NEUROPROTECTIVE ACTIVITIES OF PEANUT SPROUT EXTRACTS AGAINST OXIDATIVE STRESS IN SK-N-SH CELLS

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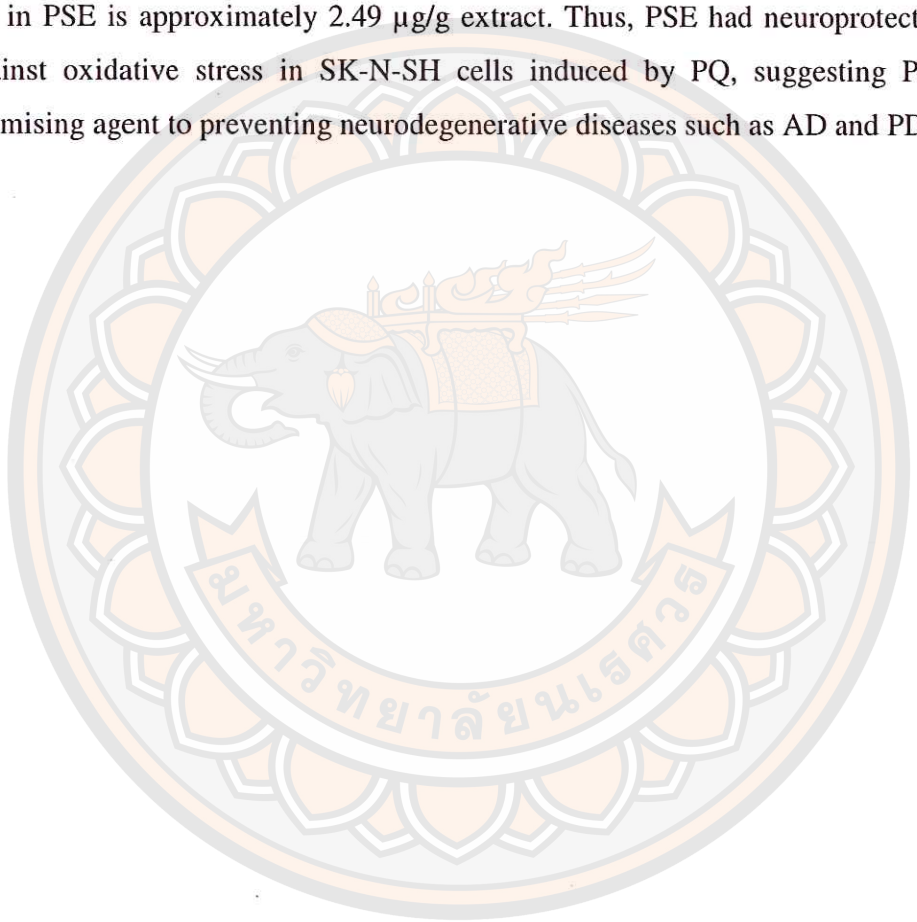
ABSTRACT

Alzheimer disease (AD) and Parkinson's disease (PD) are the most common causes of dementia, with progressive neurodegenerative processes. The important cause of neurodegenerative disease is environmental factor. A significant environmental factor is the extensive use of chemicals as pesticides and herbicides (paraquat, PQ), and for plant nutrition. Currently, there is commercially seed germination of many cereal crops with important nutrients include resveratrol (RS). RS reveals several pharmacological actions, including neurodegenerative diseases. In our research we investigated the protective effect of peanut sprout extract (PSE) against PQ induced oxidative stress in SK-N-SH cells and determined the amount of RS in peanut sprout Tainan 9 variety using HPLC. Using a MTT reduction assay, the cytotoxicity of the cells was determined after exposure to PQ and PSE. We found that PQ reduced cell viability as EC_{50} (0.75 mM) and PSE had no significant cytotoxicity at a concentration of 1.5 mg/ml. In the group of cells pre-treated with PSE cell death was significantly inhibited. The SK-N-SH treated cells were then tested for mRNA and protein expression by real-time RT-PCR and western blotting respectively. PSE significantly down regulated the mRNA expression of surtuin 1 (SIRT1) and α-synuclein, and it was found that PQ can cause significantly increased β-amyloid protein 42 levels whereas this action was inhibited by PSE. In the PQ treated group, PQ was increased in the intracellular ROS in the cells. Reactive oxygen species (ROS)

amyloid protein 42 levels whereas this action was inhibited by PSE. In the PQ treated group, PQ was increased in the intracellular ROS in the cells. Reactive oxygen species (ROS) were detected using a Muse cell analyzer. Intracellular ROS was significantly decreased in the cells treated with PSE and also those pre-treated with PSE. Finally, the amount of RS in PSE Tainan 9 variety was determined using HPLC. The concentration of RS in PSE is approximately 2.49 μg/g extract. Thus, PSE had neuroprotective activities against oxidative stress in SK-N-SH cells induced by PQ, suggesting PSE a highly promising agent to preventing neurodegenerative diseases such as AD and PD.



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ABBREVIATIONS

AD	=	Alzheimer disease
AICD	=	APP intracellular domain
Aβ	=	β-amyloid
APLP	=	Amyloid precursor-like proteins
APPL	=	Amyloid precursor protein-like
APP	=	Amyloid precursor protein
APOE	=	Apolipoprotein E
ATP	=	Adenosine triphosphate
CAT	=	Catalase
CBD	=	Corticobasal degeneration
cDNA	=	Complementary Deoxyribonucleic acid
CO_2	=	Carbon dioxide
DAB	=	3,3'-Diaminobenzidine
DDT	=	Dichlorodiphenyltrichloroethane
DHE	=	Dihydroethidium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
EO-FAD	=	Early onset familial Alzheimer disease
ER	=	Endoplasmic reticulum
FAO	=	Fatty acid oxidation
FBS	=	Fetal bovine serum
FOXO3	=	Forkhead box O3
FR	=	Free Radicals
FR	=	Fenton reaction
FTD	=	Frontotemporal dementia
g	=	Gram
GPX	=	Glutathione peroxidase

ABBREVIATIONS (CONT.)

HD	=	Huntington's disease
$^{\bullet}HO$	=	Hydroxyl radical
H_2O_2	=	Hydrogen peroxide
HPLC	=	High-performance liquid chromatography
h.	=	Hour
HMP	=	Hexose monophosphate pathway
HRP	=	Horseradish peroxidase
HWR	=	Haber-Weiss reaction
kDa	=	Kilodalton
LBD	=	Lewy body disease
LDL	=	Low-density lipoprotein
LNs	=	Lewy neuritis
LOAD	=	Late onset Alzheimer disease
LRP1	=	Low-density lipoprotein receptor related protein-1
MEM	=	Eagle's Minimum Essential Medium
MND	=	Motor neuron disease
mg	=	Milligram
ml	=	Milliliter
mM	=	Millimolar
mRNA	=	Messenger Ribonucleic acid
MSA	=	Multisystem atrophy
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NFT	=	Neurofibrillary tangles
NF-Kb	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	=	Nanogram
NO	=	Nitric oxide
NPH	=	Normal pressure hydrocephalus

ABBREVIATIONS (CONT.)

MPTP	=	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$O_2^{\bullet-}$	=	Superoxide radical
PCR	=	Polymerase chain reaction
PD	=	Parkinson's disease
PLK2	=	Polo-like kinase 2
PQ	=	Paraquat
PSE	=	Peanut sprout extract
PSP	=	Progressive supranuclear palsy
PVDF	=	Polyvinylidene difluoride
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species
sAPPβ	=	Soluble amyloid precursor protein β
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.M.	=	Standard error of the mean
SIRT1	=	Sirtuin 1
SK-N-SH	=	Human neuroblastoma cell line
SOD	=	Superoxide dismutase
SP	=	Senile plaques
αSyn	=	Alpha-synuclein
µg	=	Microgram
% (v/v)	=	Percentage volume per volume
VD	=	Vascular dementia

CHAPTER I

INTRODUCTION

Rationale of the study

Neurodegeneration is the progressive loss of structure or function of neurons, including death of neurons. There are many neurodegenerative diseases, including Alzheimer disease (AD), Parkinson's disease (PD) and Huntington's which are known to occur as a result of neurodegenerative processes. Alzheimer disease and Parkinson's disease were the most common cause of dementia, with progressive neurodegenerative processes (Grünblatt, et al., 2009). There are no treatments that prevent or slow the disease (Perrin, et al., 2009). Currently, Alzheimer disease (AD) is estimated to exist in a high percentage of adults over the age of 65. It is characterized by a progressive impairment of memory and intellectual functioning (Grünblatt, et al., 2009). Alzheimer disease has been defined as the presence of extracellular β-amyloid (Aβ) containing plaques and cytoplasmic neurofibrillary tangles (NFT) consisting of abnormal microtubule-associated protein tau. These proteinaceous aggregates are accompanied by synapse loss and neuronal cell death, which are thought to subserve the clinical syndrome of progressive cognitive impairment in AD (Coleman and Yao, 2003, Kopeikina, et al., 2012). Parkinson's disease is associated with progressive loss of dopaminergic neurons in the substantia nigra, as well as with more widespread neuronal changes that cause complex and variable motor and non-motor symptoms, decrease in dopamine level and dopaminergic transmission (Henchcliffe and Beal, 2008). However, previous studies have found that α-synuclein is a major component of Lewy bodies and Lewy neurites, the pathological hallmarks of PD and have confirmed its role in PD pathogenesis (Lücking and Brice, 2000). The important cause of neurodegenerative disease is environmental factors (Monte 2003).

Pathogenesis of PD and AD is known to be influenced by environmental factors (McCarthy, et al., 2004; Dinis-Oliveira, et al., 2006; Chen, et al., 2012). Chemicals, such as pesticides and weedicides are now a common contributor to environmental contamination. Of particular concern is paraquat which is the most popular chemical in use in agriculture and is highly toxic. Thailand is an agricultural

country, meaning that rural populations particularly risk dangerous exposure to paraquat. Exposure to paraquat leads to increase in cellular oxidative damages, specifically mitochondria of cerebral cortex and exhibits the mitochondrial dysfunction and induces β-amyloid (Aβ) formation (Chen, et al., 2012). In addition, PQ in the presence of oxygen generates the superoxide radical ($O_2^{\bullet -}$) (Dicker and Cederbaum, 1991; Busch, et al., 1998), hydroxyl radical ($^{\bullet}HO$), and hydrogen peroxide (H_2O_2) leading to deleterious effects on cell function (Youngman and Elstner, 1981; Busch, et al., 1998; Dinis-Oliveira, et al., 2006). H_2O_2 induces Sirt1 overexpression (Hasegawa, et al., 2008). SIRT1 has a dual effect on FOXO3 function by increasing FOXO3's ability to induce cell cycle arrest and to resist oxidative stress (Brunet, et al., 2004).

In recent years, nature has been a continuous source of pharmacologically active molecules and medicinal herbs (Dajas, et al., 2003). For example, peanut sprout is a plant which possesses antioxidant properties (Kang, et al., 2010). Peanut sprouts are produced by the germination of peanut kernels and have been used in the diet as health food for several centuries. It has been reported that peanut sprouts are rich in flavonoids and phenolic compounds, including resveratrol which may contribute to disease prevention and have health promoting properties (Kang, et al., 2010; Xiong, et al., 2014). They exhibit many biological functions such as anti-inflammatory activity attributed to inhibition of cyclooxygenase, estrogenic activity, and antiplatelet activity (Frankel, et al., 1993; Gehm, et al., 1997; Alarcon De La Lastra and Villegas, 2005). Moreover, it has been reported that flavonoids and phenolic compounds have a beneficial effect in the treatment of ischemia (Simonyi, et al., 2005) and neurodegenerative disease (Lu, et al., 2008).

The purpose of this study was to investigate the protective effect of peanut sprout extract (PSE) on paraquat-induced oxidative stress in SK-N-SH cells and to analyze resveratrol level in peanut sprout extract (*Arachis hypogaea* cv. Tainan 9). The knowledge to be gained from this study can improve and promote community health and well-being. Peanut sprout-derived products can be developed as the food supplements which can enhance the value of peanuts, further contributing to the community. Preliminary outcomes should support further medical research,

progressing the development of supplements which are effective neuroprotectants to prevent neurodegenerative disease in the elderly.

Objectives of the study

1. To investigate the protective effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells

1.1 To evaluate the effect of peanut sprout extract on α-synuclein and SIRT1 genes expression

1.2 To assess the effect of peanut sprout extract on Aβ 42 protein expression

2. To study the antioxidative effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells

3. To analyze resveratrol level in peanut sprout extract (*Arachis hypogaea* cv. Tainan 9)

The scope of the study

To study the toxicity of paraquat and peanut sprout extract on SK-N-SH cells, and to investigate of the protective effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells. Real-time PCR was used to assess the mRNA expression of α-synuclein and SIRT1in paraquat-treated group and preventive group by peanut sprout extract. Expression Aβ 42 protein was evaluated by western blot. In addition, investigation of antioxidative effect focused on the inhibition of ROS generation on SK-N-SH cells were assessed by Muse cell analyzer mini flow cytometry. Finally resveratrol level in peanut sprout extract (*Arachis hypogaea* cv. Tainan 9) was analyzed by HPLC.

The research hypothesis

Neuroprotective effect of peanut sprout extract may protect the Human neuroblastoma cell line (SK-N-SH) induced by paraquat.

Expected beneficial outcomes of the study

1. This study will provide information on neuroprotective effects of peanut sprout extract on paraquat-exposed Human neuroblastoma cell line (SK-N-SH).

2. This research will provide basic knowledge about resveratrol level and the antioxidant activity of peanut sprouts which can be used as a potential source of natural antioxidant.

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

The ageing of Thailand's population

In Thailand, the number of people classified as 'older person' for the purpose of being included in the 'aged population' has grown rapidly and will continue to do so in future decades. An 'older person' is defined as one aged 60 years or over. This age group has increased from being 7.3% of the Thai population in 1990 to being an estimated 25.1% in 2030 (National Statistical Office, 2009, and see Figure 1). By about 2020, the number of older persons will outnumber children under age 15 for the first time in Thai history.

In addition, Thai population ageing and the increasing number of older persons entered into the aging society (Knodel, et al., 2015). An increasing and disproportionate aged population has a significant effect on the health care sector. The proportion of the aged population suffering diseases such as cardiovascular disease, heart disease, cancer, hypertension, atherosclerosis, diabetes and neurodegenerative diseases is greater than in the population generally. This has been investigated and reported upon by many researchers. (Smith and Mensah 2003, Sonnenschein and Brody, 2005, Burt, et al., 1995, Dyer, et al., 1993, Floyd and Hensley, 2002.)

Of particular concern in Thailand, which is becoming an aged society, is the prevalence of dementia among the Thai aged population. Dementia is a broad category of brain diseases that affects memory, language, attention, emotions, and problem solving capabilities. The most common type of dementia is Alzheimer disease, which affects 38% of dementia cases (Sanchez-Juan, et al., 2012). According to the data of (Jitapunkul, et al., 2001), an estimate of the prevalence of dementia among Thai elderly is 3.4%. In the different age ranges, about 1% of those aged 60-64 years, and for those aged 90 years and over the prevalence is about 31.3%. The overall trend in prevalence rates of dementia in the aged population is illustrated in Figure 1.

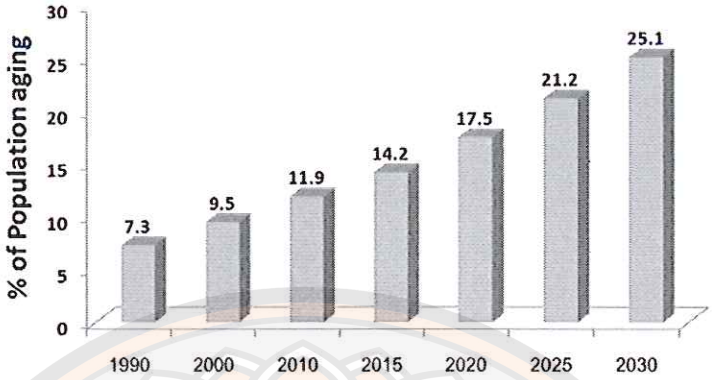


Figure 1 The percentage of Thai population aging, 1990-2030

Source: Adapted from National Statistical Office, 2009

Neurodegenerative disorders

Neurodegeneration is the progressive loss of structure or function of neurons including death of neurons affecting loss of memory, cognition, and movement. Many neurodegenerative diseases including Alzheimer disease (AD) and Parkinson's disease (PD) occur as a result of neurodegenerative processes (Grünblatt et al., 2009). Other neurodegenerative diseases include amyotrophic lateral sclerosis, Friedreich's ataxia, Huntington's disease, and spinal muscular atrophy (Figure 2). There is currently no cure for neurodegeneration, but researchers are working hard to find new treatments that can delay and potentially prevent the disease. There are important contributory factors in neurodegenerative disease such age, genetics, and environment (Masliah, 2001).

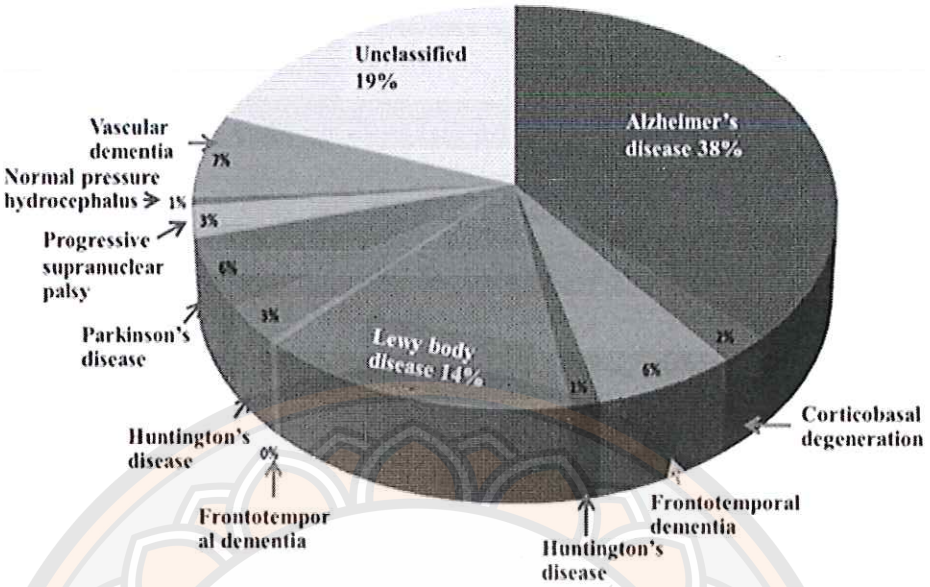


Figure 2 Distribution of neurodegenerative dementia diagnosis

Source: Stoeck, et al., 2012

Alzheimer disease, AD

Alzheimer disease, which was first discovered by Alois Alzheimer, is a physical disease that affects the brain. The major characteristic of the disease is the loss (atrophy) of brain mass (Figure 3). Specifically associated with AD, and probably somehow linked to the plaques and tangles, is the eventual degeneration of these critical areas of the brain. The symptoms of AD are memory loss and difficulties with thinking, problem-solving or language. AD is the most common cause of neurodegenerative disease (Grünblatt, et al., 2009), accounting for around 38% of all neurodegenerative disease. (Stoeck, et al., 2012). It is more commonly found in the aged population with 0.3% in the 60–69 year age group, to more than 10% in those over 80 years of age (Tilley, et al., 1998).

Alzheimer disease has been defined by the presence of extracellular β-amyloid (Aβ) containing plaques and cytoplasmic neurofibrillary tangles (NFT) consisting of abnormal microtubule associated protein tau. These proteinaceous aggregates are accompanied by synapse loss and neuronal cell death, which are thought to subserve the clinical syndrome of progressive cognitive impairment in AD (Coleman and Yao, 2003; Kopeikina, et al., 2012).

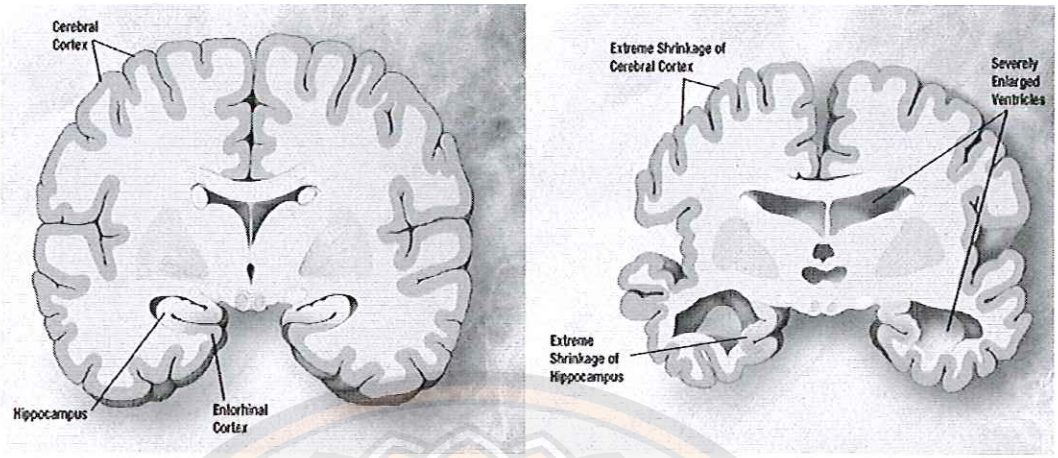


Figure 3 A representation of normal human brain on the left, and the brain affected by Alzheimer disease on the right.

