PROTECTIVE EFFECTS OF ERIODICTYOL ON HYDROGEN PEROXIDE-INDUCED CELL DEATH IN SH-SY5Y CELLS

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ฤทธิ์ปกป้องของสารอิลิโอดิกไทออลต่อการตายของเซลล์ที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์ SH-SY5Y

น.ส.เสาวรส ทองอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PROTECTIVE EFFECTS OF ERIODICTYOL ON HYDROGEN PEROXIDE-INDUCED CELL DEATH IN SH-SY5Y CELLS
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เสาวรส ทองอินทร์ : ฤทธิ์ปกป้องของสารอิลิโอดิกไทออลต่อการตายของเซลล์ที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์ SH-SY5Y. (PROTECTIVE EFFECTS OF ERIODICTYOL ON HYDROGEN PEROXIDE-INDUCED CELL DEATH IN SH-SY5Y CELLS) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร. รัชนี รอดศิริ, อ.ที่ปรึกษาร่วม : ผศ. ภญ. ดร.วริษา พงศ์เรขนานนท์

ภาวะเครียดออกซิเดชั่นและการทำงานของไมโตคอนเดรียที่ผิดปกติ เป็นสาเหตุหลักของการตายของเซลล์ประสาทในกลุ่มโรคความเสื่อมของระบบประสาท อิลิโอดิกไทออล เป็นสารกลุ่มฟลาโวนอยด์ สกัดมาจากต้น *Dendrobium ellipsophyllum* (ชื่อไทย : เอื้องทอง) ผลการศึกษาก่อนหน้ารายงานฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านอักเสบของอิลิโอดิกไทออล งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์และกลไกปกป้องของอิลิโอดิกไทออลต่อการตายของเซลล์ที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์ SH-SY5Y ฤทธิ์ปกป้องของอิลิโอดิกไทออลต่อการตายของเซลล์ที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ประเมินโดยวิธี resazurin assay ผลต่อระดับอนุมูลอิสระภายในเซลล์และผลต่อ mitochondria membrane potential ศึกษาโดยวิธี DCFH-DA assay และวิธี TMRE assay ตามลำดับ และผลของอิลิโอดิกไทออลต่อการส่งสัญญาณการตายแบบ apoptosis ผ่านทางไมโตคอนเดรีย ศึกษาโดยวิธี Western blot ผลการทดลองพบว่าอิลิโอดิกไทออล (0.1, 1 และ 10 μM) เพิ่มจำนวนเซลล์ที่มีชีวิต ($p < 0.001$) ลดระดับอนุมูลอิสระภายในเซลล์ ($p < 0.05$) และเพิ่มความเข้มแสงฟลูออเรสเซนต์ของ TMRE ($p < 0.05$) เมื่อเปรียบเทียบกับกลุ่มที่ได้รับไฮโดรเจนเปอร์ออกไซด์เพียงอย่างเดียว ไฮโดรเจนเปอร์ออกไซด์เหนี่ยวนำการส่งสัญญาณการตายแบบ apoptosis ผ่านทางไมโตคอนเดรีย โดยการลดอัตราส่วนของ Bcl-2/BAX เพิ่มการแสดงออกของ cytochrome c, caspase-3 และ cleaved caspase-3 แต่การให้สารอิลิโอดิกไทออลจะลดเหตุการณ์เหล่านี้ จึงสรุปการทดลองได้ว่าอิลิโอดิกไทออลสามารถปกป้องการตายของเซลล์ SH-SY5Y ที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ ผ่านทางฤทธิ์ต้านอนุมูลอิสระ ซึ่งส่งผลให้ยับยั้งการตายของเซลล์แบบ apoptosis ผ่านทางไมโตคอนเดรีย

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Saowarose Thongin :

PROTECTIVE EFFECTS OF ERIODICTYOL ON HYDROGEN PEROXIDE-INDUCED CELL DEATH IN SH-SY5Y CELLS. Advisor: Asst. Prof. Ratchanee Rodsiri, Ph.D. Co-advisor: Asst. Prof. Varisa Pongrakhananon, Ph.D.

Oxidative stress and mitochondrial dysfunction are the major causes of neuronal death in neurodegenerative disorders. Eriodictyol is a flavonoid extracted from the whole plant of *Dendrobium ellipsophyllum* (thai name: Ueang Thong). The antioxidant and anti-inflammatory effects of eriodictyol have been widely reported. The present study aimed to investigate the protective effect and mechanisms of eriodictyol on hydrogen peroxide (H_2O_2)-induced cell death in human neuroblastoma SH-SY5Y cells. The protective effect of eriodictyol on cytotoxicity induced by H_2O_2 was evaluated using resazurin assay. The effects on intracellular reactive oxygen species (ROS) and mitochondria membrane potential were evaluated by 2′-7’ dichlorodihydrofluorescein diacetate (DCFH-DA) assay and tetramethylrhodamine ethyl ester (TMRE) assay, respectively. The effects of eriodictyol on mitochondria-mediated apoptosis cascade were determined using western blot analysis. The results showed that pretreatment with eriodictyol (0.1, 1 and 10 μM) increased cell viability ($p < 0.001$), reduced intracellular ROS ($p < 0.05$) and increased TMRE fluorescent intensity ($p < 0.05$) compared to H_2O_2-treated alone. H_2O_2 induced mitochondria-mediated apoptotic signalling by decreasing Bcl-2/BAX ratio, increasing the expressions of cytochrome c, caspase-3 and cleaved caspase-3 while eriodictyol pretreatment reduced these events. In conclusion, eriodictyol can protect against H_2O_2-induced cytotoxicity in SH-SY5Y cells via an antioxidant effect, and consequently inhibit mitochondria-mediated apoptosis cascade.

Field of Study: Pharmacology Student's Signature .....

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Finally, I wish to express my infinite thank to my family, especially my mother, for their endless love, understanding and never-ending support throughout my life. Thank you for always being beside me.

Saowarose Thongin

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ABBREVIATIONS

α	=	alpha
β	=	beta
ºC	=	degree celsius
/	=	per
%	=	percentage
±	=	plus-minus sign
x g	=	relative centrifugal force
μg/ml	=	Microgram per milliliter
μl	=	Microliter
μM	=	Micromolar
AD	=	Alzheimer's disease
ANOVA	=	Analysis of variance
Apaf-1	=	Apoptotic protease-activating factor-1
ATCC	=	American Type Culture Collection
ATP	=	Adenosine triphosphate
ALS	=	amyotrophic lateral sclerosis
BAD	=	Bcl-2 antagonist of cell death
BAK	=	Bcl-2-antagonist/killer-1
BAX	=	Bcl-2-associated X protein

Bcl-2	=	B-cell lymphoma-2
Caspase	=	Cysteine aspartic acid specific protease
CAT	=	Catalase
CO_2	=	Carbon dioxide
DCFH-DA	=	2′, 7′- dichlorodihydrofluorescein diacetate
DMEM-F12	=	Dulbecco's Modified Essential Eagle's Medium-Ham's Nutrient Mixture F-12
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylene diamine tetraacetic acid
ETC	=	Mitochondrial electron transport chain
FBS	=	Fetal bovine serum
FDA	=	Food and drug administration
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
GSH	=	Glutathione
GST	=	Gutathione-S-transferase
GPx	=	Glutathione peroxidase
h	=	Hour
H_2O_2	=	Hydrogen peroxide
HCl	=	Hydrochloric acid
HD	=	Huntington's disease

IC50	=	50% Inhibition concentration
IL	=	Interleukin
LPS	=	Lipopolysaccharides
mg/ml	=	Milligram per milliliter
MMP	=	Mitochondria membrane potential
MPTP	=	1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine
$NaHCO_3$	=	Sodium bicarbonate
NaOH	=	Sodium hydroxide
NF-ΚB	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	=	Nanometer
Nrf2	=	The nuclear factor erythroid 2–related factor 2
$\bullet O_2^-$	=	Superoxide anion
O_2	=	Oxygen
$\bullet O_2^{-2}$	=	Peroxide
•OH	=	Hydroxyl radical
$HO^2\bullet$	=	Hydroperoxyl
PD	=	Parkinson's disease
P53	=	Tumor protein 53
PBS	=	Phosphate buffer saline

pH	=	The negative logarithm of hydrogen ion concentration
ROS	=	Reactive oxygen species
rpm	=	Round per minutes
S.E.M.	=	Standard error of mean
SOD	=	Superoxide dismutase
TNFα	=	Tumor necrosis factor alpha

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Neuronal death in neurodegenerative disorders are mainly caused by oxidative stress and mitochondrial dysfunction (Wang et al., 2014). The current pharmacological treatment of neurodegenerative diseases mainly alleviates the dysfunction of neurotransmission but they do not restore dead neurons or regenerate neurites. The neuroprotective approach is needed to slow down the disease progression.

Oxidative stress is resulted from the accumulation of reactive oxygen species (ROS) and the inadequate protective mechanisms of the cells. Hydrogen peroxide (H_2O_2), one of ROS, can induce neuronal death via an increase of ROS and the decrease of antioxidant enzymes (Feng et al., 2016; Park et al., 2015). In addition, H_2O_2 causes the translocation of cytochrome c from mitochondria to cytosol leading to apoptosis (Cai et al., 2008). Therefore, H_2O_2 is used in this study to induce oxidative stress and neuronal death in human neuroblastoma SH-SY5Y cells.

