounds that have been isolated as the principal component of *Scutellaria baicalensis*, are good examples of flavone class of flavonoids. These compounds have been demonstrated in several *in vivo* and *in vitro* models to promote neurite outgrowth, attenuate cell apoptosis, inhibit the production and accumulation of ROS, promote mitochondrial active respiration and prevent Aβ-induced impairments (Elufioye *et al.*, 2017).

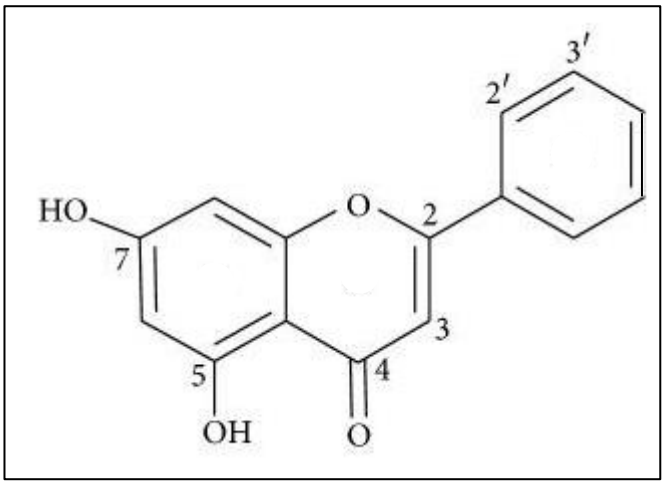

Figure 2.5: Chemical structure of flavonoids.

2.5 SH-SY5Y Cell Line as an *In Vitro* Cell Culture Model in Neuropharmacological Studies

Cell culture systems are widely used in *in vitro* study for neurological disorders including the investigation of plant-based products. Human neuroblastoma SH-SY5Y cell line was selected in the present study as it is widely used as model cell system for studying oxidative stress-induced neuronal cell death (Ruffels *et al.*, 2004). The cell line is a thrice cloned subline of the neuroblastoma cell line SK-N-SH which was established in 1970 from a metastatic bone tumour. SH-SY5Y cells had neuron-like characteristics with various biochemical and functional characteristics of dopamine and noradrenaline producing enzymes, acetylcholine, norepinephrine and various growth factor receptors (Ciccarone *et al.*, 1989). Previous studies reported that saturation density of SH-SY5Y cultures can be greater than 1×10^6 cells/cm^2 (Seoposengwe *et al.*, 2013). The mentioned characteristics makes SH-SY5Y cells a suitable cell model for the assessment of neuroprotective activity.

As for the present study, H_2O_2 was used as an oxidative stress agent to induce cell damage in SH-SY5Y cells. Generally, H_2O_2 is a physiological constituent of living cells that is continuously synthesised via diverse cellular pathways. In experimental models, H_2O_2 is often exposed to cultured cells by addition into the cultured medium. Some of the reasons are simply to investigate its physiological functions and toxic effects, oxidative stress responses of cell and cytoprotection by antioxidant agents (Gulden *et al.*, 2010). It is also known to cause oxidative stress that leads to cells growth arrest and death (Gulden *et al.*, 2010). Exogenously added H_2O_2 in SH-SY5Y cell culture was reported to induce intracellular ROS generation and affected the accompanying cellular metabolic responses (Gough & Cotter, 2011). It also known that H_2O_2 triggered apoptosis and necrosis cell death in a concentration dependant manner, thus resulting in decrease of cell viability (Norsharina *et al.*, 2012).

CHAPTER 3: METHODOLOGY

3.1 Preparation of Herbal Extracts

3.1.1 Herbal Materials

The leaves of *Piper betle* were collected from a home garden in Port Dickson, Negeri Sembilan, and the leaves of *Clitoria ternatea* were collected from a home garden in Shah Alam, Selangor, in the month of March 2017. Authentication of *P. betle* and *C. ternatea* was carried out in the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya by Dr Sugumaran Manickam. Voucher materials (Ref. No. KLU49902 & KLU49903) for this study was deposited at the same herbarium.

Upon collection, the leaves were sorted, and the fresh weights were recorded. The fresh leaves were then rinsed with tap water and ready to be used for water extract preparation. For methanolic extract preparation, the fresh leaves were oven dried at 45 to 50 °C for 4 days (Auddy *et al.*, 2003). Dried leaves then were grounded into powder and the dry weight of the herbal materials was recorded.

3.1.2 Extraction Procedures

The extraction procedures were carried out according to the method described by Auddy *et al.* (2003) with slight modifications.

i. Preparation of Methanolic Extracts

40 g of each grounded dried herbs were soaked in 400 mL of 90% methanol (Fisher Scientific, USA) (1: 10) for 3 days at room temperature (26 to 28 °C).

Thereafter, the herbs in methanol then were filtered using filter paper (Whatman No. 1) and the excess solvent were removed from the filtrate using rotary evaporator (Buchi, Switzerland). The extraction steps were repeated three times and obtained methanolic extracts were pooled, weighed and kept at 4 °C prior to analysis. Methanolic extracts were diluted with either absolute methanol or 10% DMSO depending on the type of assay to achieve final concentrations needed.

ii. Preparation of Water Extracts

20 g of fresh leaves were cut into small pieces and added into 500 mL distilled water in conical flask prior to boiling at 80 °C and placed in water bath (Memmert, German) for 1 hour. The boiled herbs were left to cool, filtered and the filtrates were freeze dried. Obtained water extracts were weighed and kept at 4 °C prior to analysis. The extracts were diluted in distilled water to prepare extract dilutions with desired concentrations.

Throughout the process of extraction, the weight of the fresh and dry herbs, and the dried extracts were recorded. Percentage of yield of extract for each herb was calculated according to the equation below as described by Ibrahim *et al.* (2011).

$$\text{Yield extract of herb (\%)} = (\text{Weight of extract} / \text{Weight of dried herb}) \times 100$$

3.2 Neuroprotective Assay

3.2.1 Human Neuroblastoma SH-SY5Y Cells

The human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (ATCC, USA) and were grown in DMEM/F12 medium (Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (TIC, Europe) in a humidified atmosphere at 37 °C with 5% CO_2. The cells were maintained by sub-culturing the cultures once in every 2 or 3 days. Upon analysis, grown cells (3×10^4 cells per well) were detached using accutase (iCT, CA) and phosphate buffered saline (PBS at pH 7.4) solution, and centrifuged (Kubota, Japan) at 100g for 5 minutes. Obtained cell pellet were then subjected to cell counting assay using tryphan blue exclusion method to attain intended concentration of cells per well. Further experiments were carried out 24 hours after the cells were seeded into the 96-well plates. Preparation method of the media, PBS and tryphan blue solution were listed in Appendix A1.

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

The toxicity of dimethyl sulfoxide (DMSO) (R&M Chemicals, UK) towards SH-SY5Y cells were determined according to the method described by Piah *et al*. (2010). Serial dilutions of methanolic extracts of the herbs in further biological assay will be carried out by dissolving the extracts in DMSO. SHSY-5Y cells seeded in 96-well plate were treated with a range of DMSO concentrations (0.1–100%) and incubated for 24 hours at 37°C, 5% CO_2. At the end of the incubation period, cell viability was determined using MTT assay as described in 3.2.6. Concentration of DMSO at which least cell death induced was selected to be used in serial dilution of the methanolic extracts.

3.2.3 Assessment of Toxicity of Hydrogen Peroxide towards SH-SY5Y Cells

Toxicity of hydrogen peroxide (H_2O_2) (Systerm Chemical, Malaysia) was assessed according to the method described by Piah *et al.* (2010) and Norfaizatul *et al.* (2010) with some modifications. For this study, H_2O_2 will be used as oxidative stress agent to induce SH-SY5Y cell death in neuroprotective assay. In order to determine the concentration of H_2O_2 to be used, SH-SY5Y cells grown in 96-well plate at 3 x 10^4 cells per well were exposed to a range of concentrations of H_2O_2; 25, 50, 75, 100, 200, 300, 400 and 500 μM (diluted in basic media) for 24 hours. Then, cell viability was determined using MTT assay (as described in 3.2.6) and the concentration of H_2O_2 providing 50% cell death (IC_{50}) was determined.

3.2.4 Assessment of Neurotoxicity Effect of the Herbal Extracts on SH-SY5Y Cells

The assessment of neurotoxicity effect of the herbal extracts towards SH-SY5Y cells was carried out based on the method adapted from Piah *et al.* (2010). SH-SY5Y cells plated in 96-well plate at 3 x 10^4 cells per well were incubated with ranges of herbal extracts concentrations (0.1–100 μg/mL) for 24 hours and cell viability was accessed by the MTT assay as described in 3.2.6.

3.2.5 Assessment of Neuroprotective Effect of the Herbal Extracts on H_2O_2-induced SH-SY5Y cells

Neuroprotective effect of the herbal extracts on H_2O_2-induced SH-SY5Y cells were carried out according to the methods described by Norfaizatul *et al.* (2010) with slight modifications. SH-SY5Y cells grown in 96-well plate at 3 x 10^4 cell per well were divided into four groups: (i) negative control (cells without addition of any test

agent), (ii) cells incubated with H_2O_2 with concentration which induced 50% of cell death (IC_{50} value obtained in 3.2.3), (iii) pre-treatment; and (iv) post-treatment group (Norfaizatul *et al.*, 2010).

For the pre-treatment group, SH-SY5Y cells were incubated with extracts at various concentrations for 24 hours before addition of H_2O_2. After the addition of H_2O_2 to the cells contained extracts, the assay plates were further incubated for 24 hours at 37°C, in a 5% CO_2 humidified incubator. In post-treatment group, cells were exposed to H_2O_2 for 24 hours and followed with incubation with herbal extracts for further 24 hours at 37°C, 5% CO_2. The concentrations tested for the extracts of *P. betle* are 0.001–1.0 µg/mL, meanwhile *C. ternatea* are 1.0–100.0 µg/mL. At the end of incubation period, the cell viability was determined by the MTT assay as described in 3.2.6.

Statistical analysis was also carried out to compare the neuroprotective activity between methanol and water extract of both herbs. Data were analysed using two-way ANOVA analysis by SPSS statistical software, version 25.0 (SPSS Inc., USA).

3.2.6 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide Assay

3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out to measure cell viability using the method described by Mickisch *et al.*, (1990) with slight modifications. The principle of the assay was based on the generation of insoluble purple coloured formazan crystals, upon the reduction of MTT by succinate dehydrogenase enzyme in the mitochondria of active cells. Addition of organic solvent to the cells will then solubilise the purple formazan which is measured photometrically (Hansen *et al.*, 1989). Hence, more colour formation indicates more cell viability. Preparation method of MTT solution was described in Appendix A1.

Prior the assay, 96-well tissue culture plates with SH-SY5Y cells with various concentrations of the test agents and incubated for a period of time were prepared. On

completion of the incubation period, 20 µL of MTT solution (5 mg/mL) was added to each wells and incubated for further 3 hours at 37°C, 5% CO_2. Thereafter, media with MTT solution in each wells were aspirated out using multichannel pipette and 200 µL of DMSO per well was added to breach the cells and dissolve the formazan crystals. Finally, absorbance was measured at 570 nm using Multiscan GO microplate reader (Thermo Fisher Scientific, USA).

Results were presented as percentage of cell viability compared to the control values (wells without addition of any test agents). The percentage of cell viability was calculated using the formula as given below:

$$\text{Percentage of cell viability (\%)} = (A_{sample}/A_{control}) \times 100$$

where $A_{control}$ is the absorbance of control group (cells without addition of any test agents); and A_{sample} is the absorbance of test samples (cells with addition of test agents).

3.3 Phytochemical Contents of the Herbal Extracts

Determination of selected phytochemical contents, total phenolic and flavonoid were carried out according to the method described by Oboh *et al.* (2016) with modifications.

3.3.1 Total Phenolic Content

Total phenolic contents (TPC) were determined using Folin- Ciocalteu method with gallic acid as the standard. In brief, 25 µL of Folin-Ciocalteau‟s reagent (R&M Chemicals, UK) (diluted to 10 fold) was added into 10 µL of herbal extract (at concentrations range from 0–1 g/L) and mixed well with the solvent; methanolic extract with methanol and water extract with distilled water, before the mixture was incubated for 5 minutes at room temperature. Then, 25 µL of 7.5 % (w/v) sodium carbonate

solution (Na_2CO_3) (R&M Chemicals, UK) and 140 µL of distilled water were added to the mixture to give a final volume of 200 µL. The mixture then was further incubated for 30 minutes at room temperature. Preparation procedures of the reagents were described in Appendix A2.

At the end of the incubation period, the absorbance readings were measured at 760 nm against blank using a Multiscan GO microplate reader (Thermo Fisher Scientific, USA). A standard curve was plotted using known concentrations of gallic acid (R&M Chemicals, UK) (at concentrations range from 0–1 g/L). TPC of each extracts were calculated from the standard curve and expressed as milligram of gallic acid equivalent (GAE) per gram of extract (mg of GAEs/ g of extract).

3.3.2 Total Flavonoid Content

Total flavonoid contents (TFC) were measured using aluminium chloride colorimetric assay with Quercetin as the standard. Briefly, 120 µL of the herbal extracts (at concentrations range from 0–0.5 g/L) dissolved with the solvent; methanolic extract with methanol and water extract with distilled water, was subsequently added with 6 µL aluminium chloride ($AlCl_3$) (Systerm Chemical, Malaysia) (10% w/v), 6 µL potassium acetate (CH_3COOK) (Sigma, UK) (1 mol/L) and 170 µL distilled water. The mixture was then left at room temperature for 30 minutes. Preparation procedures of the reagents were listed in Appendix A2.

At the end of the incubation period, the absorbance of the reaction mixture was measured at 415 nm using Multiscan GO microplate reader (Thermo Fisher Scientific, USA). The formation of yellow colour is indicative of the presence of flavonoid in the extracts. TFC was calculated based on a standard curve plot generated using known concentrations of Quercetin (Acros Organics, USA) (at concentrations

range from 0–0.1 g/L) and expressed as milligram of Quercetin equivalent (QE) per gram of extract (mg of QEs/ g of extract).

3.4 Antioxidant Activity of the Herbal Extracts

3.4.1 1, 1-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The antioxidant activity of the herbal extracts on the basis of the scavenging activity of the stable 1, 1-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined by the method described by Yen & Duh (1994) with some modifications. Briefly, 150 µL of 0.3 mM methanolic solution of DPPH (Sigma Aldrich, Germany) was reacted with 50 µL of various concentrations of the herbal extracts.The extracts of *P. betle* were tested at concentration ranges from 10 to 200 µg/mL, meanwhile the extracts of *C. ternatea* were 100 to 1000 µg/mL. Preparation method of DPPH solution was described in Appendix A3. The reaction mixture was vortexed and allowed to be incubated in dark for 30 minutes at room temperature. The absorbance then was measured at 517 nm using microplate reader (Thermo Fisher Scientific, USA) at the end of the incubation period. Ascorbic acid was used as standard and the assay was performed in triplicate. The DPPH radical scavenging activity of the extracts was calculated using following equation:

$$\text{DPPH radical scavenging activity (\%)} = [A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of DPPH radical mixed with methanol or water (depending on the type of the extract); and A_{sample} is the absorbance of DPPH radical reacted with extracts or standard. Concentration of the herbal extract at which provides 50% inhibition (depicted as IC_{50} value) of the free radical was determined and compared against standard.

3.4.2 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] Radical Scavenging Activity

2, 2‟-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] radical scavenging assay was carried out based on the method described by Re *et al.*, (1999) with slight modifications. The working stock solution for ABTS radical cation was prepared by mixing equal volumes of 7 mM ABTS solution (Sigma, USA) with 2.45 mM potassium persulfate (Sigma Aldrich, Germany) and the mixtures was allowed to react for 12 hours in dark at room temperature. Preparation procedures of the reagents are as listed in Appendix A3. At the time of measurement, the resultant working solution was diluted with distilled water to the absorbance level of 0.706 ± 0.02 at 734 nm. The assay then proceeded by adding 20 µL of different concentration of herbal extracts and allows the mixture to react with the diluted ABTS radical cation working solution for 15 minutes in dark at room temperature. The extracts of *P. betle* were tested at concentration ranges from 1 to 100 µg/mL, meanwhile the extracts of *C. ternatea* were 100 to 1000 µg/mL.

Absorbance of the mixture was measured at 734 nm and the results were compared with ascorbic acid as standard. The ABTS radical scavenging capacity of the extracts was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = [A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of ABTS radical mixed with methanol or water (depending on the type of extract); and A_{sample} is the absorbance of ABTS radical reacted with the addition of extracts or standard. The assays were carried out in three different occasions and the concentration of the extracts that provides 50% inhibition (depicted as IC_{50} value) of the ABTS radical was also calculated.

