# EFFECTS AND MECHANISMS OF *CAESALPINIA MIMOSOIDES* LEAF EXTRACT ON OXIDATIVE STRESS RESISTANCE AND ANTI-AGING ACTIVITY IN *CAENORHABDITIS ELEGANS* AND NEURITE OUTGROWTH ACTIVITY IN NEURO2A CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Clinical Biochemistry and Molecular Medicine
Department of Clinical Chemistry
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2019

# ผลและกลไกของสารสกัดจากใบผักหนามปู่ย่าต่อการต้านภาวะเครียดออกซิเดชัน และการต้านความชราใน *CAENORHABDITIS ELEGANS* และการเจริญของนิวไรท์ในเซลล์ NEURO2A

นายพันธการ รังสินธุ์



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

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| Thesis Title | EFFECTS AND MECHANISMS OF *CAESALPINIA MIMOSOIDES* LEAF EXTRACT ON OXIDATIVE STRESS RESISTANCE AND ANTI-AGING ACTIVITY IN *CAENORHABDITIS ELEGANS* AND NEURITE OUTGROWTH ACTIVITY IN NEURO2A CELLS |
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| Field of Study | Clinical Biochemistry and Molecular Medicine |
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พันธการ รังสินธุ์ : ผลและกลไกของสารสกัดจากใบผักหนามปู่ย่าต่อการต้านภาวะเครียดออกซิเดชัน และการต้านความชราใน *CAENORHABDITIS ELEGANS* และการเจริญของนิวไรท์ในเซลล์ NEURO2A. ( EFFECTS AND MECHANISMS OF *CAESALPINIA MIMOSOIDES* LEAF EXTRACT ON OXIDATIVE STRESS RESISTANCE AND ANTI-AGING ACTIVITY IN *CAENORHABDITIS ELEGANS* AND NEURITE OUTGROWTH ACTIVITY IN NEURO2A CELLS) อ.ที่ปรึกษาหลัก : ผศ. ดร.เทวิน เทนคำเนาว์

ในยุคที่ก้าวสู่สังคมผู้สูงอายุในปัจจุบัน สิ่งที่มีความจำเป็นคือการบริโภคสารอาหารที่ช่วยให้ผู้สูงอายุมีสุขภาพดี มีอายุขัยที่ยืนยาว และป้องกันการเกิดโรคต่างๆ ที่อาจขึ้นได้จากความชรา หนึ่งในโรคที่พบมากในวัยชรา คือโรคอัลไซเมอร์ ซึ่งยังคงเป็นปัญหาทางด้านสาธารณสุขที่สำคัญทั่วโลก เนื่องจากยังไม่มีวิธีการรักษาให้หายขาด มีการศึกษาในเซลล์เพาะเลี้ยงพบว่าโปรตีนต้นกำเนิดอะไมลอยด์ ซึ่งเป็นหนึ่งในโปรตีนที่สำคัญที่นำไปสู่พยาธิสภาพของโรคอัลไซเมอร์ สามารถยับยั้งกระบวนการที่สำคัญของการเจริญเติบโตของเซลล์ประสาทอย่างการงอกของนิวไรท์ ด้วยเหตุนี้ การศึกษาผลของสมุนไพรที่สามารถกระตุ้นการงอกของนิวไรท์ในสภาวะที่มีการแสดงออกของโปรตีนต้นกำเนิดอะไมลอยด์สูงจึงมีความสำคัญในการพัฒนายาเพื่อใช้ป้องกันและรักษาโรคอัลไซเมอร์ นอกจากนี้ การทดสอบหาฤทธิ์ในการต้านอนุมูลอิสระของสมุนไพรยังมีความสำคัญในการนำมาพัฒนาเป็นยา หรือสารอาหารที่ใช้บริโภคเพื่อต่อต้านความชราและโรคที่เกี่ยวข้องกับความชรา จากการทดลองหาฤทธิ์ของสารสกัดจากพืชต่างๆ ในเซลล์ประสาทเพาะเลี้ยง Neuro2a ทั้งในเซลล์ปกติและในเซลล์ที่มีการแสดงออกของโปรตีนต้นกำเนิดอะไมลอยด์สูง พบว่าสารสกัดจากใบและยอดอ่อนของผักหนามปู่ย่าสามารถกระตุ้นให้นิวไรท์งอกยาวได้มากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ เมื่อศึกษาในระดับโมเลกุลพบว่า สารสกัดผักนหนามปู่ย่าสามารถเพิ่มการแสดงออกของยีนที่สำคัญต่อการงอกนิวไรท์อย่างยีน GAP-43 และยีน Ten-4 อีกทั้งยังลดการแสดงออกของยีนที่ยับยั้งการงอกของนิวไรท์อย่างยีน Lingo-1 และยีน NgR นอกจากนี้ ในการทดลองฤทธิ์ต้านอนุมูลอิสระและต้านความชราของสารสกัดจากใบผักหนามปู่ย่าในหนอนทดลอง *Caenorhabditis elegans* พบว่าสารสกัดช่วยให้หนอนทดลองทนต่อสภาวะเครียดออกซิเดชันได้ดีขึ้น รวมถึงลดการสะสมของอนุมูลอิสระออกซิเจนในร่างกาย และเมื่อศึกษากลไกระดับโมเลกุล พบว่า เมื่อหนอนทดลองได้รับสารสกัดแล้วจะกระตุ้นให้กระบวนการ DAF-16/FOXO มีการทำงานเพิ่มมากขึ้น และเพิ่มการแสดงออกของ SOD-3 ซึ่งเป็นกระบวนการที่สำคัญในการต้านอนุมูลอิสระในระดับโมเลกุล นอกจากนี้ ยังพบว่าเม็ดสีที่บ่งชี้ถึงความชรามีปริมาณลดลงหลังจากได้รับสารสกัด งานวิจัยชิ้นนี้เป็นหลักฐานสนับสนุนฤทธิ์ในการกระตุ้นการงอกของนิวไรท์ในเซลล์ประสาทที่มีการแสดงออกของโปรตีนต้นกำเนิดอะไมลอยด์สูง และฤทธิ์ในการต้านอนุมูลอิสระ รวมถึงต้านความชราของสารสกัดจากใบผักหนามปู่ย่า ซึ่งจะมีประโยชน์อย่างยิ่งในการนำมาใช้บริโภคหรือพัฒนาเป็นยาทางเลือกเพื่อป้องกันและรักษาโรคอัลไซเมอร์และโรคชรา แต่ทั้งนี้ยังควรมีการศึกษาฤทธิ์ของสารสกัดนี้ให้ครอบคลุมยิ่งขึ้นต่อไป



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| สาขาวิชา | ชีวเคมีคลินิกและอณูทางการแพทย์ | ลายมือชื่อนิสิต ..... |
| ปีการศึกษา | 2562 | ลายมือชื่อ อ.ที่ปรึกษาหลัก ..... |

# # 5776953737 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE
KEYWORD: ALZHEIMER'S DISEASE, NEURITE OUTGROWTH, AMYLOID PRECURSOR PROTEIN, ZINGIBER MONTANUM, CAESALPINIA MIMOSOIDES, NEURO2A, CHAENORHABDITIS ELEGANS, ANTIOXIDANT, OXIDATIVE STRESS, AGING

Panthakarn Rangsinth : EFFECTS AND MECHANISMS OF *CAESALPINIA MIMOSOIDES* LEAF EXTRACT ON OXIDATIVE STRESS RESISTANCE AND ANTI-AGING ACTIVITY IN *CAENORHABDITIS ELEGANS* AND NEURITE OUTGROWTH ACTIVITY IN NEURO2A CELLS. Advisor: Asst. Prof. Tewin Tencomnao, Ph.D.

World's population of older people is rising, dietary supplements promoting healthy lifespan are needed. Moreover, Alzheimer's disease (AD), an age-related degenerative disease, becomes a public health problem in aging society. Recent reports using cell culture models of AD suggest that amyloid precursor protein (APP), a protein causally related to AD, plays an important role as an inhibitor of neurite outgrowth. Medicinal herbs with neurite outgrowth stimulatory effect may help to prevent and cure AD. Moreover, studies suggest that dietary supplements from plant sources act in preventive nutrition, since they provide antioxidant action against oxidative stress, promote healthspan and prolong lifespan. Therefore, this study aimed to investigate the neurite outgrowth promoting activity along with its possible underlying mechanisms of Thai plants using both normal and APP-overexpressing neuronal cell lines Neuro2a and Neuro2a/APPSwe, respectively .as well as antioxidant and anti-aging of the plants using in vivo model, *Caenorhabditis elegans*. The results found that the methanol extract of twigs and leaves of *Caesalpinia mimosoides* (CM) could stimulate neurite outgrowth of both normal and APP-overexpressing cells. After treatment with the extract, the mRNA expression of growth associated protein-43 (GAP-43) and teneurin-4 (Ten-4) genes, the markers of neurite outgrowth activation, were increased in both Neuro2a and Neuro2a/APPSwe cells, while the mRNA expression of neurite outgrowth negative regulators, Nogo receptor (NgR) and its co-receptor Lingo-1 were found reduced. The overall results suggest that CM extract promotes neurite outgrowth against overexpression of APP in neuronal cells via up-regulated expression of GAP-43 and Ten-4 and down-regulated expression of Lingo-1 and NgR. A leaf extract of C.M improved resistance to oxidative stress and reduced intracellular ROS accumulation in nematodes. The antioxidant effects were mediated through the DAF-16/FOXO pathway and SOD-3 expression, whereas the expression of SKN-1 and GST-4 were not altered. The extract also decreased aging pigments, while the body length and brood size of the worms were not affected by the extract, indicating low toxicity and excluding dietary restriction. The results of this study establish neurite outgrowth stimulatory effects in APP-overexpressing neurons and the antioxidant activity of CM extract in vivo and suggest its potential as a dietary supplement and alternative medicine to treatment of AD, oxidative stress and aging, which should be investigated in intervention studies.

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| Field of Study: | Clinical Biochemistry and Molecular Medicine | Student's Signature ..... |
| Academic Year: | 2019 | Advisor's Signature ..... |

# ACKNOWLEDGEMENTS

It has been a long way to achieve my goal. During this journey, various people have given me their hands and walked along with me. First of all, I would like to express my sincere gratitude to my advisor, Asst. Prof. Dr. Tewin Tencomnao, for the continuous support of my Ph.D. study and research, for his motivation, enthusiasm, and immense knowledge. He has given me many great opportunities to try, to learn, or even to travel. His ideas, perspectives, and talents always inspire me. I wish I can be a wonderful person like him someday.

I would like to thank my dissertation committee, Assoc. Prof. Dr. Rachana Santiyanont, Asst. Prof. Dr. Viroj Boonyaratnakornkit, Asst. Prof. Dr. Tewarit Sarachana, and Dr. Warunya Arunotayanun for their valuable time, interesting ideas and insightful comments which would help me approving my work, not only for this work but also for other work in the future.

I would like to express sincere thanks to Prof. Michael Wink, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany, for giving me valuable opportunities to attend his laboratory. It was a wonderful time staying in Germany, learning many new laboratory techniques, obtaining good results, making new friends, speaking English and, experiencing the snow!

I would like to thank my faculty members, department members, lecturers and lab mates for the knowledge, technical skills, presenting skills that fulfilled my Ph.D. study. I would like to give special thanks to my senior, Dr. Anchalee Prasansuklab and my friend, Chatrawee Duangjan who have provided me numerous helps and supports to complete the experiments, manuscripts, and Ph.D. I would like to thank all my co-authors who helped me to revise the manuscripts.

My Ph.D. study would not be impossible without supports from the Graduate School, Chulalongkorn University. I was financially supported for research expenses by “The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship” and “The 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund)” funding code GCUGR1125612058D No. 49 and also “The Overseas Research Experience Scholarship for Graduate Students” for the research visit in Germany.

Last but not least, I would like to thank my parents and my big family for supporting me and sharing me home throughout my life.

Panthakarn Rangsinth

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# ABBREVIATIONS

Aβ: amyloid beta

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

AC: *A. ebracteatus*: *Acanthus ebracteatus*

AD: Alzheimer's disease

APP: Amyloid precursor protein

APPSwe: Swedish mutation APP

*C. elegans*: *Caenorhabditis elegans*

CM: *C. mimosoides*: *Caesalpinia mimosoides*

BDNF: Brain-derived neurotrophic factor

DAF-16/FOXO: DAF16/Forkhead box protein O

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DPPH: Diammonium salt, 2,2-Diphenyl-1-picrylhydrazyl

EGCG: Epigallocatechin gallate

FBS: Fetal bovine serum

GAE: Gallic acid equivalents

GFP: Green fluorescence protein

GST-4: Glutathione S-transferase 4

G418: Geniticin

HPLC: High performance liquid chromatography

HSP-16.2: Heat shock protein-16.2

H2DCF-DA: 2,7-dichlorofluorescein diacetate

Juglone: (5-Hydroxy-1,4-naphthoquinone)

LC-MS: Liquid chromatography-mass spectrometry

Lingo-1: Leucine rich repeat and Immunoglobin-like domain-containing protein 1

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide

NaOCl: Sodium hypochlorite

NaOH: Sodium hydroxide

NEAA: Non-essential amino acids

NGF: Nerve growth factor

NgR: Nogo receptor

N2a: Neuro2a

PBS: Phosphate buffer saline

QE: Quercetin equivalents

ROS: Reactive oxygen species



SA: *Streblus asper*: *Streblus asper*

SKN-1/Nrf-2: SKN-1/Nuclear factor erythroid 2-related factor 2

SOD-3: Superoxide dismutase-3

Ten-4: Teneurin-4

WT: wild type

ZM*: Z. montanum*: *Zingiber montanum*

# CHAPTER I

## Introduction

### 1. Background

Alzheimer's disease (AD) is a progressive neurodegenerative disease which is the most common form of dementia among older people. Dementia is a brain disorder that seriously affects a person's memory and ability to carry out daily activities. It is characterized by loss of neurons and synapses in the brain. Although the underlying cause of this disease is still not fully understood, the amyloid beta (Aβ) has been widely accepted by the researchers as a major critical factor in AD pathogenesis. Nevertheless, the current treatment options remain ineffective to cure this disease as they only slow the symptoms of dementia but fail to rescue cognitive decline and neuronal cell death. Therefore, research efforts on development of new therapies for AD are continuously increased.

The reconstruction of the neuronal and synaptic networks for the recovery of brain functions in AD patients is considered to have a potential for the treatment. One of the neuroregeneration processes is neuritogenesis or neurite outgrowth which is a branching of neurites followed by elongation of axons and dendrites becoming mature neuron [1]. It is an important step for construct the functional networks of neurons and is thus, known to be a typical marker of neuronal differentiation [2]. Recently, overexpression and mutation of amyloid precursor protein (APP), the precursor of Aβ, is found to influence neurite outgrowth activity [3,4]. However, the molecular mechanisms how APP influence that activity are still unclear.

Normally, neurite outgrowth is induced by several neurotrophic factors including nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) [5,6]. Unfortunately, NGF level is found to decrease during aging [7-9]. Moreover, NGF is the large polypeptides that cannot cross through blood-brain barrier [10,11]. The treatment

using NGF only injects directly to the brain [12]. To find the small molecules that can promote neurite outgrowth is one of the alternative way for treatment of AD. Importantly, those molecules should affect to promote neurite outgrowth even in APP-overexpression conditions.

Dietary consumption of vegetables and fruits has been believed to implicate for human health. The attention of the researchers recently has been focused on the effects of bioactive compounds from natural products for the prevention and treatment of various diseases. In recent years, medicinal plant-derived natural compounds have received extensive attention as major sources of new therapeutic agents for treating neurodegenerative diseases or neurological disorders and they exert their neurotrophic effects by promoting neurite outgrowth [13,14]. Moreover, studies suggest that metabolites present in dietary supplements act in preventive nutrition, since they provide antioxidant action against oxidative stress generated by reactive oxygen species (ROS) which is one of the major causes of aging as well as other chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, and other degenerative diseases in humans [15-19].



*Caesalpinia mimosoides* Lamk., a small spiny and woody climbing tropical trees belonging in Fabaceae family and Caesalpinioideae subfamily, is native to Southeast Asia in addition to northern and north-eastern parts of Thailand. Young swigs and leaves are edible and are traditionally used as an anti-flatulent and a remedy against fainting and dizziness [20]. The plant has been reported to exhibit anti-inflammatory [21], anti-cancer [22], and anti-aging activities [23]. Moreover, *C. mimosoides* contains several bioactive compounds including gallic acid [22] and quercetin [24] which have been previously reported to have neurite outgrowth activity [25-27]. Recently, the isolation of

quercetin from *C. mimosoides* was shown to have neurite outgrowth and neuroprotective effects on cultured P19-derived neurons [24]. However, neurite outgrowth stimulatory effects of the plant in APP overexpression of neurons have still not been investigated. Furthermore, *C. mimosoides* has been reported to contain multiple phenolic compounds which likely give rise to its antioxidant activity [20,28]. However, in vivo study of its oxidative stress defense and anti-aging has not been reported yet.

*Caenorhabditis elegans*, a free-living soil nematode, is a well-known model organism and considered as the simplest major multicellular model organism for studying genetic and pharmacologic influences on aging and longevity. Because it has short lifespan, susceptibility to oxidative stress and the similarities with the human aging process [29] .Moreover, the genome of *C. elegans* is completely sequenced, displaying homologous to genes implicated in human diseases and the major signaling pathways that regulate longevity and stress resistance are well conserved [30-32]. Recent reports suggested that plant extracts with high phenolic and flavonoid contents exhibit antioxidant and anti-aging behavior in *C. elegans* [33-36].



In this study, the effects of APP overexpression on neurite outgrowth activity as well as the reversing effects of *C. mimosoided* (CM) leaf extract against APP-overexpressing condition in neuronal cells were investigated. Swedish mutant APP-overexpressing Neuro2a (Neuro2a/APPSwe) cells were employed to study comparing with wild-type Neuro2a cells. In addition, this study aimed to test the in vivo antioxidant and lifespan extension activity as well as the signaling pathways of the extract of *C. mimosoides* leaves and young twigs, using *C. elegans* as a model organism. The finding will have a benefit and can be developed an alternative medicine for prevention and treatment for the patients with Alzheimer's disease and used as dietary supplementation for antioxidant and anti-aging in the future.

**2. Research Questions**

2.1 What are the mechanisms how amyloid precursor protein overexpression influence neurite outgrowth activity in cultured neuronal cells?

2.2 Whether and how *C. mimosoides* leaf extracts have neurite outgrowth stimulatory effects in cultured neuronal cells even in amyloid precursor protein overexpression condition?

2.3 Whether *C. mimosoides* leaf extracts have in vivo antioxidant activity using *C. elegans* model?

2.4 Whether *C. mimosoides* leaf extracts have anti-aging effect in *C. elegans*?

2.5 What are the molecular mechanisms of antioxidant and anti-aging effects of *C. mimosoides* leaf extracts in *C. elegans*?

2.6 What are the major constituent compounds in *C. mimosoides* leaf extracts?



**3. Research Hypothesis**

3.1 The overexpression of amyloid precursor protein may influence neurite outgrowth regulatory mechanisms both positive regulatory genes including GAP-43 or Ten-4 and/or negative regulatory genes including Lingo-1 or NgR.

3.2 *C. mimosoides* leaf extracts could stimulate neurite outgrowth in cultured neuronal cells even in the amyloid precursor protein-overexpressing neurons.

3.3 *C. mimosoides* leaf extracts could reverse effects of amyloid precursor protein overexpression gene expression to enhance neurite outgrowth in amyloid precursor protein-overexpressing neuronal cells.

3.4 *C. mimosoides* leaf extract could promote oxidative stress resistance in the in vivo model *C. elegans* via stress response pathways.

3.5 Antioxidant activity of *C. mimosoides* leaf extract could subsequently exhibit anti-aging effect in *C. elegans*.

3.6 The major constituent compounds found in *C. mimosoides* leaf extract would be flavonoid and phenolic compounds, and include neurite outgrowth potentiality compounds, gallic acid or quercetin.

**4. Research Objectives**

4.1 To investigate how amyloid precursor protein overexpression influence neurite outgrowth activity in cultured neuronal cells.

4.2 To investigate the neurite outgrowth stimulatory effects of *C. mimosoides* leaf extracts in both wild type and APP-overexpressing cultured neuronal cells.



4.3 To investigate the underlying mechanisms of and *C. mimosoides* leaf extract on neurite outgrowth stimulation in both wild type and APP-overexpressing cultured neuronal cells.

4.4 To investigate in vivo antioxidant properties of *C. mimosoides* leaf extract using *C. elegans* model.

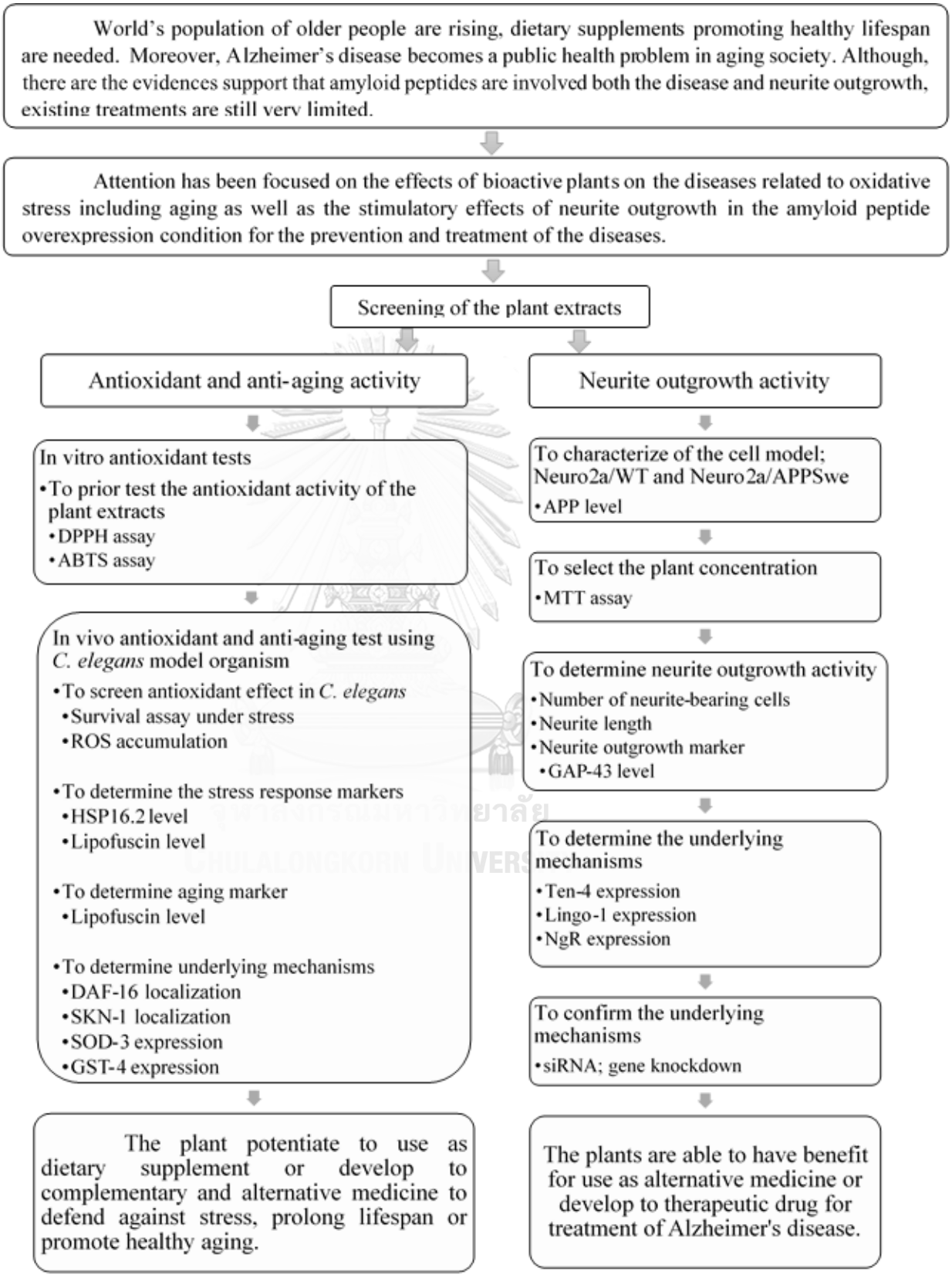
4.5 To investigate in vivo anti-aging activity of *C. mimosoides* leaf extract using *C. elegans* model.

4.6 To investigate the potential pathways mediated by *C. mimosoides* leaf extract treatment to counteract with stress and aging.

**5. Research Significance**

AD becomes one of the public health problems in aging society worldwide. Although, there are the evidences to support that amyloid peptides are involved both the disease's pathogenesis and inhibition of neurite outgrowth activity, existing treatments are still very limited. To find the stimulatory effects of Thai plant extracts on neurite outgrowth even in amyloid peptide overexpression will have benefit for use as alternative medicine or develop to therapeutic drug for treatment AD. Furthermore, it is well established that the consumption of vegetables and fruits is important for human health. Attention has been focused on the effects of bioactive of plant foods on the prevention of diseases related to oxidative stress including aging. To establish the evidences of in vivo antioxidant and anti-aging effects could potentiate to use the plants as dietary supplements or develop to complementary and alternative medicines to defend against stress and promote healthy aging.

## 6. Conceptual Framework



## 7. Experimental Design

# CHAPTER II

## Literature Review

### 1. Overview of aging

People worldwide are living longer, and it is estimated that the proportion of the world's population of older people (60 years and over of age) will nearly double by 2050 [37]. When a person becomes old, the natural changes of aging process are continuous. Aging is defined as a progressive decline in physiological functions of the cells and tissues and can cause many diseases and lead toeventually death [38]. The mechanisms leading to cellular and organismal aging still remain undefined. Many theories have been advanced to account for describe the aging process with two probable theoretical hypotheses, the genetic make-up or the damage-based theories [39]. The genetic make-up can be referred to programmed theory of aging or genetic theory of aging states that longevity is largely determined by an individual's genetics [40]. On the other hand, the damage-based theory states that a slow accumulation of cellular damage occurs over a lifetime, then causing biological system change to progressive loss of functions and impairment of the organism [2, 3].



### 2. The causes of aging

#### Oxidative stress

Oxidative stress (OS) has been implicated in the pathophysiology of aging and age-related degenerative diseases, particularly neurodegenerative diseases. OS can cause cellular damage and subsequent cell death via the reactive oxygen species (ROS) oxidize vital cellular components such as lipids, proteins, and DNA [41]. ROS are defined as atoms or groups of atoms having one free or unpaired electron. This property leads to the capacity to capture an electron from stable molecules in order to accomplish its electrochemical stability. The affected molecules, with an impaired

electron, become therefore free radicals that eventually can initiate a radical chain reaction which is normally counteracted by cellular antioxidant systems.

ROS, such as hydroxyl (OH•), superoxide ($O_2$•–) and nitric monoxide (NO•), are free radicals that significant concern for aging and oxidative stress-related conditions [17]. The production of ROS is a consequence of both, exogenous and endogenous factors. Exogenous factors include pollution, ultraviolet radiation and/or unhealthy life habits such as smoking and a diet low in antioxidants [42]. Endogenous factors are related to cellular metabolism, where most of the ROS production takes place at the mitochondrial level. The antioxidant defense system co-evolved along with aerobic metabolism to counteract oxidative damage from ROS [43]. Antioxidants include enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase have synergistic actions for removing free radicals [43]. In addition, antioxidants can be found in dietary supplements, such as, zinc, vitamin E, vitamin C vitamin A, polyphenols and flavonoids that also help to regulate the ROS generation [15,43].

When the generation of ROS is not balanced by the biologically derived antioxidants, oxidative stress can occur anddamage several biomolecules, including lipids, proteins and DNA, and leading to implicated in the development of aging as well as other chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, and other degenerative diseases in humans [17-19].

ROS are particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism is factory of ROS, which are unique to the brain and serve as sources of oxidative stress. ROS attack glial cells and neurons, which are post-mitotic cells and therefore, they are particularly sensitive to

free radicals, leading to neuronal damage [41]. It has been reported that deleterious effects of ROS on human cells may end in oxidative injury leading to programmed cell death i.e. apoptosis [44].

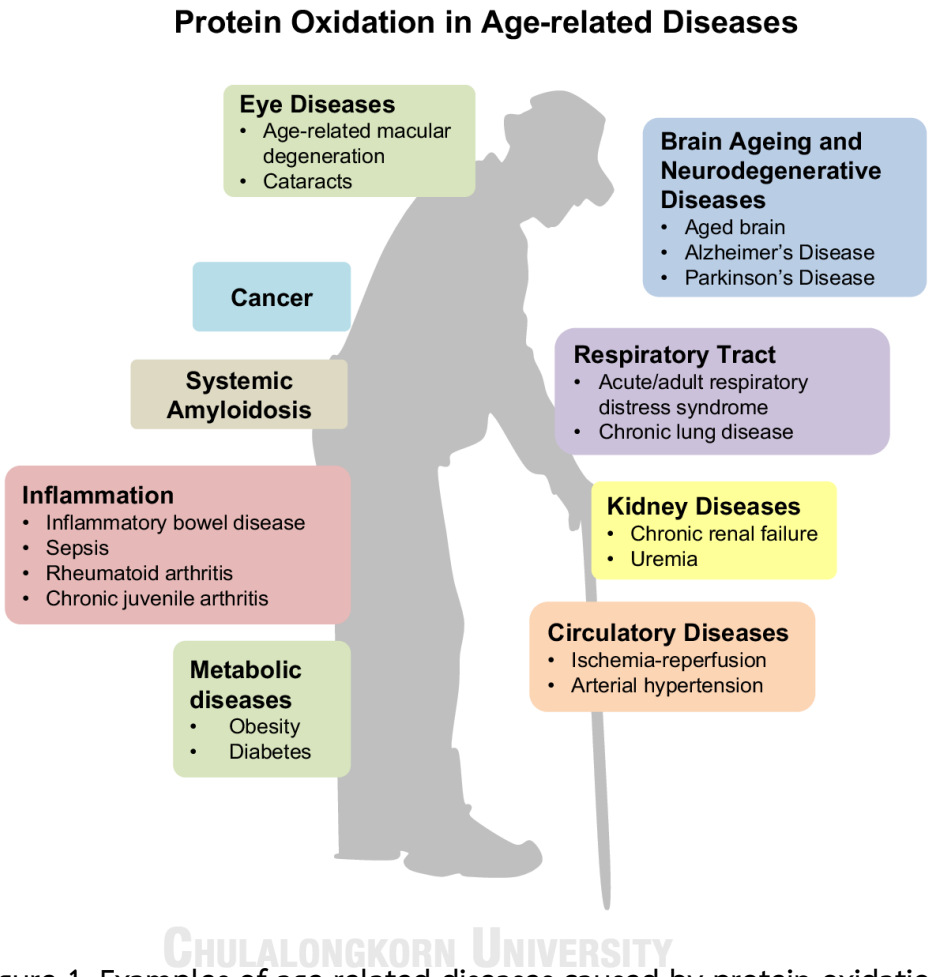





**Figure 1. Examples of age-related diseases caused by protein oxidation.**

The protein oxidation caused by ROS may be the cause or consequence of a number of diseases which affect nearly all organ systems in elderly (photo from [45]).

OS can be spread through the generation of reactive aldehydes leading to structural modifications of cellular biomolecules that in most of the cases affect their functionality. At the lipid level, polyunsaturated fatty acids (PUFA) are susceptible to be oxidized because of the multiple double bonds present in their structure, among which, the methylene groups with hydrogens particularly reactive [46]. OS also alters the structure and function of proteins by the non-enzymatic process called

carbonylation that results from the oxidation of amino acids such as Lys, Arg or Cys [47]. This reaction generates both, direct protein oxidation products and reactive aldehydes that in turn get involved in the oxidation of other biomolecules such as carbohydrates and lipids, generating the so called advanced glycation/lipoxidation end products (AGE/ALE's) [48]. Glyoxal (GO), methylglyoxal (MG) and malondialdehyde (MDA) are highly reactive carbonyl formed during the metabolism that, in excess, can increase ROS production and cause OS. GO, MGO and MDA can react with free amino groups of Lys, Arg and Cys residues leading to the formation of the AGE/ALE adducts intermolecular crosslink.