Eriodictyol is a flavonoid extracted from the whole plant of *Dendrobium ellipsophyllum* or Ueang Thong. Previous studies demonstrated the antioxidant and anti-inflammatory effects of eriodictyol (Zhu et al., 2015). However, the protective effect of eriodictyol has not yet clarified.

The present study aimed to investigate the protective effect of eriodictyol in H_2O_2-induced SH-SY5Y cell death. The mechanisms underlying the protective effect of eriodictyol involving mitochondria-mediated apoptosis.

1.2 Research questions

1. Does eriodictyol protect against the neurotoxic effect of H_2O_2 in SH-SY5Y cells?
2. What are the mechanisms underlying the protective effect of eriodictyol on H_2O_2-induced cell death in SH-SY5Y cells?

1.3 Objectives

1. To investigate the protective effect of eriodictyol on H_2O_2-induced cell death in SH-SY5Y cells.
2. To investigate the mechanisms underlying the protective effect of eriodictyol on H_2O_2-induced cell death in SH-SY5Y cells.

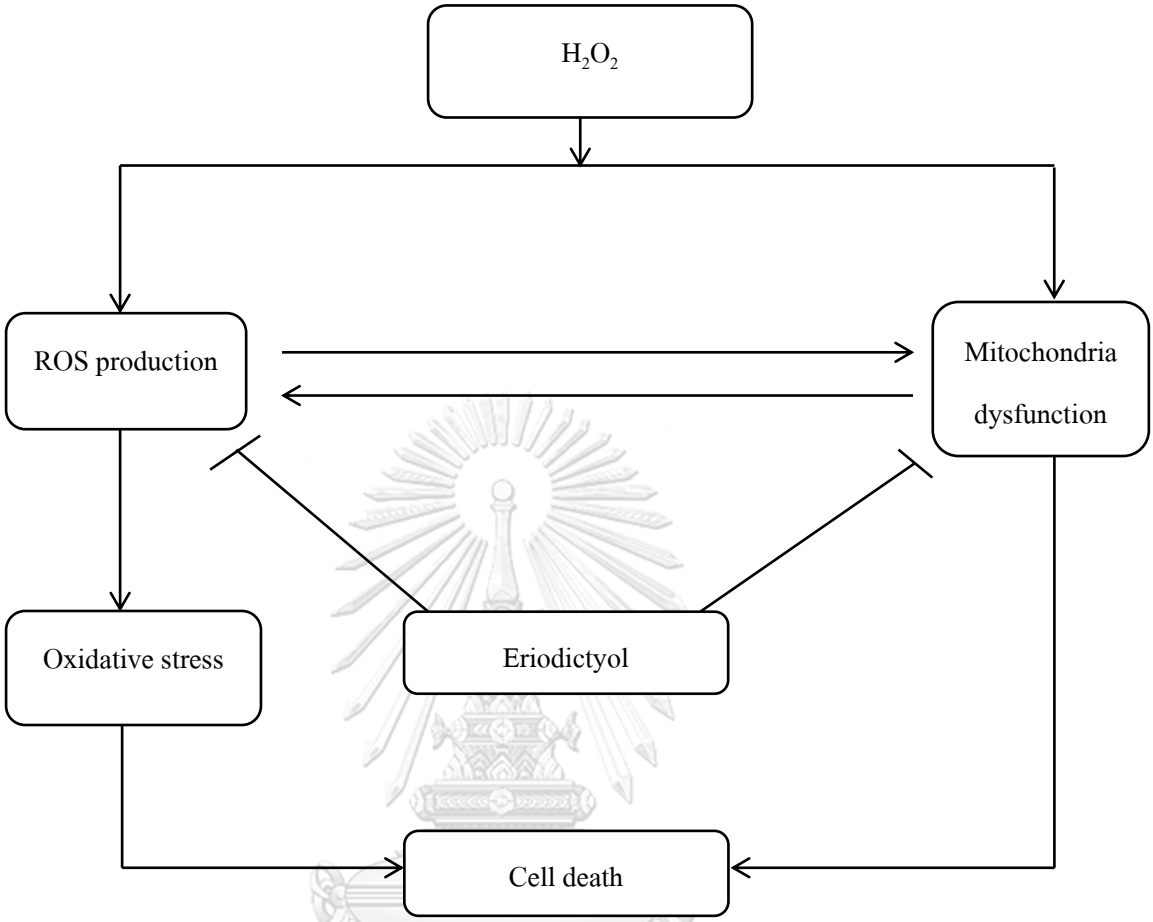
1.4 Hypothesis

Eriodictyol products against H_2O_2 -induced neuronal cell death in SH-SY5Y cells via the decrease of ROS production, the protection of mitochondria membrane potential dysfunction and the inhibition of mitochondria-mediated apoptosis cascade.

1.5 Expected Benefits

This research will provide a scientific information regarding the protective effect of eriodictyol on the neuronal cells. The data suggest further study to investigate the neuroprotective effects of eriodictyol in the *in vivo* models of neurodegenerative diseases.

1.6 Conceptual Framework



CHAPTER 2

LITERATURES REVIEW

2.1 Neurodegenerative disorders

Neurodegenerative disorders are characterized by progressive and irreversible loss of neurons in the central and peripheral nervous systems. These disorders include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). The clinical symptoms of neurodegenerative disorders are varied depending on types of neurons and brain areas defected; such as cognitive impairments in AD, and motor dysfunctions in PD, HD and ALS. Nowadays, there is no treatment for recovering neuronal death. The available treatments are to reduce the symptoms by altering neurotransmission. Therefore, the protection of neuronal death is considered as an important therapeutic approach to delay the progression of neuronal loss in neurodegenerative disorders.

The pathology of neurodegenerative disorders involves oxidative stress (Thanan et al., 2014). The increase of lipid peroxidation and the decreases of glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase, glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase, superoxide dismutase (SOD) and catalase (CAT) have been reported in the frontal cortex of AD patients (Ansari & Scheff, 2010). In addition, the transgenic mouse model of AD presented mitochondria dysfunction and the increase of ROS in the brains (David et al., 2005). To produce an animal model of PD, a mitochondrial complex I inhibitor, 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP), caused the elevation of malondialdehyde level an oxidative stress marker, and the depletion of GSH leading to parkinsonism behaviors in rats (Xu et al., 2013). Moreover, a transgenic mouse model of HD showed the increases of hydroxyguanosine (8-OHG) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels which are the markers oxidative DNA damage (Acevedo-Torres et al., 2009). Taken together, oxidative stress is an important cause of neuronal death in the neurodegenerative disorders.

2.2 Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) are defined as a group of reactive molecules derived from oxygen (O_2). ROS include peroxide ($\bullet O_2^{-2}$), superoxide anion ($\bullet O_2^{-}$), hydroxyl radical ($\bullet OH$), hydroperoxyl ($HO_2^{\bullet}$) and hydrogen peroxide (H_2O_2) (Figure 1). ROS is generally short-lived and highly reactive because of their unpaired valence electrons (Patten et al., 2010). The major source of intracellular ROS is mitochondrial electron transport chain (ETC) (Patten et al., 2010). It is estimated that 1-2% of electrons are leaked during ETC resulting in the production of superoxide anion in the mitochondrial matrix (Circu & Aw, 2010; Murphy, 2009). Superoxide anion can be transformed into H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be further transformed into the more toxic forms; hydroxyl radicals and hydroxyl anions, by Fenton reaction. Moreover, superoxide anion can be transformed into hydroperoxyl (Birben et al., 2012; Kim et al., 2015) (Figure 2). ROS cause cellular lipid, protein and DNA damage leading to oxidative stress, mitochondrial dysfunction, and cell death (Blesa et al., 2015; Kim et al., 2015; Valko et al., 2007). In normal condition, ROS production and protective mechanisms in the cells; such as antioxidants and antioxidative enzymes, are balanced. However, the excessive ROS generation and the depletion of the antioxidant system in the pathological condition resulted in oxidative stress in the cells (Kwon et al., 2014).

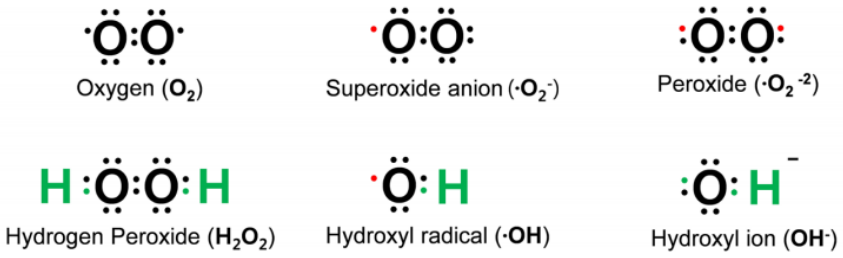


Figure 1 Types of reactive oxygen species (ROS) (Kim et al., 2015)

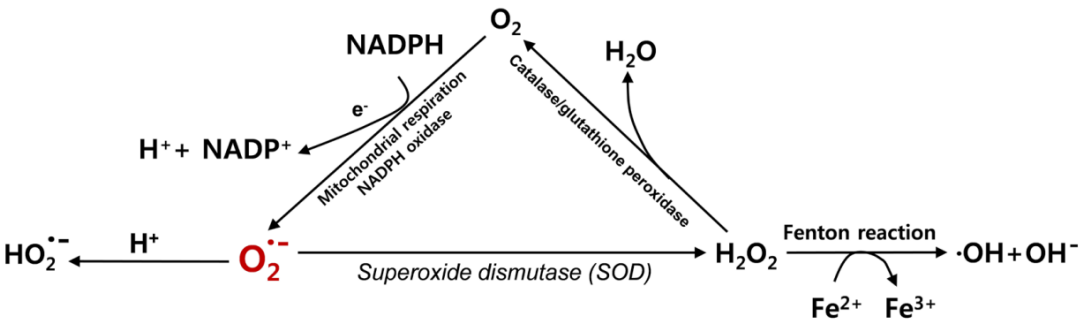


Figure 2 *The generation process of reactive oxygen species (ROS)* (Kwon et al., 2014)

