สารยับยั้งโคลีนเอสเทอเรสจากใบพญาสัตบรรณ *Alstonia scholaris* L. R. Br.

นางสาวธนวรรณ โรจน์ปิติกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHOLINESTERASE INHIBITORS FROM LEAVES OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. Br.

Miss Thanawan Rojpitikul

A Thesis Submitted in Partial Fulfillment of the Requirements
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By	Miss Thanawan Rojpitikul
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Pattara Thiraphibundet, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ธนวรรณ โรจน์ปิติกุล : สารยับยั้งโคลีนเอสเทอเรสจากใบพญาสัตบรรณ *Alstonia scholaris* L. R. Br. (CHOLINESTERASE INHIBITORS FROM LEAVES OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. Br.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. พัฒทรา ธีรพิบูลย์เดช, 75 หน้า.

สารอินโดลอัลคาลอยด์ 4 ชนิดได้ถูกแยกออกจากสิ่งสกัดอัลคาลอยด์ของใบพญาสัตบรรณ *Alstonia scholaris* L. R. Br. โครงสร้างทางเคมีของสารทั้งหมดได้ถูกพิสูจน์ด้วยเทคนิคเอ็น เอ็ม อาร์ สเปกโทรสโกปี โดยเปรียบเทียบกับรายงานที่ได้ตีพิมพ์แล้ว สารอัลคาลอยด์ที่แยกได้จากพืชชนิดนี้ คือ nareline ethyl ether (1) 19, 20-(*E*)-vallesamine (2) 6, 7-seco-19, 20-epoxyangustilobine B (3) และ 19-(*E*)-Akuammidine (4) สารที่แยกได้ทั้งหมดถูกนำไปทดสอบฤทธิ์ทางชีวภาพต่างๆ ได้แก่ ฤทธิ์ยับยั้งเอนไซม์โคลีนเอสเทอเรส และ ฤทธิ์ต้านการสะสมของแอมีลอยด์ บีต้า ผลการทดสอบพบว่าสารอัลคาลอยด์ (1 2 3 และ 4) มีค่าความเข้มข้นในการยับยั้งเอนไซม์แอซีทิลโคลีนเอสเทอเรสและบิวทิริลโคลีนเอสเทอเรสที่ร้อยละ 50 ซึ่งมีค่าใกล้เคียงกันมาก อยู่ในช่วง 0.25 ถึง 0.41 และ 0.26 ถึง 0.43 มิลลิโมลาร์ ตามลำดับ นอกจากนี้สารอัลคาลอยด์ (1 2 3 และ 4) ยังมีฤทธิ์ต้านการสะสมของแอมีลอยด์ บีต้า อยู่ในช่วง 29 ถึง 33 เปอร์เซ็นต์ และ 35 ถึง 43 เปอร์เซ็นต์ ที่ความเข้มข้น 20 และ 100 ไมโครโมลาร์ ตามลำดับ ในท้ายที่สุดพบว่าสารผสมของอัลคาลอยด์ (1 2 3 และ 4) ช่วยลดการสะสมของแอมีลอยด์ บีต้า ซึ่งเป็นสาเหตุของการเกิดแอมีลอยด์ พลาก ได้อย่างมีนัยสำคัญ โดยสังเกตด้วยภาพจากกล้องจุลทรรศน์อิเล็กตรอนแบบทรานสมิสชัน (TEM)

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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THANAWAN ROJPITIKUL: CHOLINESTERASE INHIBITORS FROM LEAVES OF INDIAN DEVIL TREE *Alstonia scholaris* L. R. Br.. ADVISOR: ASST. PROF. PATTARA THIRAPHIBUNDET, Ph.D., 75 pp.

Four indole alkaloids were isolated from the alkaloid enriched (AE) extract of *Alstonia scholaris* L. R. Br. and their chemical structures were elucidated on the basis of NMR spectroscopic data compared with the previous research. These isolated alkaloids were identified as nareline ethyl ether (1), 19,20-(*E*)-vallesamine (2), 6,7-seco-19,20-epoxyangustilobine B (3) and 19,20-(*E*)-Akuammidine (4). All isolated compounds were evaluated their anti-cholinesterase and anti-amyloid aggregation activities. The 50% inhibition concentration values of alkaloids 1-4 towards acetylcholinesterase and butyrylcholinesterase were found to be similarly which were in the range of 0.25-0.41 mM and 0.26–0.43 mM, respectively. In addition, alkaloids 1-4 inhibited amyloid aggregation in the range of 29-33% and 35-43% at the final concentrations of 20 and 100 μM, respectively. Finally, the mixing alkaloids 1-4 significantly reduced accumulation of amyloid plaques observed from TEM images.

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

Academic Year: 2014 Advisor's Signature .....

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LIST OF ABBREVIATIONS

Aβ	=	Beta-amyloid
acetone-d_6	=	deuterated acetone
AChE	=	acetylcholinesterase
A. scholaris	=	*Alstonia scholaris*
BChE	=	butyrylcholinesterase
br	=	broad (NMR)
br s	=	broad singlet (NMR)
BuOH	=	butanol
C	=	carbon
$CHCl_3$	=	chloroform
chloroform-d_1	=	deuterated chlorofrom
CH_2Cl_2	=	dichloromethane
cm	=	centimeter (s)
^{13}C-NMR	=	carbon-13 nuclear magnetic resonance
COSY	=	correlation spectroscopy
d	=	doublet (NMR)
dd	=	doublet of doublet (NMR)
EtOAC	=	ethyl acetae

g	=	gram (s)
H	=	hydrogen
^{1}H-NMR	=	proton-1 nuclear magnetic resonance
HMBC	=	heteronuclear multiple bond correlation
HSQC	=	heteronuclear single quantum coherence
Hz	=	hertz (NMR)
IC_{50}	=	concentration to inhibit enzyme active by 50 percent
in vitro	=	literally in glass
in vivo	=	within a living organism
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
n	=	sample size (statistic)
NOSEY	=	nuclear overhauser effect spectroscopy
OH	=	hydroxy group
OMe	=	methoxy group
ppm	=	part per million
R_f	=	retarding factor in chromatography
s	=	singlet (NMR)
sat. BuOH	=	saturated butanol
t	=	triplet (NMR)
U	=	enzyme unit
UV	=	ultraviolet
v	=	volume
%	=	percentage
δ	=	unit of chemical shift
δ_C	=	chemical shift of carbon
δ_H	=	chemical shift of proton
μL	=	microliter (s)
μM	=	micromolar

CHAPTER I

INTRODUCTION

Natural products compounds have been played an important role in health care and utilized to alleviate disease since early human history. In the early 19th century, before the "Synthetic Era", eighty percent of all medicines were obtained from leaves, roots, flowers and barks (McChesney *et al*., 2007) of medicinal plants. The great ancient civilization of Indians, Chinese and North Africans provide written evidence for the use of natural sources for curing various diseases (Phillipson 2001). Many drugs are derived from natural sources which are, for examples, microorganisms, plants and marine organisms. Due to the fact that the irregular and complex structures of natural products, they are difficult by chemical synthesis. Moreover, many synthetic drugs might have significant side effects in clinical use. Therefore, exploring of novel bioactive compounds from natural products is of interest for researchers and scientists.

Thailand locates in a tropical region and has abundant kinds of plants, especially herbs which are used as a great source of drugs. These herb plants have been utilized as food and folk medicine such as fever, neurodegenerative disorder, anti-oxidant, anti-diabetic, anti- malaria, anti-inflammatory, and anti-cancer activities. There are many medicinal plants in Thailand that have been reported to have an

ability to inhibit cholinesterase. The roots of *Tabernaemontana divaricate* (L.) R. Br. Ex Roem. & Schult and *Stephania suberosa* Forman (Ingkaninan *et al*., 2003), the whole parts of *Fumaria vailantii* Lois, *Fumaria capreolata* Linn, *Fumaria asepala* Bioaa and *Fumaria flabellate* Linn (Orhan *et al*., 2004), the flowers of *Quisqualis indica* L. (Wetwitayaklung *et al*., 2007) exhibited high acetylcholinesterase (AChE) inhibitory activity. The vines of *Ficus foveolata* Wall were found to exhibit a high inhibition towards butyrylcholinesterase (BChE) (Sermboonpaisarn and Sawasdee 2012). In this study, the alkaloid enriched extract from *Alstonia scholaris* leaves showed interesting anti-cholinesterase and anti-amyloid aggregation activities and there are no reports on the implementation of the cholineaterase and anti-amyloid aggregation inhibitors from *A. scholaris*. Thus, this research aimed to investigate the anti-cholineaterase activities and anti-amyloid aggregation constituents from *A. scholaris* leaves.

1.1 Botanical properties and distribution of Alstonia scholaris

Alstonia scholaris L. R. Br. (Family: Apocenaceae), commonly known in local Thai name as Paya sattaban or Teen ped, Indian devil tree (English), Chatian (Hindi), Saptaparna (Sanskrit), Chattin (Bengali), Pala (Tamil) and Saptaparni (Gujarati), is widely distributed in the tropical countries of Asia. It is an important medicinal plant in the various folk and traditional medicines (Changwichit *et al*., 2011; Dey 2011). The botanical characteristics can be described as follows:

Leaves: Leaves simple, whorled with 4-7 unequal leaves, bluntly acuminate, coriaceous, pale beneath and dark green above. Leaf stalk is 1-1.5 cm long, the lamina is elliptical, shining above, tapering towards the base, 11.5-23 × 4-7.5 cm is the size. Upper surface is dark green and the tip of the leaf is shortly pointed or rounded.

Bark: Bark is rough, lenticellate and greyish-brown. The outer blaze is cream to yellowish in color with abundant and has milky latex.

Flowers: Flowers are bisexual, greenish white flowers. They are 7-10 mm long, white or green.

Fruits: Fruits a pendulous, dehiscent follicles, two lobed, green or brown, wood or dry, 4-6 mm in diameter, spindle shaped, 15-32 cm long, containing numerous flat, linear-oblong, brown seed.

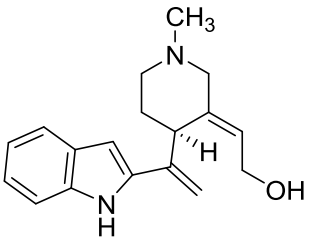
(a) (b) (c) (d)

Figure 1.1 leaves (a), barks (b), flowers (c) and fruits (d) of *Alstonia scholaris*

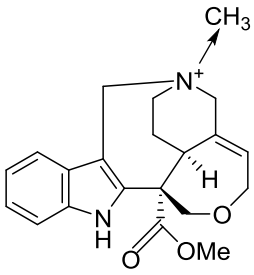
1.2 The Literature Reviews of *Alstonia scholaris*

A. scholaris is commonly found in the several regions of Thailand, and is grown all over the tropics, including India and South-East Asia. It is an important medicinal plant in the various folk and traditional medicines such as anti-stress activities from leaves (Kulkarni and Juvekar 2009), anti-malarial from barks (Warrier *et al*., 1996), anti-cancer from barks (Jahan *et al*., 2009), headache relief from leaves (Das *et al*., 2008), anti-fever from leaves and barks (Rajakumar and Shivanna 2010), anti-anxiety, anti-inflammatory and anti-depressant leaves and barks of this plant (Arulmozhi *et al*., 2012) in oriental countries.

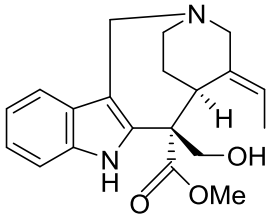
Many reports have been studied the bioactivities and phytochemicals of this plant. The phytochemical constituents of *A. scholaris* have been investigated extensively and many alkaloids have been isolated such as manilamine, *N4*-methyl angustilobine B, (19,20-(*E*)-vallesamine), angustilobine B *N4*-oxide, (20(*S*)-tubotaiwine), (6,7-*seco*-angustilobine) (Macabeo *et al*., 2005). Indole alkaloid compounds such as picrinine, vallesamine and scholaricine had isolated from ethanolic extract of *A. scholaris* and they showed the inflammatory (COX-1, COX-2 and 5-LOX) inhibitory activities (Shang *et al*., 2010). Echitamine chloride, a potent anti-cancer compound isolated from this plant, reported to possess anti-cancer activity on fibrosacoma cells *in vivo* (Saraswathi *et al*., 1998) and had potent tumor growth regression (Surya *et al*., 2012).



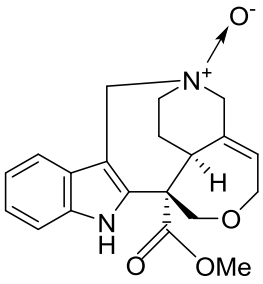
Manilamine



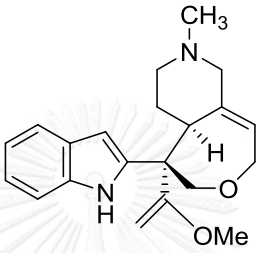
N4-methyl angustilobine B



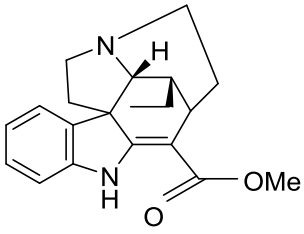
19,20-(*E*)-vallesamine



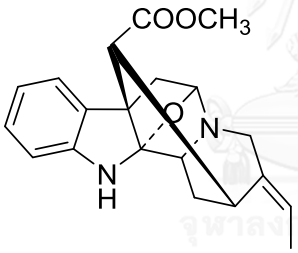
Angustilobine B *N4*-oxide



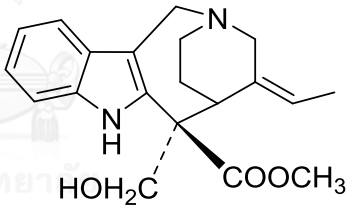
6,7-*seco*-Angustilobine



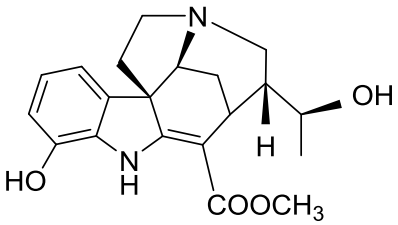
20(*S*)-Tubotaiwine



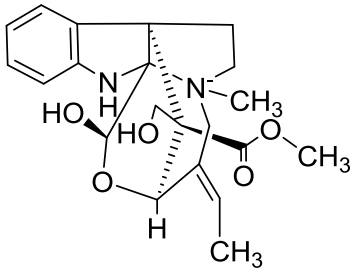
Picrinine



Vallesamine



Scholaricine



Echitamine chloride

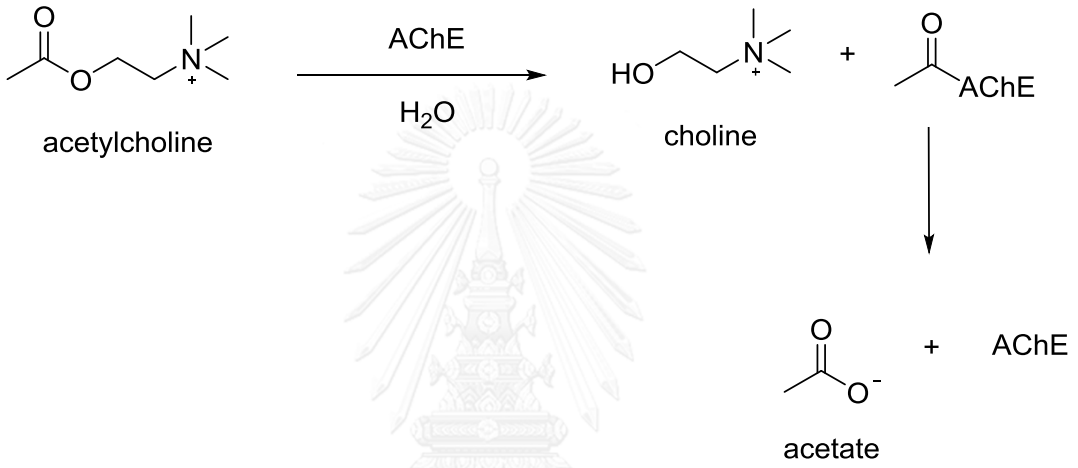
Figure 1.2 Isolated compounds from *A. scholaris*

1.3 Cholinesterase Inhibitors

Alzheimer's disease (AD) is the most regular type of dementia and a seriously damaging neurodegenerative disease that affects human health. Many reports reveal that the popular aged of people in the United States (USA) with this disease is diagnosed at more seventy years old. The man has risk to be Alzheimer's disease less than women. This disease has been determined for its causes, symptoms, risk factors and treatment only in the thirty years ago (Association 2012). Alzheimer's disease is characterized by loss of memory, cognitive dysfunction and alterations in behavior (Selkoe 2001). The most important changes observed in the brain of AD patients are the appearance of amyloid beta (Aβ) protein deposits, neurofibrillary tangles, tau protein aggregation, oxidative stress and a dramatic decreasing of the neurotransmitter acetylcholine (ACh) level in hippocampus and cortical brain. The inflammation is also thought to play important roles in the pathophysiology of AD. An affluence of evidence indicates that the pathological accumulation of Aβ in the brain relates in order to a variety of damaging events that ultimately causes neuronal destruction and cholinergic deficit (Rafii and Aisen 2009).

In a cellular brain system, the neurotransmission process begins with the acetylcholine (ACh) is synthesized by the choline acetyltransferase (ChAT) in the pre-synaptic neuron and then release into the synaptic cleft (Figure 1.3). Acetylcholine binds at an acetylcholine receptor on the post-synaptic terminal: the other side of

the synapse to transmit nerve signal, follow with rapidly broken down by an enzyme, acetylcholinesterase (AChE), and liberating choline. Choline returns to the pre-synaptic neuron for reuse. This is followed by a rapid hydrolysis of the acylated enzyme yielding acetate (Scheme 1.1), and the restoration of AChE (Soukupová *et al*., 2008).