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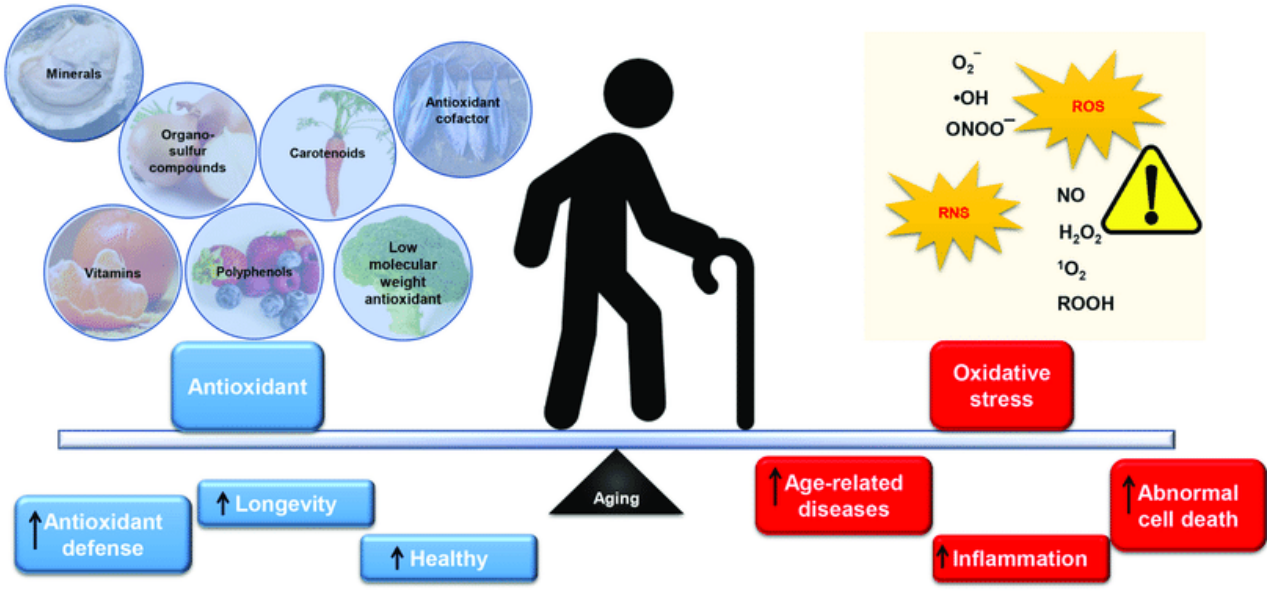



**Figure 2. The balance of oxidative stress and antioxidants in aging.**

The overproduction of ROS/RNS together with low level of antioxidants in the body may cause oxidative damage to the cellular components (protein, lipids, and DNA). This phenomenon is suffered by elderly and thereby promoted abnormal cell death, inflammation and subsequently contributes to age-related diseases. On the other hand, antioxidants intake from dietary nutrients have shown to maintain the antioxidant defense and subsequently leads to healthy longevity (photo from [50]).



## 3. Lifespan and antioxidant defense systems

### 3.1 Forkhead Box O (FoxO) transcription factor

The FoxO transcription factor is a key player in an evolutionarily conserved pathway that regulate metabolism, cellular proliferation, stress tolerance and longevity [51]. It is the important regulator of the cellular stress response and promote the cellular antioxidant defense via stimulation of the transcription of genes coding for antioxidant proteins located in different subcellular compartments, such as in mitochondria (i.e.

superoxide dismutase-2, peroxiredoxins 3 and 5) and peroxisomes (catalase), as well as for antioxidant proteins found extracellularly in plasma (e.g., selenoprotein P and ceruloplasmin) [52]. In the other hand, FoxO activity can be modulated via ROS and other stressors. The transcriptional and posttranscriptional control of the expression of genes coding for FoxO is sensitive to ROS [52]. Moreover, the inhibition of FoxO proteins activity was found and considered as a major cause of aging [53,54]

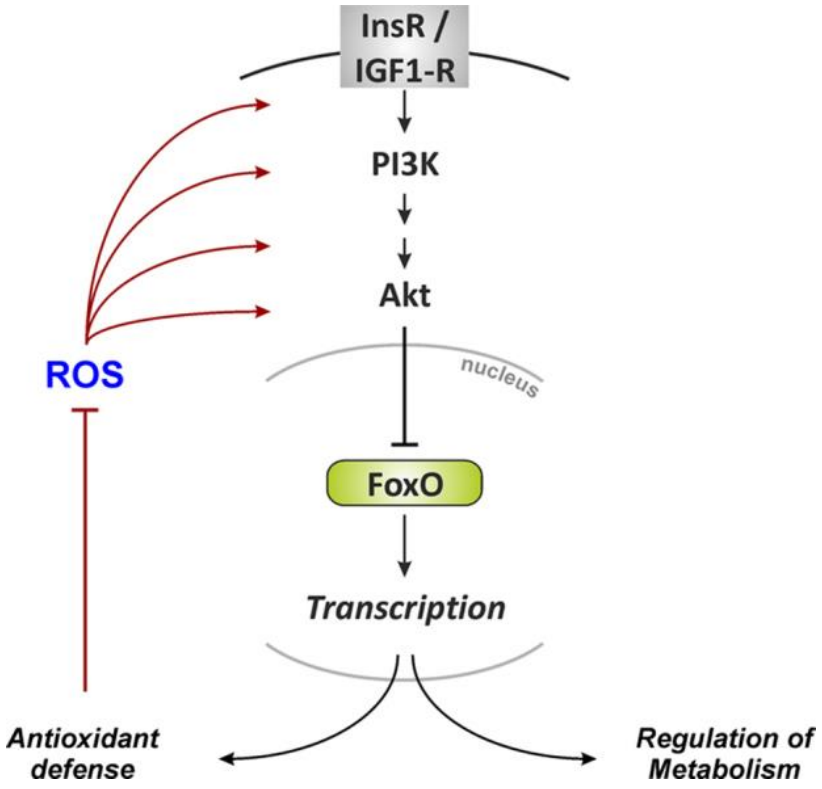



**Figure 3. Forkhead Box O (FoxO) transcription factor pathway.**

FoxO activity can be modulated via ROS to further activation of the antioxidant defense (photo from [52]).

The DAF-16 transcription factor of *C. elegans* is considered as a homologue to the FoxO transcription factor found in humans. Normally, DAF-16 is localized in the cytosol in its inactive phosphorylated form. Oxidative damage can induce its

dephosphorylation and subsequent translocalization to nucleus. Upon nuclear localization, DAF-16 activation is responsible for stress response and lifespan extension [55]. DAF-16 activation subsequently induces several genes that results in activating other stress response genes like SOD-3, which encodes mitochondrial Mn-SOD. The SOD-3 enzyme protects the worms from ROS via scavenging free radical [56].

### 3.2 The Nrf2 signaling pathway

Nrf2 signaling pathway, or the nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2, also known as NFE2L2) signaling pathway, is one of the major mechanisms in the cellular response to oxidative stress by regulation of several expression of genes involved in the detoxification and cellular antioxidant. Nrf2 is a transcription factor where the pathway is mediated by the activation of Nrf2 followed by the activation of the expression of many antioxidant and detoxifying enzymes [57] including heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), thioredoxin (Trx), glutathione S-transferase (GST) and the rate-limiting enzymes of glutathione synthesis consisting of catalytic (GCLC) and modifier (GCLM) subunits [58,59], as well as Nrf2 itself [60]. Currently, up-regulation of the Nrf2 signaling pathway such as overexpression of Nrf2 has been suggested as a potential therapeutic target for the treatment of various neurodegenerative disorders including AD [58,61].



SKN-1 transcription factor in *C. elegans* is a homologue to the human Nrf2 which targets downstream genes for Phase II detoxification mechanism [62]. GST-4 is one of the glutathione S-transferases contributing to Phase II detoxification process and activated by SKN-1 [63]. This group of enzymes is involved in the response towards oxidative stress. Moreover, SKN-1 plays a central role in the pharmacologic interventions to promote longevity in *C. elegans* [64].

**4. Age-related degenerative diseases**

Aging is a natural biological and physiological process that is known for one of the highest risk of several human disorders and diseases. One of age-related symptoms is cognitive decline which is a major segment of the population and is the primary risk factor for Alzheimer's disease and other prevalent neurodegenerative disorders [65]. Several studies suggested that working memory and short term recall, as well as the speed of processing information declines significantly in the normal aging human population [66,67]. Age-related memory loss may relate to alteration in functional activation of the prefrontal cortex and hippocampus. When presented with a task that involves executive function, regions of the prefrontal cortex activated in young adults typically exhibit reduced activation in aged adults [68,69]. Activation of the hippocampus is also reduced when healthy aged adults perform memory related tasks. And the most affected hippocampal region is dentate gyrus [70] which is an area that contributes to the formation of new episodic memories and the spontaneous exploration of novel environments [71,72], as well as is and notable as being one of a select few brain structures currently known to have high rates of neurogenesis [73].

Loss of synaptic function is also likely a contributing factor in age-related cognitive decline. Age-related alterations in dendritic spine and synapse vary among several regions of the aging hippocampus and cortex [74,75]. An example of age-related synapse loss is in the hippocampal dentate gyrus in aged rats [76]. In addition, the loss of synapse in the dentate gyrus may also account for the spatial memory deficit in aged rats [77].

**Alzheimer's disease**

Alzheimer's disease (AD) is a progressive neurodegenerative disease which is the most common form of dementia among older people. Dementia is a brain disorder that seriously affects a person's memory and ability to carry out daily activities. It is

characterized by loss of neurons and synapses in the brain. It was first described in 1906 by a German psychiatrist and pathologist Alois Alzheimer [78]. The disease most common begins in people over 65 years of age. In 2015, World Health Organization reported that there were approximately 48 million individuals worldwide with AD.

The disease has a several progressive pattern of cognitive and functional impairment. The first symptoms can be called pre-dementia. These early symptoms can affect the most complex activities of daily living [79]. The most observable deficit is minor short term memory loss, that the individual will be difficulty in remembering recently learned facts and inability to acquire new information [80,81]. When there is an increasing impairment of learning and memory, it will develop to AD. It leads to be a difficulties with language, executive functions, perception (agnosia), or execution of movements (apraxia) [82]. The progressive of AD may lead to speech difficulties due to an inability to recall vocabulary, which leads to frequent incorrect word substitutions (paraphasias). Reading and writing skills are also progressively lost [83]. During this period, the person may fail to recognize close friends relatives due to memory problems are worsen. Furthermore, long-term memory becomes impaired [82]. And in advance symptoms of the disease, the patients are completely cannot live by themselves. They will complete loss of speech. However, the patients can often understand and return emotional signals. The most common symptoms are extreme apathy and exhaustion. Moreover, aggressiveness can also be found. The patients will eventually not be able to perform even the simplest tasks independently; muscle mass and mobility deteriorate to the point where they are unable to feed themselves. The cause of death is not the disease itself. It is usually an external factor, such as infection of pressure ulcers or pneumonia [82,83].

The cause of this disease is still not clearly understood. It is believed that the disease process is associated with amyloid-β (Aβ) peptide deposited in diffuse and

neuritic plaques, and hyperphosphorylated tau protein, a microtubule assembly protein accumulating intracellularly as neurofibrillary tangles (NFTs) in the brain [84]. Both plaques and tangles are found in brains of individuals afflicted by AD [85]. Nevertheless, the exact mechanisms leading to these alterations remain to be determined and there are several competing hypothesis trying to explain the cause of the disease.

- **Amyloid hypothesis**

Amyloid beta (Aβ) is a protein that is a main component of amyloid plaques found in AD patient' brain. The peptides result from the amyloid precursor protein (APP) which locates on chromosome 21. APP is a transmembrane protein that penetrates through the neuron's membrane. There are three protease enzymes that process APP, including α-, β-, γ-secreataes. APP is first cleaved by α- or β- secreatase at N-terminus within luminal membrane. Then, it is subsequently cleaved by γ-secreatase at c-terminus within the transmembrane protein. The fragment cleaved by α- and γ- secreatase is called p3 (3kDa) peptides which is non-amyloidgenic process. Whereas, amyloidgenic process is the fragment from β- and γ- secreatase cleavage that generate Aβ (4kDa) peptides [86]. Aβ peptides have multiple length from 38 to 43 residues depended on γ- secreatase cleavage site [87]. Nearly 90% of Aβ ends in residue 40, whereas less than 10% of Aβ is Aβ1-42. Furthermore, Aβ1-37 and 1-38 have also been detected in minor amounts [87]. The formation of Aβ is a normal occurrence in the human body that has several clearance pathways. When Aβ generation and clearance are imbalance, leading to the accumulation of Aβ in the brain. Aβ can aggregate to oligomer form and develop to amyloid homeostasis alteration and apoptosis.

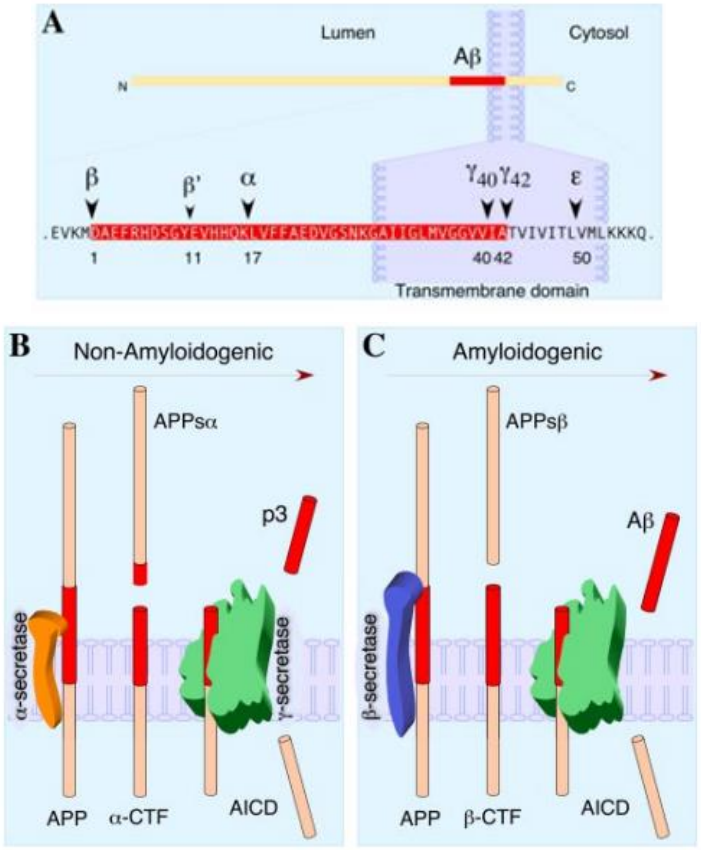



**Figure 4. Proteolytic processing of APP.**

A) the major cleavage sites by secretase, B) non-amyloidogenic processing of APP and C) amyloidogenic processing of APP (photo from [86])



Moreover, the mutation in APP gene also influence Aβ level in the brain. The well know mutation is Swedish mutation which is first found in Swedish family. It is double mutation in the β-secreatase cite of APP, resulting in a substitution of two amino acid, lysine (K) and methionine (M) to asparagine (N) and leucine (L), respectively [88]. This mutation has been reported to increase total Aβ levels both production and secretion of Aβ1-40 and Aβ1-42 [89].

- **Tau hypothesis**

Tau hypothesis relate with the abnormal of Tau proteins which are proteins stabilizing microtubules. These proteins are mostly found in neurons of CNS. The normal functions are interaction with tubulin to stabilize microtubules and promotion of tubulin assembly into microtubules [90]. In AD pathogenesis, Tau protein hyperphosphorylation is found, resulting in self-assembly of neurofibrillary tangles of paired helical and straight filaments [91]. When this occurs, the microtubules disintegrate, destroying the structure of the cell's cytoskeleton which collapses the neuron's transport system [92]. It leads to the malfunctions of biochemical communication between neurons and the death of the neurons [93]

Furthermore, there is an uncommon form of AD that usually occurs earlier in life, defined as before the age of 65, that is called Familial Alzheimer's disease (FAD) or early onset familial Alzheimer's disease (EOFAD). The cause of this type of the disease is genetic mutation including APP Swedish mutation [94].

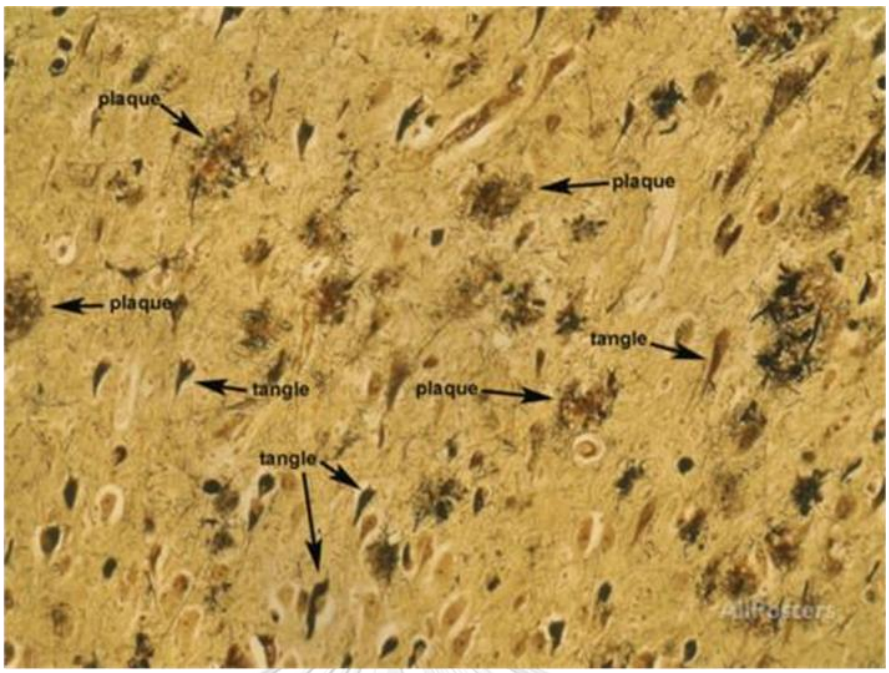



**Figure 5. Aβ plaques and neurofibrillary tangles found in Alzheimer's disease brain tissue section.**

[Photo by Thomas Deerinck].

**5. Neurite outgrowth**

Neurite outgrowth or neuritogenesis is the morphological characteristics of the branching of neurites followed by elongation of axons and dendritic arborization [1]. It is one of the important steps for neuronal development to be construction of the neuronal and synaptic networks of brain functions.

In cultured neuronal developments, the morphological changes that occur during polarization are divided into five stages. First, newly plated neurons form several thin filopodia all around the cell body (stage 1). After several hours, the neurons form a multiple of immature neurites, so-called minor neurite or monor processes (stage 2). These neurites are morphologically equal, and undergo repeated, random growth and retraction. Half a day after plating, one of these minor neuritis which is specified as an exon begins to extend rapidly, becoming much longer than the other neurites (stage

3). The other minor neurites continue to undergo brief spurts of growth and retraction, maintaining their net length, then develop into mature dendrites (stage 4). During this process, dendrites become thicker and shorter than the axon and begin to establish dendritic components and to construct premature dendritic spines (stage 5). When the axon and dendrites are mature, neurons form synaptic contacts that enable the transmission of electrical activity [95,96].

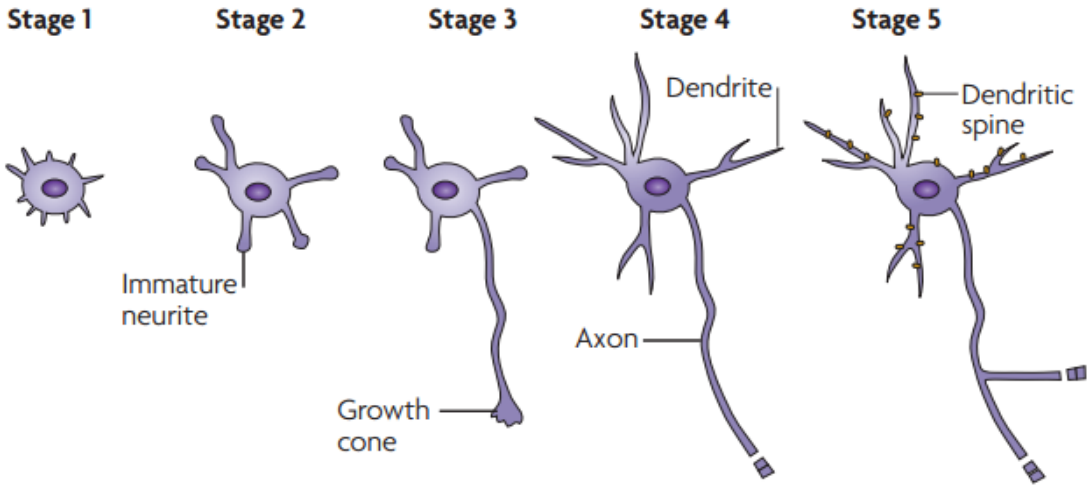



**Figure 6. Neurite outgrowth process during neural development.**

**(**photo from [96]**)**



Normally, neurite outgrowth is induced by several neurotrophic factors including nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) [5]. These factors will activate several signaling pathway to stimulate neurite outgrowth. Despite of these factors, there are another proteins that regulate neurite outgrowth activity including Teneurin-4 and Lingo-1.

**5.1 Nerve growth factor**

Nerve growth factor (NGF) is a glycoprotein that consists of three sub-units: α-NGF, β-NGF and γ-NGF. This form of NGF is also referred to as pro-NGF (NGF precursor). The gamma subunit of this complex acts as a serine protease, and cleaves

the N-terminal of the beta subunit, thereby activating the protein into functional NGF. The term NGF usually refers to β-NGF which is biologically active. NGF is synthesized as a precursor from pro-NGF and is either secreted outside the cells or cleaved intracellularly into mature NGF by furin [97]. The secretion of NGF can be a mixture of pro-NGF and mature NGF. There are three types of NGF receptors, these being; TrkA, p75 and sortilin. The trophic effect of NGF is mediated through of TrkA and p75 receptors [98], while the neurotoxic effect of pro-NGF is mediated through p75 in conjunction with sortilin [99]. Pro-NGF is the predominant form of NGF in central nervous system [100]. NGF plays an important role in proliferation, differentiation, survival, and synapse of cholinergic neurons in central nervous system[6]. It has many intracellular signal pathways transduce cellular responses to induce neurite outgrowth.

- **MAP Kinase/ERK pathway**

MAP Kinase/ERK pathway is the pathway that includes many proteins, including MAPK (mitogen-activated protein kinases, originally called ERK, extracellular signal-regulated kinases). NGF signaling pathway is activation of the mitogen-activated protein kinase pathway, which induces continuous ERK activity. In addition, ERK activation induces turn phosphorylates CREB to stimulate cyclic AMP response element-dependent transcription leading to outgrowth the neurites [101].

- **PI3/Akt Kinase pathway**

The PI3K/Akt pathway is an intracellular signaling pathway important in regulating the cell cycle. Therefore, it is directly related to cellular quiescence, proliferation, cancer, and longevity. PI3K activation phosphorylates and activates AKT, localizing it in the plasma membrane. AKT can activate CREB to induce neurite outgrowth [102].

- **Protein Kinase C pathway**

Protein kinase C or PKC is a family of protein kinase enzymes. It was found that overexpression of PCK resulted in enhanced NGF-induced neurite outgrowth, while inhibition of atypical PKC isoforms blocked NGF-induced activation [103]. The isoforms of PKC have been found that can activate by PI3 kinase-mediated generation of 3-phosphoinositides to control differentiative or survival signaling [104,105].

- **p38 MAPK pathway**

The p38 mitogen-activated protein kinase is also activated by NGF to induce neurite outgrowth. NGF activated the p38 MAPK and its downstream effector, MAPK-activated protein kinase 2 (MAPKAP kinase 2), resulting to the phosphorylation of the transcription factor CREB and the activation of immediate-early genes for neuronal differentiation [106].



**5.2 Teneurin-4**

Teneurin-4 (Ten-4) is a transmembrane protein in the family of teneurin. It is highly expressed in the CNS. Recently. It was found that Ten-4 positively regulated the formation of filopodia-like protrusion and neurite outgrowth in Neuro2a cells by activation of focal adhesion kinase (FAK) and Rho-family small GTPases, Cdc42 and Rac1, key molecules for the membranous protrusion formation downstream of FAK, respectively [107].

**5.3 Lingo-1**

Leucine rich repeat and Immunoglobin-like domain-containing protein 1 also known as Lingo-1 is the protein that negatively regulate neurite outgrowth. , a transmembrane protein which is highly expressed in the brain and is implicated in several neurodegenerative diseases [108,109]. Its action remarkably relates to the Nogo receptor (NgR) as a part of a co-receptor complex leading to activates rho-associated coiled coil-containing protein kinase (RhoA/ROCK) signaling pathways, subsequently suppression of growth cones and further axonal growth [110,111]. Lingo-1 can also directly inhibit the epidermal growth factor receptor (EGFR) using EGFR-like tyrosine phosphorylation, resulting in its internalization and degradation, and leading to a reduction in the activation of the Akt signaling pathway [108]. The inhibition of the Akt signaling pathway consequently results in a reduction in neuronal survival and neurite outgrowth, which contributes greatly to the neurodegenerative processes implicated in AD.

Moreover, Lingo-1 has been shown that Lingo-1 capable to directly bind to APP, promoting its protolytic processed via inducing β-secreatase cleavage in amyloidogenic pathway [112]. It was reported to activate β-secretase cleavage of APP, leading to the generation of Aβ in normal aging [113]. Recent study reported that HEK293 cell line overexpressing APP with the Swedish double mutation (HEK293/APPswe) shown a physical interaction between Lingo-1 and APP, facilitating its access to β-secretase and/or inhibiting its cleavage by α-secretase, resulting in an increase in the production of Aβ fragments [114].

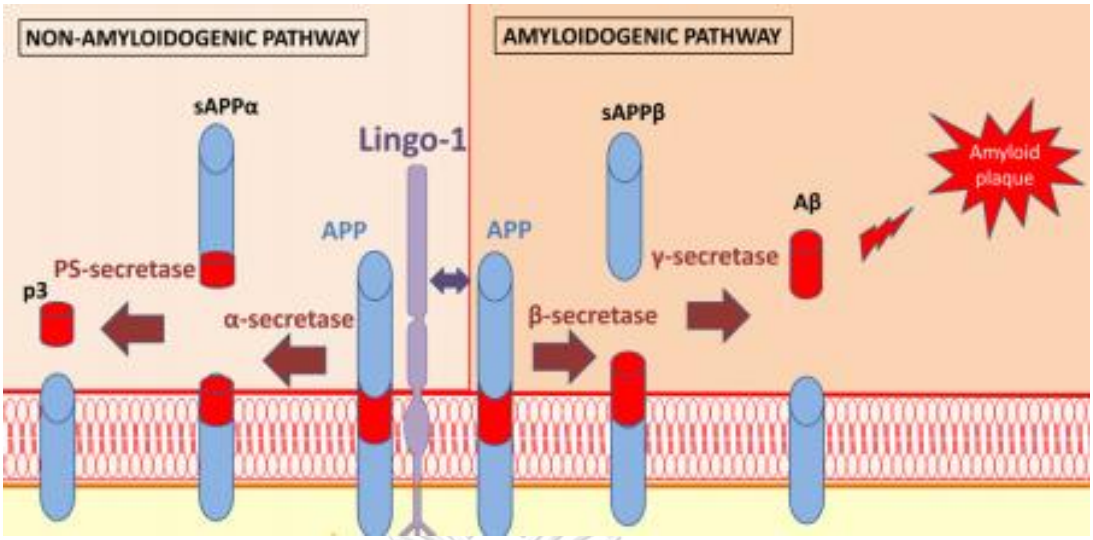



**Figure 7. Activation of β-secretase cleavage of APP by Lingo-1.**

(photo from [115])

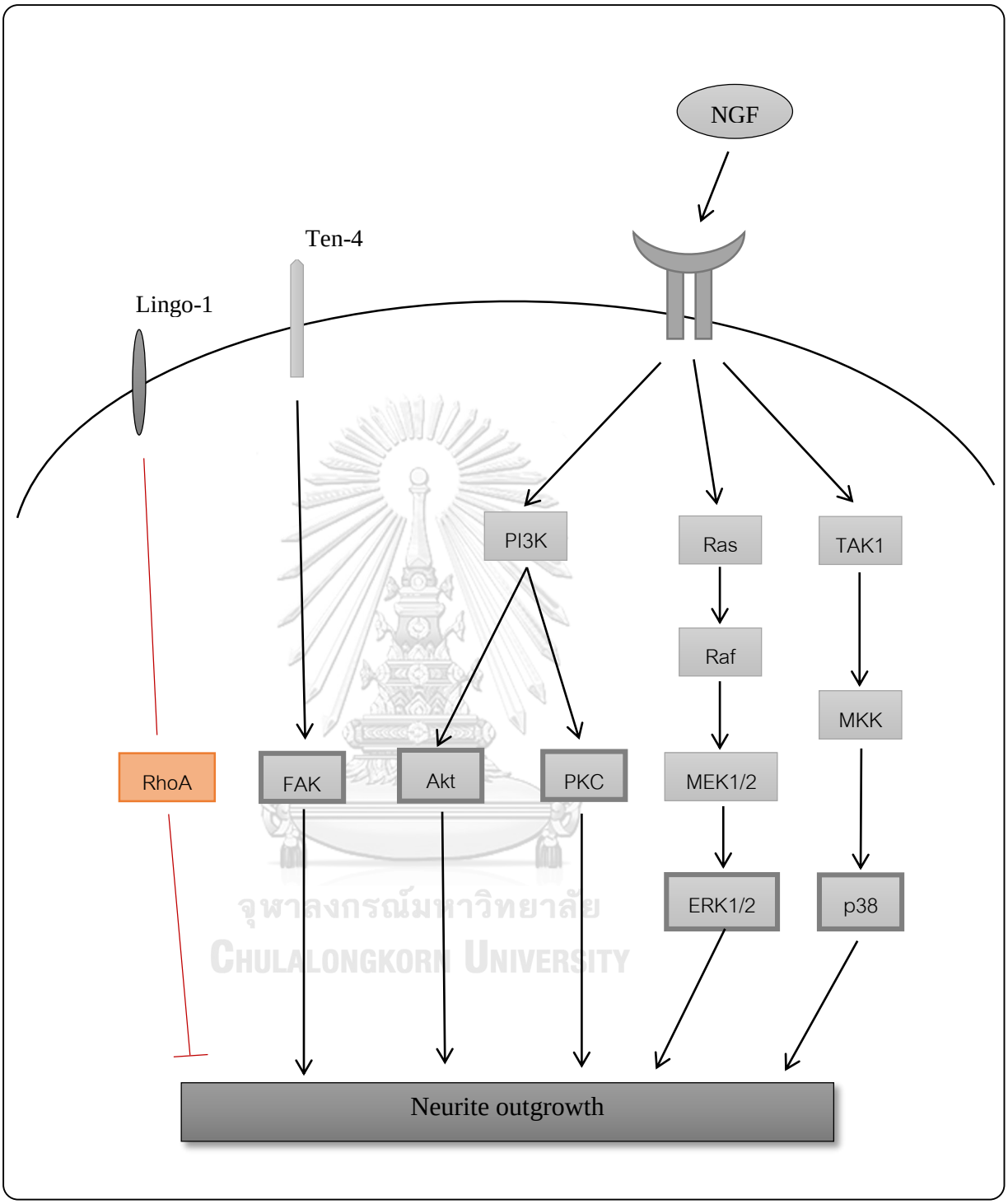





Figure 8. Neurite outgrowth regulated signaling pathways.

## 6. Experimental models

### 6.1 Neuro2a cells

Neuro2a cell also known as N2a cell is a neuroblastoma cell line which is derived from the mouse C1300 tumor and differentiates into a neuron-like cell that exhibits both cholinergic and adrenergic markers [116]. Upon the withdrawal of serum, Neuro2a cells can differentiate and elaborate neurites. It can be induced to differentiate and extend neurites after treatment with the inducing agent [117]. Recent studies found that Ten-4 is a positive regulator of cellular protrusion formation and neurite outgrowth through the FAK signaling pathway in Neuro2a cells [107]. Furthermore, neurite outgrowth is inhibited in both wild type APP-overexpressing Neuro2a and Swedish mutant APP-overexpressing Neuro2a cells [3].

### 6.2 *Caenorhabditis elegans*

*Caenorhabditis elegans*, a free-living soil nematode, is a well-known model organism and considered as the simplest major multicellular model organism for studying genetic and pharmacologic influences on aging and longevity. The worm is about 1 mm in length in the adult stage. It is a transparent self-fertilizing hermaphrodite, producing both sperm and eggs.