3.5 Correlation between Neuroprotective Activity, Polyphenols and Flavonoids Content, and Antioxidant Activity of the Herbal Extracts

The correlation between neuroprotective activity, polyphenols and flavonoids content, and antioxidant activity of the herbal extracts was determined through Pearson‟s correlation analysis using SPSS statistical software, version 25.0 (SPSS Inc., USA). The concentrations range tested for the herbal extracts in each analysis were similar to prior tests accordingly. The extracts of *P. betle* were tested at 0.001–1 µg/mL, whereas *C. ternatea* were tested at 1–100 µg/mL for the neuroprotective analysis. As for TPC, the extracts of both herbs were examined at concentrations ranged from 0–1.0 g/L. Meanwhile for TFC, both of the herbs were tested at concentrations ranged from 0–0.5 g/L.On the other hand, the extracts of *P. betle* were tested at 10–200 and 1–100 µg/mL for DPPH and ABTS respectively, wheareas *C. ternatea* at 100–1000 µg/mL for both of the antioxidant test. The results were presented in the value of correlation coefficient (*r*), where $p<0.05$ was considered significant and $p<0.01$ was highly significant.

3.6 Statistical Analysis

All experiments were repeated using three different sets of freshly prepared herbal extracts. Each of the sets was carried out in triplicate and the results were expressed as mean ± standard errors mean (SEM). Data were analysed using SPSS statistical software, version 25.0 (SPSS Inc., USA). The statistical tests in current study include one-way and two-way ANOVA ($p<0.05$) and Pearson‟s correlation analysis.

CHAPTER 4: RESULTS

4.1 Yield of Extraction

In the present study, leaves of *Piper betle* and *Clitoria ternatea* were collected from home garden and subjected to cold extraction method using methanol and water to yield methanolic and water extracts. Table 4.1 demonstrates the yield percentage of methanolic and water extracts of both herbs.

Table 4.1: Yield of methanolic and water extracts of *P. betle* and *C. ternatea*.

Herbs	Materials	Weight (g)	Yield (%)
P. betle	Fresh leaves	550.0	
	Dried leaves powder	104.31	18.96
	Methanolic extract[a]	7.72	19.30
	Water extract[b]	1.69	8.43
C. ternatea	Fresh leaves	300.0	
	Dried leaves powder	76.22	25.0
	Methanolic extract[a]	8.38	20.95
	Water extract[b]	0.8302	4.15

[a] methanol extracts were prepared from 40 g of dried leaves powder. [b] water extracts were prepared from 20 g of fresh leaves.

From the data in Table 4.1, it shows that the yield of methanolic extracts of *P. betle* and *C. ternatea* were 19.30% and 20.95% respectively. As from water extractions, the yield of extract for *P. betle* or *C. ternatea* was lower compared to the methanolic extract with the values of 8.43% and 4.15% respectively. This suggests that

the resultant yield of extraction is highly dependent on its solvent polarity as it also determines the capability of the extract in extricating important bioactive compounds.

4.2 Neuroprotective Assay

4.2.1 Human Neuroblastoma SH-SY5Y Cell Line

As obtained from Figure 4.1 (a) and (b), human neuroblastoma SH-SY5Y cells were observed to be neuroblast-like, non-polarized bodies with few, multiple and short truncated processes. The cells tend to grow in clusters and may form clumps as they appear to grow on top of one another in the central region of a cell mass. At the edges of the clusters, cells extend short neurites. SH-SY5Y cultures include both adherent and floating cells at which both types were viable. The cultures also found to be continuously proliferating with doubling time of 27 to 48 hours. Note that the hardened background in the figures was the edges of the lens of microscope.

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

Dimethyl sulfoxide (DMSO) was selected to be used as solvent to dissolve methanolic extracts of the selected nervine herbs. The purpose of this test was to identify the toxicity effect of DMSO towards human neuroblastoma SH-SY5Y cells and to determine the concentration at which it does not toxic to the cell. Hence, range of DMSO concentration from 0.1–100% was added to SH-SY5Y cells, incubated for 24 hours and the cell viability was assessed using MTT assay. It was expected that SH-SY5Y cells react towards DMSO in concentration-dependent manner, in which, DMSO causes greater cell death at higher concentrations as depicted in Figure 4.2. Raw data of the toxicity effect of DMSO towards SH-SY5Y cells was demonstrated in Appendix B1.

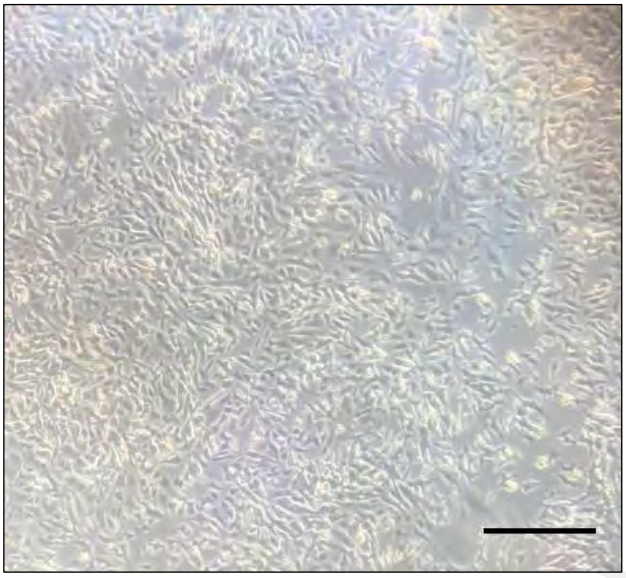

Figure 4.1: (a) SH-SY5Y cells observed under inverted microscope. Scale bar: 50 µm.

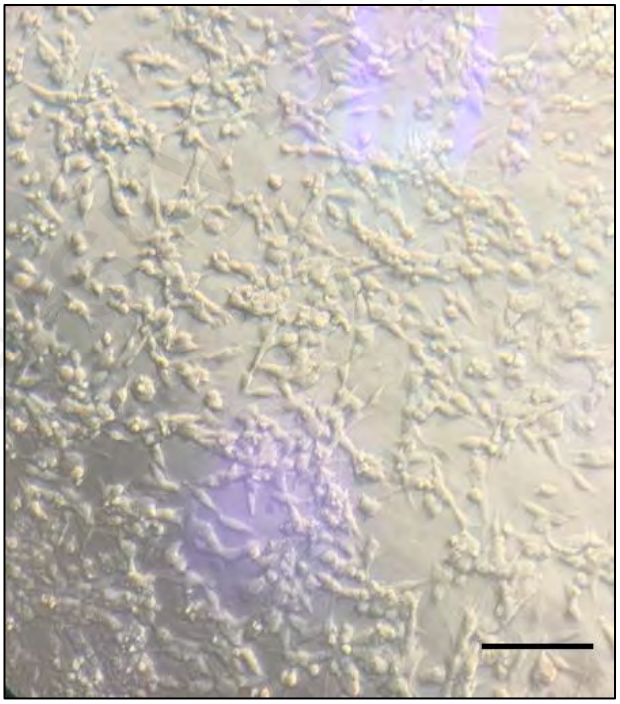

Figure 4.1: (b) SH-SY5Y cells observed under inverted microscope. Scale bar: 100 µm.

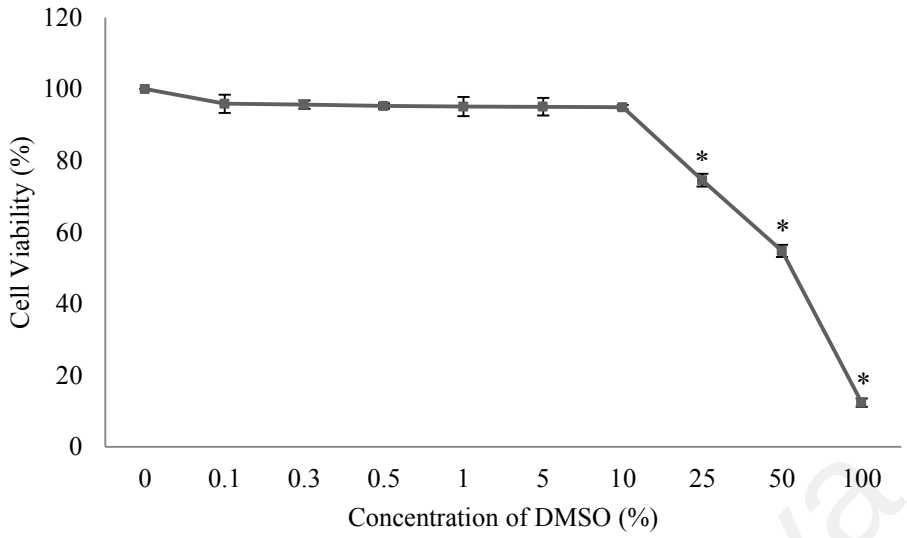

Figure 4.2: Toxicity effect of DMSO (%) towards SH-SY5Y cells. DMSO concentration range from 0.1–100% was added to SH-SY5Y cells, incubated for 24 hours and the cell viability was assessed using MTT assay. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to untreated cell group.

Figure 4.2 shows that the percentage of SH-SY5Y cell viability decreased as the concentration of DMSO increased. Cell viability started to decreased when the concentration of DMSO used is >10%. The lowest percentage of cell viability was found to be 12.39±2.03% at the highest concentration of 100% of DMSO showing that this concentration was very toxic to SH-SY5Y cells. However, there are no prominent differences in cell viability recorded at concentration of 0.1 to 10% of DMSO. The cell viability with 0.1 and 10% of DMSO are 95.88±4.44 and 94.91±1.13% respectively. This indicates that these lower concentrations of DMSO do not give apparent effect to the growth of the SH-SY5Y cells.

In order to determine the concentration of DMSO to be used in the serial dilution of methanolic extracts, the maximal concentration that did not exert any toxicity effects towards the SH-SY5Y cells was selected. Thus, methanolic extracts of the herbs will be diluted in 10% of DMSO resulting in final concentration of 0.475% per well in neuroprotective assay. 0.475% is the final concentration of DMSO in a well after being

in a series of dilution with the extracts. It was obtained through the formula $M_1V_1 = M_2V_2$.

4.2.3 Assessment of Toxicity of Hydrogen Peroxide towards SH-SY5Y Cells

Besides DMSO, toxicity test on hydrogen peroxide (H_2O_2) was also carried out to ensure the consistency of its toxicity in inducing cell death during the assessment of the neuroprotective effects of the nervine herbs in the present study. The aim of this assay was mainly to determine the concentration of H_2O_2 that provides 50% cell death (depicted as IC_{50} value) towards SH-SY5Y cells. Thus, for the toxicity assessment, seeded SH-SY5Y cells were exposed to a range of concentrations (25–500 μM) of H_2O_2 and cell viability was calculated using MTT assay after 24 hours of incubation. H_2O_2 was diluted in basic DMEM/F12 media without the supplementation of foetal bovine serum (FBS) in order to achieve desired concentrations, as the presence of catalase enzyme in FBS might deters the activity of H_2O_2 (Halliwell *et al.*, 2000).

Results were presented as the percentage of cell viability against concentration of H_2O_2. Raw data of toxicity effect of H_2O_2 towards SH-SY5Y cells was illustrated in Appendix B2. It was expected that H_2O_2 provides higher cell inhibition at higher concentrations. As shown in Figure 4.3, the results demonstrated that the percentage of cell viability drops with the increment of H_2O_2 concentration.

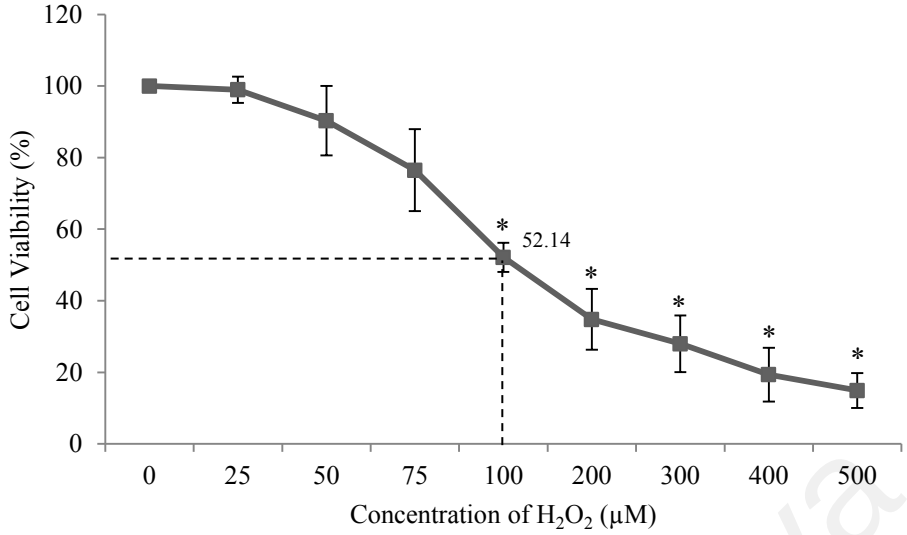

Figure 4.3: Toxicity effect of H_2O_2 towards SH-SY5Y cells. SH-SY5Y cells were exposed to a range of concentration (25–500 μM) of H_2O_2 and cell viability was calculated using MTT assay after 24 hours of incubation. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to untreated cell group.

This clearly shows that H_2O_2 induces cell death in SH-SY-5Y cells in concentration-dependant manner. The percentage of cell viability is gradually inhibited as the concentration of H_2O_2 increases. Highest concentration of H_2O_2 (500 μM) exhibited the most toxic effect towards the SH-SY5Y cells with 14.92±4.90% cell viability recorded. On the other hand, lowest concentration of H_2O_2 (25 μM) provided the least toxic effect with 98.98±3.66% cell viability. The IC_{50} value that depicted the concentration of H_2O_2 at which induced 50% of cell death of 47.86±4.10% was observed when cells are exposed to the H_2O_2 at concentration of 100 μM. Thus, the IC_{50} value of H_2O_2 towards SH-SY5Y cells is 100 μM and was selected for further neuroprotective assay.

4.2.4 Assessment of Neurotoxicity Effect of the Herbal Extracts on SH-SY5Y Cells

Prior to neuroprotective assay, toxicity test of the herbal extracts towards SH-SY5Y cells is equally important in optimizing possible parameters for the neuroprotective assay. It is also to ensure that the range of concentration of the herbal extracts used in the assay is safe and not harmful to the cells.

Thus, for the neurotoxicity test of the extracts towards SH-SY5Y cells, a range of both herbal extracts concentration (1–100 µg/mL) was added to the SH-SY5Y cells and incubated for 24 hours at 37°C before the cell viability was assessed using MTT assay. Dose response curve demonstrating the effect of various concentrations of *P. betle* and *C. ternatea* extract towards SH-SY5Y cell viability are presented in Figure 4.4 and 4.5 respectively. Raw data of the neurotoxicity effect of the extracts towards SH-SY5Y cells was demonstrated in Appendix B3.

Overall results show that the cell viability of SH-SY5Y cells reacted towards both of the herbal extracts in a concentration-dependent manner. It was found that both methanolic and water extracts of *P. betle* were toxic towards SH-SY5Y cells starting at concentration of 10 to 100 µg/mL. Hence lower range of *P. betle* concentration was tested, and results obtained from the MTT assay revealed that both methanolic and water extracts of *P. betle* were not toxic to the SH-SY5Y cells at the range of 0.01 to 1 µg/mL (Figure 4.5). The percentage of cell viability, however, was observed decreases as the concentration of *P. betle* extracts used increases. Lowest concentration of *P. betle* methanolic extract (0.01 µg/mL) provided highest cell viability at 139.90±5.58%. Meanwhile, lower cell viability at 57.43±4.94% was recorded at its highest concentration of 100 µg/mL. Similar pattern was observed for *P. betle* water extract that gives 96.18± 3.22% and 59.11±0.59% of cell viability at its lowest and highest concentration respectively. This shows that *P. betle* extracts provide neurotoxicity at

higher concentrations towards SH-SY5Y cells. The cell toxicity was believed to be highly attributable to the pro-oxidant effect of *P. betle* extracts that induces cell damage and death. Generally, concentrations of *P. betle* extracts lower than 1 µg/mL was proved not toxic and is considered safe to be used in the neuroprotective assay.