Scheme 1.1 The hydrolysis of acetylcholine

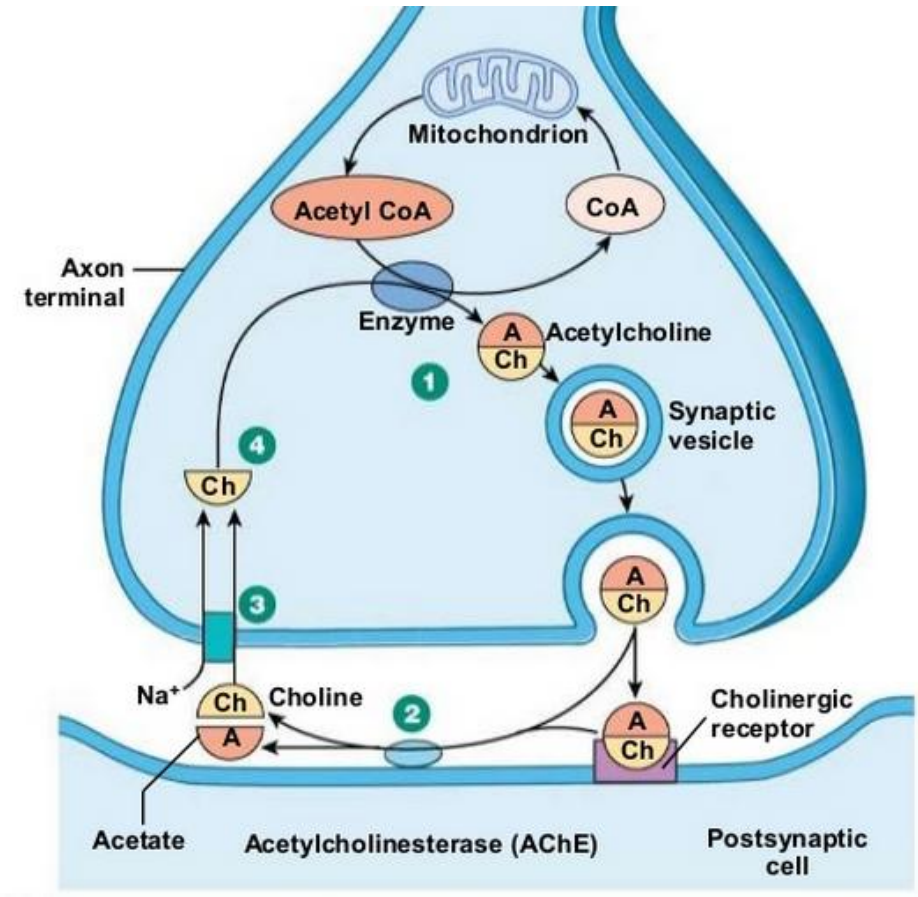


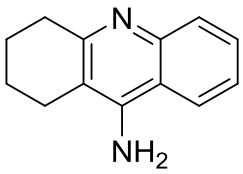
Figure 1.3 Synthesis and hydrolysis of acetylcholine (ACh) in neurons.

In Alzheimer's disease, the acetylcholine deficiency led to the formulation of the cholinergic hypothesis. Thus the primary approach in treating AD has been aimed at augmenting the cholinergic system in brain (Mukherjee *et al*., 2007). Cholinesterase inhibition is the majority of effective, extensively studied, and developed approach for the purpose of treating the symptoms. However, several cholinesterase inhibitors (ChEI) were limited mainly by reason of their some adverse effects. Therefore, the novel more effective therapy, including AChE inhibitors from plants, imperative to be alternative developed for the purpose of improving the cholinergic deficit (Menichini

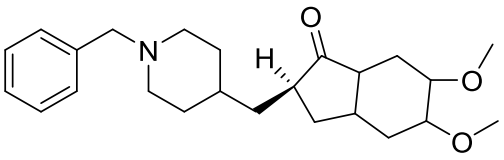
et al., 2009). The AChE inhibitors represent a well-established class of drugs for the purpose of the symptomatic treatment for AD, recent findings also point in order to BChE inhibition as further tool onto increase the cholinergic activity in AD patients affected by means of severe symptoms. Interestingly, the levels decline of AChE and BChE illustrate the predominant cholinesterase in the brain. Its performance is likewise AChE onto metabolize acetylcholine that also results a mismatching between its appropriate metabolism and acetylcholine release. So, therapeutic agents that serve as inhibitors of cholinesterase could provide additional advantages in the treatment with reference to AD.

Decline in quality of ACh-mediated neurotransmission involving ACh depletion is a key component of AD, thus the rationale behind the use of ChEI in this pathology has primarily consisted in providing an amplification of the deprived cholinergic activity. The ChEI such as tacrine, donepezil, galanthamine and rivastigmine, have been approved by the U.S. Food and Drug Administration (FDA) as shown in Figure 1.4 and represent the first line pharmacotherapy for mild in order to moderate memory and cognition in some AD patients. Tacrine was the first synthetic drug that approved by FDA for prescription use (Viegas *et al*., 2005), and showed moderate effect in relieving AD symptoms. Besides tacrine, there are three other ChEI drugs available in United Stated and Europe for treating AD. Donepezil was a selective inhibitor of AChE and individuals with AD often respond well to donepezil and few serious side effects. Rivastigmine was co-inhibited both AChE and BChE. The

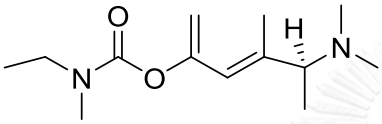
rivastigmine was not subject to hepatic metabolism, lacking the potential of adverse drug interactions with a number of agents that were commonly prescribed to the elderly and have side effects such as nausea and vomiting. Finally, galantamine was a low selectivity for AChE versus BChE and its most common side effects are nausea. Galantamine is a natural product and it has been used as a prototype in ChEI drugs drug development. However, their applications are limited due to side effects appear after several weeks of therapy. Generally, side effects have been occurred in AD patients if the dose of cholinesterase inhibitor is high enough. Their commonly side effects observed in clinical trials included nausea, vomiting, weight loss, anorexia, diarrhea, abnormal dream, headache, bradycardia and muscle cramping or weakness (Grutzendler and Morris 2001) Therefore, therapy inhibitors from a nature are a new alternative that is interesting and developing for the treatment and prevention of AD. Due to the scientists believe in the bioactive compounds from herbs less harmful than synthetic drugs. Interest in ChEI has also increased due to findings supporting cholinesterase's involvement in β-amyloid peptide fibril formation during AD pathogenesis (Inestrosa *et al*., 1996). The various studies have indeed supported that ChEI have the ability to prevent Aβ oligomerization, thus displaying both anti-amyloid and neuroprotective disease-modifying effects (Munoz-Torrero 2008). So, the research for a potent, long-term of ChEI with minimal side effects at therapeutic dose for the treatment and develop new drugs in order to combat AD.



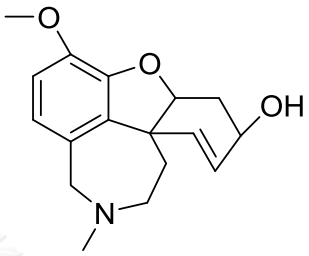
Tacrine



Donepezil



Rivastigmine



Galantamine

Figure 1.4 Chemical structures of AD drugs.

1.4 Anti-amyloid aggregation

Alzheimer's disease (AD) treating remains a challenge for the pharmaceutical community. Despite the multifactorial nature of AD, most treatment strategies have been aimed at two main targets: the cholinergic neurotransmission (Bartus *et al*., 1982) and the β-amyloid peptide (Ehehalt *et al*., 2003) and (Schenk *et* al., 1999).

Beta-amyloid peptide (Aβ) is now generally well-accepted as playing a seminal role in the neurodegenerative pathology and a cascade of harmful event rated to Alzheimer's disease (Klein *et al.*, 2004); (Lesné *et al*., 2006); (Haass and Selkoe 2007) and (Walsh and Selkoe 2007). The amyloid formation is conceptualized as a complex process of protein aggregation (Yoshiike *et al*., 2008). Recent studies

have also demonstrated that neuronal declaration to very low concentrations of soluble Aβ oligomers can initiate neurophysiological changes that are hypothesized to be a potential cause of synaptic dysfunction leading to the cognitive impairment and memory loss associated with AD (Walsh *et al*., 2002); (Ye *et al*., 2004) and (Walsh *et al*., 2005). Alleviation of the progression and/or symptoms of Alzheimer's disease might therefore be possible through inhibition of the very early stages of Aβ aggregate formation (Parihar and Hemnani 2004). Kinetic studies have suggested that misfolding of monomeric Aβ precedes formation of oligomers, which then serve as seeds or nuclei for the purpose of accelerated fibril growth followed by Figure 1.5 (Kumar *et al*., 2011).

From Fig 1.5 showed amyloid formation consists of two phases. The first phase exhibit a nucleation phase or lag phase, in which monomer undergo conformation change or misfolding and associate to form oligomeric nuclei. The second phase showed a elongation phase or growth phase, in which the nuclei rapidly grow by means of further addition of monomers and form larger polymers or fibrils until such time as saturation. The nucleation phase is thermodynamically unfavourable and occurs gradually. The elongation phase is much more favourable process and proceeds quickly. Thus, kinetics of amyloid formation is well represented by a sigmoid curve with a lag phase followed by rapid growth phase. The rate limiting step in the process is the formation of nuclei to promote

aggregation. So, amyloid formation could be substantially acceleration by the addition of performed nuclei. The addition of nuclei reduces the lag time and induces aggregate formation.

Therefore, many therapeutic efforts are targeted at finding molecules in order to prevent or disrupt Aβ aggregation in AD (Kokkoni *et al*., 2006); (Dong *et al*., 2007). Accumulating reports demonstrate that a lot of inhibitors, including small organic molecules, enzymes and antibodies, peptides and proteins (Liu *et al*., 2005); (Liu *et al*., 2004); (McLaurin *et al*., 2006); (Hoos *et al*., 2007); (Hong *et al*., 2009); (He and Shen 2009) may prevent Aβ aggregation. However, few effective inhibitors have been used for the purpose of the treatment in AD. Increasing evidence indicated that Aβ oligomers are early and intermediates in AD pathophysiological process (De Felice *et al*., 2007); (Meyer-Luehmann *et al*., 2008). Therefore, preventing Aβ oligomer formation or reducing toxicity of Aβ oligomers are the goals of all rational therapeutic.

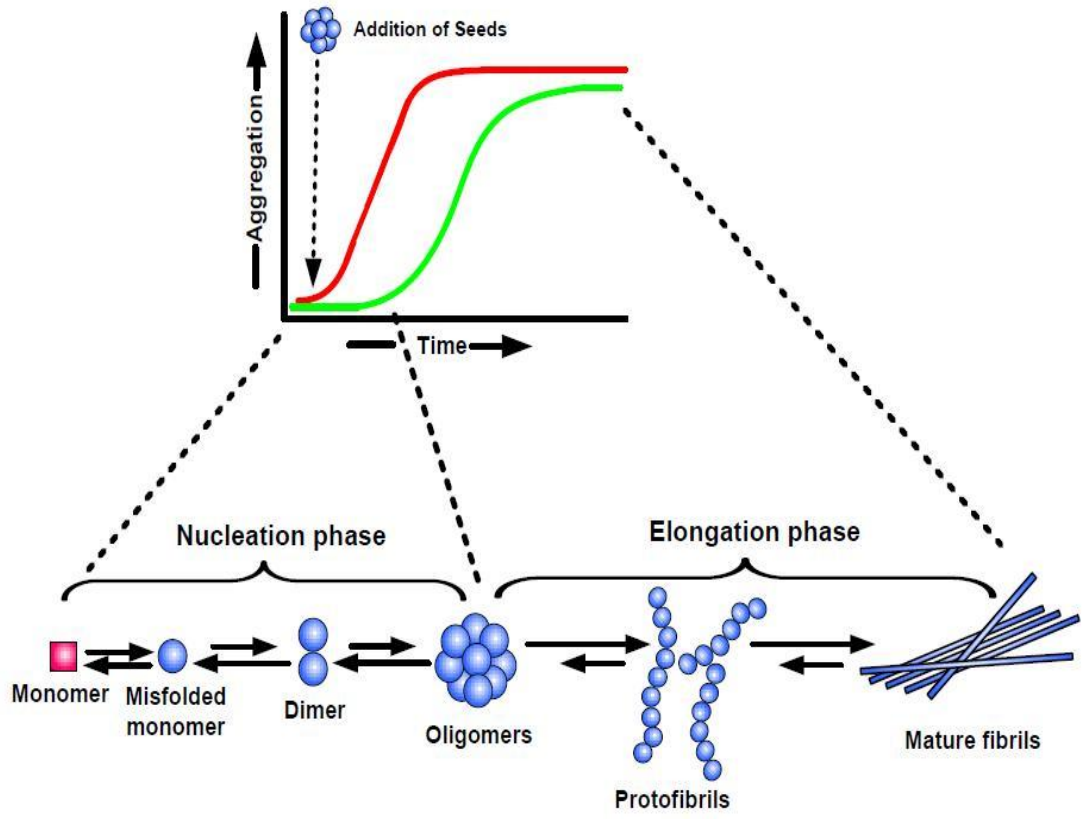


Figure 1.5: Nucleation-dependent polymerization model of amyloid aggregation

Many compounds have been found to reduce Aβ aggregation or neurotoxicity. The potential health benefits of wine consumption are mostly attributed to the polyphenol compounds. The polyphenol compounds are present in high abundance (Urquiaga and LEIGHTON 2000) and (Scalbert *et al*., 2005). Resveratrol is a polyphenolic phytoalexin and it is a main ingredient of polyphenols in wine, could mitigate or delay the onset of neurodegenerative disease (Kim *et al*., 2007). Resveratrol may exert a direct effect on Aβ to protect cells against Aβ via its anti-oxidation or other mechanism (Albani *et al*., 2009). Although many investigations concerned in resveratrol were performed in AD studies, many reports that

systematically describe the effects of resveratrol on Aβ oligomerization and the mechanism of prevention of Aβ oligomeric cytotoxicity.

Galantamine is currently prescribed as a drug treatment with reference to AD because of its activity as a moderate AChE inhibitor where it increases the response to acetylcholine in the synaptic cleft (Albuquerque *et al*., 1997). Since, galantamine shares some structural characteristics of certain benzofuran molecules previously shown to inhibit Aβ aggregation (Allsop *et al*., 2001) and (Howlett *et al*., 1999) it was decided to assess the effects of galantamine on the aggregation and toxicity of Aβ synthetic. Therefore, therapy inhibitors from a nature are a new alternative that is interesting, modifying and developing for the prevention and treatment of AD. Due to the scientists believe in the drug from the bioactive compounds have less harmful than synthetic drugs.

1.5 The Objectives of this Research

A. scholaris extract showed high anti-cholinesterase and anti-amyloid aggregate activities. Moreover, there are no reports on constituents on these activities. Thus, the objectives of this study were

1.5.1 To extract and isolate the chemical constituents from the leaves of *A. scholaris.*

1.5.2 To elucidate the chemical structures of isolated substances from the leaves of *A. scholaris.*

1.5.3 To investigate anti-cholinesterase and anti-amyloid aggregation activities of isolated compounds from the leaves of *A. scholaris.*

CHAPTER II

EXPERIMENTAL

2.1 Plant materials

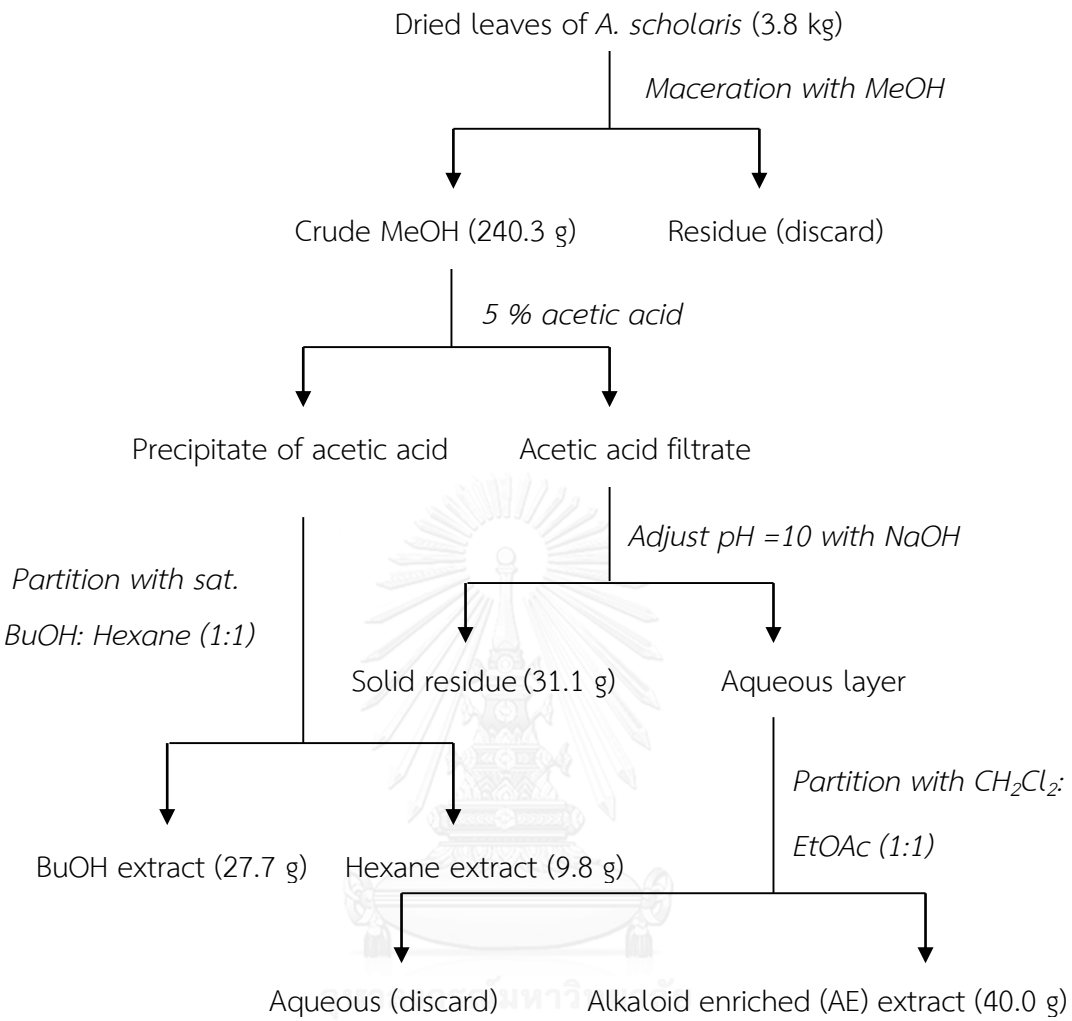
The leaves of *Alstonia scholaris* L. R. Br. were collected from Krabi Province, Thailand in March 2013. A voucher specimen (No. NPRU 0004) has been deposited at the Natural Products Research Unit, Faculty of Science, Chulalongkorn University, Thailand.