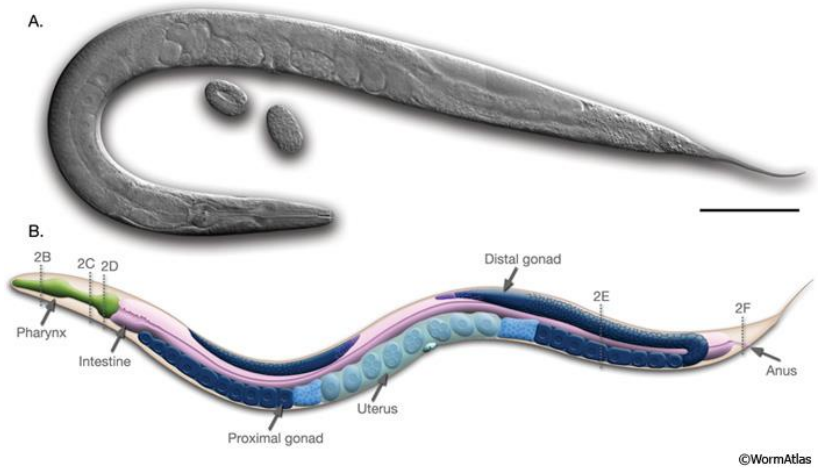



**Figure 9. Adult *C. elegans* and eggs.**

**(**A). DIC image of an adult hermaphrodite. Scale bar 0.1 mm. (B). Schematic drawing of anatomical structures [Photo from WormAtlas (https://www.wormatlas.org/)]

*C. elegans* worms have a relatively short life cycle of about 3 days and an average lifespan of 18 to 20 days when cultivated on E. coli OP50 at 20 °C which the reproductive period of a hermaphrodite generally is the first 4–5 days of adulthood [118]. The life cycle comprised of the embryonic stage, four larval stages (designated as L1-L4) and adult. The *C. elegans* first stage is L1 larvae that begin after hatching from the eggs and start feeding. Under the favorable growth conditions, e.g. abundant food, low temperature, and low population density, the larvae proceed through three more larval stages (L2, L3, and L4) before becoming reproductive adults. However, if the L1 larvae encounter the unfavorable environments for further growth, they will arrest at L1 stage when the food is limited or enter and arrest into a physiologically specialized larval stage called dauer, an alternative form of L3 larval stage when temperature is high or the population density is crowded. The dauer larvae are highly resistant to environmental stress and can survive up to 4 months without feeding. Once better growth conditions are available, they exit the L1 or dauer larval stage and resume normal reproductive development into L4 larvae and adult stage.

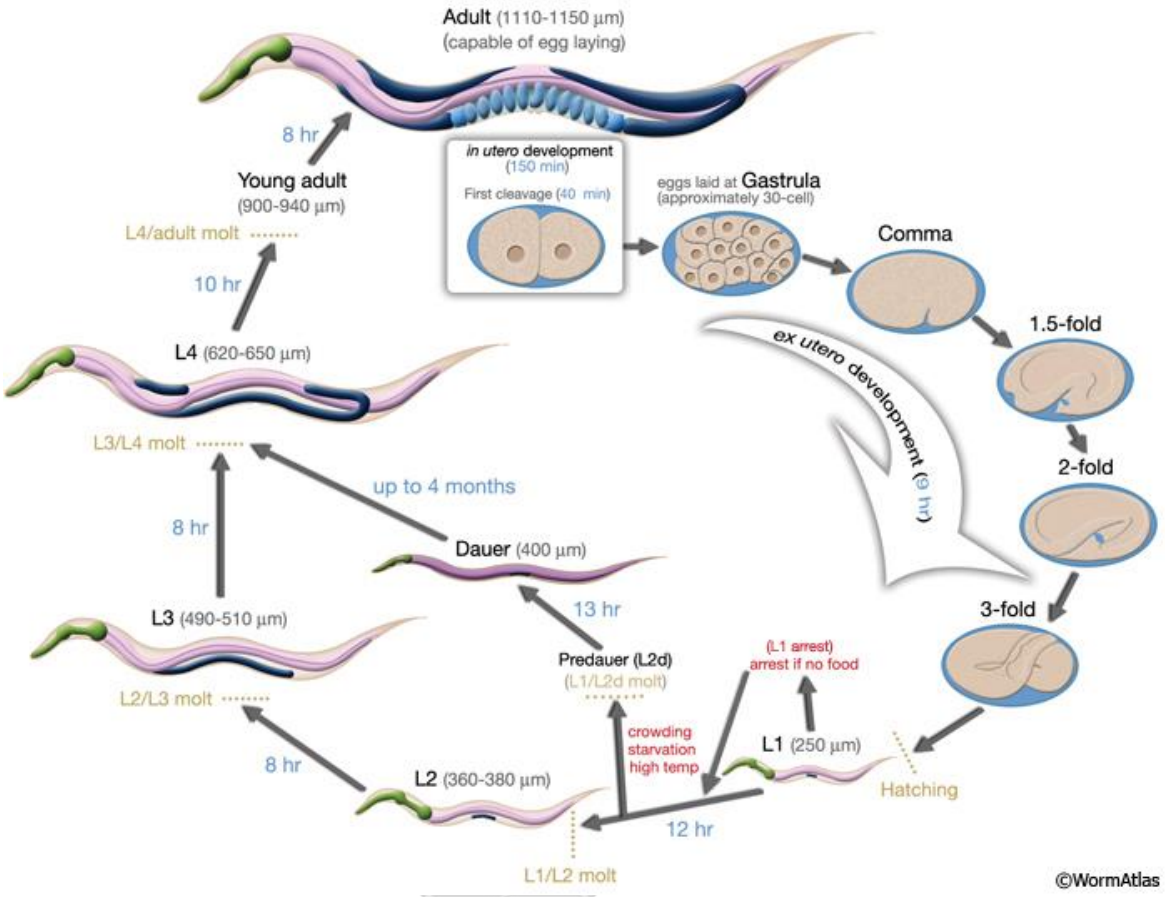



**Figure 10. The *C. elegans* life cycle.**

[Photo from WormAtlas (https://www.wormatlas.org/)]



*C. elegans* are considered one of the most powerful systems to study aging and stress response because it has short lifespan, susceptibility to oxidative stress and the similarities with the human aging process [29]. Moreover, the genome of *C. elegans* is completely sequenced, displaying homologous to genes implicated in human diseases and the major signaling pathways that regulate longevity and stress resistance are well conserved [30-32].

## 7. Effects of plants on the experimental models

### 7.1 Effects of plants on neurite outgrowth

Several plants and natural products are studied for used as herbal therapies which are one of the most commonly used forms of complementary and alternative medicine [119]. The compounds that have neurite outgrowth promoting activity may have the therapeutic efficacy in the treatment of neuronal loss and regenerate neuronal network to become the normal neuronal functions in the CNS. Therefore, the use of plants and natural products for neuroregeneration provides new insights in drug development for treating neuronal loss in AD.

Recently, a large number of plants and natural products and their isolated compounds possessing neurite outgrowth activity have been discovered. Most of the compounds evaluated for their neurite outgrowth activities are derived from plant sources. Among all the majority of in vitro models mentioned, neurite outgrowth was basically induced by compounds from natural sources alone or incombination with neurotrophic factors such as NGF. It is also evident from the reports that some compounds show a synergistic effect in their neuritogenic action when combined with NGF. Based on several reports, compounds from natural sources basically act intracellularly by causing amplification in some specific downstream MAPK signaling pathways like ERK1/2 and p38-MAPK, leading to phosphorylation of CREB and resulting in neurite formation [14].

These findings are believed that developments in the near future will allow a better understanding of the molecular underlying mechanisms of neurite outgrowth promoting compounds from several plants and natural products regulating neurite outgrowth, thereby making it possible to develop drugs promoting neuronal regeneration in AD patients.

### 7.2 Effects of plants on antioxidant and anti-aging in *C. elegans*

*C. elegans* is considered the simplest major multicellular model organism for studying genetic and pharmacological influences on aging and longevity because of its short lifespan, similarities with the human aging process and susceptibility to oxidative stress [29]. Moreover, the genome of *C. elegans* has been completely sequenced. It carries many homologous genes implicated in human diseases. Major signaling pathways that regulate longevity and stress resistance are well conserved in *C. elegans* [30,31,120]. Recent reports suggest that plant extracts with high concentrations of phenolic secondary metabolites exhibit antioxidant and anti-aging activities in *C. elegans* [33-36]. In the nematodes, oxidative damage can induce its activation by dephosphorylation and subsequent translocation into the nucleus. DAF-16 activation is responsible for stress response and lifespan extension [55]. Upon nuclear localization, DAF-16 induces the transcription of several genes involving antioxidant systems, such as SOD-3, which encodes mitochondrial superoxide dismutase (Mn-SOD). Previous studies suggested that this enzyme could protect the worms against ROS via elimination of free radicals [56]. Moreover, SKN-1/NRF2 pathway is also involved with stress response in *C. elegans*. The transcription factor SKN-1 regulates the expression of downstream genes of phase II detoxification enzymes [62]. GST-4 is an isoform of glutathione S-transferases that plays a major role in phase II detoxification process *in C. elegans* and can be activated by SKN-1[63]. Previous observations with polyphenol-rich plant extracts and isolated compounds that protect *C. elegans* against oxidative stress and prolong lifespan via both DAF-16/FOXO and/or SKN-1/NRF2 pathway [16,35,121-125].



Elevated ROS production apparently is a major contributing factor in the aging process [126]. Lipofuscin is considered as an indicator of both oxidative stress and aging in *C. elegans* [127]. It is an autofluorescent pigment that accumulates progressively over

time, particularly in lysosomes and gut granules of intestinal [128]. Several plant extract treatments were observed their antioxidant and anti-aging capacities by reducing the accumulation of lipofuscin in *C. elegans* [34,122,125,129-131].

## 8. Plant reviews

### 8.1 *Zingiber montanum* (Koenig) Link ex Dietr.

**Family:** Zingiberaceae

**Subfamily:** Zingiberoideae

**Local name[#]:** ไพล Phali (Yala); ปูลอย Pu loi, ปูเลย Pu loei (Northern); ว่านไฟ Wan fai (Central); มิ้นสะล่าง Min-sa-lang (Shan-Mae Hong Son)

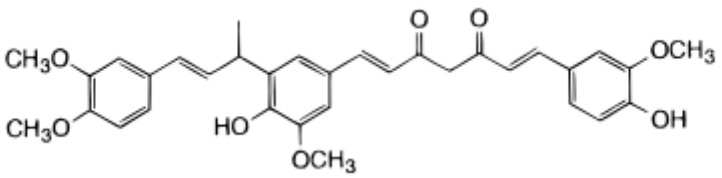
(**[#]** BGO Plant Database, the Botanical Garden Organization, Ministry of Natural Resource and Environment, Thailand)

*Zingiber montanum* Roxb., or the old name *Zingiber cassumunar*, also known as Cassumunar ginger is a species of plant in the ginger family (Family: Zingiberaceae). The rhizome is used medicinally in massage and even in food in Thailand. In aromatherapy, *Z. montanum* oil is used as an essential oil and is believed to reduce pain and inflammation.

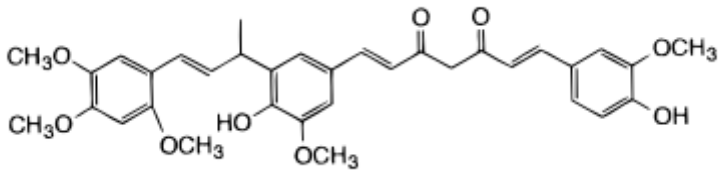
**Figure 11.** ***Zingiber montanum***

[Photo from https://www.dreamstime.com/]

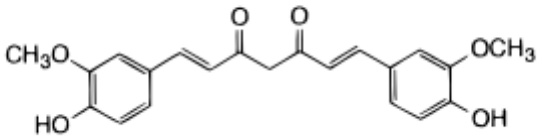
Previous studies found that (E)-1-(3,4-dimethoxyphenyl)but-1-ene, an active ingredient of *Z. montanum* rhizomes, has analgesic and anti-inflammatory properties [132]. In addition, an American study found that plai oil exhibits antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, dermatophytes, and yeasts [133]. Moreover, the plant also contains the unique curcuminoid antioxidants, namely cassumunarin types A and B which able to protect cell cultured suffering from oxidative stress [134]. Interestingly, several curcuminoid compounds have been reported to have neurite outgrowth promoting activity including curcumin, demethoxycurcumin (DMC), and bisdemethoxy-curcumin (BDMC) [135].




*Cassumunin A* [136]




*Cassumunin B* [136]




*Curcumin* [136]



**Figure 12. Constituent compounds of *Z. montanum***

**8.2 *Caesalpinia mimosoides* Lamk.**

**Family:** Fabaceae

**Subfamily:** Caesalpinioideae

**Local name[#]:** ช้าเรือด Cha rueat (General); ทะเน้าซอง Thanao song, ผักปู่ย่า Phak puya, หนามปู่ย่า Nam pyya (Northern); ผักกาดหญ้า Phakkat ya (Prachin Buri); ผักขะยา Phak khaya (Nakhon Phanom); ผักคายา Phak khaya (Loei)

(**[#]** BGO Plant Database, the Botanical Garden Organization, Ministry of Natural Resource and Environment, Thailand)

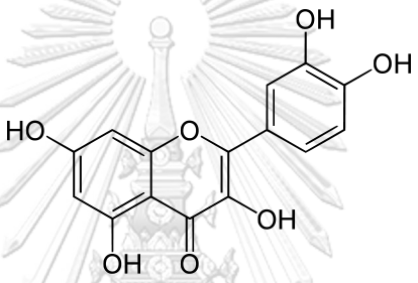
*Caesalpinia mimosoides* Lamk. [recently, reclassified as *Hultholia mimosoides* (Lam.) E. Gagnon & G. P. Lewis], is a small spiny tropical trees or climbing shrubs belonging to family Fabaceae and subfamily to Caesalpinioideae. It is native to Indian subcontinent and South-east Asia as well as in northern and north-eastern parts of Thailand. Young sprouts and leaves are edible and sour and are traditionally used as a carminative and a remedy for dizziness. In addition, the roots are used for ulcer and wound treatment, as well as for the treatment of arthritis [20].



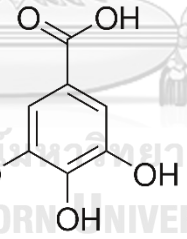
**Figure 13. *Caesalpinia mimosoides***

[Photo from Sakon Nakhon Land development Station (https://www.lddsnk.com/)]

*C. mimosoides* has several active compounds and has been reported to exhibit antioxidant [24], anti-inflammatory [21] and anticancer activities [22]. Moreover, *C. mimosoides* contains several bioactive compounds including gallic acid [22] and quercetin [24] which have been previously reported to have neurite outgrowth activity [25-27]. Recently, quercetin isolated from *C. mimosoides* was shown to process neurite outgrowth and neuroprotective properties on cultured P19-derived neurons [24]. However, the neurite outgrowth stimulatory effects of this plant on neuronal cells overexpressing APP has yet not been investigated.




*Quercetin*




*Gallic acid*



**Figure 14. Constituent compounds of *C. mimosoides.***

**8.3 *Acanthus ebracteatus* Vahl.**

**Family:** Acanthaceae

**Local name[#]:** เหงือกปลาหมอ Ngueak plaa mo, จะเกร็ง Cha kreng, นางเกร็ง Nang kreng (General); แก้มหมอ Kaem mo (Satun); แก้มหมอเล Kaem mo le (Krabi); อีเกร็ง I kreng (Central); เหลือกปลาหมอน้ำเงิน Ngueak plamo nam ngoen (General)

(**[#]** BGO Plant Database, the Botanical Garden Organization, Ministry of Natural Resource and Environment, Thailand)

*Acanthus ebracteatus* Vahl., a member of the Acanthaceae family, has been used as folk medicine for a wide range of tropical diseases. The leaves are used as a snake-bite cure, a cough medicine, a decoction against asthma, a treatment of the rheumatism as well as a purgative and anti-inflammatory in arthritis [137-140]. Previous reports revealed that the major chemical constituent of *A. ebracteatus* were polyphenolic compounds [141,142], which is well known to have the antioxidant and anti-inflammatory properties.


**Figure 15. *Acanthus ebracteatus***

[Photo by Sudarat Homhual (http://www.phargarden.com/)]

**8.4 *Streblus asper* Lour.**

**Family:** Moraceae

**Local name[#]:** ข่อย Khoi (General); กักไม้ฝอย Kak mai foi (Northern); ซะโยเส่ Sa-yo-se (Karen-Mae Hong Son); ตองขะแหน่ Tong-kha-nae (Karen-Kanchanaburi); ส้มพอ Som pho (Loei); สะนาย Sa-nai (Khmer)

(**[#]** BGO Plant Database, the Botanical Garden Organization, Ministry of Natural Resource and Environment, Thailand)

*Streblus asper* Lour. is a medicinal plant belonging to family Moraceae which inhabits in various Asian countries, such as India, Sri Lanka, Malaysia, the Philippines, China, and Thailand. Various parts of this plant are used as the folk medicines [143]. This plant is known to contain steroids, lignans [144], flavonoids and triterpenoids [145]. *S. asper* has also been shown to have several remarkable pharmacological properties such as antimicrobial [146], anti-inflammatory [147], and antioxidant activity [148]. Recently, leaf extracts of *S. asper* have been reported to protect against glutamate-induced toxicity in neuronal cells and extends lifespan of *C. elegans* [149] as well as exhibit anti-acetylcholinesterase and neuroprotective activities [150].



**Figure 16. *Streblus asper***

[Photo from Sudarat Homhual (http://www.phargarden.com/)]

# CHAPTER III

## Materials and Methods

### 1. Plant Extract Preparation

#### 1.1 List of Chemicals and Reagents

- Dimethyl sulfoxide or DMSO (Sigma-Aldrich, USA)
- Hexane (RCI Labscan, Thailand)
- Ethanol (RCI Labscan, Thailand)
- Methanol (RCI Labscan, Thailand)

#### 1.2 List of Plants

**1.2.1 *Caesalpinia mimosoides* Lamk.**

**Voucher specimen number*:** A014170 (BCU)

**Collecting location:** Local market, Chiang Rai Province, Thailand

**Plant part used:** Young twigs and leaves

**1.2.2 *Zingiber montanum* (Koenig) Link ex Dietr.**

**Voucher specimen number*:** A013701 (BCU)

**Collecting location:** HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

**Plant part used:** Rhizomes

**Figure 17.** ***Zingiber montanum***

[Photo by Miss Sakawrat Janpaijit]

**1.2.3** ***Acanthus ebracteatus*** **Vahl.**

**Voucher specimen number*:** A013422 (BCU)

**Collecting location:** HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

**Plant part used:** Leaves



**Figure 18.** ***Acanthus ebracteatus***

[Photo by Dr. Anchalee Prasansuklab]

**1.2.4** ***Streblus asper*** **Lour.**

**Voucher specimen number*:** A013419 (BCU)

**Collecting location:** HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

**Plant part used:** Leaves

**Figure 19. *Streblus asper***

[Photo by Dr. Anchalee Prasansuklab]

(***** Identified at the herbarium of Kasin Suvatabhandhu, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand)

**1.3 Plant Extraction**

All plant extracts used in this study were collected from the Dr. Tewin Tencomnao's plant extract collections that were prepared by different lab members. *C. mimosoides* (CM), *A. ebracteatus* (AE), and *S. asper* (SA) were prepared by Dr. Anchalee Prasansuklab, whereas *Zingiber montanum* (ZM) was prepared by Miss Sakawrat Janpaijit as following:

Leaves and young twigs, approximately 15-20 cm in length from the top of the tree shoots of CM and rhizomes of ZM were extracted by the Soxhlet procedure. Briefly, the used parts of plants were dried in a ventilated incubator at 40°C and ground into a fine powder. Then, approximately 40 g of the dried powder was uniformly packed into a thimble and sequentially extracted in a Soxhlet apparatus with 400 mL of three different extracting solvents (hexane, ethyl acetate, and methanol (CM)/ethanol (ZM)) for at least 24 h per solvent. The resulting supernatants were collected, filtrated and evaporated to dryness under vacuum.

AE and SA leaves were prepared using maceration method. Briefly, the plant powder was soaked in hexane or ethanol at a ratio 1:10 (w/v) at room temperature (RT) for 48 h. This process was performed twice and all resulting supernatants were

combined. Subsequently, the liquid extract obtained after maceration was collected. The extracts were filtered and evaporated to dryness under vacuum.

Finally, each extract was prepared as a stock solution of 100 mg/mL in DMSO, sterilized through a 0.2 µm pore size syringe filter, stored at -20°C, and protected from light until further use.

**2. Neurite Outgrowth Activity of Plant Extracts in APP-overexpressing Neuro2a cells**

**2.1 List of Chemicals and Reagents**

- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA)

- Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA)

- Ham's F12 medium (Sigma-Aldrich, St. Louis, MO, USA)

- Fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA)

- Geniticin (G418) was purchased from Invivogen (San Diego, USA).

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Bio Basic, Markham, Ontario, Canada)

- Trizol (Invitrogen, Carlsbad, CA, USA)

- HyClone$^{TM}$ Phosphate buffer saline (PBS) (GE Healthcare Bio-Sciences, USA)

- EmbyoMax® Non-essential amino acids (NEAA) solution (Millipore, USA)

- Penicillin/Streptomycin (Gibco, Waltham, MA, USA)

- 10X Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA)

- Anti-APP, anti-β-actin and horseradish peroxidase-coupled secondary antibodies (Cell Signaling Technology, Danvers, MA, USA)

**2.2 Cell Culture**

Mouse neuroblastoma cells used in this study included wild type Neuro2a cells, and Swedish mutant APP-overexpressing Neuro2a (Neuro2a/APPSwe) cells. Neuro2a cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). Neuro2a/APPSwe were provided by Professor Ciro Isidoro (Laboratory of Molecular Pathology and Nanobioimaging, Universita del Piemonte Orientale, Novara, Italy). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and HAMF12 medium (ratio 1:1) supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acid, 1% penicillin/streptomycin. Additional, 0.4% geniticin (G418) was added to the medium for Neuro2a/APPSwe. The cells were growth in a humidified incubator with 5% $CO_2$ at 37°C.

The ingredients for complete medium:

- 10% FBS for Neuro2a cells (100 mL)
    - DMEM/high glucose 45 mL
    - HAMF12 45 mL
    - Fetal bovine serum 10 mL
    - Non-essential amino acid 1 mL
    - Drug (Penicillin-Streptomycin) 1 mL
- 10% FBS for Neuro2a/APPswe cells (100 mL)
    - DMEM/high glucose 45 mL
    - HAMF12 45 mL
    - Fetal bovine serum 10 mL
    - Non-essential amino acid 1 mL
    - Drug (Penicillin-Streptomycin) 1 mL
    - Geniticin (G418) 400 µL



**2.3 Cell Viability Assay using MTT Assay**

To evaluate cell viability after treatment with various concentrations of the plant extracts for select the optimal concentration of the plant extracts that have not any toxic to the cells for following experiments.

*Principle*: MTT assay is a colorimetric assay for assessing cell metabolic activity. Living cells usually have NAD(P)H-dependent cellular oxidoreductase

enzymes that may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. The absorbance of this colored solution can be quantified by measuring at 550 nm wavelength by a spectrophotometer.

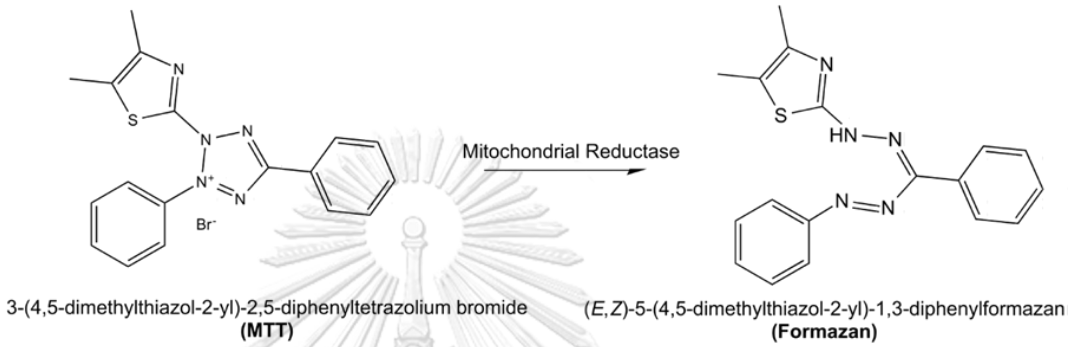



**Figure 20. MTT assay principle.**

MTT is reduced via mitochondrial enzyme to formazan.

Both Neuro2a cell types were cultured in a 96-well plate at density of 5,000 cells per well and incubated for 24 h. After that, the cells were treated with different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) of plant extracts for 48 h. After incubation, the medium was removed and MTT solution was added to the cells at a final concentration of 0.5 mg/ml. After an additional for 3 h of incubation, the solution was carefully removed, and the formazan crystal was solubilized by 150 µl DMSO. The optical density (OD) was measured using an EnSpire® Multimode Plate Reader (Perkin-Elmer, Waltham, MA, USA) at 550 nm.

### 2.4 Screening of Neurite Outgrowth Activity of Plant Extracts

To evaluate the effect of APP overexpression on neurite outgrowth and to evaluate neurite outgrowth activity of Neuro2a/WT and Neuro2a/APPSwe cells after treatment with plant extracts comparing with untreated control. The cells were seeded

in a 6-well plate at an initial density of 5,000 cells per well in 2 ml 10% FBS medium and were incubated at 37°C, and 5% $CO_2$ for 24 h. After incubation, medium was carefully removed and washed the cells by PBS. The plant extract was diluted with 1%FBS at the concentration that have cell viability effect more than 75%. The cells treated with 10%FBS and 1%FBS medium was conducted as negative controls. The cells were incubated for 48 hour at 37°C, and 5% $CO_2$. After 48 h, the cells were visualized under 10x magnification using the Differential Interphase Contrast (DIC) microscope, and were photographed using a camera and processed with an ImageJ Software (National Institutes of Health, Bethesda, MD).

The ingredients for differentiation medium:

- Differentiation medium 1% FBS (100 mL)
    - DMEM/high glucose 49.5 mL
    - HAMF12 49.5 mL
    - Fetal bovine serum 1 mL
    - Non-essential amino acid 1 mL
    - Drug (Penicillin ,Streptomycin , Amphoteracin B) 1 mL



**2.4.1 Quantification of Neurite-Bearing Cells**

For identification of neurite-bearing cells, the cells were scored positive if it bore a thin neurite extension that was double or more the length of the cell body diameter. Cells in 10-20 fields per well were randomly examined.

### 2.4.2 Quantification of Neurite Length

For neurite length determination, the longest length of neurite of the cell was measured form cell membrane of cell body to the end of growth cone and was averaged of randomly chosen more than 100 cells per well.

### 2.5 RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Neuro2a/WT and Neuro2a/APPSwe cells were seeded in a 6-well plate at an initial density of 10,000 cells per well in 2 ml 10% FBS medium and were incubated at 37°C, and 5% $CO_2$ for 24 hour. After incubation, the medium was carefully removed and washed the cells by PBS. The plant extract diluted with 1% FBS at concentration that have cell viability effect more than 75%. The cells treated with 10% FBS and 1% FBS were used as controls. The cells were incubated for 48 hour at 37°C, and 5% $CO_2$. Total RNA was extracted from the cells after treatment using Trizol reagent following the manufacturer's instructions. The amount of RNA was measured by absorbance at 260 nm. 1 µg of total RNA was used for cDNA synthesis using AccuPower RT PreMix (Bioneer Co.) and Oligo(dT) primer.



### 2.6 Quantitative RT-PCR Analysis

To determine the effect of CM extract on mRNA expression, the real-time PCR reaction was performed in the Exicycler™ version 3.0 (Bioneer Co.). The amplifications were done using SYBR Green of the Greenstar™ qPCR Premix (Bioneer Co.). The thermal cycling conditions were composed of an initial denaturation step 95° C for 10 min, followed by 40 cycles at 95° C for 15 sec, annealing for 15 sec and 72° C for 30 sec. Primer information of each gene was descripted in **Table 2**. Expression data was

normalize to β-actin to control the variability in expression levels and was analyzed using the $2^{-\Delta\Delta CT}$ method. Each sample was run in at least triplicate.

**Table 1. Primer sets and annealing temperatures used for quantitative RT-PCR.**

| Primer | Sequence (forward and reward) | Product size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| GAP-43 | 5′- AGCCTAAACAAGCCGATGTG -3′ | 157 | 62 |
| | 5′- GGTTTGGCTTCGTCTACAGC -3′ | | |
| Ten-4 [107] | 5′- GTGGACAAGTTTGGGCTCATTTA -3′ | 185 | 62 |
| | 5′- GGGTTGATGGCTAAGTCTGTGG -3′ | | |
| Lingo-1 [151] | 5′- TCTATCACGCACTGCAACCTGAC -3′ | 116 | 56 |
| | 5′- AGCATGGAGCCCTCGATTGTA -3′ | | |
| NgR | 5′- CCTGCAGAGGTCCTAATGCC -3′ | 180 | 60 |
| | 5′- GAGGCGCTTAAGATCACGGT -3′ | | |
| BACE1 | 5′- CCAGGGCTACTATGTGGAGATGA -3′ | 66 | 58 |
| | 5′- GTGTCCACCAGGATGTTGAGC -3′ | | |
| β-actin | 5'- GGCTGTATTCCCCTCCATCG -3' | 154 | 62 |
| | 5'- CCAGTTGGTAACAATGCCATGT -3' | | |

**2.7 Protein Extraction and Western Blot Analysis**

To examine the level of APP in Neuro2a/WT and Neuro2a/APPSwe cells. The cells were seeded in a 6-well plate at an initial density of 10,000 cells per well and were treated with 10% FBS, 1% FBS and CM extract for 48 hour. After 48 h, cell lysates were prepared in lysis buffer. Total protein concentrations were quantified by the Bradford assay. An equal amount of protein (15 μg) was denatured by heating in Laemmli loading buffer at 95°C for 10 min and was separated on 10% SDS polyacrylamide gel and then transferred to PVDF membranes. After blocking for 1 h with 5% skim milk in TBS-T (Tris-buffered saline, 0.1% Tween 20), the membranes were allowed to incubate overnight at 4 °C with primary antibodies specific for APP (1:5,000) or β-actin (1:5,000). Membranes were incubated with HRP-conjugated secondary antibodies (1:10,000) at room temperature for 60 min. The immune complexes were visualized using the enhanced chemiluminescence method (ECL™ Select western blotting detection reagent: GE Healthcare, Piscataway, NJ, USA). Protein bands were visualized using the DCP-165C brother scanner and evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The expression level of proteins was calculated using β-actin as the internal control.

**3. Antioxidant and Anti-Aging Activity of *Caesalpenia mimosoides* Extract in *Caenorhabditis elegans***

**3.1 List of Chemicals and Reagents**

- Dimethyl sulfoxide or DMSO (Sigma-Aldrich, St. Louis, MO, USA)

- 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt or ABTS (Sigma-Aldrich, St. Louis, MO, USA)

- 2,2-diphenyl-1-picrylhydrazyl or DPPH (Sigma-Aldrich, St. Louis, MO, USA)

- Quercetin (Sigma-Aldrich, St. Louis, MO, USA)

- Epigallocatechin gallate or EGCG (Sigma-Aldrich, St. Louis, MO, USA)

- Gallic acid was purchased from TCI America (Portland, OR, USA),

- L-ascorbic acid (Calbiochem, San Diego, CA, USA)

- 2,7-dichlorofluorescein diacetate or $H_2$DCF-DA (Fluka Chemie GmbH, Buchs, Switzerland)

- Juglone or 5-hydroxy-1,4-naphthalenedione (Sigma-Aldrich GmbH, Steinheim, Germany)



- Sodium azide (AppliChem GmbH, Darmstadt, Germany)

### 3.2 List of *Caenorhabditis elegans* Strains

- N2 - wild type (ancestral)
- BA17 (fem-1(hc17)IV)
- TJ375 (gpIs1[hsp-16.2::GFP])
- TJ356 (zIs356[daf-16p::daf-16a/b::GFP+rol-6])
- CF1553 (mu1s84[pAD76(sod-3::GFP)])
- LD1 (ldIs7 [skn-1b/c::GFP + rol-6(su1006)])
- CL2166 (dvIs19[pAF15(gst-4::GFP::NLS)])
- CF1038 (daf-16(mu86)I)

### 3.3 In Vitro Antioxidant Activity

#### 3.3.1 DPPH Assay

The 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the free radical scavenging activity of the extract based on its hydrogen atom- or electron-donating capacity to neutralize the stable radical DPPH (DPPH•), accompanied by a color change from purple to yellow. The assay was performed by using a microplate format. For the assay protocol, 100 µl DPPH• working solution (0.2 mM) was added to 100 µl of the extract at a ratio of 1:1 (v/v). The reaction mixture was incubated in the dark at RT for 30 min, and the absorbance was recorded in a microplate reader (BioTek Instruments, Winooski, VT, USA) at 517 nm. Radical scavenging activity was expressed as the percent inhibition of the DPPH• radicals calculated by the following equation: % Inhibition = 100 x [Abs of control - (Abs of sample - Abs of blank) / Abs of control]. Ascorbic acid (vitamin C) and EGCG were used as controls to study the effective concentration ($EC_{50}$) of the extract.