As for *C. ternatea*, the results (Figure 4.5) demonstrated that both of methanolic and water extracts showed no toxicity towards the SH-SY5Y cells at concentration range from 1 to 100 µg/mL. There are also no significant differences observed on the percentage of cell viability between the concentrations. At the lowest concentration tested (1 µg/mL), 98.57±2.24% and 97.45±5.65% of cell viability was recorded for methanolic and water extract accordingly. Meanwhile at the highest concentration (100 µg/mL), methanolic extract provided 98.26±2.81%, and addition of water extract to the cells recorded 100.38±1.53% cell viability. These findings show that concentration range from 1 to 100 µg/mL of *C. ternatea* extracts was not toxic to SH-SY5Y cells and is suitable to be utilized in neuroprotective ass

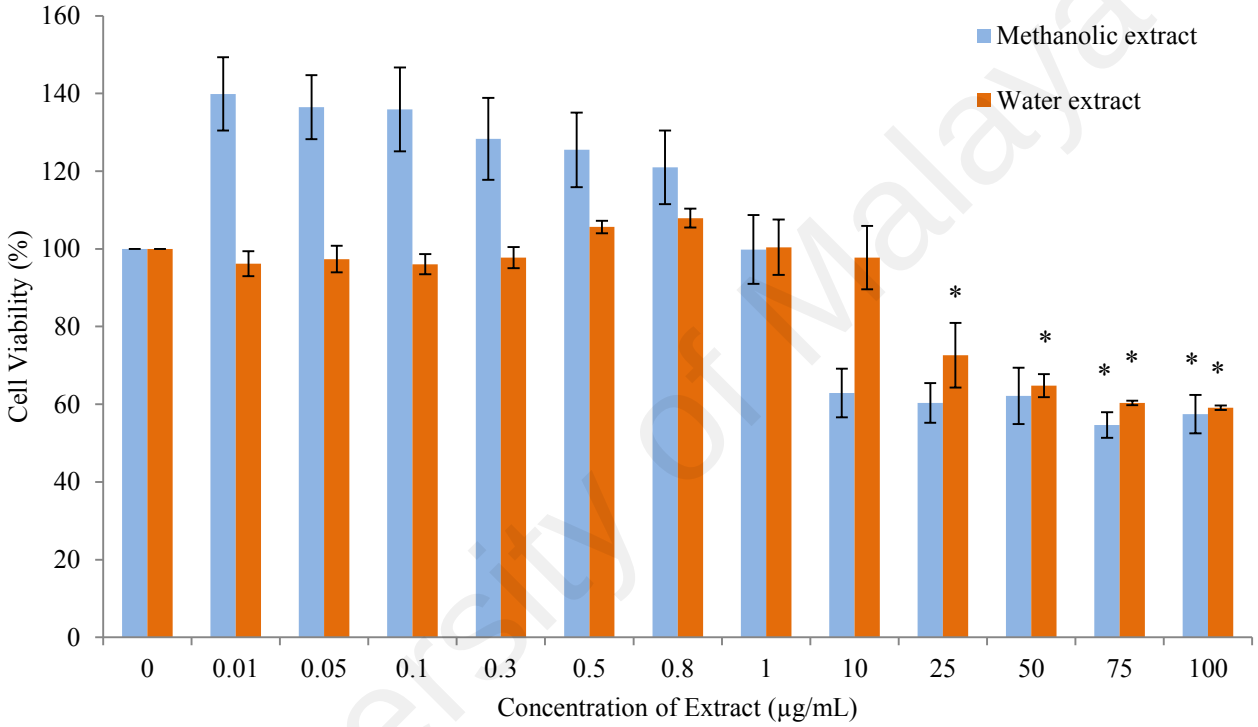

Figure 4.4: Neurotoxicity effects of methanolic and water extracts of *P. betle* towards neuroblastoma SH-SY5Y cells. Extracts with concentration of 0.01–100 µg/mL were added to SH-SY5Y cells, left for incubation for 24 hours and the percentage of cell viability was assessed using MTT assay. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to untreated cell group.

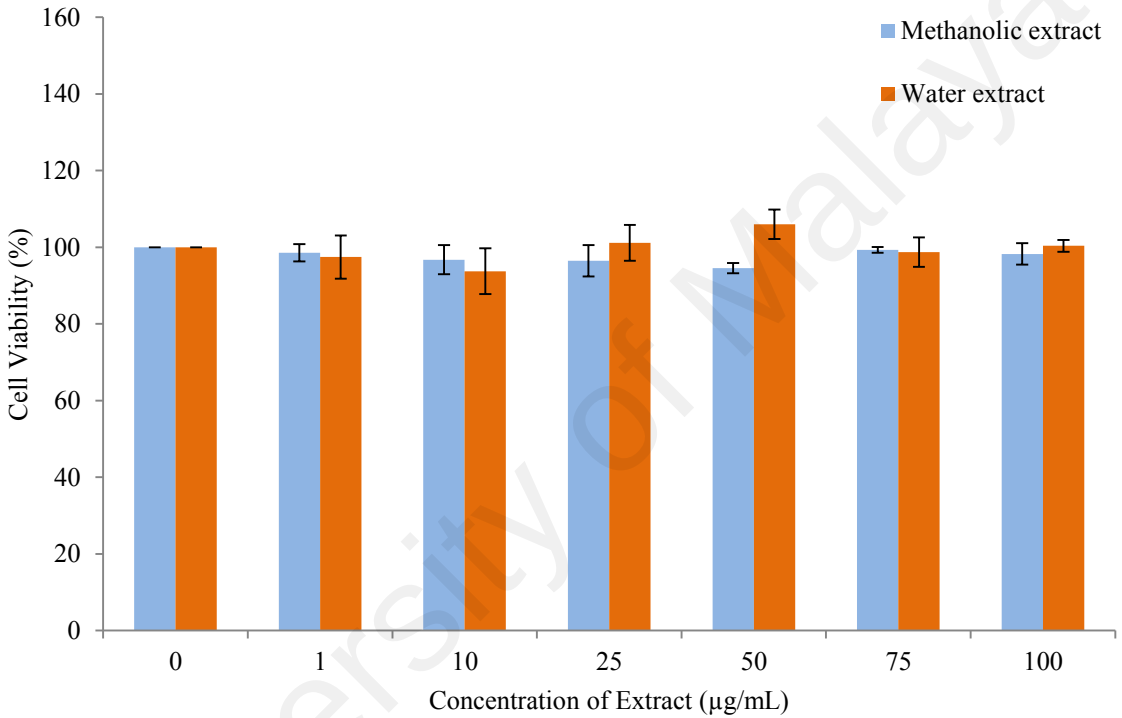

Figure 4.5: Neurotoxicity effects of methanolic and water extracts of *C. ternatea* towards neuroblastoma SH-SY5Y cells. Extracts with concentration of 1–100 µg/mL were added to SH-SY5Y cells, left for incubation for 24 hours and the percentage of cell viability was assessed using MTT assay. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to untreated cell group.

4.2.5 Assessment of Neuroprotective Effect of the Herbal Extracts on H_2O_2-induced Cell Damage in SH-SY5Y Cells

Neuroprotective effect of herbal extracts against H_2O_2-induced cell death was evaluated by the MTT assay at the end of incubation period. Seeded SH-SY5Y cells were either pre-treated or post-treated with extracts. The aim of categorizing the treated cells into pre- and post-treatment group was to determine if the herbs worked best in pre- or post-application. This also helps in providing information on the suitability of the herbs as prevention or treatment step in managing neurodegeneration diseases. For the pre-treatment group, SH-SY5Y cells in 96-well plate were treated with herbal extracts for 24 hours before being exposed to H_2O_2 (at the concentration that induced 50% cell death as was determined in Section 4.2.3) and for the post-treatment group, cells were treated with H_2O_2 for 24 hours followed by the incubation with extracts for further 24 hours at 37°C in a 5% CO_2 humidified incubator.

The neuroprotective effects for both extracts of *P. betle* and *C. ternatea* were presented in Figure 4.6–4.9, whereas the raw data was illustrated in Appendix B4. Results were presented as percentage of SH-SY5Y cell viability in conjunction with herbal extracts in various range of concentration. Neuroprotective effect exerted by the extracts was compared to the control group with H_2O_2 but without addition of any extract. Methanol was used in methanolic extract serial dilution meanwhile water extract was diluted using distilled water. Besides that, a statistical analysis was also conducted to compare the neuroprotective capability between methanolic and water extract in both herbs. The results were presented in Appendix B4 and the comparison between the two types of extract was made through the marginal means of the neuroprotective activity.

Generally, addition of extracts of *P. betle* and *C. ternatea* to H_2O_2-induced damage in SH-SY5Y cells promotes significant increment in the number of viable cells

compared to the cells exposed to H_2O_2 only (without addition of extracts). Neuroprotective effect exerted by methanolic and water extract of *P. betle* showed greater potential at lower concentrations. Figure 4.6 and 4.7 demonstrated reduction in cell viability at higher concentrations and this is in an agreement with the findings in toxicity test of methanolic and water extract of *P. betle*.

At the lowest concentration tested (0.001 µg/mL), methanolic extract of *P. betle* provided highest cell viability at 150.56±12.36% and 153.03±18.24% in both pre-treatment and post-treatment accordingly. Meanwhile for water extract, pre-treatment group recorded 124.62±2.75% and post-treatment group recorded 139.98±7.26% of cell viability at the lowest concentration of 0.001 µg/mL.

At the highest concentration of 1 µg/mL, pre- and post-treated group with methanolic extract contribute to lower cell viability of 119.34±9.54% and 89.68±18.59%, and addition of water extract produced 96.21±1.78% and 126.32±7.54% of cell viability as compared to the lowest concentration. This finding proved that *P. betle* extract induced better neuroprotective effect at lower concentrations.

Besides that, the statistical analysis between methanolic and water extract of *P. betle* revealed that its methanolic extract provided highest marginal means of cell viability by its pre-treatment group indicating greater neuroprotection towards SH-SY5Y cells followed by its post-treatment group of water extract (Appendix B4). This also shows that methanolic extract provided better neuroprotective effect among the pre-treatment groups and water extract recorded higher neuroprotective capability among the post-treatment group for *P. betle* extracts.

On the other hand, addition of extracts of *C. ternatea* at higher concentration towards SH-SY5Y cells results in better neuroprotective activity and the effects are illustrated in Figure 4.8 and 4.9. In pre-and post-treatment group with methanolic extract at lowest concentration (1 µg/mL), cell viability was recorded at 143.63±35.14%

and 111.28±10.08%, meanwhile addition of water extract recorded 115.99±25.87% and 96.59±9.42% of cell viability in the respective group.

At the highest concentration tested (100 µg/mL), pre- and post-treatment with presence of methanolic extract of *C. ternatea* resulted in 188.14±24.49% and 139.36±16.71% of cell viability respectively and in presence of water extract, cell viability was recorded at 96.16±15.20% and 106.53±3.81%, respectively. For the comparison between methanolic and water extract of *C. ternatea*, higher marginal means provided by methanolic extract indicates that this extract generates better neuroprotective potential towards damaged SH-SH5Y cells in both pre- and post-treatment group. Meanwhile in the comparison of pre- and post treatment group, both methanolic and water extract of *C. ternatea* showed higher marginal means in pre-traetment group (Appendix B4).

As a whole, the results revealed that *P. betle* prone to exert better neuroprotective capability at lower concentrations as it provides higher cell viability at lower range of concentrations, whereas for *C. ternatea*, the neuroprotective activity increases as the concentration increases. However, the methanolic extract of *P. betle* recorded higher neuroprotective effect in its pre-treatment group and water extract in its post-treatment group. Meanwhile for *C. ternatea*, the methanolic extracts provided better neuroprotection and pre-treatment groups showed higher neuroprotective capability as compared to the post-treatment group.

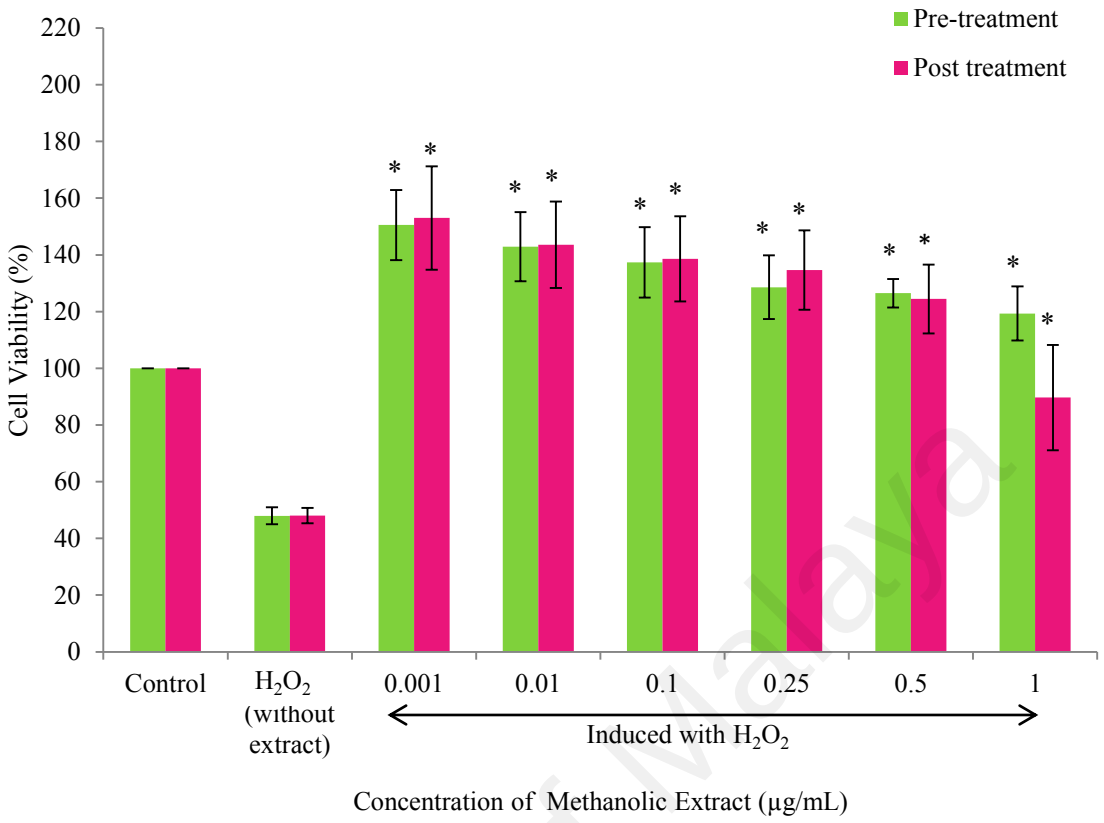

Figure 4.6: Neuroprotective effects of methanolic extract of *P. betle* against H_2O_2-induced cell damaged in neuroblastoma SH-SY5Y cells. Pre-treatment group: SH-SY5Y cells in 96-well plate were treated with extracts for 24 hours before being exposed to H_2O_2 and; post-treatment group: cells were treated with H_2O_2 for 24 hours followed by the incubation with extracts for further 24 hours. Cell viability was evaluated by the MTT assay. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to cell group treated with H_2O_2 (without addition extract).

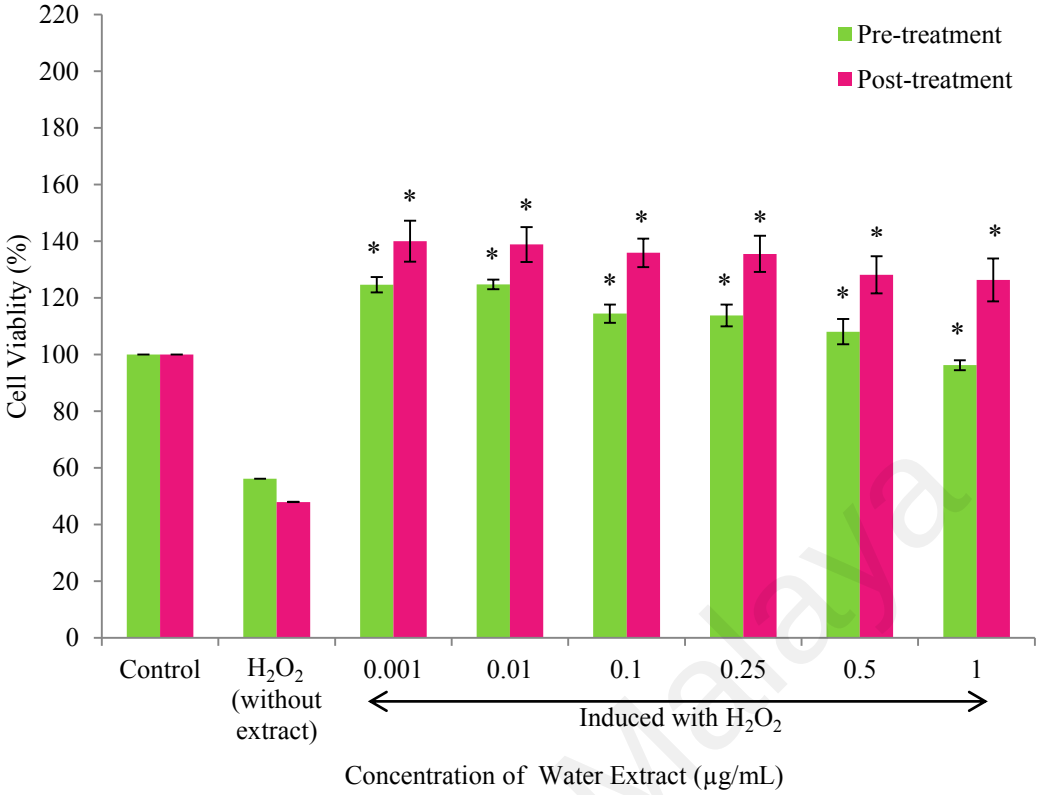

Figure 4.7: Neuroprotective effects of water extract of *P. betle* against H_2O_2-induced cell damaged in neuroblastoma SH-SY5Y cells. Pre-treatment group: SH-SY5Y cells in 96-well plate were treated with extracts for 24 hours before being exposed to H_2O_2 and; post-treatment group: cells were treated with H_2O_2 for 24 hours followed by the incubation with extracts for further 24 hours. Cell viability was evaluated by the MTT assay. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to cell group treated with H_2O_2 (without extract).