2.2 Instruments and materials

The ^{1}H, ^{13}C, and 2D NMR spectra were recorded in chloroform-d_1 ($CDCl_3$) and acetone-d_6 (C_3D_6O) on a Varian model Mercury + 400 and a Bruker Advance 400 NMR spectrometer (^{1}H 400 MHz; ^{13}C 100 MHz). The chemical shift (**δ**, ppm) was assigned with reference to the signal from the residual protons in deuterated solvent and using TMS as an internal standard in some cases. Silica gel 60 (Merck), No. 7734 was used for open column chromatography and vacuum silica gel column chromatoghaphy (VCC). Column chromatography was also carried on Sephadex LH-20. Silica gel 60 PF254 precoated on aluminium sheets (Merck Kieselgel) was used for thin layer chromatography (TLC). The spots on plates were detected under a UV light at (254 and 365 nm) and visualized by heating silica gel plates sprayed with anisaldehyde dipping reagent (MeOH:*p*-anisaldehyde:conc.H_2SO_4 = 95:3:3 (v/v) ratio).

2.3 Extraction procedure of *A. scholaris* leaves

The dried leaves of A. scholaris (3.8 kg) were extracted by maceration with MeOH three times at room temperature. The combined filtrate was concentrated in vacuo to obtain methanol extract. This methanol extract (240.3g) was dissolved in 5% acetic acid solution (pH = 3). The acetic acid filtrate was adjusted to a pH = 10 with sodium hydroxide solution and subsequently partitioned with dichloromethane and ethyl acetate (1:1). The organic layer was dried under reduced pressure to give the alkaloid enriched (AE) extract (40.0 g). In the other hand, the solid residue of the 5% acetic acid solution was further partitioned sequentially with 1:1 ratio of n-hexane and saturated butanol (sat. BuOH). The n-hexane and sat. BuOH layer were dried under reduced pressure to give the n-hexane (9.8 g) and butanol (27.7 g) extracts. The extraction procedure is summarized as displayed in Scheme 2.1.



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

Then, four extracts from *A. scholaris* leaves, e.g. sodium hydroxide precipitate, alkaloid enriched (AE), butanol and hexane extracts, were evaluated anti-cholinesterase activities by the microplate assay.

2.4 Separation of alkaloid enriched (AE) extract

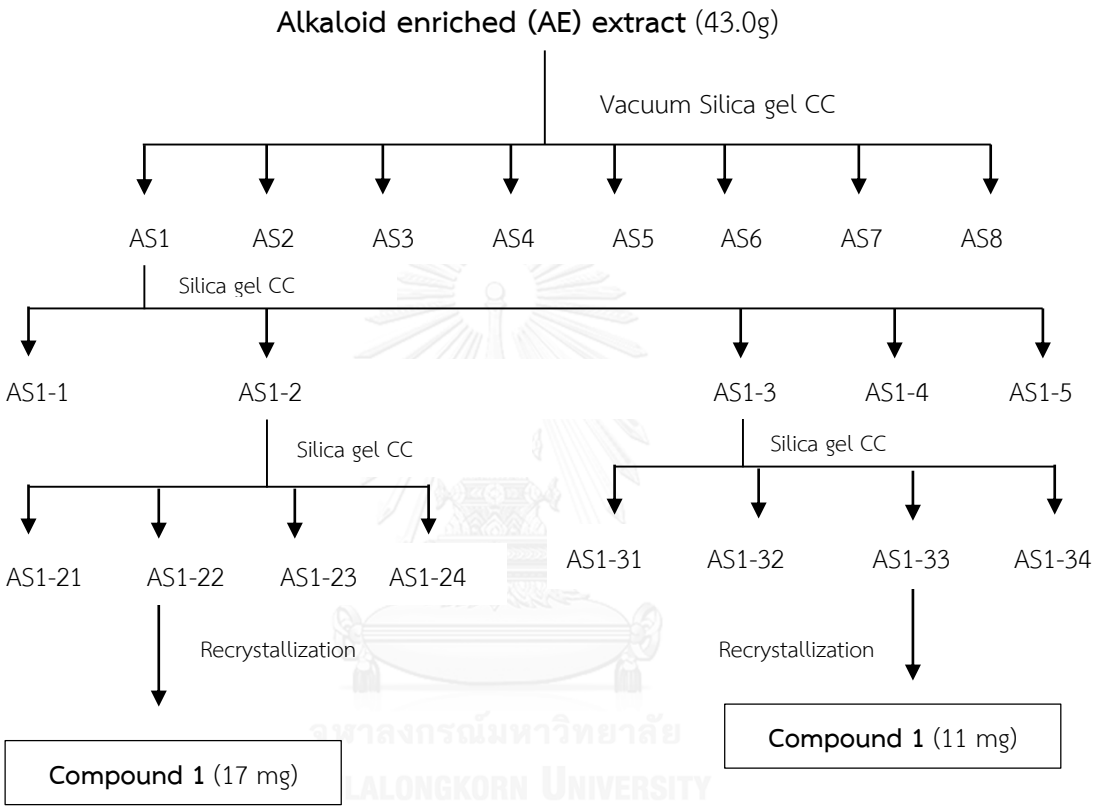
A portion of the AE extract (40.0 g) was subjected to a vacuum silica gel column chromatography (VCC) and eluted with *n*-hexane: CH_2Cl_2 gradient (100:0 to 0:100 (v/v)) and then a CH_2Cl_2: MeOH gradient (100:0 to 80:20 (v/v)). Each fraction was combined monitoring by TLC analysis to give eight fractions (AS1 to AS8). Fraction AS1 was further separated by a silica gel column chromatography, using a stepwise gradient of *n*-hexane - ethyl acetate (100:0 to 0:100) and then ethyl acetate **-** methanol (100:0 to 20:80) to give five sub-fractions (AS1-1 to AS1-5). Fraction AS1-2 was submitted to separate over a silica gel column and eluted with a stepwise gradient of *n*-hexane - ethyl acetate (60:40 to 0:100) and then ethyl acetate - methanol (100:0 to 80:20) to give four sub-fractions (AS1-21 to AS1-24). Fraction AS1-22 was purified by recrystallization using *n*-hexane- ethyl acetate (60:40) to furnish compound **1** (17 mg). Fraction AS1-3 was submitted to separate over a silica gel column and eluted with a stepwise gradient of *n*-hexane- ethyl acetate (60:40 to 0:100) and then ethyl acetate –methanol (100:0 to 80:20) to give four sub-fractions (AS1-31 to AS1-34). Fraction AS1-33 was purified by recrystallization using *n*-hexane -

ethyl acetate (60:40) to furnish compound **1** (11 mg). The separation procedure of fraction AS1 is exhibited in Scheme 2.2.

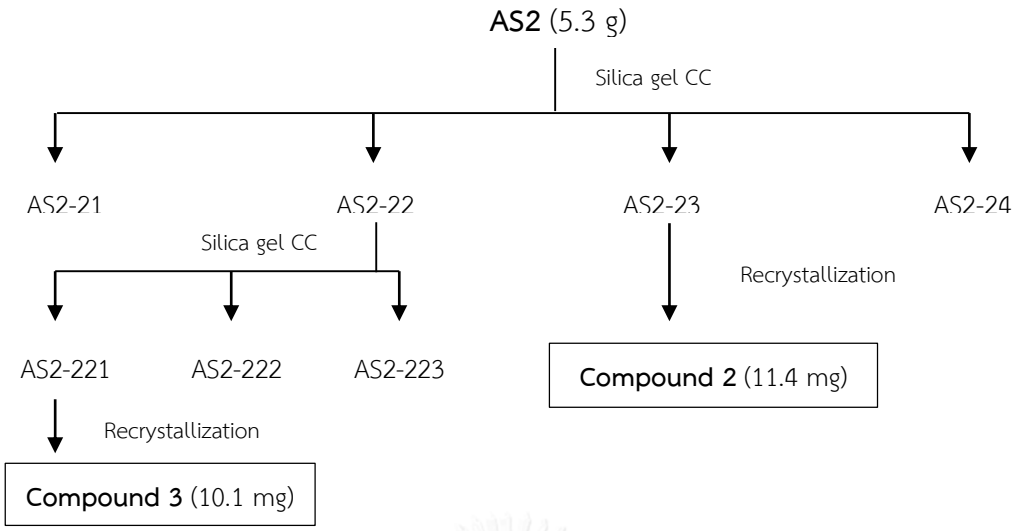
Fraction AS2 was fractionated on a silica gel column eluting with an increasing polarity of dichloromethane-methanol (100:0 to 80:20) to afford four sub-fractions (AS2-1 to AS2-4). The fraction AS2-2 was submitted to separate over a silica gel column and eluted with a stepwise gradient of dichloromethane-methanol (100:0 to 80:20) to give four sub-fractions (AS2-21 to AS2-24). Fraction AS2-23 was purified by using dichloromethane - methanol (50:50) to furnish compound **2** (11.4 mg). The fraction AS2-22 was submitted to separate over a silica gel column and eluted with a stepwise gradient of dichloromethane - methanol (100:0 to 80:20) to give three sub-fractions (AS2-221 to AS2-223). AS2-221 was purified by using dichloromethane - methanol (50:50) to furnish compound **3** (10.1 mg). The separation procedure of fraction AS2 is exhibited in Scheme 2.3.

Fraction AS4 was submitted to separate over a silica gel column and eluted with a stepwise system of n-hexane in EtOAc, EtOAc and MeOH in EtOAc, respectively, to afford five sub-fractions (AS4-1 to AS4-5). The fraction AS4-3 was subjected to silica gel column chromatography with a gradient of dichloromethane - methanol (100:0 to 80:20) to give seven sub-fractions (AS4-31 to AS4-37). The sub-fraction AS4-35 was fractionated on a silica gel column eluting with increasing polarity of dichloromethane - methanol (100:0 to 80:20) to afford four sub-fractions

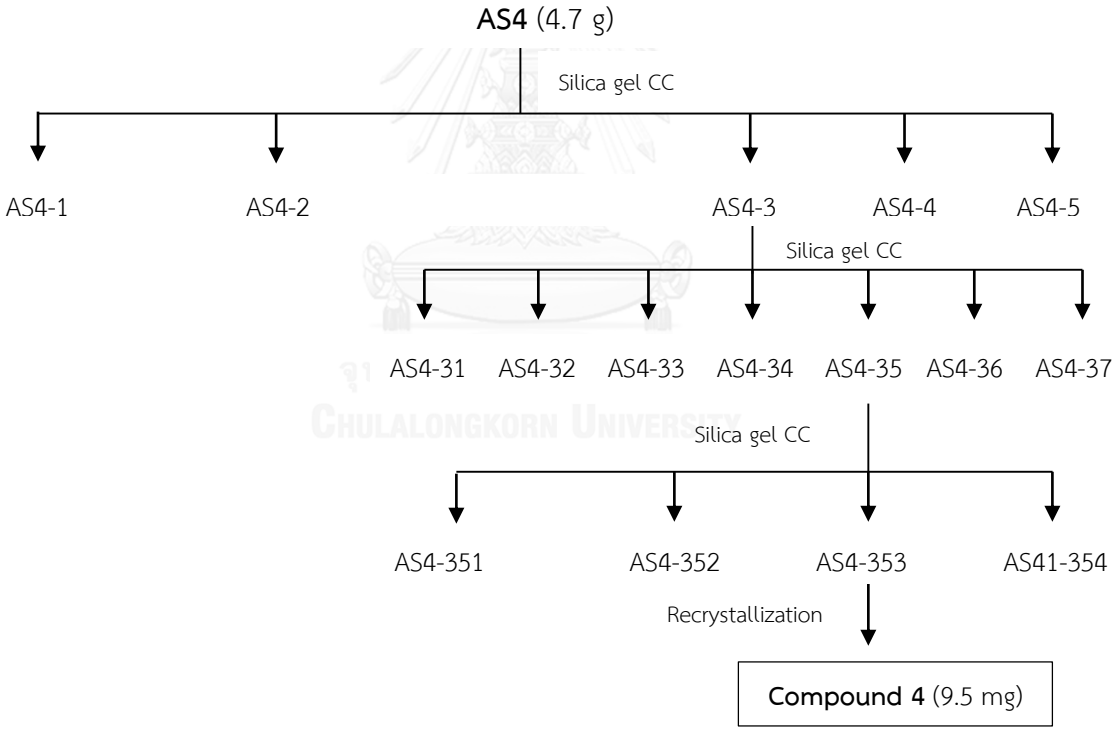
(AS4-351 to AS4-354). Sub-fraction AS4-353 was purified by recrystallization using dichloromethane - methanol (50:50) to furnish compound **4** (9.5 mg). The separation procedure of fraction AS4 is exhibited in Scheme 2.4.



Scheme 2.2 Separation diagram of compound 1 from fraction AS1.



Scheme 2.3 Separation diagram of compounds 2 and 3 from fraction AS2.

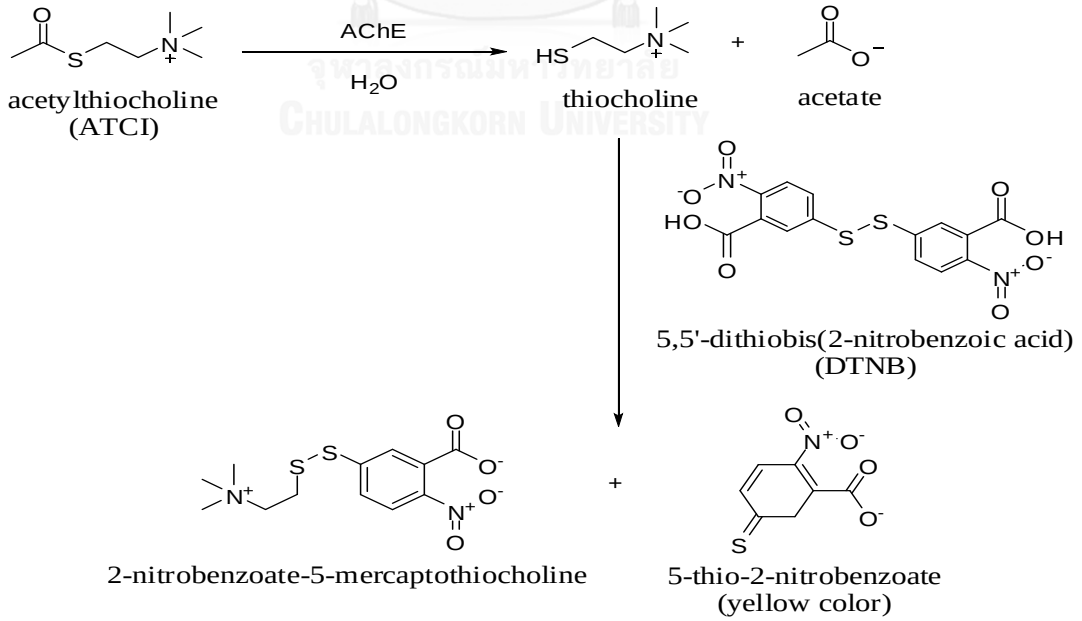


Scheme 2.4 Separation diagram of compound 4 from fraction AS4.

2.5 Bioassay procedures

2.5.1 Cholinesterase inhibitory assay

The anti-cholinesterase activity toward acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evaluated by the microplate method (Ellman *et al*., 1961). The principle of assay is the hydrolysis of substrate acetylthiocholine (ATCI) or butylthiocholine (BTCI) by AChE or BChE, respectively, and give thiocholine and acetate as products. Then, thiocholine reacts with 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) to yield yellow product of 5-thio-2-nitrobenzoate. This reaction is shown in the Scheme 2.5. If the natural product compounds or synthetic compounds are able to decrease enzyme activity or preclude the reaction between substrate molecules and enzymes, the amount of yellow products will be decreased.



Scheme 2.5 Cholinesterase catalyzed hydrolysis of acetylthiocholine

2.5.1.1 Chemical reagents

Acetylthiocholine iodide (ATCI), 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) and eserine (standard compound) were obtained from Sigma-Aldrich Co.Ltd. Albumin from bovine serum (BSA) and *Tris*-(hydroxymethyl)-aminomethane (*Tris*-HCl) were purchased from Fluka chemical company and from Merck (Darmstadt, Germany), respectively.

2.5.1.2 Chemical preparation

Buffers The following buffers were used;

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 (MgCl2.6H2O)

Enzymes

Cholinesterase enzymes (AChE; type VI-S, EC 3.1.1.7 and BChE; EC 3.1.1.8) were dissolved in buffer A to make the 113 U/mL of stock solution. These further diluted with buffer B to get 1 U/mL enzymes for assay.

Substrate

Both ATCI and BTCI were dissolved in MilliQ water to make 1.5 mM substrate for the microplate assay.

Ellman reagent

The 3 mM DTNB in buffer C was used for the microplate assay.

Test Samples and standard compound (eserine)

The concentration of extract for evaluating activity was 10 mg/mL while that for the isolated compounds and eserine were 1.0 and 0.1 mg/mL. All samples were dissolved in MeOH.

2.5.1.3 The microplate method

All extracts and isolated compounds from *A. scholaris* leaves were evaluated for their anti-cholinesterase effects by the modifying microplate assay (Ingkaninan *et al*., 2003). In the 96-well plates, 50 µL of buffer A (50 mM Tris-HCl, pH 8.0), 25 µL of 1.5 mM ATCI (or BTCI), 25 µL of sample, 125 µL of 3 mM DTNB, and 25 µL of 1.0 U/mL of enzyme (AChE or BChE) were added. The absorbance was measured at a wavelength of 405 nm for 2 min at 5 sec intervals by Sunrise™ microplate reader (P-Intertrade Equipments, Australia). Each experiment was done in triplicate. The percentage of enzyme inhibition was calculated according to the equation that shown below.

$$\% \text{ inhibition } = \left(\frac{R_{blank} - R_{sample}}{R_{blank}} \right) \times 100$$

The R_{blank} is the reaction of blank without any inhibitors and R_{sample} is the reaction of sample.

The IC_{50} value was defined as the concentration of sample that inhibited 50% of the maximum observed enzymatic activity and graphically determined from a plot of percentage inhibition versus a log final concentration value using the Graph Pad Prism 5.01 software (Graph Pad Software Inc.) as displayed in Figure 2.1. In order to obtain the sigmoidal curve of enzyme inhibition, at least ten different concentrations of inhibitors were measured. This experiment was analyzed in duplicated.

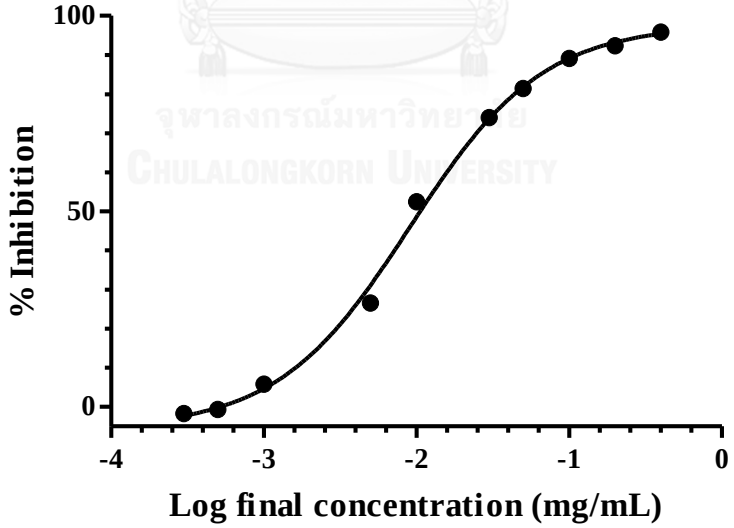


Figure 2.1 A plot of graph that showed percentage inhibition versus a log final concentration values.

