สารยับยั้งโคลีนเอสเทอเรสและสารยับยั้งการเกาะกลุ่มของบีตาแอไมลอยด์จากดอกพญาสัตบรรณ *Alstonia scholaris* L. R. BR.

นางสาวไอริณ ภูวสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2559

CHOLINESTERASE AND BETA-AMYLOID AGGREGATION INHIBITORS FROM FLOWERS OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. BR.

Miss Irin Puwasawat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
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Thesis Title	CHOLINESTERASE AND BETA- AMYLOID AGGREGATION INHIBITORS FROM FLOWERS OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. BR.
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Field of Study	Chemistry
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ไอริณ ภูวสวัสดิ์ : สารยับยั้งโคลีนเอสเทอเรสและสารยับยั้งการเกาะกลุ่มของบีตาแอไมลอยด์จากดอกพญาสัตบรรณ *Alstonia scholaris* L. R. BR. (CHOLINESTERASE AND BETA-AMYLOID AGGREGATION INHIBITORS FROM FLOWERS OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. BR.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.พัฒทรา ธีรพิบูลย์เดช, 61 หน้า.

แยกสารได้ 6 ชนิดจากสิ่งสกัดส่วนดอกของต้นพญาสัตบรรณด้วยคอลัมน์โครมาโทกราฟี พิสูจน์โครงสร้างทางเคมีของสารที่แยกได้ด้วยเทคนิค เอ็น เอ็ม อาร์ สเปกโทรสโกปี พบว่าสารทั้ง 6 ชนิด คือ α-amyrin acetate (1), stigmasterol (2), betulin (3), ursolic acid (4), β-sitosterol-3-O-β-D-glucopyranoside (5) และ nareline (6) จากนั้นนำสารเหล่านี้ไปทดสอบฤทธิ์ยับยั้งเอนไซม์โคลีนเอสเทอเรส ฤทธิ์ยับยั้งการเกาะกลุ่มของเบตาแอไมลอยด์และฤทธิ์ยับยั้งการเกิดเปอร์ออกซิเดชันของไขมัน พบว่าค่าความเข้มข้นในการยับยั้งเอนไซม์แอซีทิลโคลีนเอสเทอเรสและบิวทิริลโคลีนเอสเทอเรสที่ร้อยละ 50 อยู่ในช่วง 0.070-0.231 และ 0.282-0.721 มิลลิโมลาร์ ตามลำดับ อัลคาลอยด์ 6 มีฤทธิ์ในการยังยั้งต่อทั้งสองเอนไซม์ที่สูงที่สุด นอกจากนี้ได้ผสม α-mangostin กับสาร 5เพื่อศึกษาฤทธิ์เสริมกันต่อแอซีทิลโคลีนเอสเทอเรส ไตรเทอร์พีนอยด์ทั้ง 5 ชนิด มีฤทธิ์ระดับต่ำในการยับยั้งการเกาะกลุ่มของเบตาแอไมลอยด์และการยับยั้งการเกิดเปอร์ออกซิเดชันของไขมัน อัลคาลอยด์ 6 มีฤทธิ์ปานกลางในการยับยั้งการเกาะกลุ่มของเบตาแอไมลอยด์ด้วยค่า 28.1 เปอร์เซ็นต์ ที่ความเข้มข้นสุดท้าย 100 ไมโครโมลาร์และมีฤทธิ์ต่ำในการยับยั้งการเกิดเปอร์ออกซิเดชันของไขมัน

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

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IRIN PUWASAWAT: CHOLINESTERASE AND BETA- AMYLOID AGGREGATION INHIBITORS FROM FLOWERS OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. BR.. ADVISOR: ASST. PROF. PATTARA THIRAPHIBUNDET, Ph.D., 61 pp.

Six compounds were isolated from the flower extract of *Alstonia scholaris* by column chromatoghaphy. The chemical structures of all isolated compounds were elucidated by NMR analysis as α-amyrin acetate (1), stigmasterol (2), betulin (3), ursolic acid (4), β-sitosterol-3-O-β-D-glucopyranoside (5) and nareline (6). These compounds were further evaluated for their anti-cholinesterase, anti-amyloid aggregation and anti-lipid peroxidation activities. The IC_{50} values toward acetylcholinesterase and butyrylcholinesterase of these isolated compounds were in the range of 0.070 -0.231 and 0.282-0.721 mM, respectively. Alkaloid 6 showed the highest inhibition toward both enzymes. Moreover, α-mangostin showed the acetylcholinesterase synergistic effect with compound 5. All five triterpenoids (1-5) displayed low anti-amyloid aggregation and anti-lipid peroxidation activities. Alkaloid 6 exhibited moderate amyloid aggregation inhibition with 28.1% at the final concentrations of 100 μM and showed low anti-lipid peroxidation activity.

Department: Chemistry Student's Signature .....

Field of Study: Chemistry Advisor's Signature .....

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Irin Puwasawat

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LIST OF ABBREVIATIONS

Aβ	=	Beta-amyloid
AChE	=	acetylcholinesterase
A. scholaris	=	*Alstonia scholaris*
BChE	=	butyrylcholinesterase
br	=	broad (NMR)
C	=	carbon
$CHCl_3$	=	chloroform
chloroform-d_1	=	deuterated chlorofrom
CH_2Cl_2	=	dichloromethane
^{13}C-NMR	=	carbon-13 nuclear magnetic resonance
d	=	doublet (NMR)
dd	=	doublet of doublet (NMR)
DMSO-d_6	=	deuterated dimethyl sulfoxide
EtOAC	=	ethyl acetae
g	=	gram (s)
H	=	hydrogen
^{1}H-NMR	=	proton-1 nuclear magnetic resonance
Hz	=	hertz (NMR)

IC_{50}	=	concentration to inhibit enzyme activity at 50 percent
J	=	coupling constant
kg	=	kilogram (s)
M	=	molarity
m	=	multiplet (NMR)
Me	=	methyl
MeOH	=	methanol
mg	=	milligram (s)
MHz	=	megahertz (NMR)
min	=	minute (s)
mL	=	milliliter (s)
mM	=	millimolar
ppm	=	part per million
pyridine-d_5	=	deuterated pyridine
s	=	singlet (NMR)
t	=	triplet (NMR)
U	=	enzyme unit
UV	=	ultraviolet
v	=	volume

α	=	alpha
β	=	beta
%	=	percentage
δ	=	unit of chemical shift
δ_C	=	chemical shift value of carbon
δ_H	=	chemical shift value of proton
μL	=	microliter (s)
μM	=	micromolar

CHAPTER I

INTRODUCTION

Natural products are chemical compounds or substances produced from a various source including marine organisms, bacteria, fungi, and plants. However, plants are the major source of active compounds that can be used as traditional medicines. South East Asia region is a large source of natural products especially plants which can be used as important source of drug discovery. Nowadays, the number of neurodegenerative disease patients is increasing rapidly especially Alzheimer's disease (AD), but the treatment strategies are still limited. There are only four drugs for AD treatment that have been approved from the U.S. Food and Drug Administration (FDA). Thus, finding new drugs is a vital role to improve the treatment strategies of these disease.

There are many studies reporting cholinesterase inhibitory activity from Thai herbs. In 2003, thirty-two Thai medicinal plants were screened for acetylcholinesterase (AChE) inhibitory activity. The result revealed that the root extracts of *Stephania suberosa* and *Tabernaemontana divaricata* showed AChE inhibition more than 90% at the final concentration of 0.1 mg/mL. Dicentrine and isolaureline isolated from *S. suberosa* showed high potential to inhibit AChE with the IC_{50} values of 0.67 and 8.36 μM, respectively [1]. In addition, 19,20-dihydrotabernamine and 19,20-dihydroervahanine isolated from *T. divaricate* exhibited the AChE inhibitory activity

with the IC_{50} values of 227 and 71, respectively [2]. The stems of *Piper interuptum*, the seeds of *Piper nigrum*, the roots of *Butea superba* and the roots of *Cassia fistula* displayed 50–65% AChE inhibitory activity [3]. Furthermore, stilbenes from the vines of *Ficus foveolata* showed high butyrylcholinesterase (BChE) inhibitiory activity with the IC_{50} values in a range of 1.3 – 96.1 μM [4]. Therefore, Thai medicinal plants are the interesting source of new cholinesterase inhibitors.

From the previous study, the preliminary screening test of stems, leaves and flowers from *Alstonia scholaris* (L.) R. Br. exhibited high inhibition toward AChE. The isolation of the leaves extract gave four alkaloids which exhibited moderate cholinesterase inhibition. In this study, the flower extract of *A. scholaris* was isolated to afford the chemical constituents that will be evaluated for their anti-cholinesterase, anti-amyloid aggregation and anti-lipid peroxidation activities. Moreover, the AChE synergistic effect of the combination between the isolated compounds and α-mangostin was studied.

1.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common type of dementia. It is accounts for 60 to 80 percent of dementia cases. The World Health Organization (WHO) reported that over 47 million people worldwide are Alzheimer's [5]**.** Nowadays, the number of new patients are close to 8 million and will be almost triple in 2050. The early symptom is shorter attention span, feelings of restlessness, reading difficulty and personality changes. There are several hypotheses to explain the causes of this disease but cholinergic and amyloid hypotheses play an important role in the therapeutic strategies of AD.

1.1.1. Cholinergic hypothesis

In a central nervous system, acetylcholine (ACh) is neurotransmitter that is synthesized by choline and acetyl CoA in the axon terminal. After transmitting the nerve signal at cholinergic receptor, ACh is released into the synaptic cleft and rapidly broken down by the enzyme acetylcholinesterase (AChE) to give choline. Choline is transported back into the axon terminal and it is used for making more ACh (Figure 1.1) [6]. In AD patients, the loss of neurotransmitter is commonly observed due to the increase activity of AChE and BChE. Therefore, preventing the breakdown of acetylcholine is the key role to alleviate this disease

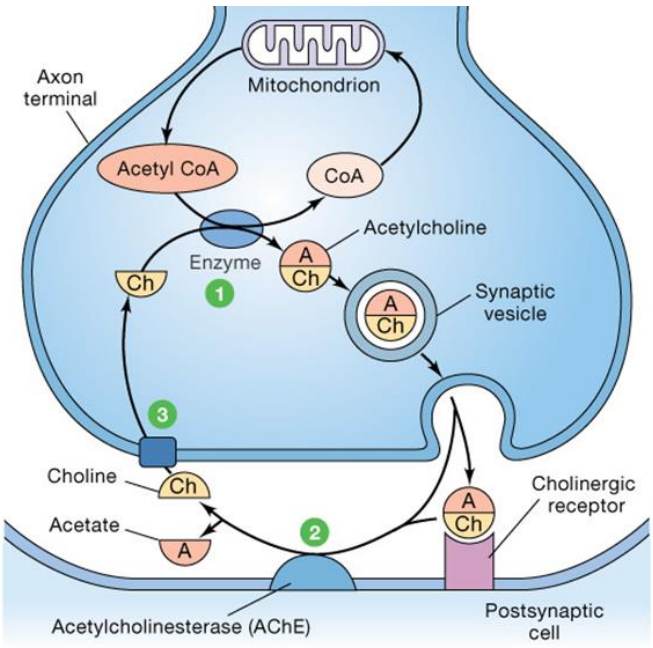


Figure 1.1 Synthesis of acetylcholine in a central nervous system

Tarcrine, donepezil, galanthamine and rivastigmine (Figure 1.2) are cholinesterase inhibitors that have been approved by the U.S. Food and Drug Administration (FDA) and represent the first line pharmacotherapy for mild to moderate AD.

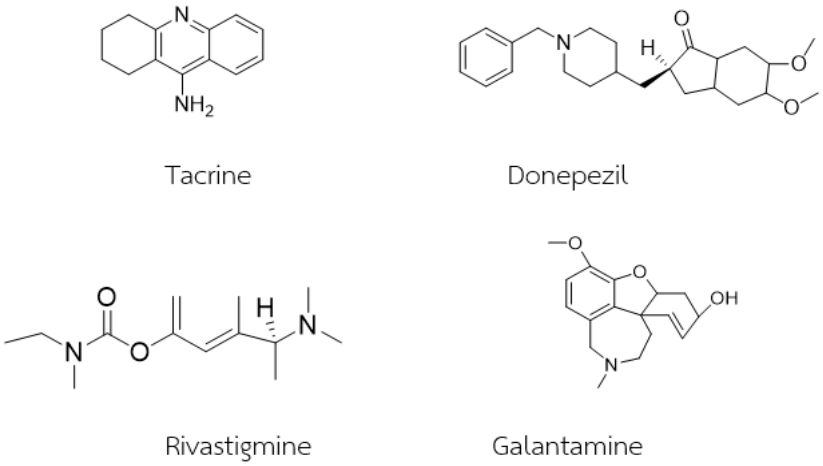


Figure 1.2 Drug for treatment Alzheimer's disease

Tacrine is the first drug for treatment AD but it causes the excessive side effects [7]. Donepezil is an AChE selective inhibitor for treatment all stages of AD. This drug is less toxic and more effective than tacrine. Rivastigmine and galantamine are both AChE and BChE inhibitors to treat mild to moderate AD symptom. However, all drugs have common side effects such as nausea, vomiting, weight loss, anorexia, diarrhea, abnormal dream, headache and bradycardia [8]. Galantamine is an alkaloid which is obtained from flowers of *Galanthus caucasicus* and *Galanthus woronowii.* This drug has been used as a prototype to develop cholinesterase inhibitor from the natural products. In some case, only one drug is not enough to treat the symptom. The combination of the cholinesterase inhibitors with another type of drug is the substitute way for treatment but the side effects will increase as well. Therefore, drug development from the natural products is an alternative way. It is believed that natural substances are less harmful than synthetic substances.

1.1.2 Amyloid hypothesis

Amyloid-beta peptide (Aβ) is the major component of amyloid plaques which play an important role in the neurodegenerative pathology rated to AD [9, 10]. The formation of amyloid plaques involves of two phases. The first phase is a nucleation phase, in which soluble monomer undergoes conformation change to form oligomeric nuclei (β-sheet nuclei). The second phase is an elongation phase which induces the fast protein assembly to form amyloid. This process causes neurodegeneration and produces insoluble plaques (Figure 1.3) [11].

The amyloid plaques also lead to abnormal hyperphosphorylation of protein tau to from neurofibrillary tangles that damage the nerve cells. Many studies have been found that the aggregation of Aβ is induced by AChE whereas BChE is not affected [12]. Therefore, Aβ aggregation and neurofibrillary tangles formation are the important target for the recently therapeutic strategies of AD.