Source: http://www.best-alzheimers-products.com/alzheimers-disease.

Pathology of Alzheimer disease

The pathological of the AD brain is characterized by two types of lesion: senile plaques (SP) or neuritic plaques and neurofibrillary tangles (NFTs) (Figure 4). Senile plaques are extracellular plaques containing beta-amyloid in the diseased brain. Beta-amyloid plaques are locally toxic and lead to significant disruption of network function, whereas NFTs are the intracellular deposition of tau protein aggregates in the diseased brain. The impact of NFTs on cellular and network function remains unclear (Tilley, et al., 1998; Kuchibhotla, et al., 2013).

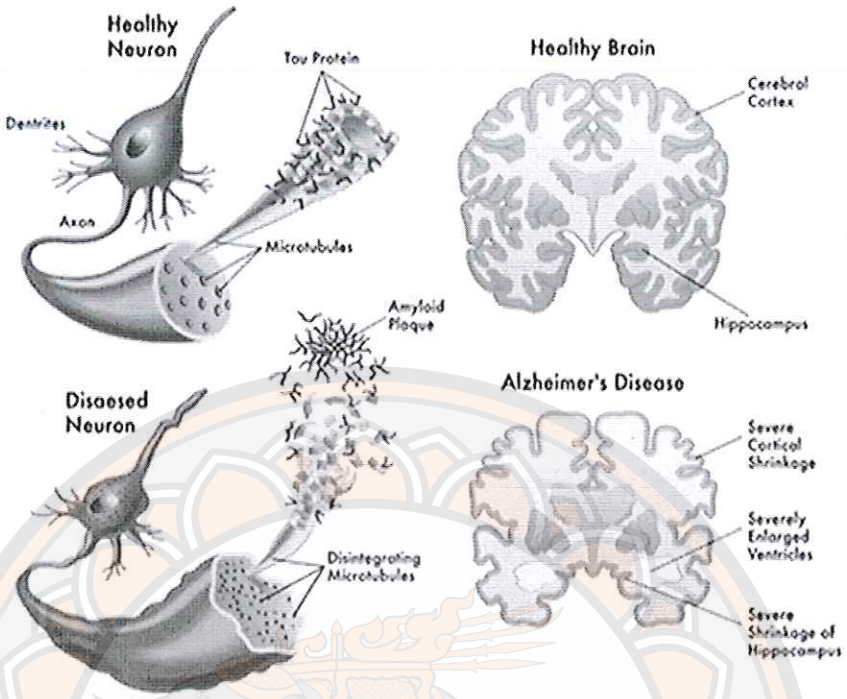


Figure 4 Pathological of normal brain (upper) and pathological of AD brain (lower)

Source: Keith, 2013

Senile plaques

Senile plaques, neuritic plaques or amyloid plaques are a hallmark lesion of AD. The extracellular aggregation of Aβ peptide (Aβ) (Figure 5) is associated with intracellular accumulation of hyper phosphorylated tau protein. Since mutations causing familial AD involve the genes of the amyloid precursor protein (APP) or of the secretases responsible for its cleavage, it has been generally accepted that accumulation of Aβ was the initiating factor of a cascade leading eventually to neuronal death (Ando, et al., 2014). The amyloid beta-protein (Abeta) is created with APP, the 38-43 amino acids residue peptide that is at the heart of the amyloid cascade hypothesis of AD (Thinakaran and Koo, 2008). Amyloid-β is cleaved from APP by the sequential activities of β-secretase and γ-secretase enzymes. It occurs in multiple forms ranging from 38 to 43 amino acids in length. Among these, Aβ40 is the most abundant species, but Aβ42 seems to be essential for initiating amyloid-β aggregation

affect to a loss of memory (Lorenzo and Yankner, 1994; Perrin, et al., 2009). Amyloid-β is toxic to neuronal cells and its excess reactive oxygen species (ROS) is toxic to biomolecules (lipid and protein) on the cell membrane. There are 2 pathways of beta-secretase in the amyloidogenic processing of APP, (1) amyloidogenic pathway and (2) non-amyloidogenic pathway.

Amyloidogenic pathway

In the amyloidogenic pathway (Figure 6), Amyloid-β peptide (Aβ) is generated by proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretase. On initiation, APP is cleaved by the transmembrane aspartic protease β-secretase BACE1, to generate the soluble amyloid precursor protein β (sAPPβ) and a carboxyl terminal fragment named CTFβ (C99), which is the substrate for γ secretase, to produce Aβ peptides and APP intracellular domain (AICD) (Canobbio, et al., 2015). Depending on the γ secretase cleavage site, there are two main isoforms of Aβ: the 42-residue Aβ42 and the 40-residue Aβ40. Amyloid-β peptide (Aβ) consists of Aβ40 and Aβ42 peptides. The amyloid plaques in AD brains consist of mostly Aβ42 and some plaques contain only Aβ42, even though Aβ40 concentration is several-fold more than Aβ42 (Zhang and Saunders, 2009; Gu and Guo, 2013).

The γ-secretase has been identified as a protein complex, consisting of at least four transmembrane proteins, presenilin1 (PS1) or presenilin2 (PS2), nicastrin, anterior pharynx defective 1 (APH1a or APH1b) and presenilin enhancer 2 (PEN2) (Grimm, et al., 2013).

Non-amyloidogenic pathway

In an alternative non-amyloidogenic pathway (Figure 7), APP may be proteolysed via the action of α- and γ-secretase. APP can be processed by α-secretases, thus precluding the generation of Aβ peptides. Cleavage within the Aβ sequence of APP by α-secretase generates a soluble N-terminal APPα fragment (sAPPα) and a carboxyl terminal fragment named CTFα, which is the substrate for γ-secretase to produce the non-amyloidogenic peptide (p3), instead of Aβ, and AICD. SAPPα has neurotrophic and neuroprotective functions (Grimm, et al., 2013).

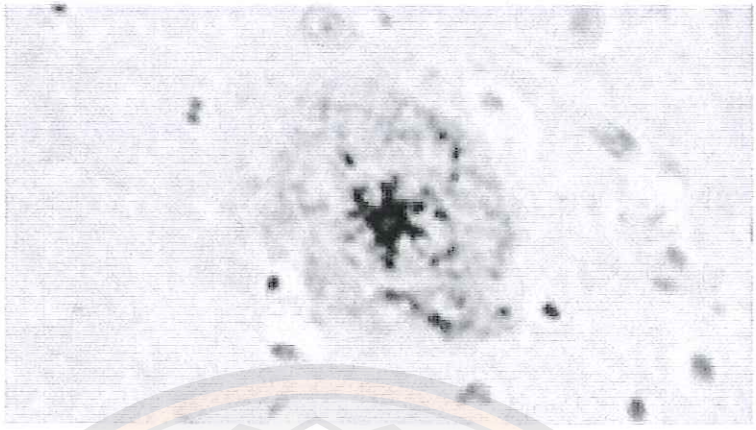


Figure 5 Amyloid beta (Aβ)

Source: L Tilley, K Morgan and N Kalsheker., 1998

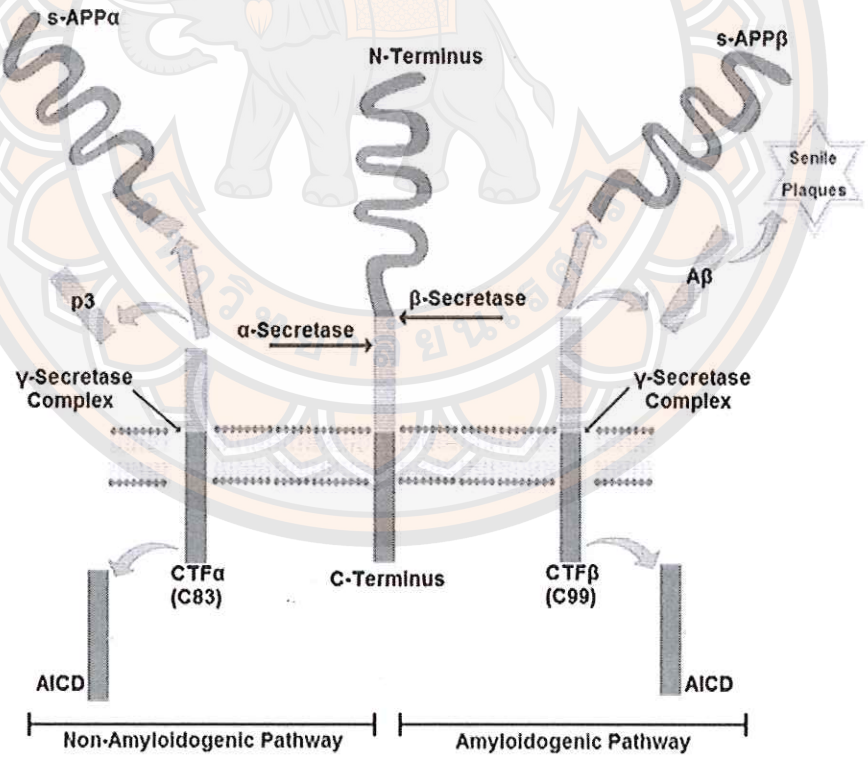


Figure 6 Amyloidogenic and non-amyloidogenic pathways of APP.

Source: Canobbio, et al., 2015

Causes and risk factors of AD

At present the causes and risk factors of AD are unknown. There are many risk factors associated with AD, including, but not limited to, age, sex, family history and genetic inheritance as well as environmental factors.

1. Age

The greatest known risk factor for Alzheimer is advancing age. Most people with AD are diagnosed at age 65 or older. For example, while one in nine people aged 65 or older has AD, nearly one in three people aged 85 or older has the disease. Advancing age is not the only risk factor for AD (Alzheimer, 2013).

2. Gender

AD has been reported to affect both men and women, with the incidence of AD being highest in women. In the United States of America, 3.2 million women and 1.8 million men aged 65 years and older had AD in 2013 (Alzheimer, 2013). It is known that mitochondria from young females are protected against amyloid-β toxicity, generate less reactive oxygen species, and release less apoptogenic signals than those from males. However, all this advantage is lost in mitochondria from older females (Viña and Lloret, 2010). This implies that there is a specific pathogenic mechanism to explain the higher incidence of AD cases in women.

3. Family history and Genetic inheritance

Family history is the second strongest risk factor for AD. Rudolph E. Tanzi reported that genetic factors are estimated to play a role in at least 80% of AD cases. AD is considered to be a genetically dichotomous disease presenting in two forms: early-onset familial cases usually characterized by Mendelian inheritance (EO-FAD), and late-onset (≥60 years), with no consistent mode of transmission (LOAD) (Tanzi, 2012). So genetic inheritance is a known risk factor for AD.

3.1 Early onset familial AD (EO-FAD)

Early-onset familial AD cases are usually characterized by Mendelian inheritance, with no consistent mode of transmission, and is usually found to strike under the age of 60 years. It is estimated that up to 80% of AD involves the inheritance of genetic factors, based on twin and family studies (Tanzi, 2012). The pathogenic mutations in amyloid precursor protein (APP), presenilin 1 and 2 (PSEN1 and PSEN2)

cause a subset of early-onset familial Alzheimer disease (EO-FAD) (Hooli, et al., 2014).

3.1.1 Amyloid precursor gene (APP)

The gene for APP is located on chromosome 21, which was first identified in 1987 by several laboratories independently using partial protein sequence information obtained by the Glenner and Beyreuther/Masters laboratories several years earlier (Thinakaran and Koo, 2008). It is one member of a family of related proteins that includes the amyloid precursor-like proteins (APLP1 and APLP2) in mammals and the amyloid precursor protein-like (APPL) in Drosophila. It is a single-pass transmembrane protein expressed at high levels in the brain and metabolized in a rapid and highly complex fashion by a series of sequential proteases, including the intramembranous γ-secretase complex, which also process other key regulatory molecules. The mammalian APP family of proteins is densely expressed in the brain (O'Brien and Wong, 2011). APP is the precursor to the amyloid β-protein (Aβ), the 38–43-amino acid residue peptide that is at the heart of the amyloid cascade hypothesis of AD. Specifically, molecular characterization of the secretases involved in Aβ production has facilitated cell biological investigations on APP processing and advanced efforts to model AD pathogenesis in animal models.

The precise physiological function of APP is not known. In various studies, APP overexpression shows a positive effect on cell health and growth. The function of APP in cell lines is to modulate cell growth, motility, neurite outgrowth, and cell survival, functions that can be reproduced by the soluble ectodomain, which is released by the cleavage of APP (O'Brien and Wong, 2011) by α-secretase which occurs within the Aβ domain, thus preventing the formation of Aβ. The main receptors for amyloid-beta peptide (Aβ), which are transported across the blood-brain barrier (BBB) from brain to blood and blood to brain, are low-density lipoprotein receptors related to protein-1 (LRP1) and receptors for advanced glycation end products (RAGE) (Deane, et al., 2009).

The amyloid cascade hypothesis

The amyloid cascade hypothesis has played a prominent role in explaining the etiology and pathogenesis of AD. This hypothesis proposes that the deposition of β-amyloid (Aβ) is the initial pathological event in AD leading to the formation of senile plaques (SPs) and then to neurofibrillary tangles (NFTs), neuronal cell death, and ultimately dementia. There are two key aspects in the amyloid cascade hypothesis (Figure 7). First, the detection of Aβ as a main constituent of the SPs and, second, mutations of the APP, PSEN1, and PSEN2 genes, which have been found in families with early-onset AD (Reitz, 2012).

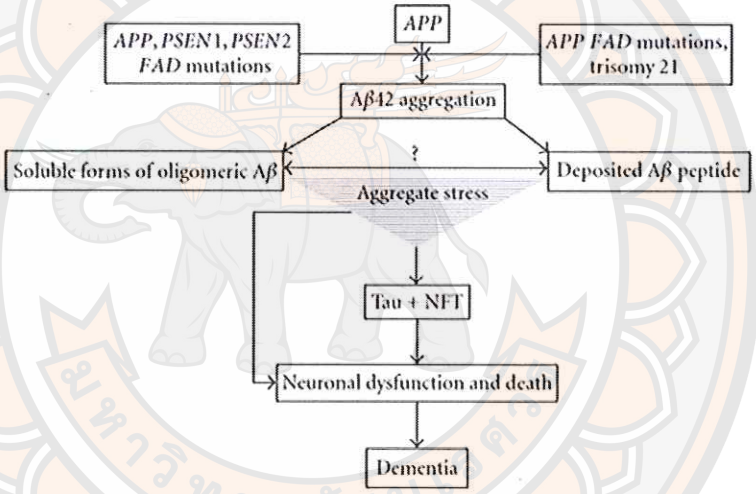


Figure 7 Amyloid cascade hypothesis

Source: Reitz, 2012

Toxicity of amyloid-β peptide

The amyloid beta (Aβ) peptide is aggregated and deposited outside the neurons in the brain tissue of Alzheimer patients, leading to the formation of neuritic plaques (also called senile or amyloid plaques) in the AD brain. Aβ is a 4.2 kDa short peptide of 40–42 amino acids, generated from the intracellular cleavage of the amyloid precursor protein (APP) by the sequential action of two proteolytic enzymes, beta- (β-) secretase and gamma- (γ-) secretase. The Aβ peptide plays an essential role at the

synapse. Aβ aggregate-mediated toxicity impairs synaptic function, which leads to the progressive memory loss and cognitive failure associated with AD. Additionally, Aβ aggregates can induce mitochondrial dysfunction, while mitochondrial dysfunction as a consequence of membrane-localized Aβ includes the inhibition of protein transport into the mitochondria, the disruption of the electron transport chain leading to impaired glucose utilization in neurons, and mitochondrial damage due to an increase in reactive oxygen species (ROS) production. Moreover, proteasome-mediated degradation of misfolded protein, including tau aggregates, is inhibited by the actions of Aβ oligomers, contributing to their enhanced accumulation (Smith, et al., 2007; Prasansuklab and Tencomnao, 2013). Interestingly, Takahashi R.H. et al., demonstrate that primary neurons from Tg2576 mice recapitulate the in vivo localization and accumulation of Aβ42 with time in culture. Furthermore, they demonstrate that Aβ42 aggregates into oligomers within endosomal vesicles and along microtubules of neuronal processes, both in Tg2576 neurons with time in culture and in Tg2576 and human AD brain. These Aβ42 oligomer accumulations are associated with pathological alterations within processes and synaptic compartments in Tg2576 mouse and human AD brains (Takahashi, et al., 2004). In addition, Aβ42 is the main component of amyloid plaques in the brains of AD patients, and the progressive formation of amyloid plaques is regarded as a key neuropathological feature of AD (Li, Zhou et al. 2011).

3.1.2 Presenilin genes

The presenilin 1 (PS1) gene on chromosome 14 and the presenilin 2 (PS2) genes on chromosome 1 were first discovered as sites of missense mutations responsible for early-onset Alzheimer disease (AD) (Hardy, 1997; De Strooper, et al., 2012). It is these genes that encode highly conserved polytopic membrane proteins (PS1 and PS2) that are required for γ-secretase activity. Most mutations in presenilins increase the relative ratio between the longer (Aβ42) and shorter (Aβ40) amyloid peptides (Aβ42/Aβ40) (Li, et al., 2011). Furthermore, presenilin proteins are also associated with neuronal differentiation, migration, neuronal survival, synaptic plasticity, synaptogenesis, memory, protein trafficking, cell adhesion, calcium dependent signaling, calcium homeostasis and apoptosis (Shimohama, 2000; Parks and Curtis, 2007)

3.2 Late onset AD (LOAD)

Late-onset Alzheimer is the most common form of the disease and is defined by onset age >65 years. It is probably modulated by genetic variants with relatively low penetrance but high prevalence (Bertram, et al., 2007, Tanzi 2012). Researchers have found that some genes that participate in amyloid-β processing (PSEN1, APOE) and methylation homeostasis (MTHFR, DNMT1) show a significant interindividual epigenetic variability, which may contribute to LOAD predisposition. The apolipoprotein E (APOE) genotype is the major susceptibility gene for LOAD in the human genome that contains the sequences for the ε4-haplotype (Wang, et al., 2008). It is the dominant apolipoprotein in the brain, primarily synthesized by astrocytes, although neurons and microglia may also contribute to its production. In addition, ApoE may affect AD pathogenesis by promoting deposition of the amyloid-β (Aβ) peptide and its conversion to a fibrillary form. It encodes three variants: APOE2, APOE3, and APOE4 (Small and Duff, 2008). The APOE ε3 allele is the most common, APOE ε4 is the strongest susceptibility gene for AD, and APOE ε2 is protective against AD. In a previous study, apoE4 had greater effects than apoE3 on Aβ burden as well as an even greater effect on fibrillar Aβ deposition and neuritic plaques in an in vivo system. This strongly supports the hypothesis that apoE's effects on Aβ deposition, fibrillogenesis, and neuritic plaque formation underlie the role apoE plays as a genetic risk modifier for AD (Holtzman, et al., 2000). APOE may also play in important role in synaptic plasticity, cell signaling, lipid transport and metabolism, and neuroinflammation (Figure 8) (Holtzman, et al., 2012).

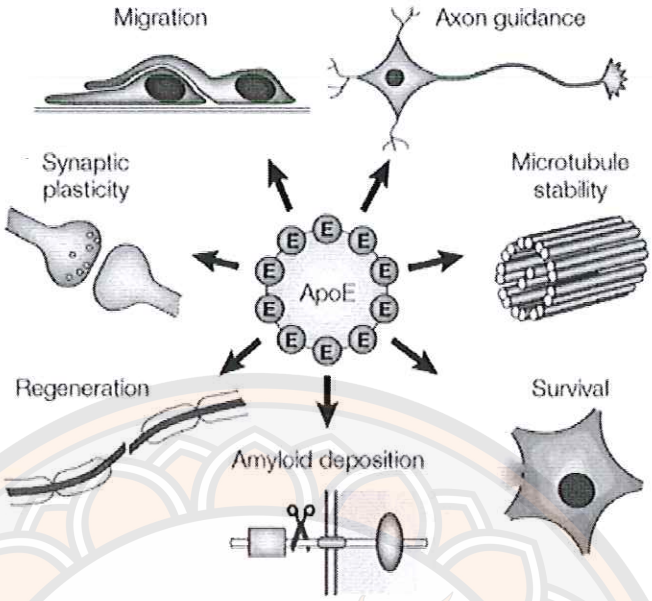


Figure 8 Role of Apolipoprotein E

Source: Herz and Beffert, 2000

4. Environmental factors

Environmental factors also play a role in the development of AD. Pesticides, food additives, air pollution and other problematic compounds are associated with risk of AD. In addition, oxidative stress and education are considered to be risk factors for AD in that the more educationally disadvantaged members of the population have a higher risk of AD than those with higher levels of education. Binge drinking and cigarette smoking have also been associated with a higher risk of AD (Lee, 1994).