2.5.2 Self-mediated amyloid beta (1-42) aggregation assay

2.5.2.1 Chemical reagents

Amyloid beta (1-42) human peptide, resveratrol and thioflavin were purchased from Sigma-Aldrich Co. Ltd. The 1,1,1,3,3,3-hexafluoro-2-propanal (HFIP) was obtained from Merck company (Darmstadt, Germany).

2.5.2.2 Preparation reagents and amyloid peptides

Amyloid beta (1-42) human peptide was prepared by the method described in the previous report (Feng *et al*., 2009). The Aβ (1-42) human peptide powder was dissolved in 100% of HFIP by sonicating in a water bath for 10 min to obtain the concentration of 1 mg/mL. The HFIP solution should then be dried under a gentle stream of nitrogen gas. Then, this HFIP-treated amyloid β (1-42) was dissolved in dimethylsulfoxide (DMSO) to adjust the concentration as 1 mg/mL. The HFIP-treated amyloid β (1-42) solution was aliqouted into 1.7 mL microcentrifuge tubes and store at -20 ºC. For a working solution, add 1x PBS (phosphate buffer saline, pH 7.4) to final concentration at 20 μM to the peptide stock solution and mixed with sample before incubating at 37 °C for 28 hours.

2.5.2.3 Self-mediated amyloid beta (1-42) aggregation method

The concentrations of extract for evaluating activity were 2, 0.5 and 0.05 mg/mL while those of the isolated compounds and standard compounds (resveratrol or curcumin) were 20 μM and 100 μM. All samples were dissolved in DMSO.

Thioflavin T (ThT) dye was used to determine the presence of amyloid-like aggregates. The fluorescence emission of ThT is shifted when ThT binds to β-sheet aggregate structures. The amyloid β (1-42) aggregation potetntial of samples was determined by the method described in a previous report (Feng, Wang et al. 2009). Briefly, 80 μL of the incubated samples (the HFIP-treated Aβ 42 mixed with sample and incubated at 37 °C for 28 hours) and 20 μL of 50μM ThT in 500 glycine-NaOH buffer (pH 8.5) were added in 96-well plates. Then the measurement of fluorescence intensity was carried out (λ_{exc}= 450 nm, λ_{em}= 485 nm) by microplate reader (Cary Eclipse Fluorescence Spectrophotometer) and values at the plateau were calculated after subtraction of the background fluorescence of the 50 μM thioflavin T solution. Each experiment was done in triplicate. The percentage of amyloid β aggregation and inhibition was calculated according to the equation that shown below.

$$\% \text{ Aggregation} = \left(\frac{A_{A\beta} - A_{sample}}{A_{A\beta}} \right) \times 100$$

$$\% \text{ Inhibition} = 100 - \% \text{ Aggregation}$$

The A_{sample} is the absorbance of sample with amyloid peptide and $A_{A}\beta$ is the absorbance of amyloid peptide aggregation.

2.5.2.4 Transmission electron microscope (TEM) imaging

TEM study (Feng *et al*., 2009); (Ramesh *et al*., 2010) was conducted to detect the presence and absence of aggregates. 20 μM amyloid β (1-42) and preformed amyloid β (1-42) were incubated in the presence or absence of inhibitors for 28 h at 37 ºC. Then a 10 μL of incubated sample was placed on carbon coated copper grid and allowed for 1 minute. The droplet then was displaced with equal volume uranyl acetate (2%, w/w) for 1 minute and the solution was wicked off, then the grid was air-dried. Sample were examined using TEM photographs were obtained dusing JEM-2100 (JEOL, Ltd, Japan) with an accelerating voltage of 120 kV in conjunction with selected area electron diffraction (SAED). TEM was performed by the Scientific and Technology Research Equipment Center, Chulalongkorn University, Thailand.

CHAPTER III

RESULTS AND DISCUSSION

3.1 The preliminary screening cholinesterase inhibitory activity of *A. scholaris* extracts

Four extracts of *A. scholaris* (Scheme 2.1 in Chapter II), which were solid residue, alkaloid enriched (AE), butanol and hexane extracts, were preliminarily tested for their anti-cholinesterase activity using microplate method according to the procedure described in Section 2.5.1. The results were shown in Table 3.1.

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

Test sample	% Inhibition[a]	
	AChE	BChE
solid residue	53.5	96.2
AE extract	75.2	97.2
butanol extract	19.0	82.8
hexane extract	36.7	37.3
eserine[b]	99.6	99.2

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

[b]*Standard compound*

Hexane extract showed the lowest inhibition while the AE extract exhibited the highest inhibition toward both AChE and BChE. The solid residue and butanol extract inhibited strongly toward BChE over AChE. Therefore, the AE extract was further investigated to afford the active chemical constituents.

3.2 Bioactivities of fractions AS1-AS8

3.2.1 Cholinesterase inhibitory activity

Table 3.2 The anti-acetylcholinesterase activity of fractions AS1-AS8 from the AE extract

Sample	% AChE Inhibition at the final concentration of (mg/mL)		IC_{50} of AChE (mg/mL)
	1	0.1	
AS1	78.2 ± 0.1	43.5 ± 0.1	128.0 ± 0.1
AS2	72.0 ± 0.3	32.0 ± 0.5	244.1 ± 0.3
AS3	79.8 ± 0.2	47.5 ± 0.5	110.7 ± 0.3
AS4	87.3 ± 0.3	41.5 ± 0.5	142.7 ± 0.3
AS5	90.7 ± 0.1	43.5 ± 0.6	160.2 ± 0.1
AS6	89.1 ± 0.6	57.9 ± 0.7	80.9 ± 0.6
AS7	90.0 ± 0.4	34.2 ± 0.5	204.3 ± 0.4
AS8	63.1 ± 0.4	18.7 ± 1.0	550.1 ± 0.4
Eserine[a]	99.6 ± 0.1	-	0.9 ± 0.1

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

[a]*Standard compound*

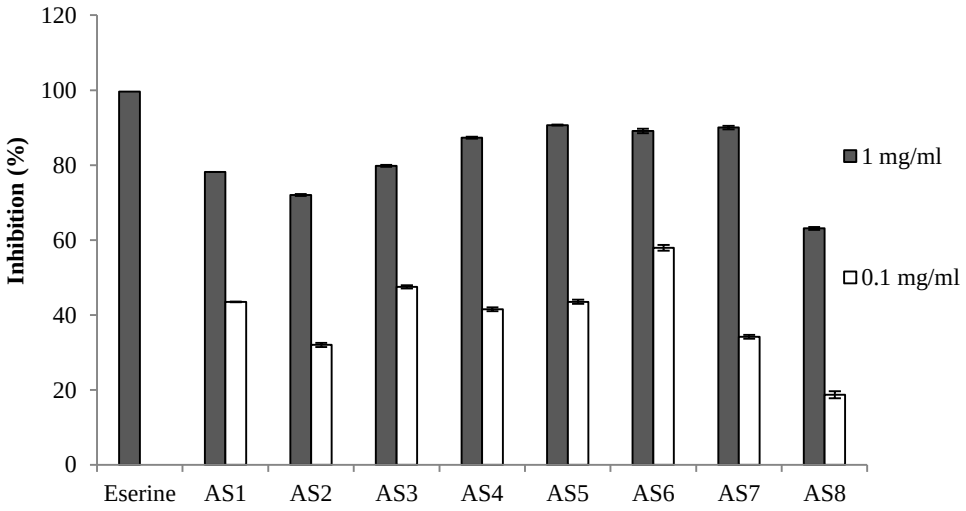


Figure 3.1 The AChE inhibitory activity of fractions AS1-AS8

The AE extract from *A. scholaris* leaves was fractionated by a column chromatography technique to obtain eight fractions (AS1 to AS8). All fractions were evaluated for their AChE and BChE inhibitory activities. The inhibitory results toward AChE shown in Table 3.2 and Figure 3.1 and those towards BChE presented in Table 3.3 and Figure 3.2. The results indicated that these fractions showed significant inhibition in dose-dependent manners towards both enzymes. Fractions AS1 to AS6 showed high AChE and BChE inhibitions at the concentration of 1 mg/mL. The inhibition towards BChE of these fractions was still high at the concentration of 0.1 mg/mL but those towards AChE exhibited only moderately.

Additionally, the IC_{50} results in Tables 3.2 and 3.3 indicated that most fractions had higher activities against BChE than AChE. The high BChE inhibitions were found in fractions AS2 - AS6 in which their IC_{50} values were in a range of 6-10 µg/mL.

Therefore, the isolation of active compound(s) from these fractions was further needed.

Table 3.3 The anti-butyrylcholinesterase activity of eight sub-fractions from the AE extract

Sample	% BChE Inhibition at the final concentration of (mg/mL)		IC_{50} of BChE (µg/ml)
	1	0.1	
AS1	92.1 ± 0.1	84.2 ± 0.1	17.5 ± 0.1
AS2	92.2 ± 0.3	84.6 ± 0.6	8.3 ± 0.2
AS3	91.3 ± 0.3	89.9 ± 0.4	10.8 ± 0.1
AS4	87.4 ± 0.3	82.6 ± 0.5	8.3 ± 0.3
AS5	94.0 ± 0.1	90.3 ± 0.6	6.6 ± 0.3
AS6	94.6 ± 0.6	88.8 ± 0.8	8.9 ± 0.5
AS7	89.0 ± 0.4	77.3 ± 0.5	36.5 ± 0.2
AS8	86.2 ± 0.4	67.7 ± 0.9	29.4 ± 0.2
Eserine[a]	98.7 ± 0.1	-	1.4 ± 0.1

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

[a]Standard compound

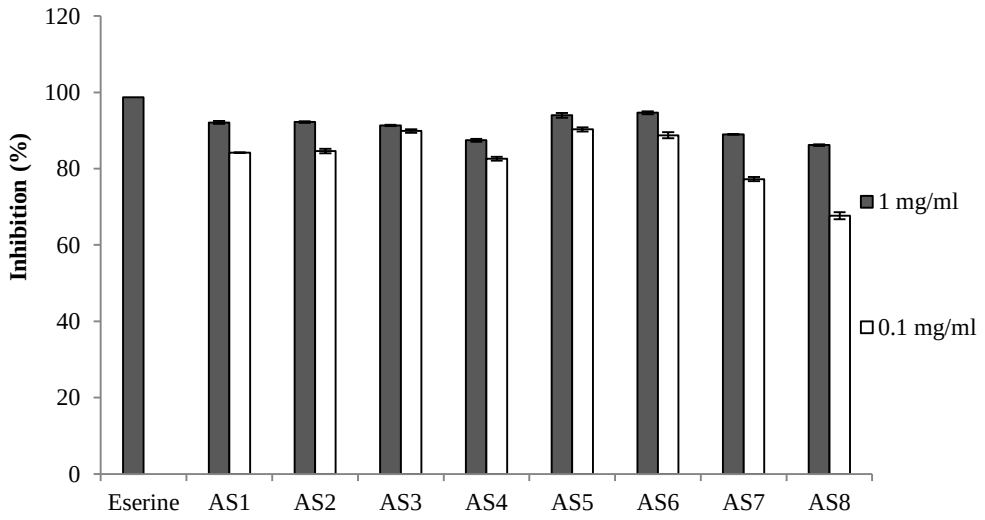


Figure 3.2 The BChE inhibitory activity of fractions AS1-AS8

3.2.2 Self-mediated Aβ (1–42) aggregation assay

Beta-amyloid (Aβ) aggregation has been strongly associated with the neurodegenerative pathology and a cascade of harmful event related to Alzheimer's disease (AD). Inhibition of Aβ assembly, destabilization of preformed Aβ aggregates and attenuation of the cytotoxicity of Aβ fibrils could be valuable therapeutics of patients with AD. Resveratrol and curcumin were reported to mitigate or delay the onset of neurodegenerative disease and significantly prevent amyloid aggregation (Yang *et al*., 2005; Feng *et al*., 2009). Thus, it is popular to use as the positive control in the amyloidogenic assay. In this study, fractions AS1-AS8 from the AE extract were evaluated for their anti-amyloid aggregation activity. The results in Figure 3.3 (and Table A-1) revealed that these fractions inhibited amyloid aggregation in dose-dependent manners. Fractions AS3 and AS8 showed the highest inhibition

percentages which were more than 70% and 50% at the final concentration of 2 and 0.5 mg/mL, respectively.

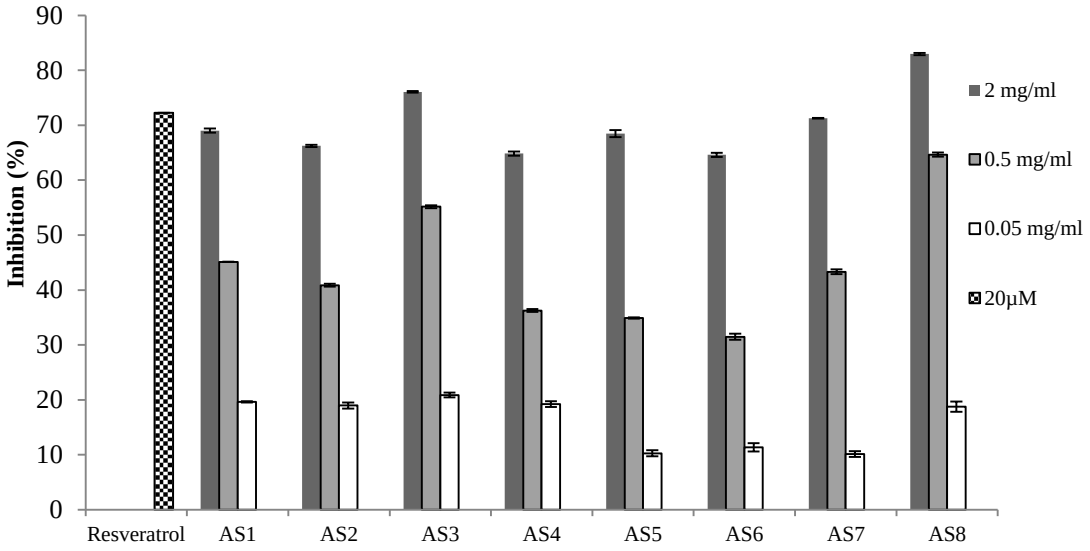


Figure 3.3 The anti-amyloid aggregation activities of fractions AS1-AS8

It has been reported that *A. scholaris* possessed a variety of pharmaceutical effects. However, the inhibitory effects towards AChE, BChE and amyloid aggregation have not been reported. The results in this study revealed that the AE extract of *A. scholaris* could be an economic alternative to treat AD. Moreover, this study was further isolated and characterized the inhibitors from these fractions.

3.3 Isolation of constituents from the AE extract

3.3.1 Elucidation chemical structures of isolated compounds

Four alkaloids (**1**-**4**) were isolated from fractions AS1, AS2 and AS4 as described in Schemes 2.2-2.4 in Chapter II. Although fractions AS3 and AS5 were subjected to separate and purify by column chromatography, pure compound (s)

was not obtained. The chemical structures of alkaloids **1**-**4** were identified based on spectroscopic data and compared with those of literature values. Alkaloids 1-4 are indole alkaloid type.

Indole alkaloids are derived from the amino acid tryptophan as can easily be discerned by comparing their nitrogen containing nucleus to the chemical structure of tryptophan. The indole system can be considered to consist of a pyrrole ring and a benzene ring fused together to form two isomeric benzopyrroles; indole and isoindole (Figure 3.4).

Figure 3.4 The structures of indole (left) and isoindole alkaloids (right)

Alkaloid **1** was obtained colorless needle, mp 225 – 227°. The 1H NMR spectral data (Figure B-1) showed the present of four aromatic protons (**δ**H 7.29 to 7.75), an ester methoxy (**δ**H 3.72, *s*; **δ**C 170.9), an ethylidene group (**δ**H 1.71, *d*, *J* = 7.0 Hz and 5.82, *br q,* J = 7.0 Hz) and an ethoxy group (**δ**H 3.09, 1H, *m*; 3.61, 1H, *m*; 1.02, 3H, *t*, *J* = 7 Hz). By comparison of spectral data, alkaloid **1** (Figure B-2) was identified as nareline ethyl ether. The spectral data of alkaloid **1** and those of nareline ethyl ether reported in the literature (Toh-Seok *et al*., 1997) are summarized in Table 3.4.

Alkaloid **2** (Figure B3-B7) was obtained light yellowish oil, mp 160 - 162º. The COSY spectrum showed a coupling system including of two pairs of methylene protons at (δ_H 3.05, *m*, H_2-3) and at (δ_H 2.00, *m*, H-14**β**) and (δ_H 2.26, *m*, H-14**α**), which correlated to the carbons at (δ_C 4.75, C-3) and (δ_C 23.9, C-14), respectively in the HMQC spectrum. In addition, three separate methylene groups were observed at (δ_H 4.94 and 4.12, *d*, *J* = 16 Hz)/ (δ_C 51.7, C-6); (δ_H 4.12 (*d*, *J* = 10.8 Hz) and (δ_H 3.813.86, *m*)/ (δ_C 70.1, C-17); (δ_H 3.60, *m*)/ (δ_C 53.3, C-21). These spectral features suggested that alkaloid **2** possessed a skeleton of the vallesamine type. Based on all of the above spectral data, alkaloid **2** was identified as 19, 20-(*E*)-vallesamine reported in the literature (Yamauchi *et al*., 1990) and that are summarized in Table 3.5.

Alkaloid **3** was obtained pale yellowish oil. The ^{13}C NMR and ^{13}C – ^{1}H COSY measurements (Figure B8-B12) were carried out and the epoxide ring was established to retain the **α** – orientation (19R, 20S) based on cross peaks at (δ_H 3.18, *m*, H – 15)/ (δ_H 2.34, *d*, *J* = 8 Hz, H - 21**α**), (δ_H 2.79, *m*, H – 19)/ (δ_H 2.59, *m*, H - 21**β**). By comparison of spectral data, alkaloid **3** was identified as 6, 7-*seco*-19, 20-epoxyangustilobine B. The spectral data of alkaloid **3** and those of 6, 7-*seco*-19, 20-epoxyangustilobine B reported in the literature (Yamauchi *et al*., 1990) are summarized in Table 3.6.