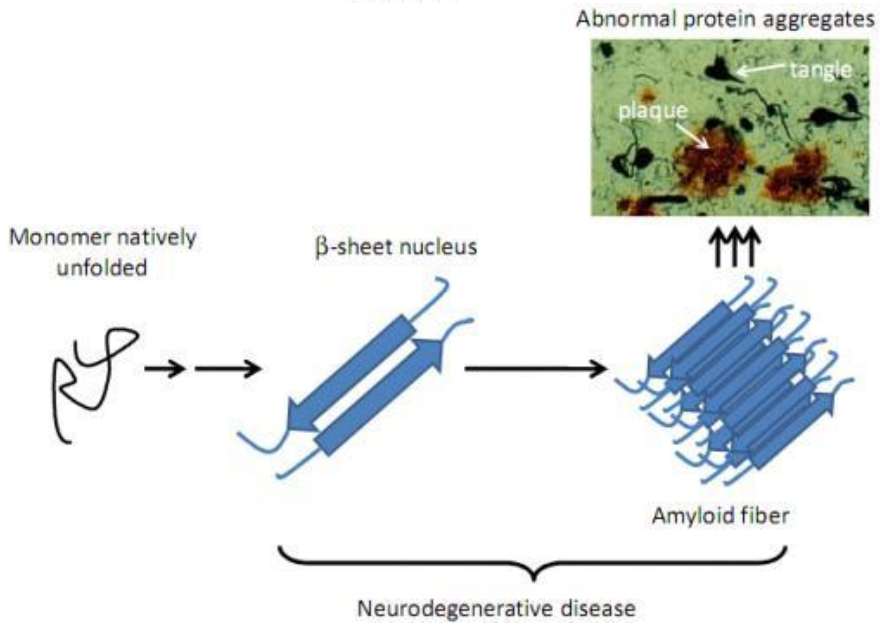


Figure 1.3 The formation of amyloid plaques

A wide range of natural compounds has been reported to inhibit amyloid fibril formation. The important class of compounds which have this activity is polyphenols. Resveratrol is a polyphenolic phytoalexin that found in wine and could reduce the initiation of neurodegenerative disease [13]. Moreover, resveratrol performed direct effect to protect cells from Aβ oligomerization [14]. Curcumin is a major active component that found in the *Curcuma longa*. This compound has been reported

several bioactivities including anti-inflammatory, anti-oxidant and anti-cancer activities [15, 16]. Furthermore, curcumin can inhibit oxidative damage, cognitive deficits and Aβ aggregation with the IC_{50} value of 0.8 μM [17]. Quercetin is a flavonoid that obtained from many fruits and vegetables such as citrus fruit, broccoli, cranberries and raspberries. Many investigations demonstrated that quercetin increased the resistance of neurons to oxidative stress and inhibited anti-amyloidogenic activity with the IC_{50} value of 2.4 μg/mL [18]. Therefore, the anti-amyloid inhibitor from natural source is a viable alternative candidate to develop for treat and prevent of AD.

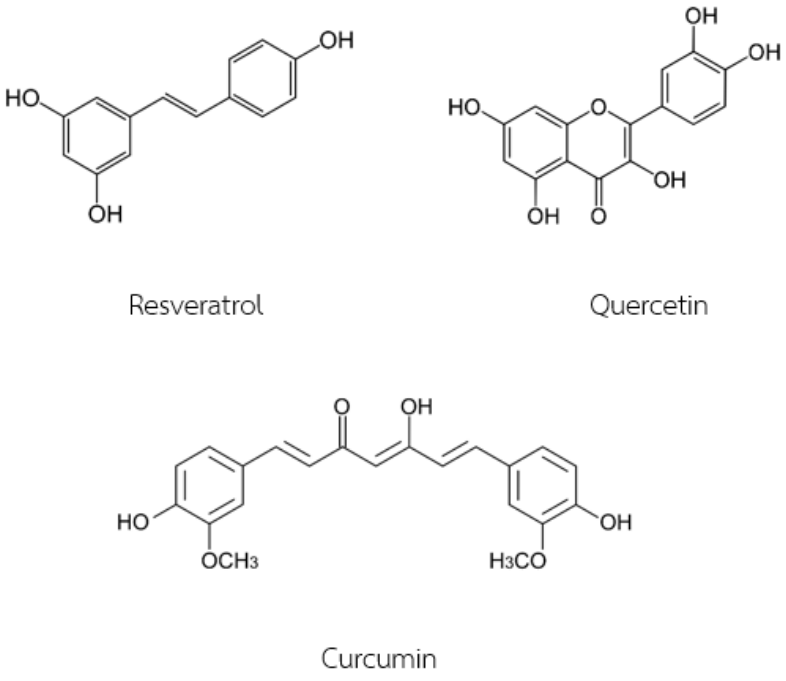


Figure 1.4 Natural anti-amyloid aggregation inhibitors

1.2 Botanical aspects of *Alstonia scholaris* (L.) R. Br.

Alstonia scholaris (L.) R. Br. (Figure 1.5) is commonly called Indian devil tree, Milkwood-pine, White cheese wood, Dita bark (English) or Thai name as Paya sattaban or Teen ped. This plant is widely distributed in the Asia-Pacific region such as India, Thailand, Malaysia, Philippines, Indonesia, Vietnam and China [19]. It is an important medicinal plant with various traditional uses such as treatment of whooping cough, malaria, asthma, and fever [20]. *A. scholaris* is a large evergreen tree and grows up to 6–10 m. Bark is rough and gray–white color but insider is yellowish and has milky latex. The leaves are occurred in whorls of 4–8 with dark green color and the upper side of leaves are glossy. Leaves are obovate to oblanceolate, coriaceous, rounded or bluntly acuminate at apex. Flowers are dense, bisexual, umbellately branched and greenish white. The flowers are bloom in October to December. Ovaries of flower are spindle shaped, follicles slender, pendulous, green or brown and 20–50 cm long.

Figure 1.5 Picture of *A. scholaris*

1.3 Phytochemical and pharmacological studies of *A. Scholaris*

The phytochemical constituents of *A. scholaris* have been investigated extensively, and many pure compounds have been isolated. Most compounds isolated from *A. scholaris* have been identified as alkaloids and triterpenoids [21]. Many alkaloids obtained from barks and leaves such as echitamine, alschomine, nareline, picraline, akuammicine *N*-oxide, *N*-demethylalstogustine and echitaminic acid (Figure 1.6). These alkaloids, especially echitamine exhibited anti-cancer activity. Moreover, echitamine showed anti-tumor effects on fibrosarcoma in rats and cytotoxic effect on Ehrlich ascites carcinoma cell cultures. Picraline and akuammicine *N*-oxide possessed anti-malarial activity [22, 23]. The examples of alkaloids obtained from the flowers and fruits of *A. scholaris* were strictamine, *N*-formylscholarine, picrinine and 19,20 -*E*-Vallesamine (Figure 1.7). Picrinine showed anti-inflammatory and analgesic effects based on several *in vivo* assays [24-26].

Some triterpenoids and flavonoids obtained from barks, leaves and flowers were betulinic acid, betulin, β-sitosterol, lupeol, α-amyrin acetate and ursolic acid (Figure 1.8). These triterpenoids showed anti-inflammatory and anti-microbial activities. Flavonoids including, kaempferol, quercetin, isorhamnetin exhibited a wide range of pharmacological activities such as anti-oxidant, anti-inflammatory, anti-microbial, anti-cancer, anti-diabetic and neuroprotective activities [27-30].

Additionally, the methanol extract of *A. scholaris* stem bark was reported to exhibit anti-stress activity [31]. And ethyl acetate extract of leaves [32] showed nootropic activities. However, the active components in these extracts have not yet been isolated to evaluated these activities.

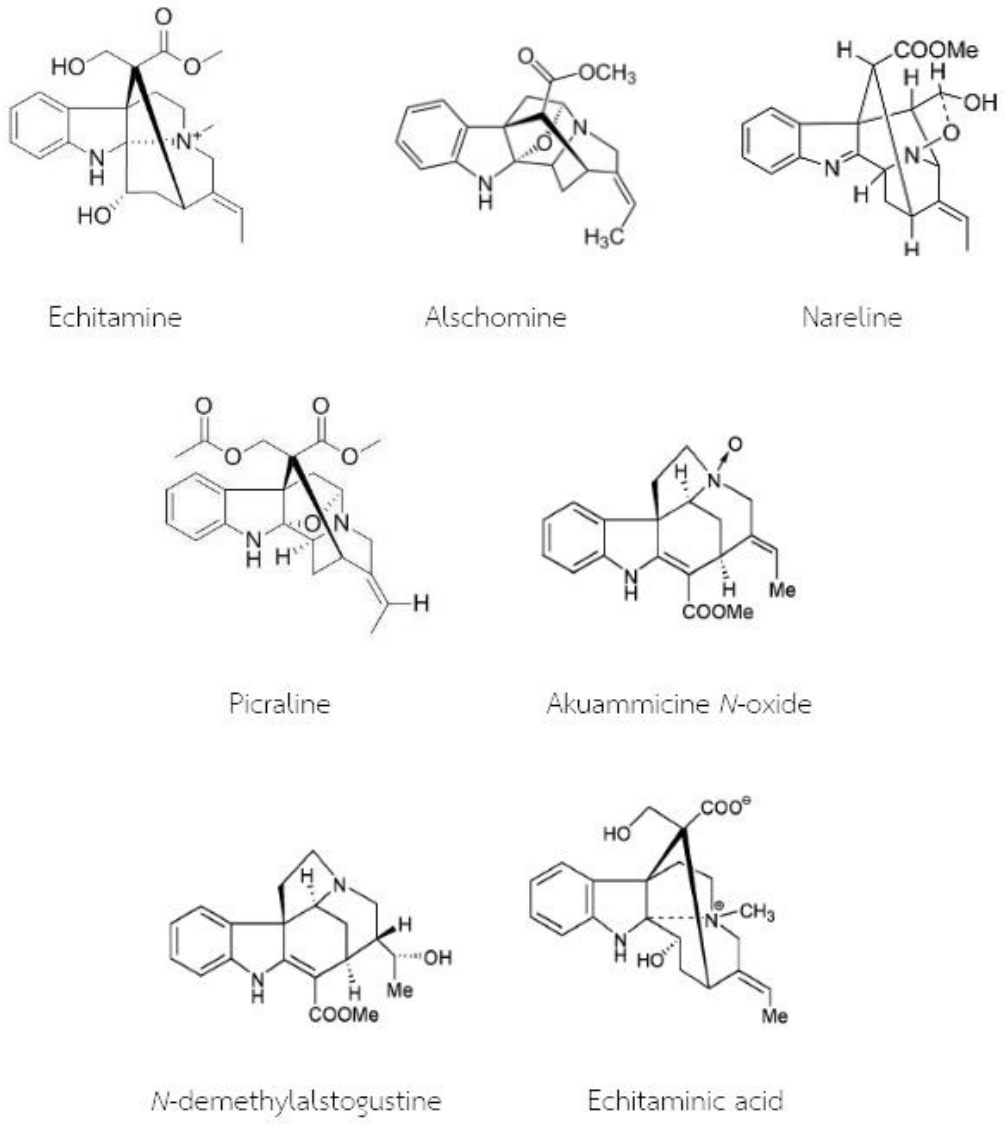


Figure 1.6 Alkaloid isolated from barks and leaves of *A. scholaris*

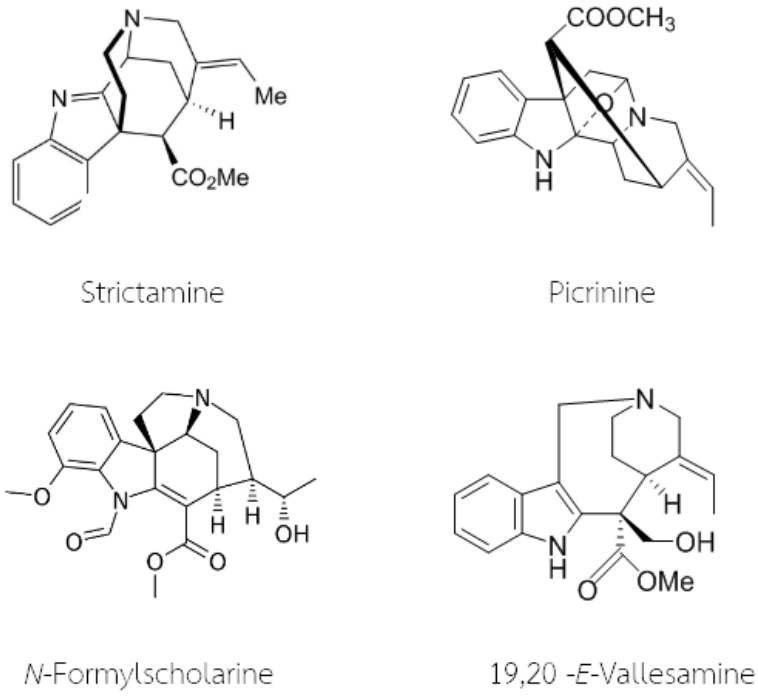
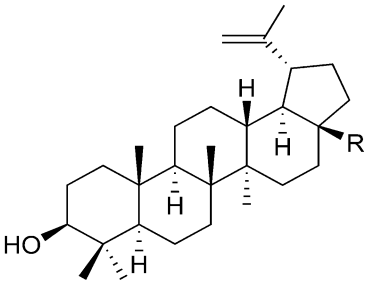


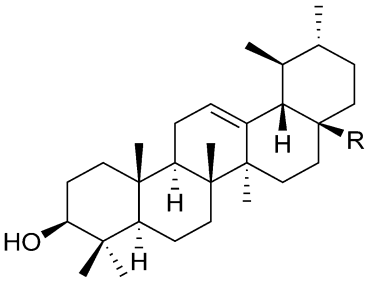
Figure 1.7 Alkaloid isolated from flowers and fruits of *A. Scholaris*



Betulinic acid R = COOH

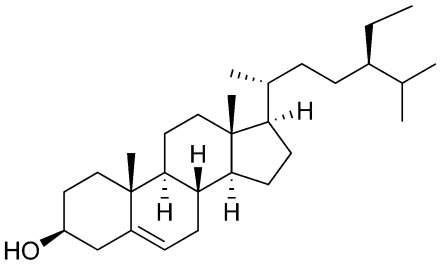
Betulin R = CH_2OH

Lupeol R = CH_3

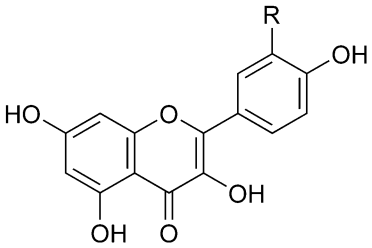


Ursolic acid R = COOH

α-Amyrin acetate R = CH_3



β-Sitosterol



Kaempferol R = H

Quercetin R = OH

Isorhamnetin R = OCH_3

Figure 1.8 Terpenoids and flavonoids isolated from *A. Scholaris*

1.4 Pharmacological activity of α-mangostin

α-Mangostin (Figure 1.9) is the major component isolated from various parts of *Garcinia mangostana* or mangosteen. This plant is widely distributed in South East Asia such as Thailand, Myanmar, Malaysia, Philippines and Indonesia. There are various traditional uses such as trauma, diarrhea, skin infection and wound treatment [33]. α-Mangostin possessed several biological activities such as anti-inflammatory, anti-tumor, anti-diabetic, anti-bacterial, anti-fungal, anti-oxidant and cardioprotective activities [34]. Moreover, α-mangostin was investigated for their cholinesterase inhibitory activity toward AChE and BChE by using Ellman's colorimetric method. This compound had high inhibition toward both enzymes with IC_{50} values of 2.14 ± 0.03 and 5.41 ± 0.17 μM, respectively [35]. Furthermore, α-mangostin was tested for anti-amyloid aggregation by using cell viability assay. This compound attenuated the neurotoxicity of Aβ (1-42) aggregation with the EC_{50} value at 4.14 nM [36].