5. Other risk factors

It has also been reported that transient ischemic attacks (TIAs), hypertension, hyperlipidemia, and male gender all tend to accelerate cerebral degenerative changes, cognitive decline, and dementia including AD (Meyer, et al., 2000). In addition, patients with arterial disease, heart disease, diabetes, high cholesterol, Down's syndrome, brain injury, and head injury are associated with risk of AD (Mayeux, et al., 1993).

Parkinson's disease

In 1817 James Parkinson was the first to describe paralysis agitans. This was later named Parkinson's disease. Parkinson's disease (PD) is associated with progressive loss of dopaminergic neurons in the substantia nigra, as well as with more widespread neuronal changes that cause complex and variable motor and non-motor symptoms (Henchcliffe and Beal, 2008). It is found that α-synuclein is a major component of Lewy bodies and Lewy neurites, the pathological hallmarks of PD, indicating its role in PD pathogenesis (Lücking and Brice, 2000). There is no preventive measure against it. Treatment is only symptomatic to relieve movement disorders associated with the disease. The symptoms of PD are resting tremor, bradykinesia, rigidity and postural instability. Symptoms of PD also include depression, Alzheimer, deterioration of the smell sense, and easy fatigue. These symptoms are also associated with gastrointestinal disorders and weight loss (Nuttiya Harnprasertpongse and Chaichan Sangdee, 2014).

PD is influenced by age, and by genetic and environmental factors. The role of genetic predisposition in PD has been increasingly acknowledged and a number of relevant genes have been identified (e.g., genes encoding alpha-synuclein, parkin, and dardarin), while the search for environmental factors that influence the pathogenesis of PD has only recently begun to gain momentum. In recent years, the investigation on paraquat (PQ) toxicity has suggested that this herbicide might be an environmental factor contributing to this neurodegenerative disorder (Dinis-Oliveira, et al., 2006).

Pathology of Parkinson's disease

The pathology of PD is not fully understood. The pathology of PD is damage to specific sub nuclei of the substantia nigra pars compacta, with severe obliteration of their neuromelanin-laden projection neurons, which is frequently considered to be the most important hallmark of PD (Figure 9). α-synuclein-immunopositive Lewy neuritis (LNs) and Lewy bodies (LBs) are necessary for neuropathological diagnosis. A major component of LNs and LBs is an aggregated form of the normally presynaptic protein α-synuclein. It is still unknown why this hydrophilic protein leaves its binding sites within synaptic boutons and, together with other components such as phosphorylated neurofilaments and ubiquitin, a heat shock protein required for the non-lysosomal

ATP-dependent breakdown of abnormal proteins, gradually transforms into virtually insoluble LNs or LBs (Braak, et al., 2003). LBs are relatively large intracytoplasmic inclusions, measuring 4–30 μm in diameter. They have a rather uniform hyaline eosinophilic appearance and at times are surrounded by a halo of paler concentric rings (Figure 10). Additionally, LBs are composed primarily of α-synuclein, a 140-amino-acid protein which is a normal constituent of the presynaptic apparatus (Perl, 2007).

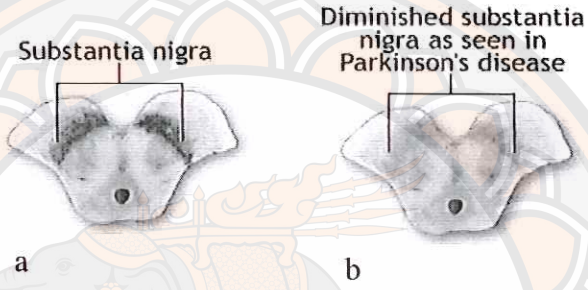


Figure 9 (a) substantia nigra pars compacta of normal (b) substantia nigra pars compacta of Parkinson's disease

Source: http://kanchanapisek.or.th

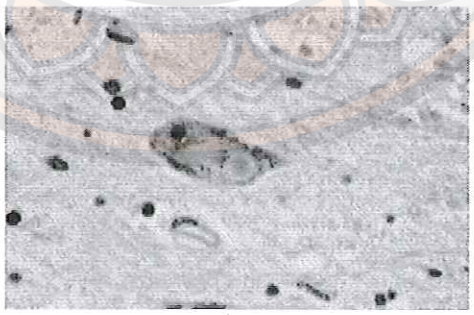


Figure 10 Parkinson's disease showing a Lewy body with prominent internal concentric ring formation. Hematoxylin and eosin stain.

Source: Perl, 2007

Alpha-synuclein (αSyn)

Alpha-synuclein (αSyn) is a presynaptic neuronal protein that is linked genetically and neuropathologically to PD (Cooper, et al., 2006). It may contribute to PD pathogenesis in a number of ways, but it is generally thought that its aberrant soluble oligomeric conformations, termed protofibrils, are the toxic species that mediate disruption of cellular homeostasis and neuronal death (Stefanis, 2012). Zhang Wei et al., reported that in a primary mesencephalic neuron-glia culture system, extracellular aggregated human α-synuclein indeed activated microglia; microglial activation enhanced dopaminergic neurodegeneration induced by aggregated α-synuclein. Furthermore, microglial enhancement of α-synuclein-mediated neurotoxicity depends on phagocytosis of α-synuclein and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase with the production of reactive oxygen species. This suggests that nigral neuronal damage, regardless of etiology, may release aggregated α-synuclein into substantia nigra, which activates microglia with production of proinflammatory mediators, thereby leading to persistent and progressive nigral neurodegeneration in PD. Finally, NADPH oxidase could be an ideal target for potential pharmaceutical intervention, given that it plays a critical role in α-synuclein-mediated microglial activation and associated neurotoxicity (Zhang, et al., 2005). Events in α-synuclein toxicity are shown in Figure 11.

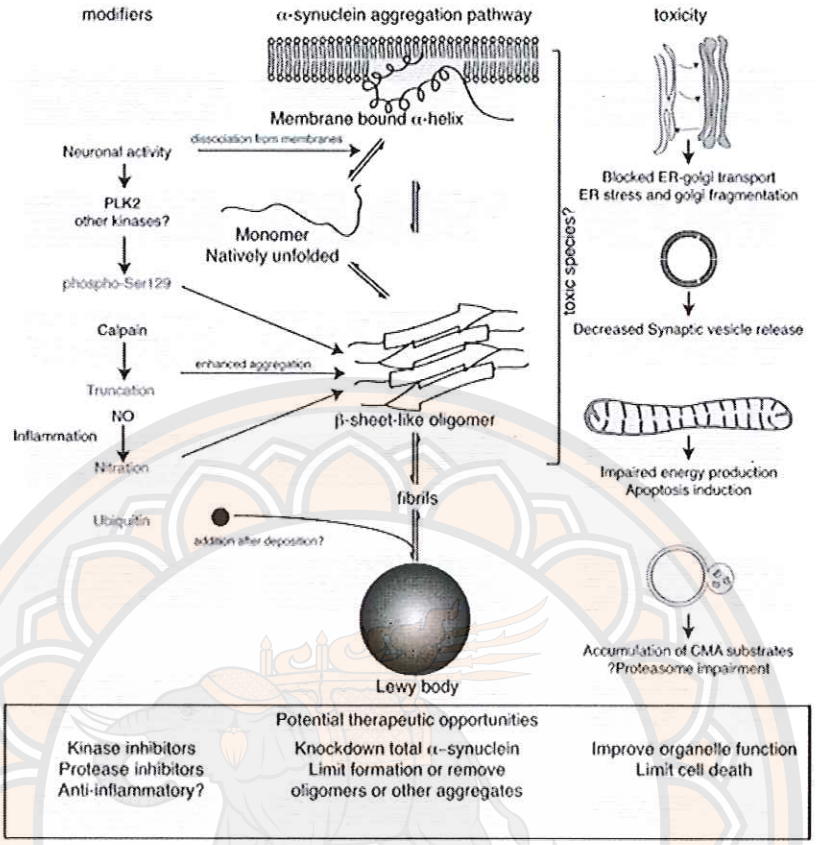


Figure 11 Events in α-synuclein toxicity

Source: Cookson, 2009

The central panel shows the major pathway for protein aggregation. Monomeric α-synuclein is natively unfolded in solution but can also bind to membranes in an α-helical form. It seems likely that these two species exist in equilibrium within the cell, although this is unproven. From in vitro work, it is clear that unfolded monomer can aggregate first into small oligomeric species that can be stabilized by β-sheet-like interactions and then into higher molecular weight insoluble fibrils. In a cellular context, there is some evidence that the presence of lipids can promote oligomer formation: α-synuclein can also form annular, pore-like structures that interact with membranes. The deposition of α-synuclein into pathological structures such as Lewy bodies is probably a late event that occurs in some neurons. On the left hand side (Figure 11) are some of the known modifiers of this process.

Electrical activity in neurons changes the association of α-synuclein with vesicles and may also stimulate polo-like kinase 2 (PLK2), which has been shown to phosphorylate α-synuclein at Ser129. Other kinases have also been proposed to be involved. As well as phosphorylation, truncation through proteases such as calpains, and nitration, probably through nitric oxide (NO) or other reactive nitrogen species that are present during inflammation, all modify α-synuclein such that it has a higher tendency to aggregate. The addition of ubiquitin (shown as a black spot) to Lewy bodies is probably a secondary process to deposition. On the right of the diagram are some of the proposed cellular targets for α-synuclein mediated toxicity, which include (from top to bottom) ER-golgi transport, synaptic vesicles, mitochondria and lysosomes and other proteolytic machinery. In each of these cases, it is proposed that α-synuclein has detrimental effects, listed below each arrow, although at this time it is not clear if any of these are either necessary or sufficient for toxicity in neurons (Cookson, 2009).

Causes and risk factors of PD

The cause of PD is unknown in the majority of cases. The current concept is that PD results from the interaction of genetic predisposition and environmental factors. Researchers have discovered 10 genes mutations, named PARK 1 to PARK 10 familial forms of PD. The gene product has been found in only four of these gene mutations. The environmental hypothesis was strongly suggested 20 years ago after the recognition that the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can cause a syndrome clinically and pathologically similar to idiopathic PD. Recently, the pesticide rotenone, a specific inhibitor of mitochondrial complex I, was shown to reproduce aspects of the disease after long exposure in animal models, which also favors the role of environmental factors in the pathogenesis of the disease (Cersosimo and Koller, 2006). Furthermore, it has been suggested that PQ might be an environmental factor contributing to neurodegenerative disorder such as PD (Dinis-Oliveira, et al., 2006; Yang, et al., 2010).

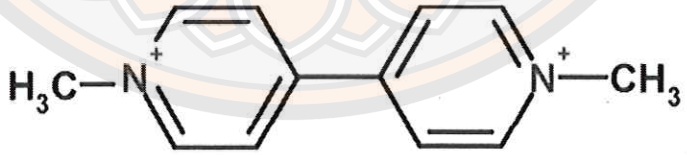
Paraquat

The paraquat 1,1'-dimethyl-4,4'-bipyridinium dichloride (PQ) molecular structure, illustrated in Figure 12, is a highly toxic quarternary nitrogen herbicide. Because of its low cost, rapid action, and environmental characteristics, paraquat is a widely used herbicide around the world (Franco, et al., 2010). It has been suggested that PQ might be an environmental factor contributing to neurodegenerative disorders such as PD. Additionally, PQ is a toxin known to target the dopaminergic neurons whose structure is similar to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Liou, et al., 1997; Di Monte, 2003; Dinis-Oliveira, et al., 2006; Chen, et al., 2010, Chin-Chan, et al., 2015). Studies using animal models have also indicated the neurotoxicity of PQ in nigrostriatal dopaminergic cells (Thiruchelvam, et al., 2003). PQ reproduces the cardinal PD pathologies such as loss of dopaminergic neurons (Kuter, et al., 2007) and protein aggregation in dopaminergic neurons as well as other pathologies that include oxidative stress, proteasome dysfunction, and mitochondrial dysfunction (Manning-Bog, McCormack et al., 2002, McCormack, et al., 2005; Castello, et al., 2007; Yang, et al., 2007). Furthermore, PQ in the presence of oxygen generates the superoxide radical ($O_2^{\bullet -}$) (Dicker and Cederbaum, 1991; Busch, et al., 1998), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2) leading to deleterious effects on cell function (Youngman and Elstner, 1981, Busch, et al., 1998). H_2O_2 induces Sirt1 overexpression (Hasegawa, et al., 2008).

Chen Qing et al., reported that PQ could damage the hippocampus of mice by oxidative stress, and that the injury was associated with hippocampal neuron mitochondrial dysfunction induced by mtDNA oxidative damage. In addition, excess ROS generation can damage a wide variety of cellular constituents including DNA, RNA, proteins, sugars and lipids, thereby compromising cell viability (Chen, et al., 2010). PQ-induced lipid peroxidation and consequent cell death of dopaminergic neurons can be responsible for the onset of the Parkinsonian syndrome, thus indicating that this herbicide may induce PD or influence its natural course. PQ has also been recently considered as an eligible candidate for inducing the Parkinsonian syndrome in laboratory animals, and can therefore constitute an alternative tool in suitable animal models for the study of PD (Dinis-Oliveira, Remiao et al., 2006).

Paraquat toxicity mechanism

PQ is a highly toxic pro-oxidant herbicide, prompting multi-organ failure, including the heart, brain, and lung injuries, although the precise underlying mechanisms remain poorly understood. To date, a number of signaling mechanisms have been postulated for paraquat toxicity, such as accumulation of free radical species and development of oxidative stress. Paraquat is believed to serve as a potent ROS generator, resulting in detrimental biological effects through oxidative stress injury and mitochondrial dysfunction (Wang, et al., 2016). The cellular toxicity of PQ is essentially due to its redox cycle (Figure 13). In previous research, PQ was reduced, mainly by NADPH cytochrome P-450 reductase NADPH-cytochrome *c* reductase, and the mitochondrial complex I also known as NADH: ubiquinone oxidoreductase, to form a PQ monocation free radical ($PQ^{\bullet+}$). It is generally accepted that PQ uses cellular diaphorases, which are a class of enzymes that transfer electrons from NAD (P) H to small molecules, such as PQ. The PQ monocation free radical is then rapidly reoxidized in the presence of oxygen generating the superoxide radical ($O_2^{\bullet-}$). This then sets off the well-known cascade of reactions leading to the generation of other reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and hydroxyl radical (HO) and the consequent cellular deleterious effects. It has been shown that hydroxyl radicals have been implicated in the initiation of membrane damage by lipid peroxidation during the exposure to PQ in vitro and in vivo (Dinis-Oliveira, et al., 2006).



PQ (1,1′-dimethyl-4,4bipyridinium ion)

Figure 12 Chemical structure of paraquat

Source: Adapted from Dinis-Oliveira, et al., 2006

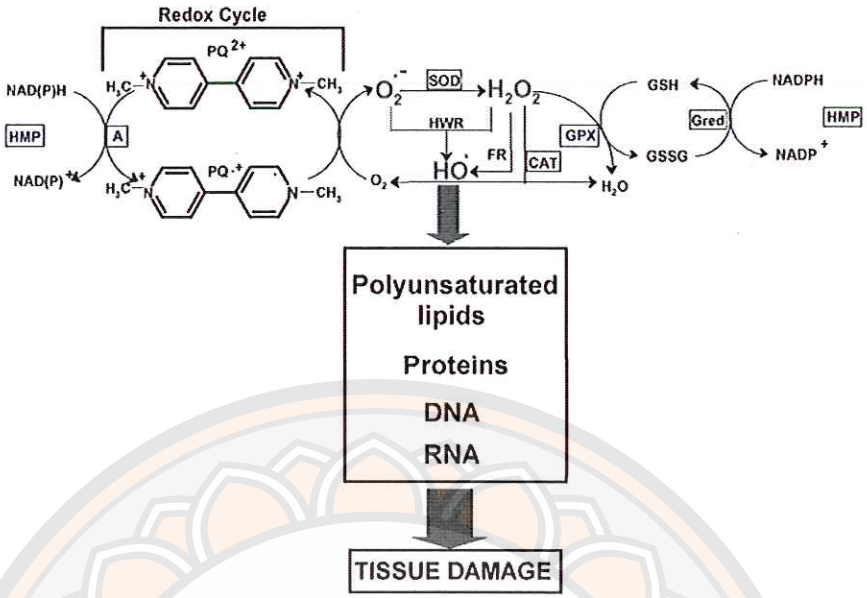


Figure 13 Schematic representation of the mechanism of paraquat toxicity A, cellular diaphorases; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; Gred, glutathione reductase; PQ^{2+}, paraquat; PQ^{+}, PQ cation radical; HMP, hexose monophosphate pathway; FR, Fenton reaction; HWR, Haber-Weiss reaction.

Source: Dinis-Oliveira, et al., 2006

Interestingly, various pesticides, herbicides and drugs have been used in animal and in vitro models of Parkinson (Cicchetti F., Drouin-Ouellet J., and Gross R. E., 2009). A common feature of many of these neurotoxic compounds, such as rotenone, paraquat, or MPTP, is the inhibition of mitochondrial complex I, followed by the overproduction of ROS, Adenosine triphosphate (ATP) exhaustion, and the induction of a wide range of abnormalities that can elicit neuronal and astrocytic cell death. Additionally, neurotoxins induce nuclear fragmentation, endoplasmic reticulum (ER) stress and unfolded protein response in catecholaminergic cells, which are associated with changes in proteasomal and chaperone activities, similar to those observed in PD. Other molecules used in PD models include the fungicide maneb, cyclodienes, organophosphates such as deltamethrin, DDT (dichlorodiphenyltrichloroethane),

2,4-dichlorophenoxyacetic acid, dieldrin, deguelin, diethyldithiocarbamate, paraquat, maneb, trifluralin and parathion (Figure 14) (Cabezas, et al., 2013).

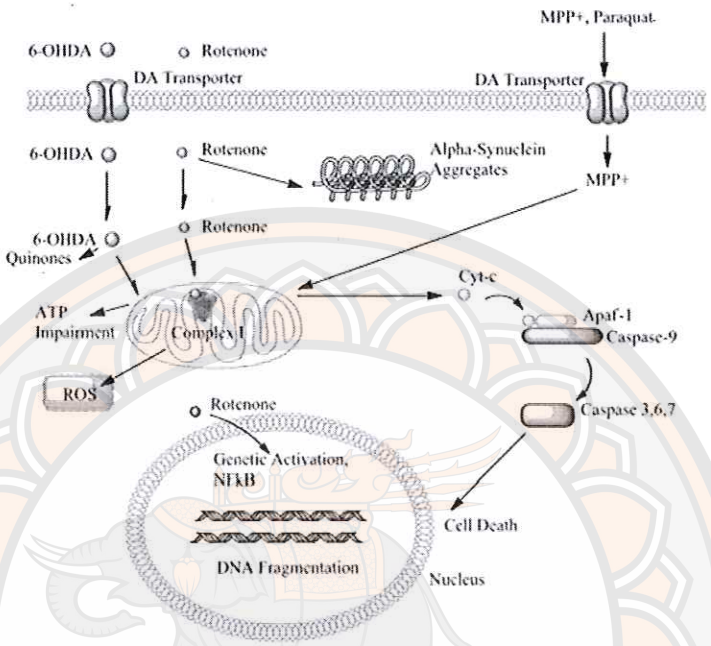


Figure 14 Experimental models in PD

Source: Cabezas, et al., 2013

Free radicals and oxidative stress

Free Radicals (FR) are substances that derive from incompletely oxidated compounds that have undergone partial burning and that have, in their structure, oxygen groups capable of initiating, at the surface of the cell membranes or even within the cells, aggressive oxidation reactions. FR results from both processes occurring in the body such as incomplete catabolism, energy production, hepatic detoxification, etc., and from the outer environment such as cigarette smoke, polluted air, foods, medicines, etc. (Butnariu and Samfira, 2012). FR mechanisms have been implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases. For example, the superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are known to be generated in the brain and nervous system in vivo, and several areas of the human

brain are rich in iron, which appears to be easily mobilizable in a form that can stimulate free-radical reactions (Aruoma, 1998).