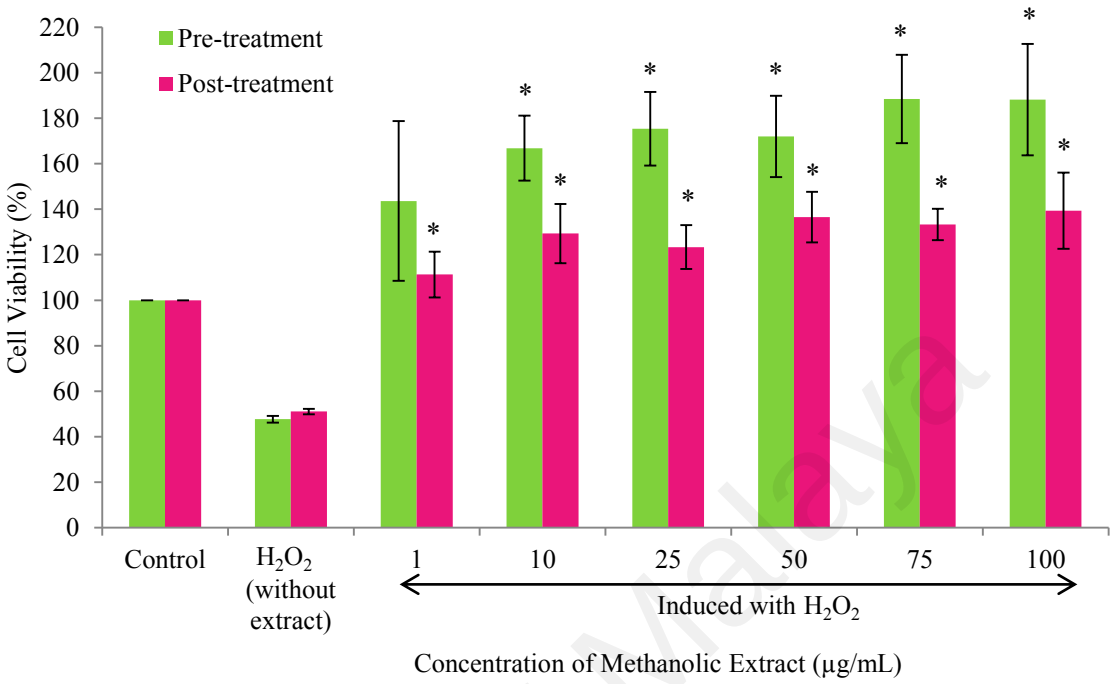

Figure 4.8: Neuroprotective effects methanolic extract of *C. ternatea* against H_2O_2-induced cell damaged in neuroblastoma SH-SY5Y cells. Pre-treatment group: SH-SY5Y cells in 96-well plate were treated with extracts for 24 hours before being exposed to H_2O_2 and; post-treatment group: cells were treated with H_2O_2 for 24 hours followed by the incubation with extracts for further 24 hours. Cell viability was evaluated by the MTT assay. Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to cell group treated with H_2O_2 (without extract).

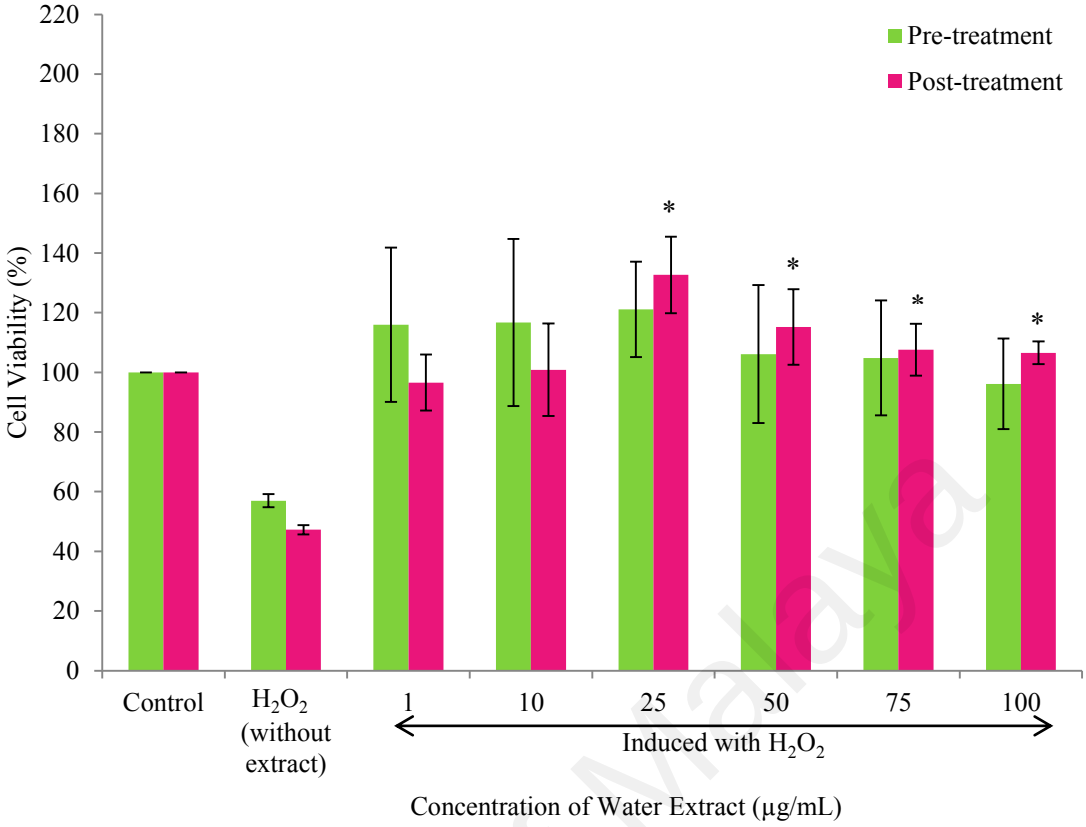

Figure 4.9: Neuroprotective effects of water extract of *C. ternatea* against H_2O_2 – induced cell damaged in neuroblastoma SH-SY5Y cells. Pre-treatment group: SH-SY5Y cells in 96-well plate were treated with extracts for 24 hours before being exposed to H_2O_2 and; post-treatment group: cells were treated with H_2O_2 for 24 hours followed by the incubation with extracts for further 24 hours. Cell viability was evaluated by the MTT assay.Data is presented as mean ± SEM (n=3), * denotes $p<0.05$ compared to cell group treated with H_2O_2.

4.3 Polyphenols and Flavonoids Content of the Herbal Extracts

4.3.1 Total Phenolic Content

Total phenolic content (TPC) for both methanolic and water extracts of *P. betle* and *C. ternatea* was determined using Folin- Ciocalteau method with gallic acid as standard. This assay based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes. In other words, it is based on the reducing power of phenolic hydroxyl groups that react with Folin-Ciocalteu phenols reagents to form chromogens, which are blue complexes that can be detected spectrophotometrically. Thus, formation of high intensity of blue colour indicates presence in high phenolic content (Saxena & Saxena, 2013). Upon phomoteric analysis, the content of phenolics was evaluated from the regression equation of the calibration curve plotted, which was illustrated in Appendix B5 (R^2= 0.9831, y= = 1.0587x + 0.066) and TPC of the extracts was expressed in GAE as miligrams per gram of extract (mg GAE/g extract). Table 4.2 depicts the results obtained for TPC of methanolic and water extracts of both herbs, and raw data was demonstrated in Appendix B5.

Table 4.2: Total phenolic content of *P. betle* and *C. ternatea* extracts.

Herbs	Extracts	Concentration of TPC (mg of GAEs/g of extracts)[a]
P. betle	Methanolic	251.82±7.07
	Water	219.52±3.62
C. ternatea	Methanolic	145.69±1.66
	Water	114.94±2.16

[a] Data are represented as mean ± SEM from three different sets of which was analysed in triplicate.

Generally, both methanolic and water extract of *P. betle* showed higher phenolic content in comparison with extracts of *C. ternatea*. TPC of methanolic and water extract of *P. betle* was recorded at 251.82±7.07 and 219.52±3.62 mg GAE/g extract respectively. TPC of methanolic and water extract of *C. ternatea*, was found to be lower than *P. betle* with the value of 145.69±1.66 and 114.94±2.16 mg GAE/g extract respectively. The observed descending order of phenolic content for the samples can be arranged as methanolic extract of *P. betle*> water extract of *P. betle* >methanolic extract of *C. ternatea*> water extract of *C. ternatea*.

4.3.2 Total Flavonoid Content

Flavonoids are well-known antioxidant components of medicinal plants and possess a wide variety of chemical and biological activity including radical scavenging ability that plays important role in neuroprotection (Miliauskas *et al.*, 2004). Therefore, the total contents of flavonoid of the extracts were assessed using aluminium chloride ($AlCl_3$) colorimetric assay with quercetin as standard. The principle behind this method is that $AlCl_3$ forms acid complexes with the ketogroups, hydroxyl group or dihydroxyl group of flavonoids, by which, can be detected spectrophotometrically. The implication of quercetin as standard has been reported to be suitable reference for determination of flavonoid content in plant extract (Bhaigyabati *et al.*, 2014). Thus, standard quercetin solution of various concentration was used to plot a calibration curve illustrated in Appendix A. Total flavonoid content (TFC) was evaluated from the regression equation of the calibration curve (R^2 = 0.9951, y = 19.957x + 0.0255), expressed in QE as milligrams per gram of extract (mg QE/g extract). Table 4.3 shows the TFC of methanolic and water extracts of *P. betle* and *C. ternatea*. Raw data on the TFC of the extracts was shown in Appendix B5.

Table 4.3: Total flavonoid content of extracts of *P. betle* and *C. ternatea*.

Herbs	Extracts	Concentration of TFC (mg of QEs/g of extracts)[a]
P. betle	Methanolic	22.27±0.23
	Water	15.15±0.36
C. ternatea	Methanolic	15.65±0.20
	Water	29.24±0.08

[a] Data are represented as mean ± SEM from three different sets of which was analysed in triplicate.

According to the data demonstrated in Table 4.3, TFC of methanolic and water extract of *P. betle* was recorded 22.27±0.2 and 15.15±0.36 mg QE/g extract respectively, meanwhile methanolic and water extract of *C. ternatea* yielded 15.65±0.20 and 29.24±0.08mg QE/g extract of TFC accordingly. *P. betle* showed higher value of TFC for in methanolic extract at about 1.5 fold higher as compared to the water extract. As for *C. ternatea*, the water extract contained 1.9 fold higher of TFC than in methanolic extract. Overall, water extract of *C. ternatea* even recorded the highest content of flavonoids. In descending order, TFC of the herbal extracts can be presented as water extract of *C. ternatea*> methanolic extract of *P. betle*> methanolic extract of *C. ternatea*> water extract of *P. betle*.

4.4 Antioxidant Activity of the Herbal Extracts

4.4.1 1, 1-diphenyl-1-picrylhydrazyl Radical Scavenging Activity

1, 1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay is a simple, rapid and convenient method to determine the antioxidant potential of plant extract. In fact, it has also been widely used to quantify antioxidant capacity of complex biological systems in many years (Esmaeili *et al.*, 2015).

The principle of this assay was based on the competency of any antioxidant molecule to donate hydrogen atom to the DPPH radical. It then resulted in the reduction of DPPH radical (1, 1-diphenyl-1-picrylhydrazyl) to its 1, 1-diphenyl-1-picrylhydrazine molecule, in which can be observed by the change of the colour from purple to yellow. This colour conversion or reduction can then be easily analysed at 517 nm spectrophotometrically. Hence, reduction in absorbance reading denotes greater antioxidant capacity in plant extract (Molyneux, 2004). The DPPH radical scavenging activity of the herbal extracts was presented in IC_{50} value; which is the concentration of the extract that inhibits the initial DPPH free radical concentration to 50% (Table 4.4). Comparison was done with ascorbic acid as standard. Raw data was demonstrated in Appendix B6.

According to the findings demonstrated in Table 4.4, ascorbic acid recorded the highest DPPH radical scavenging activity with an IC_{50} value of 13.45±0.31µg/mL. In collation between the herbs, higher scavenging activity was found in extracts of *P. betle* as compared to extracts of *C. ternatea*. Water extract of *P. betle* recorded highest DPPH scavenging activity followed by its methanolic extract with the IC_{50} value of 117.16±0.086 and 147.98±0.48 µg/mL respectively. As compared to the standard, ascorbic acid provided higher free radical scavenging activity than the water extract of *P. betle* at approximately 11 folds higher.

Meanwhile for extracts of *C. ternatea* extracts, lower scavenging activity was observed with IC_{50} values of 319.42±0.43 and 415.63±1.22 µg/mL for water and methanolic extract accordingly. In descending order, the free radical scavenging capacity of the herbal extracts can be described as ascorbic acid> water extract of *P. betle*> methanolic extract of *P. betle*> water extract of *C. ternatea*>methanolic extract of *C. ternatea*. Based on the cumulative data, it is clear that *P. betle* possess higher DPPH radical scavenging activity than *C. ternatea*. In comparison between the extracts, water extracts of both herbs provided higher DPPH radical scavenging activity.

Table 4.4: DPPH free radical scavenging activity of ascorbic acid, *P. betle* and *C. ternatea*.

Herbs/ Standard	Extracts	IC_{50} Value (µg/mL)[a]
Ascorbic Acid		13.45±0.31
P. betle	Methanolic	147.98±0.48
	Water	117.16±0.086
C. ternatea	Methanolic	415.63±1.22
	Water	319.42±0.43

[a] Data are represented as mean ± SEM from three different sets of which was analysed in triplicate.

4.4.2 2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Activity

2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay is a method used to evaluate antioxidant activity of both hydrophilic and lipophilic substances (Jaiswal *et al.*, 2014). The principle was based on the oxidation of 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) by potassium persulfate ($K_2S_2O_8$) resulting in the formation of 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation and further quenching by an antioxidant. The addition of hydrogen donating molecules by antioxidants reduced ABTS cation radicals, which are blue-green in colour to form $ABTSH^+$. Hence, the antioxidant capacity of the sample can be approximated by the decrease in the blue-green coloration at 734 nm (Miller & Rice-Evans, 1997). Table 4.5 summarized the ABTS radical scavenging activity (depicted with IC_{50} values) of the herbal extracts and ascorbic acid served as the standard. Raw data on the ABTS free radical scavenging activity of the extracts was depicted in Appendix B6.

Table 4.5: ABTS free radical scavenging activity of ascorbic acid, *P. betle* and *C. ternatea*.

Herbs/ Standard	Extracts	IC_{50} Value (µg/mL)[a]
Ascorbic Acid		51.23±0.59
P. betle	Methanolic	81.94±0.51
	Water	130.82±0.27
C. ternatea	Methanolic	399.67±0.44
	Water	414.59±0.56

[a] Data are represented as mean ± SEM from three different sets of which was analysed in triplicate.

Ascorbic acid shows the lowest value of IC_{50} at 51.23±0.59 µg/mL indication that it is the most potent antioxidant with the highest ABTS free radical scavenging activity. Generally, methanolic and water extracts of *P. betle* showed better ABTS radical scavenging capacity than the extracts of *C. ternatea*. Among all the extracts, methanolic extract of *P. betle* shows highest antiradical capacity followed by its water extract with the IC_{50} value of 81.94±0.51and 130.82±0.27 µg/mL respectively. The difference between ABTS scavenging activity recorded by the standard and methanolic extract of *P. betle* was estimated to be 1.6 fold.