Hydrogen peroxide (H_2O_2) has been used in various neurotoxic models *in vitro*. Hydrogen peroxide diffuses into plasma membrane (Sies, 2017) and is readily converted into the highly toxic hydroxyl radicals via Fenton reaction (Kim et al., 2015). H_2O_2 induced the increase of ROS, lipid peroxidation and mitochondria dysfunction leading to mitochondrial mediated apoptosis in human neuroblastoma SH-SY5Y cells (de Oliveira et al., 2018; Yang et al., 2016). Moreover, H_2O_2 stimulated apoptotic cell death in cardiomyocytes, associated with PARP cleavage, DNA fragmentation, upregulation of p53, loss of mitochondria membrane potential, BAX/BAD mitochondrial translocation, mitochondrial cytochrome c release and caspase-3 activation (Cook et al., 1999). In addition, H_2O_2 inhibit expression of Bcl-2 protein in human promyelocytic leukemia HL-60 cell line (Lee et al., 2000). Hence, H_2O_2 will be used to generate the *in vitro* neurotoxic model in this study.

2.3 Mitochondria-mediated apoptosis cascade

Apoptosis is the process of programmed cell death. Apoptosis normally occurs to maintain cell populations in tissues and to eliminate damaged cells (Elmore, 2007). However, progressive neuronal death via apoptosis leads to neurodegenerative diseases.

Apoptosis is triggered by extrinsic or intrinsic signaling pathways. Mitochondria plays an important role in the intrinsic signaling pathways of apoptosis. DNA damage, ER stress, hypoxia and metabolic stress can activate BH3-only proteins. BH3-only proteins then activates BAX and BAK resulting in translocation of BAX and BAK to the outer membrane of mitochondria.

This event leads to pore formation, mitochondrial membrane potential alteration and mitochondrial damage. Cytochrome c is subsequently released from mitochondria and then associates with adaptor protein apoptotic protease activating factor 1 (Apaf-1) to form apoptosome. Apoptosome sequentially activates caspase-9, caspase-3 and caspase-7. On the other hand, an anti-apoptotic protein, Bcl-2 proteins, inhibit cell death by inhibiting BAX and BAK (Circu & Aw, 2010; Ichim & Tait, 2016) (Figure 3).

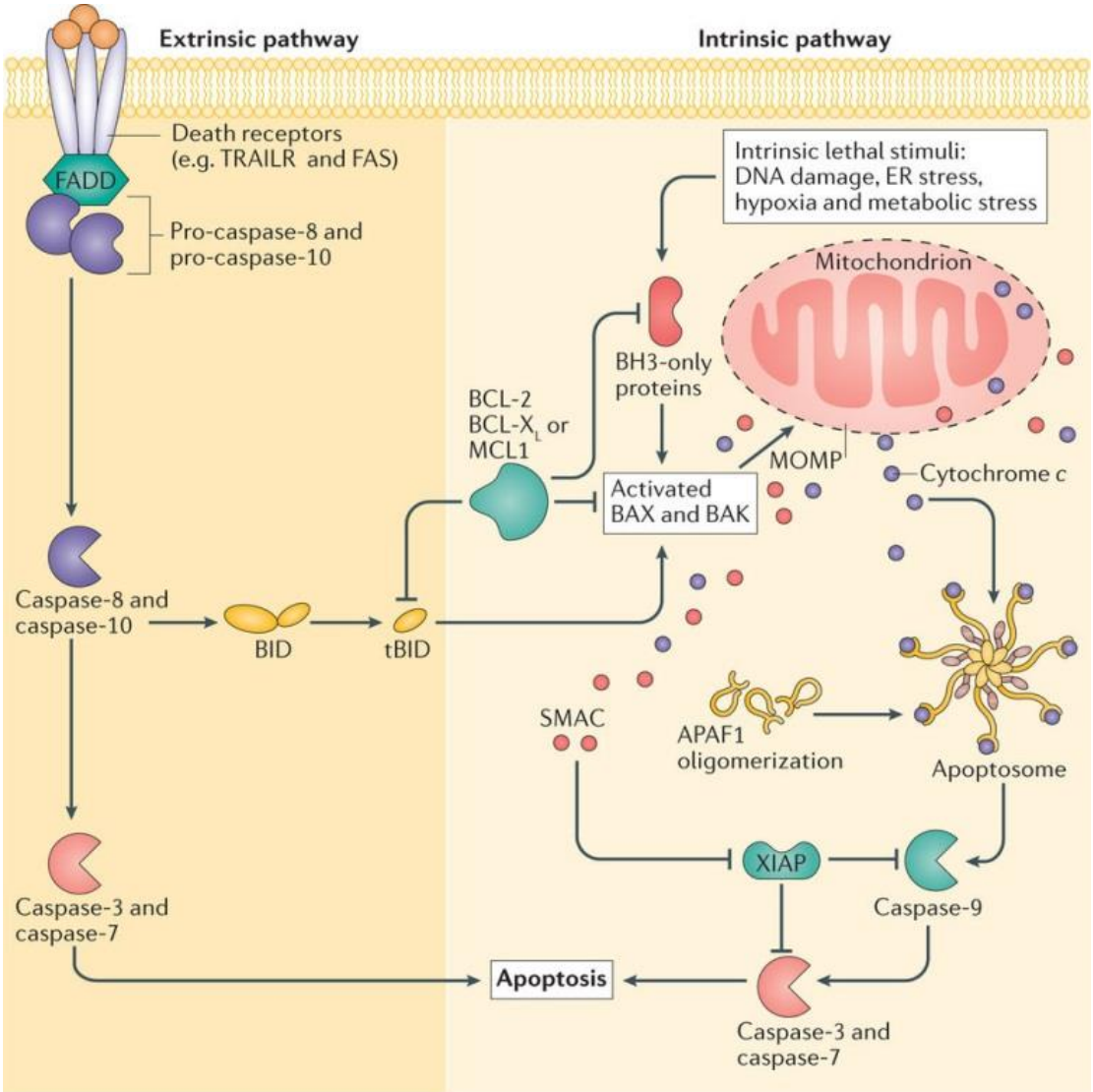


Figure 3 Apoptotic signaling pathways (Ichim & Tait, 2016)

2.4 Eriodictyol

A flavonoid, eriodictyol ($C_{15}H_{12}O_6$) (Figure 4), is extracted from the whole plant of *Dendrobium ellipsophyllum* (thai name: Ueang Thong) (Figure 5). Eriodictyol is colorless powder with molecular weight 258 g/mol, and Log P 2.02 (U.S. Environmental Protection Agency, 2018). It exhibits beneficially pharmacological properties including anti-metastatic (Tanagornmeatar et al., 2014), antioxidant and anti-inflammatory effects (Zhu et al., 2015). Eriodictyol attenuated LPS-induced acute lung injury by inhibiting the inflammatory cytokine such as TNF-α, IL-6 and IL-1β (Zhu et al., 2015). In addition, eriodictyol attenuated β-amyloid 25–35-induced oxidative cell death in primary cultured neuron by activating Nrf2 (Jing et al., 2015). Moreover, eriodictyol protected against linoleic acid hydroperoxide and H_2O_2-induced PC12 cell death (Lou et al., 2012; Sasaki et al., 2003)

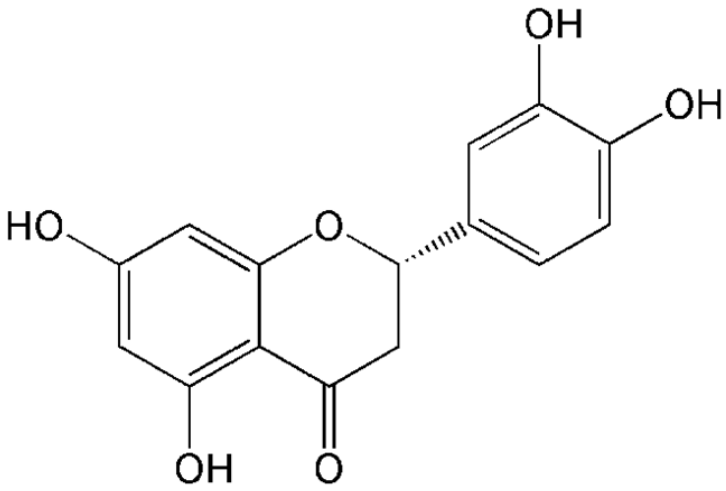


Figure 4 Chemical structure of eriodictyol

Figure 5 *Dendrobium ellipsophyllum* or Ueang Thong

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell culture and treatment

The human neuroblastoma SH-SY5Y cell line was purchased from ATCC (Manassas, VA, USA). SH-SY5Y cells were cultured in Dulbecco's Modified Essential Eagle's Medium-Ham's Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 2 days. Cells were sub-cultured at 80–90% confluent.