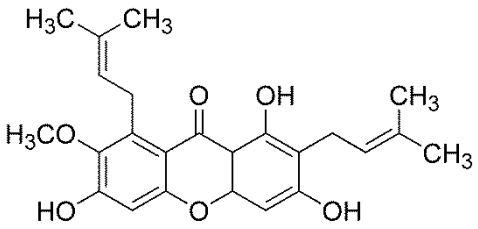


Figure 1.9 α-Mangostin

1.5 The Objectives of this Research

The flower extract of *A. scholaris* showed high anti-cholinesterase activity in our screening but there was no previous report on this activity. Thus, the objectives of this study were

1.5.1 To isolate and elucidate the chemical constituents from the flower of *A. scholaris*.

1.5.2 To investigate anti-cholinesterase, anti-amyloid aggregation and anti-lipid peroxidation activities of the isolated compounds from the flower of *A. scholaris.*

1.5.3 To study the AChE synergistic effect of the isolated compounds and α-mangostin

CHAPTER II

EXPERIMENTAL

2.1 Plant materials

The flowers of *Alstonia scholaris* (L.) R. Br. were collected from Nakhon Ratchasima Province, Thailand in December 2015. A voucher specimen No. NPRU 0004 has been deposited in the Natural Products Research Unit, Faculty of Science, Chulalongkorn University, Thailand.

2.2 Instruments

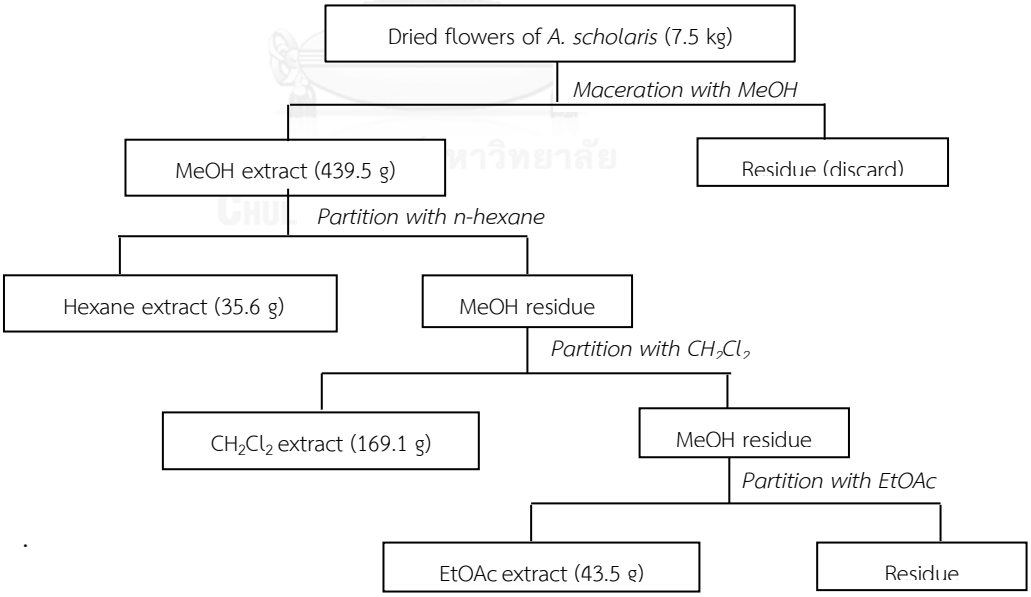
The ^{1}H and ^{13}C NMR spectroscopy (Bruker Advance 400 MHz) was used for elucidating the chemical structure of isolated compounds. The spectra were represented chemical shift (**δ**, ppm) signal from the residual protons and carbons in deuterated solvent by using tetramethylsilane (TMS) as an internal standard.

Silica gel 60 (Merck) No. 7734 and Sephadex LH-20 were used for column chromatography. In addition, Silica gel 60 F_{254} precoated on aluminium sheets (Merck Kieselgel) was used for thin layer chromatography (TLC). The spots on TLC plates were detected under a UV light at (254 and 365 nm) and visualized by using anisaldehyde reagent

2.3 Extraction of *A. scholaris* flowers

The methanol extracts of stems, leaves and flowers of *A. scholaris* were preliminarily tested for their anti-cholinesterase activity.

The dried flowers of *A. scholaris* (7.5 kg) were macerated in methanol for two days. After filtering, the residue was re-extracted with methanol. Both filtrates were combined and evaporated under vacuum to obtain a methanol extract (439.5 g). The methanol extract was partitioned consecutively with *n*-hexane, dichloromethane and ethyl acetate to obtain *n*-hexane (35.6 g), dichloromethane (169.1 g) and ethyl acetate (43.5 g) extracts, respectively. The extraction procedure is summarized as showed in Scheme 2.1.



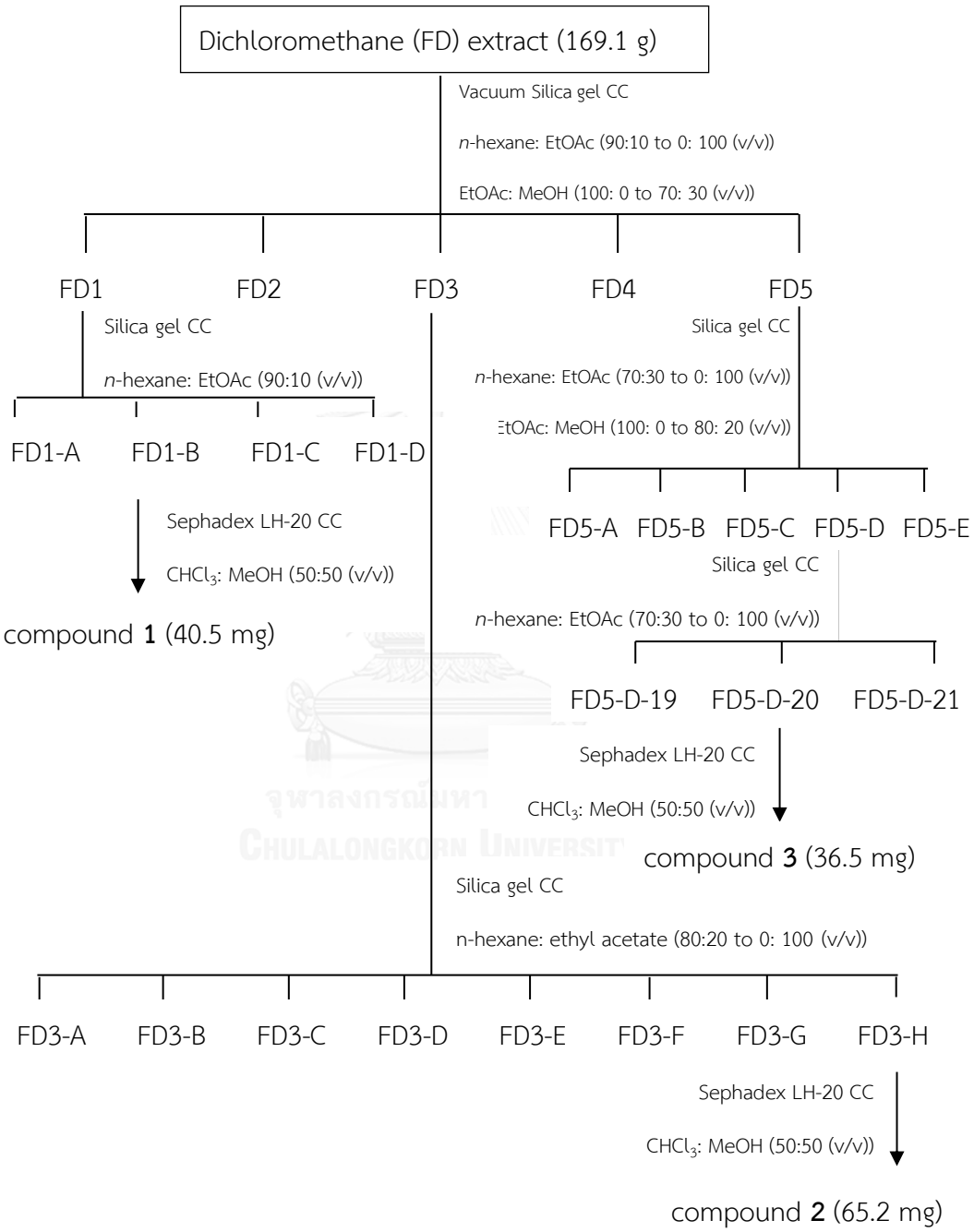
Scheme 2.1 Extraction procedure of *A. scholaris* flowers.

2.4 Isolation of the dichloromethane extract

The 150 g of dichloromethane extract (FD) was fractionated by a vacuum silica gel column chromatography (CC) eluted with a gradient of *n*-hexane and ethyl acetate (90:10 to 0: 100 (v/v)), followed by a gradient of ethyl acetate and MeOH (100: 0 to 70: 30 (v/v)). All fractions were combined on the basis of TLC result to give 11 fractions (FD1 to FD11).

Fraction FD1 was separated by a silica gel CC eluted with *n*-hexane: ethyl acetate (90:10 (v/v)) to give four fractions (FD1-A to FD1-D). Fraction FD1-B was purified by a Sephadex LH-20 CC eluted with chloroform: methanol (50:50) to obtain compound **1** (40.5 mg). Fraction FD3 was subjected to silica gel CC eluted with a stepwise gradient of *n*-hexane and ethyl acetate (80:20 to 0: 100 (v/v)) to give eight fractions (FD3-A to FD3-H). Fraction FD3-H was purified by a Sephadex LH-20 CC using the isocratic gradient of dichloromethane - methanol (50:50) to furnish compound **2** (65.2 mg). Fraction FD5 was subjected to silica gel CC eluted with a stepwise gradient of *n*-hexane and ethyl acetate (70:30 to 0: 100 (v/v)), following with a gradient of ethyl acetate and MeOH (100: 0 to 80: 20 (v/v)) to afford five fractions (FD5-A to FD5-E). Fraction FD5-D was submitted to separate over a silica gel CC using a gradient system of n-hexane and ethyl acetate to obtain FD5-D-20 fraction and then purified with a Sephadex LH-20 CC eluted with chloroform: methanol (50:50) to give compound **3**

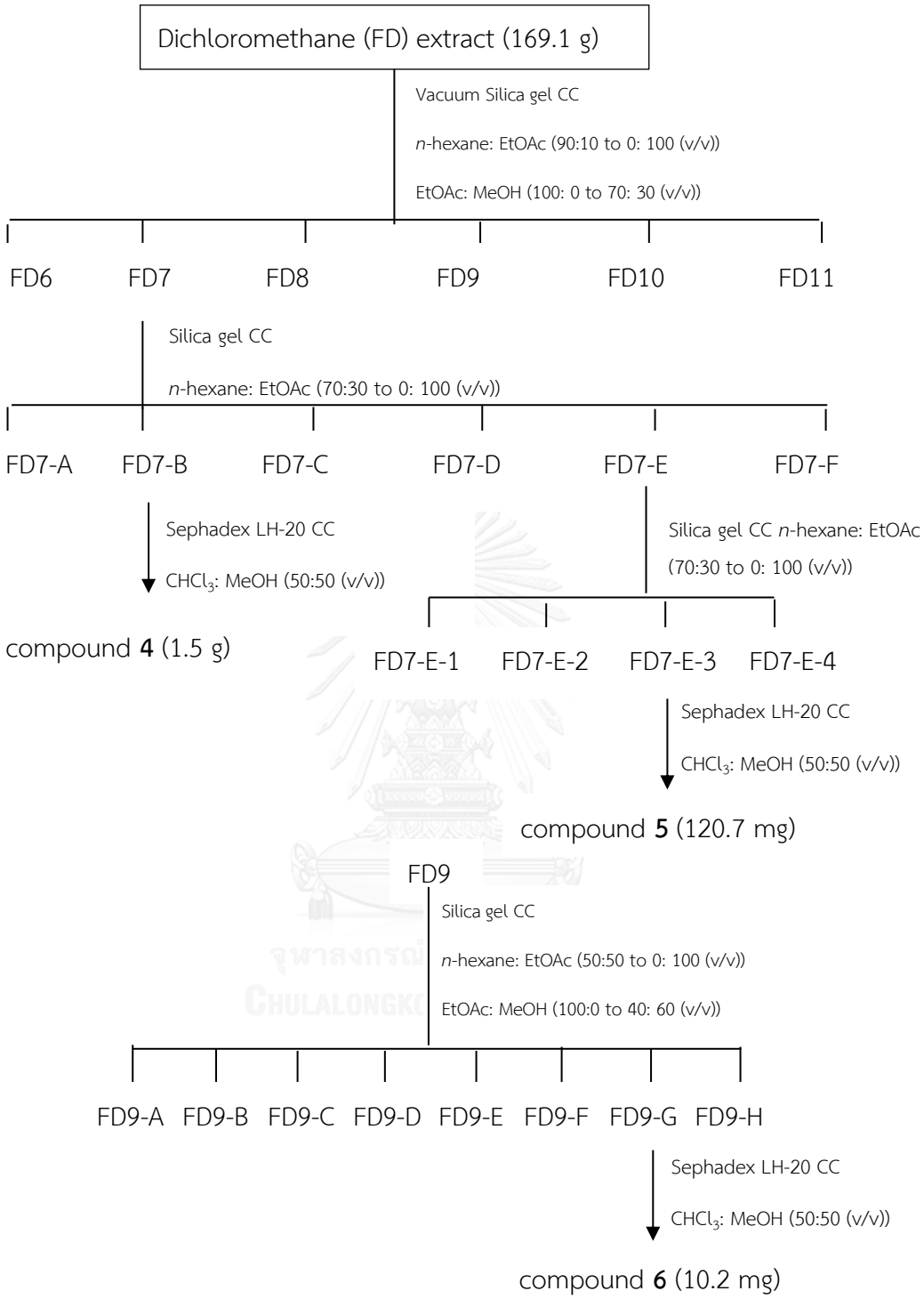
(36.5 mg). The isolation procedure of compounds **1**, **2** and **3** is displayed in Scheme 2.2.



Scheme 2.2 The isolation procedure of compounds **1**, **2** and **3**

Fraction FD7 was further separated by a silica gel CC eluted with increasing polarity of *n*-hexane and ethyl acetate to afford six fractions (FD7-A to FD7-F). Fraction FD7-B was purified with a Sephadex LH-20 CC eluted with chloroform: methanol (50:50) to give compound **4** (1.5 g). Moreover, fraction FD7-E was subjected to silica gel CC eluted with *n*-hexane and ethyl acetate (30:70 (v/v)) to give four fractions (FD7-E-1 to FD7-E-4). Fraction FD7-E-3 was purified with a Sephadex LH-20 CC eluted with chloroform: methanol (50:50) to furnish compound **5** (120.7 mg).

Additionally, fraction FD9 was subjected to silica gel CC eluted with a gradient of *n*-hexane and ethyl acetate (50:50 to 0: 100 (v/v)), following with a gradient of ethyl acetate and MeOH (100: 0 to 40: 60 (v/v)) to give eight fractions (FD9-A to FD9-H). Fraction FD9-G was purified with a Sephadex LH-20 CC eluted with chloroform: methanol (50:50) to obtain compound **6** (10.2 mg). The isolation procedure of compounds **4**, **5** and **6** is showed in Scheme 2.3.