### 3.3.2 ABTS Assay

The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was used to evaluate the free radical scavenging activity of the extract based on its hydrogen atom- or electron-donating capacity to neutralize the stable free radical cation ABTS (ABTS•+), accompanied by a color change from green to colorless. The cation radical ABTS•+ working solution was generated by the oxidation of 7 mM ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) at a 1:1 (v/v) ratio. The assay was performed by using a microplate format, in which the reaction consisted of 100 µl ABTS•+ working solution and 100 µl extracts at a 1:1 (v/v) ratio. The mixture was then incubated in the dark at RT for 45 min, and the absorbance was recorded using a microplate reader (BioTek Instruments) at 734 nm. Radical scavenging activity was expressed as the percent inhibition of the ABTS•+ radicals calculated by the following equation: % Inhibition = 100 x [Abs of control - (Abs of sample - Abs of blank) / Abs of control]. Ascorbic acid (vitamin C) and EGCG were used as controls to study the effective concentration ($EC_{50}$) of the extract.



### 3.4 Antimicrobial Activity of CM Extract using Agar Diffusion Method

CM extract was proceeded the agar diffusion test to assess the sensibility of *E. coli* OP50. The bacteria was diluted in 0.9% NaCl broth to match 0.5 in the MaFarland densitometer. The bacteria broth was then spread onto LB agar plate. After allowed to dry, sterile pasteur pipette was used to make the wells by carefully stab into the agar. The wells were filled with 60 µl of CM extract (200µg/ml), 128 µg/ml ampicillin or DMSO. The plates were incubated at 37°C for 24h.

### 3.5 *Caenorhabditis elegans* Maintenance and Synchronization and Treatment

The worms were all cultured with nematode growth medium (NGM) containing *E. coli* OP50 as a food source and kept in a 20°C incubator. All strains and *E. coli* OP50 were obtained from Caenorhabditis Genetics Center (CGC), University of Minnesota, USA.

Age synchronization of the worms was achieved by isolating eggs from gravid hermaphrodites. The eggs were prepared by adding lysis solution containing 5 M NaOH and 5% NaOCl, followed by vortexing for 10 min and centrifuging for 2 min at 1,800 rpm. Then, the supernatant was removed, and the pellet was washed once in sterile water before centrifugation for an additional 2 min. After discarding water, the remaining eggs were resuspended in M9 buffer for hatching. Larvae were then kept after hatching in S-medium containing *E. coli* OP50 ($OD_{600}$ = 1.0). Different treatments were applied according to each experiment. Agar diffusion test was performed to exclude the antimicrobial activity of CM extract against *E. coli* OP50.

For the experiment, the worms were divided into four groups. The first group was treated with 1% DMSO (solvent control group). This group served to exclude any toxicity of the solvent used for dissolving the extracts on worms. Groups two through four were treated with 25, 50 and 100 µg/ml CM extracts dissolved in DMSO (maximum 1%), respectively.

### 3.6 Survival Assay under Juglone-induced Oxidative Stress

The assay was modified as previously described [35,152]. Age synchronized L1 larvae stage of wildtype N2 and transgenic CF1038 (DAF-16 loss-of-function mutant) worms were divided into four groups of 80 worms each and treated with different concentrations of CM extracts or with DMSO diluted in S-medium and bacteria, as mentioned above. After 48 h of treatment at 20°C, the pro-oxidant juglone (a naphthoquinone from *Juglans regia*) was added to a final concentration of 80 μM, which is a lethal concentration, prior to incubation at 20°C for an additional 24 h. Afterwards, surviving and dead worms were counted.

### 3.7 Intracellular ROS Accumulation

Age-synchronized N2 and CF1038 worms (L1 stage) were treated with CM extracts or DMSO in S-medium at 20°C for 48 h. Each group contained 200-300 individuals. Then, 50 μM $H_2$DCF-DA was added and incubated for 1 h away from light at 20°C. After that, the worms were mounted on a glass slide and paralyzed by the addition of 10 mM sodium azide, and at least 30 worms were randomly photographed using a fluorescence microscope BIOREVO BZ-9000 with a mercury lamp (Keyence Deutschland GmbH, Neu-Isenburg, Germany) with $\lambda$ex 480/20 nm, $\lambda$em 510/38 nm, 10X objective lens and constant exposure time. ImageJ software version 1.50i (National Institutes of Health, Bethesda, MD, USA) was then used to measure the relative fluorescence intensity of the full body [35,152].

### 3.8 HSP-16.2 Expression

L1 age-synchronized TJ375 transgenic worms, which express a HSP-16.2::GFP reporter gene, were treated with CM extracts or DMSO as previously mentioned and incubated at 20°C for 72 h. Then, the nematodes were exposed to a nonlethal dose of 20 μM juglone for 24 h. The worms were then mounted on a glass slide with 10 mM sodium azide, and images of at least thirty worms per group were taken with a 20X objective lens at constant exposure time via fluorescence microscopy. Analysis of at least three replicates was performed by quantifying the mean relative fluorescence intensity of the pharynx using ImageJ software [152].

### 3.9 Subcellular DAF-16 Localization

TJ356 transgenic L1 worms, which express DAF-16::GFP fusion protein, were treated with CM extracts or DMSO as previously described and kept at 20°C. After 72 h, the worms were mounted on a glass slide using 10 mM sodium azide. At least thirty worms per group were imaged on a fluorescence microscope with a 10X objective lens and constant exposure time. Distribution of the transcription factor DAF-16::GFP in each worm can be in the nucleus, cytoplasm, or the intermediate region between the nucleus and cytoplasm. Worms were sorted and counted according to localization of DAF-16::GFP [35].

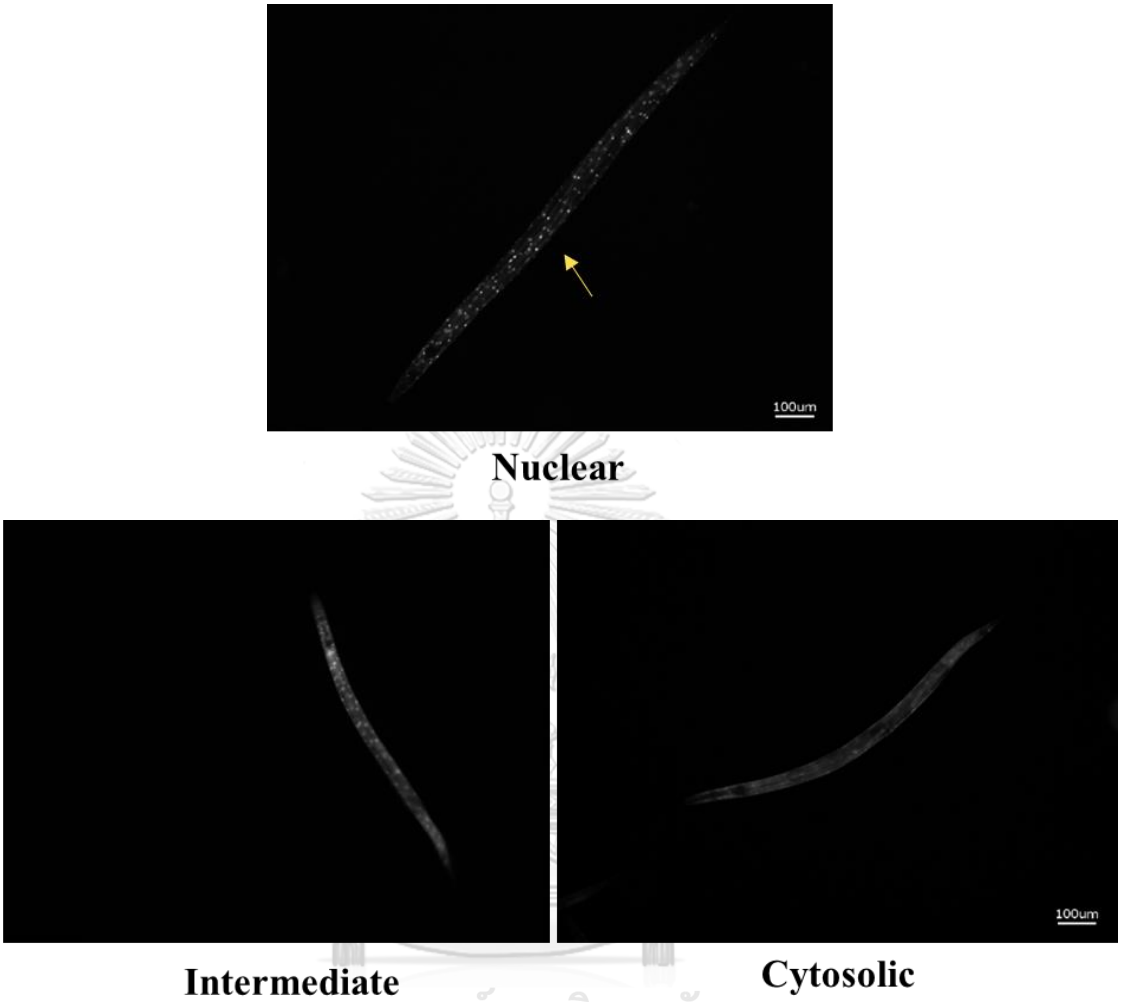





**Figure 21. DAF-16 location in *C. elegans.***

### 3.10 Subcellular SKN-1 Localization

LD-1 transgenic worms, which express a GFP reporter-fused SKN-1, were age-synchronized at the L1 stage and were treated with CM extracts or DMSO as described previously and kept at 20°C for 48 h. Fluorescence intensity was measured by fluorescence microscopy as described above. Then, the worms were mounted on a glass slide using 10 mM sodium azide for paralysis, and at least thirty worms per group were visualized under a fluorescence microscope at a 20X objective lens and constant

exposure time. The transcription factor SKN-1::GFP in each worm can be located in the nucleus, cytoplasm or the intermediate between the nucleus and cytoplasm. The nematodes were sorted and counted according to the SKN-1::GFP subcellular localization.

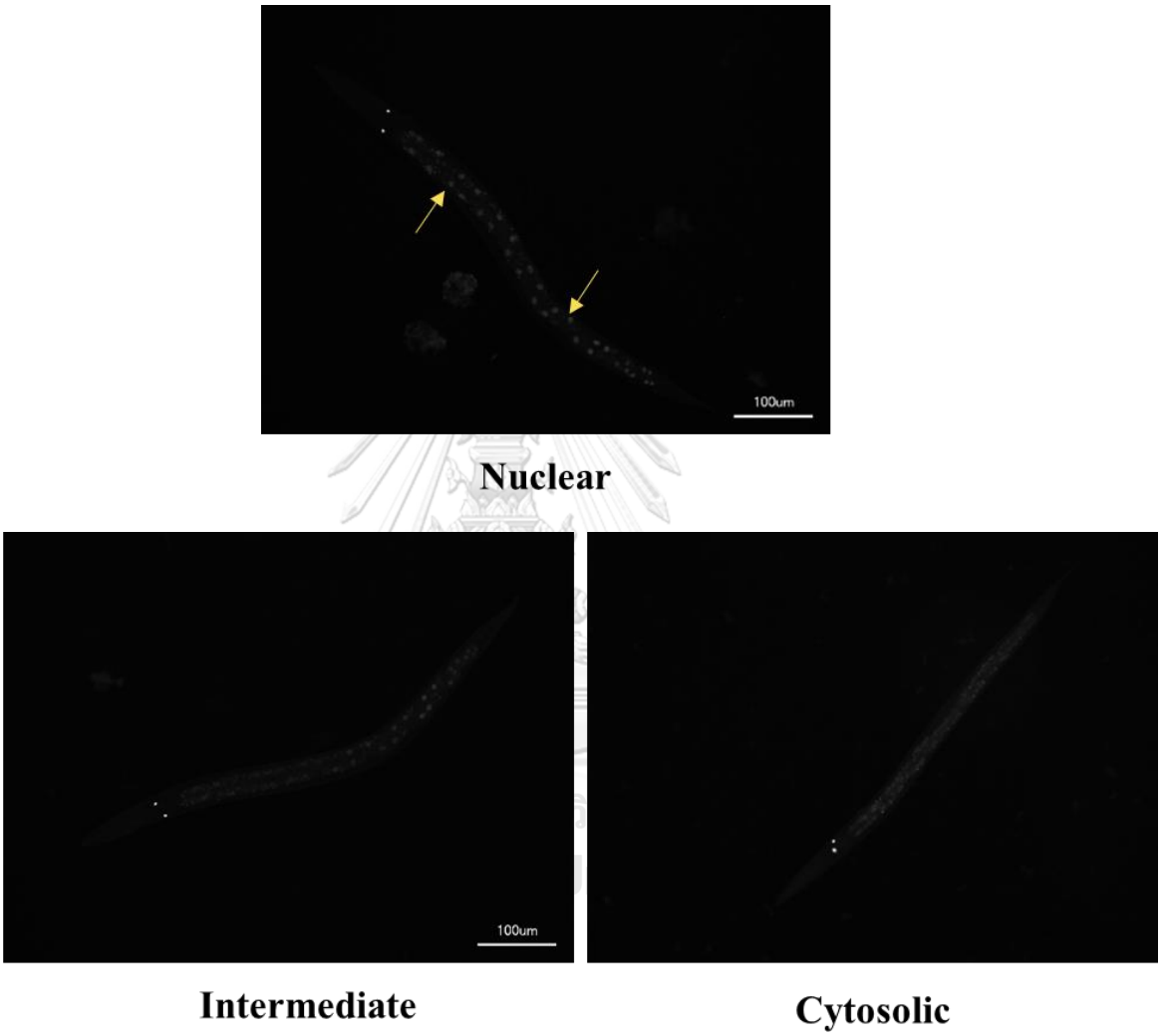



**Figure 22. SKN-1 location in *C. elegans.***

### 3.11 SOD-3 Expression

Age-synchronized CF1553 transgenic worms, expressing SOD-3::GFP fusion protein, at the L1 stage were treated with CM extracts or DMSO as described above and cultured at 20°C for 72 h. After treatment, the worms were mounted on a glass slide with 10 mM sodium azide, and at least thirty worms per group were imaged using a fluorescence microscope with a 10X objective lens and constant exposure time. The experiment was repeated at least three times, and analysis was performed by measuring the relative fluorescence intensity using ImageJ software [35,152].

### 3.12 GST-4 Expression

At the L1 stage, synchronized CL2166 worms expressing GST-4::GFP fusion protein were treated with CM extracts or DMSO as described above and kept at 20°C for 48 h. After treatment, the worms were exposed to 20 μM juglone and incubated at 20°C for 24 h. Then, the worms were paralyzed with 10 mM sodium azide on a glass slide, and at least thirty worms were imaged using a fluorescence microscope with a 10X objective lens at constant exposure time. Analysis of three replicates was performed by measuring the relative fluorescence intensity using ImageJ software.



### 3.13 Lipofuscin level

BA17 transgenic worms, which are thermosensitive and do not lay eggs at 25°C, were used to measure the expression of lipofuscin, an autofluorescent pigment that accumulates over time and thus is an indicator of aging. The worms at the L1 larval stage were treated with CM extracts or DMSO as mentioned above and cultured at 25°C for 16 days. The media and treatments were changed every second day. At day 16, the worms were paralyzed with 10 mM sodium azide on a glass slide, and at least

thirty randomly selected worms were imaged on a fluorescence microscope at a 10X objective lens and constant exposure time. Three repeat experiments were performed by measuring the relative fluorescence intensity using ImageJ software.

### 3.14 Body Length Determination

N2 worms were age synchronized by picking adult worms into NGM agar plates with *E. coli* OP50 as a food source. The adult worms were allowed to lay eggs for 2-4 h before removal, and then the remaining eggs were incubated at 20°C for 48 h. After incubation, worms at the L4 larval stage were sorted and used in the experiments. For the body length assay, 50 worms at the L4 larval stage were placed on NGM agar plates supplemented with CM extracts or DMSO in the *E. coli* OP50 lawn as a food source and cultured at 20°C for 24 h. Adult day 1 worms were paralyzed by using 10 mM sodium azide and mounted on a glass slide. At least thirty worms per group were imaged using a 10X objective lens of bright-field microscope. The software BZ-II Analyzer (Keyence Corp.) was used for the analysis of the body length.

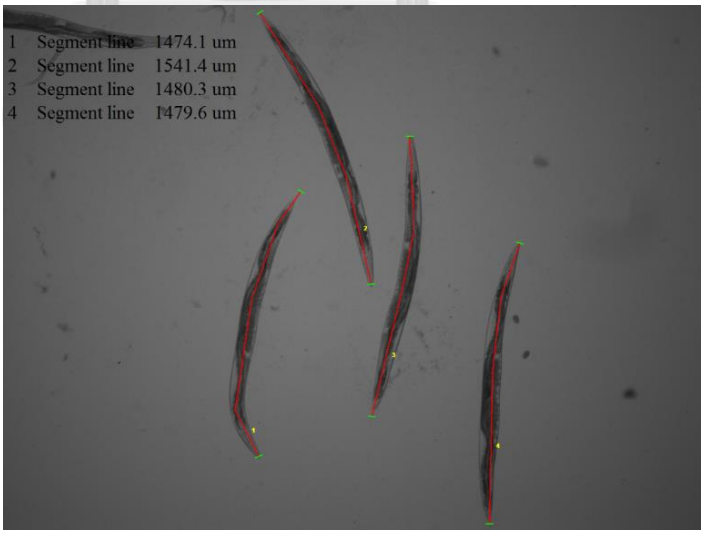



**Figure 23. The measurement of body length of *C. elegans.***

### 3.15 Brood Size Determination

For the brood size assay, each L4 larval stage worm was individually sorted, transferred onto different NGM plates and treated with CM extracts or DMSO. The worms were allowed to grow and lay eggs at 20°C and were observed under a dissecting microscope. The eggs were counted and removed to separate them from adult worms every day until the adult worm stopped laying eggs.

## 4. Phytochemical Analysis of *Caesalpenia mimosoides* Extract

### 4.1 Qualitative Phytochemical Profiling - LC-MS

The methanol extract of *C. mimosoides* was submitted to the Institute of Systems Biology (Universiti Kebangsaan Malaysia, Malaysia) to screen for phytochemicals with liquid chromatography-mass spectrometry (LC-MS) analysis. The analytical system used was a DionexTM UltiMate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a high-resolution micrOTOF-Q III (Bruker Daltonik GmbH, Bremen, Germany). The chromatographic separation was performed on an AcclaimTM Polar Advantage II C18 column (3 mm x 150 mm, 3 µm particle size) (Thermo Fisher Scientific) with a gradient mobile phase consisting of 0.1% formic acid in water (solvent A) and 100% acetonitrile (solvent B). The elution program was as follows: 5% B (0-3 min); 80% B (3-10 min); 80% B (10-15 min) and 5% B (15-22 min). The flow rate was 400 µL/min within a 22 min total run time, and the injection volume was 1 µL. The MS instrument was operated in the positive electrospray ionization (ESI) mode with the following parameters: drying gas flow at 8 L/min; drying gas temperature at 200°C; nebulizer pressure at 1.2 bar; capillary voltage at 4500 V; and m/z scan range of 50 to 1000. For identification of putative compounds, the observed (experimental) m/z values were compared with the METLIN (CA, USA) and the KNApSAcK (Keyword Search Web Version 1.000.01) databases as well as with the calculated (theoretical) mass

values from available previously published data, with an accepted difference of less than 30 parts per million (ppm). The relative abundance of a compound is expressed as the percentage of the peak area relative to the total area of all peaks observed in the chromatogram.

**4.2 HPLC Analysis**

Gallic acid and quercetin were quantified using HPLC (High-Performance Liquid Chromatography) analysis. The assay was performed at RSU Science and Technology Research Equipment Center (Rangsit University, Thailand). Gallic acid and quercetin reference compounds were accurately weighed and freshly prepared in 0.05 M perchloric acid containing 0.1 mM $Na_2EDTA$ on ice and stored at −20 °C before using. SHIMADZU LC-10 HPLC equipped with an analytical C18 reversed-phase column (ODS3 C18, 4.6 × 250 mm i.d., 5-micrometer particle size) and UV detector was used. The mobile phase consisted of 0.02 M sodium acetate, buffered to a pH of 4 with 0.0125 M citric acid, containing 0.042 M methanesulfonic acid and 0.1 mM EDTA and the flow rate was 1 mL/min. The calibration curves were prepared by injecting a series of gallic acid and quercetin standard dilutions. Gallic acid and quercetin in CM methanol extract were quantified by means of calibration curves obtained from the standard.

**5. Statistical Analyses**

All experiments were performed in at least triplicate. All the experimental data of neurite outgrowth experiments was analyzed using GraphPad-Prism version 6 (GraphPad Software, Inc., USA) and expressed as mean ± standard deviation (SD). Statistical differences between 2 groups were analyzed by T-test and more than 2 groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test from at least three independent experiments. *P* value < 0.05 was considered to be significant.

Total phenolic and total flavonoid content as well as DPPH and ABTS results are presented as the mean ± standard deviation (SD). The data from *C. elegans* experiments are presented as the mean ± standard error of the mean (SEM). The differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's method (post hoc). For the lifespan assay, the statistical significance among different groups was determined by a log-rank (Mantel – Cox) test followed by the Gehan-Breslow-Wilcoxon test. Differences with $p$ <0.05 were considered statistically significant.

# CHAPTER IV

## Results

### 1. Neurite Outgrowth Activity of Plant Extracts in APP-overexpressing Neuro2a cells

#### 1.1 APP expression in Neuro2a and Neuro2a/APPSwe Cells

The major characteristic difference between the cells is APP expression level. Neuro2a/APPSwe cells were consistently overexpression APP Swedish mutation. Western blot analysis was used to determine APP level using antibody specific to both wild type and Swedish mutant APP. The protein bands showed that level of APP was clearly different between wild-type cells and APP-overexpressing Neuro2a cells (**Figure 24**).

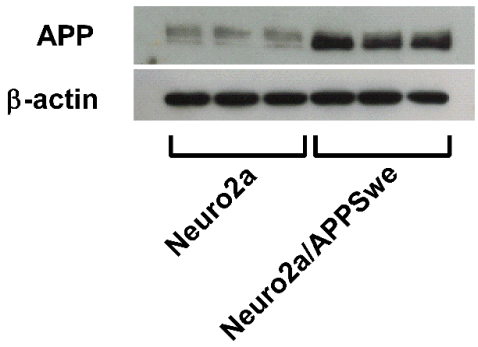





**Figure 24 APP expression level.**

Western blot analysis clearly showed that Neuro2a/APPSwe cells exhibited higher level of APP than Neuro2a cells.

**1.2 Selection of plant extract concentrations**

To select the optimal concentration of the plant extracts, MTT assay was employed to investigate cellular toxicity of the extracts at a series of different concentration. After treatment of Neuro2a and Neuro2a/APPSwe cells with the various concentrations (1, 10, 25, 50, 100 µg/ml) of *Z. montanum* (ZM), *Caesalpinia mimosoides* (CM), *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) extracts for 48 h, it was observed that the extracts exhibited a difference in concentration-dependent toxicity in both neuronal cell lines. The maximum concentrations of the extracts that produced acceptable toxicity with a minimum cell viability of 75% in both Neuro2a and Neuro2a/APPSwe cells were found at 25 µg/ml and 10 µg/ml for ZM extract and CM extract, respectively. **(Figure 25A - 25B)**. Whereas, AE and SA extract showed no toxic after treatment up to 100 µg/ml **(Figure 26A – 26B)**. Due to these results, 25 µg/ml of ZM extract, 10 µg/ml of CM extract, 100 µg/ml of AE extract and 100 µg/ml of SA extract were therefore selected for subsequent experiments.

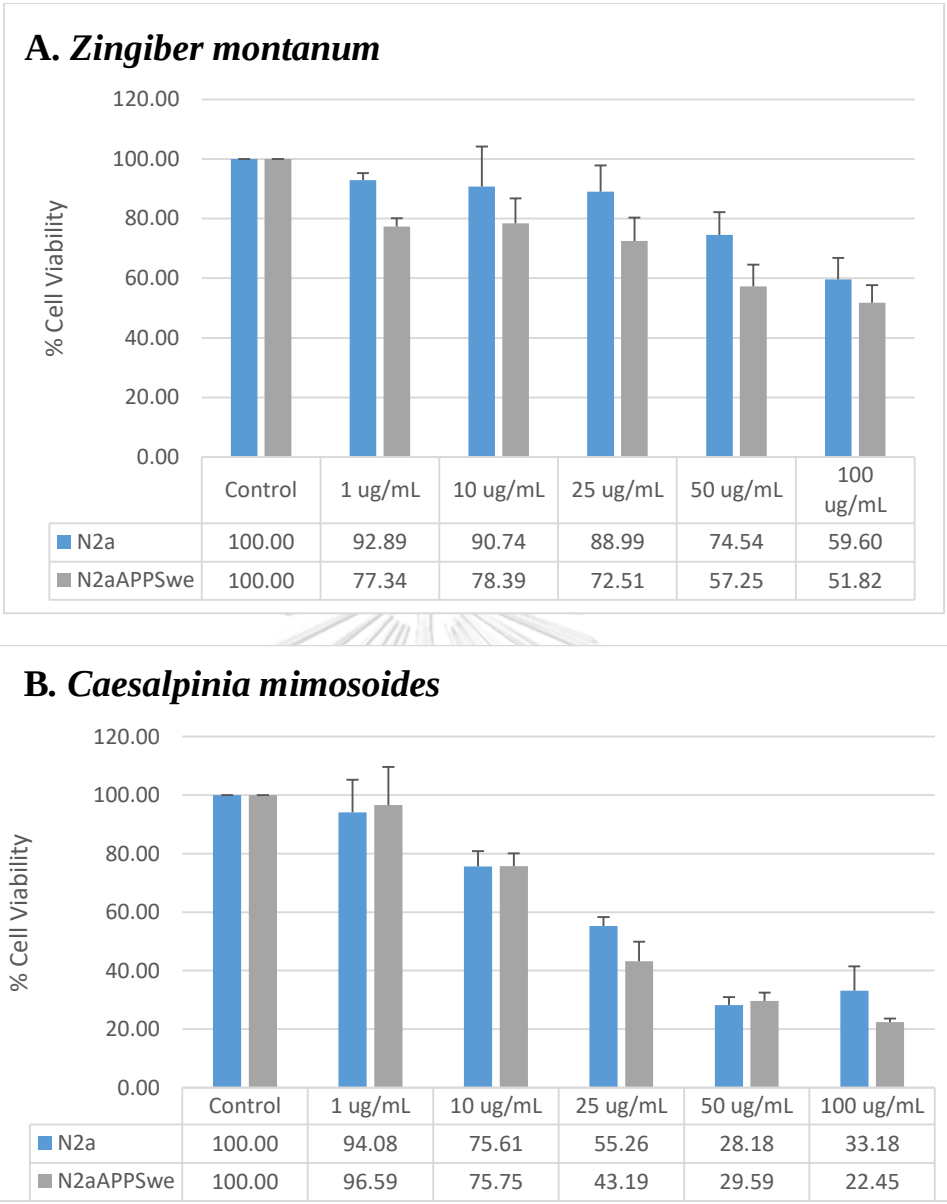



**Figure 25. Cell viability after treatment with the various concentration of ZM and CM extracts.**

MTT viability of Neuro2a and Neuro2a/APPSwe cells after treatment with the various concentrations of ZM and CM extract (A – B, respectively) for 48 h. 25 µg/ml of ZM extract and 10 µg/ml of CM extract exhibited more than 75% cell survival and were chosen as the test concentration for subsequent experiments.

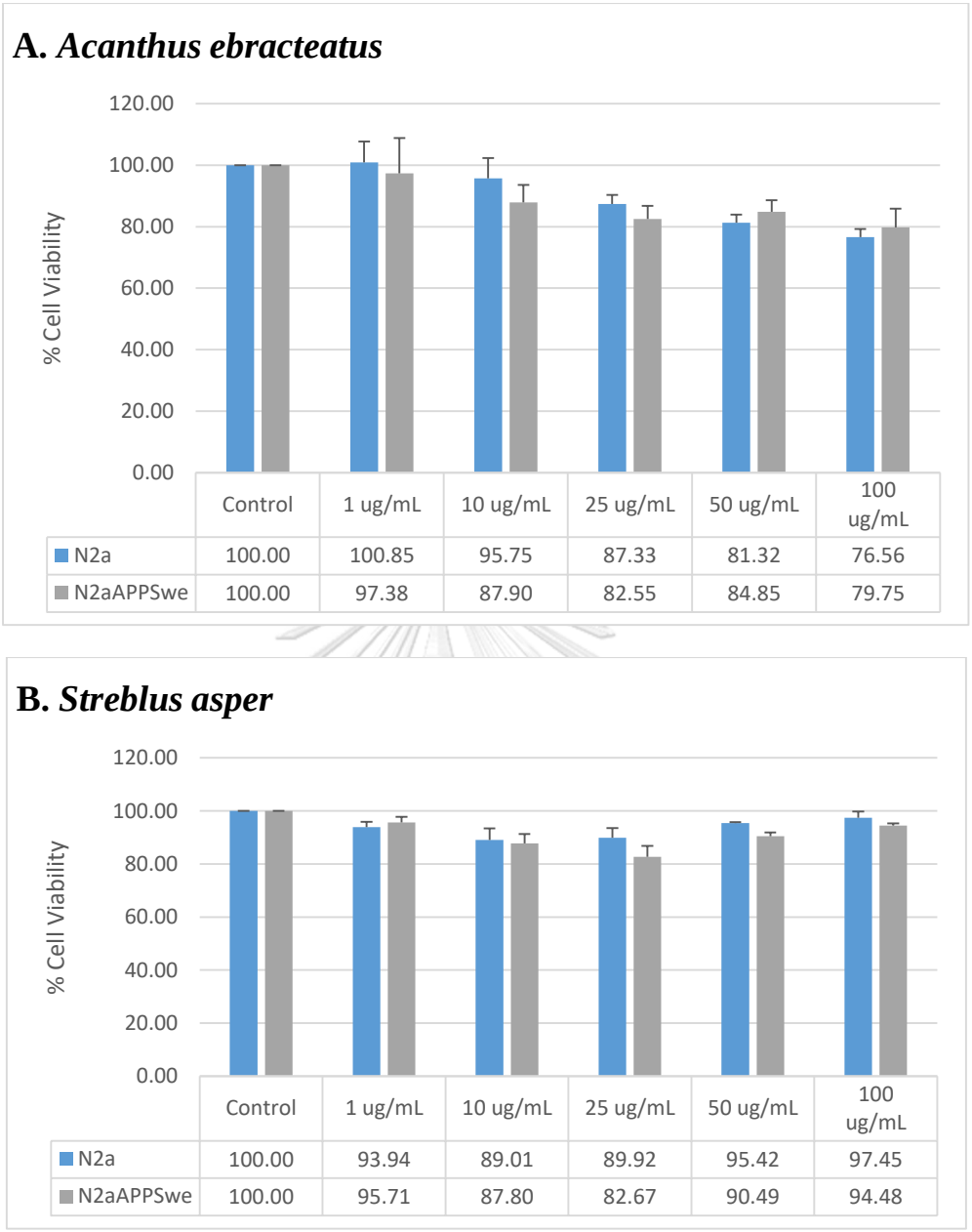



**Figure 26. Cell viability after treatment with the various concentration of AE and SA extracts.**

MTT viability of Neuro2a and Neuro2a/APPSwe cells after treatment with the various concentrations of and SA extract (A – B, respectively) for 48 h. 100 µg/ml of AE extract and 100 µg/ml of SA extract exhibited more than 75% cell survival and were chosen as the test concentration for subsequent experiments.