Meanwhile the methanolic and water extracts of *C. ternatea* recorded IC_{50} values of 399.67±0.44 and 414.59±0.56 µg/mL accordingly. In descending order, the ABTS radical scavenging capacity of the herbal extracts can be represented as ascorbic acid> methanolic extract of *P. betle*> water extract *P. betle*> methanolic extract of *C. ternatea*> water extract of *C. ternatea*. In conclusion, *P. betle* exerted better ABTS radical scavenging activity as compared to *C. ternatea* and methanolic extract of both herbs are more potent as free radical scavenger compared to the water extract.

4.5 Correlation between Neuroprotective Activity, Polyphenols and Flavonoids Content, and Antioxidant Activity of the Herbal Extracts.

4.5.1 Correlation of Neuroprotective Activity with Polyphenols and Flavonoids Content, or Antioxidant Activity of the Herbal Extracts.

The aim of the correlation analysis was to investigate the relationship between polyphenols and flavonoids content, and antioxidant activity in relevance to neuroprotective activity possessed by the herbal extracts. Researchers have been claimed that antioxidants might play important role in neuroprotection as they help in combatting free radical that contributes to neurodegeneration. Meanwhile, phenolic and flavonoid are two among many phytochemicals that carry significant antioxidant potential. Hence, this analysis not only to see whether there is or there is no interrelation between the variables, but also to see what kind and how strong the connection would be. The results of the analysis were presented in correlation coefficient (*r*), assessed using Pearson‟s correlation analysis. The $p<0.05$ was considered significant, whereas $p<0.01$ was highly significant (Table 4.6).

The interpretation of correlation coefficient takes on values ranging from +1 to -1. Value 0 means no linear relation and, symbol “+” and “-” indicates a positive and negative linear relationship respectively. Values between 0 and 0.3 indicate a weak, 0.3 and 0.7 indicate a moderate and, 0.7 and 1.0 indicate strong relationship magnitude (Cui *et al.*, 2005).

Current findings on the neuroprotective capacity *P. betle* extracts showed strong magnitude and statistically linear relationship with polyphenols and flavonoids content, and antioxidant activity. However, negative correlation was observed in methanolic extract of *P. betle*. Highest magnitude of correlation was recorded by the neuroprotective activity in relation with ABTS scavenging capacity at a very significant

r value of -0.978. Weakest correlation in methanolic extract of *P. betle* was observed on the relationship of neuroprotective activity with TFC, *r* value of -0.833.

Meanwhile for water extract of *P. betle*, inverse relationship was shown only by the pre-treatment group. Relationship of neuroprotective with DPPH scavenging activity was found to have strongest *r* value at -0.990 and with TFC was the weakest at -0.639. Apart from that, neuroprotective activity of post-treatment group of water extract of *P. betle* showed statistically positive linear relationship with TPC, TFC, DPPH and ABTS activity. Significant highest *r* value was recorded in the relationship with TFC at 0.964, whereas the lowest with DPPH at 0.81

Table 4.6: Correlation of neuroprotective activity with polyphenols and flavonoids content, and antioxidant activity of the herbal extracts.

Herbs	Extracts	Correlation Coefficient (*r* value)[a]							
		Neuroprotective vs TPC		Neuroprotective vs TFC		Neuroprotective vs DPPH		Neuroprotective vs ABTS	
		Pre[b]	Post[c]	Pre[b]	Post[c]	Pre[b]	Post[c]	Pre[b]	Post[c]
P. betle	Methanolic	-0.957**	-0.884*	-0.973*	-0.833*	-0.906*	-0.995*	-0.978**	-0.925**
	Water	-0.956**	0.900*	-0.639	0.964**	-0.990**	0.816	-0.955**	0.859
C. ternatea	Methanolic	0.834*	0.790	0.931**	0.843*	0.901*	0.813*	0.889*	0.843*
	Water	-0.806	0.204	-0.663	0.435	-0.848*	0.244	-0.879*	0.179

[a] Data is presented in r value, * denotes $p<0.05$ and ** denotes $p<0.01$. [b] Pre-treatment group of respective extract. [c] Post-treatment group of respective extract. TPC; Total Phenolic Content; TFC: Total Flavonoid Content; DPPH: 1, 1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; ABTS: 2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity.

On the other hand, correlation analysis in methanolic extract of *C. ternatea* showed direct relationship for both pre- and post-treatment group. Significant correlation magnitude was observed highest in the interrelation of neuroprotective activity and TFC by the pre-treatment group with *r* value of 0.931, meanwhile the least was not significant, with TPC at coefficient of 0.790 by the post-treatment group. Even so, in water extract of *C. ternatea*, inverse relationship was found on the pre-treatment group. Relationship between neuroprotective and ABTS provided the strongest correlation with r value of -0.879, and TFC recorded the weakest at -0.663. This finding was contrary with the post-treatment group ofwater extract of *C. ternatea*, as positive correlation was observed with *r* value ranging from 0.179 to 0.435 for ABTS and TFC respectively. However, the *r* value for *C. ternatea* water extract was considered to have low to moderate magnitude.

As a whole, the relationship of neuroprotective activity with TPC, TFC, DPPH and ABTS was observed to be vary among the extracts of the two herbs. *P. betle* showed strong inverse relationship except for the pre-treatment group of its water extract that was found to have direct relationship. As for *C. ternatea*, the neuroprotective activity was found to have positive correlation except for the pre-treatment group of its water extract that showed negative correlation towards all of the variables. Current findings also demonstrated that the correlations were observed to have high magnitude except for the post-treatment group of water extract of *C. ternatea*.

4.5.2 Correlation of Polyphenols and Flavonoids Content with Antioxidant Activity of the Herbal Extracts.

Earlier studies suggested that the biological actions of phenolic and flavonoids are related to their antioxidant activity (Saxena & Saxena, 2013). Thus, the aim of this analysis was to investigate the relationship between the variables based on the results obtained for both herbs. The results were presented in *r* value and depicted in Table 4.7. Similarly, $p<0.05$ was considered significant, whereas $p<0.01$ was highly significant (Table 4.7).

In general, the correlation between TPC or TFC with DPPH or ABTS was observed to have strong positive correlations for both *P. betle* and *C. ternatea*. For methanolic extract of *P. betle*, significant and highest *r* value was found in the relationship between TFC and ABTS scavenging activity at 0.970 and lowest was recorded between TFC and DPPH antiradical capacity at 0.842. Meanwhile for water extract of *P. betle*, strongest correlation was observed between TPC and ABTS with highly significant *r* value of 0.988. Weakest correlation recorded between TFC and DPPH at non-significant coefficient of 0.669. The *r* values mentioned for water extract of *P. betle* was observed to be the highest and lowest respectively, among the two herbs as well.

As for methanolic extract of *C. ternatea*, highest *r* value was recorded between TPC and ABTS, whereas lowest between TFC and DPPH at 0.987 and 0.955 accordingly. On the other hand, highest coefficient of 0.932 and lowest of 0.911 was observed between TFC and DPPH, and TPC and DPPH respectively for *C. ternatea* water extract. The *r* values obtained for extracts of *C. ternatea* are all significant. In a nutshell, the overall results demonstrated that polyphenols and flavonoids content of the herbal extracts were positively correlated with the antioxidant activity.

Table 4.7: Correlation of polyphenols and flavonoids content with antioxidant activity of the herbal extracts.

Herbs	Extracts	Correlation Coefficient (*r* value)[a]			
		TPC vs DPPH	TPC vs ABTS	TFC vs DPPH	TFC vs ABTS
P. betle	Methanolic	0.902*	0.959**	0.842*	0.970**
	Water	0.949**	0.988**	0.669	0.699
C. ternatea	Methanolic	0.955**	0.987**	0.948*	0.975*
	Water	0.911*	0.922**	0.932**	0.923**

[a] Data is presented in r value, * denotes $p<0.05$ and ** denotes $p<0.01$. TPC; Total Phenolic Content; TFC: Total Flavonoid Content; DPPH: 1, 1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity; ABTS: 2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity.

CHAPTER 5: DISCUSSIONS

5.1 Yield of Extraction

Extraction is a process at which it is an important step in the operation of phytochemical processing for the revelation of bioactive constituents from plant sources. Muruganandam *et al.* (2017) reported that extract yield percentage is highly attributable to some important parameters including quantity of material, quantity of solvent, temperature and extraction time.

In the present study, leaves of *Piper betle* and *Clitoria ternatea* were subjected to cold extraction method using methanol and diffusion with water to yield methanolic and water extract respectively. The latter is an extraction method whereby fresh samples were subjected to boiling with distilled water at required amount of samples, volume of water, temperature and period of time to obtain filtrates (Auddy *et al.*, 2003). The extraction methods opted were the most common method in most of the herbal preparation process. It is also based on the consideration of extraction methods that are affordable and environmentally friendly in terms of solvent and energy consumption.

One of the crucial considerations in extraction is that the ability to obtain extracts with high yield and with minimal changes to the functional properties of the extract. Extract yield and bioactivities of extract prepared using different extraction methods also have been reported to vary in several studies (Quispe Candori *et al.*, 2008). According to the data obtained, higher yield of methanolic extract was reported in both *P. betle* and *C. ternatea*. The higher yield of methanolic extract might be due to the property of methanol as the highest polarity compared to other solvent, resulting in the liberation of greater amount of bioactive compound (Sasidharan *et al.*, 2011; Yadav & Agarwala, 2011). On the other hand, Ibrahim *et al.* (2011) reported that leave samples are the best parts to be used that will produce high yield of bioactive compound. In this

study, the methanolic and water extracts of both *P. betle* and *C. ternatea* were prepared from the leaves part of the herbs.

5.2 Neuroprotective Assay

5.2.1 Toxicity Effect of DMSO towards SH-SY5Y Cells

DMSO was selected to be used in present work as solvent for extract dilution as it is one of the most commonly used solvents in cell culture due to its low toxicity to most cells (Pal *et al.,* 2012; Chen & Thibeault, 2014). It is a universal and important aprotic solvent that can solubilize a wide variety of either poorly soluble polar and non-polar molecule. Addition to its low toxicity at concentrations <10%, has led to its extensive use and widespread application (Galvao *et al.*, 2013).

In the present study, 10% DMSO (final concentration of 0.475% per well of the 96-well tissue culture plate) was chosen based on the results obtained at which this concentration was not toxic towards human neuroblastoma SH-SY5Y cells. A number of studies claimed that DMSO can be toxic towards certain cell but the specific concentration of DMSO used is often unreported. There are also a few reports on the effects of DMSO at low concentration causing toxicity in cell types other than neurons. However, based on literature survey and review by Galvao *et al.* (2013) reports on the toxicity effects of DMSO towards SH-SY5Y cells are not available up to date. A study done by Timm *et al.* (2013) reported that different cell types responded very differently to DMSO at various concentrations considering a factor related to various stimuli provided.

Generally, in cell culture, final concentration of 0.1% to 1% was reported non-toxic to most cell types. However, 0.5% final concentration of DMSO has been widely used and is recommended in cell culture. Although it has been reported that 1.4%

DMSO in cell culture medium does not affect cell growth, most studies suggest that presence of DMSO may significantly causes plasma membrane pore formation, alter the morphology and attachment of cells with significant reduction in cell viability in a dose dependent manner, and for cell culture, maximum final concentration of DMSO should be 0.5% (Schmidt *et al.,* 1989; Pal *et al.,* 2012). Previous study by Chen & Thibeault (2004) also reported that cells lost their ability at DMSO concentrations higher than a critical value; between 0.1% and 0.5%, because cells were permeabilised by DMSO. All this previous reported work justified the suitability of the final concentration of DMSO of 0.5% per well, which was selected and used in present study.

5.2.2 Toxicity Effect of Hydrogen Peroxide (H_2O_2) towards SH-SY5Y Cells

The utilization of H_2O_2 has been well known in many cell culture systems to induce cell damage and cytotoxicity (Chow *et al.*, 2005). The purpose of this assay was to evaluate a concentration of H_2O_2 at which it provides 50% cell death to SH-SY5Y cells. Even though high (>50 μM) concentration of H_2O_2 was claimed to be cytotoxic to a wide range of animal, plant and bacterial cells, some studies reported substantial variations in the concentrations of H_2O_2 determined to be cytotoxic can be found ranging from less than 10 μM to more than 1000 μM. This diversification on cytotoxic potency of H_2O_2 in cell culture can be highly attributable to some parameters including the cell type used, its iron content, length of exposure to H_2O_2, concentration of H_2O_2 used and the type of cell culture media utilized Halliwell *et al.*, 2000; Gulden *et al.*, 2010).

In this present study, the dilution was made using 30% H_2O_2 and was prepared fresh upon analysis as H_2O_2 is naturally degrades at fast rate (Halliwell *et al.*, 2000). Seeded SH-SY5Y cells in 96-well plate were exposed to H_2O_2 by directly adding the diluted H_2O_2 into the wells according to the concentrations made without removing any

media from the plate. This method has been claimed to be efficient in exposing cell culture with H_2O_2 by considering few factors including cell density and incubation period (Gulden *et al.*, 2010). As for the period of exposure, 24 hour time of exposure was found to be sufficient to determine incipient cytotoxic concentrations of H_2O_2.

In cell culture studies, the responses of proliferating mammalian cell lines towards H_2O_2 has been described as follows: very low level (3 to 15μM) causes growth stimulation, higher level (120 to 150 μM) induces a temporary growth arrest, intermediate concentrations (250 to 400 μM) cause permanent growth arrest and high concentrations (≥ 1mM) induces necrotic cell death (Wies *et al.*, 1995; Babich *et al.*, 1996; Davies, 1999). As for the results of present study, concentration of 100 μM of H_2O_2 was found to cause 50% of the SH-SY5Y cells inhibition after 24 hours‟ exposure. This concentration can be nearly categorised in higher level group, but the toxicity effect is also highly influenced by the type of cell used. The IC_{50} value of 100 μM of H_2O_2 (concentration of H_2O_2 that exerted 50% cell death) towards SH-SY5Y cells reported in this present study matches several published work (Whittermore *et al.*, 1995; Custodia *et al.*, 2013; Park *et al.*, 2015). This finding also is in agreement with previous report by Halliwell *et al.* (2000), which claimed that H_2O_2 is often cytotoxic to a wide range of cell cultures high level (>50 μM) and the toxicity effect is highly dependent on the concentration used.

On the other hand, the level of toxicity effect caused by H_2O_2 as oxidative stress agent can be minimised by the action of antioxidant defence mechanism. Thus, H_2O_2-induced cytotoxicity is a very suitable method in determining the potential of selected nervine herbs in providing neuroprotection towards induced damage SH-SY5Y cells.

5.2.3 Neurotoxicity Effect of the Herbal Extracts on SH-SY5Y Cells

In *in vitro* cell culture systems, there is an immense attention that has been dedicated in cytotoxicity studies as it is a primary step in assessing the toxicity effect of test substances including plants extracts towards wide variety of cell types. This is especially due to the close relation with screening biological activity of plant extracts and their active compounds, in which, minimal to no toxicity or selective cytotoxicity towards certain type of cells is essential to be determined. Toxicity test on plant extracts also allows the identification and prioritization of the extracts in which can be useful for further biological activity studies. It is potentially involving studies on direct cellular damage such as cytotoxicity of anticancer agent, physiological effects such as neurotoxicity in the brain, inflammation, and other systemic effects (McGaw *et al.*, 2014).

In neuropharmacological studies, herbal plants have been well known for being a rich source of significant bioactive compounds of various structures with neuroprotection properties towards neurons cells. Despite that, some of them may be toxic at certain level of doses. The information and efficacy on the usage of most herbal plants used in managing neurological disease is based on traditional knowledge conveyed through generations over years and not on pre-clinical and clinical evaluation (Nondo *et al.*, 2015). Hence, in the present study, the toxicity test on the herbal extracts could provide useful information on their toxicity effect, as neuroprotection potentials and neurocytotoxicity effect of *P. betle* and *C. ternatea* towards SH-SY5Y cells are mostly not known.