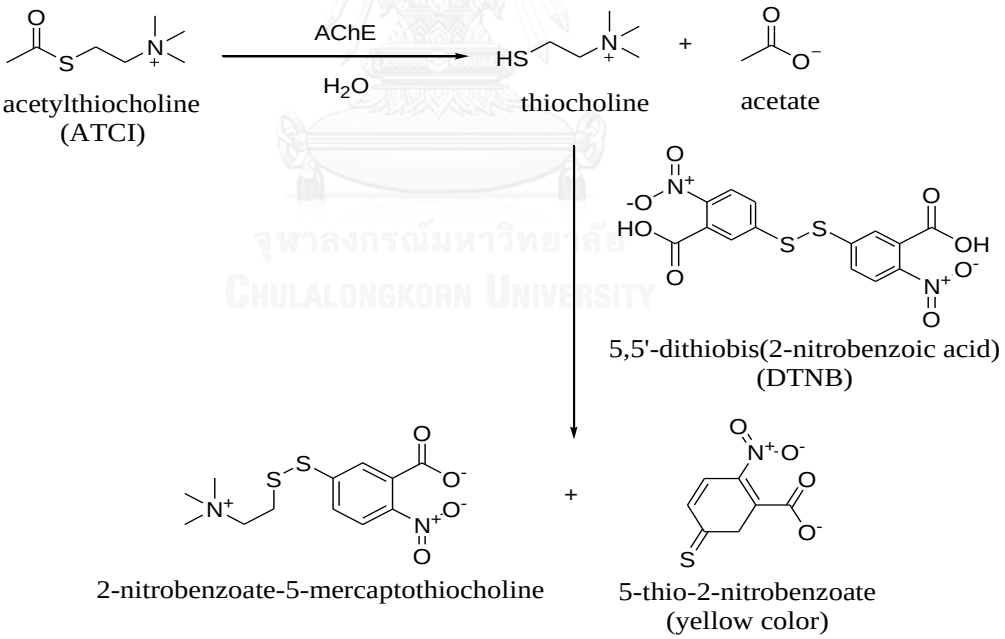


Scheme 2.3 The isolation procedure of compounds **4**, **5** and **6**

2.5 Bioactivity

2.5.1 Anti-cholinesterase activity

The evaluation of anti-cholinesterase activity is based on the Ellman's colorimetric method [37]. Acetylthiocholine (ATCI) and butylthiocholine (BTCI) are used as substrates. The substrates are hydrolysed by AChE and BChE, respectively, to produce thiocholine and acetate. Then, the reaction between thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and thiocholine produces yellow color of 5-thio-2-nitrobenzoate (Scheme 2.4). If the samples can inhibit enzyme activity, the yellow color of product will be decreased.



Scheme 2.4 The hydrolysis reaction of acetylthiocholine

2.5.1.1 Chemicals

Acetylthiocholine iodide (ATCI), bovine serum albumin (BSA), butyrylthiocholine iodide (BTCI), 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB), acetylcholinesterase (AChE) from electric eels (type VI-S, EC 3.1.1.7), butyrylcholinesterase (BChE) from horse serum (EC 3.1.1.8), eserine (standard compound), *Tris*(hydroxymethyl)-aminomethane were obtained from Sigma-Aldrich Co.Ltd.

2.5.1.2 Reagent preparation

AChE and BChE were diluted by buffer B to get 1 U/mL enzymes before assayed. Both ATCI and BTCI were dissolved in MilliQ water to adjust the concentration of 1.5 mM. DTNB was dissolved in buffer C. All buffers were prepared as shown below;

Buffer A: 50 mM Tris–HCl, pH 8

Buffer B: 50 mM Tris–HCl, pH 8 containing 0.1% bovine serum albumin (BSA)

Buffer C: 50 mM Tris–HCl, pH 8 containing 1 M sodium chloride (NaCl) and 0.2 mM magnesium chloride hexahydrate ($MgCl_2.6H_2O$)

2.5.1.3 The microplate assay

The assay was performed using a modification of the Ellman's colorimetric method. Briefly, 50 µL of buffer A was added to a 96-well plate, following by addition of 25 µL of 1.5 mM ATCI (or BTCI). After that, 25 µL of sample in buffer A was added followed by addition 125 µL of 3 mM DTNB in buffer C and 25 µL of of AChE (or BChE). The

absorbance was measured at wavelength of 415 nm for 2 min at 5 sec intervals. Each experiment was done in triplicate and eserine was used as a standard. The percentage inhibition was calculated from the equation that shown below.

$$\% \text{ Inhibition} = \left[\frac{A_{blank} - A_{sample}}{A_{blank}} \right] \times 100$$

When A_{blank} is the change in absorbance of blank without any inhibitors and A_{sample} is the change in absorbance of sample. Eserine was used as a standard.

The IC_{50} value was described as the concentration of inhibitor that reduced 50% of the enzymatic activity. The IC_{50} value was determined by the Graph Pad Prism 5.01 software using a plot of percentage inhibition versus a log final concentration value to generate a sigmoidal curve (Figure.2.1).

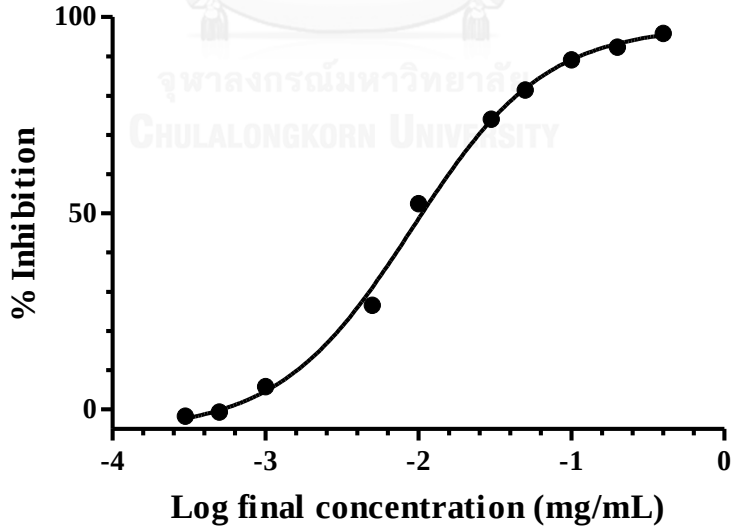


Figure 2.1 Sigmoidal curve between percentage of inhibition and log final concentration values.

2.5.2 Anti-amyloid aggregation activity

2.5.2.1 Chemical

Amyloid beta (1-42) human peptide and thioflavin T were purchased from Sigma-Aldrich Co. Ltd. The 1,1,1,3,3,3-hexafluoro-2-propanal (HFIP) was purchased from Merck Company (Darmstadt, Germany). Curcumin with 95% purity was obtained from Welltech Biotech Company (Yannawa, Bangkok)

2.5.2.2 Reagent preparation

Amyloid beta (1-42) human peptide was prepared as the previous report [38]. Briefly, 1 mg of amyloid peptide powder was dissolved in 1 mL of HFIP and incubated at room temperature for 1 hour. The HFIP/peptide solution was sonicated in a water bath for 10 minutes and then purged under a gentle stream of nitrogen gas until dry. This HFIP/peptide residue was re-suspended in 1 ml of dimethylsulfoxide (DMSO) to adjust the concentration to 220 μM. The Aβ stock solution was aliquoted into 1.5 mL microcentrifuge tubes and store at -20 ºC until use.

2.5.2.3 Thioflavin T spectroscopic assay

Thioflavin T (ThT) dye fluorescence was used to determine the formation of amyloid fibrils. When ThT binds to the aggregated amyloid, the fluorescence of ThT exhibits a dramatic shift of the maximum excitation from 385 to 450 nm and the

maximum emission from 445 to 485 nm [39]. If the compound can inhibit amyloid aggregation, the fluorescence intensity of ThT will decrease.

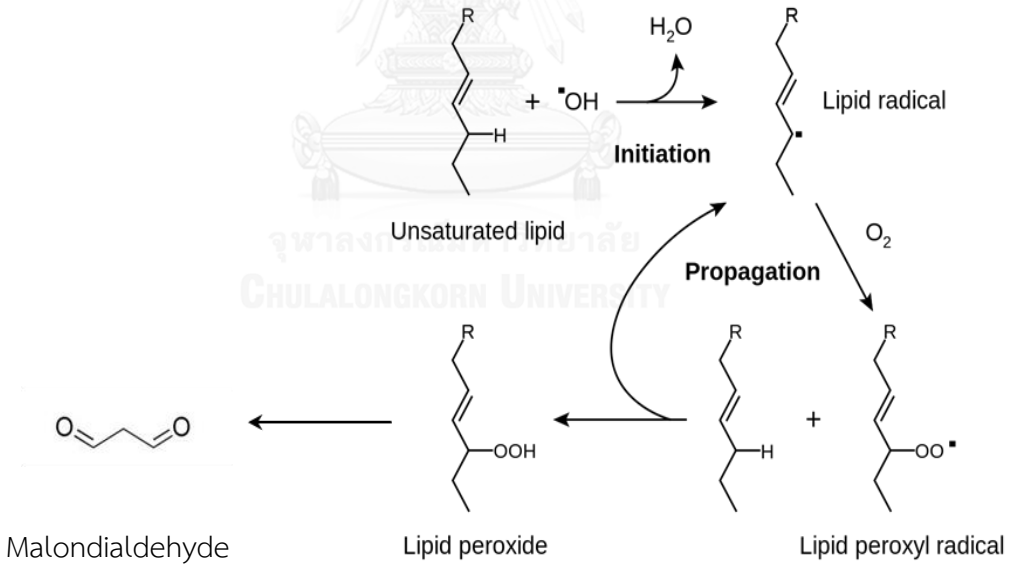
To determine this activity, 220 μM Aβ stock solution was diluted with 1x PBS (phosphate buffer saline, pH 7.4) to final concentration at 20 μM. Then, 150 μL of 20 μM Aβ solution was mixed with 150 μL of test sample. Two concentrations (20 and 100 μM) of the isolated and standard compounds (curcumin) prepared in DMSO were evaluated. The mixed solutions were incubated at 37 °C for 28 hours. After that, 80 μL of incubated sample and 20 μL of 50μM ThT in 500 glycine-NaOH buffer (pH 8.5) were added in 96-well plates. Then the fluorescence intensity was monitored with the excitation and emission at 450 and 490 nm, respectively. Each experiment was done in triplicate. The percentage inhibition was calculated from the equation that shown below.

$$\% \text{ Inhibition} = \left[\frac{F_{A\beta} - F_{sample}}{F_{A\beta}} \right] \times 100$$

When F_{sample} is the fluorescence intensity of sample with amyloid peptide and $F_{A\beta}$ is the fluorescence intensity of amyloid peptide aggregation.

2.5.3 Anti-lipid peroxidation activity

Lipid peroxidation is an oxidative process of lipid especially polyunsaturated fatty acids. Free radicals react with lipid in an organism to generate reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) as shown in Scheme 2.5. MDA can react and damage several functional cells such as proteins, lipoproteins and DNA. Increasing level of MDA is related to a wide range of chronic diseases in humans such as diabetic and neurogenerative diseases. Moreover, MDA is also used as a marker for monitoring oxidative stress and the antioxidant activities.



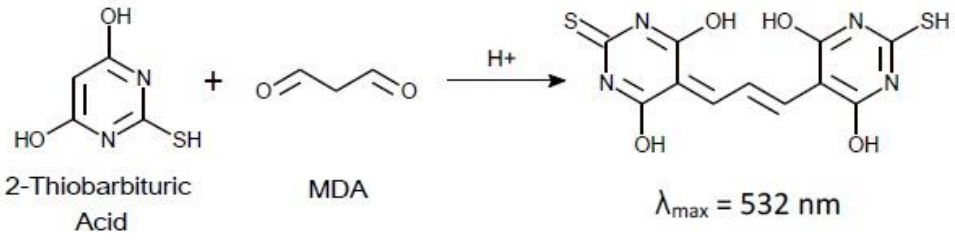
Scheme 2.5 Lipid peroxidation reaction

2.5.3.1 Reagent preparation

This activity was kindly tesed by Bio-screening Laboratory, Faculty of Pharmaceutical Sciences, Naresuan University. In this experiment, rat brain homogenates were used as sites of generation of reactive oxygen. A small piece of rat brain was mixed with 5 ml of 1xPBS buffer before homogenation and quantification of total protein was carried out by Bicinchoninic acid (BCA) assay.

2.5.3.2 Thiobarbituric acid reactive substance (TBARS) assay

This assay is based on the reaction of 2-thiobarbituric acid (TBA) and MDA [40]. One mole of MDA reacts with 2 moles of TBA via a Knoevenagel-type condensation to give a pink chromophore product which can be detected by UV-vis spectroscopy as shown below in Scheme 2.6



Scheme 2.6 The reaction of 2-thiobarbituric acid (TBA) and MDA

Briefly, 140 µl of 5.71 mg/ml rat brain homogenates were mixed with 20 µl of test sample and incubated at 37 ºC for 30 minutes. The concentrations of isolated compounds were 1 and 10 mM. Trolox was used as a positive control at 1 mM. Then 20 µl of 4 mM $FeSO_4 \cdot 7H_2O$ and 20 µl of 2 mM ascorbic acid were added into sample

and continuous incubated at 37 ° C for 1 h. After that, 200 µl of TBRA reagent (40%TCA, 1.4%TBA, and 8%HCl at ratio 1:2:1) was added, and the mixture was incubated at 90 ° C for additional 1 h. The sample was cooled to room temperature and centrifuged at 10,000 rpm for 5 min to precipitate pellet protein. Then, 100 µl of supernatant sample was added to a 96 well-plate, and its absorbance at wavelength of 532 nm was measured. Each experiment was done in triplicate. The percentage inhibition was calculated from the equation that shown below.

$$\% \text{ Inhibition} = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] \times 100$$

When A_{sample} is the absorbance of supernatant sample and $A_{control}$ is the absorbance of blank without any inhibitors

CHAPTER III

RESULT AND DISCUSSTION

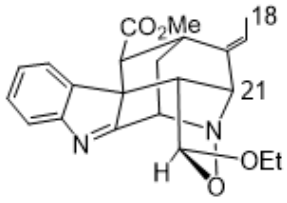
Methanol extracts of stems, leaves and flowers from *A. scholaris* were preliminarily screened for their anti-cholinesterase activity by the previous report [41]. The results are showed in Table 3.1.

Table 3.1 The anti-cholinesterase activity of *A. scholaris* extracts

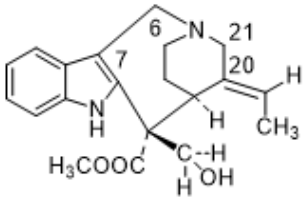
MeOH extract from	% Inhibition[a]	
	AChE	BChE
leaves	85.1 ± 0.8	95.2 ± 1.2
flowers	90.4 ± 1.0	98.1 ± 0.8
stems	70.3 ± 1.0	95.8 ± 0.5
eserine	99.6 ± 0.5	99.2 ± 0.2

[a]*The final concentration of three extracts and eserine were 1.0 and 0.1 mg/mL, respectively.*

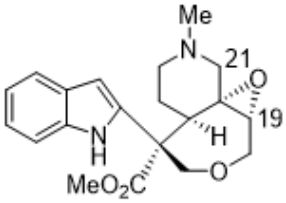
Four alkaloids were obtained from the isolation of leave extract [41] which were nareline ethyl ether, 19,20-(*E*)-vallesamine, 6,7-*seco*-19,20-epoxyangustilobine and 19,20-(*E*)-akuammidine (Figure 3.1). They inhibited AChE and BChE with the IC_{50} values in a range of 0.25-0.41 and 0.26-0.43 mM, respectively. At the concentration of 100 μM, they showed the anti-amyloid aggregation about 40%. Thus, in this study, flower extract which showed high anti-cholinesterase activity was further isolated and investigated to obtain the active constituents.