**1.3 Effects of APP overexpression and plant extracts on neurite outgrowth activity**

APP overexpression on neurite outgrowth activity was first investigated. Neuro2a and Neuro2a/APPSwe cells were cultured in differentiation medium containing 1 % FBS or complete medium containing 10 % FBS (control) for 48 h. The results found that, after culturing the cells in 1 % FBS medium, the neurite outgrowth of Neuro2a and Neuro2a/APPSwe cells were increased in both percent of neurite-bearing cells (23.62 ± 3.27 % and 16.56 ± 1.87 %, respectively) and the mean of the longest neurite length (17.01 ± 0.92 μm and 13.73 ± 1.97 μm, respectively) compared to those of cells in 10 % FBS medium. However, when the comparison was done between cell lines, both percent of neurite-bearing cells and the mean of longest neurite length after treatment of differentiation medium were found significantly lower in Neuro2a/APPSwe cells than the Neuro2a cells (*p*-value = 0.03 and 0.02, respectively) **(Figure 28A – 28B)**. When observed neuron morphology after treatment with plant extracts at the selected concentrations, ZM and CM extracts showed a potential to induce neurite outgrowth when compared with 1% FBS treatment. On the other hand, neurite outgrowth was hardly found in SA and AE extract treatments. Although ZM extract was seem to have a potential to induced neurite outgrowth, many cells seem to have an abnormal morphology. For these reasons, 10 μg/ml of CM extract was selected for the further investigation.

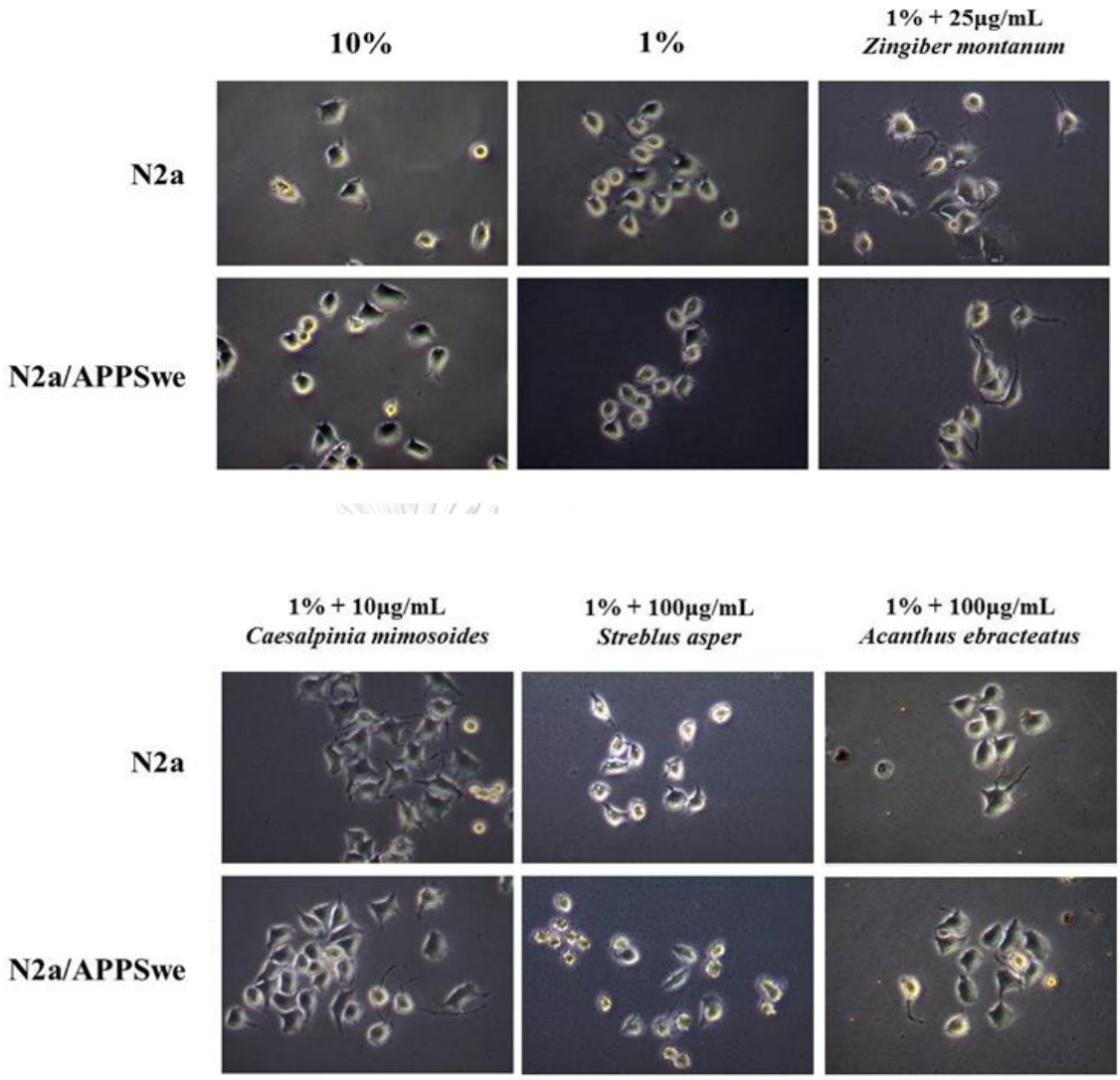





Figure 27. Cell morphology after treatment with the different conditions.

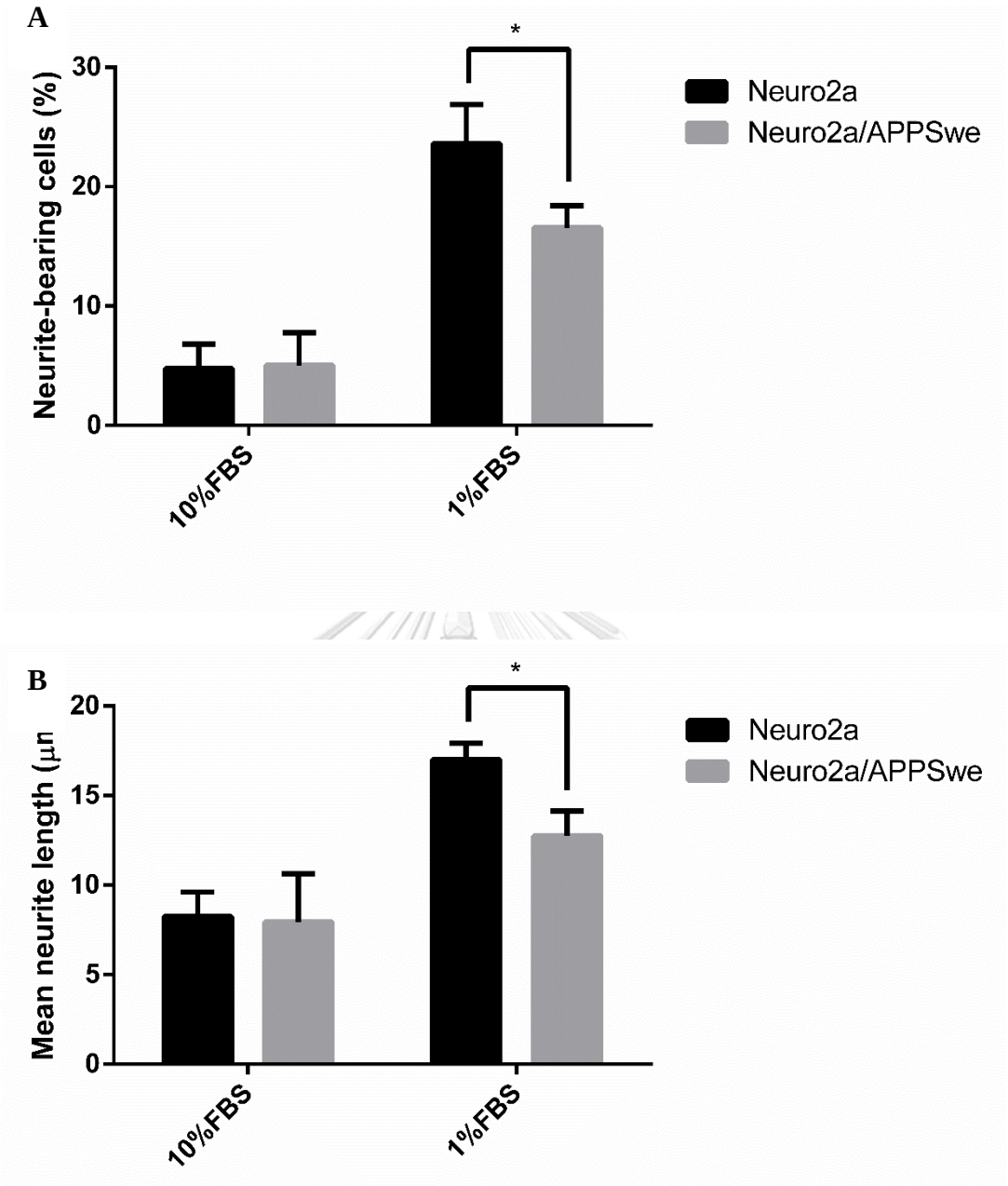



**Figure 28. Neurite outgrowth examination between Neuro2a/WT and Neuro2a/APPSwe cells.**

Neuro2a/APPSwe cells exhibited number of (A) neurite-bearing cells and (B) neurite length significant lower than Neuro2a cells after induced differentiation by 1% FBS medium for 48 h.

To investigate the ability to potentiate neurite outgrowth of CM extract, the cells were treated with the extract at the concentration of 10 µg/ml diluted in 1% FBS medium for 48 h, while cells receiving 10% FBS or 1% FBS medium treatment were used as controls. The result showed that CM extract, when compared with 1% FBS medium control, significantly increased the percent of neurite-bearing cells (43.52 ± 6.00 % vs. 23.62 ± 3.27 %, *p*-value = 0.0025) and slightly but not significantly increased the mean neurite length (21.59 ± 3.76 µm vs. 17.01 ± 0.9 µm, *p*-value = 0.12) in Neuro2a cells. Meanwhile, Neuro2a/APPSwe cells treated with CM extract have a significant increase in both percent of neurite-bearing cells (39.19 ± 3.87 % vs. 16.56 ± 1.87 %, *p*-value = 0.0025) and the mean neurite length (20.47 ± 3.56 µm vs. 13.73 ± 1.97 µm, *p*-value = 0.12) when compared with 1% FBS medium control **(Figure 29C – 29D)**.

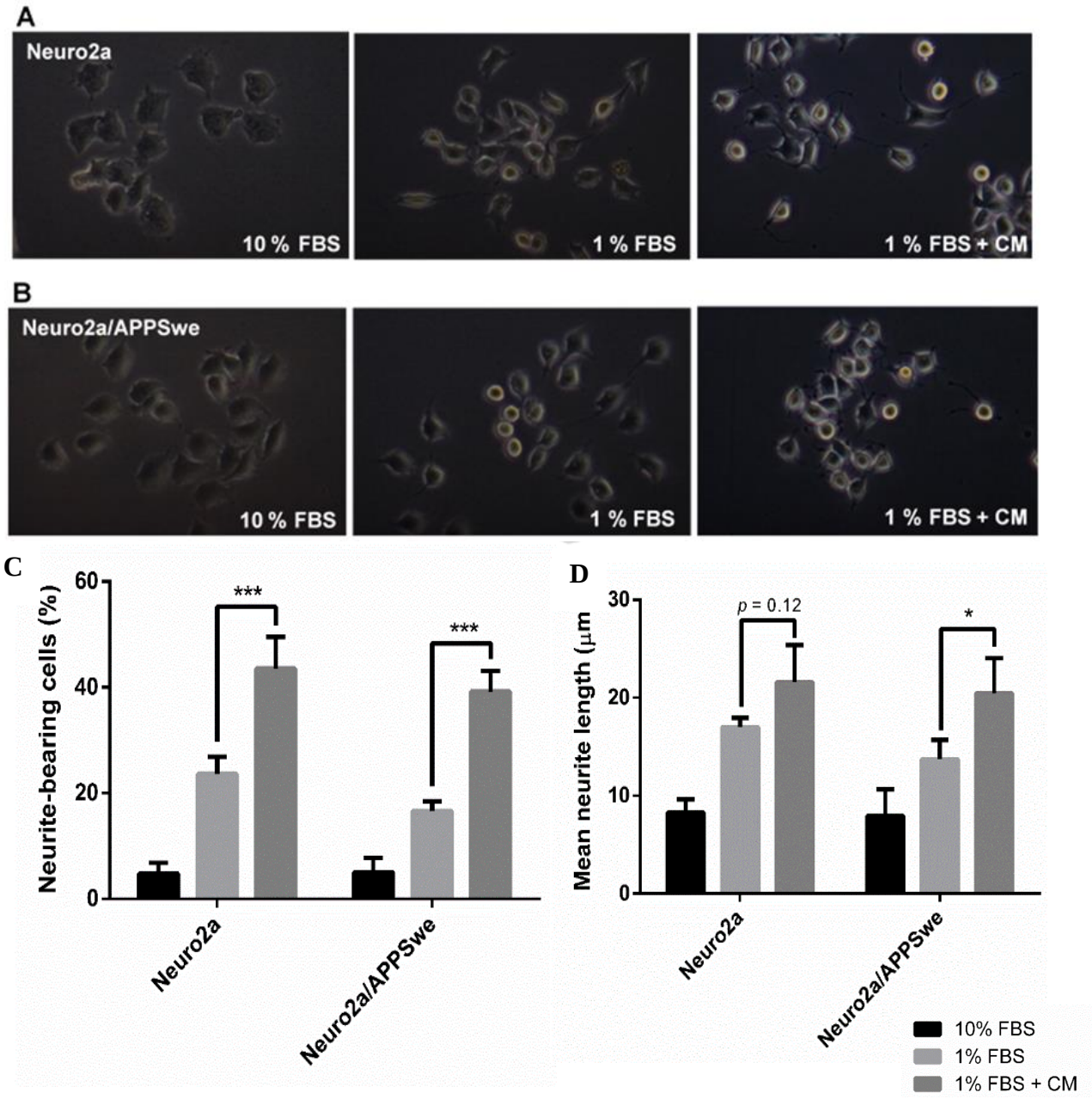



**Figure 29. Neurite outgrowth determination after treatment with CM extract.**

When treatment the cells with 10 µg/ml for 48 h, (A) Neuro2a and (B) Neuro2a/APPSwe cells significantly increased both number of (C) neurite-bearing cells and (D) neurite length when compared with 1% FBS treatment. Values are mean ± SD of at least 3 independent experiments. * = *p*-value < 0.05, *** = *p*-value < 0.001.

**1.4 Effects of APP overexpression and CM extract on GAP-43 gene expression**

To further examine the mechanism underlying neurite outgrowth activity of CM methanol extract, gene expression of the neurite outgrowth marker, GAP-43, was investigated using quantitative real-time RT-PCR. The results showed that GAP-43 mRNA levels in both Neuro2a and Neuro2a/APPSwe cells were found increased after induced differentiation by 1 % FBS medium for 48 h, when compared to the cells in 10 % FBS medium control. By comparing both cell lines under medium condition of 1 % FBS for 48 h,the results showed that the level of GAP-43 mRNA expression have significantly lower in the APP Swedish-mutant overexpressing (Neuro2a/APPSwe) cells than Neuro2a cells with normal expression (*p*-value = 0.0002) **(Figure 30A)**. However, in the presence of CM extract, both Neuro2a and Neuro2a/APPSwe cells have a significant increase of GAP-43 expression level when compared to the cells under 1% FBS medium control (*p*-value = 0.046 and 0.0008, respectively) **(Figure 30B)**.

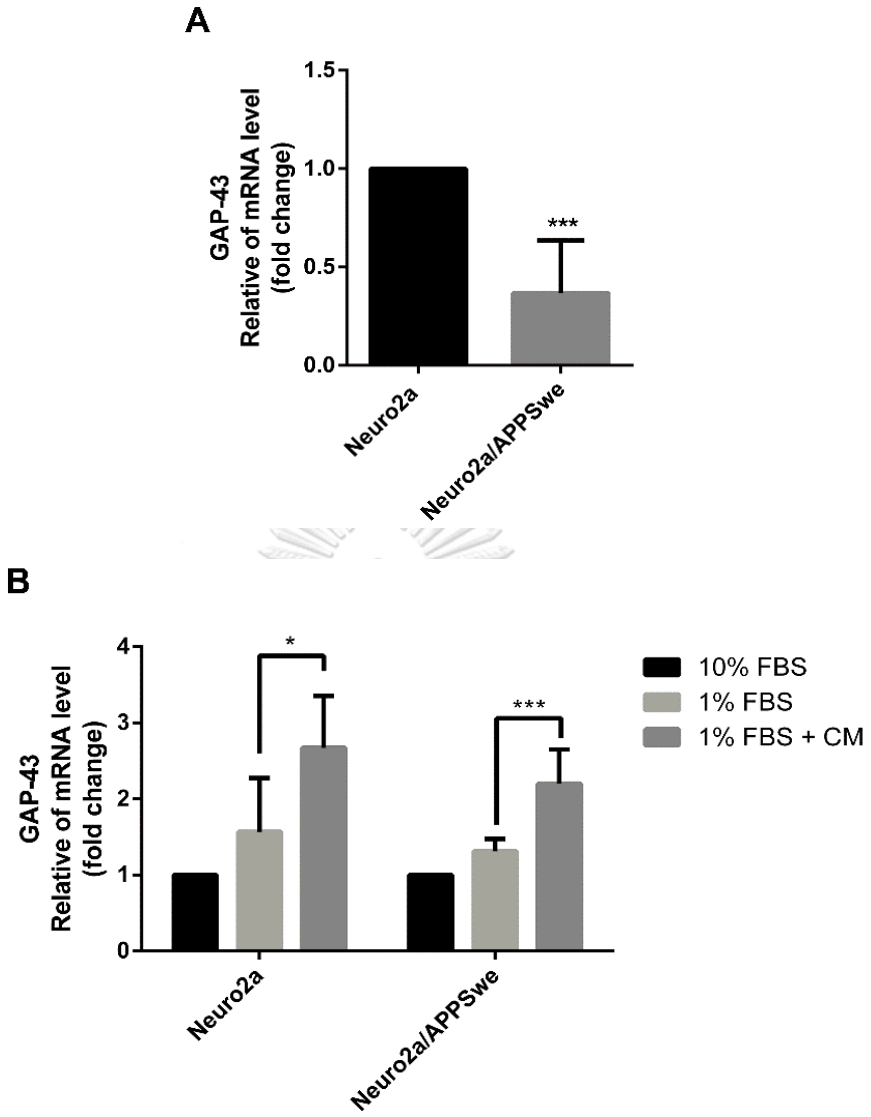





**Figure 30. GAP-43 mRNA expression level.**

(A) Neuro2a/APPSwe cells showed significant lower GAP-43 gene expression than Neuro2a cells after induced differentiation by 1% FBS medium for 48 h. (B) Neuro2a and Neuro2a/APPSwe cells significantly increased the expression of GAP-43 gene when treatment the cells with 10 µg/ml of CM extract for 48 h compared to 1% FBS treatment in each cell. Values are mean ± SD of at least 3 independent experiments. * = *p*-value < 0.05, *** = *p*-value < 0.001.

**1.5 Effects of APP overexpression and CM extract on Ten-4 gene expression**

The neuritogenesis signaling pathway of neurite outgrowth activity affected by APP overexpression and CM extract treatment was further examined. Teneurin-4 (Ten-4), which plays a key roles in neuronal development and neurite outgrowth, mRNA expression level was investigated [107]. Following treatment of the cells with 1% FBS medium control for 48 h, Ten-4 mRNA expression was found significantly lower in APP-overexpressing (Neuro2a/APPSwe) cells than normal Neuro2a cells (*p*-value < 0.0001**) (Figure 31A)**. However, treatment of the cells with 1 % FBS medium control along with 10 µg/ml CM extract could significantly increase the expression level of Ten-4 mRNA in Neuro2a/APPSwe cells as compared with 1 % FBS medium control (*p*-value = 0.007). In Neuro2a cells treated with 1 % FBS medium plus CM extract, the mRNA level of Ten-4 also tend to be increased when compared to 1 % FBS medium control alone (*p*-value = 0.58) **(Figure 31B)**. Nevertheless, in comparison to 10% FBS medium control, Ten-4 mRNA expression level after the extract treatment was found significantly higher in both Neuro2a cells (by 1.86-fold) and Neuro2a/APPSwe cells (by 2.3-fold). Notably, there was a slight increase of expression level for Ten-4 observed in APP overexpressing cells treated with 1 % FBS medium control compared to 10 % FBS medium control.

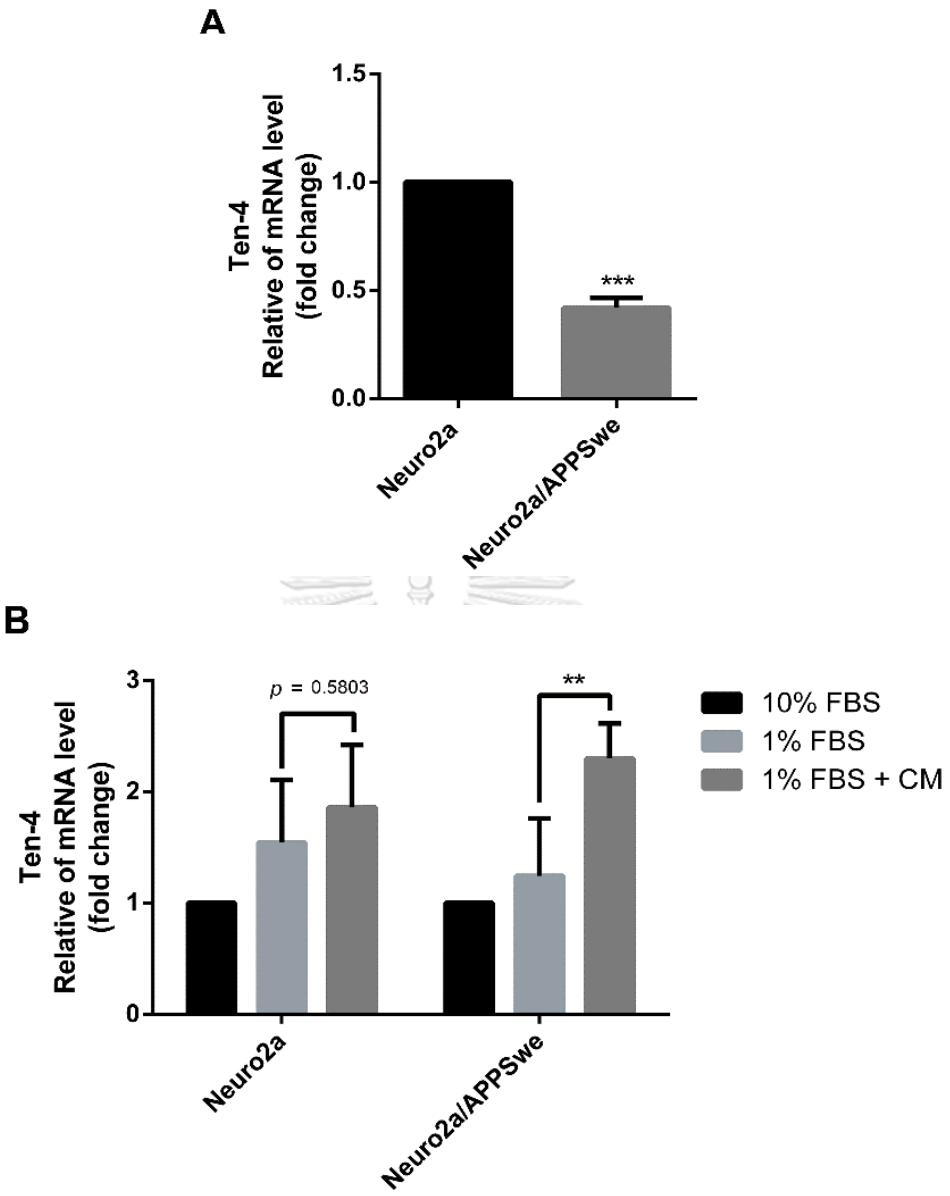



**Figure 31. Ten-4 mRNA expression level.**

(A) Neuro2a/APPSwe cells showed significant lower Ten-4 gene expression than Neuro2a cells in 1% FBS medium after 48 h treatment. (B) Ten-4 gene expression significantly increased in both Neuro2a and Neuro2a/APPSwe cells when treatment the cells with 10 µg/ml of CM extract for 48 h compared to 1% FBS treatment in each cell. Values are mean ± SD of at least 3 independent experiments. ** = *p*-value < 0.01, *** = *p*-value < 0.001.

**1.6 Effects of APP overexpression and CM extract on Lingo-1 gene expression**

Lingo-1, also known as leucine rich repeat and immunoglobin-like domain-containing protein 1, is a transmembrane signaling protein that negatively regulate neurite outgrowth [111] and is shown to have a physical interaction with APP [114]. By comparing mRNA expression level of Lingo-1 under differentiated condition induced by 1 % FBS medium control, Swedish mutant APP-overexpressing neurons exhibited a significant higher gene expression than the normal neurons expressing wild-type APP by 2.02-folds (*p*-value = 0.0002) **(Figure 32A)**. Notably, the Lingo-1 mRNA level showed no significant difference between treatment of Neuro2a or Neuro2a/APPSwe cells with 10 % FBS and 1% FBS medium control. However, the Lingo-1 expression level was found significantly decreased in both cell lines after 48 h exposure to 1 % FBS medium control along with 10 µg/ml CM extract when compared to 1 % FBS medium control alone (*p*-value = 0.0372 and 0.0086, respectively) **(Figure 32B)**.

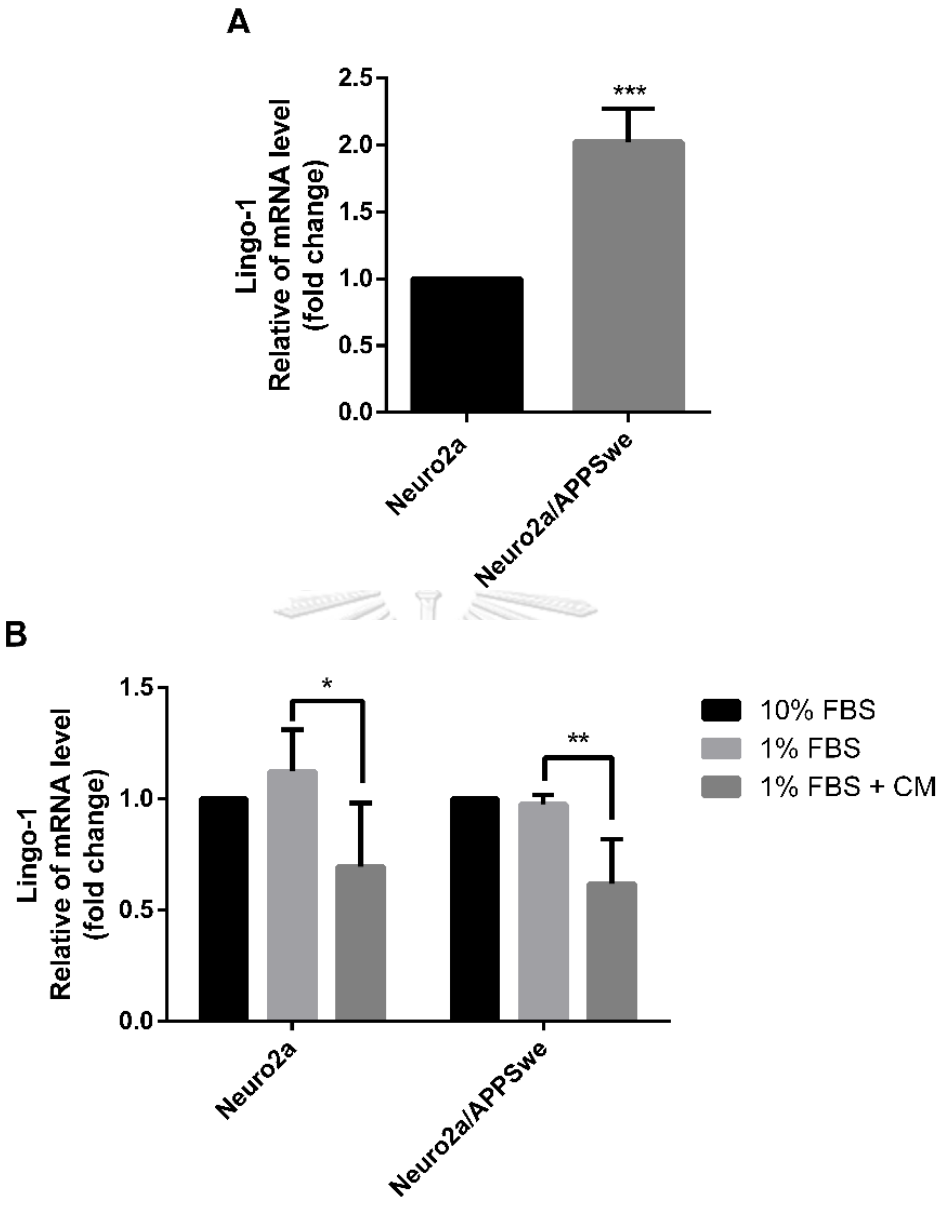



**Figure 32. Lingo-1 mRNA expression level.**

(A) Neuro2a/APPSwe cells showed significant higher Lingo-1 gene expression level than Neuro2a cells in 1% FBS medium after 48 h treatment. (B) Lingo-1 gene expression level was significantly increased in both Neuro2a and Neuro2a/APPSwe cells when treatment the cells with 10 µg/ml of CM extract for 48 h compared to 1% FBS treatment in each cell. Values are mean ± SD of at least 3 independent experiments. * = *p*-value < 0.05, ** = *p*-value < 0.01, *** = *p*-value < 0.001.

**1.7 Effects of APP overexpression and CM extract on NgR gene expression**

The gene expression of NgR or Nogo receptor which is a downstream signaling protein of Lingo-1 was further investigated. Under differentiated condition induced by 1 % FBS medium control, the APP-overexpressing Neuro2a cells exhibited a significant higher NgR mRNA expression by 2.46-fold when compared to normal neurons expressing wild-type APP (*p*-value = 0.0029) **(Figure 33A)**. Similarly to the result of Lingo-1 gene expression, the significant difference of NgR mRNA expression was not observed when compared between treatment of Neuro2a or Neuro2a/APPSwe cells with 10 % FBS and 1% FBS medium control. However, the NgR gene expression was found significantly decreased in both cell lines (*p*-value = 0.0392 and 0.0028, respectively) after exposure to 1 % FBS medium plus 10 µg/ml CM extract for 48 h, compared to 1 % FBS medium control alone **(Figure 33B)**.

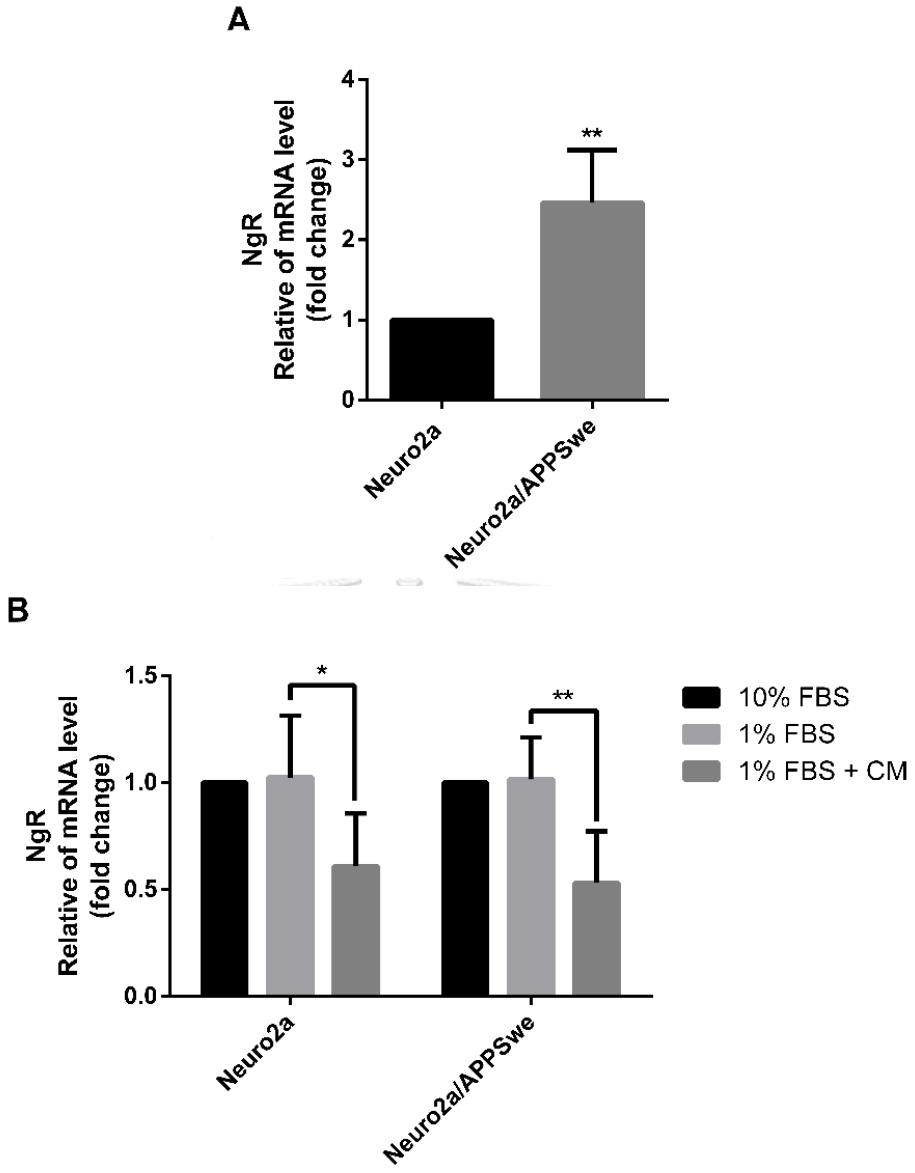



**Figure 33. NgR mRNA expression level.**

(A) Neuro2a/APPSwe cells showed significant higher NgR gene expression level than Neuro2a cells after 48 h of treatment in 1% FBS medium. (B) NgR gene expression level in both Neuro2a and Neuro2a/APPSwe cells significantly decreased when treatment with 10 µg/ml of CM extract for 48 h compared to 1% FBS treatment in each cell. Values are mean ± SD of at least 3 independent experiments. * = *p*-value < 0.05, ** = *p*-value < 0.01.