Oxidative stress is defined as an exaggerated production of oxygenated FR, accompanied by a dislocation of anti-oxidation agents. FR are oxidated derivatives of the electron deficit, unstable oxygen molecule, that cause dysfunctions of all body cells (Butnariu and Samfira 2012). Interestingly, oxidative stress, manifested by protein oxidation and lipid peroxidation, among other alterations, is a characteristic of Alzheimer disease brain (Varadarajan, et al., 2000).

Reactive oxygen species (ROS) cause damage to mitochondrial components and initiate degradative processes. Such toxic reactions form the central dogma of "The Free Radical Theory of Aging." Cadenas Enrique and Davies K.J.A. review mitochondrial DNA, RNA, and protein modifications by oxidative stress and the enzymatic removal of such oxidatively damaged products by nucleases and proteases (Cadenas and Davies, 2000). The current concepts of Reactive Oxygen Species (ROS) signaling can be grouped into two action mechanisms: alterations of intracellular redox state and protein oxidative changes (Butnariu and Samfira, 2012).

Previous reviews have explored the hypothesis that oxidative modifications, mitochondrial functional disruption, and impairment of protein degradation constitute three interrelated molecular pathways that execute neuron death including PD (Figure 15). Malkus K. A., Tsika E. and Ischiropoulos H. propose that the combined interactions of these three interrelated molecular pathways-oxidative modifications, mitochondrial dysfunction, and impaired protein degradation-constitute a "Bermuda Triangle" that ultimately induces neuron death (Malkus, et al., 2009).

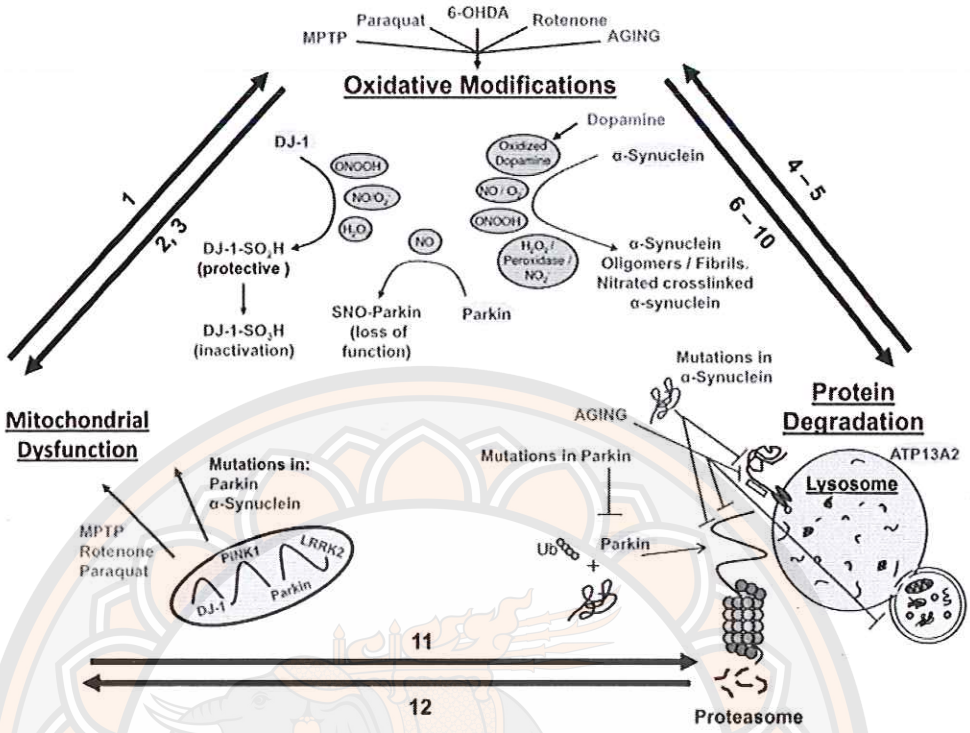


Figure 15 A "Bermuda Triangle" of insults leads to neurondeath in PD

Source: Malkus, et al., 2009

Sirtuin1 (Sirt1)

Sirtuin1 (Sirt1) is an nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase that deacetylates substrates via the consumption of NAD^+ releasing nicotinamide, O-acetyl-ADP-ribose and the deacetylated substrate. The sirtuin1 contain a 257 amino acid catalytic core domain and have differing N- and C- terminal tails and zinc-binding domains (Karagiannis and Ververis, 2012). The founding member of the family, SIRT1, promotes longevity in response to caloric restriction in species ranging from yeast to mammals, and it is believed that these protective actions may result, at least in part, from the regulation of energy homeostasis. Consistently, SIRT1 is an important regulator of metabolic processes such as lipolysis, fatty acid oxidation (FAO), mitochondrial activity, and gluconeogenesis including cellular apoptosis (Feige, et al., 2008, Hasegawa, et al., 2008). Interestingly, SIRT1 has recently been shown to attenuate amyloidogenic processing of amyloid-β protein

precursor (APP) in cell culture studies in vitro and in transgenic mouse models of Alzheimer disease. Mechanistically, SIRT1 increases α-secretase production and activity through activation of the α-secretase gene ADAM10. Because α-secretase is the enzyme responsible for the non-amyloidogenic cleavage of APP, upregulation of α-secretase shifts APP processing to reduce the pathological accumulation of the presumptive toxic Aβ species that results from β-secretase and γ-secretase activity (Bonda, et al., 2011).

In addition, previous studies found that H_2O_2 induces sirt1 overexpression. This previous work examined the role of sirt1 in renal tubular cell apoptosis by using HK-2 cells. When apoptosis was induced with H_2O_2, Sirt1 was upregulated with the concomitant increase in catalase expression. Sirt1 overexpression rescued H_2O_2-induced apoptosis through the upregulation of catalase. H_2O_2 induced the nuclear accumulation of forkhead transcription factor, FoxO3a and the gene silencing of FoxO3a enhanced H_2O_2-induced apoptosis (Figure 16). It was concluded that endogenous Sirt1 maintains cell survival by regulating catalase expression and by preventing the depletion of ROS required for cell survival. In contrast, excess ROS upregulates sirt1, which activates FoxO3a and catalase leading to rescuing apoptosis. Thus, Sirt1 constitutes a determinant of renal tubular cell apoptosis by regulating cellular ROS levels. (Hasegawa, et al., 2008). Furthermore, SIRT1 has a dual effect on FOXO3 function by increasing FOXO3's ability to induce cell cycle arrest and to resist oxidative stress (Brunet, et al., 2004).

Sirtuins play a pivotal role in various cellular processes, including gene silencing, DNA repair and life span extension in response to caloric restriction. These functions of sirtuins may be based on the fact that they can deacetylate and regulate diverse families of transcription factors including p53, FOXO, NF-κB and Ku70 (Okawara, et al., 2007).

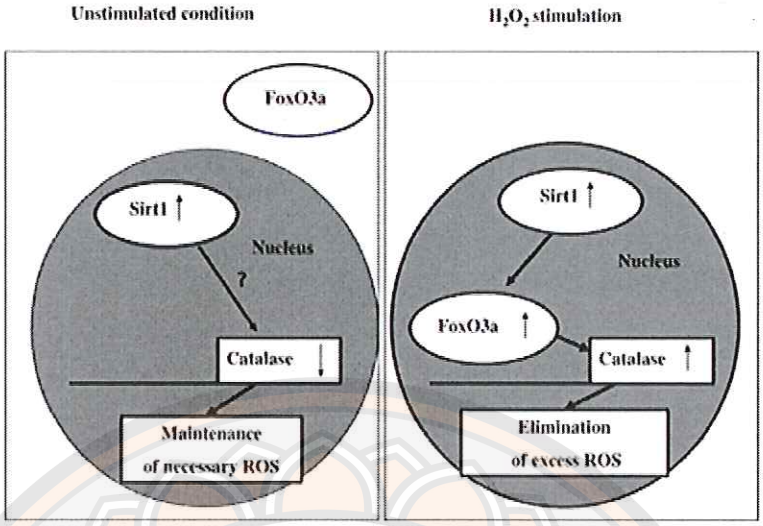


Figure 16 Schema depicting the mechanisms for Sirt1-modulated catalase expression. Sirt1 downregulates catalase expression without any ROS treatment, which functions to keep appropriate ROS levels (Left). In contrast, under excessive ROS insult, Sirt1 upregulates catalase through FoxO3a-dependent mechanisms, which leads to the decrease in ROS (Right).

Source: Hasegawa, et al., 2008

Peanuts (aka Groundnuts)

Peanuts (*Arachis hypogaea* (L.)) are a crop of global importance economically, with a total global production of about 35 million tonnes and oil production in excess of 5 million tonnes per annum (Hong, et al., 2008). The peanut cultivars recommended for growing in Thailand are Tainan 9, SK 38, Kalasin 2 and Lampang. These are commonly grown in three regions of Thailand, including Lampang, Nan, Chiang Rai, Chiang Mai, and Phayao.

Peanuts (*Arachis hypogaea* cv. Tainan 9) are an important grain legume in Thailand. It can be found cultivated in upland areas under rain-fed conditions where the amount and distribution of rainfall are relatively poor (Vorasoot, et al., 2003).

General characteristics of peanuts are bunch stems and pod clusters on the stem. Peanut flowers are usually yellow and clustered in groups of 2 to 6 flowers each. The pods are thin with a smooth external surface, with two seeds per pod (Figure 17).

Characteristics of Tainan 9: It has high yield with a high percentage of nut volume, about 32-77% of the total weight. Tainan 9 can adapt to the environment and produces about 260 kg per hectare (Department of Agricultural, 2010)

Figure 17 Peanut (*Arachis hypogaea* cv. Tainan 9)

Peanut sprout

Peanut sprouts are prepared from the germination of mature peanut kernels and have been used in the diet as health food. They are one of the most popular and personal favorite sprouts, widely eaten in Southern Thailand and used in curries, boiled, roasted, and baked. They contain important nutrients such as protein, fat, niacin, vitamin, carbohydrates, magnesium, phosphorus, and antioxidants. The main characteristic of peanut sprouts is the plump white trunk (Figure 18).

In recent years, nature has been a continuous source of pharmacologically active molecules and medicinal herbs (Dajas, et al., 2003). Peanut sprouts have been noted for their antioxidant properties and the germinated peanut kernels have been used in the diet as a health food for several centuries.

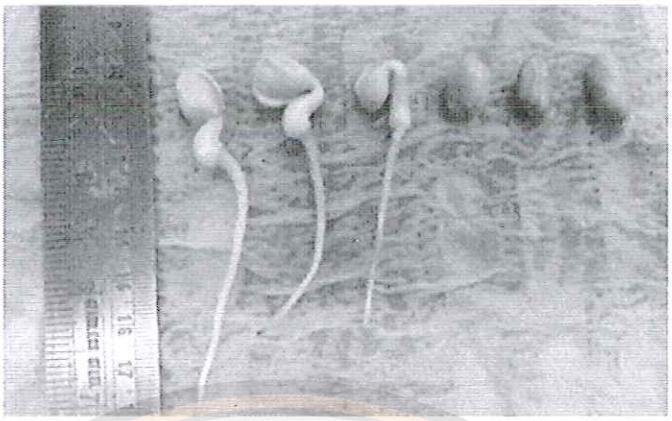


Figure 18 Peanut sprout (*Arachis hypogaea* cv. Tainan 9)

Pharmacological activities of peanut sprout

Peanut sprouts are prepared from the germination of peanut kernels. Reviews of peanuts have found that they contain bioactive compounds with antioxidant activity, such as resveratrol, flavonoids, phenolic compounds, and phytosterols. Peanut sprouts have been reported to be rich in flavonoids and phenolic compounds which may contribute to disease prevention and have health promoting properties (Kang, et al., 2010; Xiong, et al., 2014). They exhibit many biological functions such as anti-inflammatory activity attributed to inhibition of cyclooxygenase, estrogenic activity, and antiplatelet activity (Frankel, et al., 1993, Gehm, et al., 1997, de la Lastra and Villegas, 2007). They have also been reported to have beneficial anti-aging, anti-cancer and cardiovascular-protective effects, and on ischemia and neurodegenerative diseases such as Alzheimer disease (de la Lastra and Villegas, 2007; Lu, et al., 2008; Sales and Resurreccion, 2014). Choi. et al., reported that ethanol extract of peanut sprout (EPS) contained 54.2 μg/ml of *trans*-resveratrol and protected cells from ultraviolet B-induced oxidative stress. EPS has anti-oxidant properties and scavenged reactive oxygen species (ROS) and activated the Nrf2/anti-oxidant system (Choi, et al., 2015). There is a study of the neuroprotective effect of peanut sprout extract and its solvent soluble fractions on glutathione-depleted neuronal HT22 cells (629.11) That solvent fraction of PSE appears to be mediated by its potential to increase the expression of antioxidant enzyme (glutathione) rather than directly scavenging ROS (Kim, 2014).

Resveratrol

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene) is a natural phenolic compound found in many plants including vegetables, fruits, grains, roots, flowers, seeds, tea, and peanut sprouts, and also in red wine (Wendeburg, et al., 2009; Sun, et al., 2010). Peanut are a potent natural source of resveratrol. Wang Kuo-Hsi reported that germination of peanut kernels enhances resveratrol biosynthesis (Wang, et al., 2005), and also peanuts have been found to have the highest level of trans-resveratrol synthesis (Rudolf and Resurreccion, 2005).

The structure of resveratrol consists of the *cis* and *trans* forms (Figures 19 A and B). *trans*-Resveratrol was widely distributed in gymnosperms and dicotyledons including groundnuts, and was observed to occur there as a response to fungal infection or injury in 1976 (Trela and Waterhouse, 1996). Resveratrol reveals several pharmacological actions, including anti-inflammatory properties and protection against cardiovascular and neurodegenerative diseases, and cancer (Sun, et al., 2008). Recent reports have evaluated the potential protective role of resveratrol in neurodegenerative conditions. Resveratrol reduces the expression of different inflammatory mediators involved in the progression of neuropathological conditions and it has been shown to provide neuronal protection in different models (Okawara, et al., 2007). In addition, researchers have suggested that resveratrol provides beneficial influences on neuronal cells. For example, resveratrol protects cultured hippocampal neurons from cytotoxicity of nitric oxide (NO) and β-amyloid, striatal neurons from polyglutamine toxicity and dorsal root ganglion neurons from axon degeneration (Okawara, et al., 2007). Resveratrol also increased, in a dose-dependent way, brain superoxide dismutase, catalase and peroxidase activities that up regulated two catalase isoforms and a broad peroxidase band corresponding to several isoforms. Resveratrol is able to cross the brain - blood barrier and exerts potent antioxidant features. Resveratrol also exerts neuroprotective properties by upregulating several detoxifying enzymes, most of which are iron proteins. Recently, it was found that resveratrol attenuates axonal degeneration, Huntington disease's neurodegeneration and AD. These effects are mediated by the sirtuin pathway, nuclear targets of the redox signalling cascade which function as energy sensors and longevity factor (Mokni, et al., 2007).

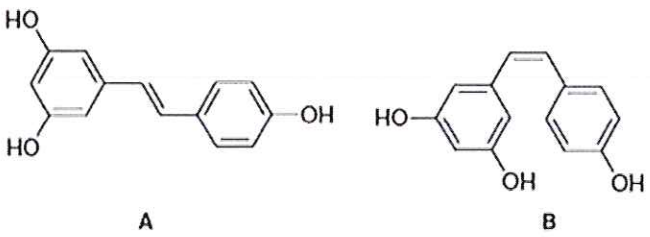


Figure 19 Chemical structure of Resveratrol (A) *trans*-isomer (B) *cis*-isomer

Source: Kolouchová-Hanzlíková, et al., 2004

In order to reveal information on absorption, metabolism, and the consequent bioavailability of resveratrol, different research approaches were performed, including in vitro, ex vivo, and in vivo models (Wenzel and Somoza, 2005). Furthermore, resveratrol-loaded solid lipid nanoparticles were functionalized with apolipoprotein E which can be recognized by the LDL receptors overexpressed on the blood–brain barrier (Figure 20) (Neves, et al., 2016).

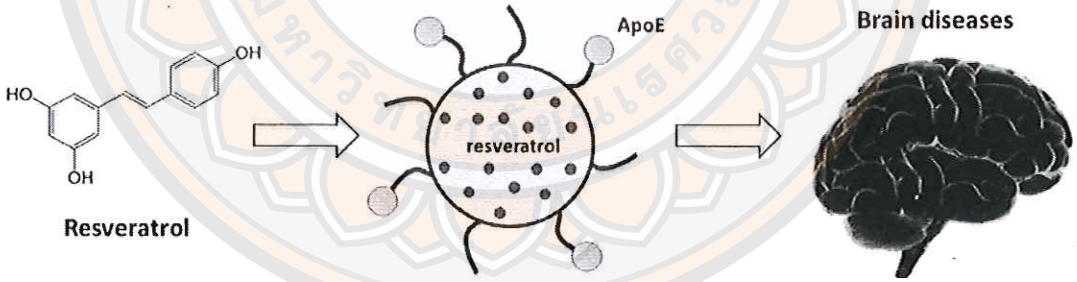


Figure 20 Brain-targeted lipid nanoparticle

Source: Neves, et al., 2016

Human Neuroblastoma Cell (SK-N-SH)

SK-N-SH (ATCC number: HBT-11) is a neuroblastoma cell line that was established in 1970 from metastatic cells found in the bone marrow aspirate of a four-year-old female of unknown ethnicity. Cells were developed by J.L. Biedler and differ from SK-N-MC (see ATCC HTB-10) in that they exhibit a longer doubling time and higher levels of dopamine-β-hydroxylase. SK-N-SH display epithelial morphology and grow (Figure 21). Eagle's Minimum Essential Medium (MEM) supplemented 10% fetal bovine serum at 37°C, 95% humidifier and 5% CO_2. Subculturing was initiated by removing from the medium, and rinsing with phosphate buffer saline, trypsinize with 0.25% trypsin, 0.03% EDTA solution at 37°C until cell detachment. Fresh culture medium was added to resuspend the population in new culture flasks. A subcultivation ratio of 1:3 to 1:8 is recommended with medium renewal, 1 to 2 times per week. In addition, SK-N-SH has been used as a target cell line in cell-mediated cytotoxicity assays and is a suitable transfection host.

SK-N-SH has been used in various research projects such as a study of estradiol protection against β-amyloid (25–35)-induced toxicity (Green, et al., 1996), the divergent role of calcium on Aβ induced cell death (Lee, et al., 2006), and the anti-apoptotic effects of red ginseng on SK-N-SH induced by hydrogen peroxide (Kim, et al., 2010).

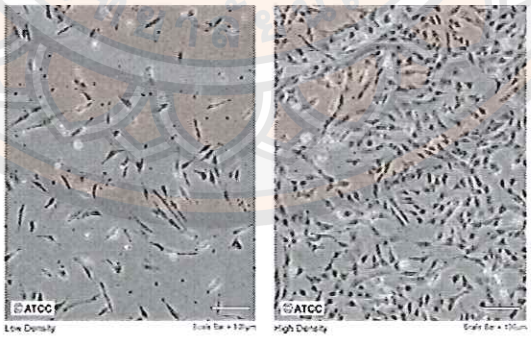


Figure 21 Morphology of neuroblastoma cell line (ATTC number: HTB-11)

Source: American Type Culture Collection (ATCC), 2014

CHAPTER III

RESEARCH METHODOLOGY

This study was designed to investigate the protective effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells and to identify resveratrol level in peanut sprout extract. The experiment was divided into three parts as follows: (1) The neuroprotective effects of peanut sprout extract were examined for cell viability and to investigate α-synuclein and SIRT1 genes expression, including the investigation of Aβ protein expression. (2) The antioxidative effect of peanut sprout extract on the intracellular reactive oxygen species (ROS) in paraquat-induced oxidative stress in SK-N-SH cells was investigated. (3) Resveratrol in peanut sprout extract (Arachis hypogaea cv. Tainan 9) was identified by High-performance liquid chromatography (HPLC).

The protective effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells

1. Germination of peanut kernels and peanut sprout extraction

Mature peanut kernels (*Arachis hypogaea* cv. Tainan 9) were soaked in normal saline for 3 hrs, washed three times with sterile water and then soaked in normal saline for 30 min. The kernels were then placed on a plastic net tray and germinated in a growth chamber for 3 days in the dark. After 1 day of incubation, the ungerminated kernels were discarded. On the third day, the peanut sprouts were weighed and dried for 72 hrs at 60°C, and then ground into powder.