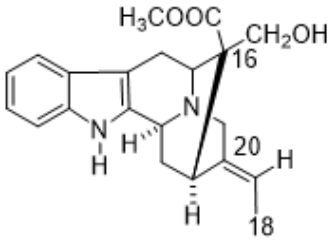
nareline ethyl ether



19, 20-(*E*)-vallesamine



6, 7-*seco*-19, 20-epoxyangustilobine



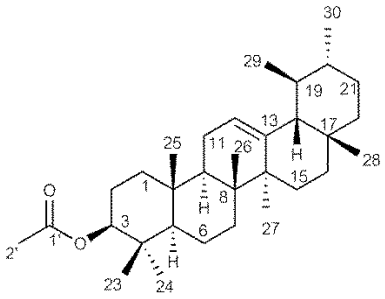
19, 20-(*E*)-akuammidine

Figure 3.1 The chemical structures of isolated compounds from the leave of *A. scholaris*

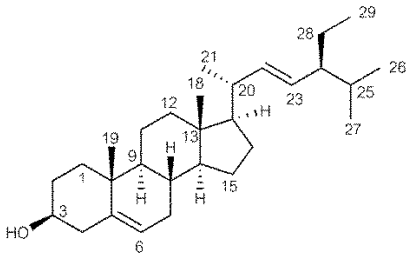
3.1 Isolation and structure elucidation of isolated compounds

Six compounds were isolated from the dichloromethane extract of *A. scholaris* flowers as described in Chapter II. All compounds were monitored by thin layer chromatography (TLC). Compounds **1** to **5** were determined as triterpenoid based on their spots on the TLC plate. Their spots could not be observed under UV light but showed the purple to green spots after dipping in 20% H_2SO_4 in methanol. Compound **6** was clearly seen under UV lamp of 254 nm. This compound gave the orange spot with dragendorff's reagent which identified itself as alkaloid. Moreover, the chemical structures of compounds **1**-**6** were further elucidated based on ^{1}H and ^{13}C NMR spectroscopic data and compared with those from the previous literatures. It was clearly that compounds **1**-**6** are α-amyrin acetate, stigmasterol, betulin, ursolic acid, β-sitosterol-3-O-D-glucopyranoside and nareline, respectively. The chemical structures of six compounds were shown in Figure. 3.2. The NMR spectrum and elucidation were shown and summarized in Figures 3.3-3.14 and Tables 3.2-3.7, respectively.

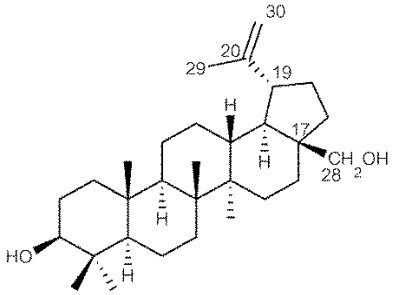
All isolated compounds had been found in *A. Scholaris.* Stigmasterol was a major component in all parts of this plant. Ursolic acid, α-amyrin acetate and betulin were the major triterpenoids obtained from the flowers of *A. scholaris*. In addition, nareline and β-sitosterol-3-O-D-glucopyranoside were found in the leaves of this plant.



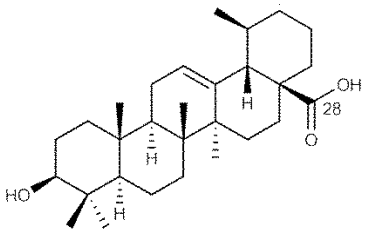
α-Amyrin acetate (**1**)



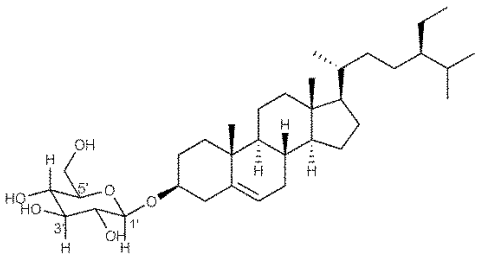
Stigmasterol (**2**)



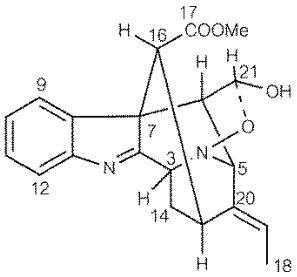
Betulin (**3**)



Ursolic acid (**4**)



β-Sitosterol-3-O-D-glucopyranoside (**5**)



Nareline (**6**)

Figure 3.2 The chemical structures of six isolated compounds form *A. scholaris* flowers

Table 3.2 The ^{1}H and ^{13}C data of compound **1** compared with **α**-amyrin acetate [42]

Position	Compound 1 ($CDCl_3$)		**α**-Amyrin acetate ($CDCl_3$)	
	δ_C	**δ_H (int., mult., *J* in Hz)**	**δ_C**	**δ_H (int., mult., *J* in Hz)**
1	38.5	-	38.6	-
2	23.6	-	23.8	-
3	81.0	4.53 (1H, *dd, J*= 6.3, 9.8 Hz)	81.2	4.48 (*dd*)
4	37.7	-	37.9	-
5	55.3	-	55.5	-
6	18.3	-	18.5	-
7	32.9	-	33.1	-
8	40.0	-	40.2	-
9	47.7	-	47.8	-
10	36.8	-	37.0	-
11	23.4	-	23.6	-
12	124.3	5.15 (1H, *t*, *J*= 3.4 Hz)	124.5	5.10 (t, 3.6)
13	139.6	-	139.8	-
14	42.1	-	42.4	-
15	28.1	-	28.3	-
16	26.6	-	26.8	-
17	33.8	-	34.0	-
18	59.1	1.31 (1H, *d, J*= 2.7 Hz)	59.3	1.29
19	39.6	-	39.8	-
20	39.7	-	39.9	-
21	31.3	-	31.5	-
22	41.5	-	41.7	-
23	28.1	0.90 (3H, *s*)	28.3	0.85 (3H, *s*)
24	16.7	0.89 (3H, *s*)	17.0	0.84 (3H, *s*)
25	15.7	1.00 (3H, *s*)	15.9	0.96 (3H, *s*)
26	17.5	1.03 (3H, *s*)	17.7	0.98 (3H, *s*)
27	23.3	1.09 (3H, *s*)	23.4	1.04 (3H, *s*)
28	28.7	0.82 (3H, *s*)	29.1	0.78 (3H, *s*)
29	16.9	0.86 (3H, *d*, *J*= 3.9 Hz)	17.0	0.77 (d)
30	21.4	0.96 (3H, *d*, *J*= 3.7 Hz)	21.6	0.83 (d)
1’	171.1	-	171.5	-
2’	21.3	2.07 (3H, *s*)	21.5	2.02 (3H, *s*)

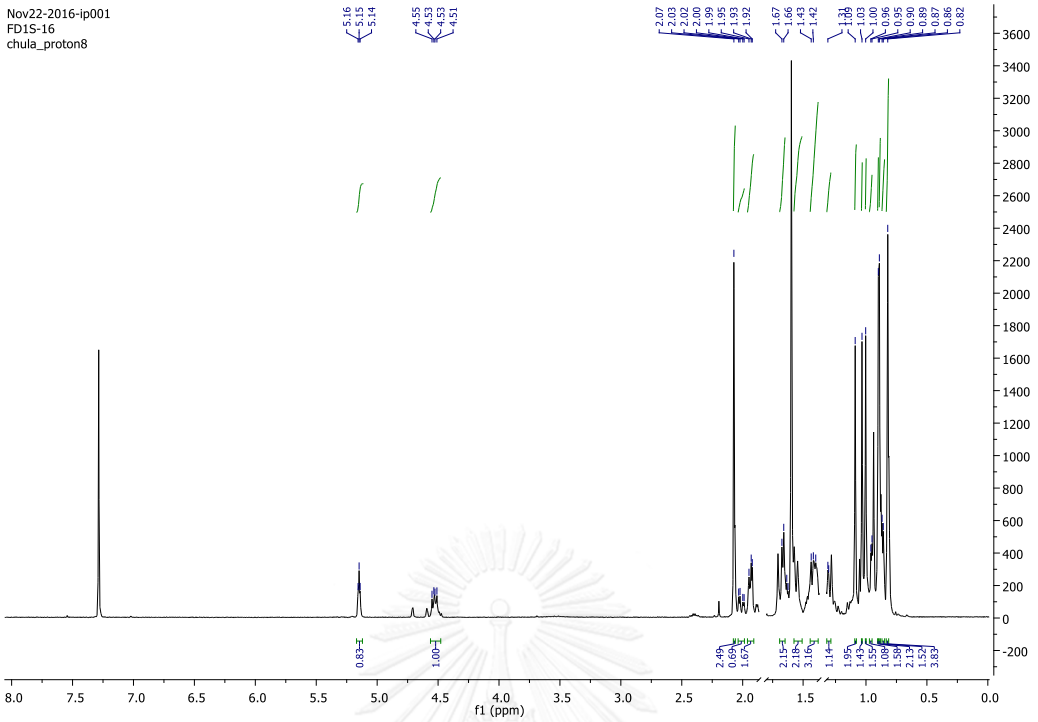


Figure 3.3 The ^{1}H-NMR spectrum ($CDCl_3$) of compound 1

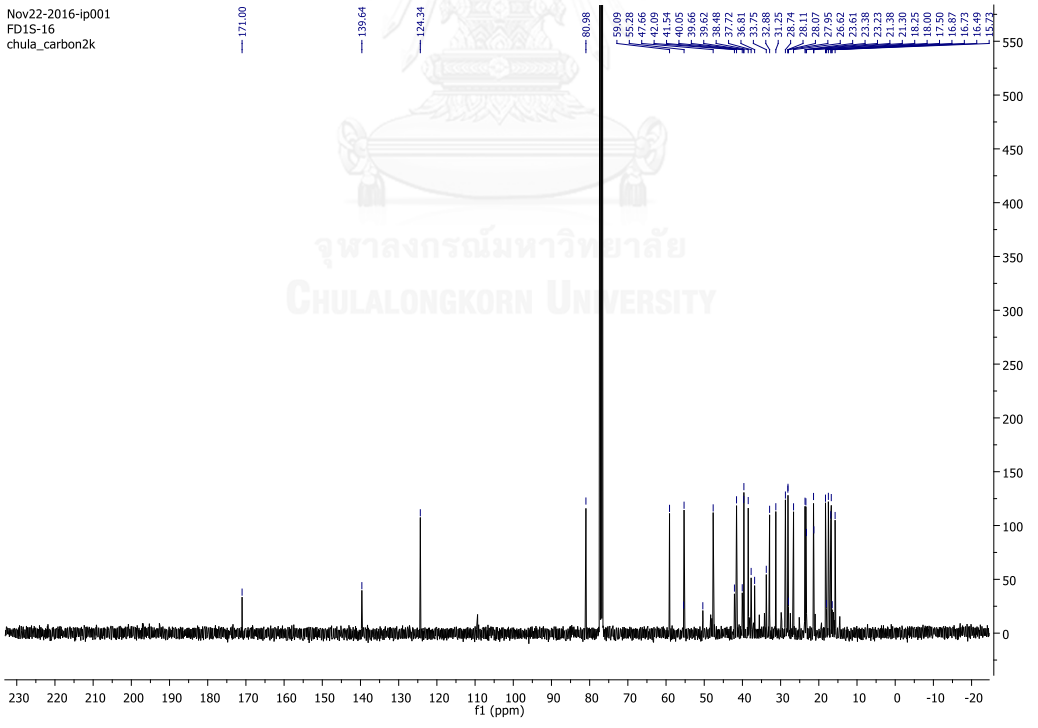


Figure 3.4 The ^{13}C-NMR spectrum ($CDCl_3$) of compound **1**

Table 3.3 The ^{1}H and ^{13}C data of compound **2** compared with stigmasterol [43]

Position	Compound 2 ($CDCl_3$)		Stigmasterol ($CDCl_3$)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	36.1	-	37.6	-
2	32.0	-	32.1	-
3	71.8	3.54 (1H, *m*)	72.1	3.51 (1H, *tdd*, *J*= 4.5, 4.2, 3.8 Hz)
4	42.3	-	42.4	-
5	140.8	5.37 (1H, *t*, *J*= 5.0 Hz)	141.1	5.31 (1H, *t*, *J*= 6.1 Hz)
6	121.7	-	121.8	-
7	29.2	-	31.8	-
8	31.7	-	31.8	-
9	50.2	-	50.2	-
10	34.0	-	36.6	-
11	19.8	-	21.5	-
12	36.5	-	39.9	-
13	39.8	-	42.4	-
14	56.8	-	56.8	-
15	23.1	-	24.4	-
16	26.1	-	29.3	-
17	56.1	-	56.2	-
18	37.3	-	40.6	-
19	21.1	0.94 (3H, *d*, *J*= 6.5 Hz)	21.7	0.91 (3H, *d*, *J*= 6.2 Hz)
20	138.3	5.03 (1H, *dd*, *J*= 15.1, 8.6	138.7	5.02 (1H, dd, *J*=15.2, 8.6 Hz)
21	129.3	Hz)	129.6	5.15 (1H, dd, *J*=15.2, 8.6 Hz)
22	45.9	5.17 (1H, *dd*, *J*= 15.1, 8.5	46.1	-
23	24.3	Hz)	25.4	-
24	11.9	-	12.1	0.83 (3H, *t*, *J*= 7.1 Hz)
25	28.2	-	29.6	-
26	19.4	0.87 (m)	20.2	0.82 (3H, *d*, *J*= 6.6 Hz)
27	19.0	-	19.8	0.80 (3H, *d*, *J*= 6.6 Hz)
28	18.8	0.84 (3H, d, *J*= 5.7 Hz)	18.9	0.71 (3H, *s*)
29	12.0	0.82 (3H, d, *J*= 5.4 Hz)	12.2	1.03 (3H, *s*)
		0.70 (3H, *s*)		
		1.03 (3H, *s*)		