**1.8 Effects of APP overexpression and CM extract on BACE1 gene expression**

When compared BACE1 gene expression between Neuro2a and Neuro2a/APPSwe cells after incubation in 1%FBS for 48 h, BACE1 mRNA level in Neuro2a/APPSwe cells was significant higher than the wild-type cells by 1.54 folds (*p*-value = 0.0002) **(Figure 34A)**. 10% FBS and 1% FBS treatments within cells were no different in BACE1 gene expression both Neuro2a and Neuro2a/APPSwe cells after incubation for 48 h. Interestingly, the CM extract treated neurons were not affected on BACE1 mRNA level both wild type and APP-overexpressing cells **(Figure 34B)**.

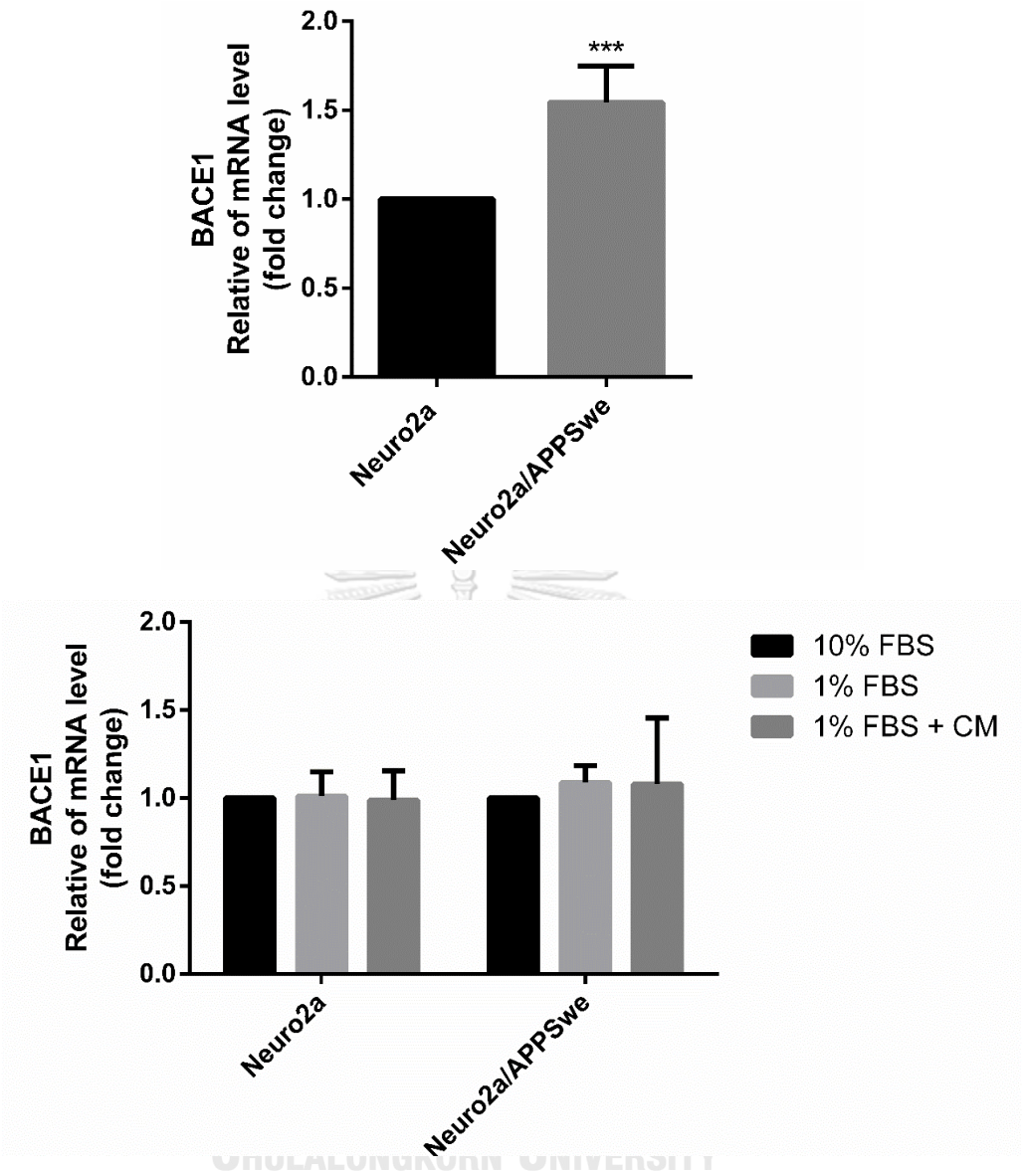



**Figure 34. BACE1 mRNA expression**

(A) Neuro2a/APPSwe cells showed significant higher level of BACE1 gene expression than Neuro2a cells after 48 h of treatment in 1% FBS medium. (B) BACE1 gene expression level in both Neuro2a and Neuro2a/APPSwe cells was not altered when treatment with 10 µg/ml of CM extract for 48 h compared to 1% FBS treatment in each cell. Values are mean ± SD of at least 3 independent experiments. *** = *p*-value < 0.001.

**2. Antioxidant and Anti-Aging Activity of *CM* Extract in *C. elegans***

**2.1 In vitro evaluation of antioxidant properties of CM young twig and leaf extracts**

DPPH and ABTS assay were used to investigate free radical scavenging capacities of CM extract in vitro. DPPH and ABTS assays were used to investigate the free radical scavenging capacities of CM extracts in vitro. The effective concentration (EG50) was recorded. Methanol extracts possessed strong antioxidant activities because they exhibited high scavenging activities against DPPH and ABTS radicals (Teble 2).

**Table 2. Free radical scavenging capacities of** CM **young twig and leaf extracts.**

| Samples | $EC_{50}$ | |
|---|---|---|
| | DPPH assay | ABTS assay |
| CM methanol (µg/ml) | 8.20 ± 0.29 | 5.16 ± 0.98 |
| Vitamin C (µM) | 40.50 ± 0.27 | 26.99 ± 0.41 |
| EGCG (µM) | 15.56 ± 0.10 | 8.95 ± 0.34 |

*Values are expressed as the mean ± SD (n=3)*

**2.2 Antimicrobial activity of CM extract**

To exclude antimicrobial activity of CM extract to kill *E. coli* OP50, a food source of *C. elegans*, agar diffusion method was used. The result found that CM extract as well as DMSO had no effect on *E. coli* OP50. While the bacteria did not allow to grow in the ampicillin well. The result may suggest that CM extracts did not interfere with E coli in the experiments. Thus, the life extension and stress resistant effects of CM extracts may not depend on the antimicrobial activity, and the extracts may promote these effects by acting directly to the worms.

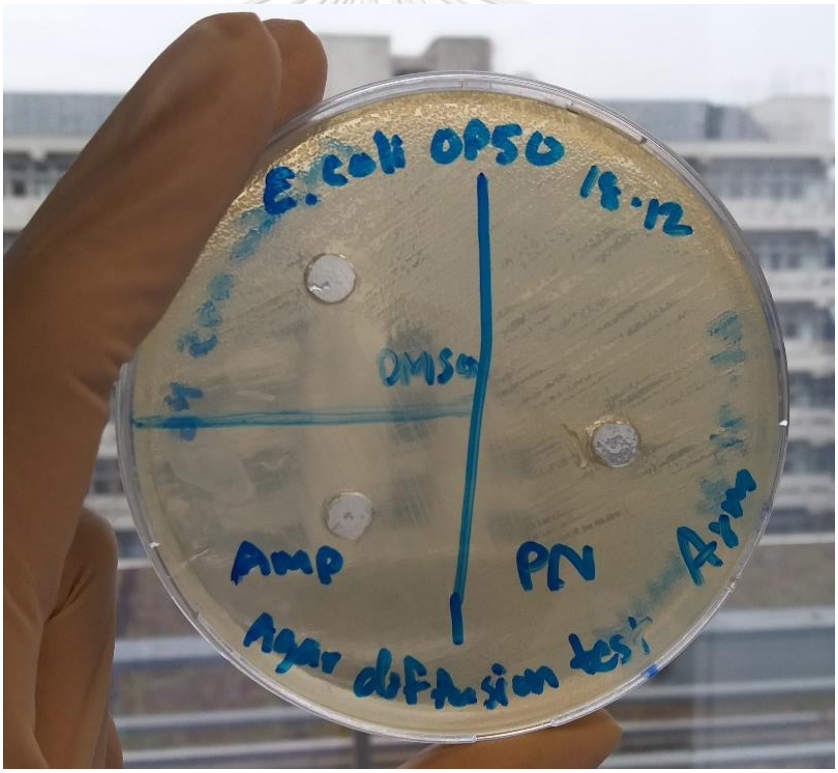



**Figure 31. Antimicrobial activity test of CM extract against *E. coli* OP50.**

CM (PN in the figure was referred to Pak Nam, its Thai name) extract at the concentration 200 µg/ml. Ampicillin and DMSO were used as positive and negative control, respectively. CM extract showed no effect on *E. coli* OP50.

**2.3 Effect of CM extract against juglone-induced oxidative stress in wild type worms**

Juglone, a yellow pigmented pro-oxidant quinone found in *Juglans regia,* is used to induce death of the nematode at a high dose.[22] Pre-treatment of the worms with CM extracts (25, 50 and 100 µg/ml) enhanced survival rates. CM extract 50 µg/ml showed the highest percentage (69.11 ± 2.36%) when compared to the DMSO solvent control (20.79 ± 0.3865) (*p-value* <0.001) **(Figure 35)**.

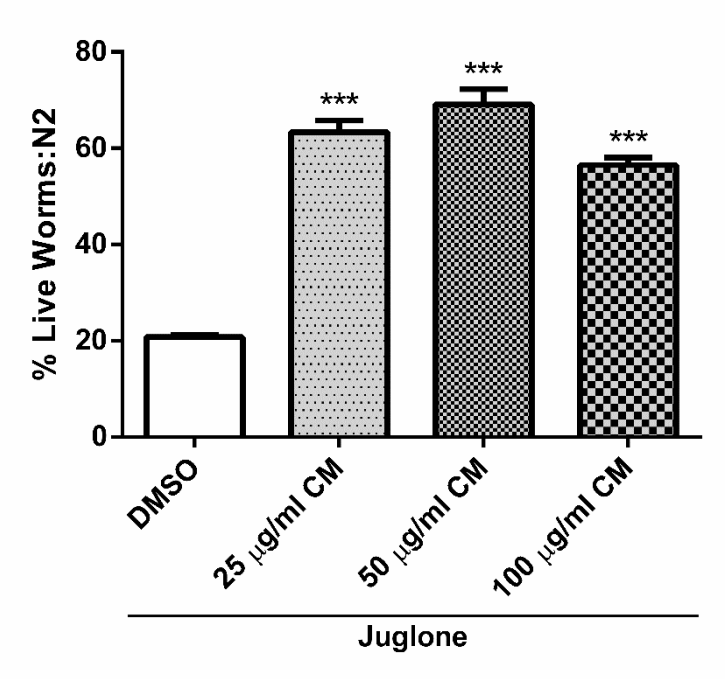



**Figure 35. Effect of Different concentrations of CM extract on survival rate under juglone-induced oxidative stress in N2 worms.**

Survival rate was significantly Increase after CM treatment. Values are mean ± SEM of at least 3 independent experiments. ***Different of DMSO control ($p < 0.001$).

**2.4 Effect of CM extract on intracellular ROS accumulation in wild type worms**

To further test the antioxidant effect of CM extract in vivo, intracellular ROS levels were evaluated using wild type N2 worms. H2DCF-DA, is a widely known marker for detecting the production of ROS inside the cell. The ROS level correlates with the fluorescence intensity resulting from deacetylated of H2DCF-DA by the intracellular esterases and forming the highly fluorescent 2'7'-dichlorofluorescin.[23] Results showed a significant decrease in the fluorescent intensity of the extract treated group (25, 50 and 100 µg/ml), up to 61.79% decrease in CM extract 50 µg/ml compared to DMSO group (*p-value* <0.001) **(Figure 36)**.

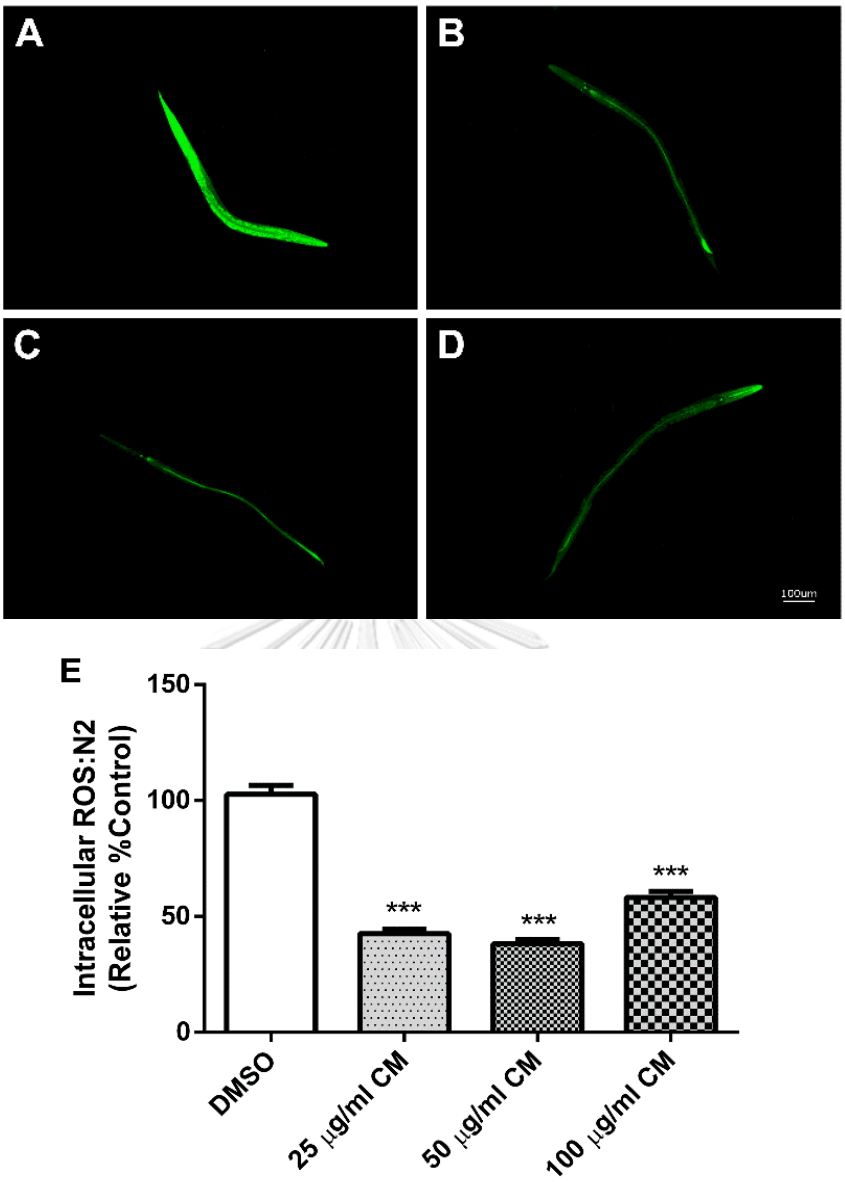



**Figure 36. Effect of CM extracts on intracellular ROS accumulation.**

N2 worms were treated with DMSO (A) and CM extracts 25, 50 and 100 μg/ml (B-D, respectively) incubated with H2DCF-DA for measurement of intracellular ROS accumulation. Intracellular ROS was significantly decrease after CM treatment (E). Values are mean ± SEM of at least 3 independent experiments. ***Different of DMSO control ($p < 0.001$).

**2.5 Effect of CM extract on HSP-16.2 expression**

TJ375 transgenic worms expressing heat shock protein (HSP)-16.2 is used as a marker of oxidative stress in the worms. To further support the antioxidant effect of the extract, HSP-16.2 level was investigated. The expression of HSP-16.2 was reduced in the worm treated with CM extract (25, 50 and 100 µg/ml) induced by juglone. CM extract 50 µg/ml showed a lowest expression of HSP-16.2 (46.63 ± 5.93 %) when compared to solvent control + juglone treated group (*p-value* <0.001) **(Figure 37)**.

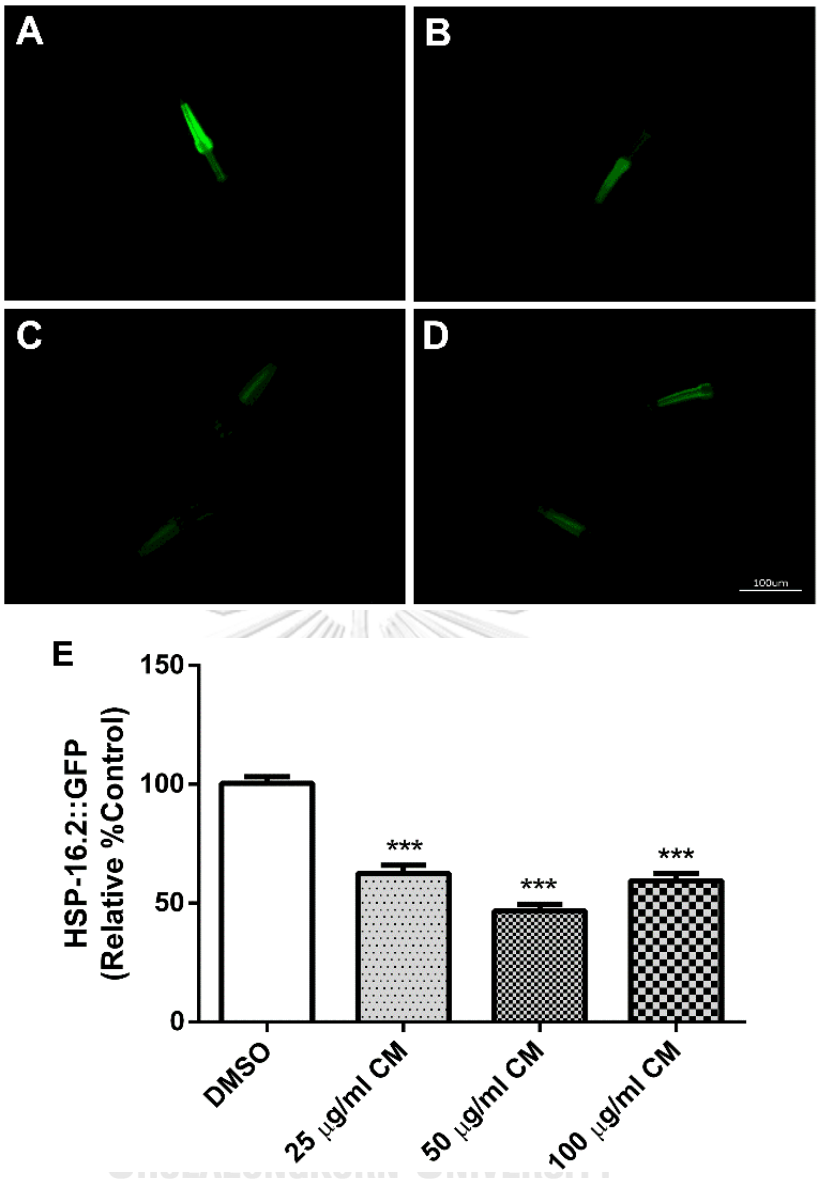



**Figure 37. To assessment of HSP-16.2::GFP expression after CM treatment.**

TJ375 worms were treated with DMSO (A) and CM extracts 25, 50 and 100 μg/ml (B-D, respectively). The expression of HSP::GFP significantly decrease after CM treatment (E). Values are mean ± SEM of at least 3 independent experiments. ***Different of DMSO control ($p < 0.001$).

**2.6 Effect of CM extract on DAF-16/FOXO pathway**

To investigate the mechanisms involved antioxidant effect of CM extract, DAF-16 transcription factor of *C. elegans*, homologue to the fork head transcription factor (FOXO) found in humans, was detected using TJ356 transgenic worms. The treatment with CM extract (25, 50 and 100 µg/ml) enhanced DAF-16 translocation into nucleus. CM extract 50 µg/ml clearly exhibited high nuclear subcellular localization of DAF-16::GFP by 65.02 ± 4.55%, compared to DMSO group with 9.96 ± 2.97% nuclear localization (*p-value* <0.001). The results highly indicated that antioxidant effect of CM extract was activated through DAF-16/FOXO pathway **(Figure 38)**.

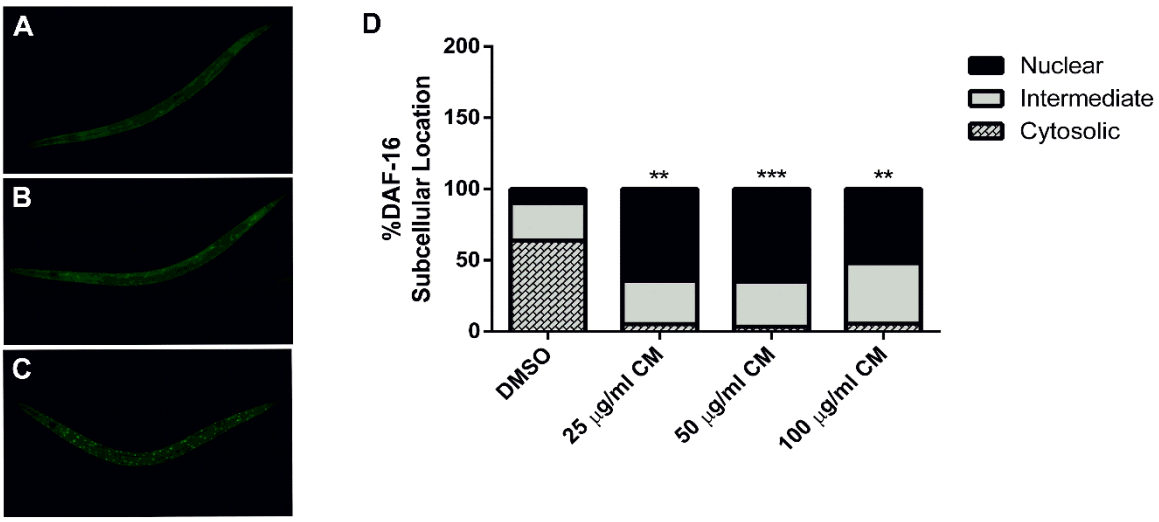



**Figure 38. Effect of CM extract on DAF-16 translocation.**

DAF-16 locations in TJ356 worms: Cytosol (A), intermediate (B) and nucleus (C). CM extract-treated worms significantly increase DAF-16 translocation to the nucleus (D). Values are mean ± SEM of at least 3 independent experiments. **Different of DMSO control ($p < 0.01$), ***Different of DMSO control ($p < 0.001$).

DAF-16 activation subsequently results in activating other stress response genes like SOD-3, which is an enzyme protecting the worms against ROS. CM extract-treated worms at the concentration of 25 and 50 µg/ml showed a higher expression of the SOD-3::GFP (119.8 ± 2.315 and 130.5 ± 2.392, respectively) compared to the solvent control group (*p-value* < 0.001). However, CM extract 100 µg/ml showed no different in fluorescence intensity compared to DMSO group **(Figure 39 - 40)**.

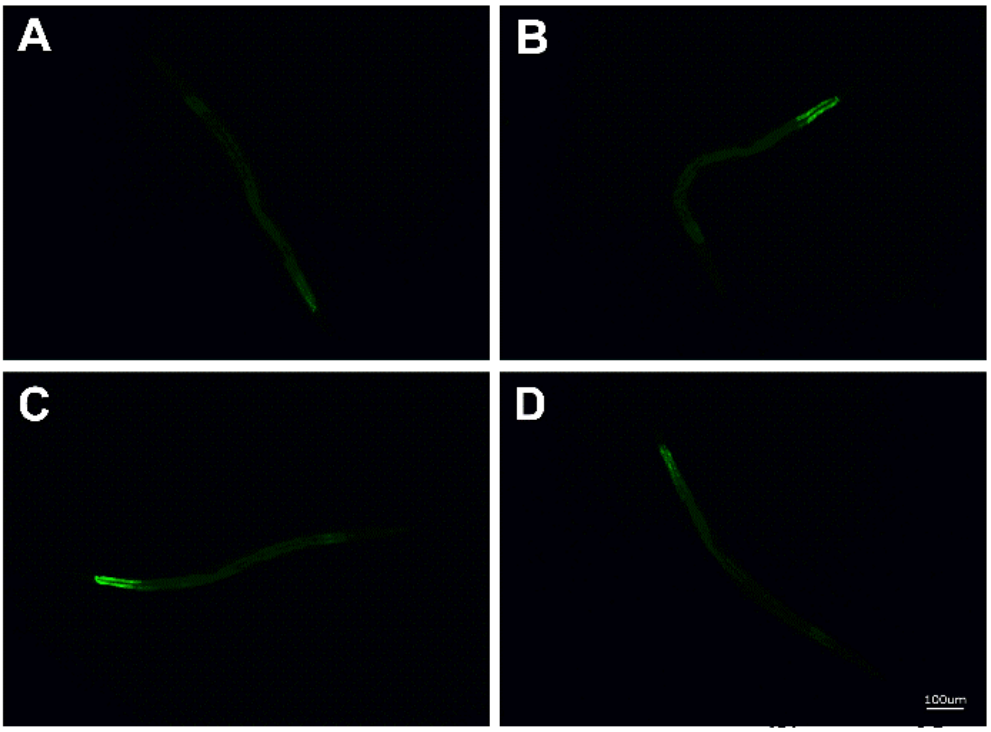



**Figure 39. Effect of CM extracts on SOD-3::GFP expression (1).**

CF1553 worms were treated with DMSO (A) and CM extracts 25, 50 and 100 µg/ml (B-D, respectively).

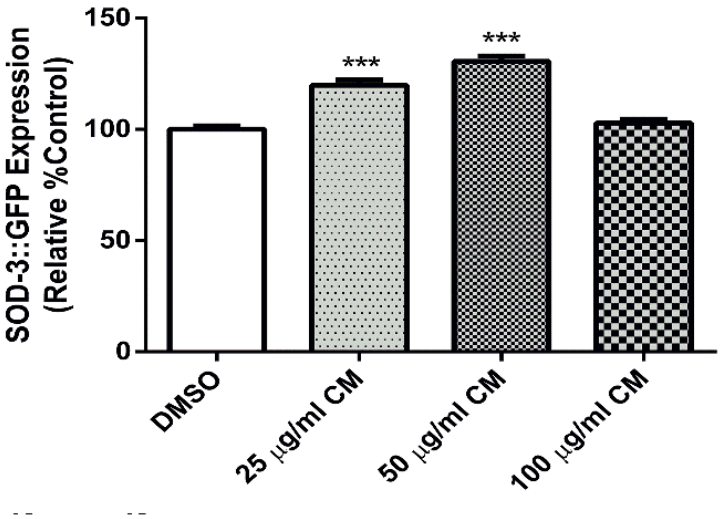



**Figure 40. Effect of CM extracts on SOD-3::GFP expression (2).**

CF1553 worms were treated with DMSO (A) and CM extracts 25, 50 and 100 μg/ml. The expression of SOD-3::GFP significantly enhance after CM treatment. Values are mean ± SEM of at least 3 independent experiments. ***Different of DMSO control ($p < 0.001$).

To further support antioxidant effect of CM extract via DAF-16/FOXO pathway *in vivo*, DAF-16 loss-of-function transgenic worms CF1038 were employed to test survival assay under juglone-induced oxidative stress and intracellular ROS accumulation. CM extract-treated DAF-16 mutant worms were unable to increase survival rate under juglone-induced oxidative stress. Similar with intracellular ROS accumulation, the difference between CM extracts and DMSO also could not be detected **(Figure 36)**.

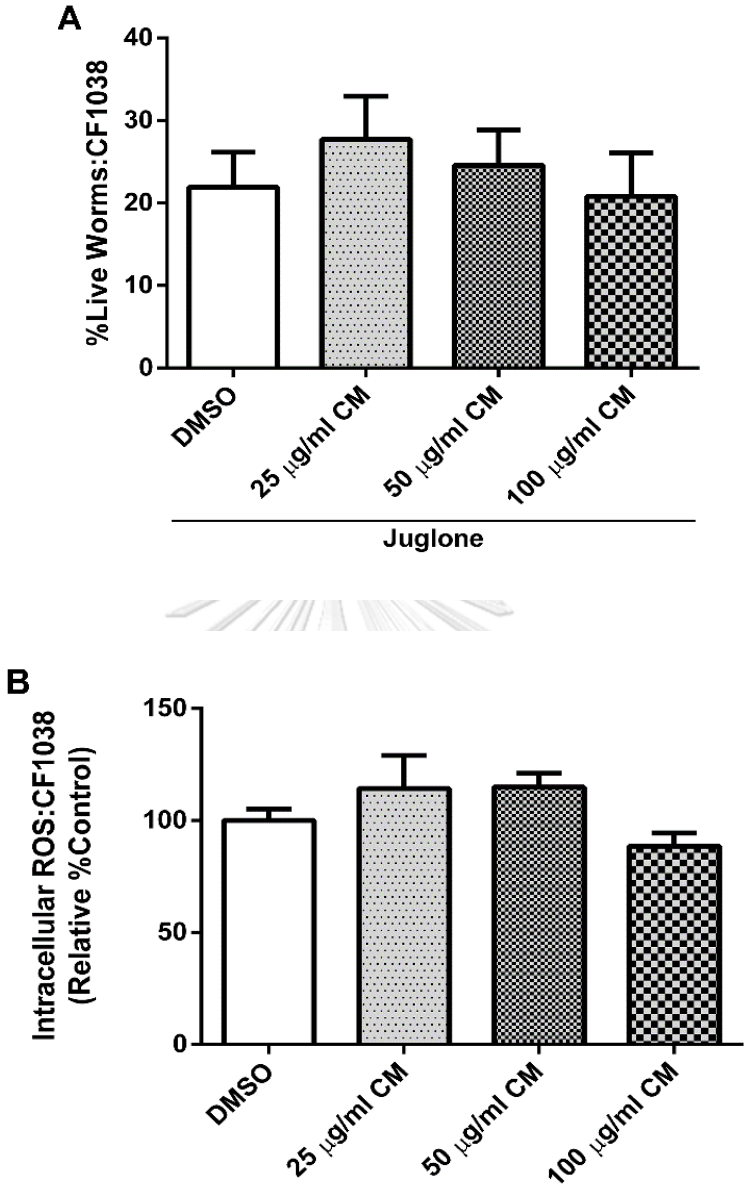



**Figure 41. The confirmation of DAF-16 pathway.**

CF1038 (DAF-16 loss of function) worms were used to confirm the effect of CM extract on DAF-16/FOXO pathway by survival assay under juglone-induced oxidative stress (A) and Intracellular ROS measurement (B) which the worms failed to counteract with stress and reduce intracellular ROS, respectively.

**2.7 Effect of CM extract on SKN-1/NRF-2 pathway**

SKN-1, a homologue to the mammalian NRF2 transcription factor, is well known as antioxidant pathway. The treatments of CM extract (25, 50 and 100 µg/ml) showed no effect to the worms in SKN-1 nuclear translocation when compared to DMSO control group **(Figure 42)**.