3.1.2 Chemicals and Reagents

Anti-cytochrom c antibody (Santa Cruz Biotechnology, CA, USA)

Anti-GAPDH antibody (Millipore, Billerica, MA, USA)

BCA protein assay kit (Thermo Scientific, Rockford, IL, USA)

2′-7′ Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, USA)

Dimethyl sulfoxide (DMSO) (Fisher Scientific, Loughborough, UK)

Dulbecco's Modified Essential Eagle's Medium-Ham's Nutrient Mixture F-12 (DMEM-F12) (Gibco, USA)

Ethanol (Merk, Germany)

Fetal bovine serum (Gibco, New Zealand)

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Millipore, Billerica, MA, USA)

Hydrogen peroxide (H_2O_2) (Ajax Finechem, Auckland, New Zealand)

Nonfat Dry Milk (Cell Signaling Technology, Inc. USA)

Penicillin/streptomycin (Gibco, USA)

Polyvinylidene difluoride transfer membrane (Immobilon, Bedford, MA, USA)

Resazurin (Sigma, USA)

Tetramethylrhodamine ethyl ester (Sigma, USA)

Phosphate buffered saline (Gibco, USA)

Protein ladder (BLUEstaint^{tm}, Gold Biotechnology, Inc. US)

Rabbit monoclonal anti-BAX antibody (Cell Signaling Technology, Inc. USA)

Rabbit monoclonal anti-Bcl-2 antibody (Sigma-Aldrich, St. Louis, MO. USA)

Rabbit monoclonal anti-caspase-3 antibody (Cell Signaling Technology, Inc. USA)

Tris-HCl (Sigma-Aldrich, St. Louis, MO. USA)

0.4% Trypan blue dye (Sigma, USA)

0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco, USA)

3.1.3 Equipment

Analytical balance (Mettler Toledo, Switzerland)

Autoclave (Hirayama, Japan)

Autopipette (Gilson, USA)

Centrifuge (Hettich, USA)

Controller pipette (Gilson, USA)

CO_2 incubator (Thermo Fisher Scientific, USA)

Light microscope (Nikon, Japan)

Micro centrifuge (Thermo Fisher Scientific, USA)

Microplate fluorescence reader (CLARIOstar®, BMG LABTECH, Germany).

pH meter (Schott, UK)

Power suppy (PowerPac^{TM}, USA)

Trans-Blot SD sem-dry electrophoretic transfer cell (Trans-Blot®, USA)

Vortex mixer (Scientific Industries, USA)

3.1.4 Materials

25 and 75 ml cell culture flask (Gibco, USA)

15 and 50 ml centrifuge tube (Corning Inc., USA)

1.7 ml micro centrifuge tube (Accumax, USA)

6-well plate (Corning Inc., USA)

96-well plate (Corning Inc., USA)

96-well plate black flat bottom polystyrene (Corning Inc., USA)

200 and 1250 µl universal grad tip (Accumax, USA)

3.2 Chemical preparation

3.2.1 Preparation of eriodictyol stock solution

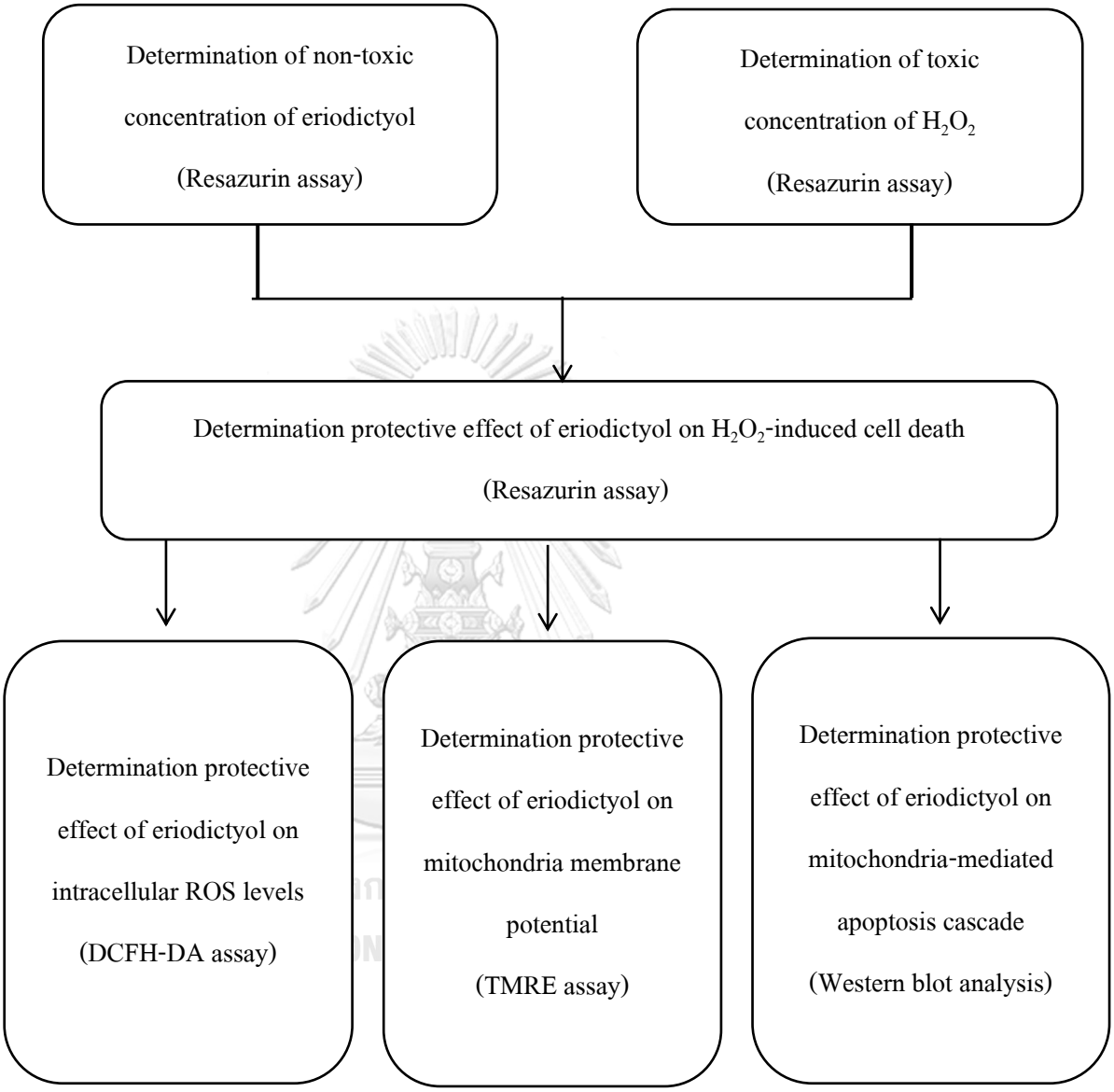
Eriodictyol was prepared as previously described (Tanagornmeatar et al., 2014) and provided by Associate Professor Dr. Boonchoo Sritularak, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Tanagornmeatar et al., 2014). Fifty-mM eriodictyol stock solution was prepared in 100% DMSO and stored at 20 °C until use. In the experiments, the stock solution was diluted in culture medium to final concentrations. The 0.2% DMSO was used as a vehicle control.

3.2.2 Hydrogen peroxide (H_2O_2)

One hundred mM H_2O_2 stock solution was freshly prepared in deionized water. The stock solution was then diluted in medium to final concentrations.

3.3 Methods

3.3.1 Experimental design



3.3.2 Determination of non-toxic concentrations of eriodictyol

SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated overnight at 37°C and 5% CO_2. Cells were treated with eriodictyol 0.001, 0.01, 0.1, 1, 10 and 100 µM, and incubated for 24 h. Cell survival was evaluated by resazurin assay.

In resazurin assay, 10 µL of resazurin (0.05 mg/ml) was added to each well 4 h prior to the end of incubation time. Viable cells with active metabolism can reduce resazurin into the resorufin product (Figure 6) detected by a microplate fluorescent reader with at 530 nm excitation wavelength and 590 nm emission wavelength. The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\% control)} = \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of control}}$$

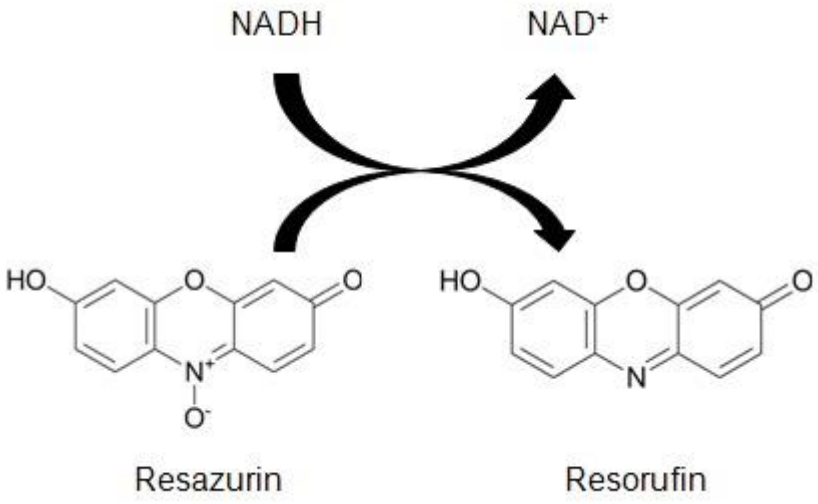


Figure 4 Principle of resazurin assay (Riss et al., 2016)

3.3.3 Determination of toxic concentrations of H_2O_2

SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated for 48 h. Cells were treated with H_2O_2 100, 200, 300, 400, 500, 600 and

1000 μM. Cell survival was evaluated by resazurin assay. The 50% inhibitory concentration (IC_{50}) was calculated and selected as a toxic concentration of H_2O_2.