100g of the peanut sprout powder was mixed with hexane 100 ml and incubated overnight on a hot plate stirrer. The mixture was then passed through filter paper. 80% ethanol 100 ml was added and incubated overnight on a hot plate stirrer. The mixture was again filtered prior to rotary evaporation at 50°C and 50 mmHg. The resulting peanut sprout extract was dried at 50°C before use.

2. Cell Culture

Human neuroblastoma cells (SK-N-SH) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution and maintained at 37°C in an humidified incubator with 5% CO_2.

3. Cell viability/cytotoxicity assay

An MTT reduction assay was used to assess the cell viability. The SK-N-SH cells were seeded in 96-well plates at a density of 3 x 10^4 cells per well and incubated overnight at 37°C in 5% CO_2. The cells were treated with paraquat (0–1 mM) and peanut sprout extract (0.25-1.5 mg/ml) for 24, 48 and 72 hrs. In one group, referred to as the prevention group, the cells were pretreated with PSE for 4 hrs, following 0.75 mM paraquat exposing for 48 hrs. The media were switched to media containing 1 mg/ml MTT, incubating for 4 hrs at 37°C, 5% CO_2 and the media were then discarded and the purple formazan crystals in the MTT were dissolved in DMSO. The product was measured by a microplate reader (Science THE CO., LTD, USA) at 540 nm. The percentage of cell viability was normalized to the control group.

$$\%\text{Cell Viability} = \frac{\text{Mean Absorbance of Sample x 100}}{\text{Mean Absorbance of control}}$$

4. Cell culture and treatment

The cells were plated at a density of 1 x 10^6 cells per well in 6-well plates and incubated at 37°C in 5% CO_2 for 24 hrs. The cells were classified into 4 groups. The cells in Group 1 were treated with serum free media for 48 hrs. Group 2 cells were treated with 0.75mM paraquat for 48 hrs. Group 3 cells were treated with 1mg/ml peanut sprout extract for 48 hrs, and the cells in group 4 were first treated with peanut sprout extract for 4 hrs and then treated with 0.75 mM paraquat and then incubated at 37°C in 5% CO_2 for 48 hrs. After treatment, the cells were used to study the expression of SIRT1 and α-synuclein genes, and amyloid beta 42 protein, including intracellular ROS.

5. RNA analysis

Total RNA was extracted from the SK-N-SH cells using RiboZol™ RNA Extraction Reagent. (Amresco, USA) according to standard procedures. Briefly, after culturing and treatment, the cells were washed with PBS and then incubated with 1ml of RiboZol™ RNA Extraction Reagent at room temperature for 10 min. The extract was then rapidly mixed with 200 µl of chloroform and incubated at room temperature for 3 min. After incubation, six tubes containing a sample of the extract were centrifuged at 12,000xg for 15 min at 4 °C. The RNA was collected from the aqueous phase and precipitated with 500 µl of isopropanol, and incubated at room temperature for 10 min. This precipitate was then centrifuged at 12,000xg for 10 min at 4 °C, and the resulting pellets were washed with 1 ml of 70% ethanol and then centrifuged at 75000xg for 5 min at 4 °C and air dried. Finally, the RNA pellets were dissolved in 20 µl of DEPC treated water. The resulting solution, Total RNA, contained only the discarded genomic DNA.

500 ng of the Total RNA was treated with DNase I. First-strand cDNA was synthesized from the Total RNA (250 ng) by Tetro reverse transcriptase and Oligo $(dT)_{18}$ primer with incubation at 45°C for 30 min. This reaction was terminated by incubating the treated Total RNA at 85°C for 5 min. The synthesized cDNAs were further utilized for quantitative PCR analysis.

The gene expression levels were determined by quantitative PCR using Light Cycler® 96 (Roche Diagnostics) and SensiFAST™ SYBR® Kits. The sequences of the primers used in this study are as follows: SIRT1 (forward: 5'-TCAGTGGCTGGAA CAGTGAG-3' /reverse: 5'-AGCGCCATGGAAAATGTAAC-3'), α-synuclein (for ward: 5'-GTGCTCAGTTCCAATGTGCC-3' /reverse: 5'-TGGGGGCAGGTACA GATACT-3') and β-actin (forward: 5'-CCACCATGTACCCTGGCATT-3' /reverse: 5'-CCAACTCGTCATACTCCTGC-3'). The real-time-qPCR was carried out in a 10 µl reaction volume containing 1x SensiFAST SYBR No-ROX mix buffer, 125 nM of both forward and reverse primers and 500 ng of cDNA template. The reaction was initially polymerase activation at 95°C for 10 min, then cycled 40 times at 95°C for 5 sec, 60°C for 10 sec and 72°C for 20 sec, and finally melting curve at 95°C for 15 sec and 60°C for 1min. The data were expressed as the mean ± standard error of the mean

(S.E.M.) from three independent experiments. Transcription levels of all genes were normalized to the level of the β-actin gene used as the internal control.

6. Western blotting

After culturing and treatment as described above, the cells were washed with PBS and then suspended in 100 µl of RIPA lysis buffer and lysed 3 times by sonication on ice at pulse 10 sec/ 10 sec, amp 60% for 15 sec, using a probe sonicator (Becthai Bankok Equipment &Chemical, USA) and incubated at 4°C for 30 min. Soluble and insoluble protein were separated by centrifugation at 12,000xg for 20 min at 4°C. The protein concentration was determined using a BCA Protein Assay. The proteins were separated using 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrically transferred to a PVDF transfer membrane, blocking with 5% skim milk. Target proteins were immunodetected using specific antibodies. Primary antibodies were composed of anti-β-actin, anti-beta-amyloid 1-42. Horseradish peroxidase-conjugated anti-rabbit IgG was applied as the secondary antibody, and bands were detected using Chromogenic detection of horseradish peroxidase (HRP) activity based the action of 3,3'-diaminobenzidine (DAB) in western blot methods.

Intracellular reactive oxygen species (ROS) determination

The cells undergoing oxidative stress defined by the presence of reactive oxygen species (ROS), namely superoxide, were determined using a Muse® Oxidative Stress Kit (Merck Millipore, Germany). Briefly, after culturing and treatment, the cells were re-suspended at a concentration of 1 x 10^6 cells per ml in 1X assay buffer (Muse® Oxidative Stress Kit), after which the samples were incubated for 30 min at 37 °C and then the ROS positive cells were examined using a Muse® cell analyzer (Merck Millipore, Germany).

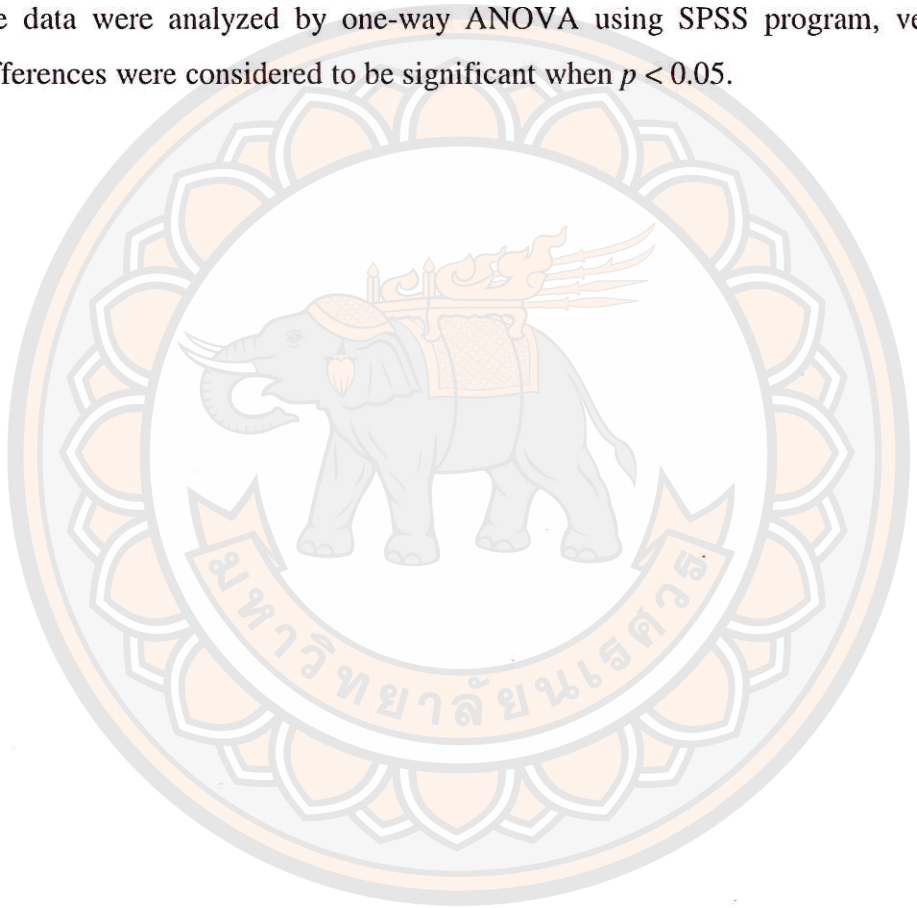
High-performance liquid chromatography (HPLC)

1 mg of peanut sprout extract was mixed with 1 ml of 80% ethanol and filtered through 0.22 µm PVDF membranes. 20 µl of mixture was injected and separated on a reversed-phase HPLC system consisting of a pump equipped with a UV detector and Luna 5U C18 (2) 100A column (250 x 46 mm) (Phenomenex Inc., USA). The isocratic mobile phase contained acetonitrile/water (35:65, v/v) and the flow rate was 1ml/min. The wavelength was set at 306 nm (Lee, Lee., et al., 2004).

Standard resveratrol was prepared in ethanol at 6 concentrations 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 µg. The concentration of resveratrol in the samples was calculated using the peak area in the same or similar retention time to the standard solutions.

Statistical analysis

All results were expressed as the mean ± standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA using SPSS program, version 17.0. Differences were considered to be significant when $p < 0.05$.



CHAPTER IV

RESULTS

The protective effect of peanut sprout extract on cytotoxicity induced by paraquat in SK-N-SH cells

1. Paraquat induced cytotoxicity in SK-N-SH cells

SK-N-SH cells were treated with paraquat at concentrations of 0, 0.25, 0.5, 0.75 and 1 mM, for 24 hrs. The results showed cell viability as 100±0%, 99±3.0%, 99±2.9%, 95±3.2%, and 96±2.1% respectively (Figure 22). These results indicate that paraquat induced cytotoxicity in SK-N-SH cells produces no significant difference in cell viability after 24 hrs.

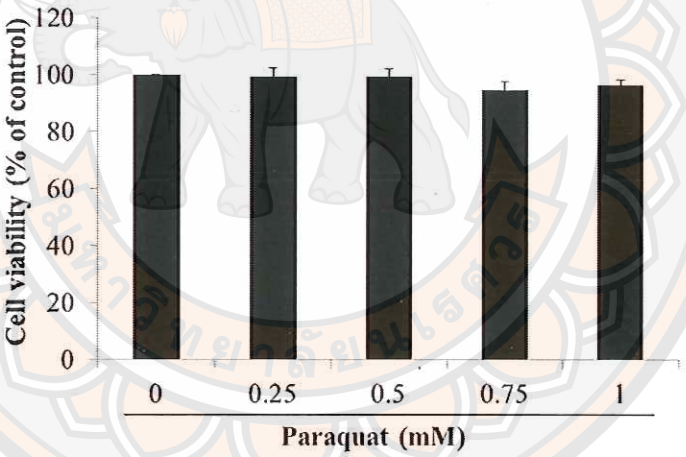


Figure 22 Effect of paraquat on the cell viability of SK-N-SH cells (24 hrs). Values are mean with standard error of triplicate experiments.

These SK-N-SH cells were then treated with the same concentrations of paraquat (0, 0.25, 0.5, 0.75 and 1 mM), but for 48 hrs. The results showed that cell viability was decreased in a concentration-dependent manner as 100±0%, 90±5%, 76±2%, 58±3%, and 35±6% respectively (Figure 23). Also, the presence of 0.5-1 mM paraquat significantly reduced cell viability over 75% ($*p<0.05$). This result indicates that paraquat enhanced the death of SH-N-SH cells. The concentration 0.75 mM was selected for use in our further studies.

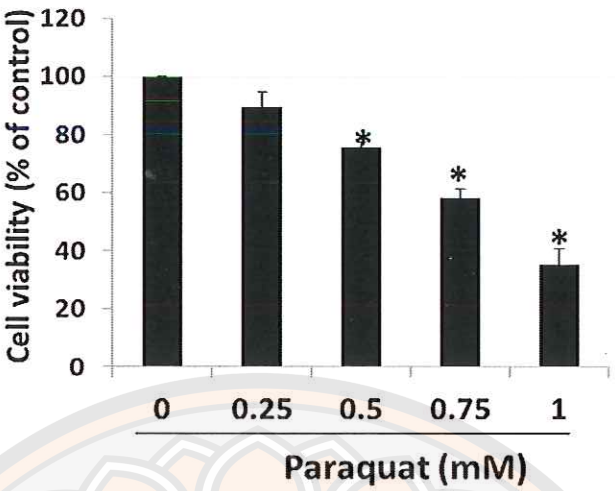


Figure 23 Effect of paraquat on the cell viability of SK-N-SH cells (48 hrs), *p<0.05 as compared with 0 mM. Values are mean with standard error of triplicate experiments.

Another sample of SK-N-SH cells was treated with paraquat at the same concentrations (0, 0.25, 0.5, 0.75 and 1 mM) for 72 hrs. The results showed cell viability was decreased in a concentration-dependent manner as 100±0%, 52±0.8%, 40±0.8%, 36±0.8%, and 32±1.2% respectively (Figure 24).

The presence of 0.25-1 mM paraquat significantly reduced cell viability over 75% (*p<0.05). This result indicated that paraquat enhanced the death of SH-N-SH cells.

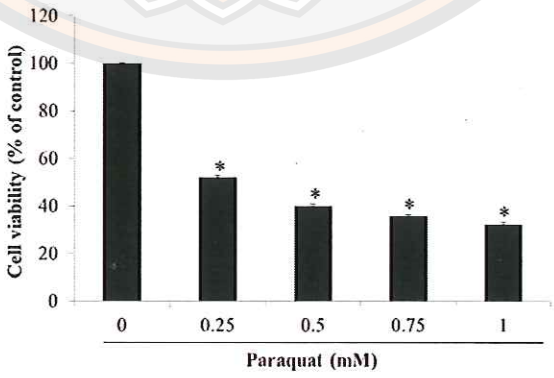


Figure 24 Effect of paraquat on the cell viability of SK-N-SH cells (72 hrs). *p < 0.05 as compared with 0 mM. Values are mean with standard error of triplicate experiments.

2. Effect of peanut sprout extract on cytotoxicity levels on untreated SK-N-SH cells

Cells were treated with peanut sprout extract at concentrations of 0.25, 0.5, 1.0, and 1.5 mg/ml for 24 hrs. Cell viability was promoted to about 125±17%, 111±5%, 124±20%, and 139±18% at these concentrations of peanut sprout extract, respectively (Figure 25). These values are not statistically significant when compared to the cell viability observed in the cultures in the control medium, without peanut sprout extract.

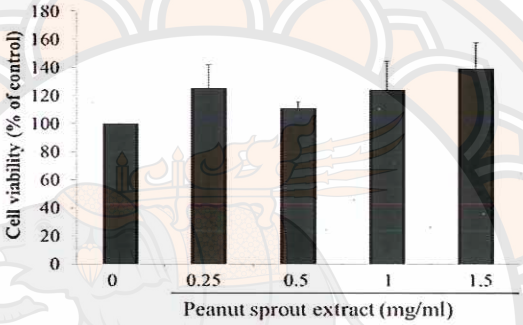


Figure 25 Effect of peanut sprout extract on the viability of SK-N-SH cells (24 hrs). Values are mean with standard error of triplicate experiments.

Other SK-N-SH cells were treated with peanut sprout extract at concentrations of 0.25, 0.5, 1.0, and 1.5 mg/ml for 48 hrs. The peanut sprout extract at concentrations of 0.25, 0.5, 1.0, and 1.5 mg/ml promoted cell viability up to about 100.89±2.00%, 104.57±1.67%, 116.29±7.98%, and 126.29±5.35% respectively (Figure 26). The presence of peanut sprout extract at the concentration of 1.5 mg/ml significantly reduced cell viability by about 26% ($*p<0.05$) as compare to cells cultured without peanut sprout extract.

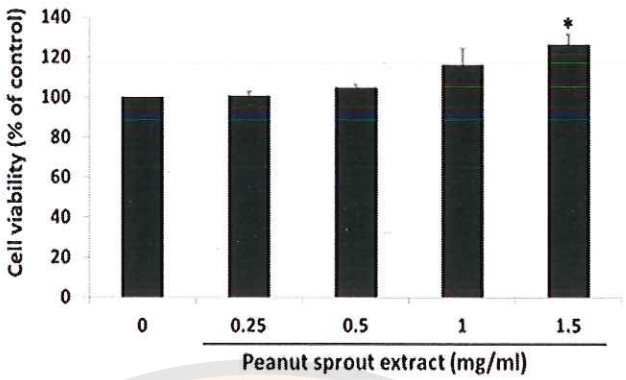


Figure 26 Effect of peanut sprout extract on the viability of SK-N-SH cells (48 hrs).*p<0.05 as compared with 0 mg/ml. Values are mean with standard error of triplicate experiments.

Cells were treated with peanut sprout extract for 72 hrs at the concentrations of 0.25, 0.5, 1.0, and 1.5 mg/ml. The peanut sprout extract at concentrations of 0.25, 0.5, 1.0, and 1.5 mg/ml promoted cell viability up to 101±3.96%, 109±3.25%, 119±4.49%, and 125±9.15% respectively (Figure 27). The presence of peanut sprout extract at the concentration of 1.5 mg/ml significantly reduced cell viability up to 25% (*p<0.05) as compared to the cells cultured in the medium without peanut sprout extract.

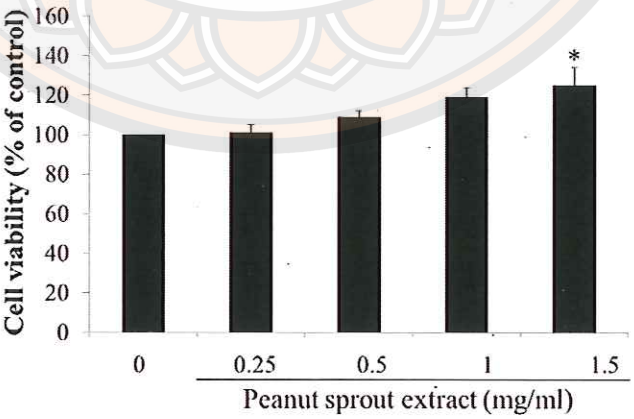


Figure 27 Effect of peanut sprout extract on the viability of SK-N-SH cells (72 hrs).*p<0.05 as compared with 0 mg/ml. Values are mean with standard error of triplicate experiments.

3. Effect of peanut sprout extract on cytotoxicity levels induced by paraquat in SK-N-SH cells

Six samples of SK-N-SH cells were cultured for 4 hrs in medium containing peanut sprout extract at concentration levels of 0, 0.25, 0.5, 1, and 1.5 mg/ml. One sample was untreated, as the control. A second sample was treated with paraquat only, 0.75 mM, for 48 hrs. The 4 samples treated with the various concentrations of peanut sprout extract were also similarly treated with paraquat, 0.75 mM, for 48 hrs. The peanut sprout extract significantly promoted cell viability at concentrations of 1 and 1.5 mg/ml up to 16.5% (**p<0.005) and 23.6%% (**p<0.005) as compared to the sample treated only with paraquat (Figure 28). This result indicates that peanut sprout extract has a protective effect against cell cytotoxicity indiced by paraquat in SH-N-SH cells.

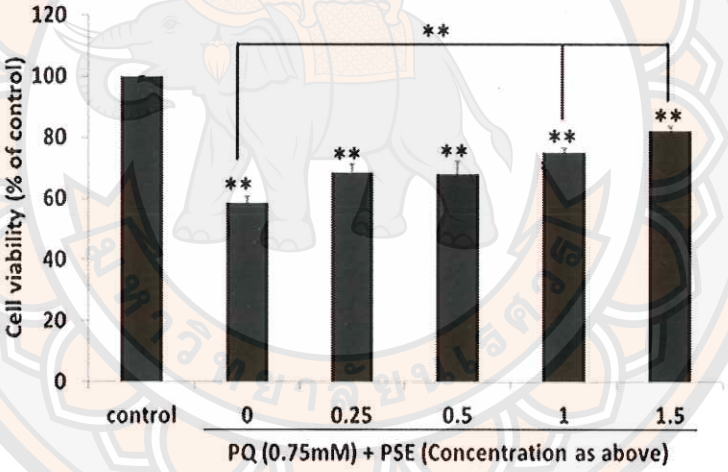


Figure 28 Neuroprotective activity of peanut sprout extract in SK-N-SH cells. **p<0.005 as compared with the control. The antioxidative effect of peanut sprout extract on SK-N-SH induced by paraquat

4. Effect of peanut sprout extract on SIRT1 and α-synuclein expression

The effects of peanut sprout extract on SIRT1 and α-synuclein genes expression induced by paraquat that genes expression were investigated in SK-N-SH cells by quantitative RT-PCR. Cells were treated with peanut sprout extract at a concentration of 1 mg/ml for 4 hrs and then treated with paraquat at a concentration of 0.75 mM for 48 hrs. The paraquat significantly increased SIRT1 and α-synuclein mRNA levels to 2.1±0.5 and 1.6±0.01 of the control, respectively (Figure 29 and 30).