Alkaloid **4** was obtained colorless needles, mp 235 - 237°. The ^{13}C NMR and HMQC spectra (Figure B-14) revealed the presence of 14 protonated carbons which included a methoxyl and a methyl carbon, in addition to four methylenes and eight methines. In the ^{1}H NMR spectrum of alkaloid **4** (Figure B-13), characteristic signals for a sarpagine structure of the corynanthean type were observed at (**δ** 4.23, *br d*, *J* = 12 Hz), (**δ** 3.05, *m*), (**δ** 2.89, *dd*, *J* = 16, 5 Hz), (**δ** 3.31, *dd*, *J* = 16, 1.5 Hz), (**δ** 5.41, *br q*, *J* = 7 Hz), and (1.64, *ddd*, *J* = 7, 2, 2 Hz). Based on all of the above spectral data, alkaloid **4** was identified as 19, 20-(*E*)-Akuammidine reported in the literature (Wongseripipatana *et al*., 2004) are summarized in Table 3.7. The chemical structures of alkaloids **1**-**4** were shown in Figure 3.5

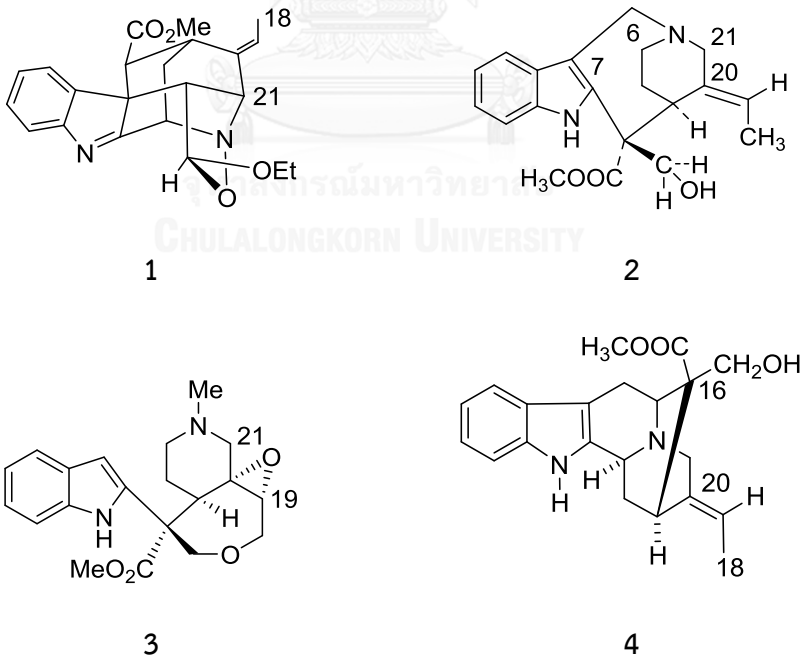


Figure 3.5 The chemical structures of nareline ethyl ether (**1**), 19, 20-(*E*)-vallesamine (**2**), 6, 7-*seco*-19, 20-epoxyangustilobine (**3**) and 19, 20-(*E*)-akuammidine (**4**)

Table 3.4 The ^{1}H and ^{13}C data of alkaloid 1 compared with those of nareline ethyl ether (Toh-Seok, Kok-Tih et al. 1997)

Position	alkaloid 1 ($CDCl_3$)		nareline ethyl ether ($CDCl_3$)	
	δ_C	**δ_H (int., mult., *J* in Hz)**	**δ_C**	**δ_H (int., mult., *J* in Hz)**
2	184.2	-	184.5	-
3	62.9	4.67 (*m*)	62.9	4.66 (*t*, 3)
5	106.7	4.01 (*s*)	105.4	3.91 (*s*)
6	55.9	3.80 (*d*, 3)	55.3	3.79 (*d*, 3)
7	54.3	-	55.2	-
8	139.5	-	139.5	-
9	125.5	7.75 (*br d*, 7.5)	125.5	7.75 (*br d*, 7.5)
10	125.8	7.29 (*m*)	125.8	7.26 (*t d*, 7.5, 1)
11	128.9	7.42 (*t d*, 7.5, 1)	128.9	7.43 (*t d*, 7.5, 1)
12	121.0	7.70 (*br d*, 7.5)	121.0	7.72 (*br d*, 7.5)
13	157.9	-	157.5	-
14	35.2	2.11 (*d t*, 14, 3)	35.2	2.10 (*d t*, 14, 3)
15	31.6	3.36 (*s*)	31.6	3.35 (*br d*, 3)
16	53.9	2.28 (*s*)	53.9	2.26 (*d*, 3)
17	170.9	-	170.7	-
18	12.7	1.71 (*d*, 7)	12.7	1.69 (*d*, 7)
19	122.9	5.82 (*br q*, 7)	122.9	5.81 (*br q*, 7)
20	130.6	-	130.7	-
21	65.7	4.12 (*d*, 3)	66.0	4.10 (*d*, 3)
CO_2Me	51.8	3.72 (*s*)	51.8	3.71 (*s*)
OCH_2CH_3	-	3.61 (*m*)	-	3.55 (*d p*, 14, 7)
OCH_2CH_3	63.5	3.09 (*m*)	63.4	3.11 (*m*)
OCH_2CH_3	14.9	1.02 (*t*, 7)	14.9	1.02 (*t*, 7)

Table 3.5 The ^{1}H and ^{13}C data of alkaloid 2 compared with those of 19, 20-(*E*)-vallesamine (Yamauchi, Abe et al. 1990)

Position	alkaloid 2 ($CDCl_3$)		19, 20-(*E*)-vallesamine ($CDCl_3$)	
	δ_C	**δ_H (int., mult., *J* in Hz)**	**δ_C**	**δ_H (int., mult., *J* in Hz)**
1	-	9.53 (*br s*)	-	9.50 (*br s*)
2	134.6	-	133.6[b]	-
3α	47.4	3.05 (*m*)	47.5	2.96-2.85 (*m*)
3β	-	3.05 (*m*)	-	2.96-2.85 (*m*)
6α	51.7	4.94 (*d*, 16)	51.2	4.82 (*d*, 17.1)
6β	-	4.12 (*d*, 16)	-	4.09 (*d*, 17.1)
7	102.0	-	109.2	-
8	127.4	-	128.1	-
9	117.9	7.19 (*br d*, 6.9)	118.4	7.17 (*br d*, 6.9)
10	120.3	7.10 (*t*, 7.0)	119.1	7.07 (*t*, 7.0)
11	123.3	7.22 (*t*, 7.9)	122.4	7.30 (*t*, 7.9)
12	111.3	7.31 (*br d*, 7.9)	110.7	7.30 (*br d*, 7.9)
13	135.0	-	137.4	-
14α	20.7	2.26 (*m*)	23.8	2.33 (*m*)
14β	-	2.00 (*m*)	-	1.89 (*m*)
15	34.9	3.68 (*m*)	36.3	3.63 (*m*)
16	48.6	-	48.5	-
17α	70.1	4.12 (*d*, 10.8)	70.2	4.19 (*d*, 10.8)
17β	-	3.86 (*m*)	-	3.81 (*d*, 10.8)
18	14.3	1.75 (*d*, 6.9)	14.0	1.74 (*d*, 6.9)
19	125.2	5.64 (*q*, 7.2)	124.1	5.56 (*q*, 6.6)
20	130.4	-	132.4	-
21α	53.3	3.60 (*m*)	54.0	3.60 (*m*)
21β	-	3.60 (*m*)	-	3.60 (*m*)
$\underline{C}OOCH_3$	173.5	-	175.2	-
$COO\underline{C}H_3$	51.7	3.76 (*s*)	52.9	3.74 (*s*)

Table 3.6 The ^{1}H and ^{13}C data of alkaloid 3 compared with those of 6, 7-*seco*-19, 20-epoxyangustilobine (Yamauchi, Abe et al. 1990)

Position	alkaloid 3 (CD_3COCD_3)		6,7-*seco*-19,20-epoxyangustilobine B ($CDCl_3$)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
2	136.0	-	134.1	-
3	56.5	1.96 (*m*)	56.3	1.95 (*t d*, 12, 3)
		2.82 (*m*)		2.84 (*br d* 12)
6	46.0	2.33 (*s*)	45.6	2.27 (*s*)
7	100.7	6.17 (*s*)	100.6	6.19 (*s*)
8	127.2	-	127.8	-
9	120.4	7.41 (*d* ,8)	120.4	7.52 (*d*, 8)
10	120.2	7.19 (*m*)	120.1	7.17 (*t*, 8)
11	122.4	7.08 (*m*)	122.4	7.08 (*t*, 8)
12	111.2	7.31 (*d*, 8)	111.0	7.33 (*d*, 8)
13	136.6	-	135.8	-
14	27.7	1.21 (*m*)	26.7	1.17 (*m*)
		1.57 (*m*)		1.60 (*m*)
15	46.3	3.18 (*m*)	46.1	3.17 (*br d*, 13)
16	54.0	-	53.2	-
17	69.4	3.73 (*m*)	70.4	3.73 (*d*, 12)
		4.66 (*dd*, 12, 1)		4.76 (*dd*, 12,1)
18	67.8	4.09 (*d*, 14)	67.1	3.95 (*d*, 14)
		4.35 (*m*)		4.37 (*dd*,14,3)
19	63.6	2.79 (*m*)	62.9	2.85 (*d*,3)
20	71.4	-	74.3	-
21	67.2	2.34 (*d*, 8)	66.1	2.32 (*dd*, 10,1)
		2.59 (*m*)		2.58 (*br d*, 10)
$\underline{CO_2}Me$	172.6	-	173.0	-
$CO_2\underline{Me}$	52.6	-	53.0	-

Table 3.7 The ^{1}H and ^{13}C data of alkaloid 4 compared with those of 19,20-€-Akuammidine (Wongseripipatana, Chaisri et al. 2004)

Position	alkaloid 4 (CD_3OD)		19-*E*-Akuammidine (CD_3OD)	
	δ_C	δ_H (int., mult., *J* in Hz)	δ_C	δ_H (int., mult., *J* in Hz)
1	-	7.75 (*br s*)	-	7.70 (*br s*)
2	136.6	-	136.6	-
3	51.3	4.23 (*br d*, 12)	51.4	4.22 (*br d*, 11)
5	58.0	3.05 (*m*)	58.0	3.1 (*m*)
6α	24.7	2.89 (*dd*, 16, 5)	24.8	2.91 (*dd*, 16, 5)
6β	-	3.31 (*dd*, 16, 1.5)	-	3.29 (*dd*, 16, 1.5)
7	106.2	-	106.2	-
8	126.9	-	127.0	-
9	118.0	7.43 (*d*, 7.4)	118.1	7.43 (*d*, 7.3)
10	119.4	7.05 (*t*, 10.3)	119.4	7.05 (*dd*, 7.3, 7.3)
11	121.5	7.11 (*t*, 7.3)	121.5	7.11 (*dd*, 10.3, 7.3)
12	110.9	7.28 (*d*, 10.3)	110.9	7.28 (*d*, 10.3)
13	136.7	-	137.2	-
14α	29.2	1.86 (*ddd*, 12, 11, 2)	29.2	1.86 (*ddd*, 12.5, 11, 2)
14β	-	2.67 (*ddd*, 12.2, 3, 2)	-	2.66 (*ddd*, 12.5, 3, 2)
15	29.4	2.94 (*d*, 11)	29.4	3.1 (*m*)
16	50.7	-	51.4	-
17	68.8	3.66 (*d*, 11)	68.7	3.68 (*d*, 11)
17’	-	3.83 (*d*, 11)	-	3.84 (*d*, 11)
18	12.9	1.64 (*ddd*, 7, 2, 2)	13.0	1.65 (*ddd*, 7, 2, 2)
19	116.8	5.41 (*br q*, 7)	116.7	5.41 (*br q*, 7)
20	137.1	-	137.4	-
21α	55.5	3.59 (*m*)	55.5	3.60 (*br d,* 16.0)
21β	-	3.61 (*s*)	-	3.58 (*br d,* 16.0)
$CO_2\underline{Me}$	50.6	2.89 (*s*)	50.6	2.94 (*s*)
$\underline{CO_2}Me$	173.8	-	174.1	-

3.3.2 Cholinesterase inhibitory activity of isolated compounds

All isolated compounds (alkaloids **1, 2, 3** and **4**) were evaluated for their cholinesterase inhibition by the colorimetric Ellman's method (Ellman *et al*., 1961). This experiment used eserine, a clinical drug, as a standard.

The AChE and BChE inhibition percentages of all isolated substances, at the final concentration of 0.3, 0.1 and 0.05 mg/mL, as well as their IC_{50} values were displayed in Tables 3.9 and 3.10, respectively. The results indicated that alkaloids **(1-4)** inhibited both enzymes in dose-dependent manners. All compounds showed the AChE and BChE inhibition percentages more than 70% at final concentration of 0.3 mg/mL. Alkaloids **1-4** exhibited the moderate AChE and BChE inhibitory activities with the similarity of IC_{50} values. Among of them, alkaloid **4**, having hydroxyl group, possessed the highest inhibitory activity towards both AChE and BChE with the IC_{50} value of 0.25 ± 0.03 and 0.26 ± 0.04 mM, respectively. These values were quite high comparing with those of eserine (IC_{50} 0.02 ± 0.01 and 0.01 ± 0.01 mM of AChE and BChE, respectively). More interestingly, alkaloid **1-4** showed inhibitory effect against these unequal owing to the effect of their structure and structure-activity relationships (SAR) cannot be identified. However, alkaloid 2 and 4 are structure isomers, differ only fused ring, bonding different position and be influenced for the risk assessment of anticholinesterase clearly.(Geissler *et al*., 2010).

Table 3.8 The AChE inhibitory activity of all isolated substances from the leaves of *A. scholaris* at final concentrations of 0.3, 0.1, and 0.05 mg/mL

Alkaloids	% AChE Inhibition at the final concentrations of (mg/mL)[a]			IC_{50} of AChE (mM)
	0.3	0.1	0.05	
1	76.1 ± 0.1	54.3 ± 0.2	51.3 ± 0.3	0.26± 0.06
2	72.9 ± 01	52.3 ± 0.3	31.1 ± 0.2	0.41± 0.03
3	77.3 ± 0.2	55.7 ± 0.2	34.3 ± 0.3	0.37± 0.07
4	82.7 ± 0.4	58.0 ± 0.3	46.1 ± 0.3	0.25± 0.03
Eserine[a]	-	96.2 ± 0.4	95.7 ± 0.1	0.02± 0.01

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

[a]Standard compound

Table 3.9 The BChE inhibitory activity of all isolated substances from the leaves of *A. scholaris* at final concentrations of 0.3, 0.1, and 0.05 mg/mL

Alkaloids	% BChE Inhibition at the final concentrations of (mg/mL)			IC_{50} of BChE (mM)
	0.3	0.1	0.05	
1	70.2 ± 0.2	46.1 ± 0.2	39.0 ± 0.3	0.36± 0.03
2	71.0 ± 0.3	50.1 ± 0.3	29.9 ± 0.3	0.43± 0.03
3	73.3 ± 0.1	51.3 ± 0.3	35.1 ± 0.2	0.37± 0.02
4	80.5 ± 0.2	54.4 ± 0.3	45.2 ± 0.1	0.26± 0.04
Eserine[a]	-	95.6 ± 0.1	92.9 ± 0.1	0.01± 0.01

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

[a]Standard compound

According to the highest activity of of 19, 20-(*E*)-Akuammidine (**4**), this alkaloid was further determined the mode of AChE and BChE inhibitions. The Lineweaver–Burk plots of alkaloid **4** (Figure 3.6 and 3.7) displayed the different K_m values (the negative reciprocal of the X-intercept) and the unchanged $\mathbf{v}_{max}$ values (the reciprocal of the Y-intercept). These results indicated the competitive inhibition at the catalytic site of AChE and BChE. Moreover, this study was the first report of 19, 20-(*E*)-Akuammidine (**4**) as the competitive inhibitor towards AChE and BChE

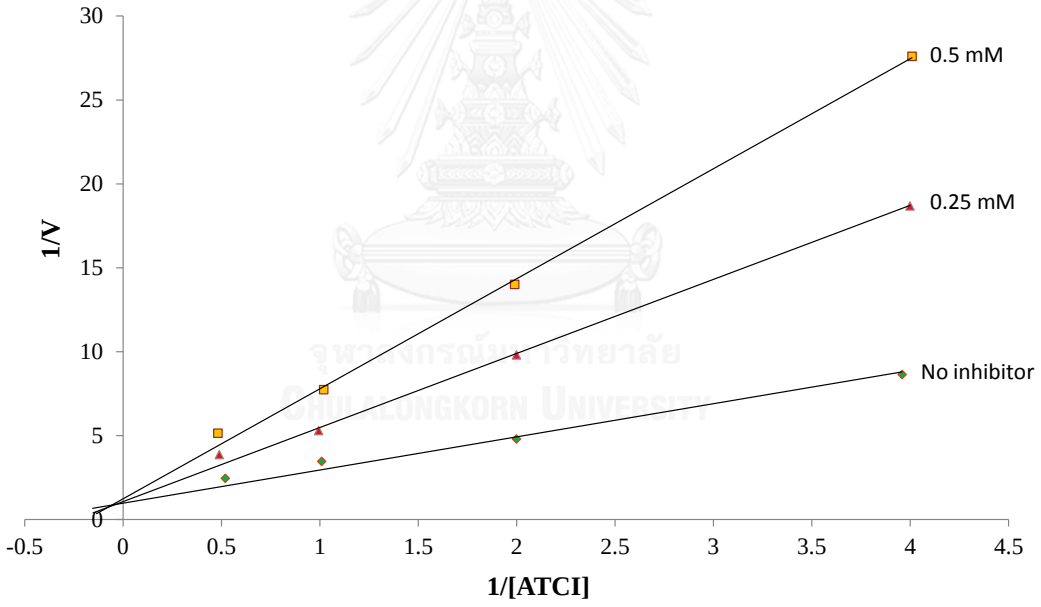


Figure 3.6 Lineweaver–Burk plots resulting of 19, 20-(*E*)-Akuammidine (**4**) from the substrate velocity curves of AChE inhibitory activity.