Current findings found that the incubation of SH-SY5Y cultures with the extracts of *P. betle* at high concentration for 24 hours resulted in concentration-dependent reduction in cell viability. This finding positively correlates with previous reported studies, in which *P. betle* extract was shown to be toxic to SH-SY5Y cells at high

concentrations (Norfaizatul *et al.*, 2010). It was claimed that *P. betle* extracts exerted a pro-oxidant effect that adversely affects the rate of cell growth and causes cells to die. It was also believed that plant with higher antioxidant potential, even some antioxidants such as vitamin E may be more likely to act as pro-oxidants. However, no significant decrease in cell viability was observed in the groups treated with methanolic and water extract of *C. ternatea* and this confirmed that the concentration ranges of *C. ternatea* used was not toxic on SH-SY5Y cells. Although very little studies have been reported on the toxicity effects on these nervine herbs on neuron cells, generally it can be concluded that the extracts of *P. betle* is more toxic as compared to the extracts of *C. ternatea*. Thus, lower concentrations of *P. betle* extracts were implied on the neuroprotective studies to ensure that the toxicity effect does not impede the neuroprotective properties of the extracts.

Even so, it is essential to emphasize that this finding was determined in *in vitro* model. *In vivo* situation may be affected by many confounding factors including absorption, metabolism and distribution of the active compounds which will affect its bioavailability (Norfaizatul *et al.*, 2010). For instance, earlier reports using animal model had shown *P. betle* was not cytotoxic at low dosage (Choudhary & Kale, 2002; Arambewela *et al.*, 2005) Thus, it must be stressed that it is important to establish a safe concentration of the plants extracts and taking into considerations of all the possible factors.

5.2.4 Neuroprotective Effect of the Herbal Extracts on H_2O_2-induced Damage in SH-SY5Y cells

Nowadays, there has been intense interest emphasized on natural products with neuroprotective capability that may be promising therapeutics as alternative or complementary remedy in neurological disorders‟ management. It has been claimed that a number of medicinal herbs contain a variety of valuable potential bioactive compounds that responsible for the protective mechanism against neurodegeneration. The specification of which part of the plant components that has biologically active for special discourse is difficult to be determined, but the use of medicinal herbs as neuroprotective has been well known since the ancient times (Phani *et al.*, 2015). In a number of traditional medicine systems, *P. betle* and *C. ternatea* has been known for its tremendous curative and health benefits including the improvement of neurological disorders among society. *P. betle* was traditionally used as anti-stress agent and treatment for anxiety, meanwhile *C. ternatea* has been well known to be used as memory enhancer, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative agent (Jain *et al.*, 2008; Mukherjee *et al.*, 2008; Battarcharjee *et al.*, 2016). Thus, this present study could emphasize the medical importance of these herbs as neuroprotective in overcoming the emerging of neurodegenerative diseases.

Findings of present study showed that presence of both of the herbs; *P. betle* and *C. ternatea* in induced-damaged of SH-SY5Y cells significantly increase the percentage of cell viability. However, extracts of *P. betle* was found to be a neuroprotective agent at much lower concentration as compared to the extracts of *C. ternatea*. This correlates well with the toxicity test of *P. betle*, in which, both of its methanolic and water extract suppressed the cells growth at high concentrations. This suggests that *P. betle* extracts are potentially neuroprotective agent at lower concentration, but becoming toxic towards SH-SH5Y cells at high concentrations.

Study done by Norfaizatul *et al*. (2010) also found concentration-dependent effect for *P. betle* with greater neuroprotective potential at lower concentrations due to the pro-oxidant effect of this herb that induces oxidative stress and damage in SH-SY5Y cell. In addition to that, pre-treatment with methanolic and post-treatment with water extract of *P. betle* exerted better neuroprotective effect as compare to other groups. This suggests that this herb might be suitable to be used as treatment or prevention in managing neurodegenerative diseases. Due to the fact that it provides better improvement on the viability of damaged cell, it is possible that this herb restores or regenerates damaged neurons in a way to suppress injury caused by neurotoxic agent (Phani *et al.*, 2015).

On the other hand, extracts of *C. ternatea* provide neuroprotection effect towards the neuroblastoma cells at higher concentrations as compared to the extracts of *P. betle*. This finding is in an agreement with the extract toxicity test as *C. ternatea* extracts does not exert toxicity as *P. betle* extracts does at higher concentrations. The neuroprotective effect recorded for this herb was generally better at higher concentrations. Pre-treated group with methanolic extract of *C. ternatea* showed better protective potential indicating that this extract might be acceptable to be utilized in the prevention of neurological disorders as neuron cells conceivably shielded against injury before the damage is done by neurotoxic species. As for the water extract of *C. ternatea*, pre-treatment group also showed better neuroprotective effect. This proposed that the water extract of this herb also perhaps suitable for prevention in the management of neurological disorders.

Although there is not much study reported on the neuroprotective effect of *P. betle* and *C. ternatea* in SH-SY5Y *in vitro* model, a number of *in vivo* studies were done on the nootropic effect of these herbs. Chan and Wong (2014) reported on the neuroprotective effect of aqueous leaf extract of *P. betle* towards the brain of ethanol-

treated rats. The finding of the study demonstrated that the treatment of induced-damage brain with extract of *P. betle* exhibited reduced level of lipids, lipid peroxidation and disturbances in antioxidant defence. Meanwhile for *C. ternatea*, Shahnas and Akhila (2014) reported that extract of the leaf of *C. ternatea* was beneficial in the management of Alzheimer"s disease and the isolated compounds had the potential as lead compounds in identifying new derivatives that could be implied for improving memory. Earlier studies also revealed the capability of *C. ternatea* in a number of neuroprotective properties including memory enhancing, anxiolytic, antidepressant, antistress and improving cognitive behaviour (Mukherjee *et al.*, 2008; Gupta *et al.*, 2010; Malik *et al.*, 2011).

5.3 Phytochemical Contents of the Herbal Extracts

Phenolic and flavonoids compounds carry significant antioxidant potential. Consumptions of these phytochemicals are reported to be very helpful in reducing the adverse effect of free radicals in the body that makes them useful in combating wide variety of diseases (Ross & Kasum, 2002). They are claimed to express substantial free radical scavenging activities through their reactiveness as hydrogen- or electron-donating agents and metal ion chelating abilities (Esmaeili *et al.*, 2015). These compounds have also been well-known as natural antioxidants that are increasingly attracting researches attention of their naturally disease preventing and health promoting properties. Thus, Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assay were carried out on the extracts of *P. betle* and *C. ternatea* to investigate the total phenolic and flavonoids content and further determining the correlation with neuroprotective activity towards induced damage neuron cells.

Phenolics are an important class of secondary plant metabolites that being a symbol of antioxidant properties by having an impressive array of pharmacological

activity (Saxena & Saxena, 2013). The present study shows that both of the herbal extracts contain phenolic compound. *P. betle* was found to have higher content of phenolic as compared to both of methanolic and water extract of *C. ternatea*. Previous studies on *P. betle* reported total phenolic content ranging from 2.12±0.50 to 133.27±2.62 mg GAE/g extract (Ibrahim *et al.*, 2011; Kaur & Mondal, 2014). As for *C. ternatea*, there are reports on total phenolic content ranging from 18.26±1.65 to 204±1.2 mg/g extract (Saxena & Saxena, 2013; Vats, 2014).

Findings of current study possess considerably higher TPC for both of the herbs when compared with reported works. This difference could be highly attributable to extraction methods, time of collecting samples, environment and genetic differences between tested herbs (Shan *et al.*, 2005). In earlier studies, the effect of certain factors on the extraction yield of total phenolic has been elucidated by some researchers. For instance, Wong *et al.* (2006) have optimized the extraction conditions for the maximum recovery of phenolic content as phenolics were claimed to be an important aspect in the field of natural antioxidant for preservation of food. Maisuthisakul (2008) also reported on differences in value of TPC in *P. betle* due to the use of different solvent with different polarity. The study found different phenolic value recorded on the use of different polar solvent. This also clears out the reason on the higher TPC estimated in methanolic extract in this present study as compared to water extract for both of the herbs. It might be due to difference in polarity of water as solvent in comparison to methanol. This finding matches the results reported by Abrahim *et al.* (2012), in which water extract of *P. betle* showed lower yield of TPC.

Flavonoids, on the other hand, are other classes of phenolic compounds. They are also effective antioxidants with known properties such as antiradical and oxidative enzymes inhibition (Zingare *et al.*, 2013). The overall finding of present study demonstrated that the TFC to be lower than TPC in both *P. betle* and *C. ternatea*. This

is because phenolics are the major contributor with higher value of total content in plant antioxidant activity and flavonoids are just one of the groups of polyphenol compounds (Jaiswal *et al.*, 2014). Previous studies reported comparable TFC of *P. betle* extracts ranging from 3.984±0.15 to 33.771±0.62 mg QE/g extract (Kaur & Mondal, 2014). As for *C. ternatea*, reported TFC was found to be in the range of 5.76±2.12 to 80.0±1.6 mg QE/g extract. Similar with TPC, the value of TFC is highly dependent on the solvent polarity, extraction methods and genetic differences of the plants.

5.4 Free Radical Scavenging Activity of the Herbal Extracts

Interest in natural antioxidants of plant origin has tremendously increased as critics have been emphasized on the possible toxicity of synthetic antioxidants. Antioxidant study has been considered as an important topic not only in neuropharmacological field, but other medical fields and food industry. The role of antioxidants in scavenging free radicals may have great relevance in the prevention and therapeutics of neurological disorders, in which, free radicals are implicated. They may serve task of reducing oxidative damage in humans induced by the overproduction of the free radicals and ROS under stress conditions. So, researchers suggested that frequent consumption of plant-derived phytochemicals from greens and herbal plants may contribute to the shift of balance towards an adequate antioxidant status (Saha *et al.*, 2008). Hence, antioxidant capacity of the herbal extracts is considered as one of the most important parameters related to neuroprotection in neuron cells.

1, 1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is an easy, rapid and sensitive method to screen for the antioxidant capacity of plant extracts (Saxena & Saxena, 2013). In this present, extracts of *P. betle* was shown to exert better DPPH free radical scavenging capacity than the extracts of *C. ternatea*. This finding was found comparable with earlier studies reported, in which the IC_{50} value (concentration of the

extracts that inhibit 50% of the free radical) ranging from 78.12 to 179.5±93.1 μg/mL (Rathee *et al.*, 2006; Sazwi *et al.*, 2013). Meanwhile the IC_{50} ranging from 319.42±0.43 to 415.63±1.22 μg/mL recorded by *C. ternatea* extracts was considered in agreement with a study done by Vats (2014) with IC_{50} of 480.00±1.5 μg/mL. Overall results also demonstrated that water extracts of both samples provided better DPPH scavenging activity. This finding suggests that antioxidant assay is highly influenced by the variety of the samples, polarity of solvent used and also extraction procedure implicated.

P. betle extracts also showed better 2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity as compared to *C. ternatea*. While DPPH assay is used most in lipophilic antioxidant system, ABTS assay is suitable to be used for both cases of hydrophobic and lipophilic antioxidant system (Jaiswal *et al.*, 2014). According to the results obtained, greater antiradical capacity in methanolic extracts of both herbs as compared to water extracts was observed.

On the other hand, in comparison between both assays, ABTS radical scavenging activity was found greater exerted by methanolic extract, meanwhile better DPPH scavenging activity recorded by water extract of both herbs. This could be due to the different mechanisms carried by ABTS and DPPH assay. Previous study reported that DPPH method provides lower values than the ABTS method due to the higher stability, and thus lower reactivity of the DPPH radical. For example, it is known that DPPH radical reacts with polyphenols, but not with phenolic acids and sugars. However, the reactivity of antioxidant compounds also is dependent on the chemical structures of radicals (Marecek *et al*., 2016). This proposed that the radical scavenging activity is also highly influenced by the reactivity of the radicals and the properties of phytochemicals contained in the herbs.

In a nutshell, both of the extracts possess good radical scavenging activity. However, *P. betle* showed better antiradical activity compared to *C. ternatea*.

Significant antioxidant potential of these herbs has been demonstrated in earlier reports. Jaiswal *et al.* (2014) reported that antioxidant action of *P. betle* leaf is very high and have been proved to be preservative for food. It is also claimed that *P. betel* was a better source of antioxidant as compared to synthetic antioxidants; butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). As for *C. ternatea*, this herb has been used in Thailand as a component of cosmetics and proved to have antioxidant activity (Lijon *et al.*, 2017). Even so, the potential neuroprotective by antioxidant capacity of the herbal extract can be further described in the correlation study.

5.5 Interrelation of Neuroprotective Activity, Polyphenols and Flavonoids Content, and Antioxidant Activity of the Herbal Extracts

The neuroprotective potential of nervine herbs can be attributed to the polyphenol and flavonoid contents, by which, were reported to exert some biological actions that are related to their antioxidant activity (Gryglewski *et al.*, 1987; Vats & Alam, 2013). These dynamic relationships can be well explained through the correlation analysis and thus determine the relevance of polyphenol and flavonoid contents, and antioxidant activity towards the neuroprotective potential possessed by the herbs.

Strong and negative correlation found for most of the extracts of *P. betle* in different assay was believed to be attributable to the dual effect of its neuroprotective and neurocytotoxic potential towards SH-SY5Y cells, as this herb expressed better neuroprotective potential at low concentrations (Norfaizatul *et al.*, 2010; Norfaizatul *et al.*, 2011). This negative relationship indicates that the variables reacted inversely with the neuroprotective activity possessed by the herb. At increasing concentration of the extracts, the polyphenol and flavonoid contents, and antioxidant capacity increased, but the neuroprotective potential decreased. This also suggests that there might be other

phytochemicals responsible for the neuroprotective action of the extracts. For instance, phytochemicals such as terpenoids, saponins, alpha-tocopherol and beta carotene were claimed to possibly carry roles in neuroprotection mechanism (Phani *et al.*, 2015). Furthermore, it was claimed that extracts are very complex mixtures of many different compounds with distinct activities, thus, different neuroprotective capacities (Kaur & Mondal, 2014).

On the other hand, positive correlation shown by most of the extracts of *C. ternatea* indicates that the neuroprotective potential was highly attributable to polyphenol and flavonoid, and antioxidant capacity of the extracts. This finding is in agreement with Cui *et al.* (2005), who reported that extract with polyphenolic compounds showed strong antioxidant activity, and possessed the most potent cytoprotective effects against H_2O_2-induced cell damage. Strongest positive correlation shown between neuroprotective activity and TFC of both herbs resembles a finding by Sazwi *et al.* (2013), who reported a positive correlation between aforementioned variables, indicating that extracts with flavonoid content contained cytoprotective capability.

Apart from that, current findings also demonstrated strong positive correlations between polyphenolic and flavonoid contents, and antioxidant capacity for both of the nervine herbs. The strongest correlation was found between TPC and ABTS scavenging capacity recorded by *P. betle* water extract followed by *C. ternatea* methanolic extract of the same relationship. This proposed that phenolic compounds have the capability in scavenging free radical and thus, enhancing the antioxidant capacity. These good correlations between polyphenolics, flavonoids and antioxidant activity can be supported by a claim made by Saha *et al.* (2008), who reported that polyphenolic compounds, including flavonoid and phenolic acids in herbal plants have wide spectrum of biological effects especially antioxidant activity. The latter effect was believed to be

mainly influenced by phenolic components due to their redox properties that play important role in absorbing and neutralizing radicals, quenching singlet and triplet oxygen or decomposing peroxides.

The result of present study is also in agreement with a number of earlier studies done on the phytochemical properties and antioxidant potential of *P. betle* and *C. ternatea*. Ibrahim *et al.* (2011) reported a significant relationship between the high amount of phenolic compound and the antioxidant activity of *P. betle*. This herb was found to have high phytochemical compounds such as tannins, saponin, terpenoid and others. *P. betle* also have been reported for its phenolic compounds such as chavicol, allylprotocatechol, chavibetol and eugenol as the major pungent component that is responsible for its antioxidant role (Jaiswal *et al.*, 2014; Aishwarya *et al.*, 2016). Apart from that, study on *C. ternatea* done by Vats (2014) suggests that antioxidant potential of *C. ternatea* was due to its significant phenolic and flavonoid contents which make this plant a better source of dietary antioxidant. This claim is in relation with a report by Zingare *et al.* (2013), which demonstrated that free radical scavenging activity expressed by *C. ternatea* is highly influenced by the amount of phenolics and flavonoids of the herb.

Even so, a limitation in this correlation study must be noted. Different range of concentration was used to assess different biological activities. The concentrations used to determine the phytochemical content and antioxidant activity were not similar to the cocnentrations that were obtained in the neuroprotective assay for this correlation analysis. This is due to the fact that some range of concentrations might generate negative readings as most of the results are depending on respective standard curve. Besides that, for certain lower range of extract concentrations, the bioactivity might be undetectable.