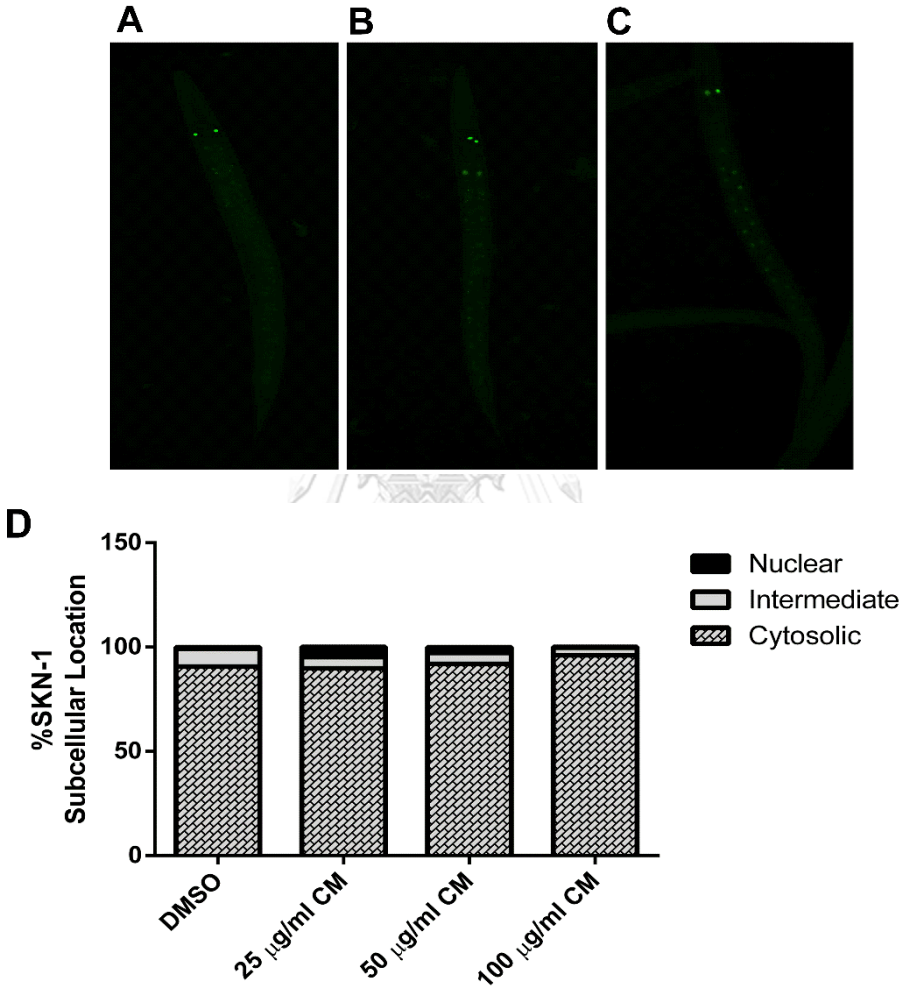



**Figure 42. Effect of SKN-1 translocation after treatment with CM extracts.**

SKN-1 locations in LD1 worms: Cytosol (A), intermediate (B) and nucleus (C). No alteration of SKN-1 subcellular localization was observed (D). Values are mean ± SEM of at least 3 independent experiments.

GST-4 is one of the glutathione S-transferases that is involved in the response towards oxidative stress. This enzyme is regulated by the SKN-1 transcription factor. Results also showed no effect of CM extract-treated worms (25, 50 and 100 µg/ml) in the expression of GST-4 when compared to DMSO control group **(Figure 38)**.

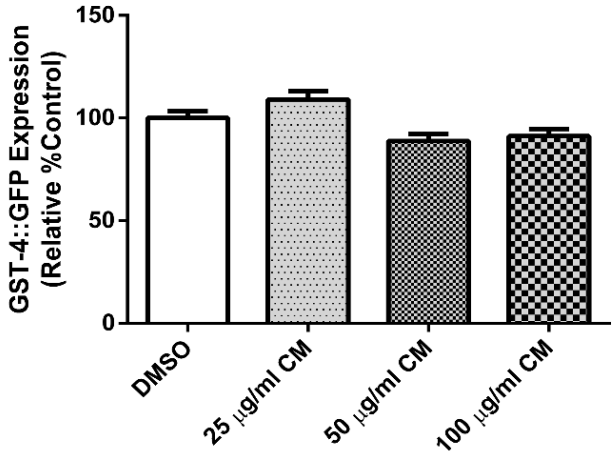



**Figure 43. Effect of CM extracts on GST-4::GFP expression.**

CL2166 worms were treated with DMSO and CM extracts 25, 50 and 100 µg/ml for measurement of GST-4::GFP expression (E). Values are mean ± SEM of at least 3 independent experiments.



**2.8 Effect of CM extract on lipofuscin level**

Intestinal cells of *C. elegans* contain lysosomes and gut granules called lipofuscin which can express autofluorescence. Lipofuscin accumulation can increase during oxidative stress and aging. The expression of lipofuscin was reduced in the worm treated with CM extract (25, 50 and 100 µg/ml). CM extract 50 µg/ml showed the highest decrease of autofluorescence (16.60 ± 1.10 %) when compared to solvent control treated group (*p-value* <0.001) **(Figure 44)**.

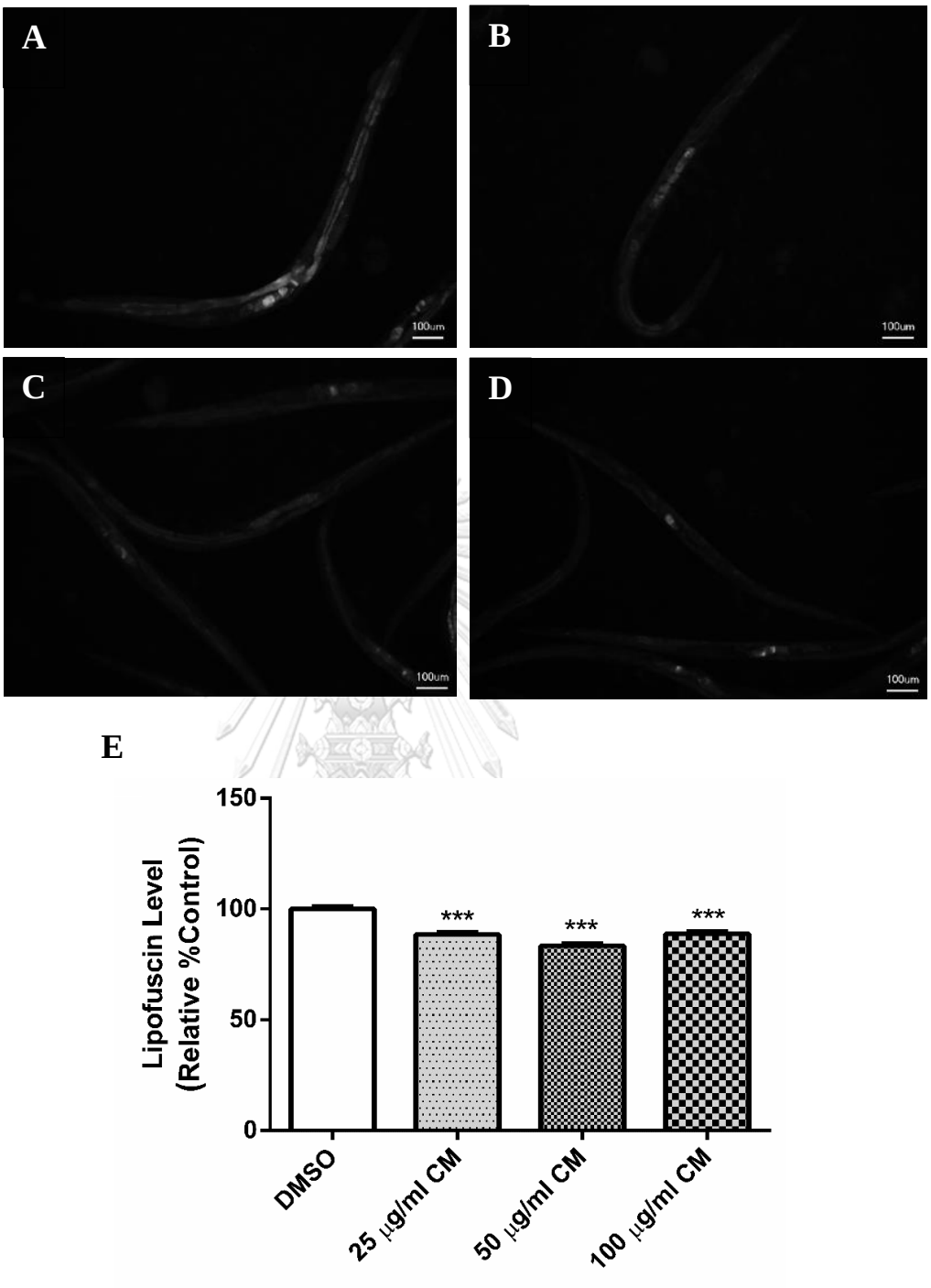



**Figure 44. Effect of CM extract on aging pigment lipofuscin level.**

BA17 worms were treated with DMSO (A) and CM extracts 25, 50 and 100 μg/ml (B-D, respectively). CM-treated worms significantly attenuate lipofuscin level. Values are mean ± SEM of at least 3 independent experiments. ***Different of DMSO control ($p < 0.001$).

**2.9 Effect of CM extract on body length and brood size**

To investigate toxicity of CM extract treatment, body length and brood size were performed to test development and fertility rate of the worms, respectively. Analyses of the body length revealed no difference of mean length per worm treated with 25, 50 and 100 µg/ml CM extract when compared with the DMSO control group **(Figure 46A)**. The brood size also did not show alteration of number of eggs laid after CM extract treatment **(Figure 46B)**.

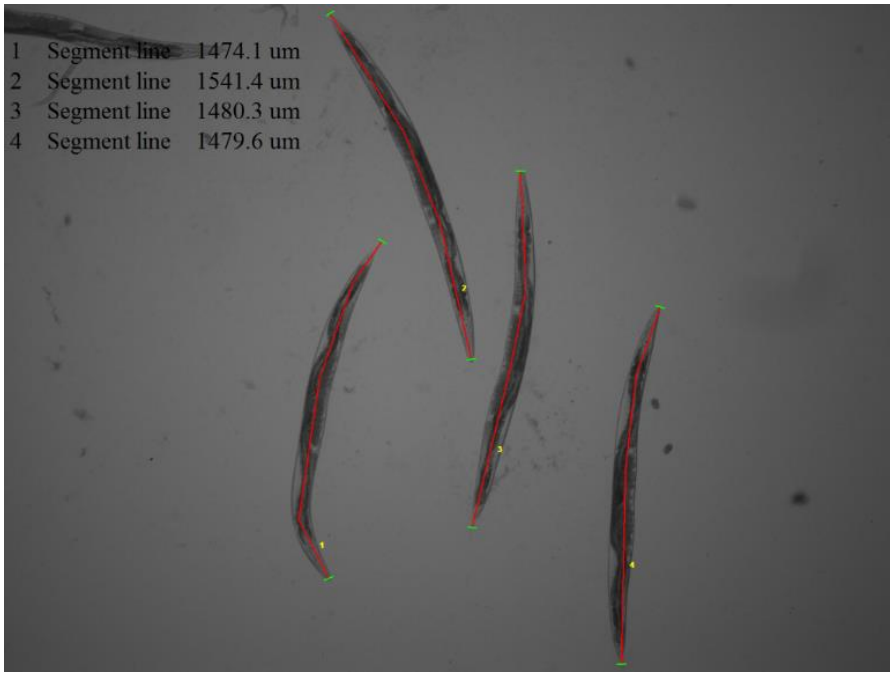



**Figure 45. The measurement of the worm's body length.**

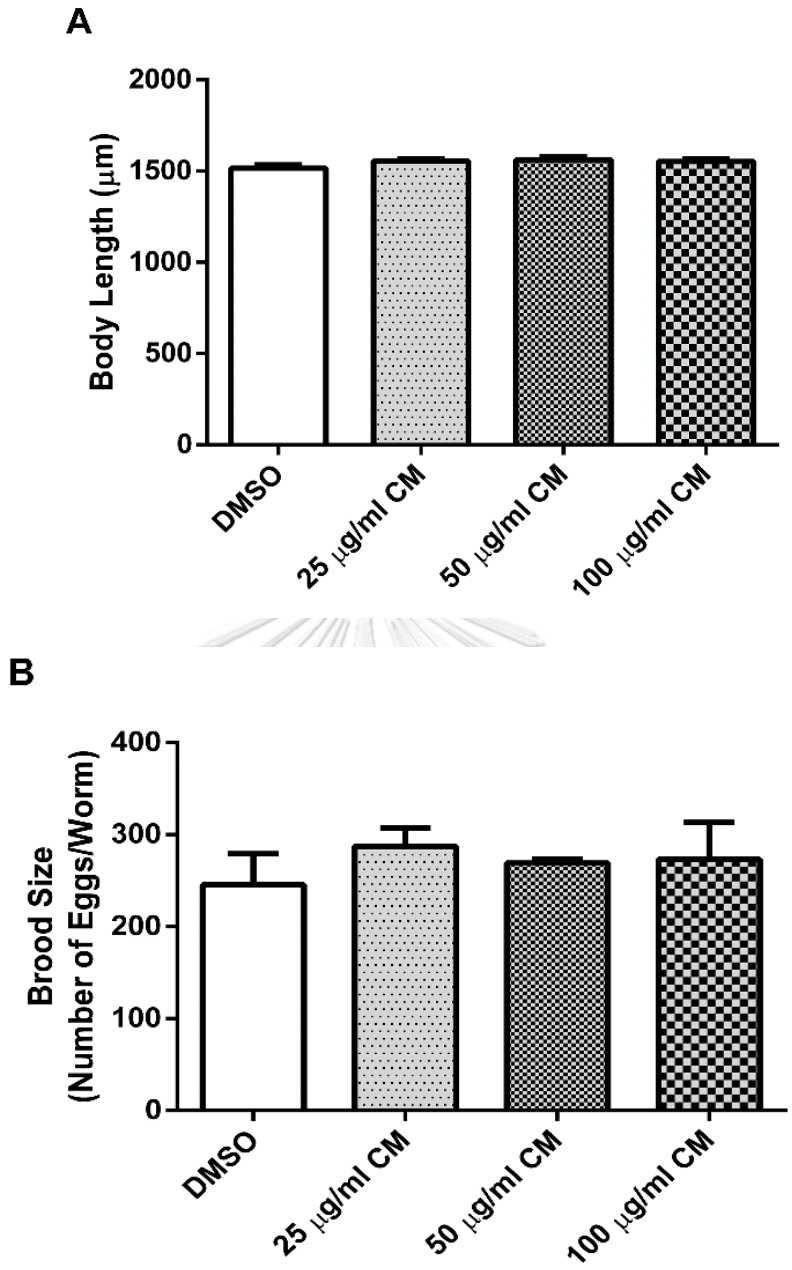



**Figure 46. Toxicity test of CM extract treatment.**

Effect of CM extracts on body length (A) and brood size (B) in N2 worms. Values are mean ± SEM of at least 3 independent experiments.

**3. Phytochemical Analysis of *Caesalpenia mimosoides* Methanol Extract**

**3.1 Chemical characterization of CM methanol extract using LC-MS**

The candidate phytochemical constituents in methanolic extract of *C. mimosoides* young twigs and leaves were predicted and proposed using LC-MS. The result showed the isolated peaks in the chromatogram of CM extract **(Figure 47)**. Chromatographic peaks were identified by comparison with databases for proposed compounds based on the search of m/z values of molecular ion peaks in the positive mode $[M + H]^+$. The five major phytochemical compounds in CM methanol extract were 3-O-Methylgallate, 4-Aminomethylindole, Emmotin A, Theogallin and Gallic acid (**Table 3**).

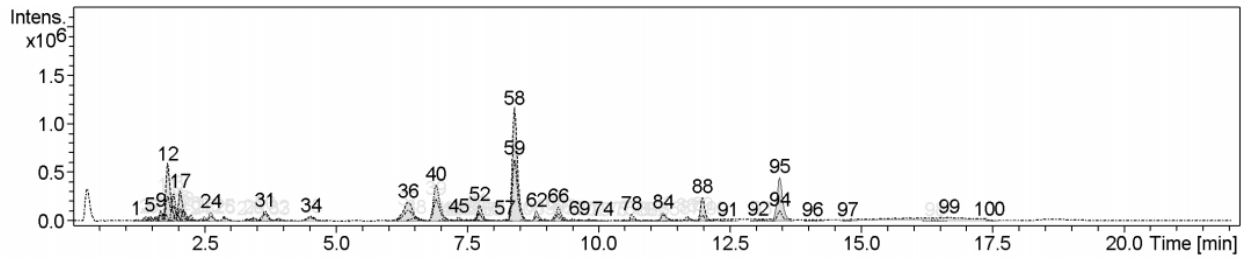



**Figure 47. LC-MS profile of CM methanol extract.**

The total ion chromatogram (TIC) was generated by LC-MS under positive electrospray ionization. Peak numbers correspond to those in **Table 3**.

**Table 3. Proposed phytochemical constituents in methanolic extract of CM young twigs and leaves using LC-MS**

| Peak No. | Rt (min) | $[M + H]^+$ (m/z) | Area (%) | Proposed compound | Theoretical mass | Mass error (ppm) |
|---|---|---|---|---|---|---|
| 12 | 1.8 | 146.0824 | 5.9 | 4-Aminomethylindole | 145.0766 | 9 |
| 17 | 2.0 | 256.1181 | 3.0 | N-D-Glucosylarylamine | 255.1107 | 0 |
| 36 | 6.4 | 171.0288 | 4.4 | Gallic acid | 170.0215 | 0 |
| 40 | 6.9 | 345.0818 | 5.6 | Theogallin | 344.0743 | 0 |
| 52 | 7.7 | 329.0869 | 1.6 | Bergenin | 328.0794 | 0 |
| 58 | 8.4 | 185.0461 | 13.0 | 3-O-Methylgallate | 184.0372 | 8 |
| 59 | 8.4 | 185.0445 | 7.2 | 3-O-Methylgallate | 184.0372 | 0 |
| 62 | 8.8 | 481.0979 | 1.1 | Quercetin-3'-glucuronide | 480.0904 | 0 |
| 66 | 9.2 | 465.1032 | 1.6 | Quercetin-3-O-glucoside | 464.0955 | 0 |
| 88 | 12.0 | 415.2117 | 2.3 | Clausarinol | 414.2042 | 0 |
| 95 | 13.4 | 279.1588 | 5.7 | Emmotin A | 278.1518 | 1 |

*Database: METLIN (CA, USA) and KNApSAcK Keyword Search Web Version 1.000.01*

**3.1 Quantification of gallic acid and quercetin of CM extract using HPLC**

Gallic acid and quercetin, previously reported to increase neurite outgrowth activity [24-27], were quantified in methanol extract of twigs and leaves of CM. The HPLC chromatograms of CM methanol extract showed peak representing gallic acid and quercetin at the retention time of 11.56 and 41.73 min, respectively **(Figure 48)**. Based on calculation of the external standard curves, the CM methanol extract contained high amount of gallic acid with small quercetin content at 7,815.17 ± 25.09 mg and 36.33 ± 0.51 mg s per 100 g crude extract, respectively **(Table 4)**.

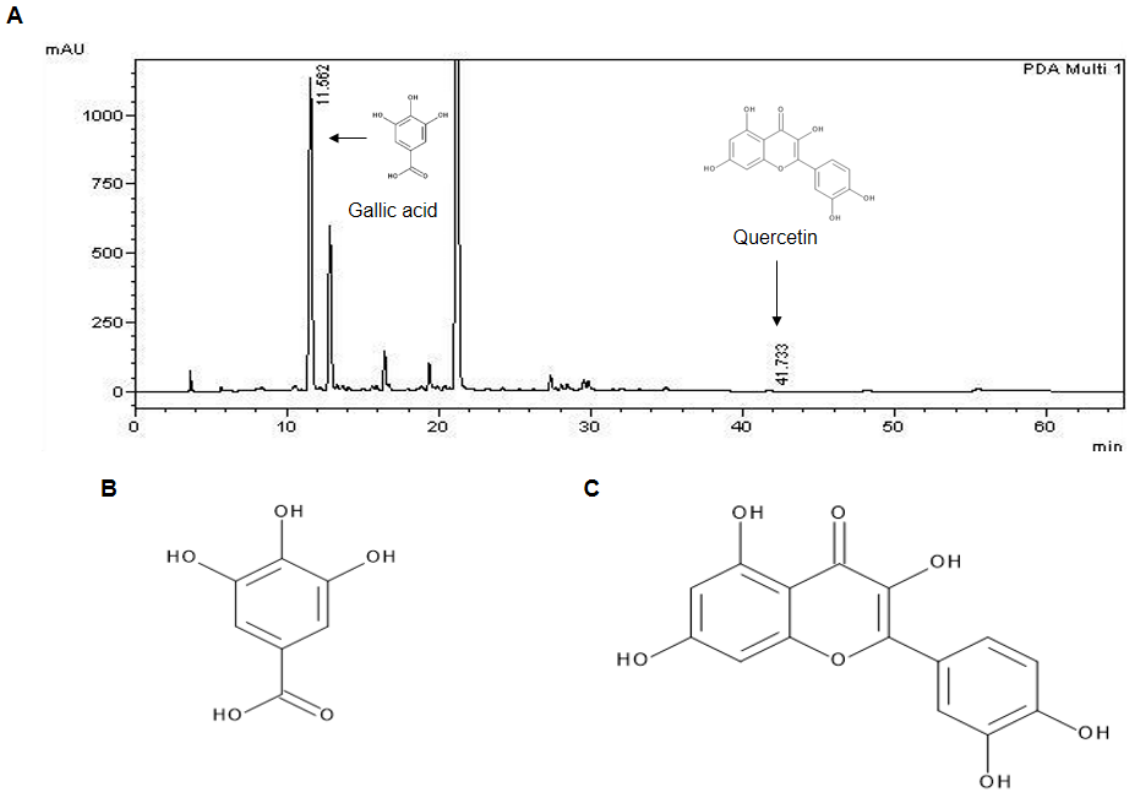



**Figure 48. HPLC analysis of CM extract.**

**Figure 43.** HPLC chromatogram (A). Gallic acid and quercetin in CM methanol extract were quantified using HPLC analysis. The peaks at the retention time of 11.56 and 41.73 min represented as gallic acid and quercetin, respectively. (B) Gallic acid. (C) Quercetin.



**Table 4. Gallic acid and quercetin content in CM methanol extract.**

| Compounds | Content (mg) in 100 g of crude extract |
|---|---|
| Gallic acid | 7,815.17 ± 25.09 |
| Quercetin | 36.33 ± 0.51 |

# CHAPTER V

## Discussion and Conclusion

Several plants and natural products have been studied for used as herbal therapies which were one of the most common used forms of complementary and alternative medicine [119]. Recently, a large number of plants and natural products and their isolated compounds possessing neurite outgrowth activity have been discovered for the treatment of neurodegenerative diseases. Here we established an alternative strategy for a discovery of Alzheimer's disease (AD) treatment. Since APP overexpression has negatively affected neurite outgrowth activity [3], *C. mimosoides* (CM) extract treatment was able to ameliorate the activity against APP overexpression in cultured neurons.

The major compounds of the extract identified using LC/MS included gallic acid, theogallin, bergenin, 3-O-methylgallate, quercetin-3'-glucuronide, quercetin-3-O-glucoside, clausarinol and emmotin A. The results showed that the methanol extract of *C. mimosoides* (CM) contains the neurite outgrowth stimulatory compounds including gallic acid and quercetin. To support this finding, HPLC analysis of CM methanol extract was performed and found that it exhibited high gallic acid content by 7.8% of the extract, while quercetin was found only by 0.04%. According to LC-MS results of CM methanol extract, it showed a high content of quercetin glycosides, including quercetin-3'-glucuronide and quercetin-3-O-glucoside. Because the structure and component of quercetin and quercetin glycosides are different leading to a difference in retention time in HPLC analysis. It is possible that CM methanol extract mainly contain quercetin glycosides rather than quercetin.



Although, the cause of AD is still not clearly understood, it is believed that the disease process is associated with hyperphosphorylated tau protein, a microtubule assembly protein accumulating intracellularly as neurofibrillary tangles (NFTs), and

amyloid-beta (Aβ) peptide deposited in diffuse and neuritic plaques in the brain [84]. Both tangles and plaques are found in brains of individuals afflicted by AD [85,153]. It is still unclear that what are the causes leading to abnormal hyperphosphorylation of tau, or Aβ accumulation occurs due to its overproduction or defect in the clearance [154-156].

Aβ, a hallmark protein found in patients with AD, is proceeded from a protein called amyloid precursor protein (APP) that is generated the Aβ fragment via β- and γ-secretase cleavage [86]. The mutation in APP gene also influence Aβ level in the brain. The well-known mutation in APP is Swedish mutation which has been reported to increase total Aβ level both production and secretion [88,89]. Moreover, overexpression of APP was reported to inhibit cell differentiation and neurite outgrowth in cultured Neuro2a cells [3]. Therefore, the therapeutic intervention of AD by inducing neuroregeneration or neurite outgrowth should also affect in the condition of APP overexpression.

The results found that neurite outgrowth was inhibited in Swedish mutation APP-overexpressing Neuro2a (Neuro2a/APPSwe) cells when compared to wild-type Neuro2a (Neuro2a/WT) cells. Furthermore, neurite outgrowth positively regulation gene, GAP-43 and Ten-4, were also decreased in Neuro2a/APPSwe cells. GAP-43 or Growth Associated Protein 43 plays a key role in neurite outgrowth that is expressed in neuronal growth cones during development [157,158]. Several evidences support that the up-regulation of GAP-43 by natural product treatment potentiate to induce neurite outgrowth [159-161]. Another neurite outgrowth marker in this study is Ten-4 or Teneurin-4, a transmembrane protein in the family of teneurin. It was positively regulated the formation of filopodia-like protrusion and neurite outgrowth [107]. Ten-4 activate neurite outgrowth by activation of focal adhesion kinase (FAK) and Rho-family small GTPases, Cdc42 and Rac1, key molecules for the membranous protrusion

formation downstream of FAK, respectively in Neuro2a cells [27]. Recently, herbal extract, *Mucuna pruriens,* was also reported to increase neurite outgrowth dependent on Ten-4 expression [162].

Recently, CM extract and its constituent compounds, quercetin and gallic acid, were reported to have neurite outgrowth enhancement activity in several cultured neuronal cells [24-27]. This study is the first report of neurite outgrowth inducing activity in APP-overexpressing neurons when treatment the cells with CM extract. Furthermore, the evidences of gene expression level of GAP-43 is up-regulated in both wild-type and APPSwe-overexpressing neurons after treatment indicated neurite outgrowth activity of the plant extract. However, there is a difference of the neurite outgrowth activity between normal and APPswe-overexpressing cells. In neuro2a cells, the number of neurite-bearing cells were significantly increased after treatment with CM extract, whereas neurite length was found to slightly increase but not significant. While both % of neurite bearing-cells and neurite length were significantly increased in Neuro2a/APPSwe cells after treatment with CM extract. It is possible that CM extract is major involve in neurite formation process rather than neurite elongation process in normal Neuro2a cells. Nevertheless, the difference of the effect of CM extract on neurite outgrowth processes should be further investigated.

Ten-4 mRNA level was significantly increased in CM extract-treated Neuro2a/APPSwe cells, whereas was slightly increased but not significant in CM extract-treated Neuro2a cells. Since Ten-4 regulate the formation of filopodia-like protrusion which is in the neurite formation process before further elongation of the neurite [107], it was able to support the evidence that CM extract has the major effect on neurite formation in Neuro2a cells. However, the difference of the effect of CM extract on Ten-4 gene expression in normal Neuro2a and Neuro2/APPSwe should be further clarified.

Lingo-1, or leucine-rich repeat neuronal protein 1, a transmembrane protein which is highly expressed in the brain and is implicated in several neurodegenerative diseases [108,109]. Its action remarkably relates to the Nogo receptor (NgR) as a part of a co-receptor complex leading to activates rho-associated coiled coil-containing protein kinase (RhoA/ROCK) signaling pathways, subsequently suppression of growth cones and further axonal growth [110]. It has been shown that Lingo-1 capable to directly bind to APP, promoting its proteolytic processed via inducing **β**-secreatase cleavage in amyloidogenic pathway [112]. According to our finding, APP Swedish mutant overexpressing neurons exhibited the up-regulation of Lingo-1, NgR and **β**-secreatase gene expression level. Interestingly, gene expression level of Lingo-1 and NgR were suppressed after neurite outgrowth induced by CM extract treatment. On the other hand, **β**-secretase gene expression level was found no alteration which its protein level and/or enzyme activity should be further investigated. Several evidences support that natural products can counteraction with NgR. Its expression level was inhibited and consequently promote neuritogenesis or neurite outgrowth when treatment with green tea and a variety of Chinese medicines [163-165]. Moreover, there was recently report that isoquercitrin a compound in the group of quercetin was found to promote neurite elongation via reducing RhoA activity [166].



Antioxidant compounds are considered for their free radical scavenging activity, which helps to ameliorate oxidative stress. Medical plants and natural products containing phenolic compounds are well-known to exhibit antioxidant activity. Current studies demonstrated that methanolic extract of *C. mimosoides* (CM) rich in phenols and flavonoids exhibit powerful free radical scavenging capacity *in vitro* via DPPH and ABTS assay. Accorging to LC-MS report, CM extract contained several compounds that were previously report to have antioxidant and anti-aging in *C.elegans* including gallic acid [167] and quercetin [33,168,169].

To further investigate antioxidant activity of CM extract *in vivo*, *C. elegans* was employed as a model organism. Wild-type worms treated with CM extract exhibited increasing survival rate under juglone-induced oxidative stress and lowering intracellular ROS level. In addition, HSP-16.2 which is a marker of oxidative stress was also significantly lower in CM treated worms. HSP-16.2 is a small heat shock protein that is induced by heating and oxidative stress in *C. elegans* [170,171]. Taken together, the treated worms showed and supported an effective antioxidant *in vivo* of CM extract.

In regard to investigate the mechanism underlying the antioxidant effect of CM extract, DAF-16/FOXO and SKN-1/NRF-2 pathways were observed in this study. The DAF-16 transcription factor of *C. elegans* is considered as a homologue to the fork head transcription factor (FOXO), found in humans. Normally, DAF-16 is localized in the cytosol in its inactive phosphorylated form. Oxidative damage can induce its dephosphorylation and subsequent translocalization to nucleus. Upon nuclear localization, DAF-16 activation is responsible for stress response and lifespan extension [55]. DAF-16 activation subsequently induces several genes that results in activating other stress response genes like SOD-3, which encodes mitochondrial Mn-SOD. The SOD-3 enzyme protects the worms from ROS via scavenging free radical [56].



SKN-1 transcription factor is a homologue to the human NRF2 which targets downstream genes for Phase II detoxification mechanism in *C. elegans* [62]. GST-4 is one of the glutathione S-transferases contributing to Phase II detoxification process and activated by SKN-1 [63]. This group of enzymes is involved in the response towards oxidative stress.

The CM extract-treated worms can up-regulate DAF-16 nuclear translocation whereas the localization of SKN-1 was unable to detect the alteration. When observe the down-regulated gene responding to DAF-16 and SKN-1, SOD-3 and GST-4

expression were detected, respectively. SOD-3 expression was found to increase in the worms after treatment with CM extract. In the other hand, GST-4 did not show the different after treatment. These results indicate antioxidant activity of CM extract through the activation of DAF-16/FOXO pathway. Similar results were also reported from previous studies using polyphenol rich plant extracts or isolated compounds protect *C. elegans* against oxidative stress via DAF-16/FOXO pathway [16,35,121-123].

The increase of ROS production is a contributory factor to the aging process [126]. Lipofuscin, is an indicator of oxidative stress and aging [127]. It is an autofluorescent pigment material that accumulates progressively over time and can be found accumulated in lysosomes and gut granules of intestinal cells of *C. elegans*. Lipofuscin inhibition is considered as a pathway counteracting against oxidative stress [172]. CM extract-treated worms demonstrated the lower expression of autofluorescent pigment. It is possible that the reduction of lipofuscin level was occurred during antioxidant activity and demonstrate a great evident to support its potential anti-aging capacity of CM extract. Several findings suggest that antioxidant compounds are able to prolong lifespan in *C. elegans*.[34,129-131] The treatment of CM extract also found to extend lifespan of the worms, demonstrating that CM extract has potent antioxidant and anti-aging activities with low toxicity according to no significant impairment in development and fertility rate.