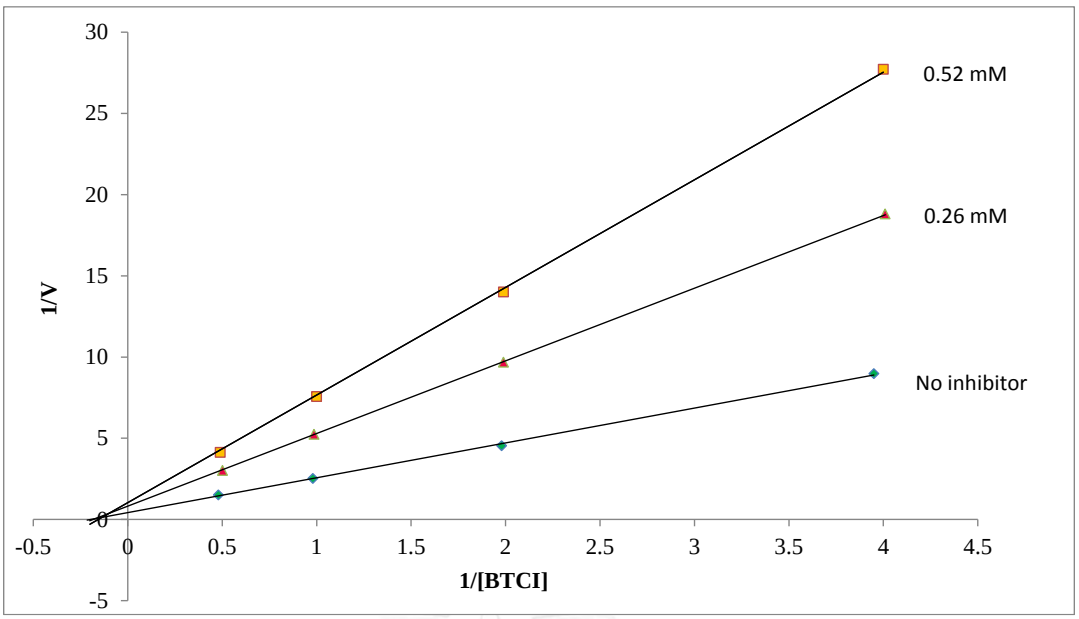


Figure 3.7 Lineweaver–Burk plots resulting of 19, 20-(*E*)-Akuammidine (**4**) from the substrate velocity curves of BChE inhibitory activity.

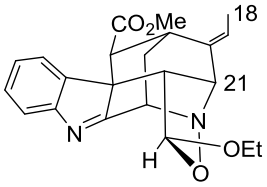
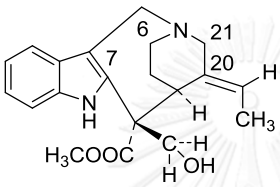
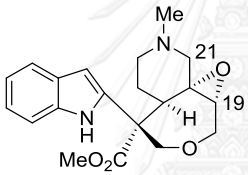
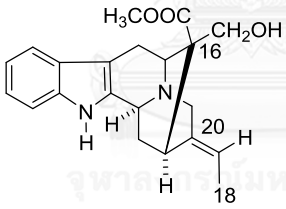
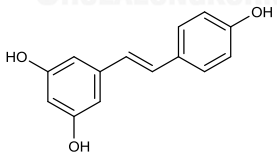
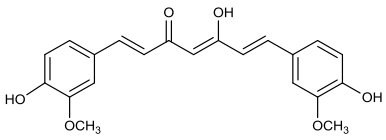
3.3.2 Anti-amyloid aggregation of the isolated compounds

The isolated alkaloids **1**-**4** were further determined their anti-amyloid aggregation activity by the fluorometric method. Resveratrol and curcumin were used as a standard compound (Feng *et al*., 2009) (Yang *et al*., 2005).

The anti-amyloid aggregation activity of all isolated substances, at the final concentrations of 20 and 100 μM were displayed in Table 3.11 and Figure 3.6. The results indicated that all isolated alkaloids inhibited amyloid aggregate in a dose-dependent manner. All alkaloids showed medium inhibition toward amyloid aggregation. Alkaloid **4** exhibited the highest anti-amyloid aggregation activity and closed to the activity of curcumin.

The anti-amyloid aggregation of mixed isolated substances, at the final concentrations of 20 μM, was found to be higher than that of single component (Figure 3.6). The inhibition percentages of the mixed compounds **3** and **4** was 40.64 (± 0.25) at the final concentration of 20 μM while those of compounds **3** and **4** were 29 and 33, respectively. In addition, the activity of mixed compounds **1-4** (46.32%) was significantly higher than that of compound **4**. The TEM images according to the procedure described in Section 2.5.2.4 and Figure 3.7, clearly proved that mixing each component together can increase the potential to inhibit the accumulation of A β-amyloid fibrils.Although, all substances from *A. scholaris* showed medium anti-amyloid aggregation comparing to the standard compounds but they were purified from natural plant which might be have low side effect and they were developed core-structure for increased inhibit cholinesterase.. However, the inhibitory effects of amyloid aggregration have not been reported. So, the results suggested that all substances from *A. scholaris* might be the promising candidate to the development for AD drugs.

Table 3.10 The anti-amyloid aggregation of all isolated substances from the leaves of *A. scholaris* at final concentrations of 20 and 100 μM

Substance	Structure	% Inhibition at the final concentrations of (μM)[a]	
		20	100
1		30.6 ± 0.1	40.7 ± 0.5
2		28.5 ± 0.1	35.0 ± 0.3
3		29.3 ± 0.1	40.3 ± 0.7
4		33.4 ± 0.1	43.6 ± 0.7
Resveratrol[a]		40.8 ± 0.2	65.4 ± 0.3
Curcumin[a]		31.2 ± 0.4	51.1 ± 0.3

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

[a]*Standard compound*

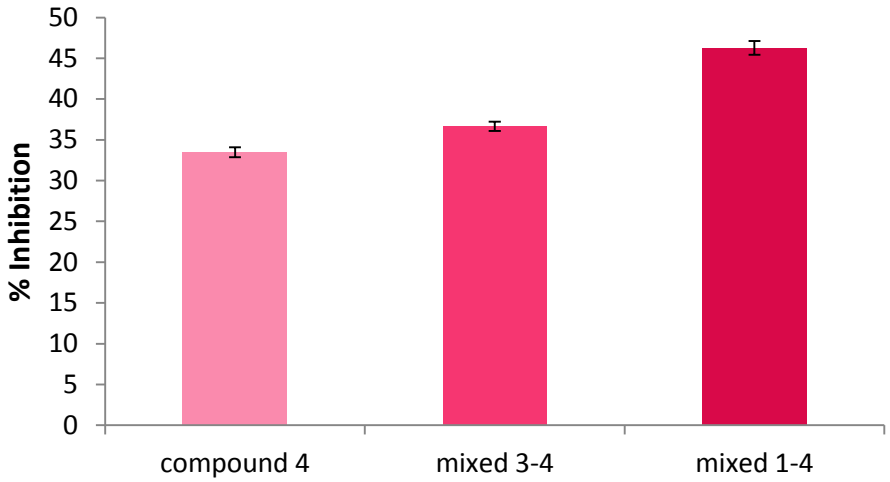


Figure 3.8 The anti-amyloid aggregation activities of compound 4, mixed compounds 3 and 4 (mixed 3-4), and mixed compounds 1-4 (mixed 1-4) at the concentration of 20 μM

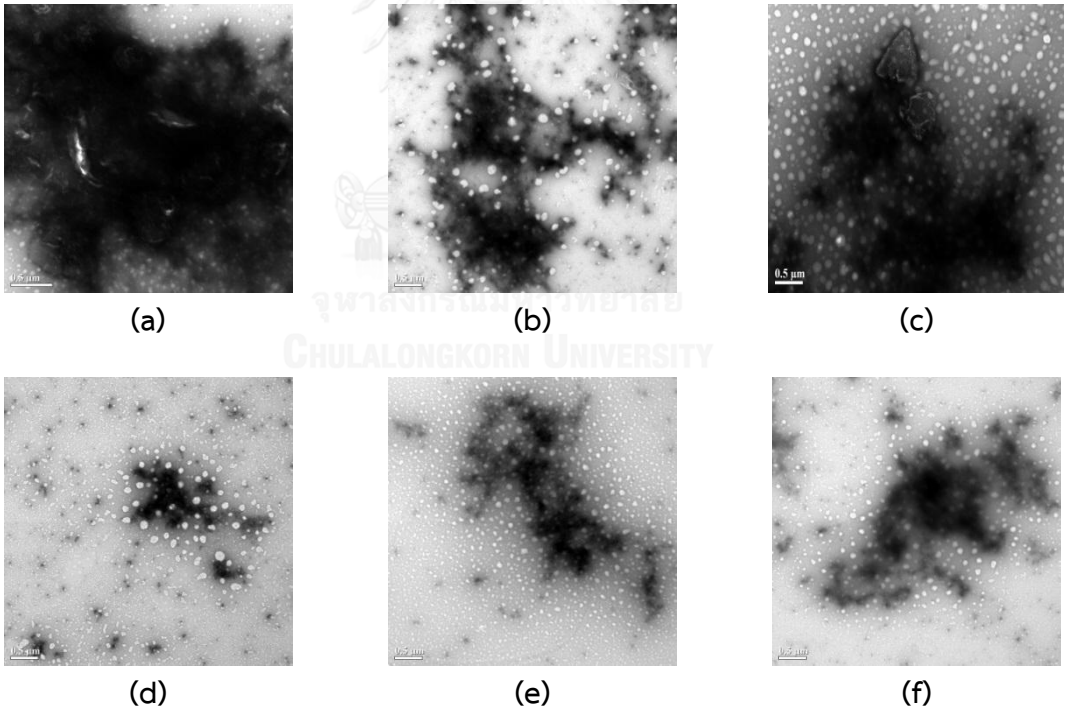


Figure 3.9 TEM images of amyloid fibrillation incubating with (a) no inhibitor, (b) curcumin, (c) resveratrol, (d) mixed compounds 1-4, (e) mixed compounds 3 and 4 and (f) compound 4

CHAPTER IV

CONCLUSION

In this research, the alkaloid-enriched extract of *A. scholaris* leaves was fractionated to give eight fractions (AS1-AS8). All fractions showed potent inhibition towards AChE, BChE and Aβ-amyloid aggregation. By column chromatography, four indole alkaloids (**1**-**4**) were obtained from separation fractions AS1, AS2 and AS4. The chemical structures of these isolated compounds were elucidated based on the NMR data (^{1}H NMR and ^{13}C NMR) and compared with these previously reported. These isolated substances were nareline ethyl ether (**1**), 19, 20-(*E*)-vallesamine (**2***), 6, 7-seco-*19, 20-epoxyangustilobine B (**3**) and 19, 20-(*E*)-Akuammidine (**4**). The chemical structures and their physical properties of the isolated substances were summarized in Table 4.1.

Alkaloids **1**-**4** were evaluated for their anti-cholinesterase and anti-amyloid aggregation activities (Table 4.2). Alkaloid **1-4** exhibited the moderate AChE and BChE inhibitory activities, with the similar IC_{50} values in the range of 0.25–0.41 mM and 0.26–0.43 mM, respectively. Moreover, all alkaloids have the same potential to inhibit amyloid aggregation in the ranges of 28.5–33.4% and 35.0–43.6% at the final concentrations of 20 and 100 μM, respectively. Mixing alkaloids **3**-**4** and **1**-**4** could clearly reduce Aβ-amyloid fibrillation observed from TEM images.

This study is the first report of anti-cholinesterase and anti-amyloid aggregation activities of the chemical constituents from *A. scholaris*. Although most isolated compounds showed only moderate activities, the core skeleton of these compounds might be useful for modification and drug development for treatment neurodegenerative disease

Table 4.1 Physical data of alkaloids 1-4 isolated from *A. scholaris* leaves

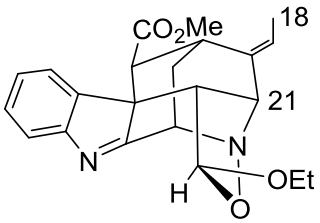
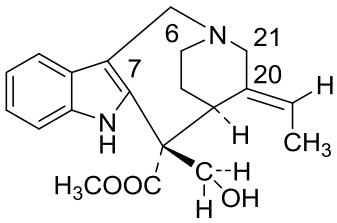
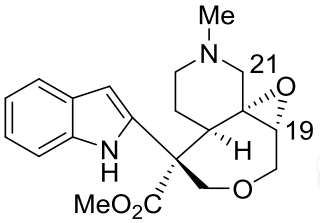
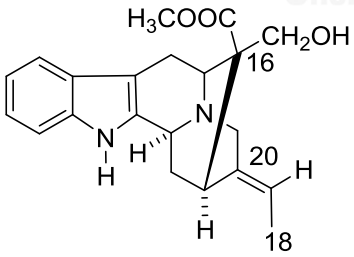
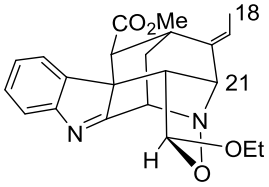
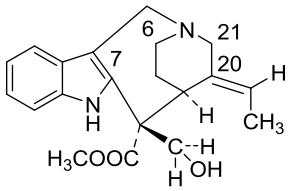
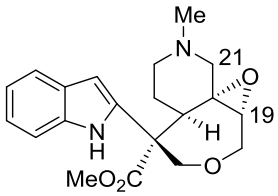
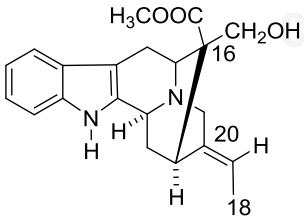
Alkaloid	Chemical formula, Molecular weight	Physical appearance	Melting point (°C)
 nareline ethyl ether **(1)**	$C_{22}H_{24}N_2O_3$, 380.44	Colorless needles	28.0, 7.0×10^{-2}
 19, 20-(*E*)-vallesamine **(2)**	$C_{20}H_{24}N_2O_3$, 340.42	pale yellowish oil	11.4, 2.9×10^{-2}
 *6, 7-seco*-19, 20-epoxyangustilobine B **(3)**	$C_{20}H_{24}N_2O_4$, 356.42	pale yellowish oil	10.1, $2.5. \times 10^{-2}$
 19, 20-(*E*)-Akuammidine **(4)**	$C_{21}H_{24}N_2O_3$, 352.43	colorless needles	9.5, 2.4×10^{-3}

Table 4.2 The anti-cholinesterase and anti-amyloid aggregation activities of alkaloids 1-4 from *A. scholaris* leave

Substance	IC_{50} of cholinesterase inhibition (mM)		% Inhibition of amyloid aggregation at (μM)	
	AChE	BChE	20	100
 (1)	0.26± 0.21	0.36± 0.31	30.6 ± 0.1	40.7 ± 0.5
 (2)	0.41± 0.42	0.43± 0.30	28.5 ± 0.1	35.0 ± 0.3
 (3)	0.37± 0.30	0.37± 0.21	29.3 ± 0.1	40.3 ± 0.7
 (4)	0.25± 0.40	0.26± 0.21	33.4 ± 0.1	43.6 ± 0.7
Eserine[a]	0.02± 0.02	0.01± 0.06	N/T	N/T
Resveratrol[a]	N/T	N/T	40.75 ± 0.25	65.36 ± 0.30
Curcumin[a]	N/T	N/T	31.18 ± 0.45	51.06 ± 0.36

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

N/T = not test,

[a]Standard compound

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APPENDIX

APPENDIX A

Table A-1 The AChE inhibitory activity of all isolated substances from the leaves of *A. scholaris* at IC_{50} concentrations (mM)

Alkaloids	IC_{50} of AChE (mM)			Average	SD
	1	2	3		
1	0.23	0.33	0.21	0.26	0.06
2	0.38	0.39	0.45	0.41	0.03
3	0.38	0.29	0.43	0.37	0.07
4	0.22	0.28	0.24	0.25	0.03
Eserine[a]	0.01	0.04	0.02	0.02	0.02

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

[a]Standard compound

Table A-2 The BChE inhibitory activity of all isolated substances from the leaves of *A. scholaris* at IC_{50} concentrations (mM)

Alkaloids	IC_{50} of BChE (mM)			Average	SD
	1	2	3		
1	0.32	0.39	0.36	0.36	0.03
2	0.43	0.40	0.46	0.43	0.03
3	0.35	0.39	0.37	0.37	0.02
4	0.22	0.3	0.27	0.26	0.04
Eserine[a]	0.01	0.02	0.01	0.01	0.01

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

[a]Standard compound

Table A-3 The anti-amyloid aggregation activity of fractions AS1-AS8

Sample[a]	% Inhibition at the concentration of (mg/ml)		
	2	0.5	0.05
AS1	69.0 ± 0.1	45.1 ± 0.1	19.6 ± 0.1
AS2	66.2 ± 0.4	40.9 ± 0.3	189.0 ± 0.5
AS3	76.1 ± 0.3	55.2 ± 0.3	20.9 ± 0.5
AS4	64.9 ± 0.6	36.3 ± 0.3	19.2 ± 0.5
AS5	68.5 ± 0.8	34.9 ± 0.1	10.3 ± 0.5
AS6	64.6 ± 0.5	31.5 ± 0.5	11.4 ± 0.8
AS7	71.3 ± 0.1	43.3 ± 0.6	10.1 ± 0.6
AS8	83.00 ± 0.2	64.6 ± 0.4	18.8 ± 0.9

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

[a]Resveratrol and curcumin are standard compounds showed 65.36 ± 0.30 and 51.06 ± 0.36%, respectively, at the concentration of 100 μM.