The cells pre-treated with peanut sprout extract and those treated with peanut sprout extract, and then treated with paraquat, had significantly decreased SIRT1 and α-synuclein mRNA levels to 2.1±0.2 and 1.1±0.05 of paraquat, respectively. This result indicates that both peanut sprout extract and paraquat effect SIRT1 and α-synuclein genes expression.

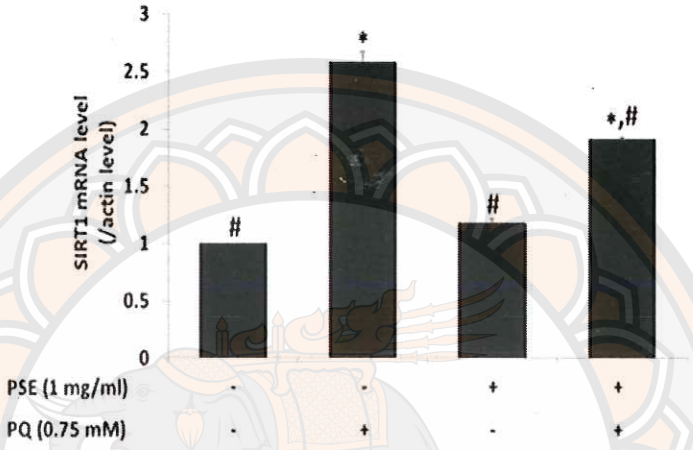


Figure 29 Expression of SIRT1 in SK-N-SH cells, relative SIRT1 mRNA levels calculated by the $2^{(-\Delta\Delta Ct)}$, normalized to β-actin mRNA level. $^{\#}p<0.05$ as compared with 0.75 mM. $^{*}p<0.05$ as compared with control.

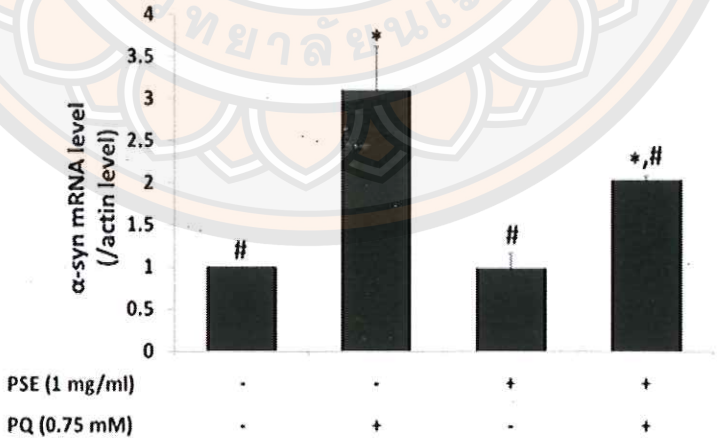


Figure 30 Expression of α-synuclein in SK-N-SH cells, relative α-synuclein mRNA levels calculated by the $2^{(-\Delta\Delta Ct)}$, normalized to β-actin mRNA level. $^{\#}p<0.05$ as compared with 0.75 mM. $^{*}p<0.05$ as compared with control.

5. Effect of peanut sprout extract on amyloid beta 42 (Aβ42) protein expression

The effects of peanut sprout extract on the protein level of Aβ42 were investigated in SK-N-SH cells by western blotting (Figure 31A and B). Cells were first treated with peanut sprout extract at a concentration of 1 mg/ml for 4 hrs, after which they were treated with paraquat at the concentration 0.75 mM for 48 hrs. The results show that these cells showed a statistically significant increase in Aβ42 protein levels as compared with medium culture without peanut sprout extract and paraquat (*p<0.05). A sample of cells treated only with peanut sprout extract showed significantly decreased Aβ 42 protein levels to 0.39±0.0 (Figure 31B). These results indicate that peanut sprout extract decreases the Aβ 42 protein levels induced by paraquat in SK-N-SH.

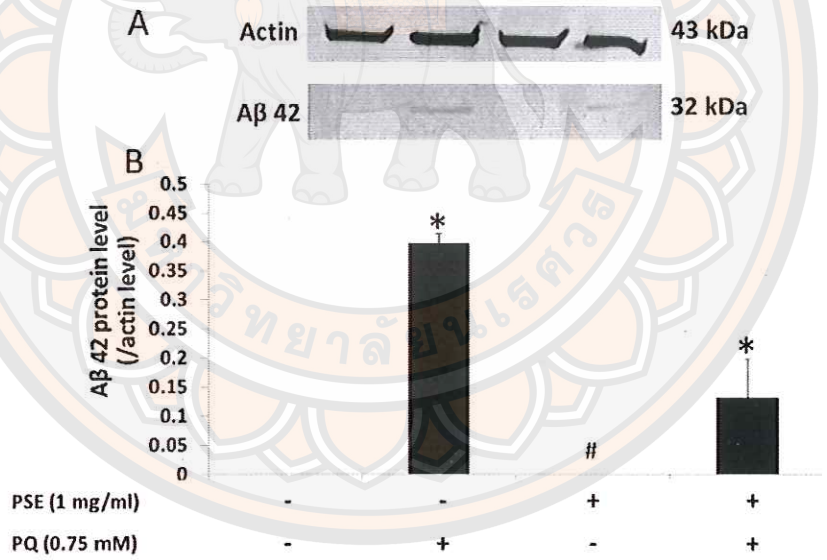


Figure 31 Effect of peanut sprout extract on protein level of Aβ 42. (A) Representative photograph of Aβ 42. (B) Protein level of Aβ42. The level of protein was quantified by ImageJ and normalized to actin level. *p<0.05 as compared with control and $^{\#}p$<0.05 as compared with 0.75 mM.

Investigation intracellular reactive oxygen species (ROS)

A significant increase in intracellular ROS was found in SK-N-SH cells after being treated with 0.75mM of paraquat as compared to control (*p<0.05) (Figure 35A and B). However, the sample of cells pre-treated with peanut sprout extract showed a significant decrease in intracellular ROS as compared to the sample treated with 0.75mM paraquat ($^{\#}p$<0.05) (Figure 32A and B). These results suggest that paraquat increases the generation of intracellular ROS in SK-N-SH cells.

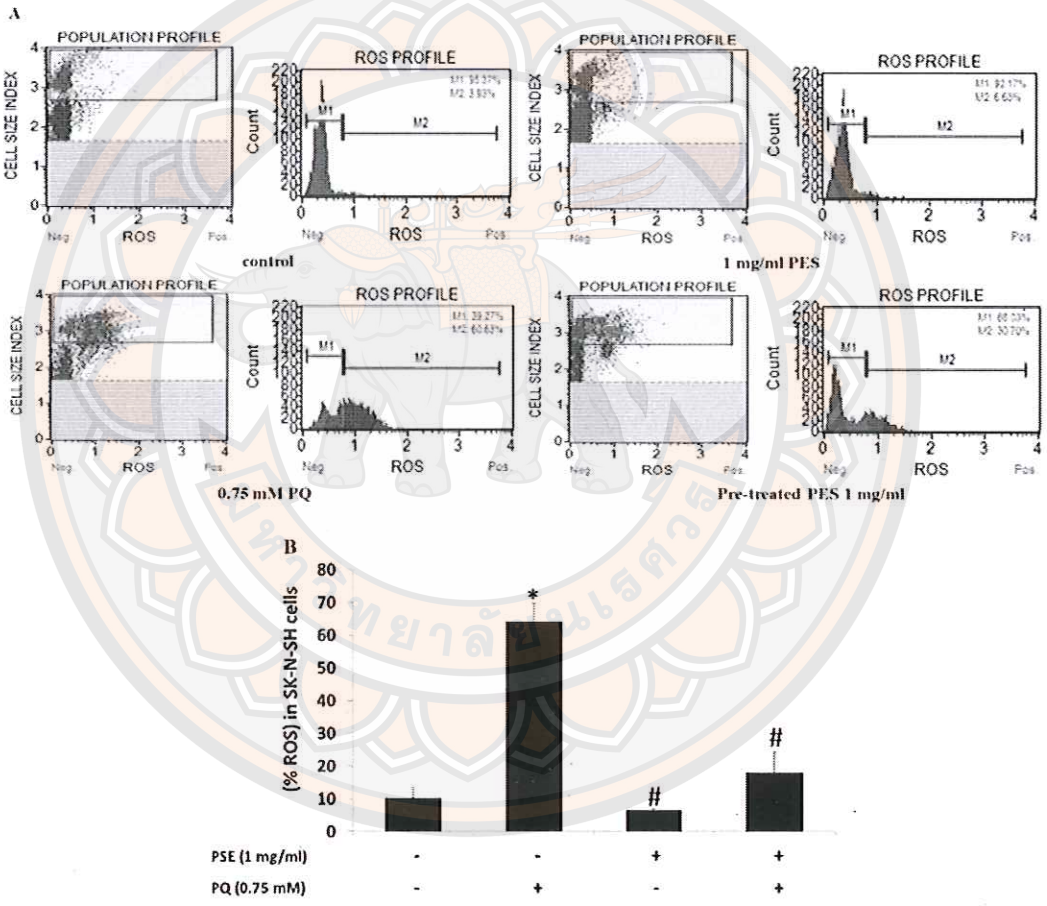


Figure 32 Effect of peanut sprout extract on intracellular ROS in SK-N-SH cells. (A) The typical picture of ROS detected by Musem cell analyzer. (B) Percentages of ROS. *p<0.05 as compared with control and $^{\#}p$<0.05 as compared with 0.75 mM.

Determination of *trans*-resveratrol in peanut sprout extract Tainan 9 variety

A modified HPLC method (Lee, et al., 2004) for the analysis of *trans*-resveratrol in peanut sprout extract from the Tainan 9 variety was developed. *trans*-resveratrol was identified by its retention times in comparison with that of standard resveratrol, identified by adding standard resveratrol to the samples. Figure 33 and 34 are the chromatograms of the peanut sprout extract monitored at 306 nm. The chromatograms show that the Tainan 9 peanut variety has no peak of *trans*-Resveratrol (Figure 33a and b), whereas a 3-day old peanut sprout extract from the Tainan 9 variety shows a peak of *trans*-Resveratrol (Figure 34a and b).

The analytical results of peanut sprout extract indicated the concentrations of *trans*-resveratrol compared with standard *trans*-Resveratrol (Figure 35). Sanders reported that the concentration of resveratrol in peanut sprout extract Tainan 9 variety was 2.49 µg/g extract.

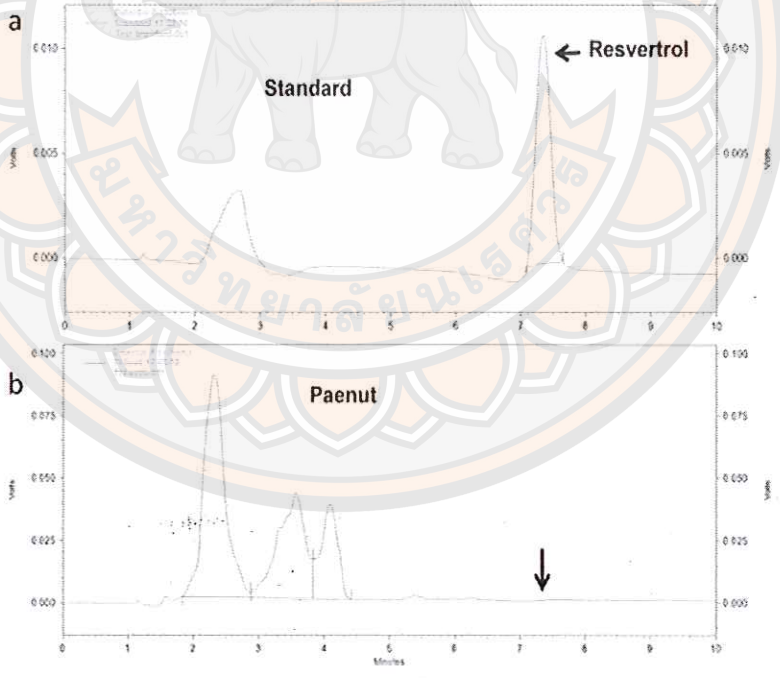


Figure 33 Representative (a) Chromatograms of standard. (b) Representative chromatograms of *trans*-resveratrol in peanut.

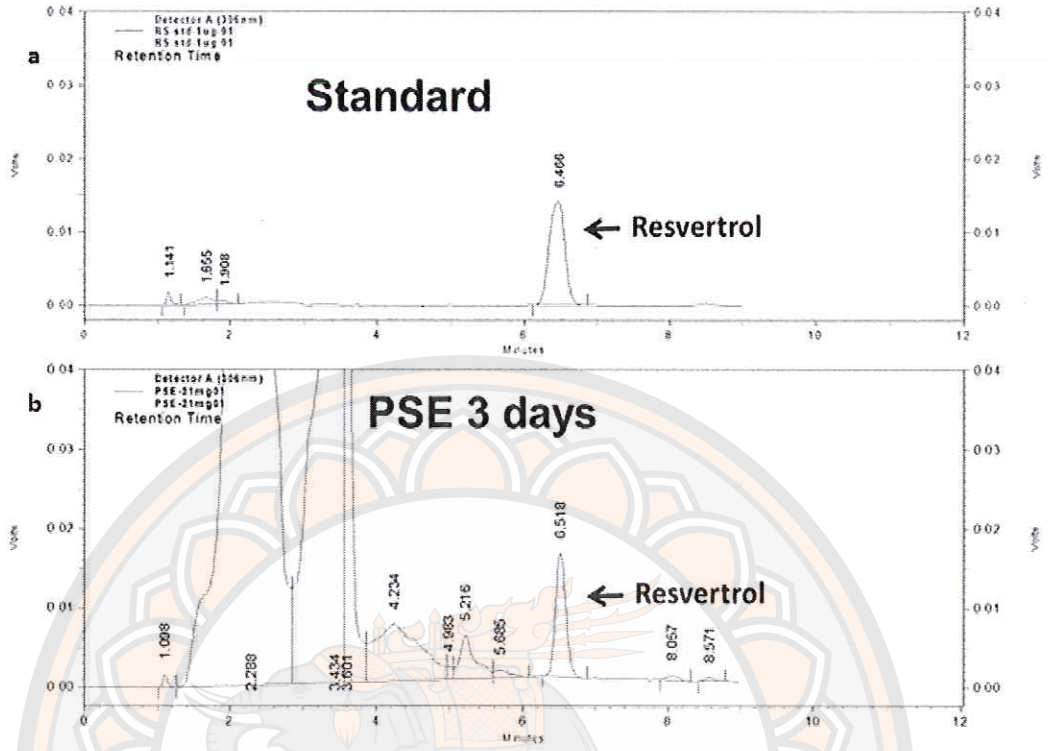


Figure 34 Representative (a) Chromatograms of standard. (b) Representative chromatograms of *trans*-resveratrol in 3 days peanut sprout extract.

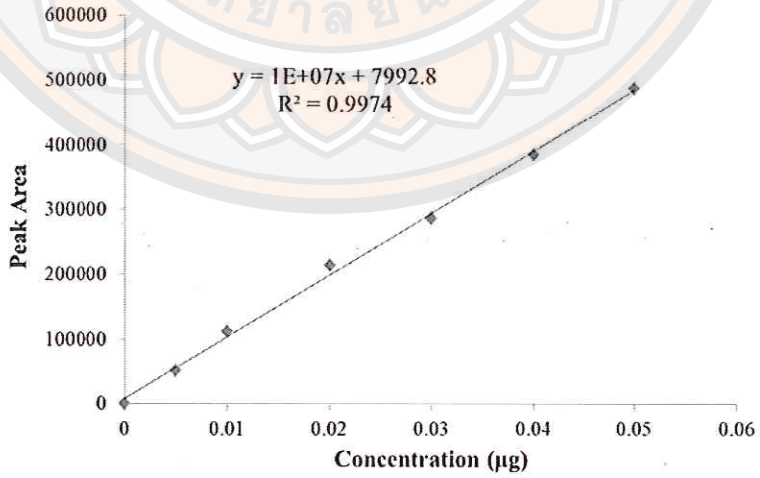


Figure 35 Standard curve *trans*-resveratrol concentrations were 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 µg

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

1. Investigation of the protective effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells

In this study, we observed a protective effect of peanut sprout extract against paraquat-induced oxidative stress in SK-N-SH cells. It has been reported that peanut sprout extract protects against UVB-induced oxidative stress by the activation of Nrf2 and the up-regulation of Nrf2-relating antioxidants. As well, peanut sprout extract plays an important role in enhanced resveratrol biosynthesis (Choi, et al., 2013; Wang, et al., 2005). Peanut sprout extract contains more polyphenols, especially resveratrol, than non-sprout peanut extract (Kang, et al., 2010). Antioxidants from nutritional sources can protect against the death of neuronal cells in AD and also modulates Aβ-induced oxidative stress (Butterfield, et al., 2002). Thus, it has been postulated that peanut sprout extract has antioxidant properties that can act to protect against oxidative stress-induced cell death.

2. Evaluation the effect of peanut sprout extract on α-synuclein and SIRT1 genes expression

Sirtuin1 (Sirt1) has been implicated in the activation of anti-apoptotic, anti-inflammatory, anti-stress responses, and aggregation of proteins involved in neurodegenerative disorders (Outeiro, et al., 2008). The action of SIRT1 is regulated through p53, NF-kB, MyoD, PGC-1, and FOXO3 (Feige and Auwerx, 2008). Our study showed that paraquat-treated SK-N-SH cells overexpressed SIRT1 mRNA. Paraquat induces ROS generation, according to (Castello, et al., 2007). It has been reported that ROS causes a high expression of Sirt1 (Figure 36) (Hasegawa, et al., 2008). Paraquat may trigger mechanisms of antioxidative defenses through SIRT1 overexpression to rescue paraquat-treated SK-N-SH cells. SIRT1 may function through FOXO3 to affect the antioxidant system. Paraquat has been found to cause neurodegenerative diseases and induced lipid peroxidation and consequential cell

death of dopaminergic neurons that are observed in the onset of the Parkinsonian syndrome (Dinis-Oliveira, et al., 2006). Further, α-synuclein is the major protein component of Lewy bodies, a cardinal pathological feature of the degenerating Parkinsonian brain. Paraquat induces the conformational change in the α-synuclein structure and significantly accelerates the formation rate of α-synuclein fibrils in vitro (Uversky, et al., 2001; Berry, et al., 2010), and α-synuclein has been found to induce mitochondrial dysfunction and oxidative stress (Hsu, et al., 2000). These actions of paraquat have been implicated in the formation of aggregated α-synuclein (Paxinou, et al., 2001). In our study, we found that paraquat not only possesses a potent toxic effect in SK-N-SH cells but also induces overexpression of α-synuclein genes. Interestingly, peanut sprout extract can down-regulate the α-synuclein gene, as has been demonstrated by Caruana, Högen et al., who found that the inhibition and disaggregation of α-synuclein oligomers is a result of natural polyphenolic compounds (Caruana, et al., 2011). Our study demonstrated that peanut sprout extract can down-regulate α-synuclein and protect against cell death.

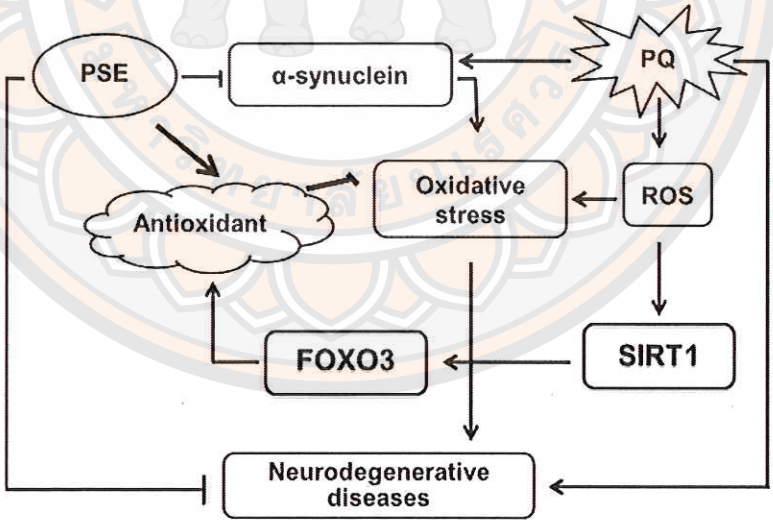


Figure 36 Schematic illustration of proposed peanut sprout extracts on α-synuclein and SIRT1 genes expression paraquat-induced oxidatrive stress in SK-N-SH cells.