In conclusion, CM extract can counteract with APP overexpression to stimulate neurite outgrowth involving with up-regulation of GAP-43 and Ten-4 gene expression as well as down-regulation of Lingo-1 and NgR gene expression. Further investigation at protein level after treatment with CM extract should be considered. In addition, the current study demonstrates the antioxidant and potential anti-aging activities of the *C. mimosoides* extract in *C. elegans.* The extract was able to increase stress resistance and able to decrease intracellular ROS level and the expression of HSP stress gene

after exposure to oxidative stress. The extract was also able to enhance nuclear localization of DAF-16 transcription factor and the expression SOD-3 gene, demonstrating antioxidant activity through DAF16/FOXO pathway, while the SKN-1/NRF2 pathway was not observed significant alteration. In addition, oxidative stress has been correlated with aging, and therefore, the observed effect of prolonged lifespan via the CM extract might be due to its antioxidant behaviors. These results suggest that *C. mimosoides* could have a potential as dietary supplement for antioxidant and anti-aging. However, higher model organisms are required for further investigation.

**Limitations of the stdudy**

1. The cell models used in this study were normal Neuro2a and Neuro2a/APPSwe cells. The major characteristic differences between these cells are level of APP expression level and Aβ level. In present study, APP level was measured using western blot analysis and was found clearly different between normal neuro2a and Neuro2a/APPSwe cells. However, Aβ level, the product in the amyloidosis pathway of APP cleavage and the major pathogenesis of AD, was not measured. To clarify that Neuro2a/APPSwe cells used in this study was a proper cell model representing as an AD cell model that has the ability to express the high level of Aβ, Aβ level should be further investigated. Moreover, Neuro2a/APPSwe used in this study was stably overexpressed Swedish mutation APP. To maintain the level of APPSwe expression in this cell line, the promoter used for stable protein expression have to strong enough to highly express APP. For this reason, CM extract treatment may not affect to APP level and gene that closely relate to APP like BACE1 gene in Neuro2a/APPSwe cells.

2. In this study, neurite outgrowth activity was determined by the measurement of % neurite bearing-cells and neurite length, which define as neurite formation and

neurite elongation process, respectively. To identify of neurite-bearing cells, the cells were scored positive if it bore a thin neurite extension that was double or more the length of the cell body diameter. To measure neurite length, the longest length of neurite of the cell was measured form cell membrane of cell body to the end of growth cone. However, in case of plant extract affect only neurite formation and has no ability to extend neurite length that was double or more the length of the cell body diameter, the neurite outgrowth will be scored false negative. To improve neurite outgrowth activity assay, the extent of branching (total number of branches and mean number of branches per cell) should be considered to investigate.

3. To improve the quality of the study, neurite outgrowth markers, MAP-2 or β-tubulin, should be stained using immunofluorescent staining for clarifying and distinguishing between neurite and pseudo-neurite.

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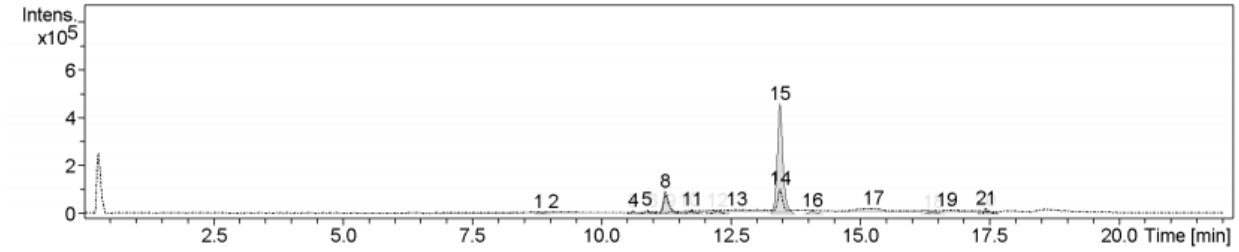
# APPENDIX

## LC-MS analysis

- Blank

**Acquisition Parameter**

| | | | | | |
|---|---|---|---|---|---|
| Source Type | ESI | Ion Polarity | Positive | Set Nebulizer | 1.2 Bar |
| Focus | Not active | Set Capillary | 4500 V | Set Dry Heater | 200 °C |
| Scan Begin | 50 m/z | Set End Plate Offset | -500 V | Set Dry Gas | 8.0 l/min |
| Scan End | 1000 m/z | Set Collision Cell RF | 200.0 Vpp | Set Divert Valve | Waste |

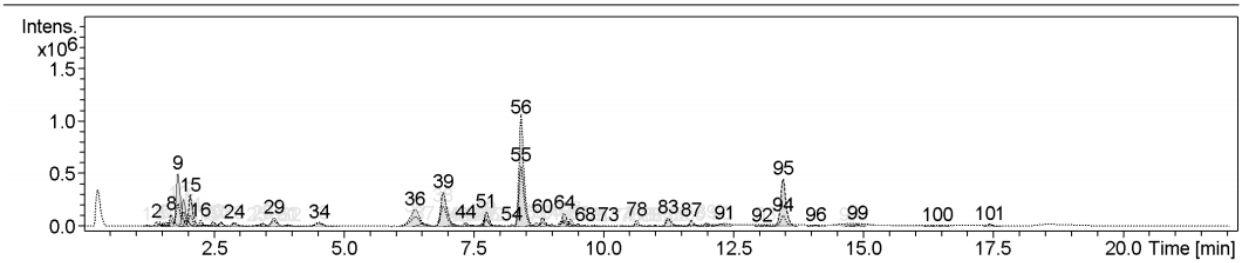



- CM extract repeat 1



**Acquisition Parameter**

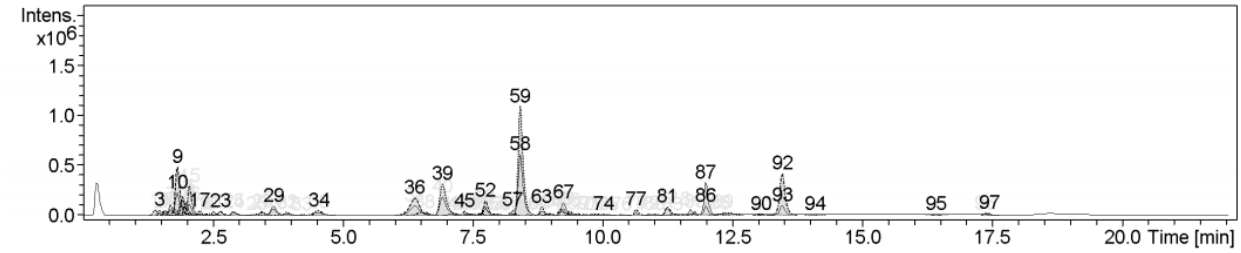
| | | | | | |
|---|---|---|---|---|---|
| Source Type | ESI | Ion Polarity | Positive | Set Nebulizer | 1.2 Bar |
| Focus | Not active | Set Capillary | 4500 V | Set Dry Heater | 200 °C |
| Scan Begin | 50 m/z | Set End Plate Offset | -500 V | Set Dry Gas | 8.0 l/min |
| Scan End | 1000 m/z | Set Collision Cell RF | 200.0 Vpp | Set Divert Valve | Waste |



- CM extract repeat 2

**Acquisition Parameter**

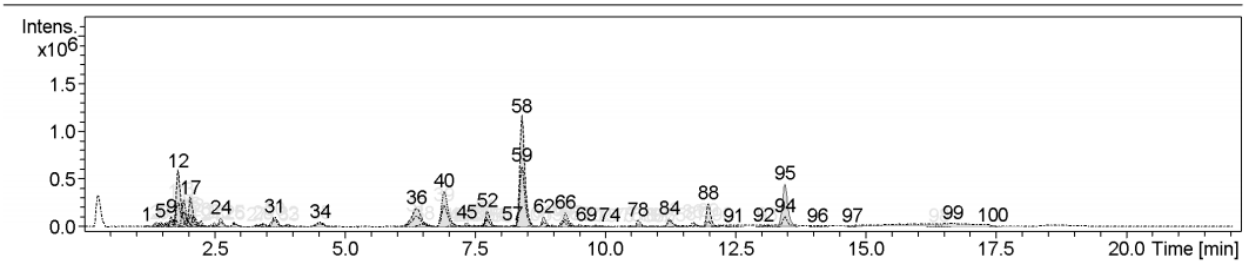
| | | | | | |
|---|---|---|---|---|---|
| Source Type | ESI | Ion Polarity | Positive | Set Nebulizer | 1.2 Bar |
| Focus | Not active | Set Capillary | 4500 V | Set Dry Heater | 200 °C |
| Scan Begin | 50 m/z | Set End Plate Offset | -500 V | Set Dry Gas | 8.0 l/min |
| Scan End | 1000 m/z | Set Collision Cell RF | 200.0 Vpp | Set Divert Valve | Waste |




- CM extract repeat 3

**Acquisition Parameter**

| | | | | | |
|---|---|---|---|---|---|
| Source Type | ESI | Ion Polarity | Positive | Set Nebulizer | 1.2 Bar |
| Focus | Not active | Set Capillary | 4500 V | Set Dry Heater | 200 °C |
| Scan Begin | 50 m/z | Set End Plate Offset | -500 V | Set Dry Gas | 8.0 l/min |
| Scan End | 1000 m/z | Set Collision Cell RF | 200.0 Vpp | Set Divert Valve | Waste |



**HPLC analysis**

**Condition :**

Mobile phase : Acetonitrile : 1% Acetic acid

Column :Inertsil C18 (4.6 x 250 mm I.D.,5 um)

oven : 35 ℃

Flowrate : 1 .0 mL/min

Detector : DAD

Instrument : SHIMADZU LC10

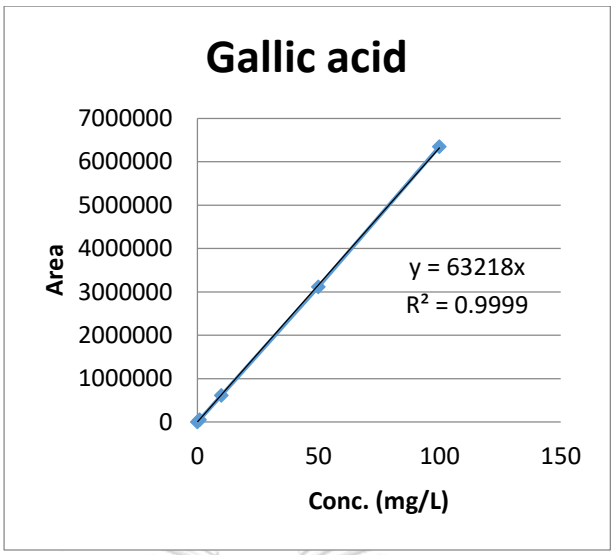
Gradient :

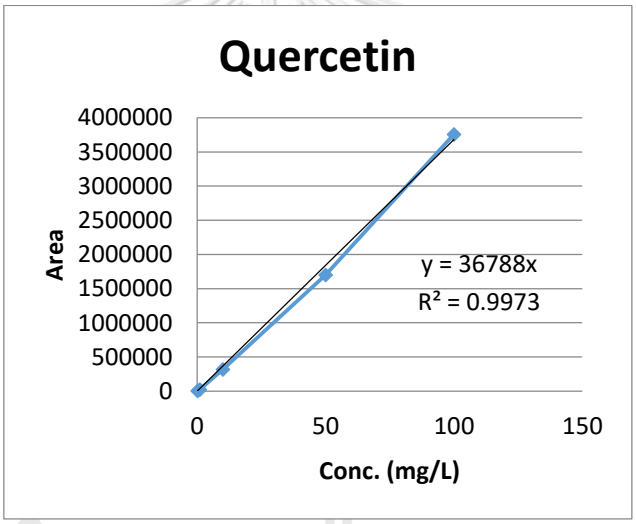
| Time | % Acetonitrile |
|---|---|
| 0 | 0 |
| 10 | 10 |
| 30-40 | 30 |
| 50 | 40 |
| 51-55 | 80 |
| 56-65 | 0 |



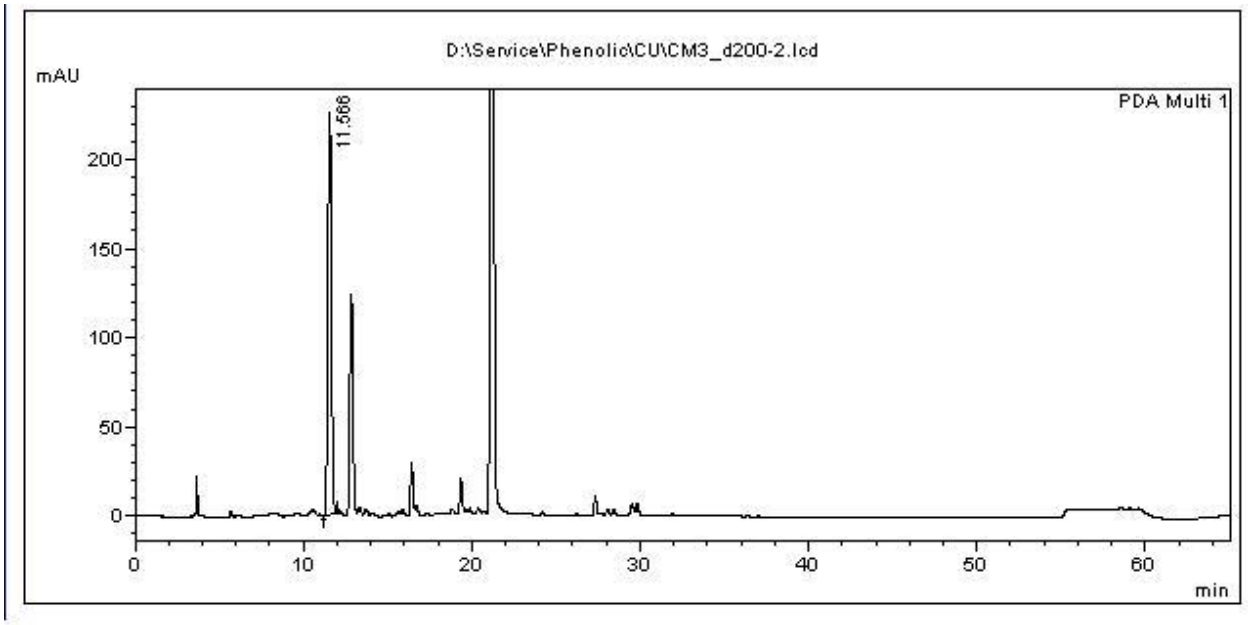
**Standard**

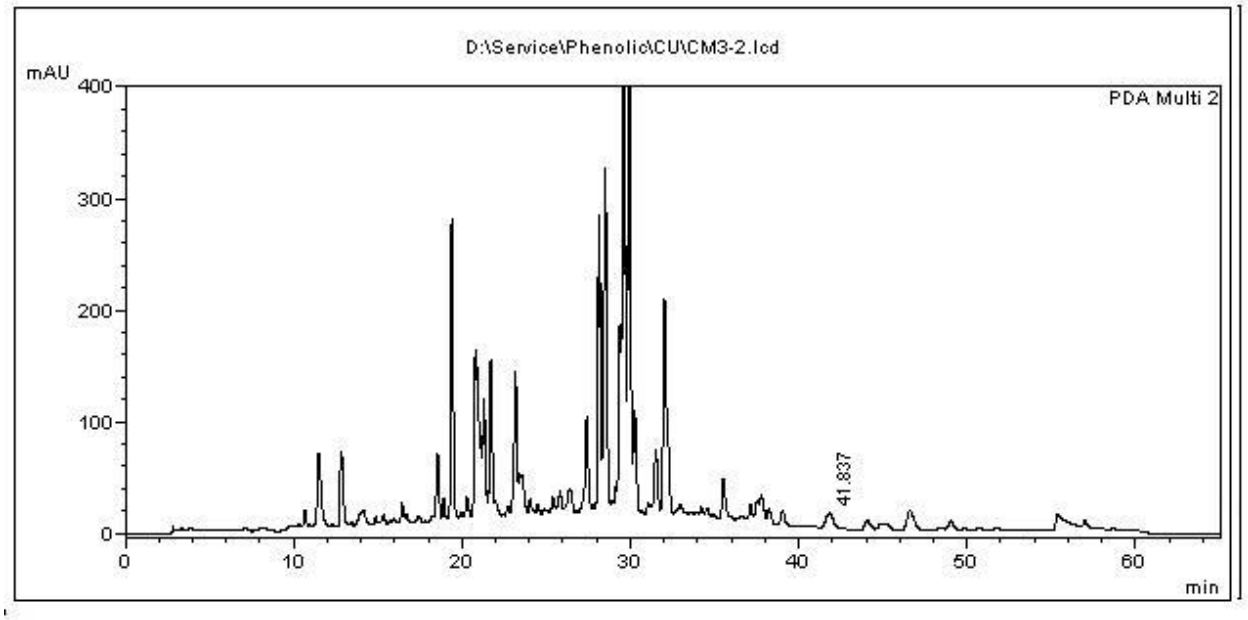
| Gallic acid ( tR = 11.7, 270 nm) | | Quercitin ( tR = 41.8, 331 nm) | |
|---|---|---|---|
| Conc.(mg/L) | Peak area | Conc.(mg/L) | Peak area |
| 0.1 | 4182 | 0.1 | 2384 |
| 1 | 54386 | 1 | 23303 |
| 10 | 612604 | 10 | 317342 |
| 50 | 3115153 | 50 | 1698832 |
| 100 | 6346751 | 100 | 3754244 |



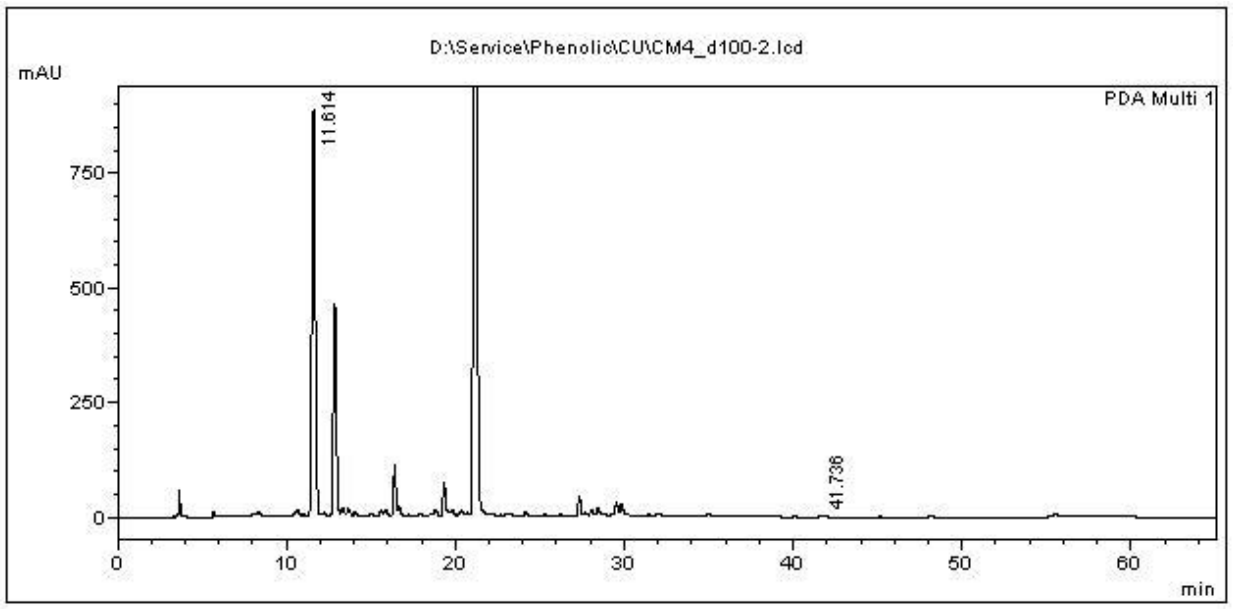



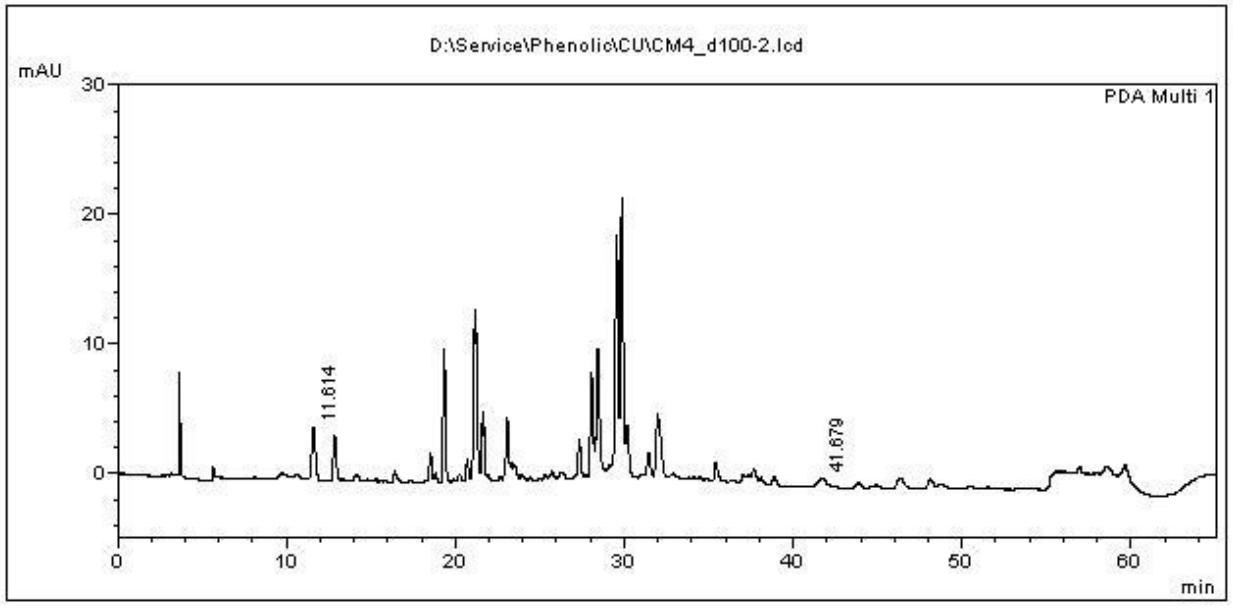
- CM extract repeat 1



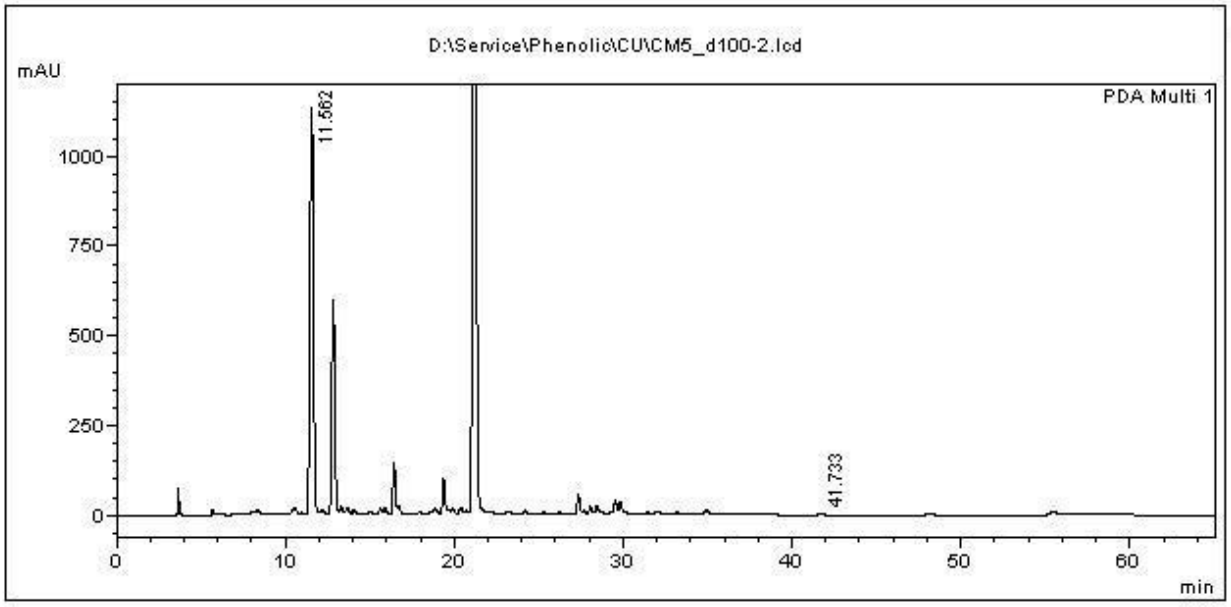



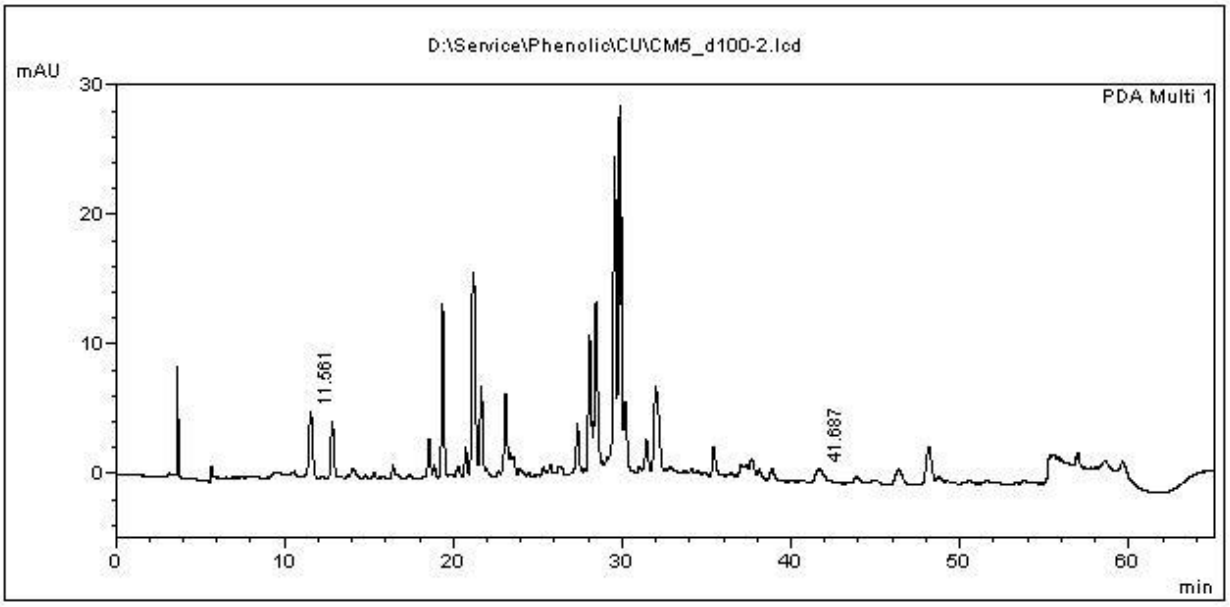
- CM extract repeat 2



- CM extract repeat 3



| Sample name | Gallic acid | | | | |
|---|---|---|---|---|---|
| | Area | Conc.(mg/L) | x df | mg /100g crude extract | Average |
| **CM3** | 310462 | 4.9110 | 9821.9494 | 5062.86 | 5056.32 |
| | 310042 | 4.9043 | 9808.6621 | 5056.01 | |
| | 309679 | 4.8986 | 9797.1780 | 5050.09 | |
| **CM4** | 1056246 | 16.7080 | 16707.99456 | 7844.13 | 7815.168 |
| | 1050473 | 16.6167 | 16616.67563 | 7801.26 | |
| | 1050320 | 16.6143 | 16614.25543 | 7800.12 | |
| **CM5** | 1403680 | 22.2038 | 22203.80271 | 7630.17 | 7652.943 |
| | 1417848 | 22.4279 | 22427.91610 | 7707.19 | |
| | 1402079 | 22.1785 | 22178.47765 | 7621.47 | |

| Sample name | Quercetin | | | | |
|---|---|---|---|---|---|
| | Area | Conc.(mg/L) | x df | mg /100g crude extract | Average |
| **CM3** | 369811 | 10.0525 | | 5.18 | 4.87 |
| | 365367 | 9.9317 | | 5.12 | |
| | 308404 | 8.3833 | | 4.32 | |
| **CM4** | 2887186 | 78.4817 | | 36.85 | 36.326 |
| | 2845582 | 77.3508 | | 36.31 | |
| | 2806636 | 76.2922 | | 35.82 | |
| **CM5** | 2846975 | 77.3887 | | 26.59 | 25.944 |
| | 2765631 | 75.1775 | | 25.83 | |
| | 2719636 | 73.9273 | | 25.40 | |

# VITA

| | |
|---|---|
| **NAME** | Panthakarn Rangsinth |
| **DATE OF BIRTH** | 8 December 1991 |
| **PLACE OF BIRTH** | Chachoengsao, Thailand |
| **INSTITUTIONS ATTENDED** | Clinical Biochemistry and Molecular Medicine Program, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand |
| **HOME ADDRESS** | 23/3 M.5 Bang Nam Priao, Bang Nam Priao, Chachoengsao, Thailand, 24150 |
| **PUBLICATION** | Publications |

1. Chatrawee Duangjan, Panthakarn Rangsinth, Xiaojie Gu, Michael Wink and Tewin Tencomnao: “Lifespan extending and oxidative stress resistance properties of a leaf extracts from Anacardium occidentale L. in Caenorhabditis elegans”, Oxidative Medicine and Cellular Longevity, 2019, 9012396, doi.org/10.1155/2019/9012396.



2. Panthakarn Rangsinth, Anchalee Prasansuklab, Chatrawee Duangjan, Xiaojie Gu, Krai Meemon, Michael Wink and Tewin Tencomnao: “Leaf extract of Caesalpinia mimosoides enhances oxidative stress resistance and prolongs lifespan in Caenorhabditis elegans.”, BMC Complementary and Alternative Medicine, 2019, 19(1):164, doi:10.1186/s12906-019-2578-5.

3. Chatrawee Duangjan, Panthakarn Rangsinth, Xiaojie Gu, Shaoxiong Zhang, Michael Wink and Tewin Tencomnao: “Glochidion zeylanicum leaf extracts exhibit

lifespan extending and oxidative stress resistance properties in Caenorhabditis elegans via DAF-16/FoxO and SKN-1/Nrf-2 signaling pathways", Phytomedicine, 2019, 64:153061. doi:10.1016/j.phymed.2019.153061

4. Chatrawee Duangjan, Panthakarn Rangsinth, Xiaojie Gu, Shaoxiong Zhang, Michael Wink and Tewin Tencomnao: "Data on the effects of Glochidion zeylanicum leaf extracts in Caenorhabditis elegans", 2019, doi.org/10.1016/j.dib.2019.104461

Presentations

1. Poster presentation on "3'untranslated region (3'UTR): a novel marker for diagnosis of G6PD deficiency"at the 8th conference in medical technology student research, Faculty of Allied Health Sciences, Thammasat University, PathumThani, Thailand, October 2013

2. Oral presentation on "Neurite outgrowth activity of Zingiber montanum, Caesalpinia mimosoides, Streblus asper and Acanthus ebracteatus extracts in Alzheimer's disease cell models" at INBIOSIS Graduate Symposium (IGS2018), Institute of System Biology, Universiti Kebangsaan Malaysia, Malaysia, November 1, 2018

3. Poster presentation on "Antioxidant and lifespan extension effects of Caesalpinia mimosoides extracts in nematode Caenorhabditis elegans" at the 30th International Symposium on the Chemistry of Natural Products and the 10th International Congress on Biodiversity (ISCNP30 & ICOB10), Athens, Greece, November 25 – 29, 2018

**AWARD RECEIVED**

- Full scholarship by the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship

- Overseas Academic Presentation Scholarship for Graduate Students from Graduate School, Chulalongkorn University (November 2018) Location: The 30th International Symposium on the Chemistry of Natural Products and the 10th International Congress on Biodiversity (ISCNP30 & ICOB10)

- Overseas Research Experience Scholarship (ORES) for graduate students from the Graduate School, Chulalongkorn University (November 25, 2017 – May 31, 2018) Location: Professor Doctor Michael Wink Laboratory, Institute of Pharmacy and Molecular Biotechnology (IPMB), Heidelberg University, Heidelberg, Germany

- Excellent poster presentation (October 9, 2013) "3'untranslated region (3'UTR): a novel marker for diagnosis of G6PD deficiency" at the 8th conference in medical technology student research, the Faculty of Allied Health Sciences, Thammasat University, Pathum Thani, Thailand