Table A-4 The anti-amyloid inhibition activity of alkaloids 1-4 at concentration 20 μM

Inhibition	AB	Cont	A1 (20 μM)	A2 (20 μM)	A3 (20 μM)	A4 (20 μM)
% inhibition 1	0	31.70	30.72	28.68	29.42	33.52
% inhibition 2	0	30.90	30.62	28.42	29.36	33.39
% inhibition 3	0	31.00	30.45	28.51	29.17	33.38
average	0	31.2	30.59	28.53	29.32	33.43
se	0	0.45	0.14	0.13	0.13	0.08

Note: Alkaloid 1 (A1), Alkaloid 2 (A2), Alkaloid 3 (A3), Alkaloid 4 (A4) and Cont (curcumin)

Table A-5 The anti-amyloid inhibition activity of alkaloids 1-4 at concentration 100 μM)

Inhibition	AB	Cont	A1 (100 μM)	A2 (100 μM)	A3 (100 μM)	A4 (100 μM)
% inhibition 1	0	51.12	38.95	37.58	43.07	34.25
% inhibition 2	0	50.80	41.19	41.25	43.19	35.05
% inhibition 3	0	51.39	41.84	42.04	44.47	35.75
average	0	51.1	40.66	35.02	40.29	43.58
se	0	0.30	0.51	0.32	0.74	0.73

Note: Alkaloid 1 (A1), Alkaloid 2 (A2), Alkaloid 3 (A3), Alkaloid 4 (A4) and Cont (curcumin)

Table A-6 The anti-amyloid inhibition activity of mix alkaloids at concentration 20 μM

Inhibition	A1:A2	A1:A3	A1:A4	A2:A3	A3:A4	A2:A4
% inhibition 1	36.13	36.01	37.09	36.05	36.52	37.19
% inhibition 2	36.08	36.02	36.91	36.03	35.89	37.43
% inhibition 3	36.01	35.97	36.82	35.71	37.16	36.39
average	36.07	36.00	36.94	35.93	36.52	37.63667
se	0.06	0.02	0.14	0.19	0.63	0.54

Note: Alkaloid 1 (A1), Alkaloid 2 (A2), Alkaloid 3 (A3) and Alkaloid 4 (A4)

Table A-7 The anti-amyloid inhibition activity of alkaloid 4 and mix alkaloids at concentration 20 μM

Sample	1st	2nd	3rd	Average	SD
compound 4	33.43	34.21	32.65	33.43	0.78
mixed 3-4	36.52	35.89	37.16	36.52	0.63
mixed 1-4	46.84	46.14	45.99	46.32	0.45