3. Evaluation the effect of peanut sprout extract on Aβ protein expression

The neuropathology of Alzheimer disease (AD) is characterized by the presence of extracellular neuritic plaques (amyloid plaques), intracellular neurofibrillary tangles and loss of cholinergic neurons in the basal forebrain (Schenk, et al., 1999; Huang and Jiang, 2009). Oxidative stress leads to the formation of amyloid plaques and neurofibrillary tangles (NFT) (Nunomura, et al., 2001). In our study, paraquat treated cells showed overexpression of Aβ 42. Chauhan reported that paraquat induced production of reactive oxygen species (ROS) which could induce β- and γ-secretases leading to an increase in Aβ production from APP (Chauhan and Chauhan, 2006). Our results showed that Aβ 42 protein was in oligomer, protofibril, and eventually amyloid fibril aggregates (Figure 37). Oligomer and protofibril are putative toxic species that drive neuronal dysfunction (Huang and Jiang, 2009; Zako and Maeda, 2014). Resveratrol in peanut sprout extract expresses antioxidant properties and also down-regulates the Aβ 42 protein (Wang, et al., 2005; Kang, et al., 2010). Resveratrol has also been shown to be able to prevent and alleviate the numerous neurodegenerative disorders and age-related neurological decline (Levine, et al., 2008).Therefore, it can be postulated that peanut sprout extract down-regulates Aβ 42 protein and decreases cell death.

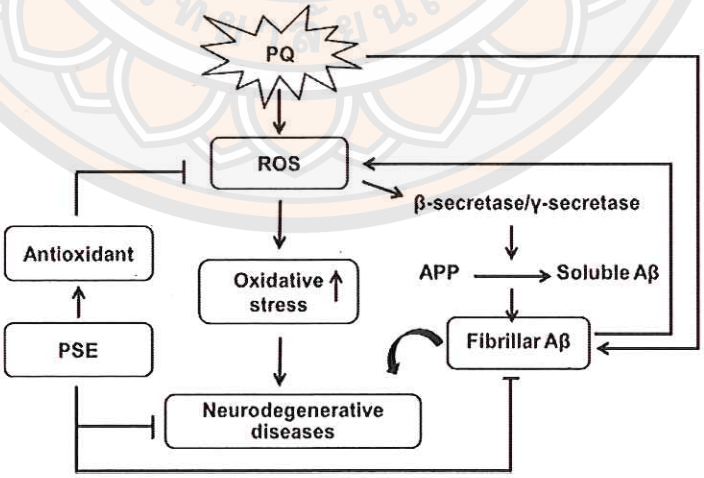


Figure 37 Schematic illustration of proposed neuroprotective of peanut sprout extract on paraquat-induced oxidatrive stress in SK-N-SH cells

4. Investigation the antioxidative effect of peanut sprout extract on paraquat-induced oxidative stress in SK-N-SH cells

Mitochondria are the major source of oxidative stress because the unavoidable electron leakage during electron transfer leads to the constant production of superoxide anions which, despite the presence of an efficient mitochondrial/cellular antioxidant system, is responsible for 90% of the endogenous ROS. Mitochondrial dysfunction is likely involved in the initiation and/or amplification of oxidative stress during the onset and progression of Alzheimer disease (Wang, et al., 2014). A previous study indicates that free radicals are possibly involved in the pathogenesis of neuron death in AD (Markesbery, 1997). In addition, other researchers have found that paraquat-induced ROS production adversely effects mitochondrial function and consequently causes dysfunction of the mitochondria (Rumsey and Katchur 2013; Huang, Lee, et al., 2012). In our study, we found that paraquat induced the generation of ROS in SK-N-SH cells. This finding is consistent with paraquat-induced oxidative stress in brain mitochondria, via increased ROS such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) (Figure 36 and 37) (Castello, et al., 2007; Vesely, et al., 1979; Mollace, et al., 2003). However, paraquat generates superoxides which produce redox cycling with intracellular diaphorases and molecular oxygen (Day, et al., 1995). In our study, however, we found that PSE can reduce paraquat-induced intracellular ROS in SK-N-SH cells. According to (Kim, et al., 2014) the proposed 8, 13-dihydroxy-9, 11-octadecadienoic acid (PSE-1) from peanut sprouts reduces the maximum inhibition of H_2O_2-induced DNA damage in the human leukocytes.

5. Determination the resveratrol in peanut sprout extract (*Arachis hypogaea* cv. Tainan 9)

Resveratrol is one of the major stilbene phytolexins found in various families of plants. This has provoked interest due to the association with reduce cardiovascular disease and reduced cancer risk, probably due to its antioxidative and anti-proliferative activities, including being a therapeutic agent for neurodegenerative diseases (Sun, et al., 2010). In our study, the concentration of *trans*-resveratrol in peanut sprout extract was similar to that reported by (Lee, et al., 2004) who investigated the concentration of *trans*-resveratrol in peanuts and peanut butter. In addition, biosynthesis (Wang, et al., 2005) reported that germinating peanut kernels

can enhance resveratrol. However, these levels were at lower concentrations of *trans*-resveratrol than reported in other studies (Wang, et al., 2005). In that study, when the rehydrated kernels of the three peanut cultivars were germinated at 25 °C and relative humidity 95% in the dark for 9 days, resveratrol contents increased significantly from the range of 2.3 to 4.5 μg/g up to the range of 11.7 to 25.7 μg/g depending upon the peanut cultivar. Further, they reported that methanol extracts of the freeze-dried sprouts exhibited potent 1,1-diphenyl-2-picryl-hydrazyl scavenging activity and antioxidative potency against linoleic acid oxidation. In our study, we found lower concentrations of *trans*-resveratrol of about 2.49 μg/g extract in Tainan 9 variety peanut sprout extract, substantially lower than the Wang study. However, this might be due to differences in the process of peanut sprout germination and extraction in the two studies. In addition, the resveratrol contents deviated considerably in the different cultivars and individual sprouts. Factors affecting the germination of plant seeds consist of nutrition, light, time, temperature, pH, and hormones (Arditti J., 1967; Vanstraelen M., & Benková E., 2012). It has been reported that the amount of resveratrol in peanut plants and peanuts has been found to increase by external stimuli including microbial infection, wounding, UV light irradiation, ultrasonication, yeast extract treatment and by plant stress hormones (Hasan, M. M., et al., 2013). Tainan 9 variety peanut sprout extract may contain phenolic compound such as Quercetin. This is suggested by (Kang, et al., 2010) who reported that peanut sprouts have total polyphenol content and the antioxidant activity. Thus, peanut sprout extract has *trans*-resveratrol and may enhance polyphenol contents. However, the bioactive compound in peanut sprout extract should be further determined using LC/MS/MS.

Conclusion

The overall conclusion of this study is that exposure to paraquat leads to oxidative stress in SK-N-SH cells and ROS causes a high expression of SIRT1. Paraquat may trigger mechanisms of antioxidative defenses through SIRT1 overexpression to rescue paraquat-treated SK-N-SH cells. Furthermore, ROS may be relevant in the activation of β- and γ-secretases to increase Aβ production from APP. Aβ and APP may also directly induce the production of ROS. In addition, ROS activates α-synuclein which may directly induce the production of ROS in

neurodegenerative diseases. Conversely, peanut sprout extract, which possesses antioxidant properties, can protect against cell death and inhibits paraquat-induced ROS production leading to a decrease in progressive neurodegeneration. Moreover, peanut sprout extract can moderate the expression of SIRT1 thereby providing resistance to oxidative stress and apoptosis. In addition, we found that peanut sprout extract can down-regulate the α-synuclein gene and Aβ 42 protein affecting a decrease in ROS production, which can protect against neurodegenerative diseases.

Our study shows that peanut sprout extract demonstrates neuroprotective activities against paraquat-induced oxidative stress in SK-N-SH cells, suggesting that peanut sprout extract is a highly promising agent for the prevention of neurodegenerative diseases such as AD and PD.

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APPENDIX

Chemicals and molecular reagents

1. 100 bp DNA ladder (Fermenttas, USA)
2. SensiFAST™ SYBR® Kits (A Meridian Life Science, USA)
3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biobasic, Canada)
4. 3,3'-Diaminobenzidine (DAB) (Biobasic, Canada)
5. N,N′-dimethyl-4,4′-bipyridinium dichloride (Paraquat) (Sigma, USA)
6. TEMED (BioRad, UAS)
7. Resveratrol (Sigma, USA)
8. Minimum Essential Medium (MEM) (Invitrogen, USA)
9. Fetal Bovine Serum (FBS) (Invitrogen, USA)
10. 0.25% trypsin–EDTA (Invitrogen, USA)
11. 1% penicillin-steptomycin (Invitrogen, USA)
12. Dimethyl sulfoxide (DMSO) (Sigma, USA)
13. Muse® Oxidative Stress Kit (Merck Millipore, Germany)
14. Muse™ Annexin V & Dead Cell Kit (Merck Millipore, Germany)
15. RiboZol RNA extraction reagent (Amresco ,USA)
16. DNase I (fermentas, USA)
17. 50 mM EDTA (fermentas, USA)
18. Tetro reverse transcriptase (Bioline, UAS)
19. Anti-body (actin and beta-amyloid 1-42) (Merck Millipore, Germany)
20. Ethyl alcohol (Tedia Company, INC., USA)
21. *N,N′*-dimethyl-4,4′-bipyridinium dichloride (Paraquat) (Sigma, USA)
22. Sodium chloride (Amresco ,USA)
23. 0.4% trypanblue (Gibco, USA)
24. Methanol (TEDIA company INC., USA)
25. Acetonitrile (RCI Labscan Limited, Thailand)
26. Phosphate-buffered saline (PBS) (Amresco ,USA)
27. Water-DEPC treated (Biobasic, Canada)
28. Guannidine thiocyanate (Biobasic, Canada)
29. Sodium dodecyl sulfate solution (SDS) (Applichem, Germany)
30. Tris (USB Corporation, USA)

31. Glacial acetic acid (RCI Labscan Limited, Thailand)
32. Sodium phosphate dibasic (Sigma, USA)
33. Agarose (Invitrogen, USA)
34. BCA Protein Assay Kit (Novagen,USA)
35. Sonicator (Becthai Bankok Equipment &Chemical, USA)

Instruments

1. CO_2 incubator (Sanyo model MCO-20AIC, Japan)
2. Laminar flow hood (Affiutech CO.,LTD. Thailand)
3. Refrigerated centrifuge (Heraeus, Germany)
4. Microtiter plate reader (Science THE CO, USA)
5. Water bath (Julabo sw 20, Germany)
6. Thermocycler (PCR) (BioRad, USA)
7. Pipette aid (BioRad, USA)
8. Micropipette (Corning, USA)
9. High Performance Liquid Chromatography (HPLC) (Bara Scientific Co., Ltd., Thailand)
10. Autoclave (Astell model AMA260s, England)
11. Haemacytometer (Boeco, Germany)
12. pH metter (Mtter Toledo,USA)
13. Vortex (Finepcr model finvortex, Korea)
14. Refrigerators -80°C (Becthai Bangkok Equipment & Chemical CO., Thailand)
15. Refrigerators -50°C (Thermo scientific, puffer hubbard, USA)
16. Refrigerators -20°C (Sanden intercool, Thailand)
17. Refrigerators -4°C (Sanden intercool, Thailand)
18. Liquid nitrogen storage (minnesuta, USA)
19. Incubator 37°C (Memmert, Germany)
20. Heat block (Labnet model D1100, USA)
21. DNA electrophoresis apparatus (Major science, Taiwan)
22. Hot air oven (Contherm, USA)
23. Inverted microscope (Leica, Germany)

24. Mini centrifuge (Wealtec, Taiwan)
25. Gel documentation (Image Quant™ LAS 500) (GE Health care life sciences, Sweden)
26. Nano Drope 2000/2000C (Thermo scientific, puffer hubbard, USA)
27. Muse® cell analyzer (Merck Millipore, Germany)
28. Light Cycler® 96 (Roche Diagnostics)
29. Heat box (Labnet International, Inc., USA)
30. Protein Transfer (BioRad, USA)
31. SDS-Polyacrylamide Gel Electrophoresis (BioRad, USA)
32. Balane 0.000 (Mettler Toledo, Switzerland)
33. Shaker (Major Science, Taiwan)

Buffer solutions

1. Complete media (100 ml)
    1. Eagle's minimum essential medium (MEM) 90 ml
    2. Heat inactivated fetal bovine serum 10 ml
    3. Penicillin-Streptomycin 1 ml

2. 10X Phosphate-buffered saline (PBS) pH 7.2-7.4 (400 ml)
    1. 1.37 mM NaCl 32 g
    2. 27 mM KCl 0.8 g
    3. 100 mM Na_2HPO_4 6 g
    4. 18 mM KH_2PO_4 0.8 g

    Add water 400 ml

3. 0.25% trypsin EDTA (200 ml)
    1. 5% trypsin EDTA (0.5%, 10X) 10 ml
    2. 1X PBS 190 ml

4. Frozen media (10 ml)
    1. Complete media 9 ml
    2. DMSO 1 ml

5. 5 mg/ml MTT Solution (5 ml)
    1. MTT powder 5 mg
    2. 1X PBS 1 ml

6. 50X TAE buffer (1,000 ml)
    1. 40mM Tris base 242 g
    2. 20mM Glacial acetic acid 57.1 ml
    3. 0.5 M EDTA (pH 8.0) 100 ml

    Add water up to 1,000 ml

7. 1% Agarose gel (100 ml)
    1. Agarose 1 g
    2. 1XTAE buffer100 ml

8. 0.5 M Tris-HCl pH 6.8 1,000 ml
    1. Tris base 65.55 g

    Adjust pH to 6.8 using concentrated HCl Make up total volume to 1,000 ml with distilled water

9. 1.5 M Tris-HCl pH 8.8 (300 ml)
    1. Tris base 54.5 g

    Adjust pH to 8.8 make up total volume to 300 ml with distilled water

10. Tris-glycine buffer (1,000 ml)
    1. 25 mM Tris base 3 g
    2. 192 mM Glycine 14.4 g
    3. (0.1% w/v) SDS 1 g

    Add water 1,000 ml

11. Transfer buffer (1,000 ml)

1. 25 mM Tris base	3 g
2. 192 mM Glycine	14.4 g
3. 10% SDS	1 g
4. 20% Methanol 200 ml	

Adjust pH to 8.3 make up total volume to 1,000 ml with distilled water

12. 5X sample buffer (5 ml)

1. SDS	1 g
2. β Mercapto Ethanol	2.56 ml
3. 0.5 M Tris HCl pH 6.8	2.13 ml
4. 1% Bromo Phenol Blue	250 µl

13. Staining solution (250 ml)

1. (0.1% w/v) Coomassie brilliant blue R-250	0.25 g
2. 50% Methanol	125 ml
3. 10% Acetic acid	25 ml

Add water 250 ml

14. Destaining solution (500 ml)

1. 40% Methanol	125 ml
2. 10% Acetic acid	50 ml

Add water up to 500 ml

15. Gel Buffer (50 ml)

1. 3 M Tris HCl pH 8.45	18.15 g
2. 0.3% SDS	150 mg
3. H_2O up to 50 ml	

16. 10X Cathode buffer (200 ml)

1. 1M Tris 24.2 g
2. 1 M Tricine 35.84 g
3. 1% SDS pH 8.25 2 g
4. H_2O up to 200 ml

17. 10X Anode buffer (200 ml)

1. 2.1 M Tris pH 8.9 48.4 g
2. H_2O up to 200 ml

18. Sample preparation for treatment of SK-N-SH cells

100 mg/ml of stock solution of peanut sprout extract was prepared by mixing 100 mg of peanut sprout extract in 80% ethanol 1 ml and filtered through 0.22 µm PVDF membranes. The stock solution was diluted with serum-free media, providing 60 mg/ml for treatment at final concentration of 1 mg/ml containing 0.8 % ethanol.

Table 1 Separating and Stacking Gel

Solutions	Separating (4%)	Stacking (15%)
Acrylamide Solution 30%	3.26 ml	0.31 ml
Gel Buffer	3.26 ml	0.74 ml
Glycerol 70%	1.30 ml	-
H_2O	2.02 ml	1.99 ml
10% APS	100 µl	80 µl
TEMD	5 µl	5 µl

Table 2 Effects of peanut sprout extract on cell viability in SK-N-SH cells (24 hrs)

Conc. (mg/ml)	1	2	3	mean	% cell viability	S.E.M.
0.0	100	100	100	300	100	0
0.3	106	158	111	376	125	17
0.5	106	120	106	332	111	5
1.0	104	164	105	373	124	20
1.5	125	176	117	418	139	18

Table 3 Effects of peanut sprout extract on cell viability in SK-N-SH cells (48 hrs)

Conc. (mg/ml)	1	2	3	mean	% cell viability	S.E.M.
0.0	100	100	100	300	100	0.00
0.3	105	100	98	303	101	2.00
0.5	102	104	108	314	105	1.67
1.0	103	131	115	349	116	7.98
1.5	116	131	133	379	126	5.35

Table 4 Effects of peanut sprout extract on cell viability in SK-N-SH cells (72 hrs)

Conc. (mg/ml)	1	2	3	mean	% cell viability	S.E.M.
0.0	100	100	100	300	100	0
0.3	94	107	102	303	101	3.96319
0.5	114	111	103	327	109	3.24651
1.0	113	128	117	358	119	4.48454
1.5	107	137	131	375	125	9.14667

Table 5 Effects of paraquat on cell viability in SK-N-SH cells (24 hrs)

Conc. (mM)	1	2	3	mean	% cell viability	S.E.M.
0.00	100	100	100	100	100	0
0.25	95	105	98	99	99	3.0
0.50	97	105	96	99	99	2.9
0.75	91	101	92	95	95	3.2
1.00	92	97	99	96	96	2.1

Table 6 Effects of paraquat on cell viability in SK-N-SH cells (48 hrs)

Conc. (mM)	1	2	3	mean	%cell viability	S.E.M.
0.00	100	100	100	300	100	0
0.25	98	80	90	269	90	5
0.50	79	72	76	227	76	2
0.75	64	53	59	175	58	3
1.00	26	45	35	106	35	6

Table 7 Effects of paraquat on cell viability in SK-N-SH cells (72 hrs)

Conc. (mM)	1	2	3	mean	%cell viability	S.E.M.
0.00	100	100	100	100	100	0.0
0.25	54	52	51	52	52	0.8
0.50	39	42	39	40	40	0.8
0.75	35	37	35	36	36	0.8
1.00	34	31	31	32	32	1.2

Table 8 Neuroprotective activity of peanut sprout extract paraquat-induced oxidative stress in SK-N-SH cells

Conc. (mg/ml)		1	2	3	mean	%cell viability	S.E.M.
control		100	100	100	300	100	0
0.0	0.75 mM PQ	56	56	63	175	58	2
0.25	0.75 mM PQ	71	72	62	205	68	3
0.5	0.75 mM PQ	75	60	69	204	68	4
1.0	0.75 mM PQ	77	76	72	225	75	1
1.5	0.75 mM PQ	81	85	80	246	82	2

Table 9 Effect of peanut sprout extract on intracellular ROS in SK-N-SH cells (48 hrs)

Sample	ROS(+) 1	ROS(+) 2	ROS(+) 3	Mean± S.E.M.
control	4	15	12	10±3
0.75 mM (PQ)	61	75	57	64±6
1 mg/ml (PSE)	7	6	7	7.0±0
Pretreated with PSE	31	11	12	18±6