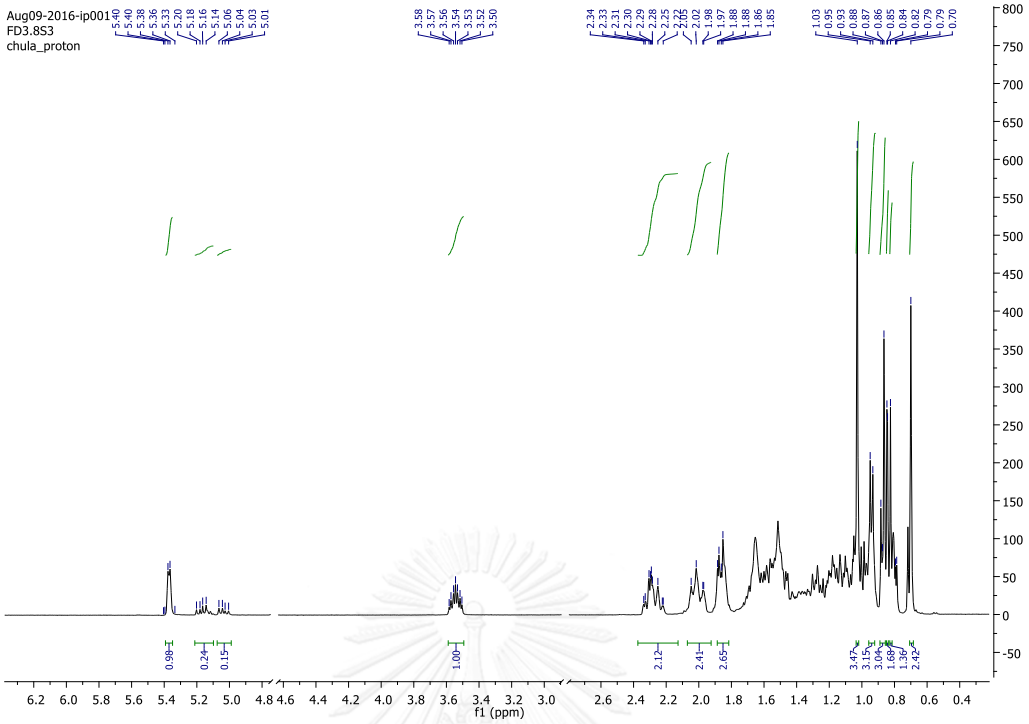


Figure 3.5 The ^{1}H-NMR spectrum ($CDCl_3$) of compound **2**

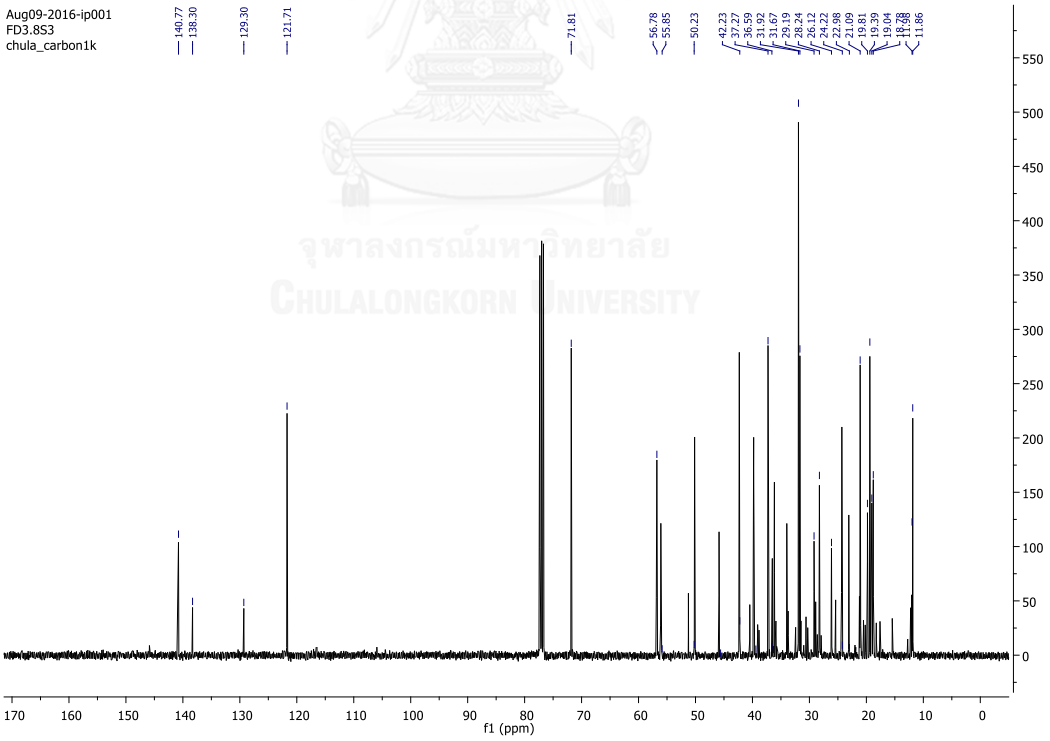


Figure 3.6 The ^{13}C-NMR spectrum ($CDCl_3$) of compound **2**

Table 3.4 The ^{1}H and ^{13}C data of compound **3** compared with betulin [44].

Position	Compound 3 ($CDCl_3$)		Betulin ($CDCl_3$)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	37.3	-	38.6	-
2	27.4	-	27.3	-
3	79.0	3.26 (1H, *dd*, *J*= 12.0, 6.3 Hz)	78.9	3.18 (1H, *dd*, *J*= 11.4, 4.8 Hz)
4	38.7	-	38.8	-
5	55.3	0.71 (1H, *d*, *J*= 12.0 Hz)	55.2	0.67 (1H, *d*, *J*= 9.0 Hz)
6	18.3	-	18.2	-
7	32.8	-	34.1	-
8	38.8	-	40.8	-
9	50.2	-	50.3	-
10	34.3	-	37.1	-
11	23.3	-	20.7	-
12	25.2	-	25.1	-
13	35.1	-	37.2	-
14	42.4	-	42.6	-
15	27.0	-	26.9	-
16	29.2	-	29.1	-
17	47.8	-	47.7	-
18	48.8	-	48.7	-
19	47.8	2.17 (1H, *m*)	47.6	2.37 (1H, *m*)
20	150.4	-	150.4	-
21	29.7	-	29.6	-
22	34.0	-	34.1	-
23	28.0	0.90 (3H, *s*)	27.9	0.96 (3H, *s*)
24	15.6	0.75 (3H, *s*)	15.3	0.76 (3H, *s*)
25	16.1	0.88 (3H, *s*)	16.0	0.82 (3H, *s*)
26	16.0	0.95 (3H, *s*)	15.9	1.02 (3H, *s*)
27	15.3	0.93 (3H, *s*)	14.7	0.97 (3H, *s*)
28	60.6	3.73 (1H, d, *J*= 10.2 Hz)	60.5	-
		3.46 (1H, d, *J*= 10.7 Hz)		
29	109.7	4.58 (2H, *brs*)	109.6	4.68 (*brs*)
30	20.8	1.61 (3H, *s*)	19.0	1.68 (3H, *s*)

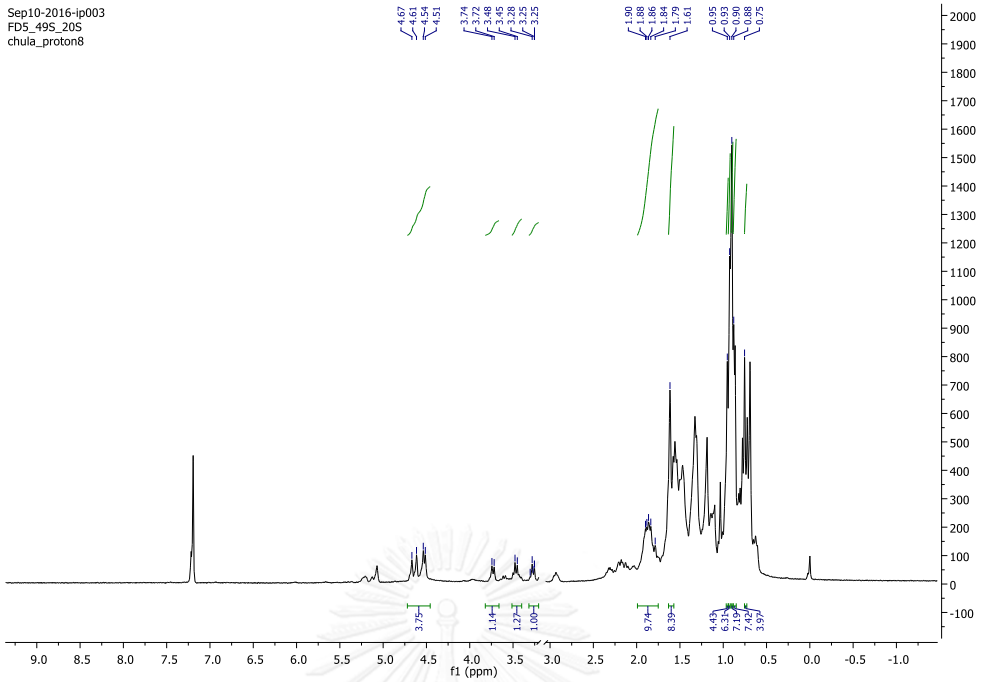


Figure 3.7 The ^{1}H-NMR spectrum ($CDCl_3$) of compound **3**

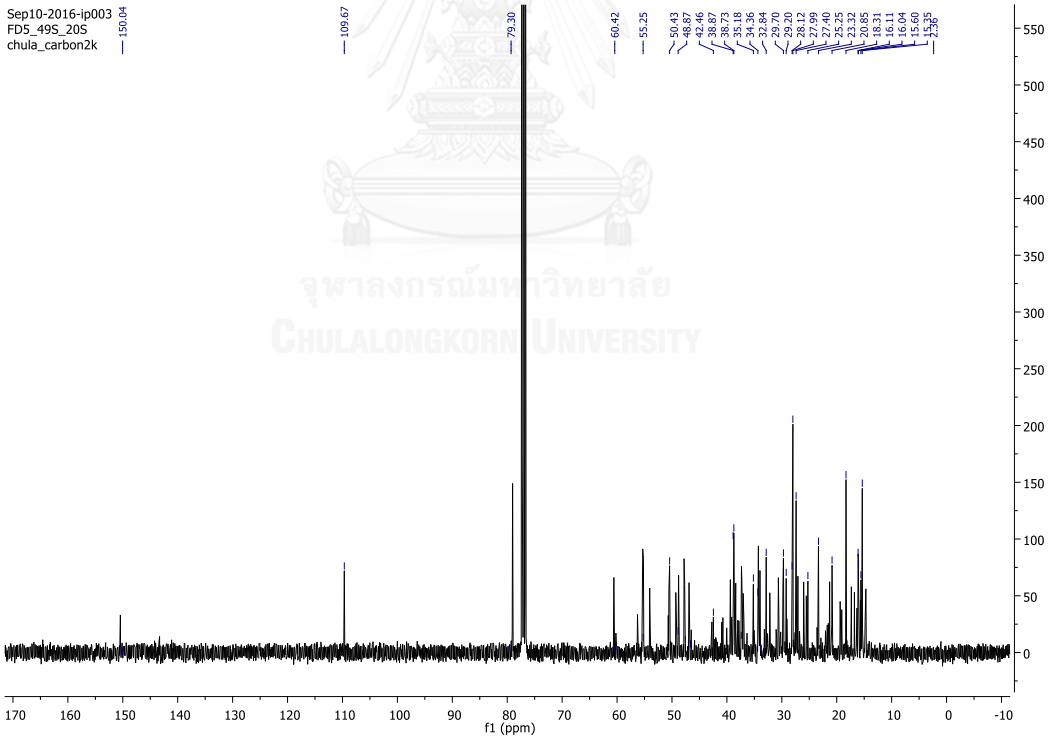


Figure 3.8 The ^{13}C-NMR spectrum ($CDCl_3$) of compound **3**

Table 3.5 The ^{1}H and ^{13}C data of compound **4** compared with ursolic acid [45]

Position	Compound 4 (DMSO-d_6)		Ursolic acid (DMSO-d_6)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	36.5	-	38.4	-
2	26.8	-	28.1	-
3	76.8	3.03 (1H, m)	78.1	3.43 (1H, brs)
4	38.2	-	38.4	-
5	54.8	-	55.8	-
6	18.0	-	18.8	-
7	30.1	-	33.6	-
8	38.5	-	40.0	-
9	47.0	-	48.3	-
10	36.3	-	37.4	-
11	22.6	-	23.6	-
12	124.6	5.20 (1H, m)	125.6	5.50 (1H, *brs*)
13	138.2	-	139.7	-
14	41.6	-	42.5	-
15	27.0	-	28.7	-
16	23.3	-	24.9	-
17	46.8	-	48.0	-
18	52.4	2.11 (1H, d, *J*= 11.2)	53.5	2.52 (1H, d, *J*= 11.0)
19	38.5	-	39.5	-
20	38.4	-	39.1	-
21	28.7	-	31.1	-
22	32.7	-	37.3	-
23	27.5	1.05 (3H, *s*)	28.8	1.24 (3H, *s*)
24	15.1	0.87 (3H, *s*)	15.7	1.02 (3H, *s*)
25	16.0	0.68 (3H, *s*)	16.6	0.93 (3H, *s*)
26	16.9	0.90 (3H, *s*)	17.4	1.05 (3H, *s*)
27	22.8	0.92 (3H, *s*)	23.8	1.22 (3H, *s*)
28	179.2	-	180.0	-
29	17.0	0.74 (3H, *d*, *J*= 6.14 Hz)	17.5	0.97 (3H, *d*, *J*= 6.10 Hz)
30	21.0	0.82 (3H, *d*, *J*= 6.14 Hz)	21.4	0.99 (3H, *d*, *J*= 6.10 Hz)

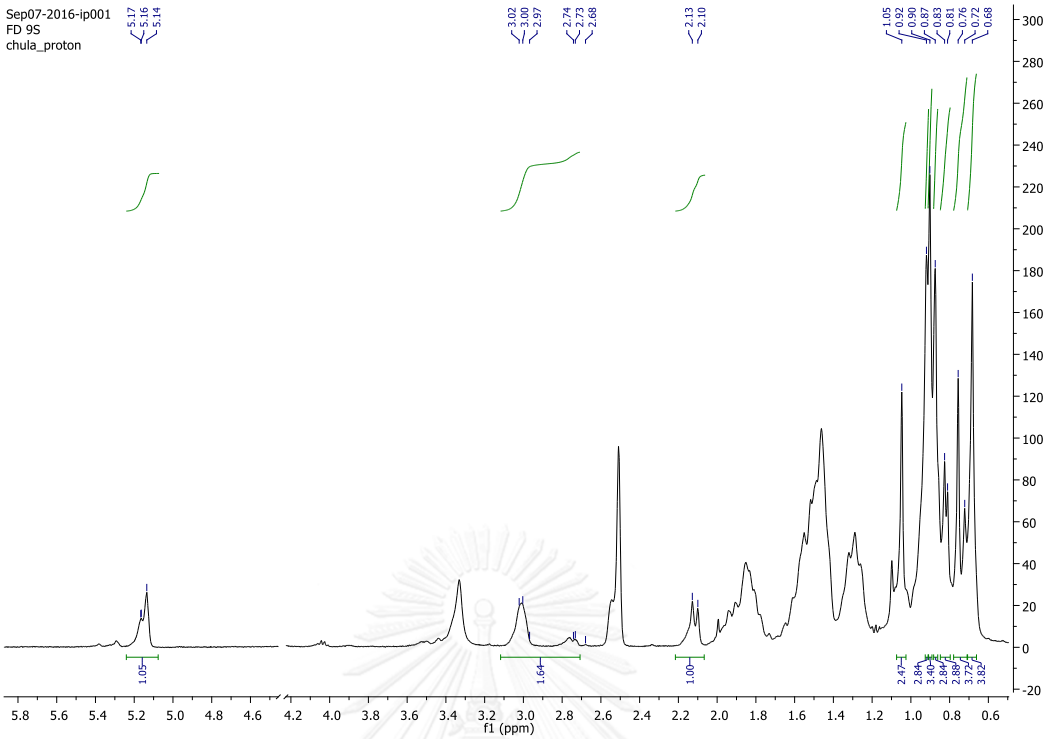


Figure 3.9 The ^{1}H-NMR spectrum (DMSO-d_4) of compound **4**

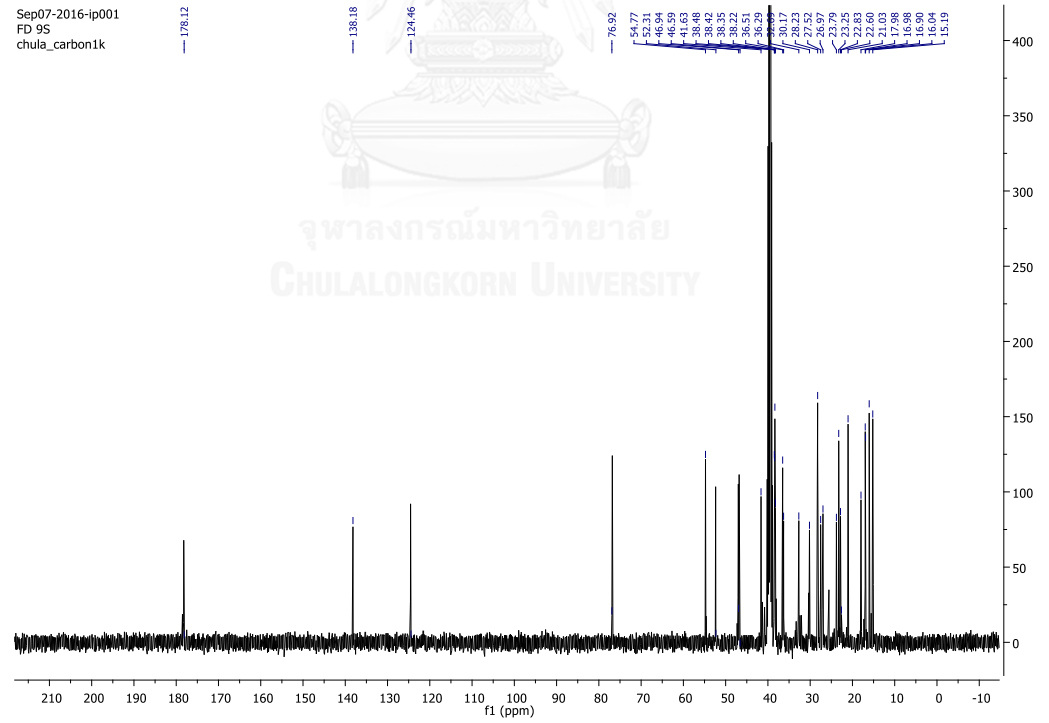


Figure 3.10 The ^{13}C-NMR spectrum (DMSO-d_6) of compound **4**

Table 3.6 The ^{1}H and ^{13}C data of compound **5** compared with β-Sitosterol-3-O-D-glucopyranoside [46]