3.3.4 Determination of the protective effect of eriodictyol on H_2O_2-induced cell death

SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated overnight at 37°C and 5% CO_2. Cells were treated with eriodictyol 0.1, 1, and 10 μM for 24 h. Then, the medium was removed. Cells were then treated with 200 μM H_2O_2 for 12 h. Cell viability was measured using resazurin assay.

3.3.5 Determination of the effects of eriodictyol on intracellular ROS levels

SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated overnight at 37°C and 5% CO_2. Cells were treated with eriodictyol 0.1, 1, and 10 μM for 24 h. Then, the medium was removed and cells were treated with 200 μM H_2O_2 for 1 hour. After incubation period, cells were washed twice with PBS and 100 μL of DCFH-DA was added (Hu et al., 2015; Kwon et al., 2014). The fluorescence intensity was measured by microplate reader.

In the DCFH-DA assay, 100 μL of DCFH-DA (25 μM) was added to each well and incubated for 30 minutes. DCFH-DA is the nonfluorescent dye. Oxidation of DCFH-DA produces a fluorescent molecule, dichlorofluorescein (DCF) (Figure 7). DCF was quantified using a microplate reader, with 488 nm excitation and 575 nm emission wavelengths. Intracellular ROS levels were expressed as fluorescence intensity and further calculated as a percentage of control.

$$\text{Intracellular ROS levels (\% control)} = \frac{\text{Fluorescent intensity of sample}}{\text{Fluorescent intensity of control}}$$

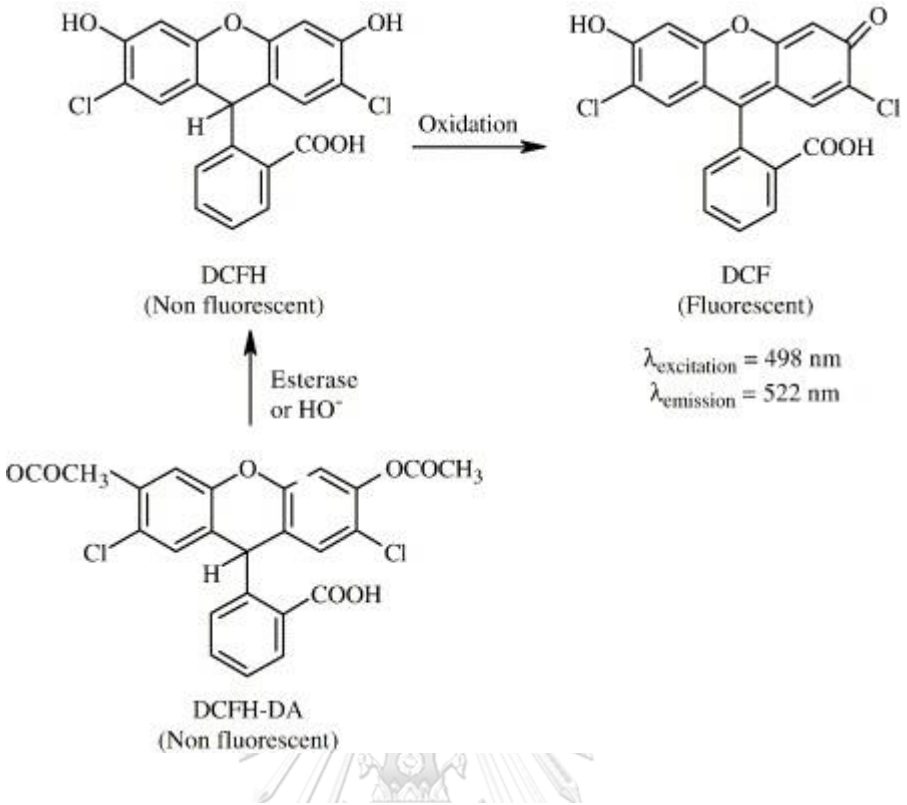


Figure 5 Principle of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay (Gomes et al., 2005)

3.3.6 Determination of the effect of eriodictyol on mitochondrial membrane potential

SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated overnight at 37°C and 5% CO_2. Cells were treated with eriodictyol 0.1, 1, and 10 µM for 24 h. Then, the medium was removed and cells were treated with 200 µM H_2O_2 for 12 h. Cells were washed with PBS once time. Mitochondrial membrane potential was detected using tetramethylrhodamine ethyl ester (TMRE) assay.

In TMRE assay, 100 µL of TMRE (100 nM) was added to each well and incubated for 15 min. Fluorescent intensity of TMRE was measured using a microplate reader at excitation wavelength 585 mm and emission wavelength 535 nm. TMRE, a red-orange positive charge dye, can accumulate inside a healthy mitochondrion which exhibits negative membrane potential.

3.3.7 Determination of the effects of eriodictyol on mitochondria-mediated apoptosis cascade

SH-SY5Y cells were seeded in 6-well plates at a density of 2×10^6 cells/well and incubated with eriodictyol 0.1, 1, and 10 μM for 24 h. Then, medium was removed and cells were treated with 200 μM H_2O_2 for 12 h. Cell lysates were then collected. A proapoptotic protein, BAX, an anti-apoptotic protein, Bcl-2 and the markers of apoptosis, cytochrome c, cleaved caspase-3 and caspase-3 expression levels were detected using western blot analysis.

In the western blot analysis, cells are washed with PBS, harvested and centrifuged at 16,000 g for 15 min at 4 °C. The protein concentrations were determined using a BCA protein assay kit. Eighty-μg protein were separated on an 15% SDS–polyacrylamide gel, and then transferred onto a polyvinylidene difluoride membrane. The nonspecific proteins were blocked with 5% skim milk containing 0.5 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature. The membrane was subsequently incubated with the primary antibodies (1:1000 anti-Bax, anti-Bcl-2, anti-cytochrome c, and anti-caspase-3 antibodies) overnight at 4 °C. After washes with TBST (Tris-buffered saline with 0.1% Tween-20), the blots were incubated with a secondary antibody (1:1000 in TBST with 5% skim milk) for 2 hours at room temperature. The blots were then washed and developed using the enhanced chemiluminescence detection method by a luminescence-image analyzer. Protein bands were quantified by densitometric analysis using Image J software.

3.3.8 Statistical Analysis

Each experiment was repeated three times. All results were presented as mean ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA), followed by LSD post-hoc for comparisons between group means. Differences were considered statistically significant if p value was lesser than 0.05 ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 The effect of eriodictyol on cell viability

Eriodictyol (0.001-100 μM) did not affect cell viability (Figure 8). The highest concentration of eriodictyol (100 μM) slightly decreased cell viability by 17.36% but this was not significantly different from control. Therefore, three concentrations of eriodictyol, 0.1, 1 and 10 μM, were selected for further study.

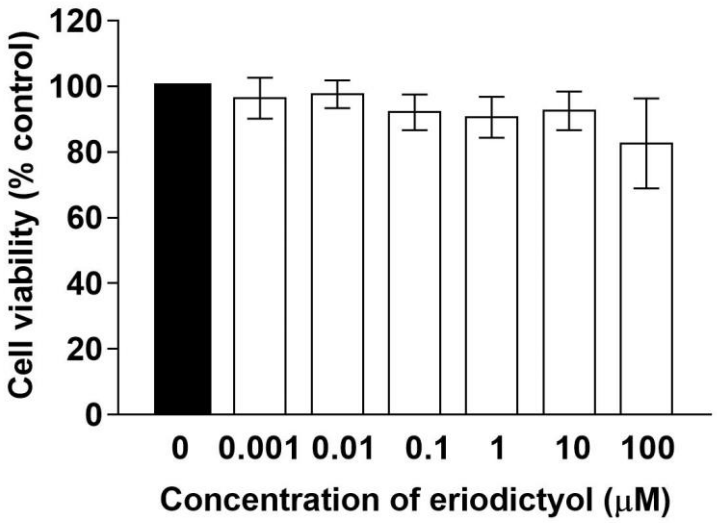


Figure 6 The effect of eriodictyol (0.001-100 μM) on cell viability in SH-SY5Y cells. Data are presented as mean ± S.E.M of three independent experiments.

4.2 The effect of H_2O_2 on cell viability

To determine the effects of H_2O_2 on cell viability in SH-SY5Y cells, cells were exposed to H_2O_2 (100-1000 μM) for 12 h. H_2O_2 (100-1000 μM) significantly reduced cell viability ($p < 0.01$ VS control) (Figure 9). The IC_{50} was 163±0.12 μM. Since H_2O_2 200 μM decreased cell viability to 52.56 ± 3.60%, this concentration was selected to induce cytotoxicity.