APPENDIX B

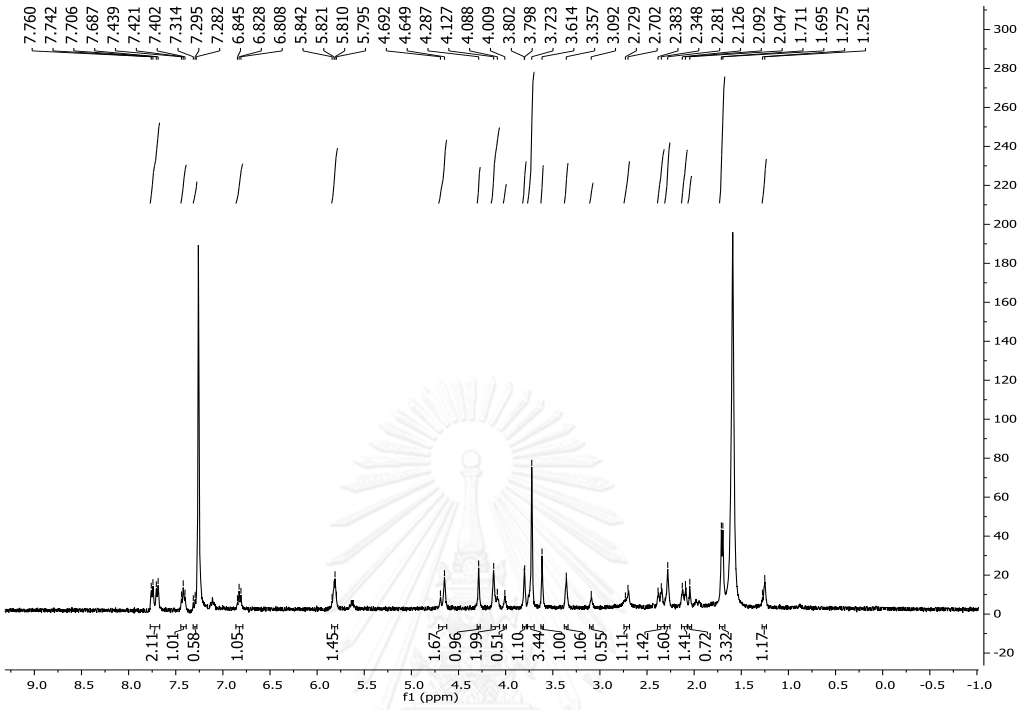

Figure B-1 The ^{1}H-NMR spectrum ($CDCl_3$) of alkaloid **1**

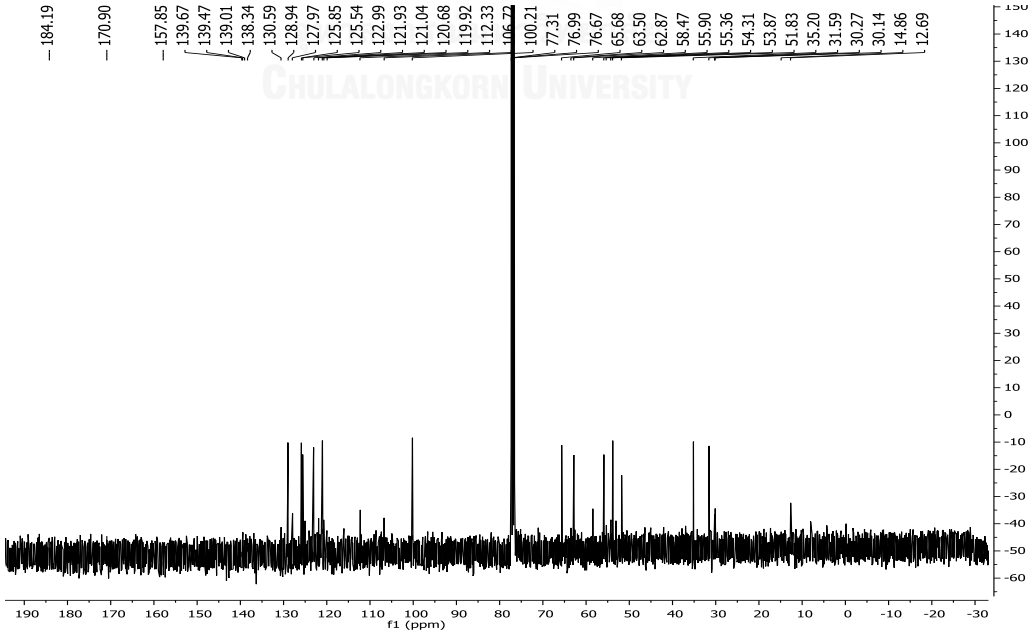

Figure B-2 The ^{13}C-NMR spectrum ($CDCl_3$) of alkaloid **1**

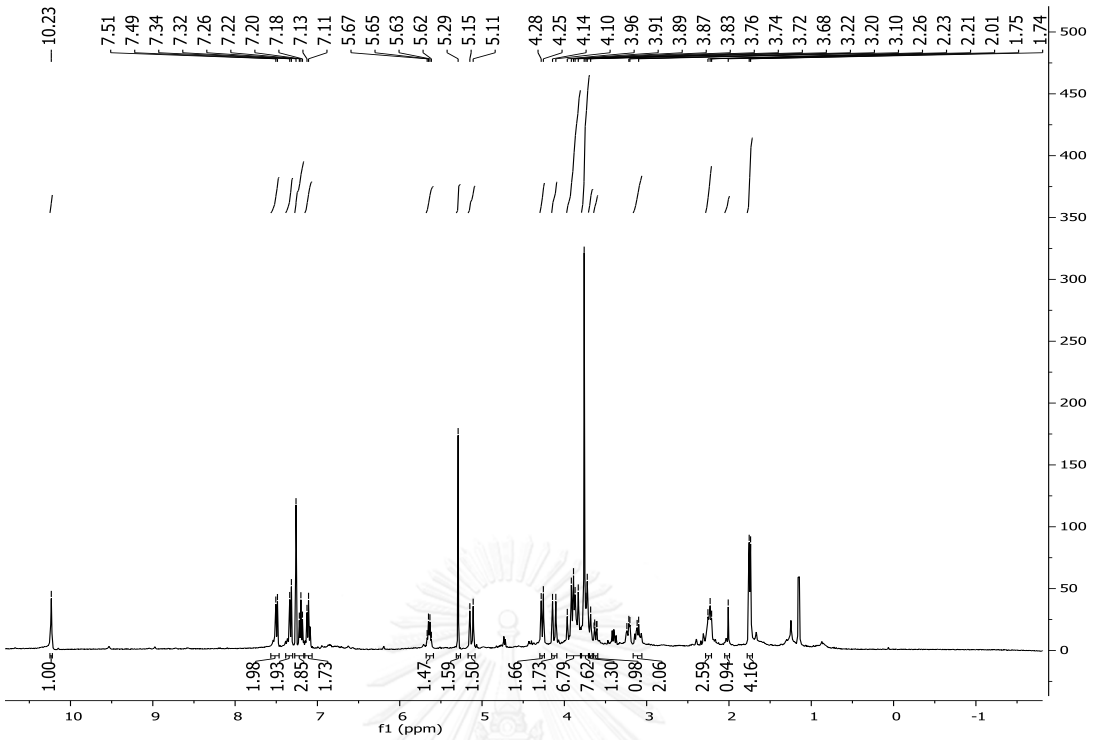

Figure B-3 The ^{1}H-NMR spectrum ($CDCl_3$) of alkaloid **2**

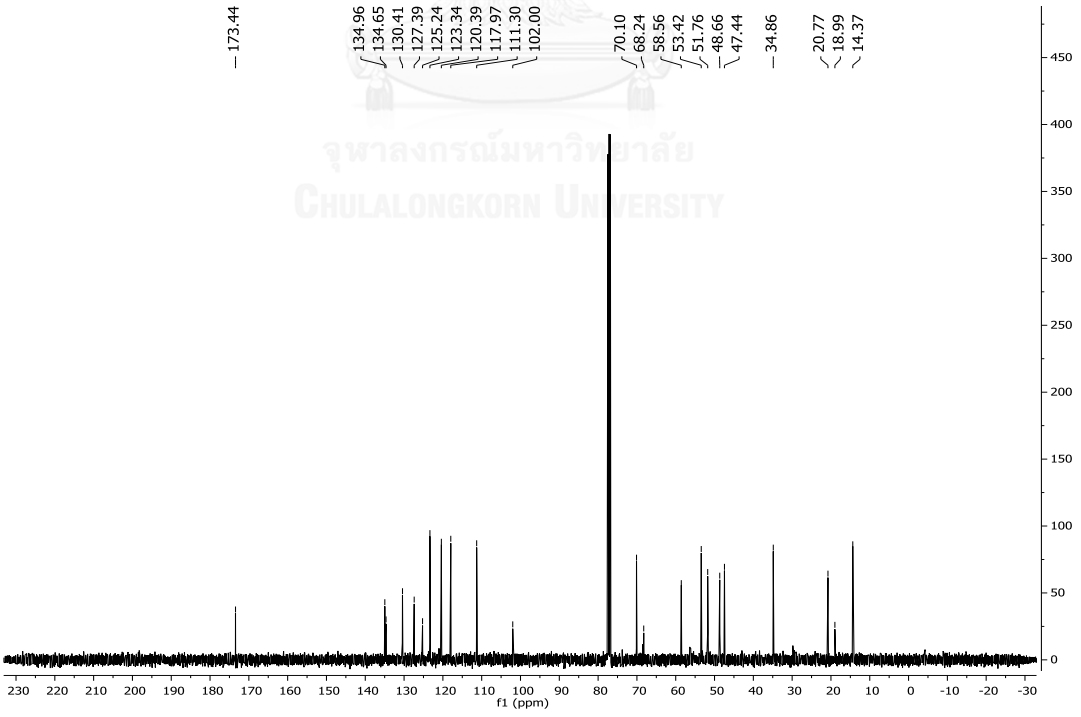

Figure B-4 The ^{13}C-NMR spectrum ($CDCl_3$) of alkaloid **2**

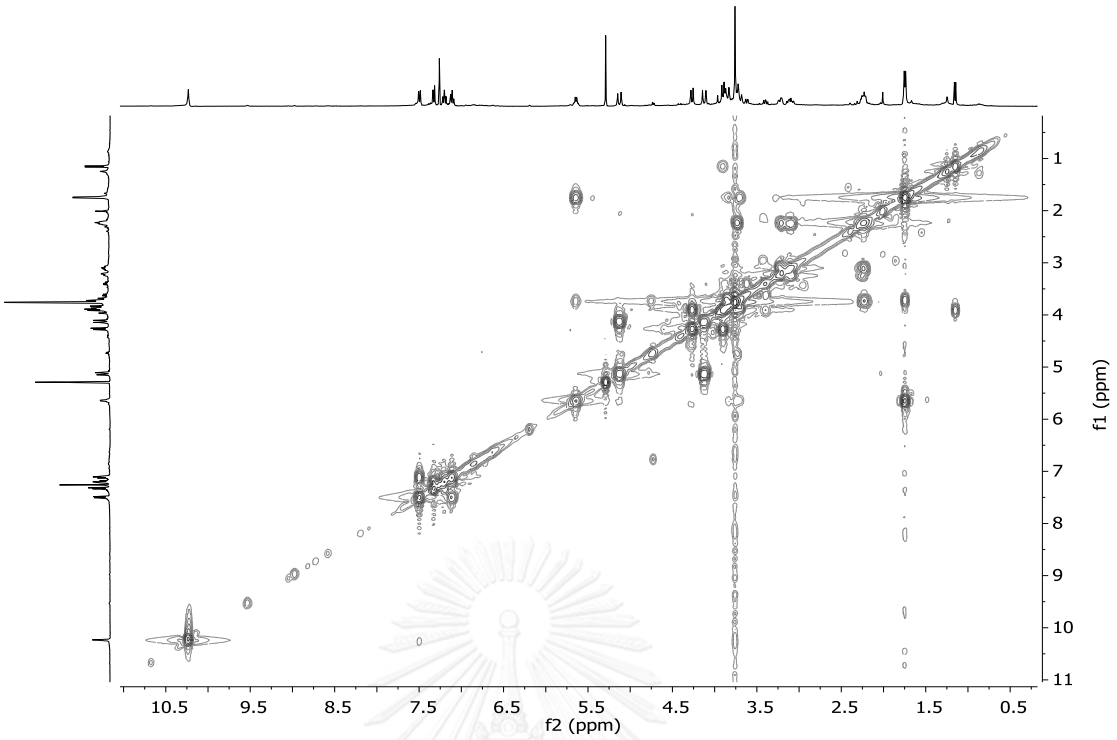

Figure B-5 The COSY spectrum ($CDCl_3$) of alkaloid **2**

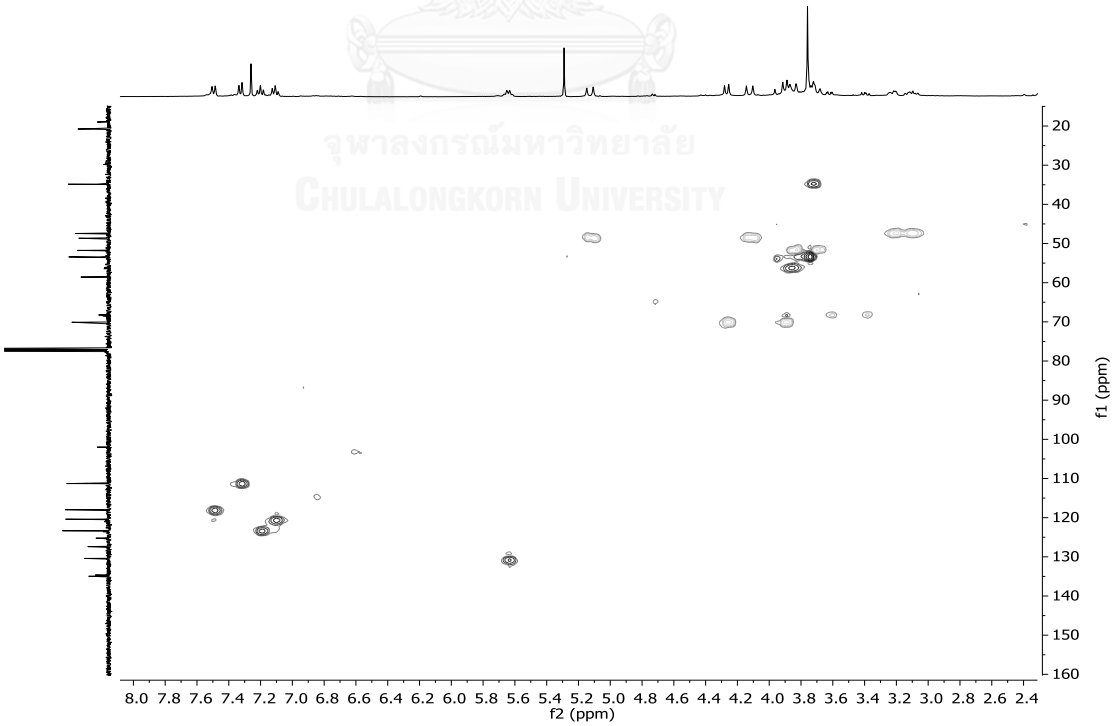

Figure B-6 The HSQC spectrum ($CDCl_3$) of alkaloid **2**

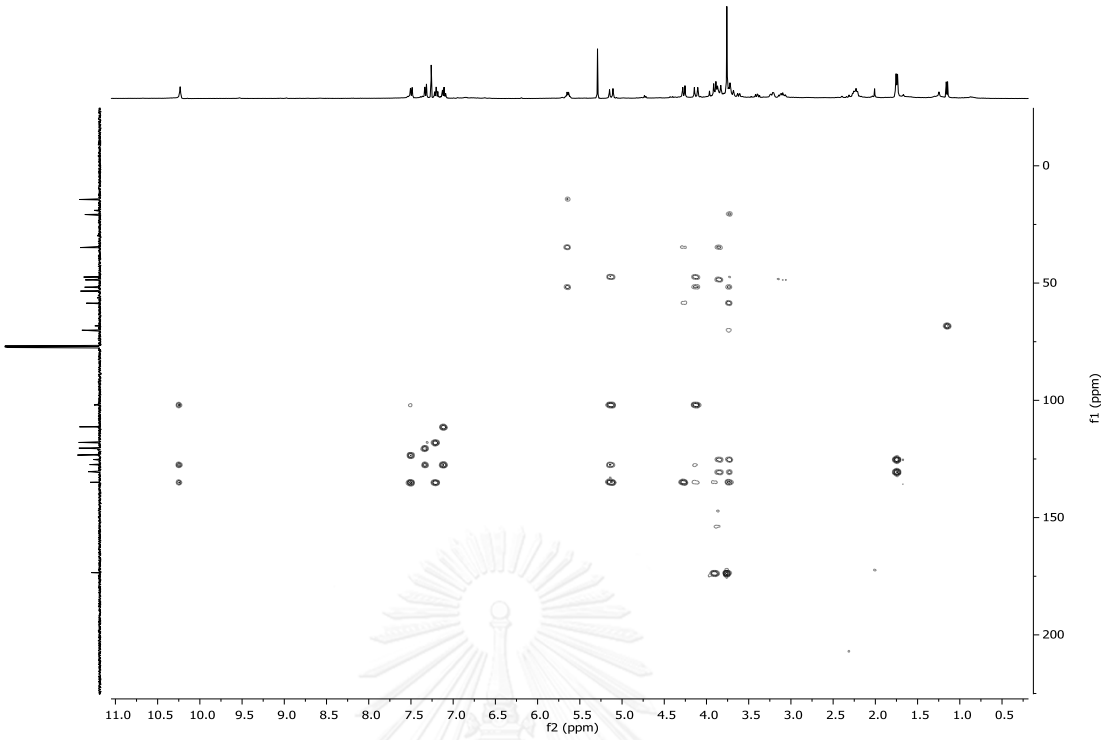

Figure B-7 The HMBC spectrum ($CDCl_3$) of alkaloid **2**

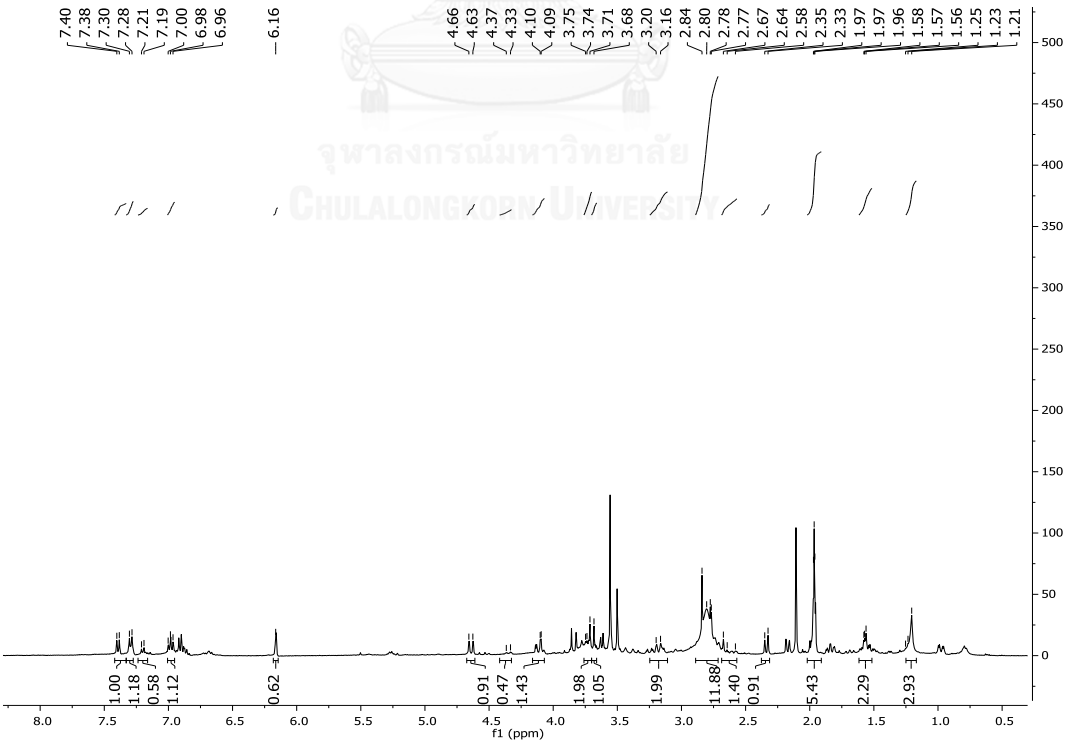

Figure B-8 The ^{1}H-NMR spectrum (CD_3COCD_3) of alkaloid **3**

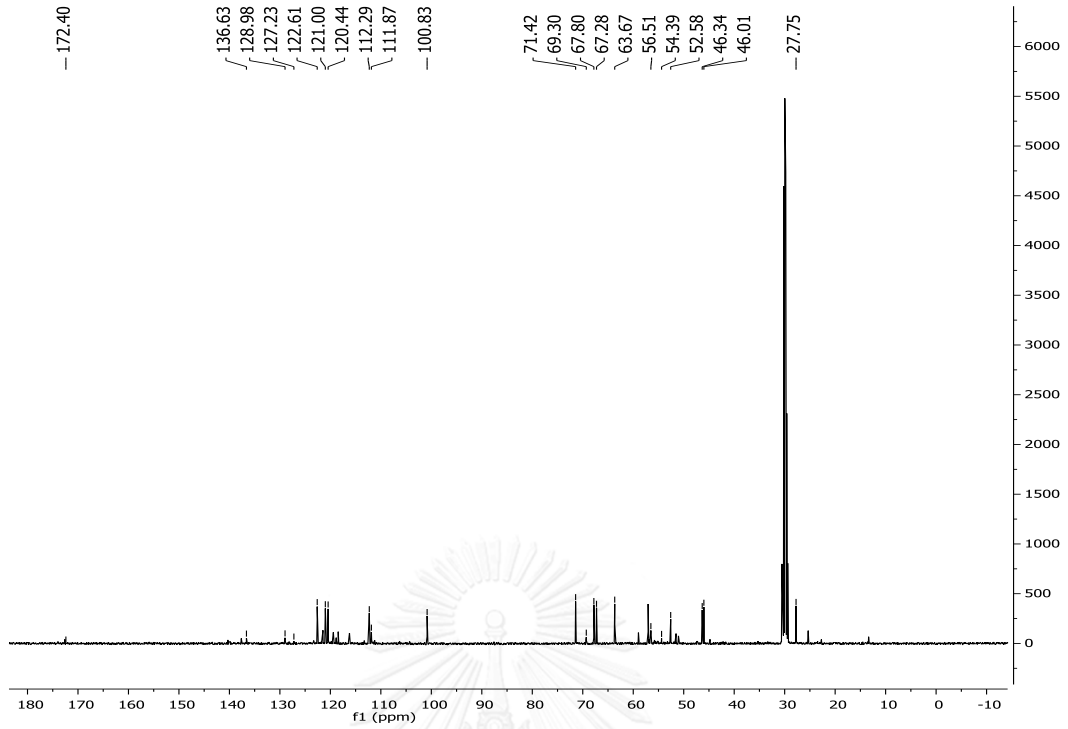

Figure B-9 The ^{13}C-NMR spectrum (CD_3COCD_3) of alkaloid **3**

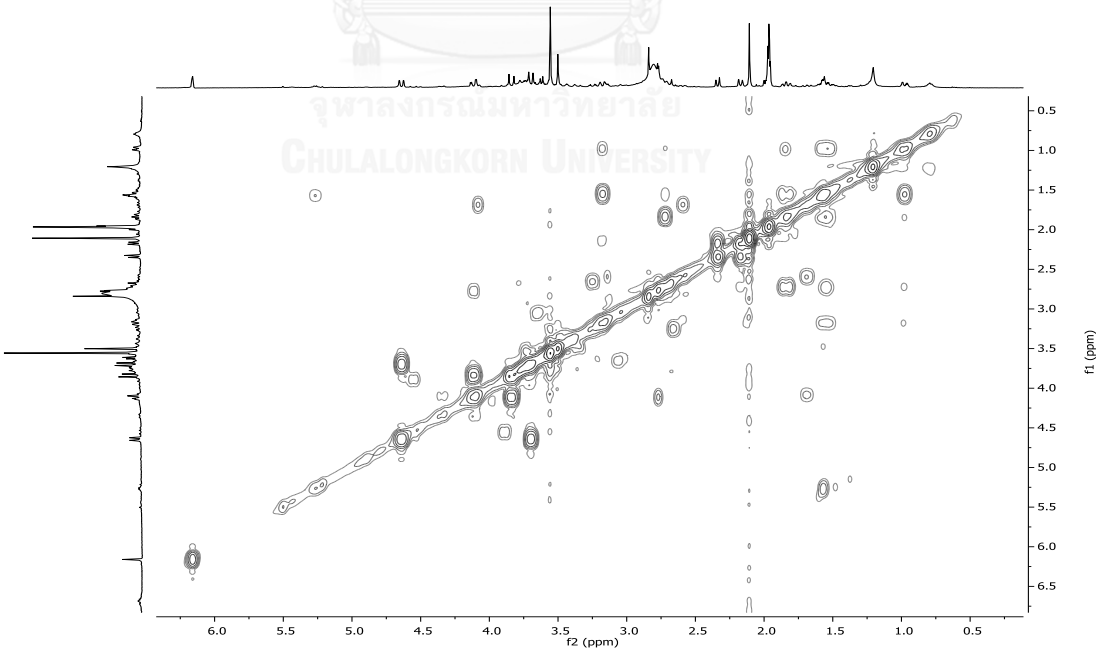

Figure B-10 The COSY spectrum (CD_3COCD_3) of alkaloid **3**

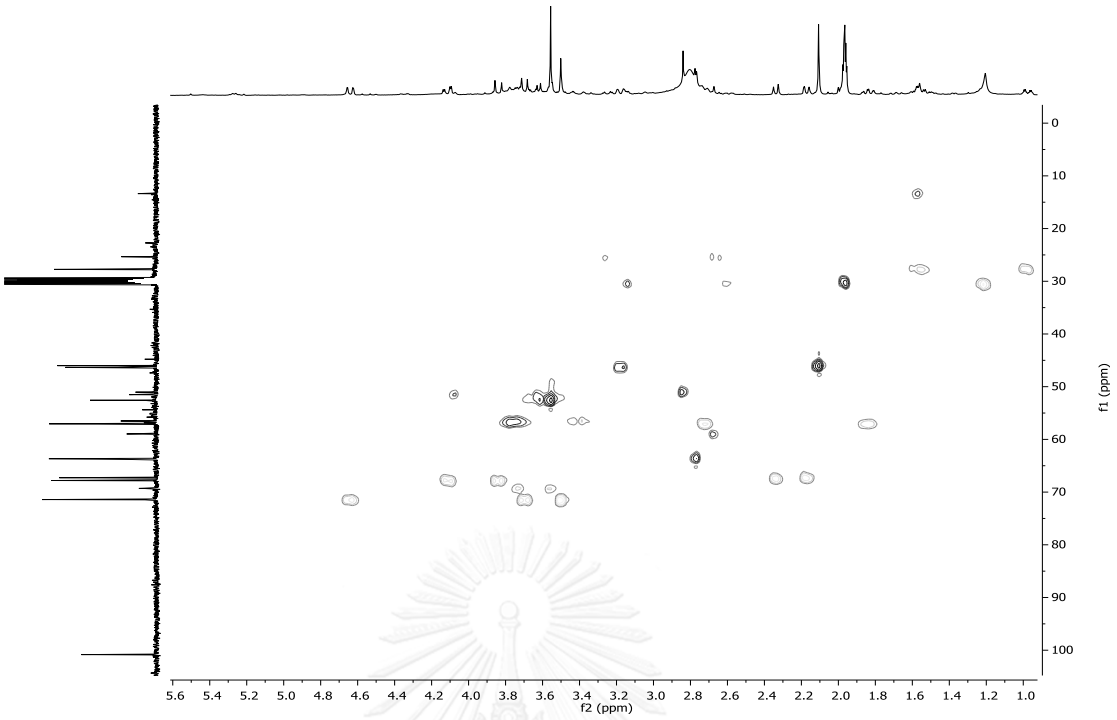

Figure B-11 The HSQC spectrum (CD_3COCD_3) of alkaloid **3**

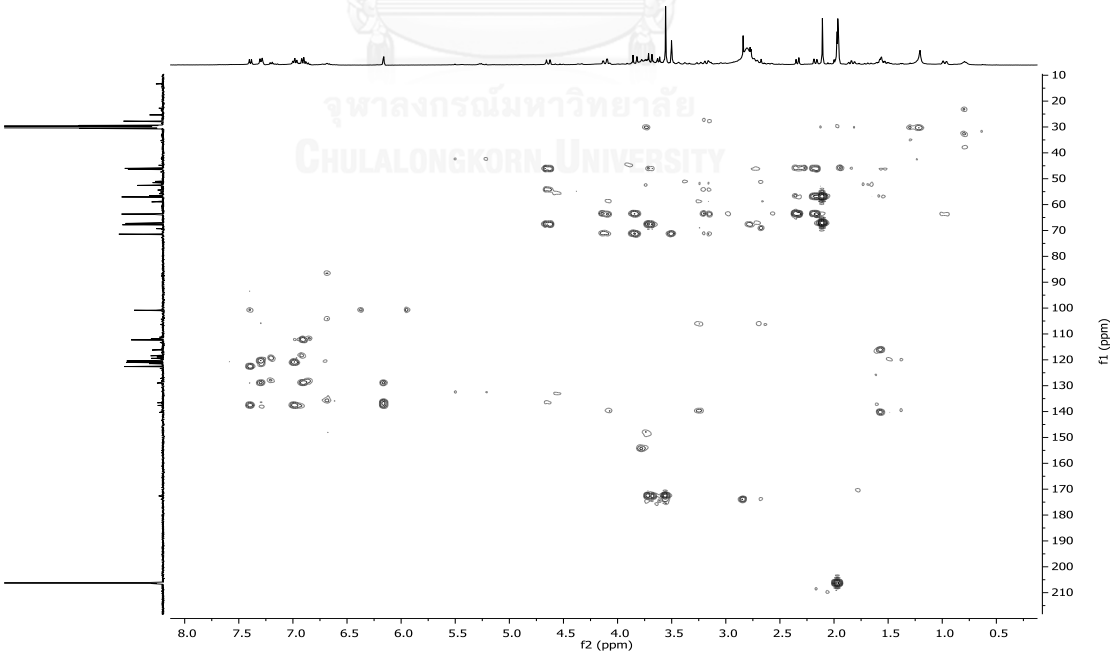

Figure B-12 The HMBC spectrum (CD_3COCD_3) of alkaloid **3**

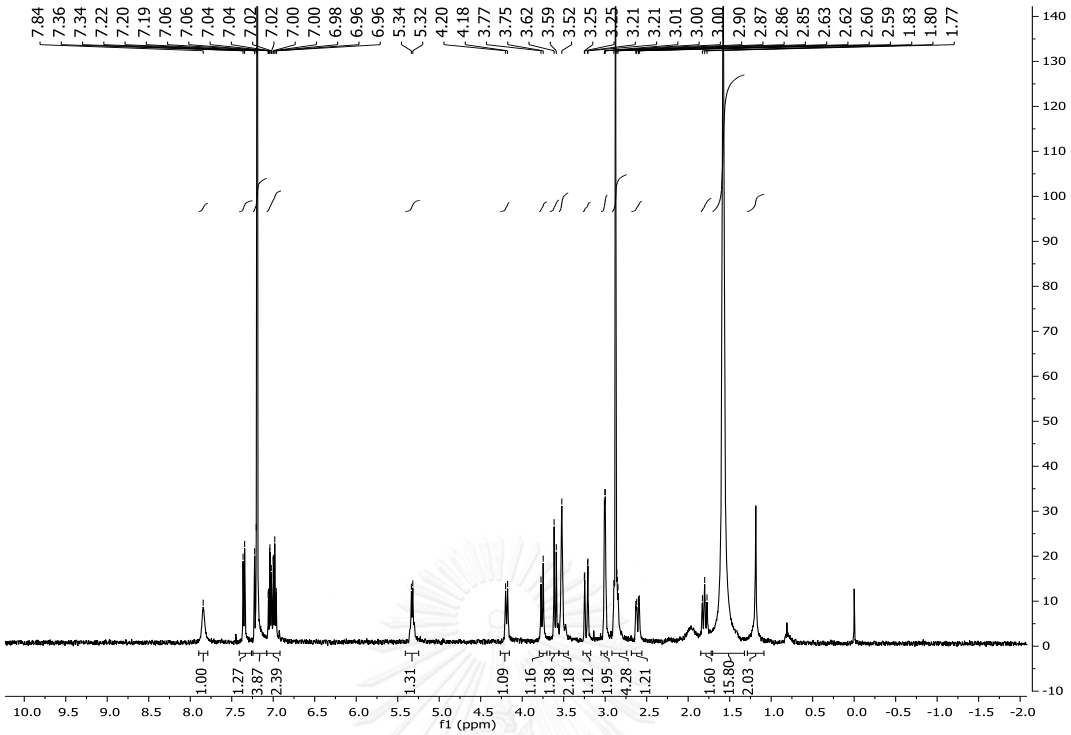

Figure B-13 The ^{1}H-NMR spectrum (CD_3OD) of alkaloid **4**

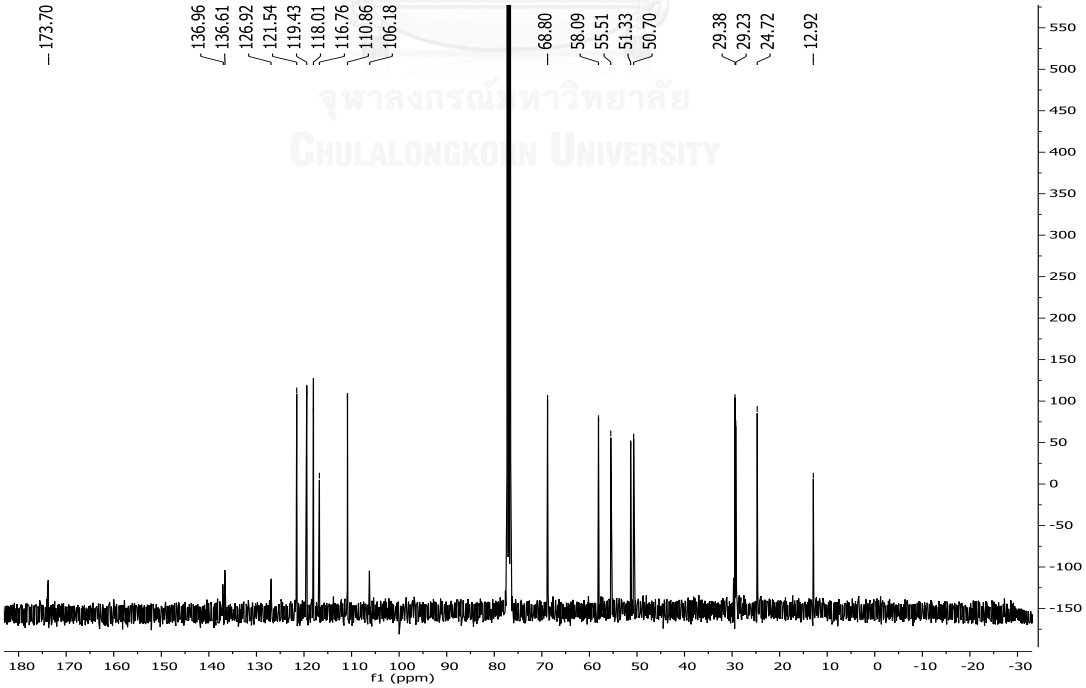

Figure B-14 The ^{13}C-NMR spectrum (CD_3OD) of alkaloid **4**

VITA

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Academic presentation;

1) Thanawan Rojpitikul,1 Pattara Sawasdee2,* ANTI-CHOLINESTERASE AND ANTI-AMYLOIDOGENIC ACTIVITIES FROM LEAF EXTRACT OF Alstonia scholaris L. R. Br. In "The 40th Congress on Science and Technology (Science and Technology towards ASEAN Development)" 2-4 December 2014, Hotel Pullman Khon Kaen Raja Orchid, Khon Kaen, Thailand (Proceeding book)