Position	Compound 5 (pyridine-d_5)		β-Sitosterol-3-O-D-glucopyranoside (pyridine-d_5)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	37.9	-	37.5	-
2	30.7	-	30.3	-
3	79.1	3.94 (1H, *m*)	78.5	3.13 (1H, *m*)
4	42.9	-	42.1	-
5	141.4	-	140.9	-
6	122.3	5.28 (1H, *brs*)	121.9	5.09 (1H, *brs*)
7	32.7	-	32.2	-
8	32.6	-	32.1	-
9	50.8	-	50.3	-
10	37.4	-	36.9	-
11	21.7	-	21.3	-
12	39.8	-	39.3	-
13	40.4	-	41.9	-
14	57.3	-	56.8	-
15	24.9	-	24.5	-
16	29.0	-	28.6	-
17	56.7	-	56.2	-
18	12.4	0.64 (3H, *s*)	12.0	0.62 (3H, *s*)
19	20.1	0.92 (3H, *s*)	19.43	0.94 (3H, *s*)
20	36.8	-	36.4	
21	19.9	0.97 (3H, *d*, *J*= 6.4 Hz)	19.0	0.84 (3H, *d*, *J*= 6.3 Hz)
22	34.7	-	34.2	-
23	26.9	-	26.4	-

Table 3.6 The ^{1}H and ^{13}C data of compound **5** compared with β-Sitosterol-3-O-D-glucopyranoside **(**continue**)**

Position	Compound 5 (pyridine-d_5)		β-Sitosterol-3-O-D-glucopyranoside (pyridine-d_5)	
	δ_C	**δ_H (int., mult., *J* in Hz)**	**δ_C**	**δ_H (int., mult., *J* in Hz)**
24	46.5	-	46.0	-
25	29.9	-	29.4	-
26	19.5	0.86 (3H, *d*, *J*= 7.5 Hz)	19.2	0.75 (3H, *d*, *J*= 7.7 Hz)
27	19.8	0.81 (3H, *d*, *J*= 2.6 Hz)	20.0	0.73 (3H, *d*, *J*= 1.6 Hz)
28	23.8	-	23.4	-
29	12.77	-	12.1	-
1’	103.1	4.56 (1H, *d*, *J*= 6.8 Hz)	102.5	4.11 (1H, *d*, *J*= 7.8 Hz)
2’	75.8	4.34 (1H, *m*)	75.3	3.14 (1H, *m*)
3’	78.9	4.34 (1H, *m*)	78.5	3.14 (1H, *m*)
4’	72.2	4.34 (1H, *m*)	71.6	3.14 (1H, *m*)
5’	78.6	4.28 (1H, *m*)	78.1	3.06 (1H, *m*)
6’	63.3	4.06 (1H, *m*)	62.8	2.94 (1H, *m*)

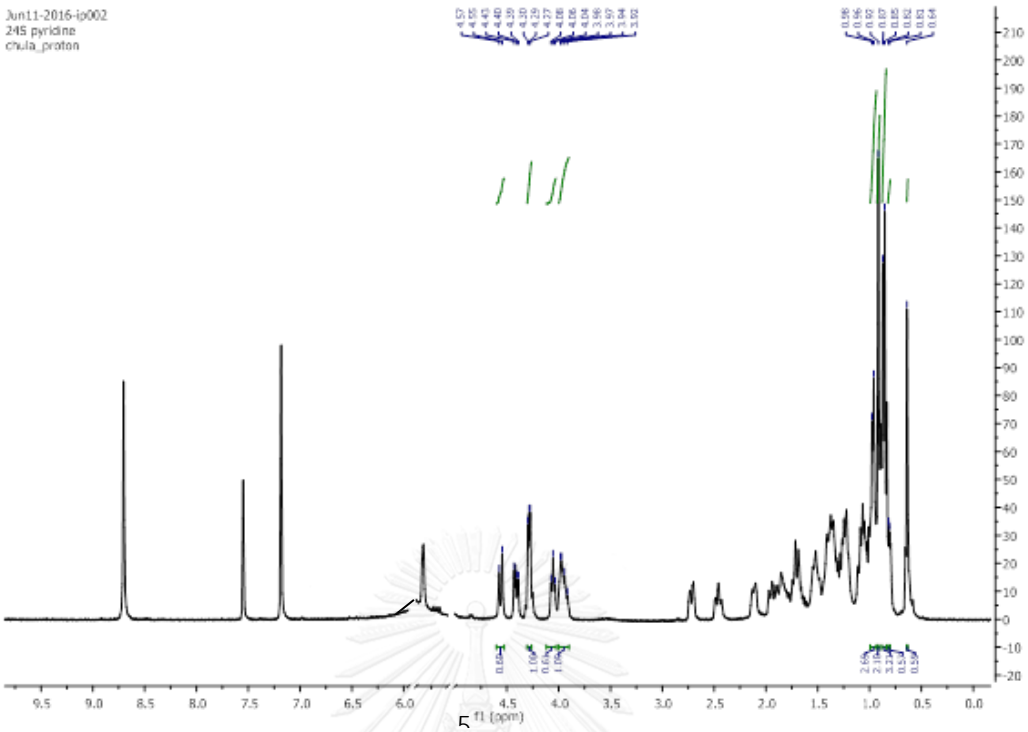


Figure 3.11 The ^{1}H-NMR spectrum (pyridine-d_5) of compound **5**

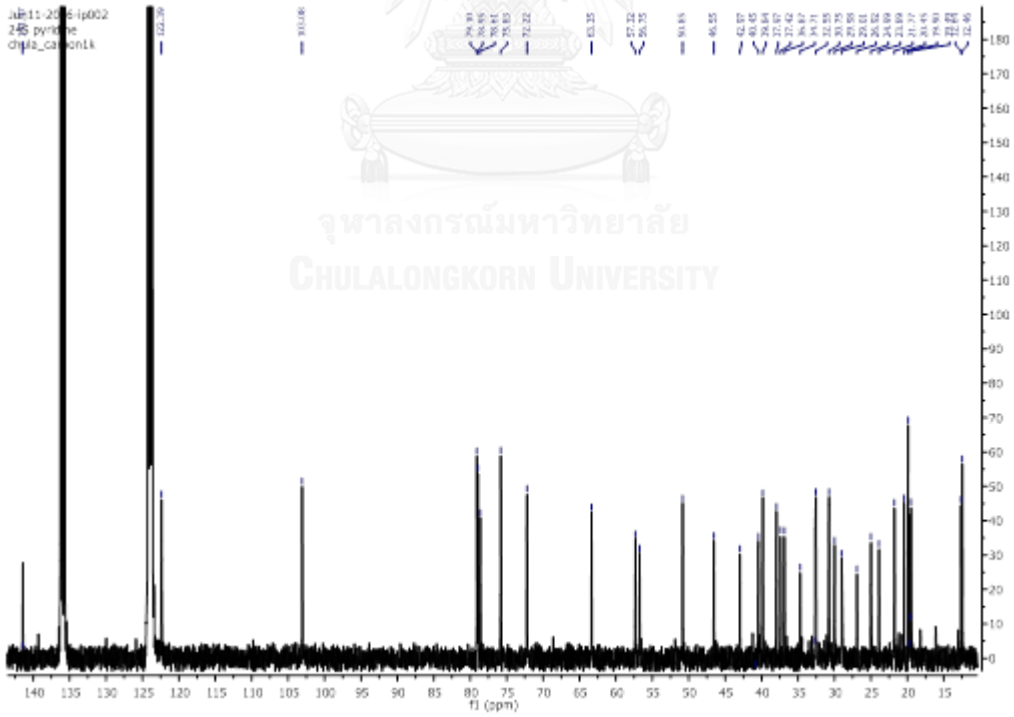


Figure 3.12 The ^{13}C-NMR spectrum (pyridine-d_5) of compound **5**

Table 3.7 The ^{1}H and ^{13}C data of compound **6** compared with [47]

Position	Compound 6 (DMSO-d_6)		Nareline (DMSO-d_6)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
2	184.2	-	184.4	-
3	61.9	4.40 (1H, *t*, *J*= 2.9 Hz)	62.1	4.57 (1H, *t*, *J*= 3 Hz)
5	99.4	4.09 (1H, *s*)	99.6	4.18 (1H, *s*)
6	55.7	3.89 (1H, *d*, *J*= 2.9 Hz)	55.4	3.74 (1H, *d*, *J*= 3 Hz)
7	54.9	-	55.0	-
8	139.8	-	138.9	-
9	124.9	7.66 (1H, d, *J*= 7.8 Hz)	125.0	7.77 (1H, d, *J*= 7 Hz)
10	125.4	7.27 (1H, t, *J*= 7.4 Hz)	125.6	7.29 (1H, t, *J*= 7 Hz)
11	128.7	7.44 (1H, t, *J*= 7.6 Hz)	128.5	7.45 (1H, t, *J*= 7 Hz)
12	120.4	7.62 (1H, d, *J*= 7.6 Hz)	119.8	7.66 (1H, d, *J*= 7 Hz)
13	157.6	-	156.2	-
14	34.2	2.14 (2H, m)	34.5	2.14 (dd, *J*= 14, 3 Hz), 2.36 (dd, *J*= 14, 2 Hz)
15	31.0	3.44 (1H, m)	31.9	3.41 (1H, q, *J*= 3 Hz)
16	52.6	2.38 (1H, d, *J*= 2.7 Hz)	53.3	2.35 (1H, d, *J*= 2 Hz)
17	170.9	-	170.5	-
18	12.3	1.65 (3H, d, *J*= 6.8 Hz)	11.6	1.73 (3H, d, *J*= 7 Hz)
19	121.5	5.76 (1H, q, *J*= 6.7 Hz)	122.6	5.86 (1H, q, *J*= 7 Hz)
20	131.5	-	130.1	-
21	64.8	3.97 (1H, d, *J*= 2.8 Hz)	65.1	4.13 (1H, d, *J*= 3 Hz)
-COOMe	51.6	3.68 (3H, *s*)	51.0	3.75 (3H, *s*)

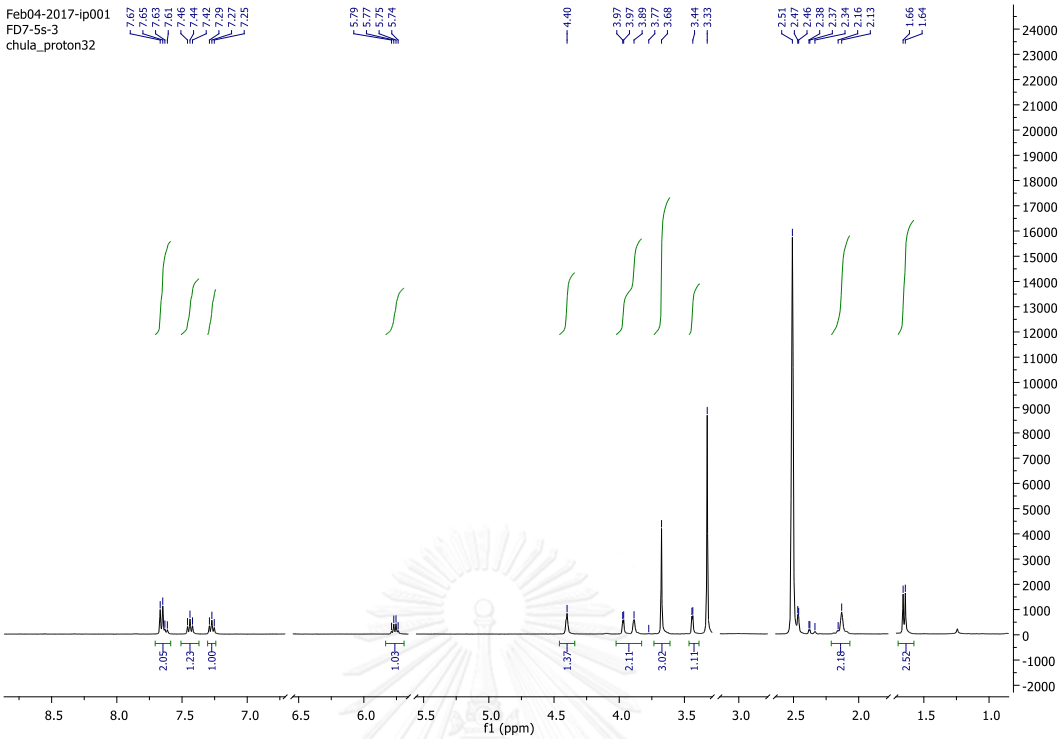


Figure 3.13 The ^{1}H-NMR spectrum (DMSO-d_6) of compound **6**

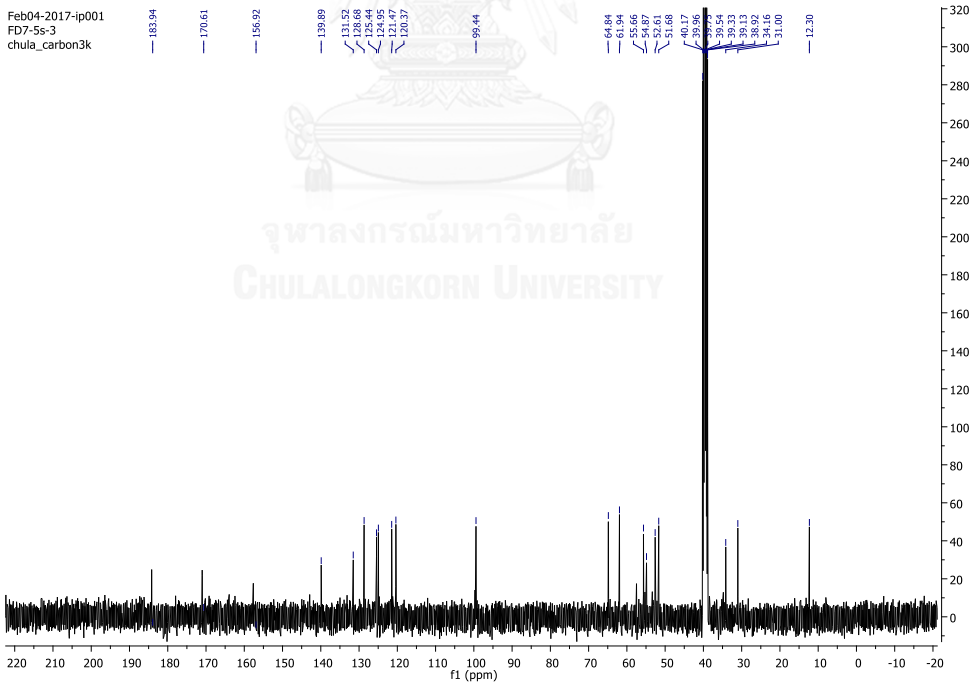


Figure 3.14 The ^{13}C-NMR spectrum (DMSO-d_6) of compound **6**

3.2 Bioactivities of isolated compounds

3.2.1 Anti-cholinesterase inhibitory activity

All isolated compounds were evaluated for their cholinesterase inhibitory activity. The experiment was done with different five final concentrations to evaluate their IC_{50} values toward AChE and BChE. Eserine was used as a standard. The results are shown in Table 3.8.