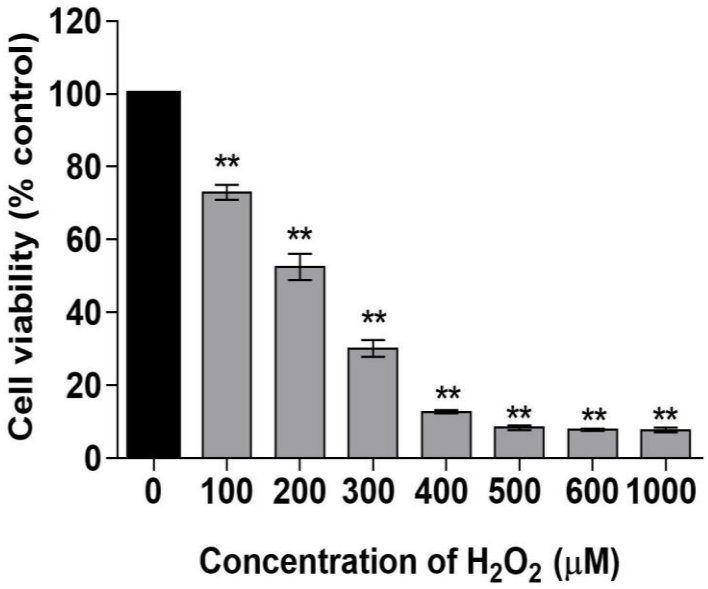


Figure 7 The effect of H_2O_2 (100-1000 μM) on cell viability in SH-SY5Y cells. Data are presented as mean ± S.E.M of three independent experiments. $^{**}p < 0.01$ compared to control.

4.3 The protective effect of eriodictyol on H_2O_2-induced cell death

H_2O_2 (200 μM) significantly decreased cell viability compared to control group ($p < 0.01$). Pretreatment with eriodictyol (0.1, 1 and 10 μM) significantly increased cell viability compared to H_2O_2-treated alone ($p < 0.01$) (Figure 10), indicating the protective effect of eriodictyol.

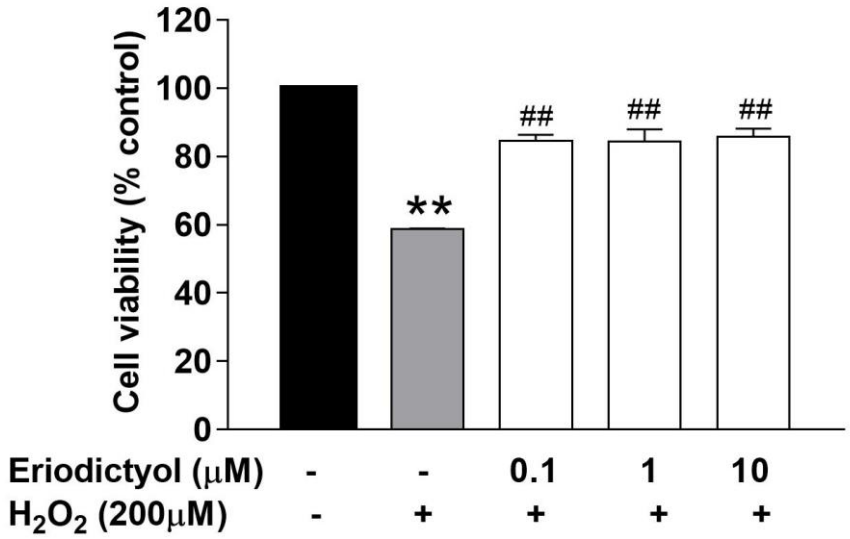


Figure 8 The protective effects of eriodictyol on H_2O_2-induced cell death in SH-SY5Y cells. Data are presented as mean ± SEM of three independent experiments. $^{**}p < 0.01$ compared to control; $^{\#\#}p < 0.01$ compared to H_2O_2 -treated alone.

4.4 The protective effects of eriodictyol on H_2O_2-induced ROS generation

H_2O_2 significantly increased intracellular ROS levels ($p < 0.05$) VS control. Eriodictyol pretreatment significantly decreased intracellular ROS levels induced by H_2O_2 compared to H_2O_2-treated alone ($p < 0.05$) (Figure 11).

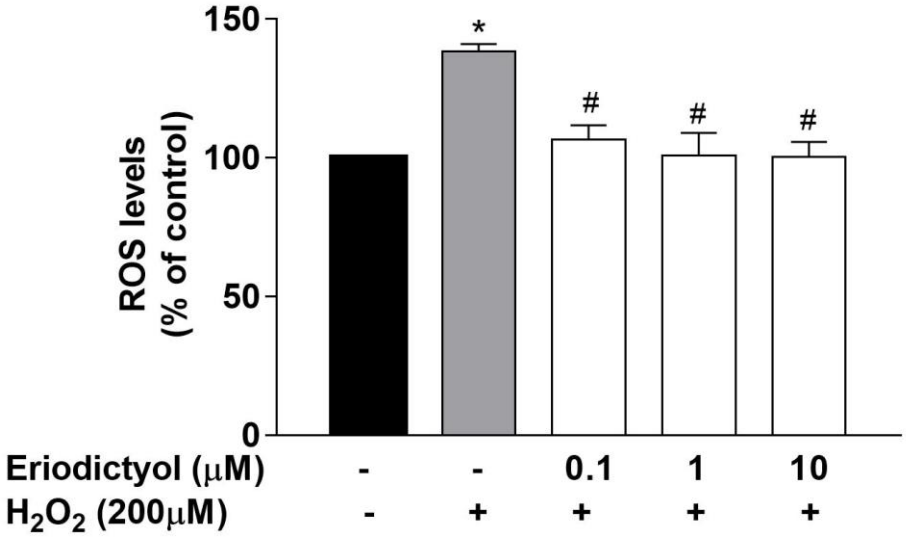
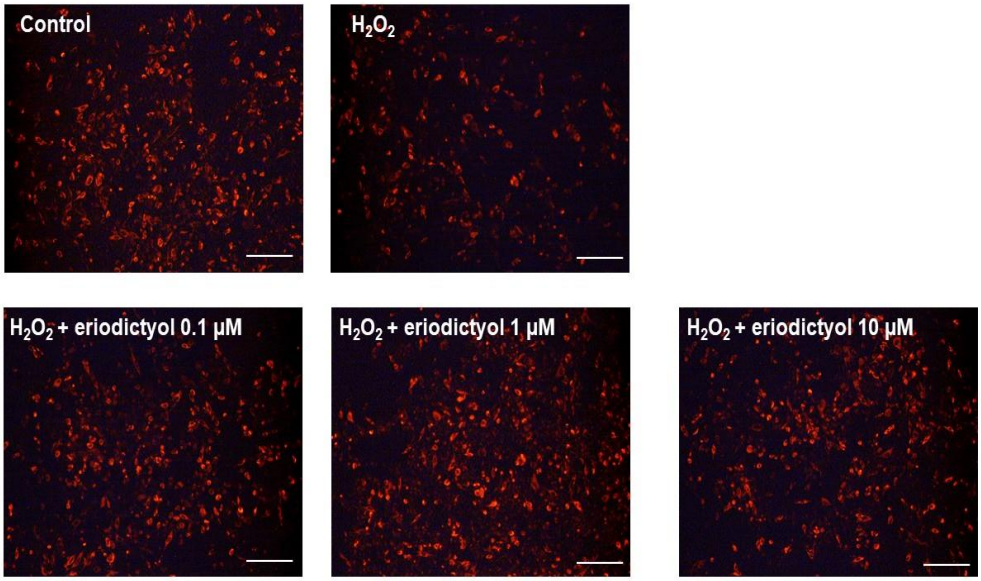


Figure 9 The effects of eriodictyol on intracellular ROS levels induced by H_2O_2. Data are presented as mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to H_2O_2-treated alone.

4.5 The effect of eriodictyol and H_2O_2 on mitochondrial membrane potential

Mitochondrial membrane potential was determined by TMRE fluorescent intensity. Figure 12A showed the reduction of TMRE intensity in H_2O_2-treated group while eriodictyol (0.1,1 and 10 μM) pretreated cells increased TMRE intensity. The quantitative analysis by a microplate reader showed a significant decrease of TMRE intensity in H_2O_2-treated cells compared to control ($p < 0.05$). Eriodictyol (0.1, 1 and 10 μM) pretreatment significantly increased TMRE intensity ($p < 0.05$ vs H_2O_2-treated alone), indicating the protection of mitochondria dysfunction (Figure 12B).

A



B

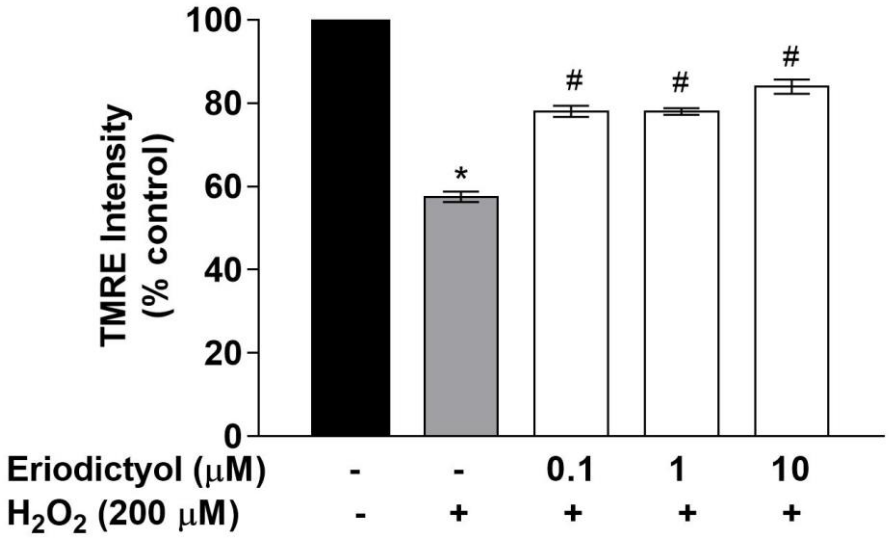
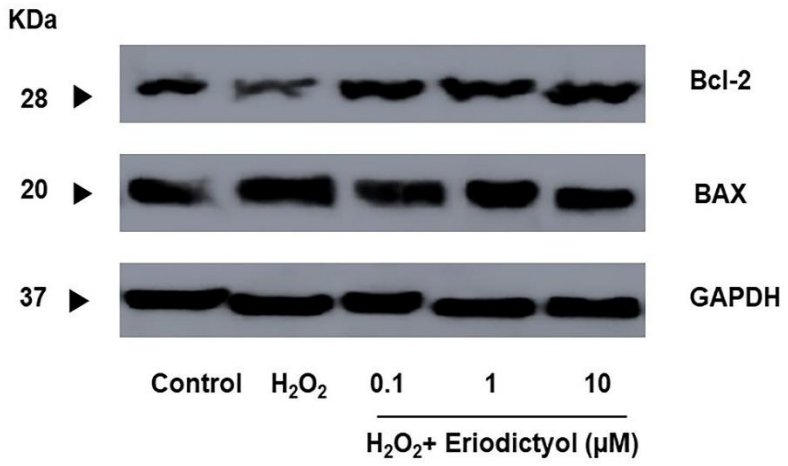


Figure 10 The effects of eriodictyol and H_2O_2 on mitochondrial membrane potential presented as TMRE fluorescent imaging (A) and TMRE fluorescent intensity (B). The fluorescent intensity are presented as mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared to control; #$p < 0.05$ compared to H_2O_2-treated alone.