As a whole, most of the *P. betle* extracts showed negative and *C. ternatea* expressed positive correlation in the relationship of neuroprotective potential with polyphenolics, flavonoids, and antioxidant activity. The results are in relation with the toxicity effect of the extracts, in which, *P. betle* was toxic towards the cell at high and neuroprotective at low concentrations. As for *C. ternatea*, the neuroprotective potential is highly attributable to the polyphenolics, flavonoids and antioxidant capacity. Even so, both of the herbs possessed strong positive correlation between polyphenolics, flavonoids and antioxidant activity which contribute to their important medicinal effects.

CHAPTER 6: CONCLUSION

The management of neurodegenerative diseases through natural remedies using medicinal plants has been a part of human culture and it continues to play a major role in the neurological health maintenance practice worldwide. Aside from being a rich source of bioactive compounds to counteract oxidative damage and provide neuroprotection, the use of natural products in neurological disorders management has been encouraged due to less or no side effects and cost effectiveness as compared to the conventional synthetic drugs. Two nervine herbs; *P. betle* and *C. ternatea*, were selected for this study based on the documented ethno-medicinal uses in improving memory, as neuroprotective agent, possess good antioxidant profile and other neurological related advantages. The herbs were screened for their neuroprotective potential, total phenolic and flavonoid content, and antioxidant capacity in prior to the correlation analysis of the variables. Therefore, present study provides support to the herbs‟ traditional and alternative use in the administration of various neurological related diseases and ailments.

Collectively, the present study demonstrated that the extracts of *P. betle* and *C. ternatea* ameliorated H_2O_2-induced cell damage in SH-SY5Y cells in dose-dependent manner. The extracts of *P. betle* provide better neuroprotection towards the neuron cells at lower concentration, meanwhile the extracts of *C. ternatea* at higher concentration. This finding also proved that *P. betle* extracts are potentially neuroprotective agent at lower concentration, but becoming toxic towards SH-SY5Y cells at high concentrations. Addition to that, this present study suggests that the methanolic extracts of *P. betle* might be suitable to be used as treatment and water extracts as prevention in neurodegenerative diseases management. As for *C. ternatea*, the extracts of this herb

can be potentially well utilized as prevention in the management of neurological disorders.

The extracts of *P. betle* and *C. ternatea* were also found to exhibit different degrees of phytochemicals content and antioxidant capacity. Overall results showed that the herbs can be considered as a good source of phytochemicals and natural antioxidants. In particular, the extracts of *P. betle* provide higher phenolic content, DPPH and ABTS free radical scavenging activity, meanwhile the extracts of *C. ternatea* recorded higher flavonoid content.

Further correlation analysis between exerted neuroprotective activity by the extracts and the variables revealed that *P. betle* and *C. ternatea* were highly reacted in concentration-dependent manner. Most of the *P. betle* extracts exerted negative and *C. ternatea* showed positive correlation in the relationship of neuroprotective potential with phenolic, flavonoids, and antioxidant activity. This confirmed the toxicity effect of the extracts of *P. betle*, which was toxic towards the SH-SY5Y cells at high and neuroprotective at low concentrations. Meanwhile for *C. ternatea*, the neuroprotective potential is highly creditable to the polyphenolics, flavonoids and antioxidant capacity. Strong positive correlation between polyphenolics, flavonoids and antioxidant activity were also found for both *P. betle* and *C. ternatea*, which holds their medicinal importance as an alternative source for treating various diseases. As a whole, the results obtained in the present study have provided scientific support for the ethno-medicinal use of *P. betle* and *C. ternatea*, which might be promising in neurodegenerative diseases management.

However, some limitations must be noted. Considering the first attempt on the SH-SY5Y cell line and the herbs, longer time has been consumed on stabilizing and optimizing the culture work. Other than that, a few mechanism studies on cell cycle in order to deepen the understanding on neuroprotective effect of the herbs cannot be

carried out due to time and technical problems. Examples of mechanism study that can be carried in the future are flow cytometric analysis for detection of apoptotic cells and measurement of of inhibitory enzyme activity. Additional investigations also should be carried out on the dietary intake of the herbs, and bioavailability analysis needs to be elucidated in future. Further research in identifying, isolating and determining the role of individual phytochemicals involved in the neuroprotective activity of the herbs could be beneficial for their use as alternative source of medicine in neuropharmaceutical industry. Detailed studies also will be needed to clarify the halflife of H_2O_2 at the temperature and time of the assay, and level of active peroxidase in extracts preparation. Besides that, more refined separation or purification steps towards the crude extracts can be incorporated in future, in order to improve results interpretation.

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APPENDIX A1: PREPARATION OF MEDIA AND SOLUTIONS FOR CELL CULTURE

Basic DMEM/F12 Media

12 g of DMEM/F12 powder (Sigma, USA) was dissolved in 1 L of sterilized distilled water. 2.44 g of sodium bicarbonate (NaHCO3, R&M Chemicals, UK) and 0.5206 g of (4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid) (HEPES) was added into the media. The mixture was stirred well before the pH was adjusted to 7.4 and subjected to filter sterilization using 0.22 μm filter membrane (Orange Scientific, Europe). Filtered media was stored in 4 °C up to 4 months use.

10% Supplemented DMEM/F12 Media

90 mL of basic DMEM/F12 media was added with 10 mL of Foetal Bovine Serum (FBS, PAA Lab, Austria) and the mixture was filter sterilized using 0.22 μm filter membrane (Orange Scientific, Europe). 10% DMEM/F12 media was stored in 4 °C up to 2 weeks use.

Phosphate Buffered Saline (PBS) pH 7.4

1.52 g of sodium phosphate anhydrous ($NaHPO_4$) (Merck, USA), 0.58 g of potassium dihydrogen orthophosphate (KH_2PO_4) (Merck, USA) and 8.50 g of sodium chloride (BDH AnalaR, UK) were dissolved into distilled water to made up a volume of 1 L. The mixture was stirred well before the pH was adjusted to 7.4 and filter sterilized

using 0.22 μm filter membrane (Orange Scientific, Europe). The buffer was autoclaved and stored and room temperature.

0.4% (w/v) Tryphan Blue Solution

0.2 g of tryphan blue powder was dissolved in 50 mL sterilized distilled water and mixed well. Tryphan blue solution was stored at room temperature.

5 mg/mL 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Solution

50 mg of 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) powder was dissolved in PBS to made a volume of 10 mL and mixed well. The solution was kept in dark at 4°C upon analysis.

APPENDIX A2: PREPARATION OF SOLUTIONS AND REAGENTS FOR PHYTOCHEMICAL SCREENING ASSAYS

10% Folin-Ciocalteaou reagent

10 mL of Folin-Ciocalteaou reagent (R&M Chemicals, UK) was added into 90 mL of distilled water and mixed well. The mixture was stored in dark and at room temperature.

7.5% sodium carbonate (Na_2CO_3) Solution

7.5 g of sodium carbonate (R&M Chemicals, UK) was dissolved in 100 mL of distilled water and stirred well. The solution was kept at room temperature.

10% Aluminium Chloride ($AlCl_3$) Solution

1.0 g of aluminium chloride ($AlCl_3$) (Systerm Chemical, Malaysia) was dissolved in distilled water and made a volume of 10 mL. The mixture was mixed well and stored and room temperature.

1 M Potassium Acetate (CH_3COOK) Solution

0.98 g of potassium acetate (CH_3COOK) (Sigma, UK) was dissolved into 10 mL distilled water and mixed well. The solution was kept at room temperature.

APPENDIX A3: PREPARATION OF SOLUTIONS AND REAGENTS FOR ANTIOXIDANT ASSAYS

0.3 mM 1, 1-diphenyl-1-picrylhydrazyl (DPPH) Solution

0.001 g of 1, 1-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich, Germany) was dissolved in 8.45 mL 90% methanol (Fischer Scientific, USA) and mixed well. The solution was kept in dark at room temperature.

7 mM 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] Solution

0.096 g of 2, 2"-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] (Sigma, USA) was dissolved in 25.0 mL of distilled water and mixed well. The solution was stored in dark at room temperature.

2.45 mM Potassium Persulfate ($K_2S_2O_8$) Solution

0.017 g of potassium persulfate ($K_2S_2O_8$) (Sigma Aldrich, Germany) was dissolved in 25.0 mL of distilled water and mixed well. The solution was kept at room temperature.

APPENDIX B1: ASSESSMENT OF TOXICITY OF DIMETHYL SULFOXIDE (DMSO) TOWARDS SH-SY5Y CELLS

Toxicity effect of dimethyl sulfoxide (DMSO) towards SH-SY5Y cells raw data:

Concentration of DMSO (%)	Percentage of cell viability (%)			
	Test 1	Test 2	Test 3	Average±SEM
0	100.0	100	100	100±0.0
0.1	91.50	95.77	100.39	95.89±2.57
0.3	94.49	94.39	97.93	95.60±1.16
0.5	93.85	95.52	96.41	95.26±0.75
1.0	100.29	91.46	93.48	95.07±2.67
5.0	91.63	99.88	93.66	95.06±2.48
10.0	95.61	95.53	93.61	94.92±0.65
25.0	78.06	73.27	72.23	74.52±1.80
50.0	56.77	56.04	51.37	54.73±1.70
100.0	12.05	10.55	14.57	12.39±1.17

APPENDIX B2: ASSESSMENT OF TOXICITY OF HYDROGEN PEROXIDE (H_2O_2) TOWARDS SH-SY5Y CELLS

Toxicity effect of hydrogen peroxide (H_2O_2) towards SH-SY5Y cells raw data:

Concentration of H_2O_2 (μM)	Percentage of cell viability (%)			
	Test 1	Test 2	Test 3	Average±SEM
0	100.0	100.0	100.0	100.0±0.0
25.0	99.48	105.05	92.40	98.98±3.66
50.0	72.83	106.29	91.81	90.31±9.69
75.0	63.45	99.27	66.67	76.46±11.44
100.0	44.42	58.38	53.61	52.14±4.10
200.0	17.89	41.57	44.89	34.78±8.50
300.0	12.40	38.19	33.36	27.98±7.92
400.0	8.71	33.82	15.51	19.35±7.50
500.0	7.63	24.24	12.92	14.93±4.90

APPENDIX B3: ASSESSMENT OF TOXICITY OF HERBAL EXTRACTS TOWARDS SH-SY5Y CELLS

Neurotoxicity effect of methanolic extract of *P. betle* towards SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability (%)			
	Test 1	Test 2	Test 3	Average±SEM
0	100.0	100.0	100.0	100.0±0.0
0.01	158.79	130.45	130.45	139.90±9.45
0.05	127.23	153.97	128.22	136.47±8.25
0.1	150.50	125.08	132.06	135.88±10.81
0.3	114.71	121.30	149.24	128.30±10.56
0.5	142.73	116.20	117.54	125.49±9.62
0.8	139.95	109.84	113.12	120.97±9.49
1.0	114.43	83.82	101.28	99.85±8.67
10.0	50.41	68.33	69.96	62.90±6.26
25.0	51.10	61.04	68.89	60.34±5.15
50.0	76.62	55.72	54.16	62.17±7.24
75.0	59.17	48.18	56.56	54.63±3.32
100.0	67.27	51.79	53.23	57.43±4.94

Neurotoxicity effect of water extract of *P. betle* towards SH-SY5Y cell raw data:

Concentration of extract (µg/mL)	Percentage of cell viability (%)			
	Test 1	Test 2	Test 3	Average±SEM
0	100.0	100.0	100.0	100.0±0.0
0.01	90.96	95.51	102.06	96.18±3.22
0.05	94.67	93.30	104.14	97.37±3.41
0.1	100.62	91.51	96.07	96.06±2.63
0.3	103.13	94.31	95.86	97.77±2.72
0.5	106.86	107.54	102.50	105.64±1.58
0.8	109.06	111.42	103.32	107.93±2.40
1.0	107.23	107.85	86.19	100.42±7.12
10.0	109.05	102.34	81.87	97.76±8.17
25.0	89.25	65.06	63.60	72.64±8.32
50.0	70.19	64.28	59.92	64.80±2.98
75.0	59.30	60.48	61.92	60.35±0.58
100.0	58.60	58.44	60.28	59.11±0.59

Neurotoxicity effect of methanolic extract of *C. ternatea* towards SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability			
	Test 1	Test 2	Test 3	Average±SEM
0	100.0	100.0	100.0	100.0±0.0
1	97.55	102.86	95.29	98.57±2.24
10	101.86	89.35	99.06	96.76±3.79
25	103.96	89.98	95.47	96.47±4.07
50	92.40	97.11	94.15	94.55±1.38
75	100.01	97.80	100.18	99.33±0.77
100	103.61	97.11	94.07	98.26±2.81

Neurotoxicity effect of water extract of *C. ternatea* towards SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability			
	Test 1	Test 2	Test 3	Average±SEM
0	100.0	100.0	100.0	100.0±0.0
1	104.04	102.11	86.22	97.45±5.65
10	98.13	101.20	81.91	93.75±5.98
25	103.93	107.52	91.99	101.15±4.70
50	102.08	113.70	102.30	106.03±3.84
75	106.06	96.95	93.20	98.70±3.82
100	103.45	98.84	98.84	100.38±1.53

APPENDIX B4: ASSESSMENT OF NEUROPROTECTIVE EFFECT OF THE HERBAL EXTRACTS ON H_2O_2-INDUCED SH-SY5Y CELLS

Neuroprotective effects of methanolic extract of *P. betle* against H_2O_2-induced cell damaged in neuroblastoma SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability (%)							
	Pre-treatment group				Post-treatment group			
	Test 1	Test 2	Test 3	Average±SEM	Test 1	Test 2	Test 3	Average±SEM
No extract (without H_2O_2)	100.0	100.0	100.0	100.0±0.0	100.0	100.0	100.0	100.0±0.0
No extract (with H_2O_2)	52.34	42.23	49.36	47.97±3.0	44.81	46.01	53.38	48.07±2.68
0.001	132.12	174.05	145.52	150.56±12.36	118.80	159.20	181.08	153.03±18.24
0.01	125.53	166.45	136.61	142.86±12.22	113.31	155.29	162.05	143.55±15.25
0.1	123.27	162.13	126.73	137.38±12.42	111.66	140.55	163.64	138.61±15.04
0.25	113.66	150.55	121.50	128.57±11.22	107.62	141.96	154.30	134.63±13.97
0.5	121.62	136.44	121.36	126.47±4.98	100.29	134.43	138.62	124.45±12.14
1.0	102.20	135.17	120.67	119.34±9.54	54.29	97.54	117.21	89.68±18.59

Neuroprotective effects of water extract of *P. betle* against H_2O_2-induced cell damaged in neuroblastoma SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability							
	Pre-treatment group				Post-treatment group			
	Test 1	Test 2	Test 3	Average±SEM	Test 1	Test 2	Test 3	Average±SEM
No extract (without H_2O_2)	100.0	100.0	100.0	100.0±0.0	100.0	100.0	100.0	100.0±0.0
No extract (with H_2O_2)	56.09	56.11	56.12	56.11±0.01	47.96	47.97	47.94	47.96±0.01
0.001	129.97	120.86	123.02	124.62±2.75	127.16	152.31	140.46	139.97±7.26
0.01	121.64	125.18	127.47	124.76±1.70	131.08	150.91	134.42	138.80±6.13
0.1	107.91	118.12	117.15	114.39±3.26	125.78	142.02	139.77	135.86±5.08
0.25	106.15	118.52	116.55	113.74±3.84	123.83	145.84	136.82	135.50±6.39
0.5	99.11	112.64	112.35	108.03±4.46	118.83	140.80	124.74	128.12±6.56
1.0	99.25	93.09	96.30	96.21±1.78	118.0	141.37	119.59	126.32±7.54

Neuroprotective effects methanolic extract of *C. ternatea* against H_2O_2-induced cell damaged in neuroblastoma SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability							
	Pre-treatment group				Post-treatment group			
	Test 1	Test 2	Test 3	Average±SEM	Test 1	Test 2	Test 3	Average±SEM
No extract (without H_2O_2)	100.0	100.0	100.0	100.0±0.0	100.0	100.0	100.0	100.0±0.0
No extract (with H_2O_2)	50.51	45.72	46.79	47.68±1.45	52.52	51.92	48.76	51.06±1.17
1.0	202.10	80.61	148.16	143.63±35.14	127.53	113.46	92.83	111.28±10.08
10.0	195.37	154.13	151.05	166.85±14.29	114.41	155.18	118.33	129.31±12.99
25.0	207.61	157.54	160.74	175.30±16.18	122.06	140.70	107.27	123.34±9.67
50.0	207.66	155.72	152.63	172.0±17.85	125.28	158.83	125.50	136.53±11.15
75.0	225.19	159.0	181.23	188.47±19.45	126.93	147.15	125.73	133.27±6.95
100.0	236.92	159.85	167.64	188.14±24.49	167.67	140.59	109.82	139.36±16.71