Table 3.8 AChE and BChE inhibitory activity (IC_{50}) of all isolated compounds

Compound	IC_{50} of AChE (mM)	IC_{50} of BChE (mM)
1	NA	NA
2	0.195 ± 0.003	0.721 ± 0.007
3	0.191 ± 0.009	0.676 ± 0.015
4	0.231 ± 0.007	0.672 ± 0.006
5	0.122 ± 0.003	0.538 ± 0.077
6	0.070 ± 0.004	0.282 ± 0.004
Eserine	0.013 ± 0.001	0.011 ± 0.012

Note: Results are expressed as mean ± SD (n=3)
NA = no activity

Comparing the activity of isolated compounds with that of eserine, all triterpenoids (compounds **1-5**) showed low activity toward both cholinesterases. They inhibited AChE better than BChE. Compound **1** was less dissolved in MeOH and DMSO that might disturb the activity test by this method. Among of isolated triterpenoids, compound **5** had the highest inhibition that might due to the interaction via hydrogen bonding between the hydroxyl groups of glucose unit and the amino acid residues of the active sites of both enzymes [48]. Alkaloid **6** showed the highest activity, however,

it had significantly lower activity than that of standard. The presence of nitrogen atom in their structure aided this compound to better binding with the active site of both AChE and BChE [49].

Although these isolated triterpenoids 1-5 have been reported for their anti-AChE activity, they were evaluated by different times and conditions. In addition, there were no reports about the anti-BChE activity of these compounds excepted for ursolic acid. Table 3.9 summarized the activity of AChE and BChE inhibition of compounds 1-6 from this study and those of the previous reports. The anti-AChE activity of compounds 1 and 5 in this study was closed to the results of the previous studies. The anti-AChE activity of others was not consistent with those reported in the previous studies.

Triterpenoids **1**-**5** were previously reported a wide range of bioactivities. Ursolic acid inhibited gram-positive bacteria and showed a synergistic effect with ampicillin and tetracycline against both *Bacillus cereus* and *Staphylococcus aureus* [44]. β-Sitosterol-D-glucopyranoside had anti-bacterial activity and exhibited significant synergistic effect with ciprofloxacin [50]. Nareline had no bioactivity report.

Table 3.9 The cholinesterase inhibitory activities of triterpenoids isolated from *A. scholaris*

Compound	IC_{50} value of cholinesterase inhibitory activities in this study		Cholinesterase inhibitory activities from the previous reports	
	AChE (mM)	BChE (mM)	AChE	BChE
1	NA	NA	NA	NA
2	0.195 ± 0.003	0.721 ± 0.007	IC_{50}= 0.644 ±0.010 mM [51]	ND
3	0.191 ± 0.009	0.676 ± 0.015	IC_{50}= 0.029 ±0.001 mM [52]	ND
4	0.231 ± 0.007	0.672 ± 0.006	55.1 ± 0.34% at 0.437 mM [53]	68.1 ± 0.28% at 0.437 mM[53]
5	0.122 ± 0.003	0.538 ± 0.077	65.0 ± 1.30 % at 0.173 mM [54]	ND
Eserine	0.013 ± 0.001	0.011 ± 0.002	-	-

Note: Results are expressed as mean ± SD (n=3)

NA = no activity

Recently, drug combination therapy has been interesting to improve the treatment efficiency and lessen drug resistance. In this study, **α**-mangostin (MG) was chosen to study the synergistic AChE inhibition by 3 isolated compounds which were compounds **2**, **4** and **5**. MG had been reported as a good AChE inhibitor with the IC_{50} value of 2.14 uM [35]. However, the IC_{50} value of AChE inhibition of MG was 0.011 mM by our test. The concentration of each isolated compound in the combination test was used at the IC_{50} value of each compound. The combination result was shown in

Figure 3.15. The combination of MG with compounds **2** and **4** did not exhibit the synergistic effect. However, the combination of MG with compound **5** showed significantly enhanced AChE inhibition due to the presence of glucose moiety.

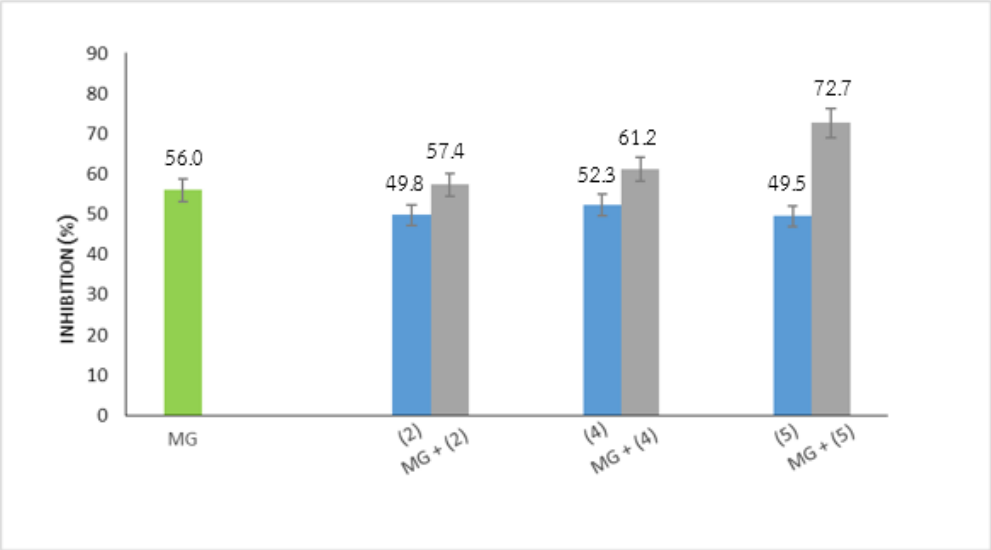


Figure 3.15 The AChE inhibitory activity of single compound and combination at the concentration of the IC_{50} value of each compound

Thus, the combination test of 2 concentrations of MG (0.011 and 0.005 mM) and 3 concentrations of compound **5** (0.180, 0.120 and 0.06 mM) was further studied. The results in Figure 3.16 showed that the inhibition percentages of mixed compounds were higher than individual compound. This result revealed that MG can enhance the AChE inhibition of compound **5** in a dose-dependent manner.

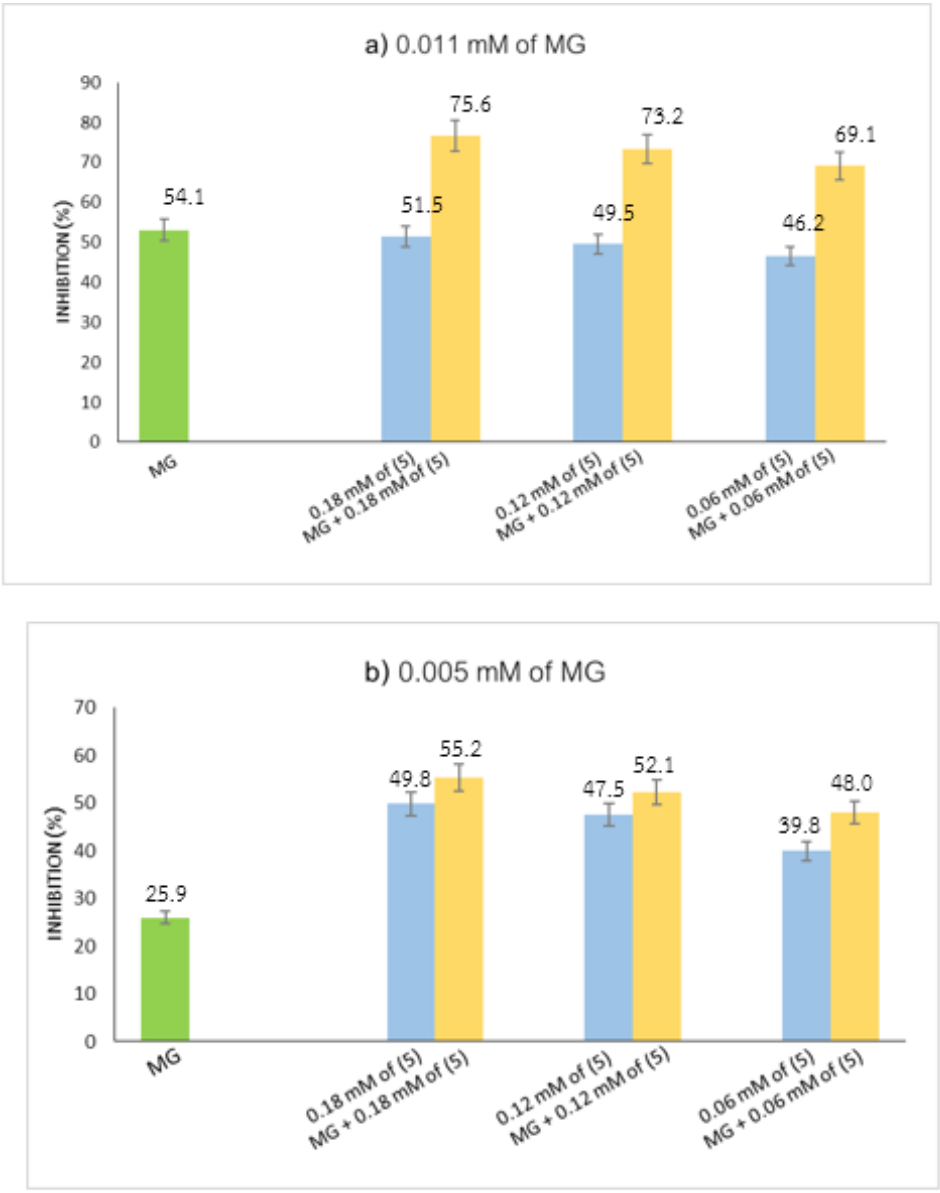


Figure 3.16 The AChE inhibitory activity of the combination of compound **5** at 0.18, 0.12 and 0.06 mM and **α**-mangostin at a) 0.011 mM and b) 0.005 mM

3.2.2 Anti-amyloid aggregation activity

All isolated compounds including MG were evaluated for their anti-amyloid aggregation activity at the final concentration of 100 μM. Curcumin was used as a standard. The results are shown in Table 3.10.

Table 3.10 The anti-amyloid aggregation activity of isolated compounds from *A. scholaris*

Compound	% Inhibition at the final concentration of 100 μM
1	14.7 ± 0.60
2	14.1 ± 0.85
3	13.3 ± 0.67
4	19.4 ± 0.40
5	14.4 ± 0.97
6	28.1 ± 0.60
Curcumin	46.8 ± 0.87
α-Mangostin	NA

Note: Results are expressed as mean ± SD (n=3)

NA = no activity

The results indicated that triterpenoids **1-5** showed inhibition lower than 20% while alkaloid **6** showed better activity (28.1%). This might be caused by the presence of aromatic ring or N-atom in core structure. According to previous research, they found that curcumins structure, β-diketone linking with two phenyl groups and the double bonds are essential structural for inhibit amyloid aggregation. Furthermore, some flavonoids and naphthoquinone also had a considerable influence on *in vitro* amyloid

aggregation inhibition. However, the mechanism in detail remains unclear [55]. Interestingly, **α**-mangostin had been reported as a good inhibitor *in vitro* anti-amyloid aggregation, determined by measuring the number of cell viability in rat neurons, with the IC_{50} value of 4.14 nM [44]. However, **α**-mangostin in this experiment had no activity and seemed to induce the aggregation of amyloid. This might be caused by high concentration of **α**-mangostin and different measurement method.

3.2.3 Anti-lipid peroxidation activity

Compounds **2**-**5** were subjected to test for anti-lipid peroxidation activity at the final concentrations of 0.1 and 1.0 mM. Trolox was used as a standard**.** The results are displayed in Table 3.11.

Table 3.11 The anti-lipid peroxidation activity of isolated compounds from *A. scholaris*

Compound	%inhibition at final concentration	
	0.1 mM	1.0 mM
2	7.42 ± 2.22	7.83 ± 2.83
3	3.20 ± 1.79	7.79 ± 2.90
4	8.72 ± 3.85	11.58 ± 2.40
5	7.65 ± 0.77	13.16 ± 5.00
Trolox	80.17 ± 2.96	NT

Note: Results are expressed as mean ± SD (n=3)

NT = not tested

All test compounds showed low activity. This was in agreement of the previous reports that phenolic moiety was important toward this activity [56].

CHAPTER IV
CONCLUSION

Five triterpenoids and one alkaloid were isolated from the dichloromethane extract of *A. scholaris* flowers by column chromatoghaphy. The chemical structures of all isolated compounds were elucidated by NMR analysis and compared with those of the previous reports. Five triterpenoids were α-amyrin acetate (**1**), stigmasterol (**2**), betulin (**3**), ursolic acid (**4**) and β-sitosterol-3-O-β-D-glucopyranoside (**5**), and one alkaloid was nareline (**6**). All isolated compounds were evaluated for their anti-cholinesterase, anti-amyloid aggregation and anti-lipid peroxidation activities. Five triterpenoids showed low AChE and BChE inhibitory activities with the IC_{50} values of 0.122 - 0.231 mM and 0.538 - 0.721 mM, respectively. On the other hand, nareline exhibited moderate inhibition toward both enzymes with the IC_{50} values of 0.070 and 0.282 mM, respectively. Furthermore, the combination of β-sitosterol-3-O-β-D-glucopyranoside (**5**) and α-mangostin had the AChE synergistic effect with a dose-dependent manner. Most isolated triterpenoids displayed low anti-amyloid aggregation and anti-lipid peroxidation activities. Nareline exhibited moderate anti-amyloid aggregation with 28.1% at the final concentrations of 100 μM. Although the flowers extract had high inhibitory activity, the isolates were found to have less potency. This might be caused by the combination of chemical constituents in extract can enhance the bioactivity.

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