4.6 The effects of eriodictyol and H_2O_2 on mitochondria-mediated apoptosis cascade

Mitochondria-mediated apoptosis cascade was determined by protein expression levels of a proapoptotic protein, Bcl-2, an anti-apoptotic protein, Bax, and the markers of apoptosis, cytochrome c, cleaved caspase-3 and caspase-3. H_2O_2 significantly reduced ratio of Bcl-2/BAX expression ($p < 0.05$) compared to the control while eriodictyol pretreatment significantly increased ratio of Bcl-2/BAX expression compared to H_2O_2-treated alone ($p < 0.05$) (Figure 13). Moreover, H_2O_2 increased cytochrome c expression ($p < 0.05$ vs control) while eriodictyol pretreatment significantly decreased cytochrome c expression ($p < 0.05$ vs H_2O_2-treated alone) (Figure 14). In addition, H_2O_2 increased caspase-3 and cleaved caspase-3 expression levels ($p < 0.05$ vs control) while eriodictyol pretreatment significantly decreased caspase-3 and cleaved caspase-3 expression levels ($p < 0.05$ vs H_2O_2-treated alone) (Figure 15).

A



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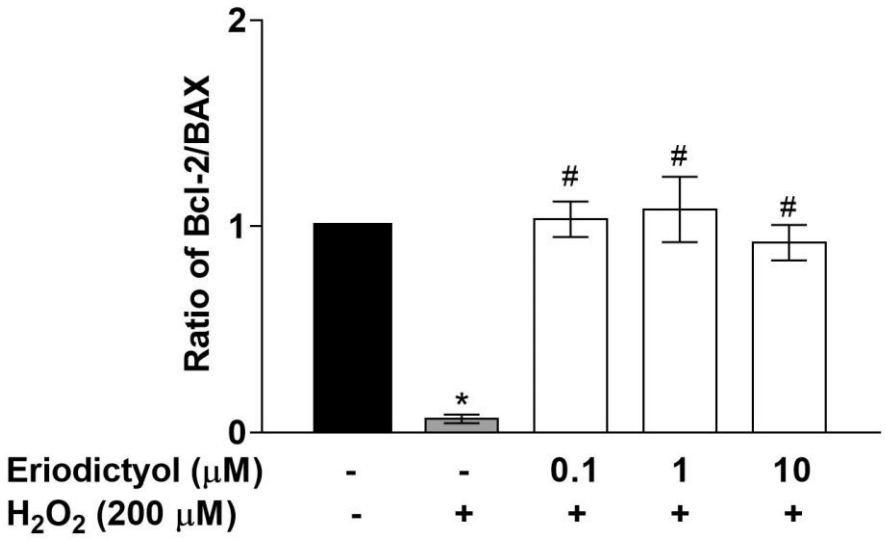
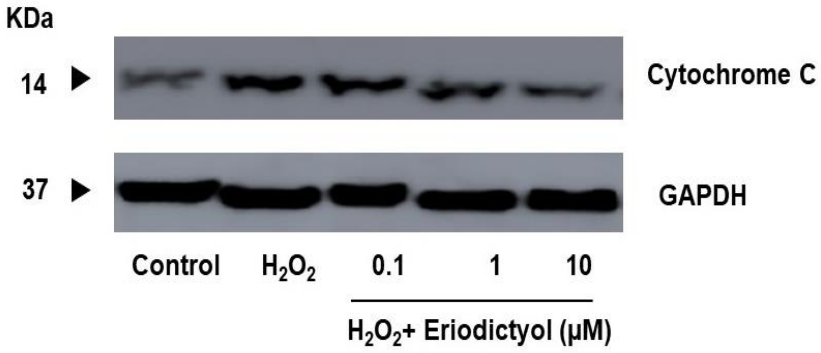


Figure 11 The effects of eriodictyol and H_2O_2 on Bcl-2 and BAX protein expression (A). Bcl-2/BAX ratio of each experimental groups were calculated and presented as mean ± S.E.M. of three independent experiments (B). *$p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to H_2O_2-treated alone.

A



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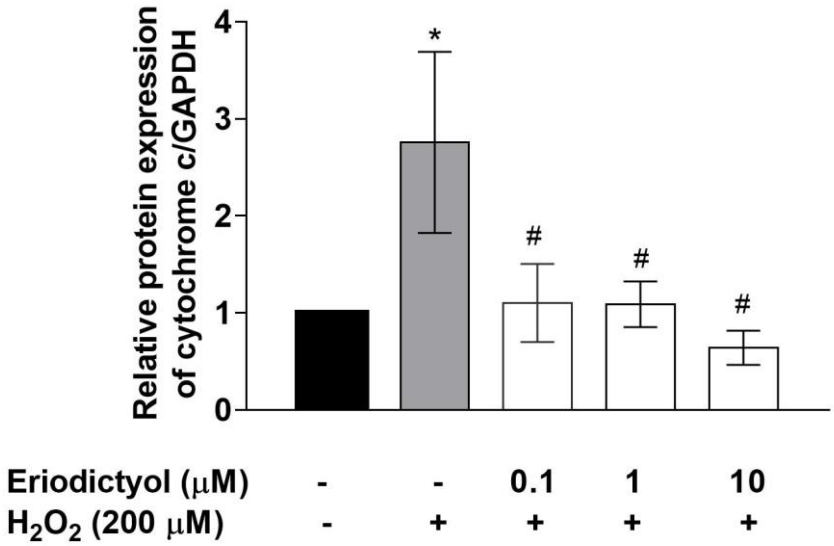
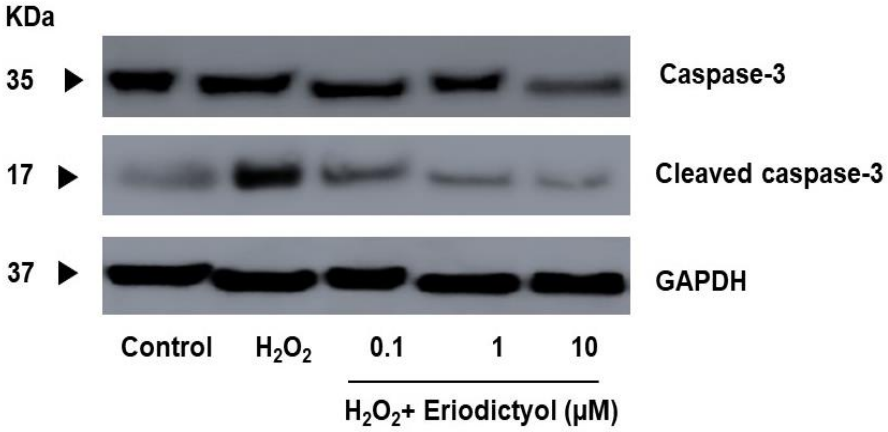
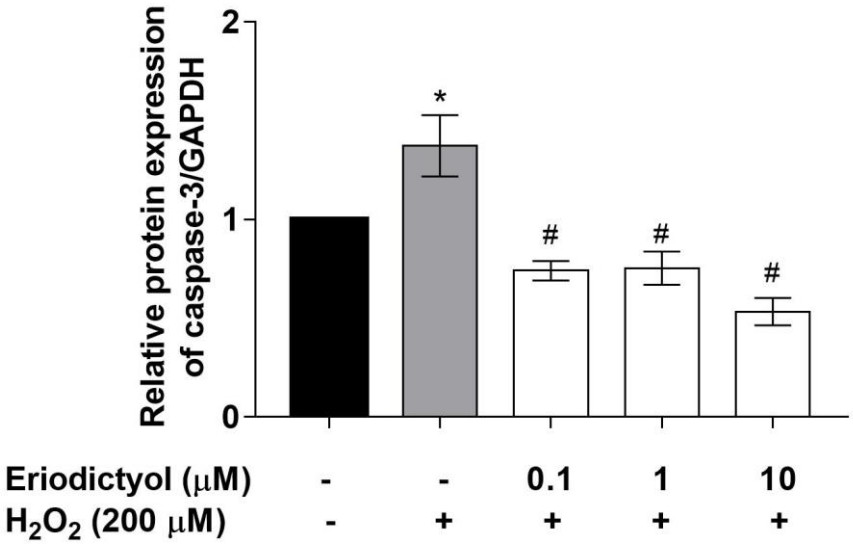


Figure 12 The effects of eriodictyol and H_2O_2 on cytochrome c expression (A). The expression levels were calculated and presented as cytochrome c/GAPDH of three independent experiment (B). *$p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to H_2O_2-treated alone.