Neuroprotective effects of water extract of *C. ternatea* against H_2O_2 –induced cell damaged in neuroblastoma SH-SY5Y cells raw data:

Concentration of extract (µg/mL)	Percentage of cell viability							
	Pre-treatment group				Post-treatment group			
	Test 1	Test 2	Test 3	Average±SEM	Test 1	Test 2	Test 3	Average±SEM
No extract (without H_2O_2)	100.0	100.0	100.0	100.0±0.0	100.0	100.0	100.0	100.0±0.0
No extract (with H_2O_2)	58.21	52.67	59.99	59.96±2.21	43.63	48.20	48.23	47.24±1.53
0.001	89.56	167.73	90.69	115.99±25.87	62.17	86.20	93.30	96.59±9.42
0.01	86.71	172.65	90.74	116.70±28.0	55.54	100.09	104.08	100.89±15.56
0.1	96.35	151.05	116.11	121.17±15.99	120.94	80.10	115.50	132.67±12.80
0.25	88.16	152.06	78.21	106.14±23.14	116.25	136.68	92.71	115.21±12.70
0.5	85.67	143.35	85.55	104.86±19.25	123.57	105.39	93.82	107.59±8.66
1.0	75.05	125.65	87.78	96.16±15.20	114.12	102.06	103.42	106.53±3.81

Two-way ANOVA analysis for the neuroprotective activity of methanolic and water extract of *P.betle*:

Dependent Variable: CellViability

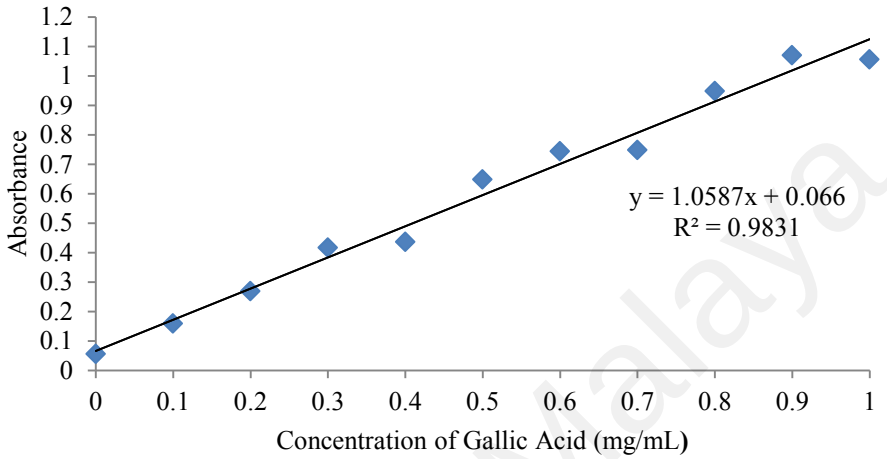
Extract	ExtractConc	Mean	Std. Deviation	N
PbMetPre	.001	150.56333	21.415126	3
	.010	142.86333	21.164587	3
	.100	137.37667	21.506709	3
	.250	128.57000	19.434678	3
	.500	126.47333	8.632365	3
	1.000	119.34667	16.524788	3
	Total	134.19889	19.071887	18
PbMetPost	.001	153.02667	31.595603	3
	.010	143.55000	26.405825	3
	.100	138.61667	26.043875	3
	.250	134.62667	24.188612	3
	.500	124.44667	21.024924	3
	1.000	89.67967	32.189205	3
	Total	130.65772	30.931230	18
PbWatPre	.001	124.61733	4.761834	3
	.010	124.76300	2.941539	3
	.100	114.39200	5.638286	3
	.250	113.73767	6.644601	3
	.500	108.03460	7.729588	3
	1.000	96.21373	3.083261	3
	Total	113.62639	11.095097	18
PbWatPost	.001	139.97467	12.582551	3
	.010	138.80233	10.615451	3
	.100	135.85633	8.796670	3
	.250	135.49600	11.062943	3
	.500	128.12033	11.369762	3
	1.000	126.31767	13.059058	3
	Total	134.09456	10.887425	18
Total	.001	142.04550	20.844178	12
	.010	137.49467	17.111879	12
	.100	131.56042	18.316594	12
	.250	128.10758	16.976070	12
	.500	121.76873	14.097027	12
	1.000	107.88943	22.952272	12
	Total	128.14439	21.150256	72

Two-way ANOVA analysis for the neuroprotective activity of methanolic and water extract of *C.ternatea*:

Dependent Variable: CellViability

Extract	ExtractConc	Mean	Std. Deviation	N
CtMetPre	1	143.62693	60.871402	3
	10	166.88077	24.714987	3
	25	175.29970	28.027363	3
	50	172.00367	30.915363	3
	75	188.47353	33.686531	3
	100	188.13680	42.423239	3
	Total	172.40357	36.057711	18
CtMetPost	1	111.27613	17.454870	3
	10	125.61840	25.848078	3
	25	123.34277	16.756080	3
	50	136.56833	19.282725	3
	75	133.26903	12.033576	3
	100	139.35800	28.946606	3
	Total	128.23878	20.039474	18
CtWatPre	1	115.99167	44.811164	3
	10	116.69800	48.499379	3
	25	121.20400	27.749986	3
	50	106.14337	40.073646	3
	75	104.85747	33.334704	3
	100	96.16257	26.320987	3
	Total	110.17618	32.896516	18
CtWatPost	1	80.55683	16.315505	3
	10	86.56700	26.944992	3
	25	105.51480	22.174803	3
	50	115.21600	21.999980	3
	75	107.59467	14.994830	3
	100	106.53433	6.606151	3
	Total	100.33061	20.627364	18
Total	1	112.86289	41.093814	12
	10	123.94104	41.122980	12
	25	131.34032	34.317286	12
	50	132.48284	36.364887	12
	75	133.54868	41.310483	12
	100	132.54793	44.883131	12
	Total	127.78728	39.279963	72

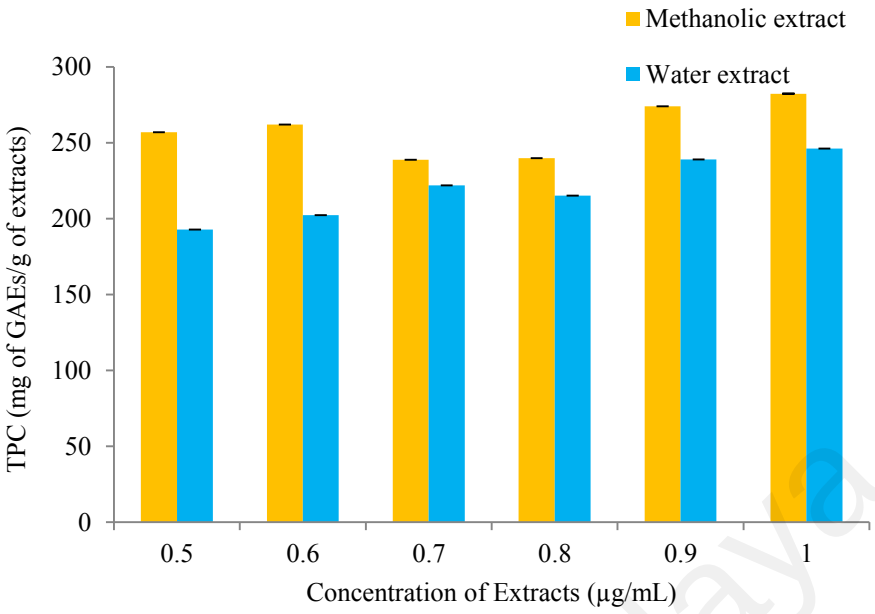
APPENDIX B5: TOTAL PHENOLIC AND FLAVONOIDS CONTENT OF THE HERBAL EXTRACTS



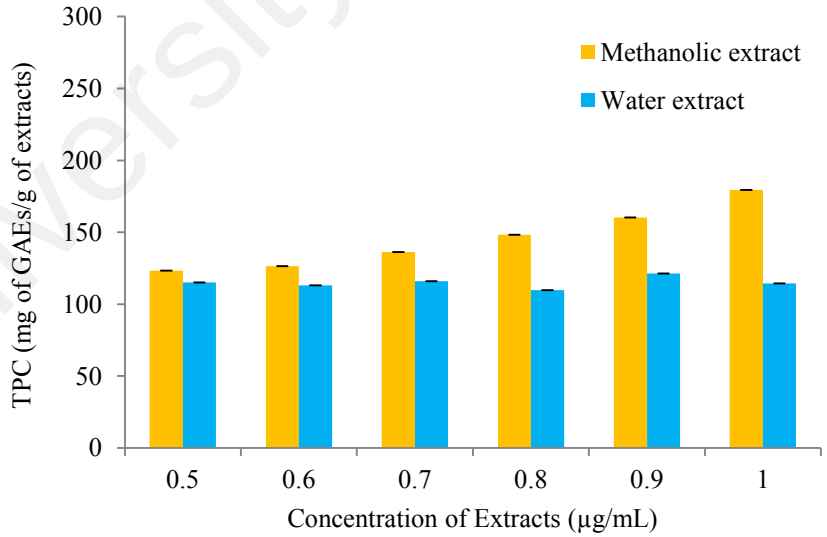
Standard Curve of Gallic Acid

Total phenolic content (TPC) of *P. betle* and *C. ternatea* raw data:

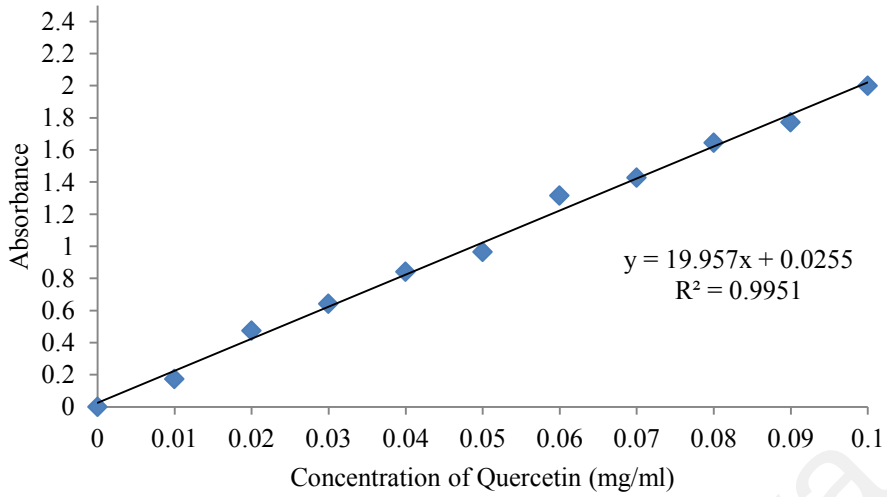
Plant species	Extract	Gallic Acid Equivalent (mg GAE/g)			
		Test 1	Test 2	Test 3	Average±SEM
P. betle	Methanolic	258.75	266.34	242.37	251.82 ±7.07
	Water	213.30	219.41	225.85	219.52±3.62
C. ternatea	Methanolic	142.37	147.28	147.42	145.69±1.66
	Water	114.19	111.65	119.0	114.94±2.16



TPC of methanolic and water extract of *P. betle* according to concentrations tested (0.5–1.0 µg/mL). Results were expressed in mg of GAEs/g of extracts ± SEM (n=3).



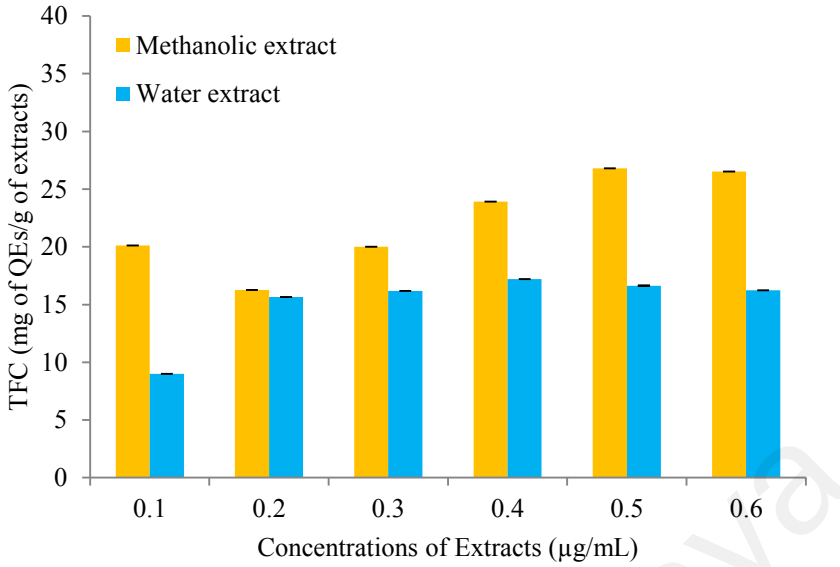
TPC of methanolic and water extract of *C.ternatea* according to concentrations tested (0.5–1.0 µg/mL). Results were expressed in mg of GAEs/g of extracts ± SEM (n=3).



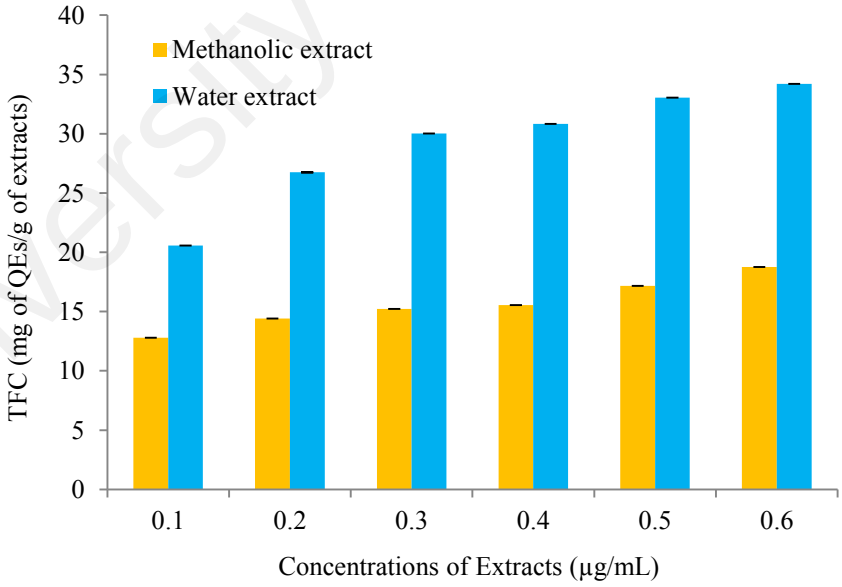
Standard Curve of Quercetin

Total flavonoids content of *P. betle* and *C. ternatea* raw data:

Plant species	Extract	Quercetin Equivalent (mg QE/g)			
		Test 1	Test 2	Test 3	Average
P. betle	Methanolic	22.66	21.87	22.30	22.27±0.23
	Water	15.83	15.01	14.60	15.15±0.36
C. ternatea	Methanolic	15.99	15.65	15.31	15.65±0.2
	Water	29.40	29.16	29.15	29.24±0.08



TFC of methanolic and water extract of *P.betle* according to concentrations tested (0.1–0.6 µg/mL). Results were expressed in mg of QEs/g of extracts ± SEM (n=3).



TFC of methanolic and water extract of *C. ternatea* according to concentrations tested (0.1–0.6 µg/mL). Results were expressed in mg of QEs/g of extracts ± SEM (n=3).

APPENDIX B6: ANTIOXIDANT ACTIVITY OF THE HERBAL EXTRACTS

1, 1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (IC_{50}) of ascorbic acid, *P. betle* and *C. ternatea* raw data:

Plant species	Extract	IC_{50} (µg/mL)			
		Test 1	Test 2	Test 3	Average±SEM
Ascorbic acid		13.74	13.77	12.83	13.45±0.31
P. betle	Methanolic	148.04	148.78	147.13	147.98±0.48
	Water	116.99	117.22	117.27	117.16±0.086
C. ternatea	Methanolic	417.76	415.602	413.53	415.63±1.22
	Water	320.26	318.861	319.15	319.42±0.43

2, 2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging activity (IC_{50}) of ascorbic acid, *P. betle* and *C. ternatea* raw data:

Plant species	Extract	IC_{50} (µg/mL)			
		Test 1	Test 2	Test 3	Average±SEM
Ascorbic acid		51.165	52.284	50.246	51.232±0.589
P. betle	Methanolic	82.816	81.063	81.926	81.935±0.506
	Water	131.130	130.279	131.049	130.82±0.271
C. ternatea	Methanolic	398.886	400.42	399.713	399.673±0.443
	Water	415.538	414.479	413.588	414.588±0.564