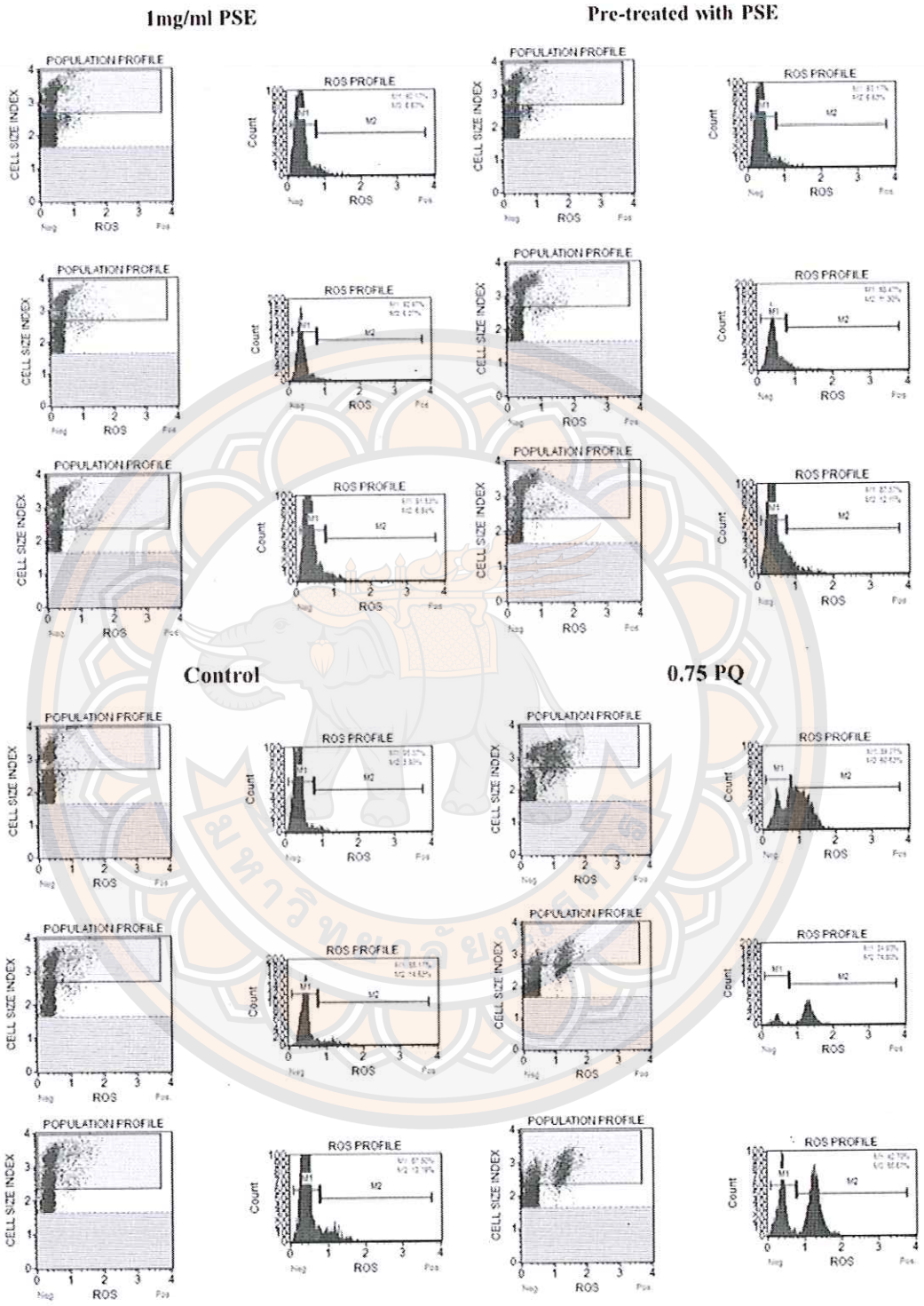


Figure 38 Effect of peanut sprout extract on intracellular ROS in SK-N-SH cells

Table 10 Expression of α-synuclein in SK-N-SH cells (No.1)

Sample	Actin (mean Ct)	α-Syn (mean Ct)	ΔCt	ΔΔCt	$2^{\wedge-\Delta\Delta Ct}$	S.E.M.
control	16.51	23.97	7.46	0.00	1.00	0.00
0.75mM PQ	21.17	26.78	5.61	-1.85	3.60	0.20
1mg/ml PN	16.25	23.87	7.62	0.16	0.90	0.04
Pre-treated with PSE	17.31	23.75	6.44	-1.01	2.02	0.10

Table 11 Expression of α-synuclein in SK-N-SH cells (No.2)

Sample	Actin (mean Ct)	α-synuclein (mean Ct)	ΔCt	ΔΔCt	$2^{\wedge-\Delta\Delta Ct}$	S.E.M.
control	16.43	25.05	8.62	0.00	1.00	0.00
0.75mM PQ	20.40	27.17	6.77	-1.85	3.61	0.18
1mg/ml PN	16.27	24.49	8.22	-0.40	1.32	0.07
Pre-treated with PSE	17.35	24.90	7.55	-1.07	2.10	0.18

Table 12 Expression of α-synuclein in SK-N-SH cells (No.3)

Sample	Actin (mean Ct)	α-synuclein (mean Ct)	ΔCt	ΔΔCt	2^-ΔΔCt	S.E.M.
control	16.	24.	7.	0.	1.00	0.
0.75mM PQ	20.	26.	6.	-1.	2.05	0.
1mg/ml PN	16.	24.	8.	0.	0.71	0.
Pre-treated with PSE	17.	24.	6.	-0.	1.95	0.

Table 13 Expression of α-synuclein in SK-N-SH cells

	α-synuclein				
Sample	**1**	**2**	**3**	**Mean**	**S.E.M.**
control	1	1	1	1	0
0.75mM PQ	3.61	3.61	2.08	3.10	0.88
1mg/ml PN	0.90	1.32	0.73	0.98	0.31
Pre-treated with PSE	2.02	2.12	1.95	2.03	0.08

Table 14 Expression of SIRT1 in SK-N-SH cells (No.1)

Sample	**Actin (mean Ct)**	**SIRT1 (mean Ct)**	**ΔCt**	**ΔΔCt**	**$2^{\wedge-\Delta\Delta Ct}$**	**S.E.M.**
control	16.67	24.05	7.39	0.00	1.00	0.00
0.75mM PQ	21.33	27.34	6.02	-1.37	2.58	0.09
1mg/ml PN	16.53	23.67	7.14	-0.24	1.19	0.03
Pre-treated with PSE	17.40	23.85	6.45	-0.93	1.91	0.02

Table 15 Expression of SIRT1 in SK-N-SH cells (No.2)

Sample	**Actin (mean Ct)**	**SIRT1 (mean Ct)**	**ΔCt**	**ΔΔCt**	**$2^{\wedge-\Delta\Delta Ct}$**	**S.E.M.**
control	21.06	25.57	4.51	0.00	1.00	0
0.75mM PQ	23.55	26.82	3.27	-1.24	2.54	0.61734
1mg/ml PN	19.74	24.85	5.11	0.59	0.72	0.17638
Pre-treated with PSE	19.69	23.54	3.85	-0.66	1.70	0.40415

Table 16 Expression of SIRT1 in SK-N-SH cells (No.3)

Sample	Actin (mean Ct)	SIRT1 (mean Ct)	ΔCt	ΔΔCt	$2^{\wedge-\Delta\Delta Ct}$	S.E.M.
control	16.61	24.08	7.47	0.00	1.00	0.00
0.75mM PQ	20.89	27.45	6.56	-0.91	2.13	0.64
1mg/ml PN	16.48	23.59	7.11	-0.36	1.28	0.07
Pre-treated with PSE	17.48	23.83	6.35	-1.12	2.18	0.12

Table 17 Expression of SIRT1 in SK-N-SH cells

	SIRT1				
Sample	1	2	3	Mean	S.E.M.
control	1.0	1.0	1.0	1.0	0.00
0.75mM PQ	2.6	2.5	2.1	2.4	0.15
1mg/ml PN	1.2	0.7	1.3	1.1	0.17
Pre-treated with PSE	1.9	1.7	2.2	1.9	0.14

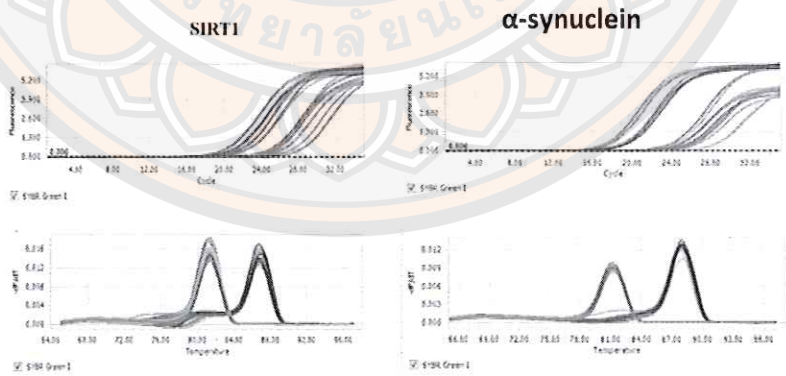
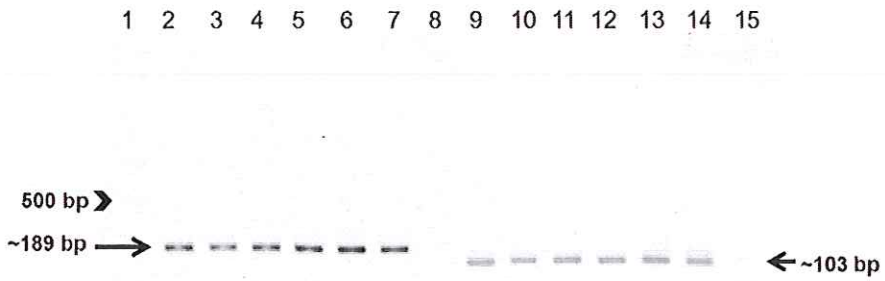


Figure 39 Melting curves of SIRT1 and α-synuclein genes



Lane 1 = 100 bp Ladder
Lane 2 = control Actin
Lane 3 = 0.75 mM PQ Actin
Lane 4 = 1 mg/ml PSE Actin
Lane 5 = 1 mg/ml PSE & PQ Actin
Lane 6 = 1.5 mg/ml PSE Actin
Lane 7 = 1.5 mg/ml PSE & PQ Actin
Lane 8 = Negative Actin
Lane 9 = control α-syn
Lane 10 = 0.75 mM PQ α-syn
Lane 11 = 1 mg/ml PSE α-syn
Lane 12 = 1 mg/ml PSE & PQ α-syn
Lane 13 = 1.5 mg/ml PSE α-syn
Lane 14 = 1.5 mg/ml PSE & PQ α-syn
Lane 15 = Negative α-syn

Figure 40 Agarose gel electrophoresis of the real-time PCR product for α-synuclein genes

Lane 1 = 100 bp Ladder
Lane 2 = control Actin
Lane 3 = 0.75 mM PQ Actin
Lane 4 = 1 mg/ml PSE Actin
Lane 5 = 1 mg/ml PSE & PQ Actin
Lane 6 = 1.5 mg/ml PSE Actin
Lane 7 = 1.5 mg/ml PSE & PQ Actin
Lane 8 = Negative Actin
Lane 9 = control SIRT1
Lane 10 = 0.75 mM PQ SIRT1
Lane 11 = 1 mg/ml PSE SIRT1
Lane 12 = 1 mg/ml PSE & PQ SIRT1
Lane 13 = 1.5 mg/ml PSE SIRT1
Lane 14 = 1.5 mg/ml PSE & PQ SIRT1
Lane 15 = Negative SIRT1

Figure 41 Agarose gel electrophoresis of the real-time PCR product for SIRT1 genes

Table 18 %Efficiency of Actin, SIRT1 and α-synuclein genes

Genes	Primer Sequence (5' → 3')	%Efficiency
Actin	F CCA CCA TGT ACC CTG GCA TT R CCA ACT CGT CAT ACT CCT GC	98
SIRT1	F TCA GTG GCT GGA ACA GTG AG R AGC GCC ATG GAA AAT GTA AC	106
α-synuclein	F GTG CTC AGT TCC AAT GTG CC R TGG GGG CAG GTA CAG ATA CT	96

Table 19 %Efficiency of Actin gene

Actin			
Dilute	**Quantity**	**log Quantity**	**Mean (Ct)**
un	1	0	21.971
10x	0.1	-1	25.129
100x	0.01	-2	28.719
%Efficiency		97.87	

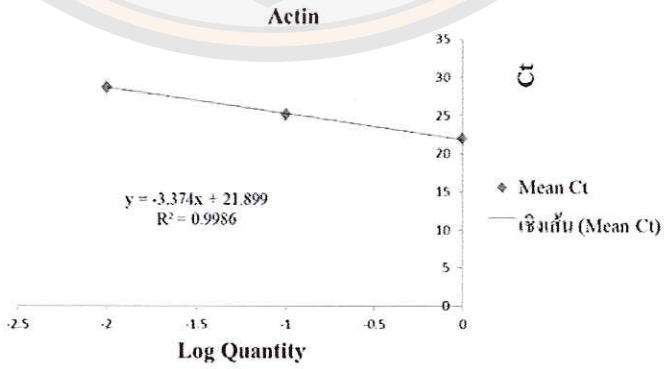


Figure 42 %Efficiency of Actin gene

Table 20 %Efficiency of SIRT1 gene

SIRT1			
Dilute	**Quantity**	**log Quantity**	**Mean (Ct)**
un	1	0	26.69
10x	0.1	-1	30.05
100x	0.01	-2	33.07
%Efficiency		**106**	

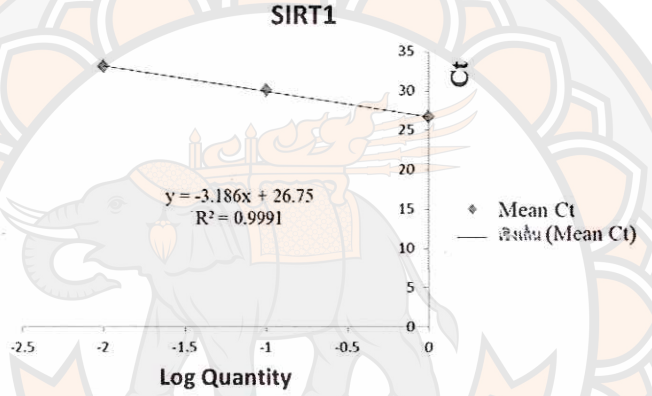


Figure 43 %Efficiency of SIRT1 gene

Table 21 %Efficiency of α-synuclein gene

α-synuclein			
Dilute	**Quantity**	**log Quantity**	**Mean (Ct)**
un	1	0	21.424
10x	0.1	-1	24.762
100x	0.01	-2	28.255
%Efficiency		**96**	

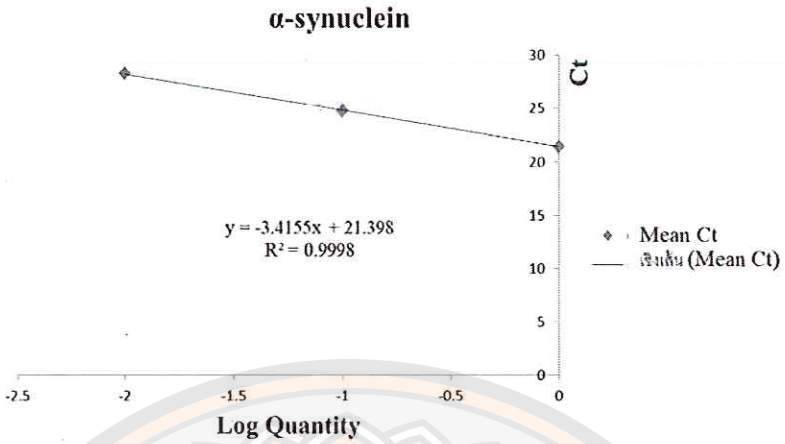


Figure 44 %Efficiency of α-synuclein gene

Table 22 Concentration of BSA protein standard

Conc. (µg)	Standard	OD 1	OD 2	OD 3	Mean
0.0	0 µg/ml	0.12	0.11	0.11	0.11
1.563	62.5 µg/ml	0.19	0.19	0.18	0.19
3.125	125 µg/ml	0.28	0.29	0.30	0.29
6.25	250 µg/ml	0.47	0.47	0.47	0.47
12.5	500 µg/ml	0.77	0.79	0.81	0.79
25.0	1000 µg/ml	1.75	1.80	1.69	1.74

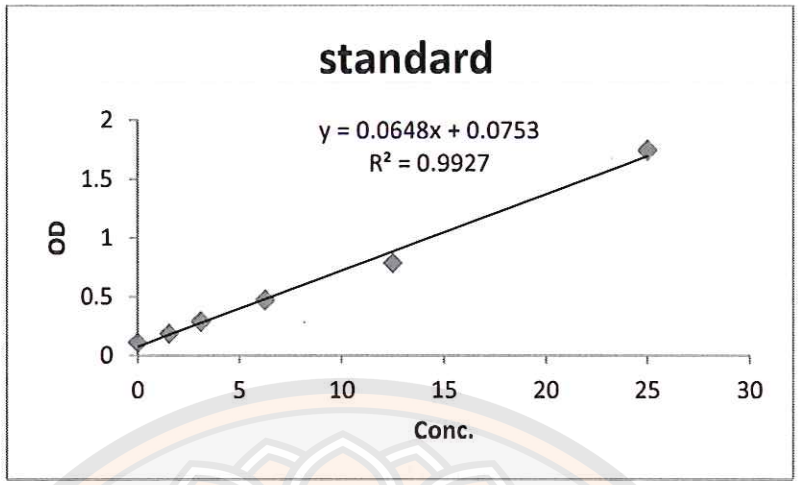


Figure 45 Standard protein

Table 23 Concentration of protein

Sample	OD 1	OD 2	OD 3	Mean	µg/25µl	µg/25µl*10	Conc. (µg/µl)
control	0.27	0.27	0.26	0.27	2.95	29.53	1.18
0.75mM PQ	0.22	0.20	0.23	0.22	2.20	21.97	0.88
1mg/ml PN	0.34	0.34	0.34	0.34	4.05	40.54	1.62
Pre-treated with PSE	0.30	0.30	0.30	0.30	3.42	34.16	1.37

Table 24 Effect of peanut sprout extract on protein level of Aβ 42 (No.1)

Sample	Area (actin)	Area (Aβ 42)	Aβ 42/Actin
control	22825.74	0.00	0.00
0.75mM PQ	22938.75	8625.49	0.38
1mg/ml PN	18743.82	0.00	0.00
Pre-treated with PSE	14952.64	0.00	0.00

Table 25 Effect of peanut sprout extract on protein level of Aβ 42 (No.2)

Sample	Area (actin)	Area (Aβ 42)	Aβ 42/Actin
control	19646.53	0.00	0.00
0.75mM PQ	25203.55	10530.94	0.42
1mg/ml PN	22441.36	0.00	0.00
Pre-treated with PSE	22387.54	5916.84	0.26

Table 26 Effect of peanut sprout extract on protein level of Aβ 42

Sample	1	2	Mean ± S.E.M.
control	0.00	0.00	0.00 ± 0.00
0.75mM PQ	0.38	0.42	0.40 ± 0.02
1mg/ml PN	0.00	0.00	0.00 ± 0.00
Pre-treated with PSE	0.00	0.26	0.13 ± 0.07

Table 27 Standard of resveratrol

Standard (µg/ml)	Mean area	Mean area/1000
0.00	51370.67	0.00000
0.25	112248.3	51.37067
0.50	214566.7	112.2483
1.00	286316.7	214.5667
1.50	384450.3	286.3167
2.00	487393.7	384.4503
2.50	51370.67	487.3937

Table 28 The amount of resveratrol in peanut sprout extract

Conc. (mg/ml) PSE	Mean area	Y=1E+07x+7992.8	Resveratrol in PSE (µg/g extract)
21	155520.33	0.015	2.49

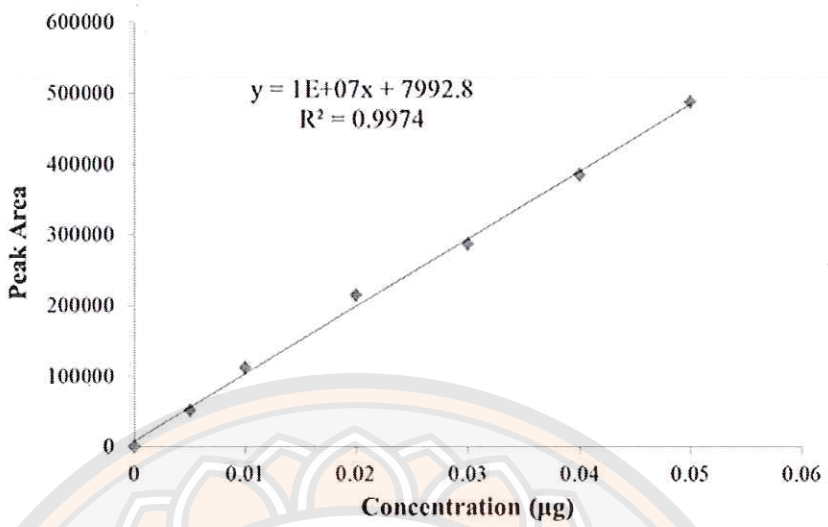


Figure 46 Standard of resveratrol