A


KDa
35
17
37
Caspase-3
Cleaved caspase-3
GAPDH
Control
H_2O_2
0.1
1
10
H_2O_2+ Eriodictyol (μM)

B


Relative protein expression of caspase-3/GAPDH
2
1
0
*
#
#
#
Eriodictyol (μM) - - 0.1 1 10
H_2O_2 (200 μM) - + + + +

C

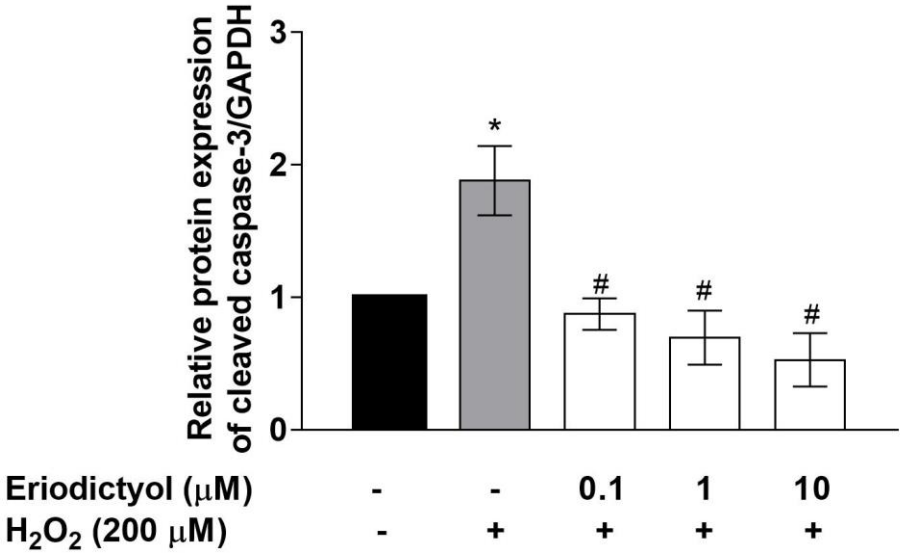


Figure 13 The effects of eriodictyol and H_2O_2 on caspase-3 and cleaved caspase-3 expression (A). The expression levels were calculated and presented as caspase-3/GAPDH (B) and cleaved caspase-3/GAPDH of three independent experiment (C). *$p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to H_2O_2-treated alone.

CHAPTER 5

DISCUSSION AND CONCLUSION

Oxidative stress and mitochondrial dysfunction are the important causes of neuronal death in neurodegenerative disorders (Kim et al., 2015). The excessive ROS generation and the depletion of the antioxidant system result in oxidative stress in neurons (Kwon et al., 2014). Therefore, the neuroprotective approaches in the treatment of neurodegenerative disorders, such as a decrease of ROS production, an increase of antioxidant system and the protection of mitochondria function have gained more interested. In this study, pretreatment of eriodictyol can increase cell viability in H_2O_2-treated cells as concentration, indicating the protective effect of eriodictyol in H_2O_2-induced cytotoxicity in SH-SY5Y cells.

The protective effect of erioductyol was concentration-independent as similar percentage cell viability was observed in cells pretreated with 0.1, 1 and 10 μM of eriodictyol (Figure 8). This might be due to the maximum effect of eriodictyol presented at a concentration of 0.1 μM. Either pretreatment with the lower concentrations of eriodictyol or increasing H_2O_2 concentration can be used to clarify this point. In addition, as resazurin assay detects mitochondrial reductase activity in living cells (Riss et al., 2004), this assay cannot distinguish between apoptosis and necrosis cell death. Using Annexin V-FITC and PI staining together with flow cytometry can elucidate whether the maximum effect of eriodictyol is caused by the maximum protection of either death pathway.

Flavonoids exert a direct ROS scavenging effect by reacting with $\bullet O_2^-$, $HO_2^{\bullet-}$, nitric oxide ($NO^{\bullet}$), alkoxyl and peroxyl radicals (Bors et al., 1990). The ROS scavenging-mediated antioxidant activity of flavonoid is primarily attributed to the benzene ring-bound hydroxyl groups that are capable of donating either one hydrogen atom or a single electron to the ROS, and consequently stabilize ROS (Amic et al., 2007; Bors et al., 1990). Scavenging activity of eriodictyol was reported in the *in vitro* DPPH assay (Chanput et al., 2016). Taken together, eriodictyol, as a flavonoid, can reduce intracellular ROS partly due to its direct scavenging effect. Previous studies also demonstrated that eriodictyol activated Nrf2 transcription factor (Johnson et al., 2009; Xie et al., 2017). Nrf2 is responsible for the induction of various genes encoding the protective antioxidant proteins, and thus promoting the regulation of an optimal intracellular

redox environment (Ma, 2013). Therefore, the protective mechanisms of eriodictyol involving the increase of antioxidant enzyme expression should be further investigated.

Mitochondria dysfunction has been implicated in neurodegenerative diseases as the reductions of mitochondria complex I and IV activity have been reported in patients with Alzheimer's disease and Parkinson's disease, respectively (Sullivan & Brown, 2005). Moreover, mitochondria complex I inhibitors, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone have been used to produce parkinsonism behaviors in animal models (Betarbet & Greenamyre, 2008; Bové et al., 2005). Excessive ROS generation directly impairs mitochondrial function as it causes an decrease of Bcl-2/BAX ratio resulting in the impairment of mitochondria membrane potential and the decrease of adenosine triphosphate (ATP) levels (Alvarino et al., 2017; Redza-Dutordoir & Averill-Bates, 2016; Waseem et al., 2017). In addition, ROS induces Bcl-2 down-regulation via the ubiquitin-proteasomal degradation pathway (Chanvorachote et al., 2009). This study showed that H_2O_2 markedly decreased Bcl-2/BAX ratio, and consequently decreased mitochondria membrane potential while pretreatment with eriodictyol can reverse these events. These results suggest that the mitochondria protection effect of eriodictyol is partly due to a decrease of an intracellular ROS. Further study should focus on the direct effect of eriodictyol on mitochondria function, for example the protection of mitochondria electron transport complex and the effect on mitophagy.

The decrease of mitochondria membrane potential causes cytochrome c release, and consequently induces apoptosis cascade (Green & Kroemer, 1998). Cytochrome c and caspase-3 expressions were used as indicators of mitochondria-mediated apoptosis in this study, H_2O_2 increased cytosolic cytochrome c, caspase-3 and cleaved caspase-3 expression while pretreatment with eriodictyol decreased the expressions of proteins. This result is in agreement with previous study showing that eriodictyol decreased pro-caspase-3 and cytochrome c expression in human keratinocyte HaCaT cells (Lee et al., 2011).

In conclusion, eriodictyol protect H_2O_2-induce cell death by reducing an intracellular ROS leading to the inhibition of mitochondria-mediated apoptosis cascade (Figure 16). The effect is partly due to the direct ROS scavenging effect of eriodictyol. The effect of eriodictyol on the increase of cellular defensive mechanism and the protection of mitochondria should be further investigated. Overall, this study suggests the possible benefit of eriodictyol in the protection neuronal death in neurodegenerative disorders.

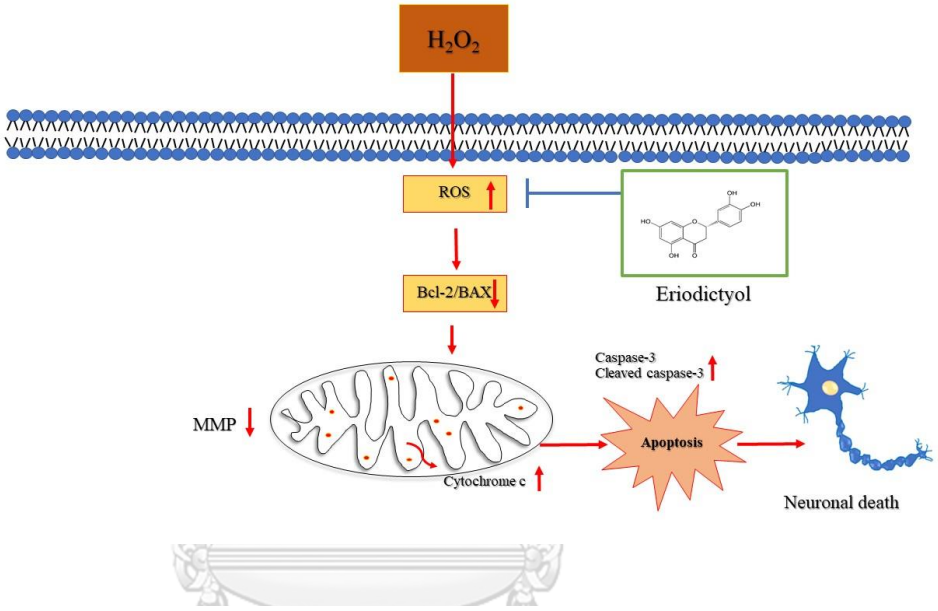


Figure 14 The action mechanisms of eriodictyol on H_2O_2-induced cell death in SH-SY5Y cells

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